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The highest affinity DNA element bound by Pbx complexes in t(1;19) leukemic cells fails to mediate cooperative DNA-binding or cooperative transactivation by E2a-Pbx1 and Class I Hox proteins – evidence for selective targetting of E2a-Pbx1 to a subset of Pbx-recognition elements

Paul S Knoepfler and Mark P Kamps

Department of Pathology, University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, California 92093, USA

Oncoprotein E2a-Pbx1 contains the N-terminal transactivation domains of E2a and the majority of the homeodomain protein, Pbx1. Using recombinant proteins, both Pbx1 and E2a-Pbx1 heterodimerize with Hox proteins on bipartite elements, Pbx1 binding a 5′ TGAT core and Class I Hox proteins binding adjacent 3′ TAAT, TTAT, or TGAT cores. In contrast to these *in vitro* results, nuclear extracts from E2a-Pbx1-transformed cells assemble an abundant Pbx-containing complex on TGATTGAT that excludes E2a-Pbx1, suggesting that an uncharacterized *in vivo* partner discriminates between E2a-Pbx1 and Pbx proteins, distinguishing it from Hox proteins. Here, we describe the DNA-binding properties of this complex, and identify TGATTGAC (PCE; *P*bx *C*onsensus *E*lement) as its optimal recognition motif. *In vitro*, the PCE fails to bind heterodimers of Class I Hox proteins plus either Pbx1 or E2a-Pbx1. Likewise, *in vivo*, the PCE fails to mediate cooperative transactivation by E2a-Pbx1 plus Class I Hox proteins. Thus, the PCE binds a Pbx dimer partner that behaves unlike Class I Hox proteins. Competition analysis indicates that the Pbx-containing complex that binds the PCE also binds the TGATTGAT Pbx-Hox element and binds promoter elements required for tissue-specific expression of a number of cellular genes. Thus, different Pbx partners dictate targetting of Pbx heterodimers to related DNA motifs that differ in the sequence of their 3′ half-sites, and E2a-Pbx1 heterodimerizes with only a subset of Pbx partners, restricting its potential DNA targets.

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

Introduction

Pbx1 is a homeodomain (HD) protein first identified as the chromosome one participant of the t(1;19) translocation, which is found in 20% of pediatric pre-B cell acute lymphocytic leukemia (ALL; Kamps *et al*., 1990; Nourse *et al*., 1990). The 3′ portion of the PBX1 gene, including the homeobox, is fused to the 5′ portion of the E2A gene, which encodes two transactivation domains. The resulting chimeric protein, E2A-Pbx1, induces myeloblastic and T-lymphoblastic leukemias in mice, blocks differentiation of primary cultured myeloblasts without altering factor dependence, and induces foci in NIH3T3 fibroblasts (Kamps *et al*., 1990; Kamps and Baltimore, 1993; Dedera *et al*., 1993). In the same assays, Pbx1 has no transforming potential, and because disruption of the transactivation function of E2a abolishes all forms of transformation by E2a-Pbx1, E2a-Pbx1 is suggested to induce transformation by transcriptional activation of specific cellular genes (Van Dijk *et al*., 1993; LeBrun and Cleary, 1994; Lu *et al*., 1994). Interestingly, DNA-binding by the Pbx1 HD is not required for the focus-forming ability of E2A-Pbx1 nor for its ability to induce T-cell ALL (Monica *et al*., 1991; Kamps *et al*., 1996), even though it remains important for the efficient ability of E2a-Pbx1 to block myeloid differentiation. This suggests that E2A-Pbx1 activates transcription of two distinct subsets of genes, one that mediates growth stimulation and a second that disrupts differentiation.

The Pbx gene family is comprised of Pbx1, Pbx2, and Pbx3, all of which exhibit ubiquitous expression except for Pbx1, which is not expressed in the B and T cell lineages (Monica *et al*., 1991; Roberts *et al*., 1995). While the internal 75% of Pbx proteins, which includes the HD, are highly conserved, the N- and C-terminal ends of Pbx proteins are divergent, implying that these regions mediate unique functions. Extradentical (Exd) is a Pbx homolog in *D. Melanogaster* that binds DNA cooperatively with several homeotic selector proteins (Rauskolb *et al*., 1993; Rauskolb and Wieschaus, 1994; van Dijk and Murre C, 1994), and together with these HD proteins, induces appropriate transcription of differentiation genes. This observation laid the foundation for the subsequent discovery that Pbx1 exhibits cooperative DNA-binding with the mammalian homologs of the HOM-C proteins, the Hox proteins, as well as with the Engrailed family of HD proteins. To date, all DNA-binding properties of E2A-Pbx1 have been found to be identical to those of normal Pbx proteins (Lu *et al*., 1995). Both Pbx1 and E2A-Pbx1 dimerize with Class I Hox proteins on half-sites in the motifs, TGATTGAT, TGATTAAT, and TGATTTAT, in which the Pbx1 protein binds the 5′ TGAT core, and the Hox protein occupies the 3′ TGAT, TAAT, or TTAT cores (Chang *et al*., 1996; Knoepfler *et al*., 1996; Lu and Kamps, 1997). Both Pbx1 and Pbx2 exhibit identical DNA-binding profiles for various DNA motifs. Separation of the tetrameric cores by even a single base insertion disrupts complex formation for combinations of all Hox proteins with either E2a-Pbx1 or Pbx1 (Chang *et*

