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The role of E2A-PBX1 in leukemogenesis

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Introduction

A significant number of the malignancies of the immune system are the result of nonrandom chromosomal translocations that cause either the overexpression of endogenous genes or the production of novel chimeric factors that can promote uncontrolled cell growth and block differentiation. Translocations which fuse the gene encoding the basic helix–loop–helix transcription factor E2A with either the gene encoding the homeodomain protein PBX1 or the bZIP protein HLF result in the generation of chimeric proteins that can cause pre- and pro-B cell acute lymphoblastic leukemias (ALL), respectively. What is currently known about the E2A-HLF fusion protein is reviewed elsewhere in this issue. This review focuses on the mechanisms and models of E2A-PBX1-mediated pre-B cell transformation.

E2A is a regulator of lymphocyte differentiation

The E2A genetic locus encodes two gene products, E12 and E47, which are produced by differential splicing events (Murre *et al*., 1989b). Both proteins belong to the class I family of basic helix–loop–helix (bHLH) proteins, and contain two characterized activation domains, termed the AD1 and AD2 (or loop–helix domain) (Aronheim *et al*., 1993; Massari *et al*., 1996; Quong *et al*., 1993). These proteins are widely expressed and can heterodimerize with a variety of tissue-specific bHLH proteins including myogenic and neurogenic factors (Lassar *et al*., 1991; Lee *et al*., 1995; Naya *et al*., 1997). In B lymphocyte populations, E2A functions predominantly as homodimeric complexes (Bain *et al*., 1993; Shen and Kadesch, 1995). E12 and E47 were initially characterized by their ability to bind to E box motifs identified in the immunoglobulin (Ig) promoter and enhancer elements and, as such, were considered likely candidates to be regulators of Ig locus activation and B cell development (Murre *et al*., 1989a; Sen and Baltimore, 1986). A definitive role for the E2A gene products in directing B cell lineage commitment was demonstrated by targeted disruption of the locus in mice (Bain *et al*., 1994; Zhuang *et al*., 1994). In these E2A-deficient animals, B cell development was arrested at the early pro-B cell stage, prior to the onset of Ig heavy chain rearrangement. These studies clearly demonstrated a requirement for E2A function at the earliest detectable stages of B cell commitment.

In addition to this early requirement for E2A in B lineage commitment, mounting evidence indicates that E2A plays multiple roles at later stages of B cell development and maturation (reviewed in Kee *et al*., 2000). The importance of E2A as a master regulator of B lymphopoiesis was demonstrated by its ability to upregulate B cell-specific gene transcription in non-lymphoid cells. For example, ectopic expression of E2A in fibroblast cells activates the transcription of B cell-specific Ig heavy chain transcripts (Choi *et al*., 1996). Expression of E12 in a macrophage cell line also directs the activation of a variety of B lineage genes, including the transcription factor EBF-1, the surrogate light chain $\lambda 5$ and Rag-1 (Kee and Murre, 1998). In addition to activating the expression of B cell-specific genes, E2A also plays a direct role in Ig gene rearrangement, which constitutes a critical step in B cell development (Bain *et al*., 1999; Romanow *et al*., 2000; Schlissel *et al*., 1991). At later stages of B cell maturation, E2A functions to regulate Ig class switch recombination in peripheral mature B cells (Goldfarb *et al*., 1996; Quong *et al*., 1999). E2A is also an important regulator of T cell development, and E2A-deficient mice showed defects in multiple stages of thymocyte selection and differentiation (Bain *et al*., 1997; Barndt *et al*., 2000; Rivera *et al*., 2000). The wide variety of roles that E2A plays in directing lymphocyte differentiation and activation likely contribute to its ability to transform cells when expressed as part of the oncoprotein E2A-PBX1.

PBX1 is a homeodomain-containing HOX cofactor

The chromosomal translocation t(1;19) (q23;p13.3) is detected in approximately 23% of all pediatric pre-B cell ALL cases (Carroll *et al*., 1984; Williams *et al*., 1984). This translocation is not observed in other forms of leukemia and typically correlates with poor patient responses to standard chemotherapeutic re-

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gimes (Crist *et al*., 1990). The fusion event results in the expression of a protein which combines the transactivation domains of E2A with the DNA binding homeodomain region of another protein, initially called PRL (Kamps *et al*., 1990; Nourse *et al*., 1990) but later renamed PBX1 (*P*re-*B* cell leukemic homeobo*X1*) (Hunger *et al*., 1991; Kamps *et al*., 1991). Two forms of E2A-Pbx1 are detected in human primary tumors cells; E2A-Pbx1a and the shorter E2A-Pbx1b (Kamps *et al*., 1991). Both of these alternatively spliced forms contain the PBX1 homeodomain, but differ at the C-terminus, and both E2A-PBX1a and E2A-PBX1b are capable of cellular transformation. Whereas E2A has many roles during the normal development of B cells, Pbx1 is not expressed in the lymphoid compartment. However, two closely related genes, Pbx2 and Pbx3, are transcribed in lymphocytes (Monica *et al*., 1991). Insights into the function of Pbx1 were revealed by genetic studies of its *Drosophila* homolog *extradenticle (exd)* (Flegel *et al*., 1993; Rauskolb *et al*., 1993).

