The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A–Pbx1 fusion proteins with differing transforming potentials

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The t(1;19) translocation that characterizes 25% of pediatric pre-B cell acute lymphoblastic leukemias (pre-B ALL) produces a chimeric gene, joining 5′ sequences that encode a transcriptional activator domain of E2A with 3′ sequences that, in part, encode a homeo box domain of a new gene called *pbx1*. Two *E2A–pbx1* transcripts have been cloned. They encode the putative fusion proteins, $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$, which differ in Pbx1 sequences alone, containing unique carboxyl termini whose sequences diverge after the Pbx1 homeo box. In this study, an antiserum to Pbx1 was used to investigate the identity and abundance of E2A–Pbx1 fusion proteins in both the pre-B ALL cell line, 697, and in cryopreserved leukemic bone marrow cells, obtained from six children with t(1;19)-positive pre-B ALL. Five species of E2A–Pbx1 proteins were identified in all cells containing t(1;19), two of which were indistinguishable from in vitro-translated $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$. To assess the biological properties of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ in fibroblasts, the cDNAs encoding these proteins were cloned into retroviral vectors, and each was introduced into NIH-3T3 cells. Both $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ are localized in the nucleus, and expression of either resulted in malignant conversion of NIH-3T3 cells as assayed by tumor formation in nude mice. When scored by focus formation, density-independent growth, and growth in agar assays, $p77^{E2A-Pbx1}$ was a much more potent transforming protein than was $p85^{E2A-Pbx1}$. Because subtle mutations in $p85^{E2A-Pbx1}$ converted its transforming activity into that of $p77^{E2A-Pbx1}$, we suggest that a sequence within the unique carboxyl terminus of $p85^{E2A-Pbx1}$ serves to negatively regulate its biochemical activity.

[*Key Words*: t(1;19) translocation; pre-B ALL; homeo box domain; nuclear fusion proteins]

Received July 18, 1990; revised version accepted January 14, 1991.

The binding of potential transcription factors to specific DNA sequences in the immunoglobulin heavy- and light-chain enhancers was first observed by footprinting studies both in vitro and in vivo (Church et al. 1985; Ephrussi et al. 1985). The κE2 DNA sequence motif, contained in the immunoglobulin κ light-chain enhancer, was subsequently shown to be important in the transcriptional activation of this gene, because mutation of this sequence diminishes the strength of the κ enhancer (Sen and Baltimore 1986; Lenardo et al. 1987). Both the *E2A* and *ITF-2* genes encode proteins that bind the κE2 motif, each behaves as a transcriptional activator (Murre 1989a; Henthorn et al. 1990), and together they share considerable amino acid identity. Two domains in the E2A protein have been characterized—an amino-terminal domain that activates transcription and a carboxy-terminal domain involved in sequence-specific DNA-binding and dimer formation with other cellular transcription factors (Murre 1989a,b; Davis et al. 1990; Henthorn et al. 1990; Voronova and Baltimore 1990). In contrast to the normal role of E2A in transcriptional regulation, the occurrence of a mutant form of E2A in human pre-B ALL (Kamps et al. 1990) suggests that it may also be involved in aberrant transcription and leukemic tumorigenesis.

The breakpoint of the t(1;19)(q23;p13.3) translocation, which is evident cytogenetically in 25% of pediatric pre-B ALL (Carroll et al. 1984; Williams et al. 1984) and is associated with a poor prognosis (Crist et al. 1990; Raimondi et al. 1990), has been mapped within the *E2A* gene, which resides at 19p13.3 (Mellentin et al. 1989). Using an *E2A* cDNA probe, chimeric cDNAs containing sequences from both *E2A* and a second uncharacterized gene, previously designated *prl*, but now renamed *pbx1*, were isolated from the t(1;19)-positive pre-B ALL cell lines, 697 (Kamps et al. 1990) and SUP-B27 (Nourse et al. 1990). *prl* was renamed *pbx1* to avoid confusion with the prolactin locus, which is also designated PRL. The fusion

proteins predicted by *E2A–pbx1* transcripts contain the amino-terminal *trans*-activation domain of E2A but have the carboxy-terminal DNA-binding and dimerization domain of E2A replaced with a homeo box encoded by *pbx1*. The sequence of the Pbx1 homeo box is not identical to that of any other but does contain a remarkable 19 of 27 amino acid identity in its carboxy-terminal half to that of the yeast MATa1 protein, a transcription factor that, together with the MATα1 protein, represses haploid-specific gene expression and allows sporulation to occur (Miller et al. 1985; Nasmyth and Shore 1987). The reciprocal translation product, composed of the 5′ portion of *pbx1* and the 3′ portion of *E2A*, is not involved in the development of t(1;19)-containing pre-B ALL because it is contained on the der(1) chromosome, which is lost from the leukemic cells in >80% of cases examined (Mellentin et al. 1989) and because transcriptional regulation of this locus, when it remains intact, is mediated by the *pbx1* promoter, which is silent in pre-B and mature B cells (Kamps et al. 1990; Nourse et al. 1990). The domain structure of E2A–Pbx1 proteins suggests that their potential contribution to the development of pre-B ALL is the activation of Pbx1-responsive genes by the E2A *trans*-activation domain in either pre-B cells or their progenitors.

Two *E2A–pbx1* transcripts, likely the result of alternative splicing, have been cloned and sequenced (Kamps et al. 1990; Nourse et al. 1990). They are designated type I and type II and encode the putative fusion polypeptides, $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$, which differ at their carboxy-terminal ends. The goals of this study were to identify the forms of E2A–Pbx1 proteins contained in leukemic cells having the t(1;19) translocation and investigate the transforming potential of these fusion proteins in NIH-3T3 fibroblasts. We find that a family of E2A–Pbx1 proteins, including $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$, arise as a consequence of the t(1;19) translocation, and that E2A–Pbx1 proteins are oncoproteins whose specific activities appear dependent on their unique carboxyl termini.

Results

Antibodies to Pbx1 bind five species of E2A–Pbx1 fusion proteins

Previously (Kamps et al. 1990), we reported that 697 cells, which contain the t(1;19) translocation, contain a new, 90-kD, E2A-related polypeptide, in addition to normal E2A proteins. This novel protein was predicted to be the translocation product of the cloned type I *E2A–pbx1* fusion transcript. At this time, the putative 77-kD product of the type II *E2A–pbx1* transcript was not identified in immunoprecipitates from 697 cells using antibodies reactive with E2A. To separate E2A–Pbx1 fusion proteins from normal E2A proteins in 697 cells and then compare their primary structures to that of in vitro-translated $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$, a rabbit antiserum was raised against Pbx1 (see Materials and methods) and used to isolate Pbx1-containing polypeptides from 697 cells by immunoprecipitation. Five proteins were immunoprecipitated by the anti-Pbx1 serum (Fig. 1, lane 6), two of which comigrated with in vitro-translated $p77^{E2A-Pbx1}$ and $p85^{E2A-Pbx1}$ (lanes 7 and 8). None of the five proteins was immunoprecipitated from Nalm-6 cells, a human pre-B cell line that lacks the t(1;19) translocation (lane 3) or by a nonspecific antiserum to the retinoblastoma protein, $p110^{Rb}$ (lanes 1 and 4). Although the two proteins that comigrated with in vitro-translated $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ are detectable in immunoprecipitates from 697 cells using an antiserum to E2A, the remaining three were obscured by polypeptides reactive with antibodies to E2A (lane 5) and, therefore, were not observed earlier. These results demonstrate that a family of at least five proteins, expressed specifically in 697 cells, are immunoprecipitated by anti-Pbx1 serum because they either contain Pbx1, are associated with a Pbx1-containing protein, or cross-react with anti-Pbx1 antibodies.