Correspondence: PS Knoepfler
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al., 1996; Knoepfler *et al*., 1996). Heterodimerization of Hox proteins with either Pbx1 or E2a-Pbx1 requires the Hox HD and a tryptophane-containing N-terminal peptide motif (Johnson *et al*., 1995; Chang *et al*., 1995; Phelan *et al*., 1995; Knoepfler and Kamps, 1995; Neuteboom *et al*., 1995; Peers *et al*., 1995) and requires the Pbx HD and 17 C-terminal residues, which are retained in E2a-Pbx1 and are highly conserved among Pbx family members (Chang *et al*., 1995; Lu and Kamps, 1996). In these complexes, interaction of the Hox N-terminal peptide motif with either Pbx1 or E2a-Pbx1 alters the DNA-binding specificity of the Hox protein at position 2 of its four base-pair core, from TAAT to either TGAT or TTAT (Chang *et al*., 1996; Lu and Kamps, 1997); however, the Hox protein retains inherent DNA-binding specificity for the dinucleotide GG, GA, TG, or TA 3′ to the Hox core (Knoepfler *et al*., 1996).

Pbx/Hox motifs are proposed to be bound by Pbx/Hox heterodimers *in vivo* and are required for expression of tissue specific genes. Pbx/Hox heterodimers are thus hypothesized to be important regulators of differentiation. For example, the HoxB1 promoter contains three repeats – TGATGGAT, AGATTGAT, and TGATTGAA – that are required for expression in rhombomere 4 of the hindbrain, and are bound by Pbx and a factor proposed but not proven to be HoxB1 itself (Popperl *et al*., 1995). During retinoic acid (RA)-induced neuronal differentiation of embryonal carcinoma cells, both Hox gene expression and Pbx protein production is strongly upregulated, suggesting that RA controls the formation of potential Pbx and Hox heterodimers by regulating their temporal and spatial coexpression (Knoepfler and Kamps, 1997). In the somatostatin promoter, a Pbx-STF-1 heterodimer binds the sequence TGATTAAT, an element that contributes to expression of somatostatin in pancreatic islet cells and cotransfection of vectors expressing E2A-Pbx1 and STF-1 activates reporter gene expression synergistically through this element. *In vitro*, both Pbx1 and E2a-Pbx1 heterodimerize with STF-1 on this TGATTAAT element (Peers *et al*., 1995). Pbx1 and a hitherto unidentified partner also bind a cAMP-inducible regulatory element (TTGATGGACA) within the promoter of the human CYP17 gene that is related to both the PRS and the elements within the HoxB1 promoter (Kagawa *et al*., 1994).

In spite of the fact that E2a-Pbx1 and normal Pbx proteins exhibit identical abilities to heterodimerize with Hox proteins *in vitro*, as well as identical DNA-binding specificities as heterodimers with Hox proteins *in vitro*, the Pbx-Hox motif, TGATTGAT binds an abundant Pbx-containing complex in nuclear extracts from t(1;19) cells, but fails to bind E2a-Pbx1 (Lu *et al*., 1994), and the alternative Pbx-Hox motifs, TGATTAAT and TGATTTAT, fail to bind this complex substantially. This suggested that a Pbx heterodimer partner exists in pre-B cells that is biochemically distinct from Class I Hox proteins by virtue of its ability to discriminate between E2a-Pbx1 and Pbx proteins, and opened the possibility that Pbx proteins might heterodimerize with other factors whose DNA sequence specificity differs from that of Hox proteins. At the level of leukemogenesis by E2a-Pbx1, the existence of such an activity would indicate that the biochemical targets of E2a-Pbx1 in pre-B cells may be restricted to a subset of genes transcriptionally regulated by Pbx motifs in which the heterodimer partner retains an efficient ability to dimerize with E2a-Pbx1.