The *exd* mutation was identified during a screen for mutations in genes that regulate early events in embryonic patterning which produce changes in the pattern of hairs (known as denticles) on the surface of *Drosophila* larvae. In the case of *exd*, the larvae were covered in additional denticles (Jürgens *et al*., 1984). The phenotypes characterized in *exd* mutants resemble those caused by alterations in the expression of *Hox* genes, even though in the absence of *exd, Hox* genes are expressed normally. EXD is a cofactor for the HOX homeodomain proteins, and is required for HOX activity (Peifer and Wieschaus, 1990). When *exd* was cloned, sequence similarity identified it as a highly conserved homolog of Pbx1 (Flegel *et al*., 1993; Rauskolb *et al*., 1993).

Following this genetic analysis of *exd*, the *Drosophila* HOX proteins UBX and ABD-A were shown by gel shift analyses to co-operatively bind DNA with EXD (van Dijk and Murre, 1994). Vertebrate HOX proteins demonstrated similar cooperativity with both PBX1 and E2A-PBX1 (Lu *et al*., 1995; van Dijk *et al*., 1995). A tryptophan containing pentapeptide motif in HOX proteins from paralogous groups 1–10, lying N-terminal to the HOX homeodomain, contacts PBX1 (Chang *et al*., 1995; Knoepfler and Kamps, 1995; Neuteboom *et al*., 1995; Phelan *et al*., 1995). The portions of PBX1 required for interactions with HOX proteins are the homeodomain and a region C-terminal to the homeodomain termed the HOX Co-operativity Motif (HCM), both of which are retained in E2A-PBX1 (Chang *et al*., 1997a; Peltenburg and Murre, 1997). PBX1 also interacts with a range of other homeodomain containing transcription factors including members of the ENGRAILED family (Peltenburg and Murre, 1996).

The chromosomal translocation linking E2A and Pbx1 has two primary effects. First, it disrupts one allele of both E2A and Pbx1. Second, it generates the novel fusion gene E2A-Pbx1 expressed under the control of E2A regulatory sequences.

The effect of E2A heterozygosity on tumor development

Given the importance of E2A in B lymphopoiesis, there is evidence that the oncogenic potential of the t(1;19) translocation may be due, in part, to the reduction of the levels of wild-type E2A (Figure 1). Perturbation of E2A levels could affect several aspects of tumor formation. Clearly, E2A plays an important role as a mediator of B cell differentiation, regulating the expression of many factors important for lymphoid development (Kee and Murre, 1998; Sigvardsson *et al*., 1997). Reduced levels of E2A activity may disrupt the maturation of lymphoid lineages and expand undifferentiated progenitor populations. Functional heterozygosity at the E2A locus in mice does in fact reduce the steady state populations of developing lymphocytes in both B and T cell lineages, indicating that E2A levels are critical for proper lymphocyte differentiation (Bain *et al*., 1994; Zhuang *et al*., 1994).

In addition to its role as a transcriptional activator of lymphocyte-specific genes, there is growing evidence that E2A can function as a tumor suppressor. Experiments in NIH3T3 cells demonstrate that over-expression of E47 prevents foci development in soft agar assays and that this growth suppression maps to the N-terminus of the polypeptide (Peverali *et al*., 1994). More recent data indicates that E2A activates transcription of the p21 CIP/WAF cyclin dependent-kinase inhibitor and is a target of inactivation by cyclinG1-dependent kinases, suggesting a direct link between E2A activity and cell cycle regulation (Chu and Kohtz, 2000; Prabhu *et al*., 1997). The most convincing evidence that E2A functions as a negative regulator of tumorigenic cell growth *in vivo* is the development of thymomas in E2A-deficient mice (Bain *et al*., 1997; Yan *et al*., 1997). These lymphomas were observed in nearly all E2A null mice, typically arising by 75 days after birth. Cell lines derived from these tumors rapidly undergo cell death following the ectopic expression of E12 or E47 (Engel and Murre, 1999; Park *et al*., 1999). As such, the chromosomal translocation t(1;19) may act to decrease the amount of functional E2A available to act as a tumor suppressor.

The E2A-PBX1 fusion protein: novel biochemical properties and functional activities

The E2A-PBX1 fusion protein is capable of cellular transformation in a wide variety of *in vitro* and *in vivo* assays. The ability of this chimeric protein to mediate oncogenesis is due to its unique biochemical properties and altered association with regulatory protein complexes relative to either wild-type E2A or PBX1. These novel functional properties of E2A-PBX1 and their potential contribution to tumor formation are described below.

