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An inhibitory switch derepressed by Pbx, Hox, and Meis/Prep1 partners regulates DNA-binding by Pbx1 and E2a-Pbx1 and is dispensable for myeloid immortalization by E2a-Pbx1

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The Pbx/Exd family of homeodomain (HD) proteins contribute to the transcriptional and developmental roles of other Hox and Meis/Prep1/Hth HD proteins through heterodimer formation. E2a-Pbx1 is an oncogenic derrivative of Pbx1 produced by the t(1;19) translocation in pediatric pre-B cell acute lymphoblastic leukemia. E2a-Pbx1 heterodimerizes with Hox but not with Meis/Prep1 proteins, produces acute myeloid leukemia in mice, and blocks differentiation of cultured murine myeloid progenitors. Here, we characterize negative and positive regulatory sequences that flank the Pbx1 HD and determine their importance for myeloid immortalization by E2a-Pbx1. A 25 residue predicted α helix preceding the Pbx1 HD bound the HD and prevented both its binding to DNA and its ability to heterodimerize with Hox proteins. Addition of 39 residues N-terminal to this inhibitory helix exposed a Pbx dimerization interface that orchestrated cooperative DNA-binding of E2a-Pbx1 and all Pbx proteins as homodimers and heterdimers. Sequences inhibiting DNA-binding and mediating Pbx dimerization coincided with those reported to have nuclear export function. An additional 103 residues N-terminal to the Pbx dimerization interface restored heterodimerization with Hox and Meis1/Prep1 proteins. This negative switch domain – comprised of the inhibitory helix and N-terminal regions required for its partner-mediated derepression – was dispensable for myeloid immortalization by E2a-Pbx1. While stabilizing the heterodimer, the 3_{10} helix C-terminal to the Pbx1 HD was also dispensable for the ability of E2a-Pbx1 to heterodimerize with Hox proteins and immortalize myeloblasts. Retention of myeloid immortalization by E2a-Pbx1 proteins lacking all Pbx1 sequences N- or C-terminal to the HD indicates that Hox proteins, or a yet undefined factor that binds the Pbx1 HD and derepresses DNA-binding by the HD, cooperate with E2a-Pbx1 in myeloid immortalization.

Keywords: Pbx; E2a-Pbx1; t(1;19) pre-B ALL; Hox; Meis; Prep1; leukemia

Introduction

Twenty per cent of pediatric pre-B cell acute lymphoblastic leukemia (ALL) contain E2a-Pbx1, a chimeric oncoprotein produced by the t(1;19) translocation (Carroll *et al*., 1984; Williams *et al*., 1984; Kamps *et al*., 1990; Nourse *et al*., 1990). This translocation fuses exons encoding two transactivation domains of E2a (Quong *et al*., 1993) to exons encoding the HD protein, Pbx1, beginning at residue 89. E2A encodes a basic helix–loop–helix transcription factor required for proper B cell development (Bain *et al*., 1994), and is expressed in most embryonic and adult tissues (Roberts *et al*., 1993), while PBX1 prototypes a family of three related homeobox genes, including PBX2, and PBX3 (Monica *et al*., 1991), and is expressed in most fetal and adult tissues, but not in lymphoid cell lines (Kamps *et al*., 1990; Monica *et al*., 1991; Roberts *et al*., 1995). Replacement of the DNA-binding domain of E2a with the Pbx1 HD and flanking sequences creates an oncoprotein that induces inappropriate expression of tissue-specific genes (Fu and Kamps, 1997; McWhirter *et al*., 1997), transforms NIH3T3 fibroblasts (Kamps *et al*., 1991), produces acute myeloid leukemia (AML; Kamps and Baltimore, 1993) and T-cell ALL (Dedera *et al*., 1993) in mice, and arrests differentiation of factor-dependent myeloid progenitors from marrow (Kamps and Wright, 1994). Immortalization (differentiation arrest) of mouse myeloid progenitors is the only *in vitro* hematopoietic assay of the leukemic function of E2a-Pbx1, and occurs with single-hit kinetics (Kamps and Wright, 1994).

Extradentical (EXD) is the *Drosophila* homolog of PBX genes, and EXD mutant embryos exhibit inappropriate transcriptional regulation of the genetic targets of Homeotic Complex genes (Rauskolb *et al*., 1993; Rauskolb and Wieschaus 1994). This observation led to the discovery that Pbx/Exd proteins heterodimerize cooperatively with Hox proteins (Van Dijk and Murre, 1994; Chan *et al*., 1994) and Engrailed proteins (Peletenburg and Murre, 1997) on TGATTNAT elements (Lu *et al*., 1995; Chang *et al*., 1996; Lu and Kamps, 1997; Shen *et al*., 1997) as well as with a cellular factor first dubbed NFPP (Nuclear Factor Pbx Partner; Knoepfler and Kamps, 1997) and later demonstrated to be members of the Meis (Knoepfler *et al*., 1997; Chang *et al*., 1997) and Prep1 (Knoepfler *et al*., 1997) family of HD proteins on TGATTGACAG elements. Meis/Prep1 are the eucaryotic cognates of the *Drosophila* gene, Hth, which is required for pattern formation by Hox genes (Rieckhof *et al*., 1997), binds Exd in the absence of DNA, and is required for nuclear localization of Exd (Pai *et al*., 1998). In all heterodimers, Pbx/Exd binds the 5′TGAT core (Lu *et al*., 1995; Chan *et al*., 1994) and stabilizes DNA-binding by Hox and Meis to their 3′ sites by

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more than 100-fold (Lu *et al*., 1995; Knoepfler *et al*., 1997). In the Hox-Pbx-DNA complex, the Hox protein binds the Pbx1 HD (Lu and Kamps, 1996; Green *et al*., 1998; Passner *et al*., 1999; Piper *et al*., 1999) using a four to eight residue tryptophan-containing Pbx-interaction motif (the W-PIM motif). Meis/Prep1, which fails to heterodimerize with E2a-Pbx1, binds an N-terminal Pbx domain using a region in their N-terminus that we designate the Meis/Prep1 Pbx-interaction motif (MP-PIM). Pbx3 uses a third uncharacterized interaction surface, which we will designate the Pbx-specific Pbx-interaction motif (P-PIM) to homodimerize on TGATTGATTTGAT (Neuteboom and Murre, 1997). Heterodimers of Pbx proteins with HoxB1 and Stf-1 contribute to transcriptional activation of the HoxB1 and Somatostatin genes, respectively (Pöpperl *et al*., 1995; Di Rocco *et al*., 1997; Peers *et al*., 1995). Pbx-Meis/Prep1 heterodimers cooperate with other transcription factors in activation of the uPA (Berthelsen *et al*., 1998a,b; De Cesare *et al*., 1997) and bovine CYP17a promoters (Bischof *et al*., 1998), and synergize with Oct-1 in both transcriptional activation and glucocorticoid-induced repression of the prolactin gene (Subramaniam *et al*., 1997, 1998). Synergistic interactions between Pbx, Hox and Meis proteins has recently been reported. Pbx-Prep complexes cooperate with a Stf-1 binding site in activating Somatostatin transcription through a proximal enhancer (Goudet *et al*., 1999), and Pbx-HoxB1 complexes cooperate with a Meis binding motif to activate HoxB2 transcription through the r4 enhancer (Jacobs *et al*., 1999).