To distinguish among these possibilities, a partial proteolysis assay using *Staphylococcus aureus* V8 protease was developed (Cleveland et al. 1977). A distinctive pattern of peptides resulted from partial proteolysis of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ with 5, 15, or 50 ng of *S. aureus* V8 protease, followed by resolution by polyacrylamide gel electrophoresis (PAGE). The pattern obtained by digestion with 50 ng of protease was used to typify E2A–Pbx1 proteins and to discriminate between

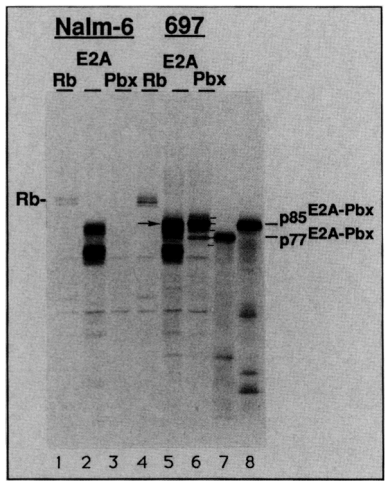

Figure 1. Immunoprecipitation with antibodies to E2A and Pbx1. Nalm-6 cells, a human pre-B cell line that does not contain the t(1;19) translocation, and 697 cells, which contain the translocation, were labeled biosynthetically with [^{35}S]methionine and subjected to immunoprecipitation with a control antiserum against the retinoblastoma protein (designated Rb), an antiserum against E2A (designed E2A), and an antiserum to Pbx1 (designated Pbx1). $p77^{E2A-Pbx1}$ and $p85^{E2A-Pbx1}$ markers were translated from RNA, transcribed in vitro from the type II and type I *E2A–pbx1* cDNAs, and analyzed in the last two lanes. The mobility of $p110^{RB}$, $p85^{E2A-Pbx1}$, and $p77^{E2A-Pbx1}$ are indicated (*left* and *right*). The location of the five bands immunoprecipitated with the anti-Pbx1 serum are indicated by dashes in lane 6. The major polypeptide that is immunoprecipitated by anti-E2A serum from 697 cells is indicated by the arrow.

$p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ (Fig. 2B). In vitro-translated $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ each contained major proteolytic fragments of 46, 33, and 20 kD (peptides A, B, and D, respectively); however, only $p85^{E2A-Pbx1}$ contained a major polypeptide of ~24 kD (peptide C). This fragment is most likely derived from the carboxyl terminus of $p85^{E2A-Pbx1}$, which contains an additional three methionines not present in $p77^{E2A-Pbx1}$ (Fig. 2A). Proteins precipitated from 697 cells with antibodies to Pbx1 were given numerical assignments indicated in Figure 2C. The partial proteolytic pattern produced by $p85^{E2A-Pbx1}$ translated in vitro was identical to the major protein precipitated from 697 cells by antibodies to Pbx1 (Fig. 2D, lanes 1 and 2), as well as to that of the new antigen precipitated from 697 cells by antibodies to E2A (data not shown). This demonstrated that translation of the type I *E2A–pbx1* transcript we cloned from 697 cells produces the major novel antigen detected both by antibodies to E2A and Pbx1. Partial proteolysis of band 4 (Fig. 2D, lane 4) produced a pattern of polypeptides identical to that of in vitro-translated $p77^{E2A-Pbx1}$ (Fig. 2B, lane 77, under the 50 ng heading), each lacking peptide C. Consequently, $p77^{E2A-Pbx1}$ is also a bona fide E2A–Pbx1 protein in 697 cells. Finally, bands 1, 3, and 5 yielded a proteolytic pattern containing the diagnostic C peptide of $p85^{E2A-Pbx1}$, and overall, that was very similar to the pattern of $p85^{E2A-Pbx1}$. A slightly larger A peptide was generated by bands 3 and 5, suggesting that these proteins are more closely related to each other than to the other E2A–Pbx1 proteins. We will refer to the proteins that comprise bands 1, 3, and 5 and $p87^{E2A-Pbx1}$, $p83^{E2A-Pbx1}$, and $p72^{E2A-Pbx1}$, respectively.

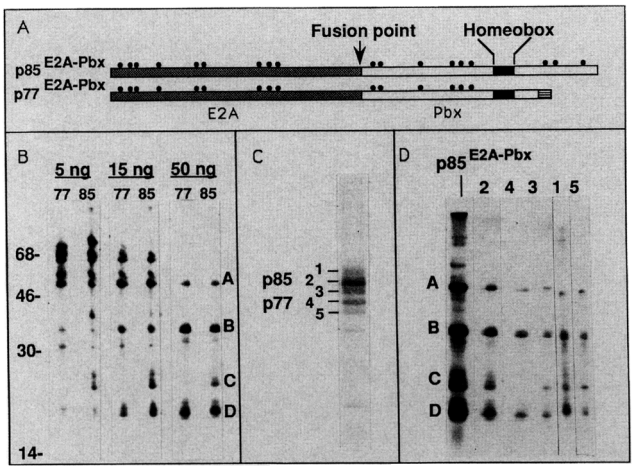

Figure 2. Proteolytic analysis of polypeptides immunoprecipitated by antibodies to Pbx1. (*A*) Comparative structures predicted for the proteins $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$. The stippled region is E2A, and the remainder of the polypeptide is Pbx1. The horizontal stripes at the end of $p77^{E2A-Pbx1}$ indicate its unique carboxyl terminus, and the black rectangle denotes the location of the homeo box in Pbx1. The solid circles represent the positions of methionine residues in each protein. (*B*) Peptide patterns of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ after digestion with the indicated amounts of *S. aureus* V8 protease and resolution by SDS-PAGE. (85) $p85^{E2A-Pbx1}$; (77) $p77^{E2A-Pbx1}$. Relative molecular masses are indicated at *left*; peptide assignments are shown at *right*. (*C*) Pattern of E2A–Pbx1 proteins obtained from an anti-Pbx1 immunoprecipitation of 697 cells that results from twice the normal time of electrophoresis in SDS-PAGE and no enhancement of the autoradiographic signal. The band assignments used in the text are indicated to the side of the proteins they represent. (*D*) Comparison of peptides generated by digestion with 50 ng of *S. aureus* V8 protease of in vitro-translated $p85^{E2A-Pbx1}$ and of bands 1 and 5 (shown in *C*, designed 1–5 at *top* of lane). Peptides A–D are designated at *left*.