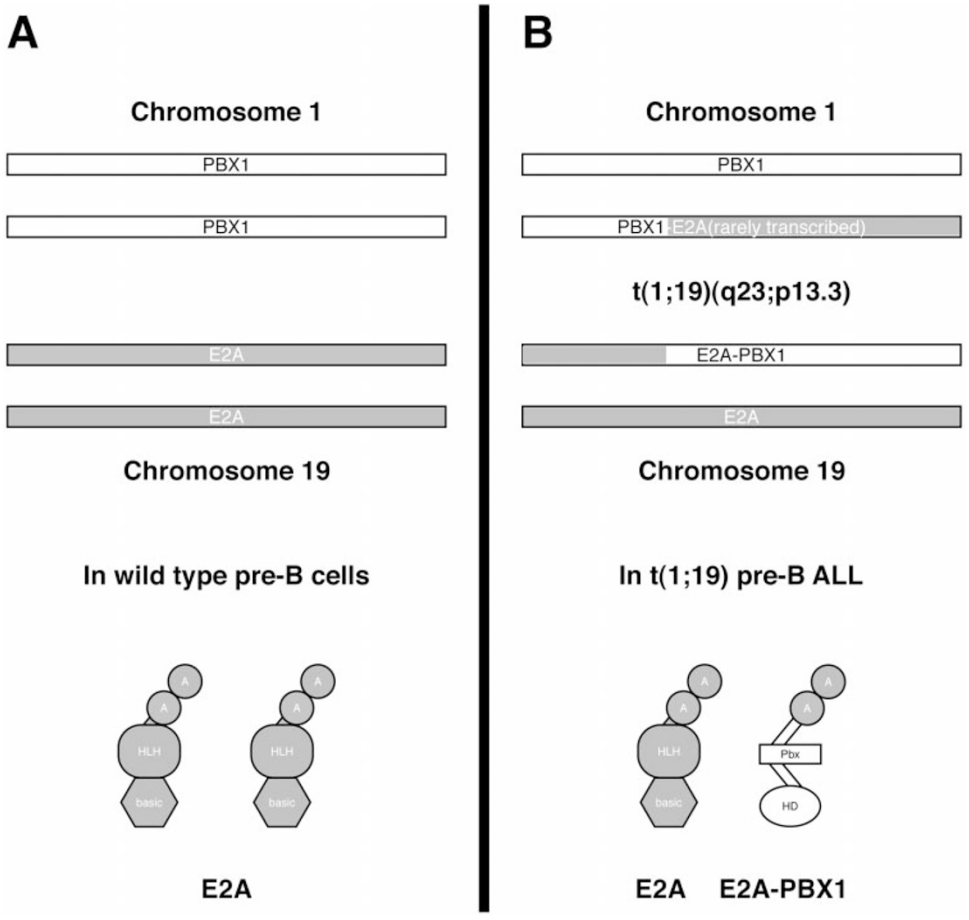

Figure 1 The t(1;19) chromosomal translocation event generates the E2A-Pbx1 fusion gene and deletes a functional allele of E2A. (**a**) Wild type cells contain two copies of the Pbx genetic locus (located on chromosome 1) and two functional copies of the E2A gene (located on chromosome 19). In wild type pre-B cells only E2A, which contains a basic helix-loop-helix domain (bHLH) and two activation domains (**a**) is expressed. (**b**) The t(1;19) translocation event fuses one copy of the E2A gene with one copy of Pbx1, creating E2A-Pbx1. The E2A-PBX1 protein contains the activation domains of E2A with the dimerization (Pbx) and the homeodomain (HD) regions of PBX. The reciprocal translocation event encodes for the PBX1-E2A chimeric protein, which is rarely transcribed in tumor cells (Van Dongen *et al*., 1999). Pre-B cells containing the translocation express both E2A and E2A-PBX1

E2A-PBX1 can activate transcription through PBX1 binding sites

Unlike PBX1, E2A-PBX1 is a transcriptional activator (LeBrun and Cleary, 1994; Lu *et al*., 1994; van Dijk *et al*., 1993). Evidence from reporter assays indicates that full-length E2A-PBX1 strongly increases expression from artificial promoters containing PBX1 binding sites, whereas PBX1 alone has no effect (LeBrun and Cleary, 1994; Lu *et al*., 1994; van Dijk *et al*., 1993). The importance of E2A activation domains for the transforming activity of E2A-PBX1 has been demonstrated in studies comparing the ability of deletion mutants of E2A-PBX1 to transform both lymphoid and fibroblast cell lines with their capacity to activate expression of the reporter activated by full-length E2A-PBX1 (Monica *et al*., 1994). Deletion of the entire E2A region of E2A-PBX1 completely abrogates both focus formation in NIH3T3 cells and the ability of E2A-PBX1 to activate transcription. Interestingly, the two characterized activation domains of E2A may play different roles in these two processes when fused to PBX1. Deletion of the AD1 domain inhibits transactivation in both lymphoid and fibroblast lines, while deletion of the loop–helix (AD2) domain primarily blocks transcriptional activation of a reporter gene in fibroblasts (Monica *et al*., 1994).

E2A-PBX1 activates expression of HOX/PBX1 target genes

As expected, in addition to E2A activation domains the homeodomain from PBX1 is required for E2A-PBX1 to activate the expression of a reporter gene. This ability is enhanced by inclusion of the flanking HCM (Kamps *et al*., 1996). However the HCM is insufficient on its own and regions of PBX1 required for homodimerization are completely expendable (Calvo *et al*., 1999). In order to transform myeloid cells in culture the same portions of PBX1 are required.

As described above, E2A-PBX1 is a strong activator of transcription. In contrast, PBX1 does not activate transcription on its own and has been shown to inhibit the activity of HOX proteins, as well as other transcription factors (Lu and Kamps, 1996). The replacement of PBX1 with E2A-PBX1 in DNA-binding complexes containing HOX proteins alters the regulation of HOX/PBX1 target genes, likely inducing ectopic activation of HOX/PBX target genes (Lu and Kamps, 1997) (Figure 2). Although the myeloid transformation experiments on their own provide evidence that this activation of HOX/PBX1 targets is oncogenic, further evidence comes from the fact that many HOX genes are themselves oncogenic when over-expressed or if they become part of chimeras containing activation domains. For example, mice reconstituted with bone marrow cells engineered to over-express HoxA10 all developed acute myeloid leukemia, which were transplantable to second recipients (Thorsteinsdottir *et al*., 1997). Furthermore, the t(7;11)(p15;p15) chromosomal translocation creates a chimeric protein linking the DNA binding domains of HOXA9 to the N-terminal part of the nuclear pore protein NUP98. In this context the N-terminal part of NUP98 acts as a strong transactivator. NUP98-HOXA9 is a probable cause of acute myeloid leukemia (Borrow *et al*., 1996; Nakamura *et al*., 1996). As will be discussed in the section on mouse models, E2A-PBX1 can co-operate with HOXA9 to cause acute myeloid leukemia (Thorsteinsdottir *et al*., 1999). The role of HOX proteins as leukemic oncogenes are reviewed in detail in (Shimamoto *et al*., 1998) and (Buske and Humphries, 2000).