Both the nuclear location and DNA-binding activity of Pbx proteins are negatively regulated by intrinsic mechanisms derepressed by interaction with other HD proteins and DNA. Despite the nuclear localization sequence (NLS) contained within its HD, Exd/Pbx1 is retained in the cytoplasm by active nuclear export sensitive to Leptomycine B, an antibiotic that blocks the CRM1/exportin1 export pathway (Abu-Shaar *et al*., 1999; Berthelsen *et al*., 1999). Nuclear export requires the function of 52 residues N-terminal of the HD (Abu-Shaar *et al*., 1999). Similar to nuclear localization, monomeric DNA-binding by Pbx proteins and E2a-Pbx1 is repressed by a domain N-terminal to the HD (Lu and Kamps, 1996). Here, we identify a negative regulatory switch preceding the Pbx1 HD that inhibits DNA-binding and is derepressed by heterodimer partners only in the presence of additional discrete N-terminal sequences. We report the existence of a common Pbx-dimerization surface within the switch domain. Sequences inhibiting DNA-binding and mediating Pbx-dimerization are located within the same 52 residues reported to mediate nuclear export, implicating their potential role in subcellular localization. The negative switch domain is dispensable for myeloid immortalization by E2a-Pbx1. We also report that both the 3_{10} helix and α-helix C-terminal to the HD (Piper *et al*., 1999; Figure 1d) are dispensable for heterodimerization of E2a-Pbx1 with multiple Hox proteins, for cooperation with Hox proteins in transcriptional activation and for blocking myeloid differentiation. Because the Pbx1 HD alone is essential for myeloid immortalization and because Hox proteins are the only known E2a-Pbx1 partners that can heterodimerize with the HD alone we predict that Hox proteins – or a yet undefined factor that binds the Pbx1 HD and derepresses DNA-binding by the HD – are obligate partners with E2a-Pbx1 in myeloid immortalization.

Results

Twenty-five amino acids N-terminal to the Pbx1 HD inhibit its binding to DNA

Formerly, we reported that addition of N-terminal sequences to S193 prevented the Pbx1 HD/C-terminus ($Pbx1_{D232-Q347}$) from binding DNA as a monomer or heterodimerizing with HoxA5 (Lu and Kamps, 1996). A panel of Pbx1 mutants containing progressive N-terminal additions to $Pbx1_{D232-Q347}$ was constructed to test the specificity of these inhibitory sequences, and to map their precise boundary (Figures 1b,c). $Pbx1_{K216-Q347}$ bound DNA monomerically almost as well as $Pbx1_{D232-Q347}$ (Figure 2a, lane 10 vs 11); however, proteins initiating at K195 or seven sites N terminal to K195 failed to bind DNA (lanes 1–9), demonstrating the specificity of the inhibitory sequence. Initiation at R206 ($Pbx1_{R206-Q347}$, Figure 2b, lane 3 vs 4) also prevented DNA-binding, and R206-L231, a predicted α-helix, was designated the inhibitory helix (IH).

We hypothesized that the IH could block DNA-binding by binding the 63 residue HD, or by binding and inactivating the stabilizing function of the cooperativity helices (CH; the 3_{10} helix and α-helix in residues 295–304; Figure 1d), which flank the C-terminal edge of the Pbx1 HD and bind the HD (Piper *et al*., 1999). To discriminate between these alternatives we tested whether the IH prevents DNA-binding by the isolated Pbx1 HD. Indeed, the IH also prevented weak DNA-binding by the Pbx1 HD ($Pbx1_{D232-G296}$; Figure 2b, lanes 6–8), indicating it suppresses DNA-binding by direct effects on the HD. Eight point mutants and ten double point mutants were introduced within the IH of $Pbx1_{S202-Q347}$ (Table 1). While $Pbx1_{S202-Q347}$ demonstrated less than 1% the monomeric DNA-binding activity of $Pbx1_{D232-Q347}$, mutations increased monomeric DNA-binding up to 15% that of $Pbx1_{D232-Q347}$; however, in no case was IH function completely abrogated. In the context of E2a-Pbx1, deleting sequences encompassing the IH increased monomeric DNA-binding (Figure 4b, top panel; E2a-$Pbx1\Delta_{487-623}$, lane 4) while deleting sequences upstream of the IH did not (E2a-$Pbx1\Delta_{487-578}$; lane 3).

The behavior of HD point mutant E28R provided independent evidence that the IH binds the HD. Previously we found that mutation E28R blocked cooperative DNA-binding by $Pbx1_{D232-Q347}$ and HoxA5. To affirm that E28R consistently alters HD conformation, its impact on heterodimerization with HoxB7, HoxB8, and HoxD10 was compared with that on HoxA5 (Figure 2c). Heterodimerization with each Hox protein was reduced over tenfold. Strikingly, mutation E28R also restored active monomeric DNA-binding to $Pbx1_{S202-Q347}$ (Figure 2b, lane 10 vs 2) while not altering DNA-binding of $Pbx1_{D232\text{-}Q347}$ (lane 9 vs 5). The fact that a conformational change in the HD disrupts IH function independently suggests the IH binds the HD (Figure 1d).