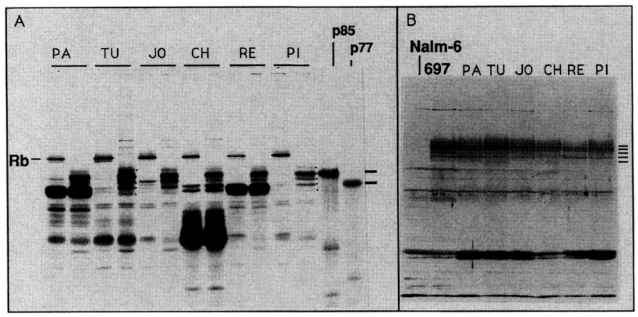

Figure 3. Identification of E2A–Pbx1 proteins in lymphoblasts from t(1;19)-positive pre-B ALL patients. (*A*) Immunoprecipitations, using an antiserum to either Rb (first of each of two lanes) or Pbx1 (second of each of two lanes), from six samples of bone marrow lymphoblasts, labeled biosynthetically with [^{35}S]methionine. Sets of immunoprecipitations are designated by the first two letters of the patient's last name. The migration of Rb is indicated at *left*, and that of in vitro-translated $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$, at *right*. The migrations of E2A–Pbx1 proteins 1–5 are indicated by dots in the samples TU and PI. (*B*) Immunoblot of total cellular extracts of bone marrow aspirates from the same six patients described in *A*, using affinity-purified antibodies to Pbx1. Designations are the same, and Nalm-6 and 697 cells were used as controls.

Each of the five E2A–Pbx1 proteins are present in bone marrow lymphoblasts from patients with t(1;19)-containing pre-B ALL

It was of interest to determine whether the five E2A–Pbx1 proteins are contained in primary tumor cells in approximately the same abundance as in 697 cells and thereby exclude the possibility that any one of the forms may have arisen uniquely in 697 cells during their establishment in tissue culture. Bone marrow aspirates comprised of >85% leukemic cells were obtained from six children with t(1;19) pre-B ALL. The identity of Pbx1-containing proteins in these cells was determined by two methods. First, cells were labeled biosynthetically with [^{35}S]methionine, and Pbx1-containing proteins were isolated from cellular extracts by immunoprecipitation (Fig. 3A). Proteins that bound nonspecifically during immunoprecipitation were identified by the use of an anti-Rb serum and are illustrated in the first of each set of lanes. The five forms of E2A–Pbx1 proteins were found in cells from patients TU, JO, and PI. Forms 1, 2, and 3 were found in cells from patients PA, CH, and RE, but forms

4 and 5 were obscured by the presence of background bands, probably monoclonal IgM heavy chain. To confirm the results of immunoprecipitation analysis and avoid the problem of comigrating nonspecific proteins, an immunoblot analysis was developed, utilizing affinity-purified anti-Pbx1 immunoglobulins and total cellular proteins from lymphoblasts derived from each of the same patients (Fig. 3B). Immunoblot analysis represented a rapid and accurate diagnostic technique for the identification of E2A–Pbx1 proteins. In this analysis, no proteins were stained in extracts from the control cell line, Nalm-6, in the region containing E2A–Pbx1 proteins in 697 cells (lanes designated Nalm-6 and 697). Each of the cells from patients contained all of the same five species of E2A–Pbx1 proteins as were found in 697 cells, and most are visible in Figure 3B. In particular, forms 4 and 5 were apparent in PA, CH, and RE. The staining of E2A–Pbx1 band 4 was approximately twofold more intense in lysates from 697 cells than in those from patient cells. The fact that all five E2A–Pbx1 proteins are observed in all cells containing the t(1;19) translocation strengthens the suggestion that the breakpoints in *E2A* and *pbx1* occur consistently in the same introns, join the same coding exons in the fully processed mRNA, and, therefore, produce the same E2A–Pbx1 proteins. We know that the 3′ *E2A* sequences in the *E2A–pbx1* transcript are 3′ sequences of exon "I" of the *E2A* gene, and, consequently, the *E2A* breakpoints lie within the adjacent 3′ intron, which is 4 kb in length (Kamps et al. 1990; S.-H. Sun, in prep.). Because 697 cells contain a fairly accurate representation of both the sizes and abundances of E2A–Pbx1 proteins that are observed in lymphoblasts of patients exhibiting t(1;19)-positive pre-B ALL, they represent an appropriate choice of cells in which to study these proteins.

The relative abundances of E2A–Pbx1 proteins, most accurately reflected by immunoblot analysis (Fig. 3B, lane 697; Fig. 4B, lane 697), were the same as observed by immunoprecipitation, in which case 697 cells were labeled biosynthetically for 3 hr (Fig. 1, lane 6, 697 cells; Fig. 2C). Each analysis suggested that form 2 ($p85^{E2A-Pbx1}$) comprises approximately half of all Pbx1-containing proteins, that form 5 is least abundant, and that forms 1, 3, and 4 are approximately equal and intermediate in abundance. Pulse-chase analysis revealed that the same relative abundance of the major forms of E2A–Pbx1—polypeptides 2-4—is found after only a 30-min label of 697 cells and that each of these proteins has a half-life of ~4 hr. Thus, all E2A–Pbx1 proteins are likely to be primary translation products of different transcripts.

Expression of $p77^{E2A-Pbx1}$, but not $p85^{E2A-Pbx1}$, induces focus formation in NIH-3T3 cells

The cDNAs encoding $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ were each inserted into a retroviral expression vector designated pGD (see Materials and methods), which expresses the neomycin resistance gene (*neo*) from an internal SV40 promoter, and the inserted cDNA from the 5′ viral

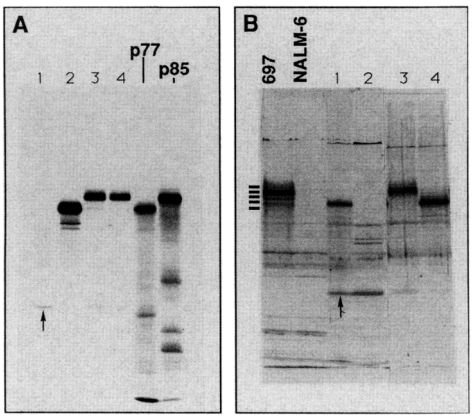

Figure 4. Expression of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ by retrovirus. (*A*) Anti-Pbx1 immunoprecipitates from NIH-3T3 cells, infected by viruses encoding $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$. (Lane *1*) Uninfected NIH-3T3-cl2; (lane *2*) NIH-3T3-cl2 infected with virus encoding $p77^{E2A-Pbx1}$; (lanes *3* and *4*) NIH-3T3-cl2 and NIH-3T3-cl3, infected with virus encoding $p85^{E2A-Pbx1}$. All cells were infected at an m.o.i. of 1 and selected for growth in the presence of G418. (*B*) Immunoblot analysis performed using equivalent amounts of cellular extract, resolved by SDS-PAGE. Analysis of a cloned focus containing $p77^{E2A-Pbx1}$ (represented in lane *3*) was included to demonstrate comigration. (Lane *1*) A clone of NIH-3T3-cl2 expressing $p77^{E2A-Pbx1}$; (lane *2*) NIH-3T3 cells infected by the *neo* virus; (lanes *3* and *4*) NIH-3T3-cl2 infected at an m.o.i. of 1 with virus encoding $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$, respectively, and selected for growth in G418. (*A* and *B*) Arrows indicate the migration of a potential normal form of the Pbx1 polypeptide.