E2A-PBX1 binds to a subset of the sites bound by PBX1

The optimal *in vitro* binding site for PBX1 and E2A-PBX1 proteins, 5′-TGATTGAT(T)-3′ was derived employing different strategies (LeBrun and Cleary, 1994; Lu *et al*., 1994; van Dijk *et al*., 1993). The first studies examining the cooperative binding of HOX proteins and either PBX1 or E2A-PBX1 used the PBX1 site linked to a consensus HOX binding site (5′-TAAT-3′) (Lu *et al*., 1995; van Dijk *et al*., 1995). The 'HOX' site was dispensable in this study as HOX and PBX1 or E2A-PBX1 proteins can form

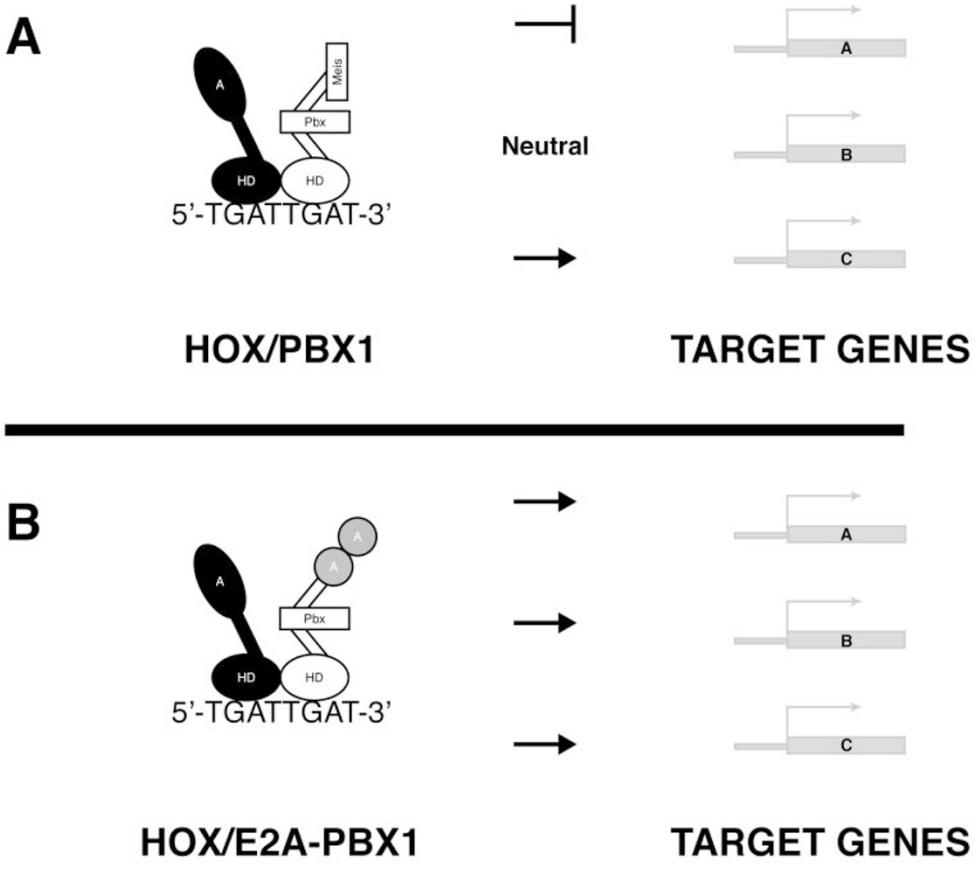

Figure 2 Association of E2A-PBX1 with HOX proteins can alter the regulation of HOX-PBX target genes. (**a**) The co-operative DNA binding of HOX proteins with members of the PBX family represses the transcriptional activation of a subset of HOX target genes, up-regulates the expression of other HOX target genes, and has no effect on the regulation of a third set of target genes. (**b**). The E2A-PBX1 oncoprotein contains two E2A activation domains and lacks transcriptional repressor domains present in wild type PBX1. As such, association of HOX proteins with E2A-PBX1 likely activates the transcription of many HOX/PBX1 target genes

heterodimers on the PBX1 homodimer site which is very similar to the HOX/PBX1 consensus site (Chang *et al*., 1996). Reporter studies examining the transcriptional activity of E2A-PBX1 use a PBX1 homodimer binding site to drive expression (LeBrun and Cleary, 1994; Lu *et al*., 1994; van Dijk *et al*., 1993).

There are three different PBX proteins that are all structurally very similar to one another. Even though Pbx1 is not expressed in lymphoid cells, Pbx2 and Pbx3 are (Monica *et al*., 1991). In cell lines transformed with E2A-PBX1, these other PBX proteins form abundant complexes that exclude E2A-PBX1. These complexes bind to the sequence 5′-TGATTGAC-3′ which differs to that preferred by E2A-PBX1 alone or complexed with a HOX partner protein (Knoepfler and Kamps, 1997). This is in fact the sequence to which PBX proteins bind with another family of homeodomain proteins, the MEIS/PREP1/pKNOX1 family. PBX1 binds to these proteins using an N-terminal motif that is deleted in E2A-PBX1 (Chang *et al*., 1997b; Knoepfler *et al*., 1997; Knoepfler and Kamps, 1997; Lu and Kamps, 1997). These data indicate that E2A-PBX1 only binds to a subset of the targets of endogenous PBX and may sequester a fraction of the normal partners of PBX proteins (Figure 3).