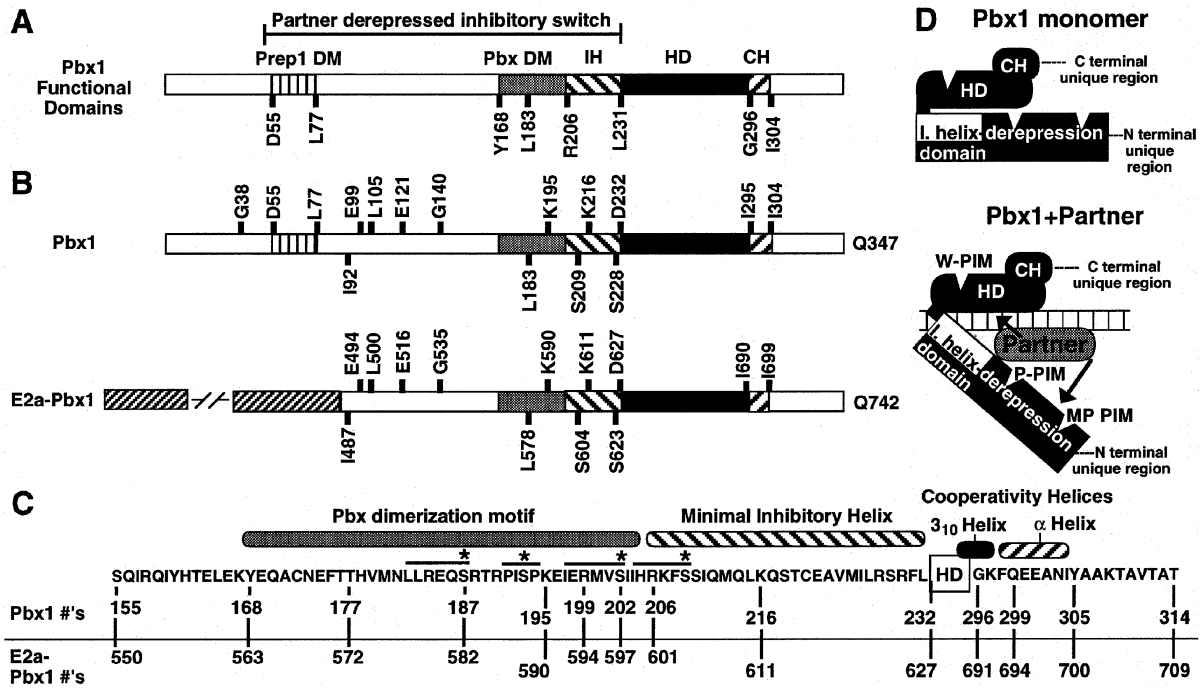

Figure 1 Functional domain designations in Pbx1 and E2a-Pbx1. (**a**) Location of boundaries defining minimal Prep1 dimerization motif (Prep1 DM; vertical bars), Pbx dimerization motif (Pbx DM; stippled), inhibitory helix (IH; left-right cross-hatched), homeodomain (HD, solid), and cooperativity helices (CH; right-left cross-hatched). (**b**) Locations of residues involved in the Pbx1 N-terminal deletion series and their cognate positions in E2a-Pbx1 (numbers designated above line diagrams) and in the E2a-Pbx1 internal deletion series and their cognate positions in Pbx1 (numbers designated below line diagrams). (**c**) Pbx1 sequence comprising the Pbx dimerization motif (stippled), the minimal inhibitory helix (IH; left-right cross hatch), and cooperativity helices (the 3_{10} helix [solid] and α-helix [right to left cross-hatched]). Numbered residues designate the first or last amino acids in truncation proteins used to identify the N-terminal boundaries of the Pbx dimerization motif and the minimal IH, as well as the position of C-terminal truncation proteins used in determination of heterodimerization potential with Hox, Prep1, and Pbx partners. Lines overhead sequence and asterisks above serine residues indicate the recognition sequences and phosphorylation sites, respectively, for MAP-KAP (I/L-X-R-X-X-**S**) and MAP (P-X-**S**-P) kinases. (**d**) Model for the structure of Pbx1 monomers and heterodimers. HD; homeodomain, CH; cooperativity helices, W-PIM; binding site of tryptophan-containing Pbx1-interaction motif of Hox proteins, P-PIM; binding site for Pbx-interaction motif of Pbx proteins, MP-PIM; binding site of the Pbx-interaction motif of Meis-Prep1. Triangular depressions in the surface of Pbx1 denote sites bound by the W-PIM, the P-PIM, and the MP-PIM

The IH is a dominant inhibitor of heterodimerization, requiring distinct N-terminal extensions to permit partner-dependent DNA-binding

Addition of the IH to $Pbx1_{D232-G296}$ (Figure 2d, lane 8 vs 10) or to $Pbx1_{D232-Q347}$ (lane 5 vs 7) nearly abolished heterodimerization with HoxA5. Among the 18 point and double point mutants introduced within the IH of $Pbx1_{S202-Q347}$ (Table 1), those that increased monomeric DNA-binding also increased dimerization with HoxA5, indicating that the IH blocks heterodimerization by blocking DNA-binding (data not shown).

To map the minimal sequences required to derepress IH function, N-terminal extension mutants in Pbx1b were evaluated for their ability to heterodimerize with Hox and Prep1 partners. Restoration of full cooperative DNA-binding with HoxA5, HoxB7, HoxB8, and HoxC8 required Pbx1 sequences initiating at D55 (Figure 2e). Initiation at D55 restored both DNA-independent binding to Prep1 (co-IP in Figure 2e) and heterodimerization with Prep1 on TGATTGAC. Thus, Hox proteins, like Prep1, required a specific determinant N-terminal to the HD to evoke a conformational change permitting DNA-binding. The IH and N-terminal domain required for dimerization with Hox and Prep1 proteins functions as an inhibitory switch, where a switch in conformation coincident with heterodimerization permits DNA-binding.

The inhibitory switch contains a general Pbx dimerization motif, previously reported to exist specifically in Pbx3

In contrast to reports that Pbx1 and Pbx2 lack the novel ability of Pbx3 to homodimerize on TGATT-GATTTGAT (Neuteboom and Murre, 1997), we found that both differentially spliced forms of Pbx1 (1a and 1b), which differ in sequences C-terminal to the HD, homodimerized on TGATTGATTTGAT as efficiently as did Pbx3 (Figure 3a, lanes 2–4). The amount of E2a-Pbx1 produced in coupled transcription/translation was one-quarter that of Pbx1, and it also bound well as a homodimer (lane 6), and formed heterodimers with Pbx3, Pbx1a, or Pbx1b (lanes 7–9). Antibodies to E2a failed to super-shift Pbx1a homodimers (lane 12 vs 11), supershifted monomers and homodimers of E2a-Pbx1 (lane 14 vs 13), and shifted heterodimers of E2a-Pbx1 and Pbx1a without affecting Pbx1a homodimers (lane 16 vs 15), confirming the predicted dimer compositions. The properties of $Pbx1_{K216-Q347}$ and $Pbx1_{D232-Q347}$ confirmed that Pbx1, like Pbx3, binds TGATT-GATTTGAT as a dimer. Each protein formed a single complex with TGATTGAT and yielded no