LTR. Constructs were transfected into NIH-3T3 cells, and cells expressing *neo* were selected in the presence of G418 (Geneticin). Defective viruses encoding each E2A–Pbx1 protein were rescued by superinfection with Moloney murine leukemia virus (MLV), and their titers were assayed by transmission of G418 resistance. Populations of cells, selected for expression of each virus, were shown to contain proteins that comigrated with $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$, translated in vitro (Fig. 4A). The morphological effects of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ expression in fibroblasts were analyzed by infection of NIH-3T3-cl10 cells with each virus at a multiplicity of one. After 12 days of growth in normal medium (no selection), many foci of varying sizes formed on monolayers infected with the virus encoding $p77^{E2A-Pbx1}$ (Fig. 5, photo 3; Fig. 6, photos 3 and 6), but only tiny areas of slightly increased cell density formed on monolayers infected by the virus encoding $p85^{E2A-Pbx1}$ (Fig. 5, photo 2; Fig. 6, photos 2 and 5). A different subclone of NIH-3T3, NIH-3T3-cl2, developed larger foci after infection by virus encoding $p77^{E2A-Pbx1}$ (Fig. 5, photo 4). Therefore, the focus-forming potential of $p77^{E2A-Pbx1}$ is influenced somewhat by clonotypic variations in NIH-3T3. On NIH-3T3 cl10, low multiplicity of infection (1000 neo-forming units/5×10^5 infected cells) with virus encoding $p77^{E2A-Pbx1}$ produced mostly small foci-containing cells that were not as refractile as those resulting

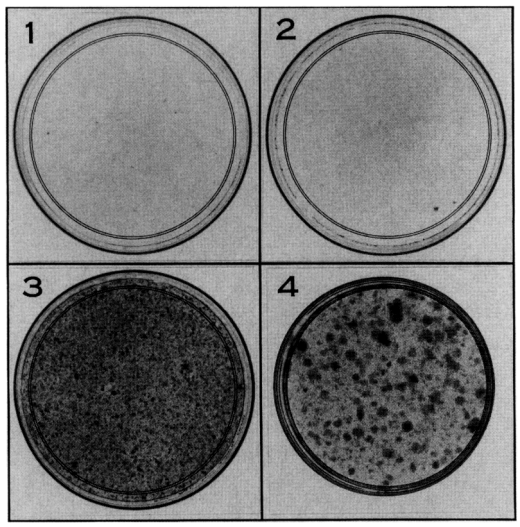

Figure 5. Focus-forming activity of viruses encoding p85$^{E2A-Pbx1}$ and p77$^{E2A-Pbx1}$. (Photos *1–3*) 100-mm plates of NIH-3T3-cl10, infected at an m.o.i. of 1 with *neo* virus (*1*), virus encoding p85$^{E2A-Pbx1}$ (*2*), and virus encoding p77$^{E2A-Pbx1}$ (*3*), all of which were stained with crystal violet after 12 days. (Photo *4*) A 35-mm dish of NIH-3T3-cl2, infected at an m.o.i. of 0.02 with virus encoding p77$^{E2A-Pbx1}$, and stained with crystal violet after 12 days.

from higher multiplicity of infection. However, when cells were infected at low multiplicity with virus expressing p77$^{E2A-Pbx1}$ and selected for growth in G418, 40% of NIH-3T3-cl1 colonies exhibited the transformed, refractile phenotype (data not shown). Therefore, normal cells induced suppression of the transformed phenotype. Three subclones of NIH-3T3 were evaluated for focus formation by virus encoding p85$^{E2A-Pbx1}$. All produced the same subtle morphological changes depicted in photos 2 and 5 of Figure 6.

Mutations in p85$^{E2A-Pbx1}$ convert its behavior to that of p77$^{E2A-Pbx1}$

While cells infected with virus encoding p85$^{E2A-Pbx1}$ were being passaged, large foci reminiscent of those produced by cells containing p77$^{E2A-Pbx1}$ arose after 8–10 weeks. Virus from these isolated foci now induced overt focus formation on unselected NIH-3T3-cl1, mimicking the effect of virus encoding bona fide p77$^{E2A-Pbx1}$. The identity of E2A–Pbx1 protein encoded by these focus-forming variants was analyzed from eight clones (Fig. 7A). All eight virus-encoded E2A–Pbx1 proteins had molecular masses quite similar to that of p85$^{E2A-Pbx1}$, and none was identical to p77$^{E2A-Pbx1}$. Therefore, subtle mutations in p85$^{E2A-Pbx1}$, some of which slightly alter the mobility of p85$^{E2A-Pbx1}$, can change its biochemical activity to mimic that of p77$^{E2A-Pbx1}$. Because focus-forming variants of p85$^{E2A-Pbx1}$ were produced by cells productively infected by virus encoding p85$^{E2A-Pbx1}$, an accurate analysis of the ability of p85$^{E2A-Pbx1}$ to induce growth in agar and tumors in nude mice could not be performed using these cells. Therefore, nonproducer lines of NIH-3T3 containing p77$^{E2A-Pbx1}$ or p85$^{E2A-Pbx1}$ were generated.

Both p77$^{E2A-Pbx1}$ and p85$^{E2A-Pbx1}$ are oncoproteins

NIH-3T3-cl1 cells were infected with helper-free stocks of virus encoding p77$^{E2A-Pbx1}$ or p85$^{E2A-Pbx1}$ (Materials and methods) and selected in G418 for 3 weeks. As shown by immunoblot analysis, these NIH-3T3 cells contained equivalent quantities of p77$^{E2A-Pbx1}$ and p85$^{E2A-Pbx1}$ (Fig. 7B) and did not produce virus (<1 G418 resistance unit/10 ml of supernate vs. 2×10^6/ml for producer cells). After extended passage, no foci formed in cultures of nonproducer cells containing p85$^{E2A-Pbx1}$, indicating that the activating mutations that arose in p85$^{E2A-Pbx1}$ in cultures containing helper virus occurred