Deregulated association of E2A activation domains promotes uncontrolled cell division

A series of experiments which used deletion mutants of the PBX1 portion of E2A-PBX1 revealed that transformation of NIH3T3 cells requires at least part of PBX1, as the truncated portion of E2A alone had no effect (Monica *et al*., 1994). These experiments demonstrated that either the PBX1 homeodomain or everything but the homeodomain is sufficient for foci formation. This was a surprising result given the original model for transformation by E2A-PBX1. This model postulated that the PBX1 homeodomain was required to localize E2A activation domains to DNA binding sites, where they would deregulate the expression of target genes of wild type PBX proteins (Monica *et al*., 1994). In fact, DNA binding mutants of E2A-PBX1 induce foci formation in fibroblasts more efficiently than full-length E2A-PBX1 (Kamps *et al*., 1996). Later analyses showed that the HCM, a small region C-terminal to the PBX1 homeodomain, known to interact with HOX, was sufficient for transformation of fibroblasts. *In vitro* this portion of PBX1 does not tether the resultant chimeric protein to DNA (Chang *et al*., 1997a). In addition, the GAL4 or GCN4 DNA-binding/dimerization domains when linked to E2A can cause foci formation in fibroblasts (Bayly and LeBrun, 2000). Taken to the

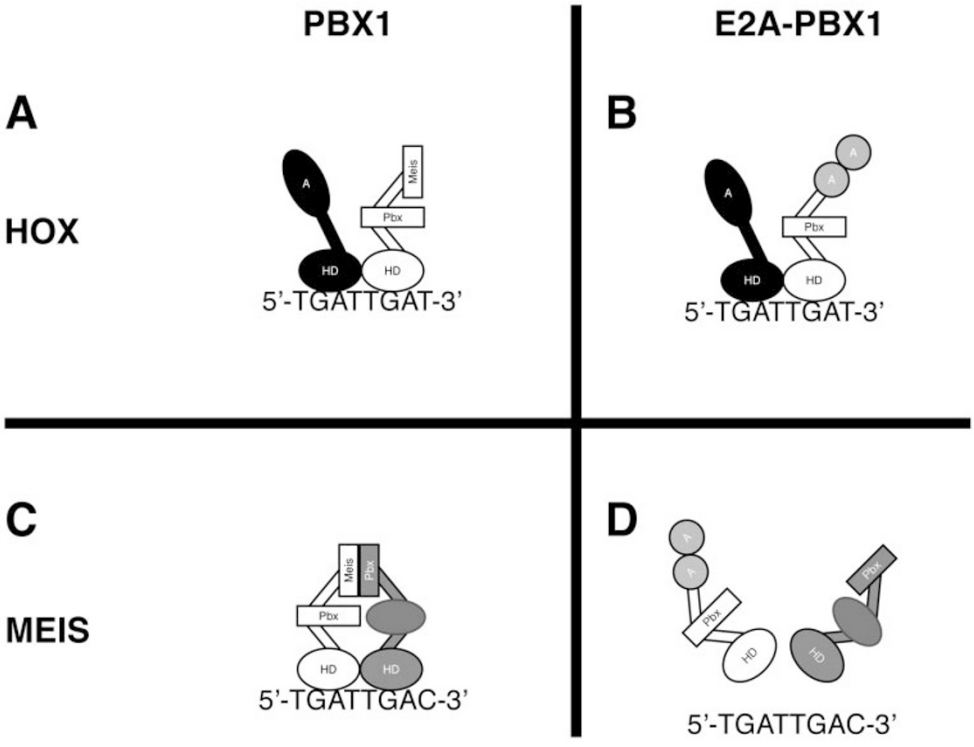

Figure 3 E2A-PBX1 dimerizes with HOX proteins but does not form complexes with the MEIS/PREP family of homeodomain proteins. (**a**) PBX1 binds the DNA sequence 5′-TGATTGAT-3′ cooperatively with HOX proteins. (**b**) E2A-PBX1 retains the ability to bind DNA complexed with HOX proteins (**c**) PBX proteins dimerize with members of the MEIS/PREP family of homeodomain transcription factors using a motif located at the N-terminus of PBX. PBX/MEIS complexes preferably bind to 5′-TGATTGAC-3′, a DNA sequence that differs from that bound by PBX/HOX dimers. (**d**). E2A-PBX1 lacks the MEIS/PREP interacting domain and as such is unable to associate with these proteins

very extreme, dimerization of a peptide containing tandem FKBP domains linked to the truncated part of E2A is sufficient for NIH3T3 transformation (Bayly and LeBrun, 2000). This indicates that an E2A-PBX1 homodimer may act as a dominant negative molecule which sequesters potential E2A co-activators, such as p300/CBP and the SAGA complex (Massari *et al*., 1999; Qiu *et al*., 1998) (Figure 4).