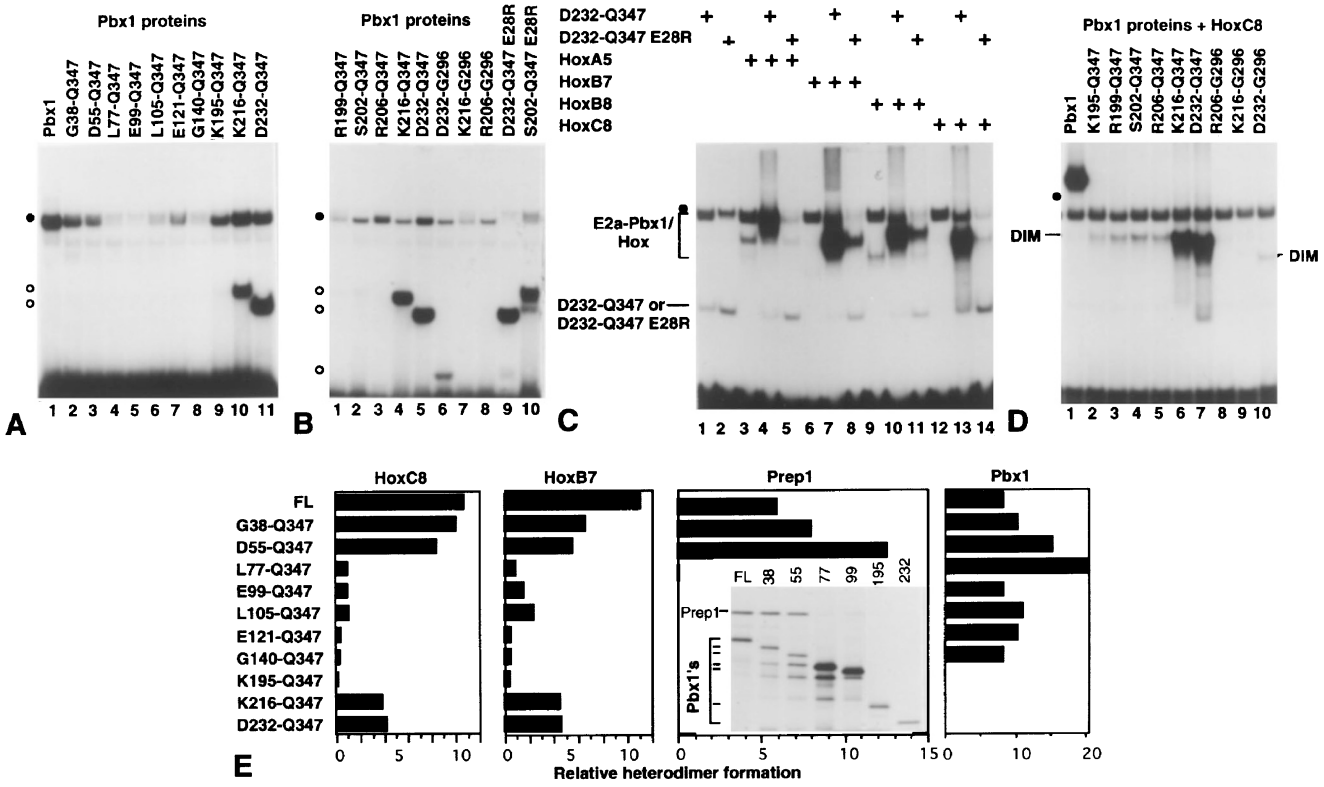

Figure 2 Mapping the IH and derepression domains of Pbx1. All panels represent electrophoretic mobility shift analysis of proteins produced *in vitro* by coupled transcription/translation. Equivalent molar quantities of all recombinant proteins were used based on analysis of parallel transcription/translation reactions performed in the presence of ^{35}S-methionine. All forms of Pbx1 are in the context of the Pbx1b C-terminus. (**a**) Mapping the location of minimal N-terminal sequences required to prevent strong monomeric DNA-binding by $Pbx1_{D232-Q347}$. The complex bound by $Pbx1_{D232-Q347}$ is designated as a monomer based on the fact that it comigrates with recombinant $Pbx1_{D232-Q347}$ bound to a true half-site probe that contains TGATT as the only recognition element. The protein in this complex also fails to form complexes of intermediate size when mixed with $Pbx1_{K216-Q347}$ (see Figure 3). (**b**) The minimal IH prevents DNA-binding by the Pbx1 HD and is functionally inhibited by HD mutation E28R. (**c**) Pbx1 HD mutation E28R disrupts heterodimerization of $Pbx1_{D232-Q347}$ with HoxA5, HoxB7, HoxB8 and HoxC8 without disrupting DNA-binding. (**d**) The minimal N-terminal inhibitory sequences prevent heterodimerization with Hox proteins and can be derepressed by inclusion of residues N-terminal to K195. (**e**) Efficiency of heterodimerization of N-terminal Pbx1 mutants with HoxC8, HoxB7, Prep1, and Pbx1b. FL refers to full length Pbx1b. Inset in Prep1 bar graph represents binding of Pbx1 N-terminal mutants to Prep1 in the absence of DNA, using coimmunoprecipitation with antibodies to Pbx1. DNA motifs used to identify heterodimers were TGATTTAT for Pbx1b plus HoxC8 or HoxB7; TGATTGACAG for Pbx1b plus Prep1; and TGATTGATTTGAT for Pbx1b. The identity of complexes in (**a**–**e**) are indicated at left, and migration of the non-specific complex is indicated by a filled circle

complex of intermediate mobility when mixed, indicating binding as monomers (Figure 3b, lanes 2–4). By contrast, each protein bound TGATT-GATTTGAT both as monomers and as a higher order complex (lanes 6 and 7), and formed one new complex of intermediate mobility when mixed, indicating that the higher order complex is a non-cooperative dimer (lane 8). The same N-terminal extension series used to identify the minimal domain required to derepress IH function for Hox and Prep1 partners was used to identify the minimal domain required for Pbx dimerziation. N-terminal residues required for cooperative homodimerization were mapped to Y168 (Figure 3c; lanes 1–12, 16–19). In E2a-Pbx1, deletion of Pbx1 sequences from the E2a/Pbx1 junction to the equivalent of L183 or S209 (E2a-$Pbx1\Delta_{487-578}$ and E2a-$Pbx1\Delta_{487-604}$, respectively) suppressed, then abolished dimerization (Figure 3d, lanes 2 and 3), indicating that Y168-S209 contains an essential Pbx dimerization sequence (designated P-PIM; Figure 1d). As predicted, further deletion of the IH (E2a-$Pbx1\Delta_{487-623}$; Figure 3d, lane 4) revealed strong monomeric DNA-binding by E2a-Pbx1 and the same degree of non-cooperative dimeric DNA-binding as observed for $Pbx1_{D232-Q347}$ (Figure 3c, lane 12).

The inhibitory switch in E2a-Pbx1 is dispensable for cooperative transactivation with Hox proteins and for myeloid immortalization

E2a-Pbx1 exhibits two distinct transactivation functions, each detected in Nalm-6 pre-B cells. E2a-Pbx1 requires coexpression of Hox proteins to activate transcription through 6×TGATTTAT but activates transcription through 6×TGATTGAT independent of Hox protein coexpression through an unidentified mechanism (Lu and Kamps, 1997). Independent activation through 6×TGATTGAT is not likely to be mediated by cooperation with endogenous Hox proteins. All Hox proteins tested to date – even if they prefer to heterodimerize with E2a-Pbx1 on TGATT-GAT elements – activate transcription strongly with E2a-Pbx1 on the 6×TGATTTAT reporter in Nalm-6

Table 1 Monomeric and heterodimeric DNA-binding potential of $Pbx1_{233-347}$ or $Pbx1_{202-347}$ containing mutations in the IH. Assays were performed using HoxA5 as a Pbx1 partner and TGATTGAT as the binding site

Control protein	*Mutant version of control*	*Monomeric DNA-binding* (% $Pbx1_{233-347}$)	*Heterodimeric DNA-binding* (% $Pbx1_{233-347}$)
$Pbx1_{233-347}$		100	100
$Pbx1_{233-347}$		<1	14
	K207A F208A	2	21
	I211A Q212A	<1	10
	M213A Q214A	<1	9
	L215A K216A	2	13
	Q217A S218A	<1	5
	C220A E221A	<1	6
	V223A M224A	1	12
	I225A L226A	9	29
	R227A S228A	<1	8
	R229A F230A	15	32
	I211R	3	11
	I211P	2	25
	I211G	2	23
	L215R	2	9
	L215P	7	24
	L215G	2	8
	C220P	3	25
	L226P	7	26

pre-B cells. Therefore, if activation of the 6×TGATTGAT reporter by E2a-Pbx1 were accomplished in conjunction with an endogenous Hox protein, these factors should also activate transcription through the 6×TGATTTAT reporter. Because no activation of the 6×TGATTTAT reporter is observed by expression of E2a-Pbx1 alone, we hypothesize activation through the 6×TGATTGA is accomplished by a Hox-independent mechanism. The inhibitory switch in Pbx1 was excised in E2a-$Pbx1\Delta_{487-623}$, producing a protein similar to that of an alternative t(1;19) E2a-Pbx1 chimera in pre-B ALL (Numata *et al*., 1993). The only known partners whose interaction surface on Pbx1 is retained in E2a-$Pbx1\Delta_{487-623}$ are Hox proteins. E2a-$Pbx1\Delta_{487-623}$ bound DNA actively as a monomer and bound cooperatively with Hox proteins *in vitro* (Figure 4b, lane 4), activated transcription cooperatively with Hox proteins in Nalm-6 cells, and immortalized myeloid progenitors (Figure 4a). In an attempt to eliminate heterodimerization with Hox proteins, a second inhibitory switch mutant retaining the IH (E2a-$Pbx1\Delta_{487-578}$) was characterized. E2a-$Pbx1\Delta_{487-578}$ exhibited poor cooperative DNA-binding with Hox