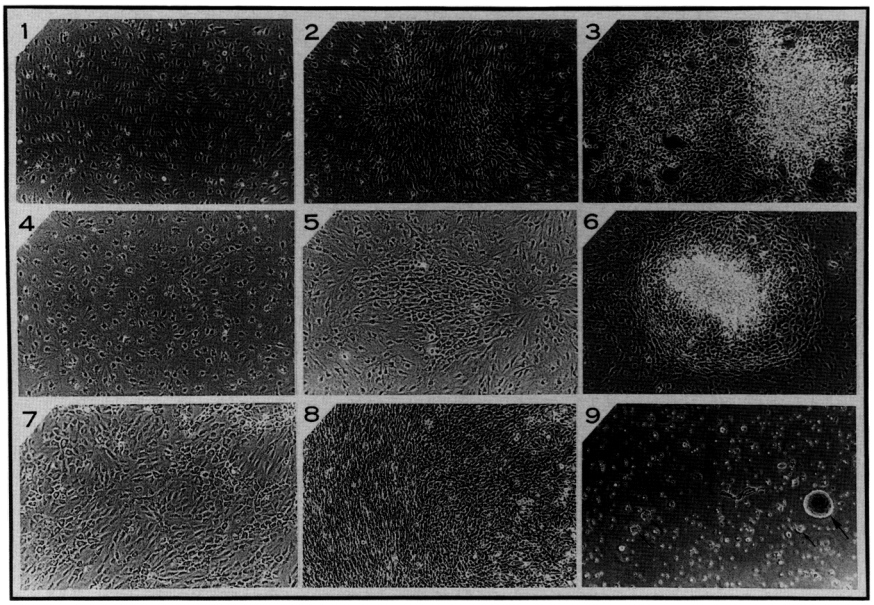

Figure 6. Focal induction and growth in agar associated with expression of p85$^{E2A-Pbx1}$ and p77$^{E2A-Pbx1}$. (Photos *1–3*) Focus formation by infection of NIH-3T3-cl1 cells using nonselected conditions. (Photo *1*) *neo* virus; (photo *2*) virus encoding p85$^{E2A-Pbx1}$; (photo *3*) virus encoding p77$^{E2A-Pbx1}$. (Photos *4–6*) Focus formation by transfection of NIH-3T3-cl3 with retroviral vectors using nonselected conditions. (Photo *4*) pGD vector; (photo *5*) pGD encoding p85$^{E2A-Pbx1}$; (photo *6*) pGD encoding p77$^{E2A-Pbx1}$. (Photos *7–8*) Colony formation by infection of NIH-3T3-cl1 cells followed by selection in G418. (Photo *7*) *neo* virus; (photo *8*) virus encoding p85$^{E2A-Pbx1}$; (photo *9*) growth in agar by NIH-3T3, infected by virus encoding p77$^{E2A-Pbx1}$. Small colonies (3–20) of cells are designated by the small arrows, and one large colony (>100 cells) by the large arrow.

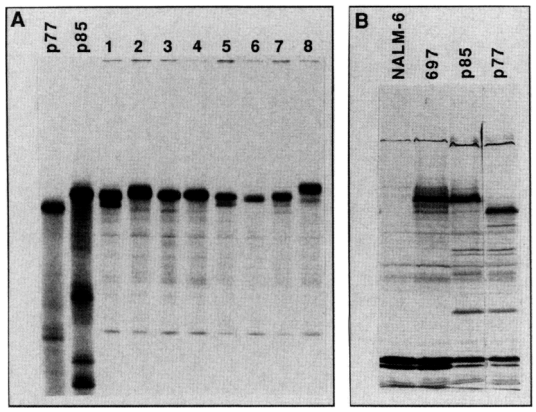

Figure 7. Generation of nonproducer cell lines avoids the formation of focus-forming variants of $p85^{E2A-Pbx1}$. (*A*, lanes *1–8*) Immunoprecipitations of E2A–Pbx1 proteins from cells infected by focus-forming variants of $p85^{E2A-Pbx1}$. Marker proteins are contained in the lanes labeled p77 and p85. (*B*) Western blot analysis of E2A–Pbx1 proteins in total cellular extracts of nonproducer NIH-3T3 cells. (p85) NIH-3T3 cells infected with helper-free virus encoding $p85^{E2A-Pbx1}$; (p77) NIH-3T3 cells infected with helper-free virus encoding $p77^{E2A-Pbx1}$. Samples from NALM-6 and 697 cells were included for verification of the molecular mass of E2A–Pbx1 proteins.

during viral replication. Nonproducer NIH-3T3 cells containing $p77^{E2A-Pbx1}$ and $p85^{E2A-Pbx1}$ were therefore used to test additional oncogenic properties of these two E2A–Pbx1 fusion proteins. Expression of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ in nonproducer cells induced a 5- and 12-fold increase in the density at which cells stopped dividing (Table 1). Although expression of either $p85^{E2A-Pbx1}$ or $p77^{E2A-Pbx1}$ permitted the growth of NIH-3T3 cells in agar, $p77^{E2A-Pbx1}$ was at least 10-fold more effective than was $p85^{E2A-Pbx1}$ (Table 1; Fig. 6, photo 9). Immunofluorescence analysis revealed that the largest colonies of cells that grew in agar also contained the most E2A–Pbx1 protein (data not shown). Tumorigenicity was tested in nude mice by subcutaneous injection of 8×10^5 cells. Nonproducer cells expressing $p77^{E2A-Pbx1}$ or $p85^{E2A-Pbx1}$ formed tumors at the site of injection within 6 weeks. Antibodies to Pbx1 heavily stained a 77-kD protein on an immunoblot containing protein extract from a tumor that arose after injection of cells expressing $p77^{E2A-Pbx1}$, indicating that the tumor grew from the introduced cells and was not a host proliferation in resonse to the introduced cells. Normal NIH-3T3 cells were not tumorigenic (Table 1). Therefore, in spite of the different transforming potentials of $p77^{E2A-Pbx1}$ and $p85^{E2A-Pbx1}$ in cell culture assays, both proteins contribute directly to the oncogenic conversion of NIH-3T3 cells.

E2A–Pbx1 proteins are nuclear

The intracellular location of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ was determined by immunofluorescence. Cells expressing both $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ exhibited nuclear fluorescence that spared the nucleoli (Fig. 8 and data not shown). We estimate the average abundance of each protein in the nucleus of selected populations of NIH-3T3 to be approximately fivefold greater than that in the nuclei of 697 cells, based on Western blot analysis of equivalent amounts of cell extract (Fig. 4, lanes 697, 3, and 4) and taking into consideration the difference in protein content per cell between fibroblasts and lymphocytes. We were unable to demonstrate strict nuclear localization of E2A–Pbx1 proteins in 697 cells, because the lower levels of E2A–Pbx1 expression required higher sensitivity, and cytosolic and membrane staining, a small amount of which is evident in the normal fibroblasts in Figure 8, became prominent features under these conditions.

Normal pbx1 *transcripts are observed in most tissues in the developing and adult mouse*

Transcriptional regulation of *pbx1* was examined by analysis of the *pbx1* transcript during mouse development, in 12-, 14-, 16-, and 18-day embryos, as well as in specific adult tissues. The size of *pbx1* transcripts are heterogeneous, having two major species of 2.2 and 6.8 kb (Fig. 9). The difference in size between the 2.2- and 6.8-kb transcripts of *pbx1* was essentially the same as for the 3.2- and 8.0-kb fusion transcripts of *E2A–pbx1*, suggesting that alternate splicing in *pbx1* accounts for this difference of ~4.8 kb. Low levels of *pbx1* expression were detected throughout embryogenesis, as well as in most adult tissues, and *pbx1* transcripts in lung and brain were approximately twofold more abundant, the 6.8-kb form predominating in both tissues. Expression of *pbx1* was extremely low in spleen, consistent with our previous observation that *pbx1* is not expressed in early

Table 1. *Summary of the transforming properties of E2A–Pbx1 proteins*

Cell type	Growth in agar (colony size) 3–20	21–100	>100	Saturation density (per 5-cm plate)	Tumor formation in nude mice (mm^3)
NIH-3T3	0	0	0	1.2×10^6	0
$+p85^{E2A-Pbx1}$	782	17	7	6.0×10^6	600
$+p77^{E2A-Pbx1}$	9350	1530	80	1.44×10^7	50

Analysis was performed on nonproducer populations of NIH-3T3 expressing either $p85^{E2A-Pbx1}$ (designated $+85^{E2A-Px1}$) or $p77^{E2A-Pbx1}$ (designated $+p77^{E2A-Pbx1}$). The number of colonies formed in agar was measured from plates seeded with 200,000 cells. The size of tumors formed in nude mice was measured 6 weeks after injection of cells.