The nuclear-cytoplasmic localization of PBX1 is regulated, but E2A-PBX1 is constitutively nuclear

EXD encoded by the *Drosophila* homolog of Pbx1 (Flegel *et al*., 1993; Rauskolb *et al*., 1993) moves between the nucleus and cytoplasm in a highly regulated fashion (Aspland and White, 1997; Mann and Abu-Shaar, 1996). This is controlled by its interaction with another homeodomain protein, HOMOTHORAX (HTH), whose function can be mimicked in *Drosophila* using mouse MEIS1a which has large areas of homology with HTH (Kurant *et al*., 1998; Pai *et al*., 1998; Rieckhof *et al*., 1997). The intracellular localization of PBX1 is regulated in the same way by MEIS1a during limb development in mice (González-Crespo *et al*., 1998). All proteins of the PBX family (including EXD) contain both a nuclear localization and a nuclear exclusion signal. The nuclear exclusion signal is dominant in unbound protein, which

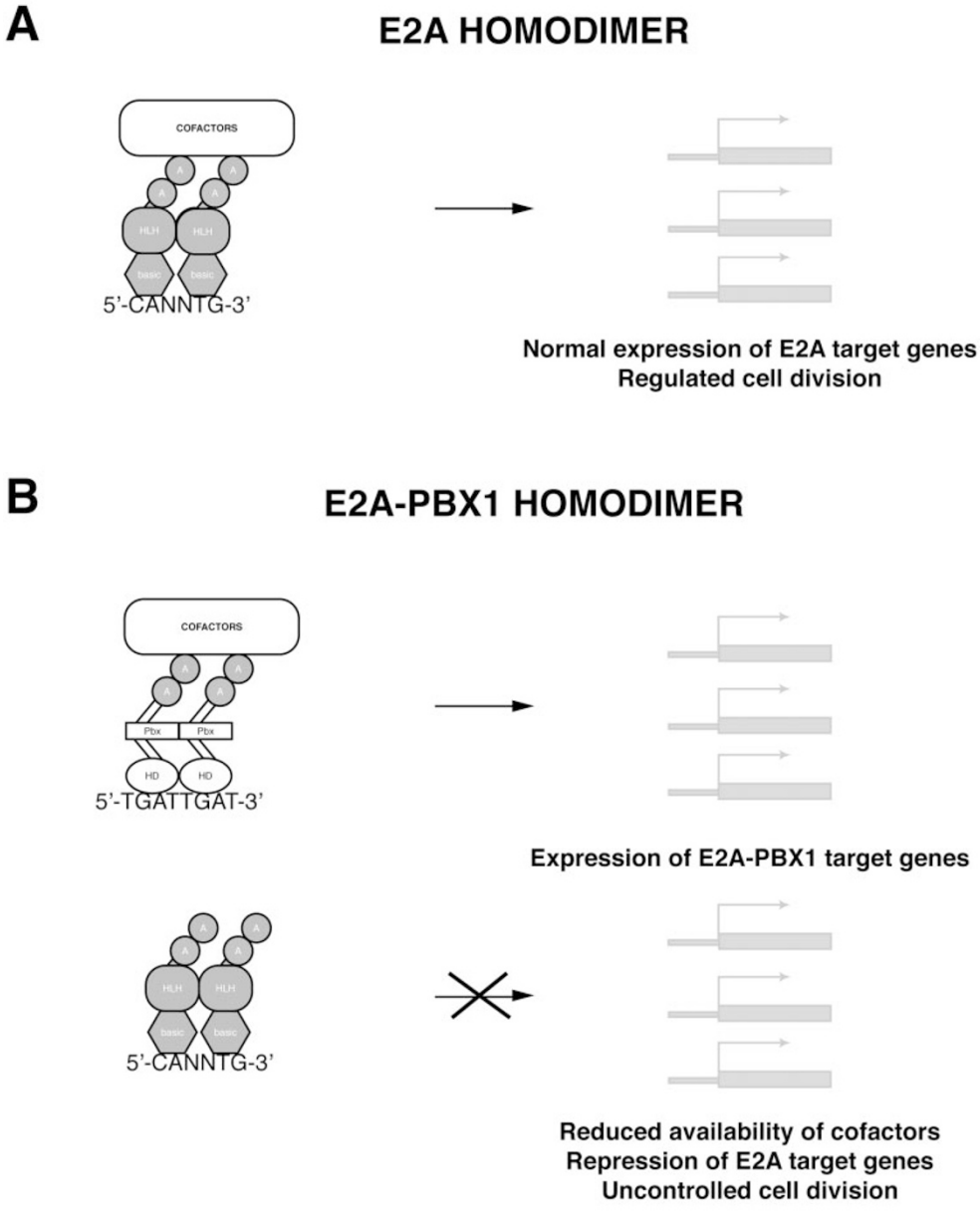

Figure 4 E2A-PBX1 may deregulate cell division by sequestering coactivators of E2A. (**a**) Appropriate expression of E2A target genes promotes regulated cell growth. (**b**) E2A-PBX1 dimerization may sequester cofactors required by endogenous E2A to regulate the cell cycle

is retained in the cytoplasm where it has no known function. When PBX proteins bind to MEIS/PREP proteins they undergo a conformational change, the nuclear localization signal becomes dominant and the complex moves to the nucleus (Abu-Shaar *et al*., 1999; Berthelsen *et al*., 1999). As mentioned earlier, the N-terminal domain in PBX1 required for this interaction is deleted in E2A-PBX1. In transfected COS cells, ectopically expressed PBX1 protein is diffuse throughout the cell, but E2A-PBX1 is localized exclusively to the nucleus (LeBrun *et al*., 1997). As such, while the availability of a PBX partner for HOX proteins may be regulated by shuttling PBX proteins in and out of the nucleus, the constitutively nuclear E2A-PBX1 is always available for dimerization with HOX proteins (Figure 5).