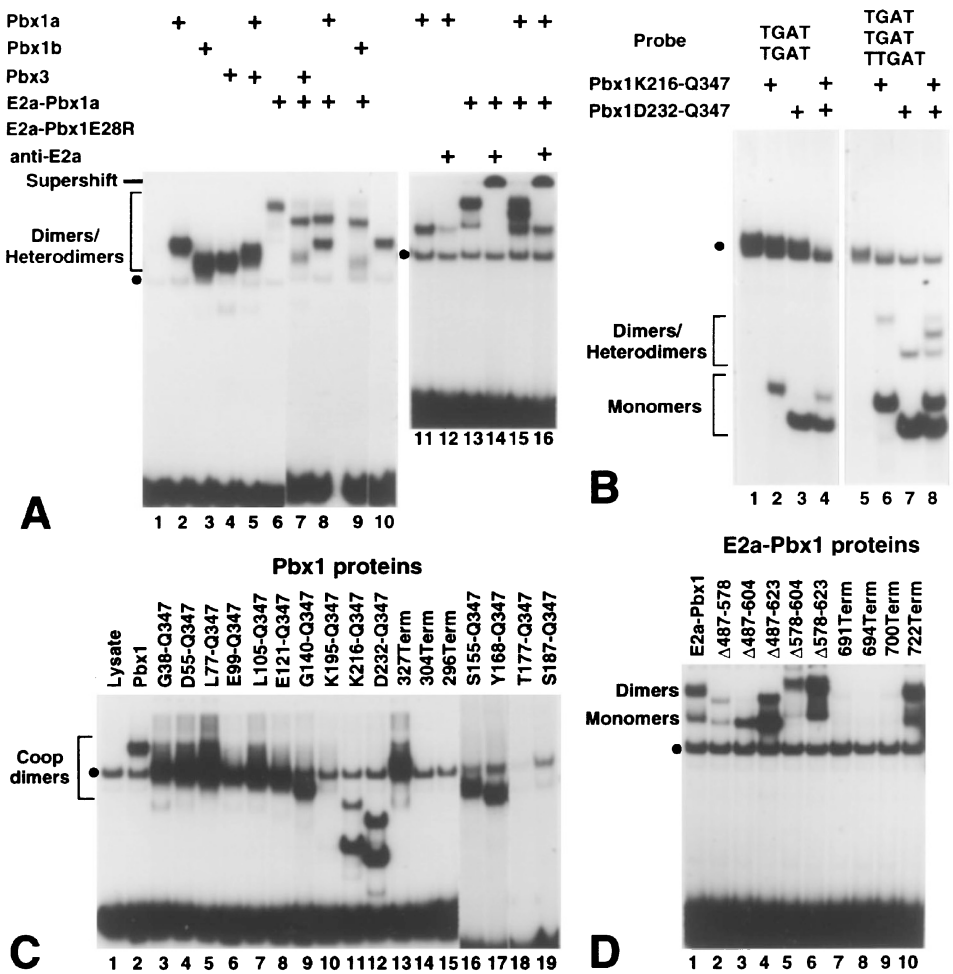

Figure 3 Homodimerization and heterodimerization potential of Pbx proteins and E2a-Pbx1. All panels represent electrophoretic mobility shift analysis of proteins produced *in vitro* by coupled transcription/translation. Equivalent molar quantities of all recombinant proteins were used based on analysis of parallel transcription/translation reactions performed in the presence of ^{35}S-methionine. When unspecified, C-terminal sequences of Pbx are those encoded by Pbx1b. (**a**) Homodimerization and heterodimerization by Pbx1, Pbx3, and E2a-Pbx1 proteins on TGATTGATTTGAT. (**b**) Fragments of Pbx1b containing the HD and C-terminus bind TGATTGAT as monomers and TGATTGATTTGAT as noncooperative dimers. (**c**) Mapping minimal sequences N-terminal to the HD required for homodimerization of Pbx1b on TGATTGATTTGAT. (**d**) Monomeric and homodimeric DNA-binding by E2a-Pbx1 and mutants of E2a-Pbx1b on TGATTGATTTGAT, a subset of which were used for analysis of myeloid immortalization

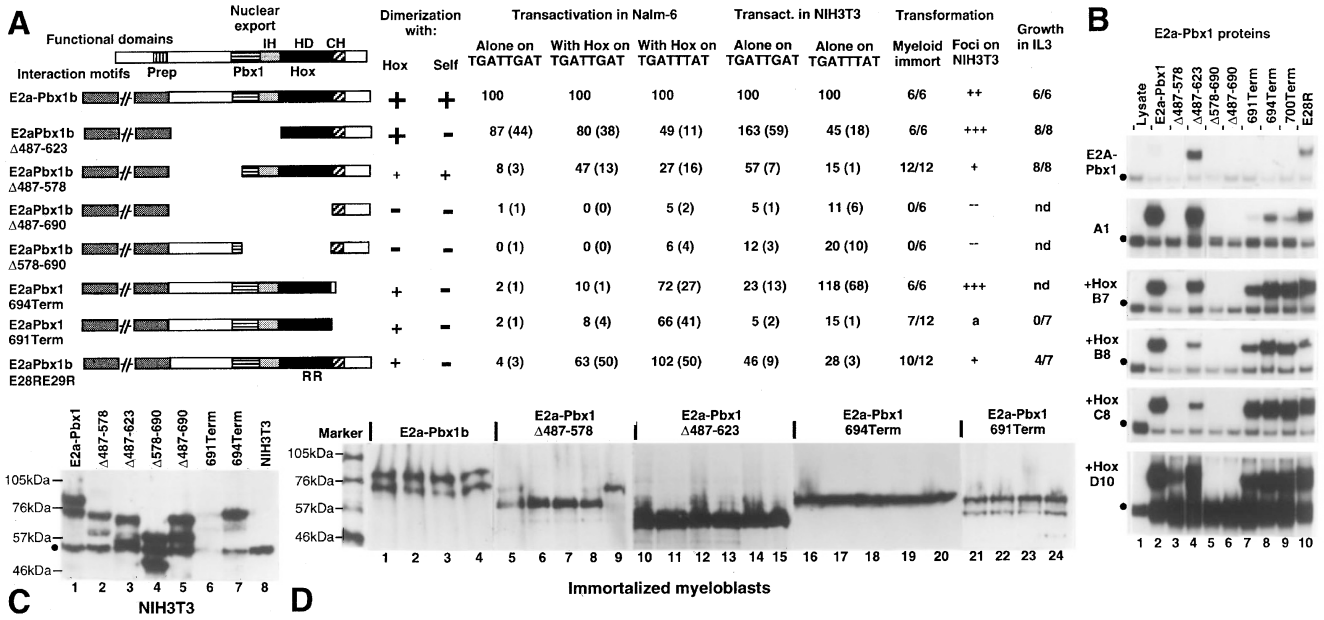

Figure 4 Abilities of E2a-Pbx1 and mutants of E2a-Pbx1 to activate transcription, immortalize myeloid progenitors, and induce foci on NIH3T3 cells. (**a**) Transactivation and transformation properties of wild-type and mutant E2a-Pbx1b proteins. nd; not determined. a; protein detectable only at very low levels in stably-expressing NIH3T3 fibroblasts. The conclusion that E2a-$Pbx1_{\Delta 487-690}$ and $Pbx1_{\Delta 578-690}$ fail to activate reporter gene transcription because they lack their HD rather than because they are expressed at extremely low levels is based on the observation that their stability is equal to that of wild-type E2a-Pbx1 in NIH3T3 fibroblasts (**c**). (**b**) Heterodimerization potential of mutants of E2a-Pbx1b with indicated Hox proteins. The migration of a non-specific complex is indicated at left by a filled circle. Equivalent molar quantities of all recombinant proteins were used based on analysis of parallel transcription/translation reactions performed in the presence of ^{35}S-methionine. (**c**) Western blot analysis of wild-type and mutant E2a-Pbx1b proteins expressed in NIH3T3 cells. These cells were used as a source of virus for marrow infections. Two forms of E2a-Pbx1 are detected in all samples. The lower form arises by an internal mRNA splicing event within E2a, as Yae (Santa Cruz Biotechnology), a specific monoclonal antibody that binds residues 195–208 of E2a, binds the upper but not the lower species. (**d**) Anti-E2a immunoblot analysis of total cellular extracts from myeloblasts immortalized by virus produced from NIH3T3 fibroblasts in (**c**). The two different sizes of the fusion proteins arise from alternative splicing within the E2a mRNA, as described in (**c**)

proteins (Figure 4b, lane 3), yet retained a mediocre degree of transcriptional activation with Hox proteins, and immortalized myeloid progenitors (Figure 4a).

The CH cannot block myeloid differentiation in the absence of the HD and is fully dispensable for blocking differentiation in the presence of the HD

The Pbx1 CH alone when fused to E2a has been reported to mediate fibroblast transformation (Chang *et al*., 1997). We tested the biochemical and myeloid immortalizing potential of Pbx1 containing the CH but lacking the HD. E2a-$Pbx1\Delta_{487-690}$ and E2a-$Pbx1\Delta_{578-690}$ failed to bind DNA cooperatively with Hox proteins (Figure 4b, lanes 5 and 6), failed to activate transcription cooperatively with Hox proteins, and failed to immortalize myeloblasts (Figure 4a). E2a-$Pbx1\Delta_{487-690}$ and E2a-$Pbx1\Delta_{578-690}$ are presumed to be expressed in myeloid progenitors at levels commensurate with that of wild-type E2a-Pbx1 because their abundance after G418-selection in NIH3T3 fibroblasts is equal to that of wild-type E2a-Pbx1 (Figure 4c, lanes 4 and 5 vs lane 1). The CH is therefore insufficient for myeloid immortalization and the HD is essential.