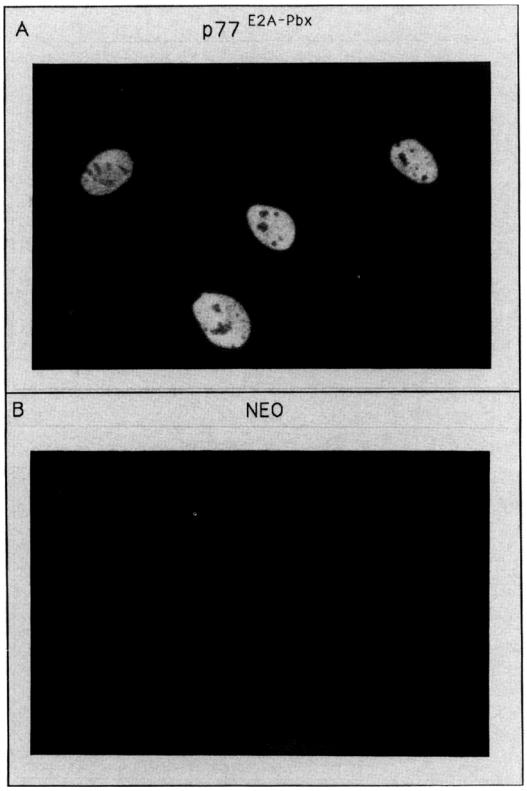

Figure 8. Nuclear localization of Pbx1 by indirect immunofluorescence using anti-Pbx1 serum. (*A*) NIH-3T3-cl2 infected by virus encoding $p77^{E2A-Pbx1}$ and selected for growth in G418. (*B*) Uninfected NIH-3T3-cl2.

or mature B or T cells (Kamps et al. 1990; Nourse et al. 1990). *pbx1* was also not expressed in adult testes. Therefore, although Pbx1 contains a homeo box, its expression pattern suggests that it is neither a tissue- nor a developmental stage-specific transcription factor. Instead, the widespread, low-abundance expression of *pbx1* indicates that the Pbx1 protein performs a more ubiquitous function.

Discussion

In this study we identify five forms of E2A–Pbx1 proteins in lymphoblasts having the t(1;19) translocation, and characterize the activities of the proteins encoded by two cloned *E2A–pbx1* cDNAs. Immunoprecipitation and Western blot analysis were used to demonstrate that each of the five forms of E2A–Pbx1 proteins is contained in both the pre-B ALL cell line, 697, and in lymphoblasts from the marrow of children having t(1;19)-positive pre-B ALL. Immunoblotting with affinity-purified antibodies to Pbx1 provided a rapid and sensitive screen for the presence of E2A–Pbx1 proteins in t(1;19)-positive lymphoblasts. Comigration in SDS–PAGE and identical patterns of partial proteolysis with *S. aureus* V8 protease suggested that two of the five E2A–Pbx1 proteins were identical to the polypeptides encoded by each of two transcripts cloned earlier (Kamps et al. 1990; Nourse et al. 1990). Partial proteolytic cleavage patterns of the other three polypeptides were related to that of $p85^{E2A-Pbx1}$, indicating that each of these proteins represents either a post-translation modification of $p85^{E2A-Pbx1}$ or a highly related protein translated from an alternatively spliced *E2A–pbx1* mRNA. Because none of the other three forms of E2A–Pbx1 proteins was observed in NIH-3T3 cells expressing either $p85^{E2A-Pbx1}$ or $p77^{E2A-Pbx1}$, we favor the hypothesis that each additional form results from alternative splicing. The suggestion that alternate splicing produces transcripts encoding these proteins is not unlikely because two different sequences have been found to initiate normal E2A proteins (S.-H. Sun, unpubl.). In addition, the existence of a second major 8.0-kb transcript in all t(1;19)-containing lymphoblasts examined to date (Mellentin et al. 1989; Kamps et al. 1990; Nourse et al. 1990) suggests that at least one additional variation within the Pbx1 portion of E2A–Pbx1 proteins is also possible.

The fact that $p77^{E2A-Pbx1}$ was more transforming than $p85^{E2A-Pbx1}$ in tissue culture assays and the observation that subtle mutations in $p85^{E2A-Pbx1}$ dramatically elevate its transforming activity suggest that a biochemical activity of $p85^{E2A-Pbx1}$ may be regulated by a normal cellular mechanism and that such regulation may require the unique 97-amino-acid carboxyl terminus of $p85^{E2A-Pbx1}$. If so, alternate splicing would represent a cellular mechanism that generates an activated E2A–Pbx1 protein, p77, and might be a method used by normal cells to regulate the activity of endogenous Pbx1.

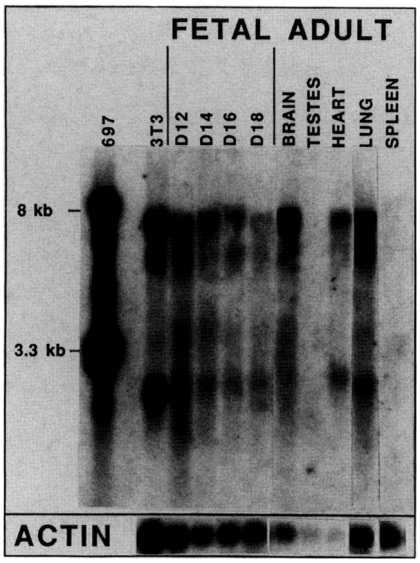

Figure 9. Expression of *pbx1* in mouse development. (*Top*) A Northern blot, using a *pbx1* cDNA probe, and samples of poly(A)-selected RNAs from the indicated stages and tissues in fetal and adult mice. RNAs from 697 cells and NIH-3T3 cells are included as controls for the size of *E2A–pbx1* fusion transcripts and normal *pbx1* transcripts. (*Bottom*) Hybridization of the same blot with a probe to actin.

Although the absolute activities of $p85^{E2A-Pbx1}$ were lower than those of $p77^{E2A-Pbx1}$, $p85^{E2A-Pbx1}$ was at least as good as $p77^{E2A-Pbx1}$ in contributing to the tumorigenic conversion of NIH-3T3, as assayed by tumor formation in nude mice. Therefore, both forms of E2A–Pbx1 proteins may well alter the growth or development of pre-B lymphocytes, coincident with acquisition of the t(1;19) translocation.