Mouse models for human E2A-PBX1 pre-B ALL

In vitro experiments have proven that E2A-PBX1 is capable of transforming many cell types. However attempts to assess the oncogenic potential of E2A-PBX1 in primary murine B lineage cells have yielded diverse results. Transgenic mice in which either E2A-Pbx1a or E2A-Pbx1b expression was driven by an Ig heavy chain promoter/enhancer cassette died from T cell-derived lymphoid tumors by 5 months of age (Dedera *et al*., 1993). In reconstitution experiments where mouse bone marrow cells retrovirally transduced to express E2A-Pbx1 were transferred to lethally irradiated recipient mice, the recipients developed acute myeloid leukemias by 5 months post-transfer (Kamps and Baltimore, 1993). Tumor cells derived from these mice readily grew as Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) or Interleukin-3 (IL-3) dependent cell lines. Co-expression of HoxA9 with E2A-Pbx1 in a similar experimental system induced aggressive myeloid tumor formation by 39 days post-transfer (Thorsteinsdottir *et al*., 1999). In addition, dual expression of E2A-Pbx1 and HoxA9 promoted growth factor-independent proliferation in culture, reconfirming the importance of E2A-PBX1-HOX interactions for tumor formation *in vivo* (Thorsteinsdottir *et al*., 1999).

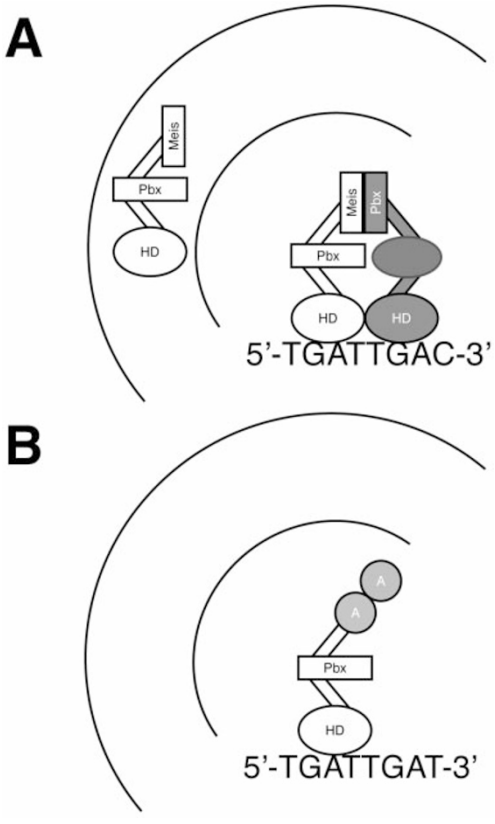

Figure 5 Cellular localization of E2A-PBX1 differs from that of PBX1. (**a**) Unbound PBX1 is cytoplasmic. Nuclear localization of this protein requires binding to MEIS/PREP family proteins. (**b**). E2A-PBX1 does not dimerize with MEIS/PREP proteins. The constitutively nuclear localization of this protein is due to the retention of nuclear localization sequences from the E2A fusion partner

Taken together, these results from tumor models in mice illustrate the oncogenic potential of E2A-PBX1 in multiple hematopoietic lineages. However, the lack of B cell transformation in these experimental systems has been difficult to reconcile. Technical difficulties may exist in targeting the expression of E2A-Pbx1 to the appropriate B cell precursor population. Indeed, expression of the E2A-Pbx1 transgenes under the control of Ig heavy chain transcriptional regulatory sequences was preferentially directed to thymocytes despite the use of these B cell specific elements (Dedera *et al*., 1993). Retroviral transduction of murine bone marrow introduces expression of E2A-Pbx1 to multiple hematopoietic lineages, even when cells are cultured under conditions which favor B cell differentiation (Kamps and Baltimore, 1993; Thorsteinsdottir *et al*., 1999). It is possible that a myeloid progenitor population transformed by E2A-Pbx1 in the radiation chimera experiments is more rapidly transformed than B lineage cells, and as such, the mice succumb to myeloid tumors prior to the onset of B cell transformation.

In addition to potential technical explanations for the lack of precursor B cell transformation in mice, observations from E2A-Pbx1 transgenic mice and B cell lines may provide a more salient explanation. These transgenic animals exhibited a 60–80% reduction in the numbers of B lineage cells in the bone marrow (Dedera *et al*., 1993). Reductions in developing lymphocytes in these mice indicated a cytotoxic effect of E2A-PBX1 expression in primary B (and T) lineage cells. Importantly, it has been demonstrated that E2A-PBX1 does indeed activate apoptosis in primary and transformed pre-B cells and that this cell death can be abrogated by expression of the anti-apoptotic gene Bcl-2 (Smith *et al*., 1997). Unlike Myc and E1A, E2A-PBX1-mediated programmed cell death is p53-independent (Smith *et al*., 1997).

Lastly, all of the previously described transgenic studies examining E2A-PBX1 function have used

full-length protein expression in various compartments. A mutant form of E2A-Pbx1 lacking the homeobox has also been studied using transgenic mice (Monica *et al*., 1994). Expression of this truncated protein in the T cell compartment reduced the number of thymocytes, and ultimately, resulted in the development of T cell lymphomas to a comparable extent as that observed for full-length E2A-PBX1 (Monica *et al*., 1994). This result demonstrates that a mutant form of E2A-PBX1 that does not bind DNA with HOX proteins was able to transform T cells in transgenic models with a similar efficiency as compared to full-length E2A-PBX1. Thus, these data indicate that such transgenic mouse models may primarily address the dominant negative aspect of E2A-PBX1 activity. Perhaps other components of E2A-PBX1-mediated transformation, such as the ectopic expression of HOX/PBX targets, can be addressed in mouse models utilizing a potent transactivator other than the AD domains of E2A, such as the VP16 transactivation domain. Experiments of this type will help to dissect the relative contributions of the various functions of E2A-PBX1 in primary cell transformation.