The CH contains both a 3_{10} helix (residues 690–692) and an α-helix (694–699) that pack against the HD (Piper *et al*., 1999) and increase its DNA-binding affinity (Lu *et al*., 1996). Earlier we found that termination before (E2a-$Pbx1_{700Term}$) or elimination of (E2a-$Pbx1_{694Term}$) the α-helix did not disrupt myeloid immortalization. E2a-$Pbx1_{691Term}$ was made to disrupt the 3_{10} helix and therein examine its function. E2a-$Pbx1_{691Term}$ formed heterodimers with HoxA5 and HoxB7 that were less stable than those formed by E2a-$Pbx1_{700Term}$ or E2a-$Pbx1_{694Term}$ (Figure 5a, lanes 4 vs 2 and 3; lanes 10 vs 8 and 9; lanes 15 vs 13 and 14; lanes 20 vs 18 and 19). E2a-$Pbx1_{691Term}$ retained significant heterodimerization ability with Hox proteins on TGATTTAT – heterodimers of E2a-Pbx1 with HoxA5, HoxB7, HoxB8 or HoxC8 exhibited off-rates of 110, 130, 125, and 100 min, respectively, while those containing E2a-$Pbx1_{691Term}$ had off-rates of 23, 14, 10, and 25 min. Cooperativity with E2a-$Pbx1_{691Term}$ was dependent on the Hox W-PIM, as mutation of the esssential tryptophan in the W-PIM of HoxA5 or HoxB8 strongly suppressed cooperative DNA-binding with E2a-Pbx1 (Figure 5b, lanes 1 vs 3, and 5 vs 7, respectively), and eliminated it with E2a-$Pbx1_{691Term}$ (lane 2 vs 4, and 6 vs 8, respectively).

E2a-$Pbx1_{691Term}$ retained Hox-dependent transcriptional activation through 6×TGATTTAT (Figure 4a) and immortalized myeloblasts. These myoblasts were GM-CSF-dependent, exhibited myeloid-specific nuclear morphology and azurophilic granules, and contained less than 1% of cells exhibiting a 'donut' nuclear morphology indicative of neutrophilic differentiation, demonstrating that a strong differentiation block is maintained in the absence of the 3_{10} helix. However, their cell cycle was distinctly longer (41 h) than those

immortalized by E2a-Pbx1 or by E2a-Pbx1$_{694Term}$ (22 h), and they failed to proliferate in IL3 while those transformed by all other versions of E2a-Pbx1 responded well to IL3. Thus, the 3_{10} helix contributes to a subset of the phenotypic properties of myeloblasts immortalized by E2a-Pbx1.

Hox-independent transcriptional activation through TGATTGAT correlates with the ability of E2a-Pbx1 to bind DNA as a homodimer

Deletion of the CH abolished homodimerization on TGATTGATTTGAT of both Pbx1 (Figure 3c, lanes 14 and 15 vs lane 13) and E2a-Pbx1 (Figure 3d, lanes 7–9 vs 10) and abolished transcriptional activation on 6×TGATTGAT (Figure 4a). A different mutant, E2a-Pbx1$_{\Delta 487-578}$, which lacks much of the Pbx dimerization motif, homodimerized weakly on TGATTGATTTGAT (Figure 3d, lane 1) and activated transcription poorly on 6×TGATTGAT (Figure 4a). Surprisingly, HD mutations E28R and E28RE29R provided additional evidence for a homodimeric mechanism of activation through 6×TGATTGAT. Both mutants failed to homodimerize on TGATTGATTTGAT (Figure 3a lane 10 and not shown) and failed to activate transcription autonomously through 6xTGATTGAT (Figure 4a, and data not shown). Therefore, the effects of mutations within three distinct Pbx1 domains – N-terminal to the HD, within the HD, and C-terminal to the HD – support a homodimeric mechanism of transcriptional activation of TGATTGAT. Each of these three categories of mutants retained significant cooperative transactivation with Hox proteins and immortalized myeloid progenitors, suggesting that homodimeric activation by E2a-Pbx1 is dispensable for myeloid immortalization, and that heterodimerization with Hox proteins may be essential.

The CH and inhibitory switch mediate clone-specific fibroblast transformation

There are conflicting reports concerning the ability of DNA-binding and HD mutants of E2a-Pbx1 to

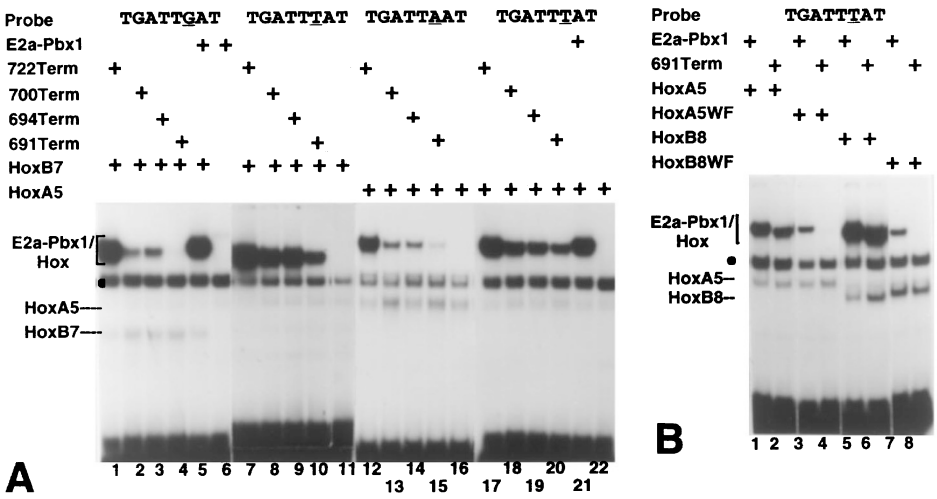

Figure 5 The 2,3 helix is dispensable for W-PIM-dependent heterodimerization of E2a-Pbx1 with Hox proteins. Both panels represent electrophoretic mobility shift analysis of proteins produced by coupled transcription/translation *in vitro*. (**a**) CH-mutants of E2a-Pbx1b actively heterodimerize with HoxB7 or HoxA5 on TGATTTAT but not on TGATTGAT motifs. (**b**) CH-independent heterodimerization of E2a-Pbx1b is dependent on the W-PIM of Hox proteins. The identify of complexes are indicated at left of panels. Migration of a complex containing a factor that binds DNA non-specifically is indicated by a filled circle