The transformation potential of E2A–Pbx1 proteins may also differ when they are expressed in fibroblasts and pre-B lymphocytes. Because normal *pbx1* is transcribed in NIH-3T3 fibroblasts but not in pre-B cells, E2A–Pbx1 protein would presumably compete for binding DNA with endogenous Pbx1 proteins in fibroblasts but would bind unchallenged in pre-B cells. As a result of such competition, more E2A–Pbx1 protein might be needed to induce penetrance of the tumorigenic phenotype in fibroblasts than in pre-B cells. The positive correlation between the abundance of E2A–Pbx1 protein and the ability of cells to grow as colonies in agar may reflect one example of behavior consistent with such a hypothesis. Our observation that larger foci are formed when NIH-3T3 cells are exposed to higher titers of virus encoding $p77^{E2A-Pbx1}$ also supports this contention. Examination of the effects of E2A–Pbx1 expression in pre-B lymphoid cells will be required to further clarify this question.

In an effort to clarify the effects of $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ in lymphocytes, we expressed each in the IL3-dependent cell lines BA/F3 and LyD-9. Neither protein affected either the absolute or relative dependence of these cell lines on IL3 for growth. Consequently, we conclude that these two E2A–Pbx1 proteins do not behave as does BCR-ABL, or activated cytosolic or transmembrane tyrosine protein kinases, whose expression obviates the growth factor dependence of these and similar IL3-dependent cells (Daley and Baltimore 1988; Kipreos and Wang 1988; Pierce et al. 1988; Sherr 1988). Experiments are now under way to determine whether $p85^{E2A-Pbx1}$ or $p77^{E2A-Pbx1}$ themselves, or in combination with growth factors or other oncoproteins, can induce lymphoid transformation.

The normal *pbx1* gene is clearly transcribed in many cell types. Major transcripts of 2.2 and 6.8 kb, as well as a variety of intermediate, less abundant forms, were expressed at approximately equal levels in many tissues. Because *pbx1* is also expressed in 12-, 14-, 16-, and 18-day embryos with little variation in abundance, we conclude that Pbx1 performs a widespread function that is either not required or detrimental to the development of B and T cells. We are now cloning the normal *pbx1* transcript to determine the size and properties of the amino-terminal domain of Pbx1 that is not present in the E2A–Pbx1 fusion protein. Immunoprecipitation analysis suggests that a normal Pbx protein may be ~42 kD, because an antigen of this size reacts with anti-Pbx1 sera in NIH-3T3 fibroblasts, but not in pre-B cells (Fig. 4). This would predict that 55 amino-terminal residues of Pbx1 are not contained in E2A–Pbx1. However, in our efforts to clone a full-length *pbx1* transcript, we have cloned a highly related cDNA, denoted *pbx2*, that is transcribed from a clearly distinct genetic locus and would most likely also react with anti-Pbx antisera. Therefore, we do not know whether the 42-kD protein is Pbx1, Pbx2, or another member of a potentially larger family of Pbx proteins. Cloning of the 5′ end of *pbx1* will permit us to address two key questions in understanding the biological activity of E2A–Pbx1 proteins: Does the E2A amino-terminal domain contribute an active or passive role in the biochemistry of E2A–Pbx1 fusion proteins, and must the normal amino-terminal sequences of Pbx1 be removed to create a transforming protein? It is possible that the role of E2A in t(1;19) pre-B ALL may be simply to induce the production of Pbx1 in these cells. Alternatively, the amino-terminal transcriptional activation domain of E2A may be indispensable for transformation-associated properties of E2A–Pbx1 proteins. It is also possible that the amino-terminal sequences of Pbx1 may repress its activity and would need to be deleted to form an oncoprotein. Although these questions can, in part, be addressed by expression of proteins in NIH-3T3 cells, an animal model involving E2A–Pbx1-mediated leukemogenesis in mice will inevitably be required to evaluate fully the functional domains essential in the induction of pre-B ALL by E2A–Pbx1 proteins.

Materials and methods

Production and purification of GST–Pbx1 fusion protein

The oligonucleotide, 5′-GTGTTTTGAGAATTCGAGGAGC-CC-3′, was used to introduce an *Eco*RI site 6 nucleotides to the 3′ side of the junction between *E2A* and *pbx1* sequences in the *E2A–pbx1* cDNA clone, pBSK:*E2A–pbx1-II*. The *pbx1* sequences, now flanked by *Eco*RI sites, were ligated into the *Eco*RI site of pGEX-2T (Amrad, Australia), a bacterial vector that expresses cloned cDNAs as glutathione S-transferase (GST) fusion proteins. The resulting GST–Pbx1 protein contains 28 kD of Pbx1 and has a predicted molecular mass of 56 kD. $p56^{GST-Pbx1}$ was isolated from the insoluble fraction of bacterial extracts for immunization (see Preparation of antisera to Pbx1, below) and by adsorption to glutathione agarose (Sigma) and eluted with free glutathione (Sigma) for the purification of soluble $p56^{GST-Pbx1}$ as specified by Amrad. A majority of $p56^{GST-Pbx1}$ was insoluble, but 5% could be isolated as soluble protein. Once purified, the DNA-binding properties of these GST–Pbx1 proteins was tested by adsorption to calf thymus DNA–agarose (Sigma), followed by elution with increasing concentrations of salt. A total of 1.3 mg of GST–Pbx1 protein was incubated with 0.4 ml of DNA–agarose (containing 1.0 mg/ml of bound DNA) in a total of 3 ml of 50 mM Tris (hydroxymethyl) aminomethane (Tris; pH 7.1), 100 mM NaCl (TN) with continuous agitation for 2 hr at 4°C. After 2 hr, the resin was allowed to settle in the column, a flowthrough fraction was collected, and the column was washed three times with 1.5 column volumes (CV) of TN. Proteins that bound the DNA were eluted by washing the resin with 1.5 CV of TN containing NaCl concentrations that were increased by 100 mM increments.

Preparation of antisera to Pbx1

Fifty milliliters of bacteria containing the pGEX-2T–*pbx1* expression plasmid were induced for 2 hr with 100 μM IPTG, as

specified by Amrad. The bacteria were pelleted by centrifugation, resuspended in 15 ml of 100 mM EDTA, 1% NP-40 (Sigma), 1% aprotinin (Sigma), and disrupted by sonication. At this point, 90% of the GST–Pbx1 fusion protein was contained in the insoluble material. Insoluble GST–Pbx1 was isolated by centrifugation at 10,000*g* for 10 min at 4°C. The yield of insoluble GEX–Pbx1 fusion protein was ~1200 μg/50 ml of initial bacterial culture. This pellet was solubilized in 2 ml of SDS containing sample buffer, and the equivalent of 600 μg of GST–Pbx1 protein was fractionated by SDS-PAGE, using a gel 2 mm thick. After completion of electrophoresis, GST–Pbx1 was visualized as a band of precipitated protein–SDS complexes after incubation in 2.0 M KCl for 30 min at 4°C. The band was excised and equilibrated in water for 30 min. One-third of this gel slice was emulsified with two volumes of complete Freund's adjuvant and one volume of water. Rabbits were injected with a total of 100 μg of GST–Pbx1 at six intradermal locations on their backs and boosted 4 weeks later using the same protocol with the substitution of incomplete for complete Freund's adjuvant. Four weeks after the first boost, the serum was tested for its ability to immunoprecipitate Pbx1-containing proteins from 697 cells. Each of two rabbits subjected to this protocol produced a titer of anti-Pbx1 antibodies by which 1 μl of serum was capable of immunoprecipitating all of the five E2A–Pbx1 proteins from 5×10^6 cells.