E2A-PBX1 pre-B cell ALL may require secondary mutations

The long incubation time and monoclonal nature of tumors observed in E2A-Pbx1 transgenic mice indicate that secondary genetic events may be required for leukemic transformation (Dedera *et al*., 1993). A screen to identify other factors that might contribute to transformation induced by E2A-PBX1 was recently developed. E2A-Pbx1 transgenic mice develop T cell lymphomas in approximately half a year. E2A-Pbx1 transgenic neonates were infected with M-MulV, a virus that integrates into the genome, up-regulating and potentially truncating genes in the vicinity of the viral integration site (Feldman *et al*., 1997). Infected mice which developed tumors more rapidly than uninfected E2A-Pbx1 transgenics were examined further, as the integration of virus may have altered the expression of genes enhancing the activity of E2A-PBX1. Alterations in known oncogenes in infected mice that died prematurely were examined by Southern blot analysis revealing Pim1 as one such gene. A similar study in which the DNA flanking viral integration sites was sequenced identified Notch1 as another gene which enhanced E2A-PBX1-mediated tumorigenesis (Feldman *et al*., 2000). Viral integration caused the expression of two truncated forms of Notch1; a constitutively active intracellular version and another lacking C-terminal residues which control regulated NOTCH1 degradation called NOTCH$^{\Delta C}$. Transgenic mice expressing either Pim1 or Notch$^{\Delta C}$ alone failed to induce T cell lymphomas over the course of the experiment. However, mice engineered to express E2A-Pbx1 and either of these genes develop tumors much more quickly than those expressing E2A-Pbx1 alone. These data demonstrate that Pim1 and Notch$^{\Delta C}$ synergize with E2A-Pbx1 during tumorigenesis; however, expression of neither gene has been observed in t(1;19) pre-B ALL (Feldman *et al*., 1997, 2000).

Downstream target genes

In order to gain a detailed understanding of how E2A-PBX1 causes pre-B cell ALL, representational difference analysis (RDA) was used to identify genes whose expression is activated by E2A-PBX1 (de Lau *et al*., 1998; Fu and Kamps, 1997; McWhirter *et al*., 1999). RDA analyses performed on NIH3T3 mouse fibroblasts expressing E2A-Pbx1 compared to others containing a control vector identified a number of genes specifically activated by E2A-PBX1 in fibroblasts, but not in either mouse myeloid primary cells transformed by E2A-PBX1 or human cell lines derived from patients with the t(1;19) chromosomal translocation. (Fu and Kamps, 1997; Fu *et al*., 1999b; McWhirter *et al*., 1997). As such, this set of target genes are most likely not involved in the development of pre-B cell ALL.

In a second differential expression analysis, the pre-B cell line Reh, either stably or transiently transfected with E2A-Pbx1, was compared to control cells transfected with the empty vector (de Lau *et al*., 1998). Granulocyte-Colony Stimulating Factor receptor (G-CSFr) was specifically expressed in E2A-PBX1-containing cells. G-CSFr was also expressing in t(1;19) pre-B cell lines, but not in lines lacking E2A-Pbx1. However, these cells do not proliferate in response to G-CSF, either alone or in combination with several cytokines, suggesting that the up-regulation of this receptor does not contribute to tumor formation.

Most recently, human pre-B cell lines that contain the t(1;19) chromosomal translocation, were compared to transformed pre-B cell lines lacking this translocation. A number of genes specifically expressed in t(1;19) cell lines were identified, and two in particular have been characterized in detail; EB-1 (Fu *et al*., 1999a) and Wnt16 (McWhirter *et al*., 1999). EB-1 encodes a phosphotyrosine binding domain protein, and as such may have a role in the regulation of cell proliferation. The Wnt16 gene encodes a novel member of the WNT/WG family of growth factors (McWhirter *et al*., 1999). The WNT/WG signaling pathway is a potent activator of growth and differentiation and is frequently mutated in many human cancers (Polakis, 2000). Although Wnt16 is not normally expressed in pre-B cells (McWhirter *et al*., 1999), a role for WNT signaling has been demonstrated in early hematopoiesis and pro-B cell proliferation (Austin *et al*., 1997; Reya *et al*., 2000; Van Den Berg *et al*., 1998). As such, the specific activation of Wnt-16 in t(1;19) pre-B ALL may contribute to the development of leukemia.

Discussion

Although it has been more than 16 years since the identification of the t(1;19) chromosomal translocation and more than 10 years since the characterization of E2A-PBX1, the mechanisms by which this oncoprotein produces pre-B cell ALL remain mysterious. Extensive analyses of E2A-PBX1 deletion mutants have demonstrated different requirements for transformation in fibroblasts, myeloid cell lines, and primary T lineage cells. When examined as a whole, these experiments indicate that E2A-PBX1 contributes to oncogenic cell growth in multiple ways. In particular, E2A-PBX1 associates with HOX family members and alters the transactivation activity of these complexes. Although no direct tumorigenic function has been ascribed to the characterized target genes of E2A-PBX1, the requirement for the PBX1 homeodomain in myeloid cell transformation strongly suggests that the activation of gene transcription is an important component of E2A-PBX1-mediated tumor development. In addition, the constitutively nuclear localization of E2A-PBX1 may function to alter the expression patterns of HOX/PBX target genes. Here we propose that a reduction in wild type E2A activity also directly contributes to the development of leukemia. Although previous studies have provided much insight into potential mechanisms of transformation, a primary B cell model that accurately reflects human pre-B ALL is needed to carefully examine the molecular requirements for the development of this disease.

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