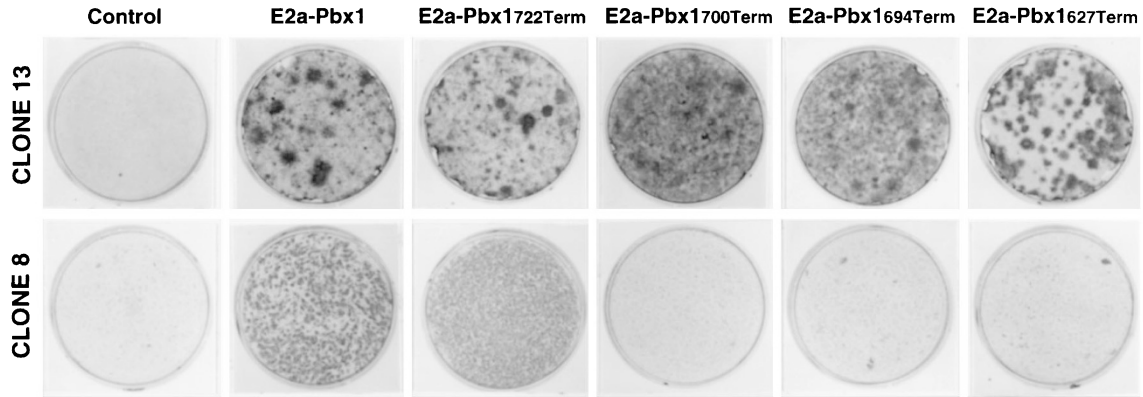

Figure 6 NIH3T3 fibroblasts exhibit clonal variation in their transformation response to mutants of E2a-Pbx1. Control cells were infected with Moloney Murine leukemia virus (MLV), and all other cells with retrovirus encoding the version of E2a-Pbx1 specified above the panel. All viruses were pseudotyped with MLV. Foci were visualized 14 days after infection using Wright stain

transform NIH3T3 fibroblasts. While we reported that both E2a-Pbx1$_{694Term}$ and E2a-Pbx1$_{626Term}$ transform NIH3T3 cells (Kamps *et al*., 1996), others demonstrated that truncation of the CH or any sequences including the CH (e.g. at residue 627) abrogates NIH3T3 cell transformation (Monica *et al*., 1994), and that the CH alone tethered to E2a transforms NIH3T3 cells (Chang *et al*., 1997). Clonal variation in NIH3T3 fibroblasts can account for at least some of these discrepancies. While terminations within the α-helical portion of the CH (E2a-Pbx1$_{700T}$, E2a-Pbx1$_{694T}$) as well as preceding the HD (E2a-Pbx1$_{627Term}$) induce overt focus formation in clone 13 fibroblasts, they have little or no effect in close 8 fibroblasts (Figure 6). Binding of the IH to the Pbx HD may explain the puzzling observation that fusions of E2a with mutually exclusive Pbx1 domains – that preceding the HD and that following the HD – are both capable of transforming NIH3T3 fibroblasts. Both these regions can bind the Pbx1 HD and both may use binding to the HD's of differentially expressed endogenous Pbx proteins as their transforming mechanisms.

Discussion

A partner-derepressed inhibitory switch regulates DNA-binding by Pbx proteins and E2a-Pbx1

Here, we demonstrate that intrinsic DNA-binding by the Pbx1 HD is repressed by the IH. We propose that the IH binds the minimal 63 residue HD because (1) it blocks DNA-binding by the minimal HD and (2) a mutation within the HD (E28R) abrogates IH function. The IH could either mask the DNA-binding surface of the HD or alter HD structure so as to destabilize the HD-DNA complex without blocking access of DNA to the binding pocket. Derepression of IH function occurs during cooperative DNA-binding and requires addition of different portions of N-terminal sequences, depending on the identity of the heterodimerizing partner. In the case of Prep1, exactly the same N-terminal sequences are required for DNA-independent binding to Pbx1 as are required for heterodimerization with Pbx1, suggesting that binding of partners to N-terminal sequences of Pbx1 may be a common requirement to derepress IH function, and indicating that the minimal N-terminal HD extensions required for homodimerization of Pbx (to Y168) and heterodimerization with Hox proteins (to D55) may delineate such interaction surfaces. Off-rate analysis supports the possibility that Hox proteins use surfaces distinct from the P-PIM to contact Pbx1 N-terminal to its HD, as heterodimers of Hox proteins with full-length Pbx1 are three times as stable as those formed with Pbx1$_{D232-Q347}$.

Both the α subunit of PEBP2/CBF and the Ets family of transcription factors also use autoinhibitory sequences to block intrinsic DNA-binding and exhibit heterodimer-induced derepression of DNA-binding. In full-length Ets-1, domains on either side of the minimal DNA-binding domain function in concert to inhibit DNA-binding (Wasylyk *et al*., 1992) by destabilizing the protein-DNA complex, not by masking the DNA-binding surface (Jonsen *et al*., 1996). In the case of the α subunit of PEBP2/CBF (also called PEBP2αB1/AML1), DNA-binding by the Runt domain is inhibited by 109 adjacent C-terminal residues. Interaction of the β-subunit with the RUNT domain relieves the mechanism that inhibits DNA-binding (Kanno *et al*., 1998). Interestingly, the ability of the α subunit of PEBP2 to cooperate with Ets-1 in transcriptional activation of the β subunit of the T-cell receptor requires mutual derepression of mechanisms blocking DNA-binding by both subunits, and is mediated by interaction of the two inhibitory domains (Kim *et al*., 1999). This mechanism parallels the proposed heterodimerization mechanism with Pbx1, where binding of partners to a domain adjacent to the IH derepresses DNA-binding inhibition. Interestingly, in Ets-1, the secondary structure of an α-helix involved in repressing DNA binding becomes disrupted when Ets-1 binds DNA while the structure of the DNA-binding domain remains unaltered (Petersen *et al*., 1995). A similar mechanism could also occur in Pbx1, where the structure of the IH is altered coincident with heterodimerization.

The inhibitory switch may cooperate with nuclear export sequences in dictating intracellular distribution of Pbx proteins

In some *Drosophila* S2 cell lines, inclusion of 52 residues N-terminal of the HD – which contains both the IH and Pbx-dimerization motifs – prevents nuclear localization (Abu-Shaar *et al*., 1999). The fact that Leptomycine B, which blocks the CRM1/exportin1-related export pathway, also inhibits cytosolic localization of Exd, suggests these sequences mediate active nuclear export rather than passive inhibition of nuclear localization mediated by the NLS within the HD. These Pbx1 sequences, however, contain no leucine repeat sequence indicative of direct targets for nuclear export by CRM1/exportin1, and in other *Drosophila* S2 cell lines, addition of these same 52 residues fails to prevent nuclear localization (Berthelsen *et al*., 1999). The derepression switch may contribute to this more complex mechanism regulating Pbx subcellular location. In addition to blocking DNA-binding, the IH could mask the function of the NLS in the Pbx1 HD, similar to the example of intermolecular masking of the p65 NLS by IkappaB in regulation of nuclear NF-κB activity (Ganchi *et al*., 1992). This would account for the fact that in the absence of a partner, the nuclear export signal is dominant over the NLS. A partner-induced conformational change could alter IH conformation, expose the NLS, and permit nuclear import. Because phosphorylation of transcription factors, such as yeast Pho4 regulates nuclear import/export (Kaffman *et al*., 1998; Komeili and O'Shea, 1999), it is also important to consider the roles of multiple potential phosphorylation sites that flank the IH. Phosphorylation of MAP-KAP kinase sites (S187, S202, and S209; consensus; I/L/M-X-R-X-X-S) or of a MAP kinase site (S193, consensus: P-X-S/T-P) could provide another mechanism for regulating the subcellular distribution and DNA-binding potential of Pbx1 and Pbx family proteins. Indeed, nuclear export of the MAPKAP kinase-2 substrate, p38, occurs in a complex with MAPKAP kinase (Ben-Levy *et al*., 1998), and nuclear export of MAPKAP kinase itself is prevented by

Leptomycin B (Engel *et al*., 1998). A link between phosphorylation and Exd-dependent gene expression does occur in *Drosophila*, where transcriptional activation of Labial by Decapentaplegic (DPP), a member of the TGF-β family of signaling molecules that activate serine-threonine kinases, requires an enhancer element that binds Exd-Labial (Grieder *et al*., 1997). DPP activity is also required for nuclear localization of Exd (Rieckhof *et al*., 1997). Analysis of Pbx1 phosphorylation during cell division and differentiation may provide further insights into the complex regulatory function of this region of Pbx proteins.