Biosynthetic labeling, immunoprecipitation, and partial proteolysis

Both lymphocytes and fibroblasts were labeled biosynthetically with 200 μCi of [^{35}S]methionine (Amersham) for 3 hr in 1 ml of methionine-free DMEM, containing 20% dialyzed fetal bovine serum and 50 μM 2-mercaptoethanol. After 3 hr, the cells were washed once in PBS and lysed in 400 μl of 50 mM Tris (pH 7.4), 300 mM NaCl, 0.1% NP-40, 1 mM PMSF, 5 mM EDTA, and 1 mM DTT (ELB buffer). Lysates were clarified by centrifugation at 10,000*g* for 1.5 hr. Portions of this cellular extract were subjected to immunoprecipitation according to published conditions (Sefton et al. 1978), using ELB buffer for all washes, and analyzed by SDS-PAGE using 10% acrylamide gels with low *bis*-acrylamide concentrations. Gels were processed using DMSO containing 20% PPO to enhance the signal from radioactive proteins. Partial proteolysis was performed in the gel according to the method of Cleveland et al. (1977), and peptides were resolved by SDS-PAGE using 20% acrylamide gels with low *bis*-acrylamide concentrations.

Affinity purification of antisera

A total of 0.75 mg of GST–Pbx1 proteins, purified from bacterial extracts by affinity to glutathione–agarose (see Production and purification of GST–Pbx1 fusion protein, above) was coupled to 0.4 ml of Affi-gel 10 in 100 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) (pH 7.0) buffer according to the manufacturer's protocol (Bio-Rad). The resin was washed twice at 4°C in 10 ml of PBS, twice in 10 ml of 100 mM glycine (pH 2.8), and twice in 10 ml of PBS. Rabbit anti-Pbx-1 serum (12 ml) was applied to the resin in a column four times. The resin was washed four times with 10 ml of PBS. Immunoglobulins were eluted by consecutive addition of ten 600-μl volumes of 100 mM glycine (pH 2.8), and each eluted fraction was collected into a tube containing 60 μl of 1.0 M Tris (pH 8.05), to immediately neutralize the pH. A total yield of 1.7 mg of immunoglobulin was obtained.

Western blotting protocol

Lymphocytes were solubilized by boiling in SDS-containing Laemmli sample buffer at a concentration of 2×10^7/ml. These samples (30 μl) were resolved by SDS-PAGE and transferred to nitrocellulose in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (pH 10.8), 20% methanol, at 0.5 amp for 45 min at 4°C. A Western blotting protocol (Promega) was followed, using 10 μg/ml anti-Pbx1 immunoglobulin to bind Pbx1-containing proteins and alkaline phosphatase-conjugated, goat anti-rabbit immunoglobulin to detect the location of rabbit antibodies bound to Pbx1.

Construction of expression vectors

The retroviral expression vector pGD, the parental vector of pGD210 (Daley et al. 1990), was obtained from Marty Scott, who had converted a non-unique *Xho*I site into a unique *Bcl*I site to facilitate the cloning of various cDNAs. The cDNAs of both type I and type II *E2A–pbx1*, encompassing sequences from the 5′ end described previously (Kamps et al. 1990) to the unique *Dra*I site located in both 3′-noncoding regions (residue 2851 in the type I transcript; Kamps et al. 1990), were ligated into the *Bcl*I site, after addition of *Bcl*I linkers to the inserts. Each plasmid was transfected into NIH-3T3 cells using the calcium phosphate technique (Shih et al. 1979).

Generation of helper-free virus

The ecotropic packaging cell line, ψ-CRE, was transfected with the retroviral constructs described above. Transfected cells, selected for construct expression by growth in G418 for 2 weeks, were cloned by growth in 96-well plates from single cells and screened for expression of virus by RNA dot blot analysis. Clones expressing the most virus were expanded, and the viral titer of supernatants analyzed. The titer of ψ24L, a clone expressing virus encoding $p85^{E2A-Pbx1}$, was 5×10^5 G418-resistance units per milliliter, whereas that of ψ81S, a clone expressing virus encoding $p85^{E2A-Pbx1}$, was 5×10^3.

Cells, viral infections, and soft agar assays

NIH-3T3 cells were obtained from Paul Kaplan. Both normal and infected NIH-3T3 cells were grown in Dulbecco–Vogt modified Eagle medium (DMEM) containing 5% calf serum. Viral infections were performed for 2 hr in 2 ml of the same medium containing 8 μg/ml Polybrene, then diluted to 10 ml and incubated overnight. The medium was changed the following morning. Selection for cells expressing the neomycin resistance gene (*neo*) was performed in DMEM containing 5% calf serum and 500 μg/ml of G418 (Geneticin, GIBCO). Incubation with selective medium was initiated 2 days after infection. Soft agar assays were performed using 200,000 cells resuspended in DMEM containing 5% calf serum, and 0.2% Noble agar, and with a base layer of 0.6% Noble agar. Colonies were scored after 20 days. 697 cells were grown in RPMI medium supplemented with 20% fetal bovine serum, 2 mM glutamine, and 50 μM 2-mercaptoethanol. Primary pre-B ALL cells were obtained from bone marrow aspirates of patients having t(1;19)-positive leukemias, based on cytogenetic analysis conducted by Dr. Susana Raimondi. Leukemic cells were isolated by Ficoll–Hypaque density gradient centrifugation and viably cryopreserved in medium containing 10% dimethylsulfoxide. Each patient or patient's

guardian was advised of the procedures and risks, in accordance with institutional guidelines, and gave informed consent.

Immunofluorescence

NIH-3T3 cells were grown on coverslips coated with fibronectin (Sigma) as follows. Fibronectin (1.0 mg) was dissolved gently without vortexing in a volume of 1 ml of water at 37°C for 30 min. Twenty milliliters of this solution was diluted to 1 ml in DMEM. Aliquots (100 μl) of the diluted fibronectin were added directly to untreated coverslips, and incubated at 37°C for 30 min in a tissue-culture incubator. Coverslips were rinsed with PBS twice and used immediately for growth of cells. Cells, grown on the fibronectin-treated coverslips, were prepared for immunofluorescence by fixation in 3% paraformaldehyde (pH 7.4) in PBS and permeabilized with 1.0% NP-40. E2A–Pbx1 proteins were identified by incubation using a 1 : 300 dilution of anti-Pbx1 serum followed by incubation with affinity-purified, rhodamine-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch Laboratories) and visualized by fluorescence microscope as described (Van Etten et al. 1989).

Acknowledgments

We thank Cornelis Murre and Sybille Mittnacht for their generous gifts of antisera to E2A and Rb, respectively. We also thank Susana Raimondi for expert cytogenetic analysis of leukemic lymphoblasts. M.P.K. is supported by a Damon Runyon-Walter Winchell Cancer Research Fund Fellowship, DRG-982. This work was supported by National Institutes of Health grant GM39458 and by grants CA-42804, CA-20180, and CA-21765 and the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital.

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