The inhibitory switch model in conjunction with mutational analysis suggests Hox proteins or other factors that bind the Pbx1 HD are essential partners in myeloid immortalization by E2a-Pbx1

A common theme is that negative regulatory domains in protooncoproteins are either dispensable or must be eliminated for the genesis of their cognate oncoproteins. This is true not only for regulation of catalytic activity, as occurs in the genesis of tyrosine protein kinase oncoproteins, but also for regulation of DNA-binding, as occurs in the Ets protein encoded by avian leukemia virus E26. The E26 Ets protein contains a deletion of C-terminal sequences that inhibit DNA-binding (Leprince *et al*., 1983). The IH of Pbx1 illustrates another such example of a negative regulatory sequence dispensable both for myeloid immortalization as well as for human pre-B cell leukemogenesis (Numata *et al*., 1993). The dispensable nature of the P-PIM motif both in myeloid immortalization and in human pre-B cell leukemogenesis furthermore indicates that specific Pbx-Pbx interactions are unnecessary. What does the dispensable nature of these sequences indicate about the mechanism of myeloid immortalization by E2a-Pbx1? Our model suggests that heterodimer partners of E2a-Pbx1 are required to permit active DNA-binding and to target E2a-Pbx1 to physiologically relevant DNA-motifs. Despite removal of all Pbx sequences on either side of the HD, E2a-Pbx1 retains its ability to target genes required for myeloid immortalization, suggesting that immortalization requires partners that interact with the Pbx1 HD, and implicating Hox proteins or other factors that bind the HD as essential cofactors. This hypothesis is consistent with observations that transformation by Hox proteins is also dependent on Pbx proteins. Fibroblast transformation by HoxB3 and HoxB4 requires Pbx coexpression (Krosl *et al*., 1998), and the W-PIM is required for fibroblast transformation by NUP98-HoxA9 (Kasper *et al*., 1999), implicating a Pbx-dependent mechanism. Our mutual analysis of the CH and the HD also support the possibility that Hox proteins function as cofactors in myeloid immortalization by E2a-Pbx1. Deletion of the C-terminal 3_{10} helix and α-helix, or HD mutation E28RE29R disrupted Hox-independent transactivation on TGATTGAT but did not prevent either Hox-dependent activation on TGATTTAT or myeloid immortalization. In an effort to disrupt Hox-dependent activation on TGATTTAT motifs we introduced mutations E28R and E28RE29R into the Δ487–623 version of E2a-Pbx1; however, both these proteins still exhibited robust cooperative transcriptional activation with Hox proteins on TGATTTAT motifs. Ultimately, proving Hox-dependence of myeloid immortalization by E2a-Pbx1 will require introductions of specific mutations within the HD that disrupt interaction with the W-PIM and determination of whether such mutations prevent myeloid immortalization by E2a-Pbx1.

Materials and methods

Construction of recombinant plasmids

All cDNA's used in coupled transcription/translation were cloned in pGEM3z, pGEM3zf-, or pGEM4z (Promega). Wild-type and mutant versions of E2a-Pbx1 were cloned in the *Eco*R1 site of pMSCV2.1. This vector was used for subsequent assessment of biochemical activities by cotransfection, as well as for generation of retrovirus used to infect primary mouse marrow and NIH3T3 fibroblasts.

Mutagenesis

Site directed mutations were created using the Muta-gene phagemid *in vitro* mutagenesis kit (Biorad) according to the manufacturers protocol. All mutations were verified by sequence analysis. Deletions were created by introducing two inframe *Mlu*I sites and excising intervening sequences, resulting in derivatives that contain the TR dipeptide at each excision junction. Amino terminal deletion mutants of Pbx1 were generated by PCR.

In vitro *transcription/translation*

Coupled transcription/translation was performed *in vitro* using the Promega TNT Coupled Reticulocyte Lysate System in accordance with manufacturer's protocol using SP6 polymerase.

Electrophoretic mobility shift assays (EMSA)

Double-stranded oligonucleotides were labeled with γ-^{32}P-ATP to equal specific activities by phosphorylation of a common reverse oligonucelotide that was annealed to the 3′ portion of oligonucleotides containing different DNA-binding motifs, filled in using dNTP's and Klenow polymerase, and isolated after separation through 15% acrylamide gels run in 0.5 × TBE (27 mM Tris; 27 mM boric acid; 0.6 mM EDTA). For EMSA, 20 000 c.p.m. of probe was incubated with 3–6 ml of *in vitro* translated proteins in the presence of 1 mg of poly (dI:dC) in a buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.1% NP-40, and 5% glycerol for 30 min at room temperature. Complexes were separated by electrophoresis in 5.0% acrylamide gels formed and run in 0.6 × TBE (27 mM Tris; 27 mM boric acid; 0.6 mM EDTA). Protein-DNA complexes were visualized by autoradiography and quantitated using a Bio-Rad GS-250 Molecular Imager. Unless otherwise noted, equal molar amounts of mutant and wild-type Pbx or E2a-Pbx1 proteins were added as assayed by parallel transcription translation reactions performed in the presence of ^{35}S-methionine, followed by quantitation of proteins resolved by SDS-gel electrophoresis using a Bio-Rad GS-250 Molecular Imager. The doubled-stranded, unlabeled oligonucleotide (TCACGG**TGATTTAT**GAGC-GACTGCTCGG) was used for off-rate analysis.

Transfections and luciferase assays

NIH3T3 cells were dispersed on 6-well plates 24 h prior to transfections in Dulbecco's modified Eagles medium containing 10% fetal bovine serum (FBS). A mixture of reporter and expression plasmids were introduced using lipofectamine (Gibco-BRL) according to the manufacturers specifications.

Cells were harvested 40–48 h post transfection and luciferase assays performed, and luciferase abundance normalized to an internal control of renilla production using a dual luciferase reporter system (Promega). Nalm-6 pre-B cells were transfected using electroporation, luciferase production measured 36 h post-transfection and normalized to renilla controls.

Western blotting

Proteins in NIH3T3 cells and primary myeloblast populations stabily expressing E2a-Pbx1 were dissolved in Laemmli sample buffer and boiled for 5 min. Proteins in 5×10^5 cells were resolved by electrophoresis through 12.5% SDS polyacrylamide gels, transferred to a PVDF-Plus membrane, and detected immunologically with mouse anti-human E12/E47 monoclonal antibody G193-86 (Pharmingen). Detection was also performed using monoclonal Yae (Santa Cruz Biotechnology), which bound the upper but not the lower form of E2a-Pbx1.

Marrow assays

Marrow was harvested from femurs and tibias of Balb/c female mice, and progenitors purified by centrifugation through Ficoll-Paque (Pharmacia), as described (Kamps and Baltimore, 1993). 1×10^6 progenitors were transferred to each well of a 6-well tissue culture plate, incubated 1.0 h with 1 ml of E2a-Pbx1 retrovirus containing 5×10^5 G418-resistance units, and 1 ml of Marrow Culture Medium (MCM; 500 mls RPMI1640, 10% FBS, 1 × antibiotics [Penicillin, Streptomycin], 1 × glutamine, 16 units/ml GM-CSF) was added. Polybrene was added to 8 μg/ml. After 1 h, 6 ml of MC media was added to dilute the polybrene to 2 μg/ml, and the infection was allowed to proceed 3 days. After 3 days, 4 ml medium was removed and replaced with 4 ml fresh MCM. Non-adherent cells were transferred every 7 days to new plates. Three to fourteen days after infection, cultures contained predominantly differentiated, adherent macrophages, differentiated non-adherent neutrophils, and proliferating non-adherent myeloid progenitors. Populations comprised of predominantly myeloid progenitors arose within 21 days for cells infected with all forms of E2a-Pbx1 that elicited immortalization with the exception of those infected with E2a-Pbx1$_{691Term}$, which arose within 30 days due to their protracted doubling time (42 vs 22 h).

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