Here we provide evidence that this widely expressed cellular factor is a nuclear protein that differs fundamentally from Class I Hox proteins by its selective affinity for heterodimerizing with Pbx proteins (one of which is Pbx2) on TGATTGAC, a sequence that is incapable of efficiently assembling Pbx-Hox heterodimers. While E2a-Pbx1 in t(1;19) cell nuclear extract failed to bind TGATTGAC in conjunction with this cellular factor, it efficiently bound the TGATTGAT motif when an exogenous Hox partner was added, demonstrating that E2a-Pbx1 is present and active in such extracts. Similarly, while

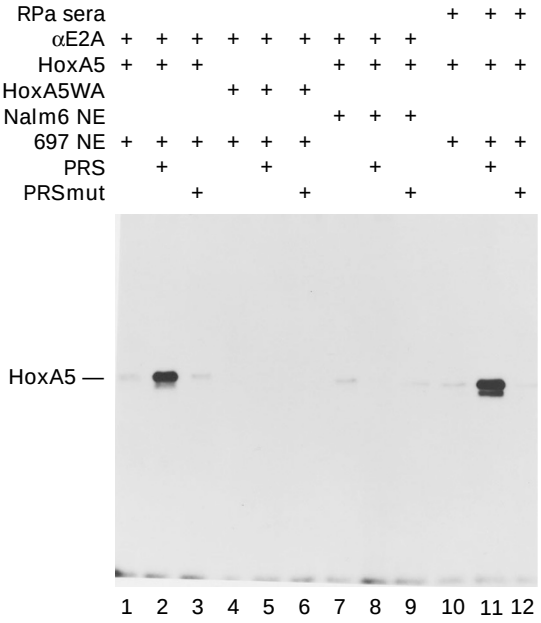


Figure 1 Antibodies to Pbx1 or E2a-Pbx1 can be used to immunoaffinity purify complexes containing DNA, Pbx proteins, and heterodimer partners. The ability of antisera to E2a (lanes 1–9) or Pbx1 (RPa; lanes 10–12) were tested for their ability to immunoprecipitate exogenously added [^{35}S]-labeled Hox-A5 from a mixture of specific wild type oligonucleotide (lanes, 2, 5, 8, and 11) or mutant oligonucleotide (lanes 3, 6, 9, and 12) and 697 pre-B cell nuclear extract (NE), which supplied endogenous E2a-Pbx1 and Pbx proteins (lanes 1–6 and 10–12), or Nalm-6 NE (lanes 7–9) which supplied endogenous Pbx proteins. Reactions were also conducted in the absence of added DNA (lanes 1, 4, 7, and 10) or with a DNA-binding mutant of Hox-A5 (Hox-A5WA), containing a typtophan to alanine conversion at residue 135 (lanes 4, 5, and 6)

Position(5'-3')

Base	-1	1	2	3	4	5	6	7	8	9	10	11	12	13
T	23	46	**91**	4	4	**59**	**96**	0	0	0	4	41	14	19
G	18	36	0	**96**	0	27	4	**100**	27	0	32	46	46	38
C	27	5	0	0	4	14	0	0	0	**96**	9	14	32	25
A	32	14	9	0	**91**	0	0	0	**73**	4	**55**	0	9	19
Consensus	N	T/G	T	G	A	T	T	G	A	C	A/G	G/T	G/C	N

Figure 2 TGATTGAC is the most common motif selected by Pbx-containing nuclear complexes from the t(1;19) pre-B cell line, 697. Representation of each nucleotide as positions -1 to 13 of the PCE consensus element. All percentages displayed in the boxes are based upon this subgroup of 22

E2a-Pbx1 exhibits strong cooperative transactivation with Class I Hox proteins through the TGATTGAT motif, it failed to cooperate with Hox proteins to activate transcription through the TGATTGAC motif. Collectively, these data suggest that different types of Pbx partners dictate targeting of Pbx heterodimers to related DNA motifs and may restrict targeting of E2a-Pbx1 to only a subset of those motifs.

Results

Antibodies to Pbx1 or E2a can be used to isolate DNA-protein complexes of endogenous Pbx proteins or E2a-Pbx1 heterodimerized with exogenously added Hox proteins

As a prelude to determining the highest affinity DNA motifs bound by E2a-Pbx1 and Pbx proteins in t(1;19) cell nuclear extract, antisera to Pbx1 or E2a were tested for their ability to immunoprecipitate an exogenously added ^{35}S-labeled Hox partner (HoxA5) from a mixture of specific oligonucleotide and nuclear extract (NE) from 697 pre-B cells, which contain the t(1;19) translocation and thus supply E2a-Pbx1 as well as endogenous Pbx proteins (Monica *et al*., 1991; Figure 1). Two antisera raised to recombinant Pbx1 designated RPa and RPb were used in this and subsequent experiments (see Materials and methods for description). RPa cross-reacted with endogenous Pbx proteins in 697 nuclear extract by Western blot analysis, did not disrupt complex formation in gel-shift experiments, and did not supershift well in gel-shift analysis. Like RPa, RPb serum cross-reacted with Pbx proteins on Western blots, but unlike RPa, RPb serum quantitatively supershifted Pbx-containing gel-shift complexes. The anti-E2a serum supershifted and did not disrupt E2a-

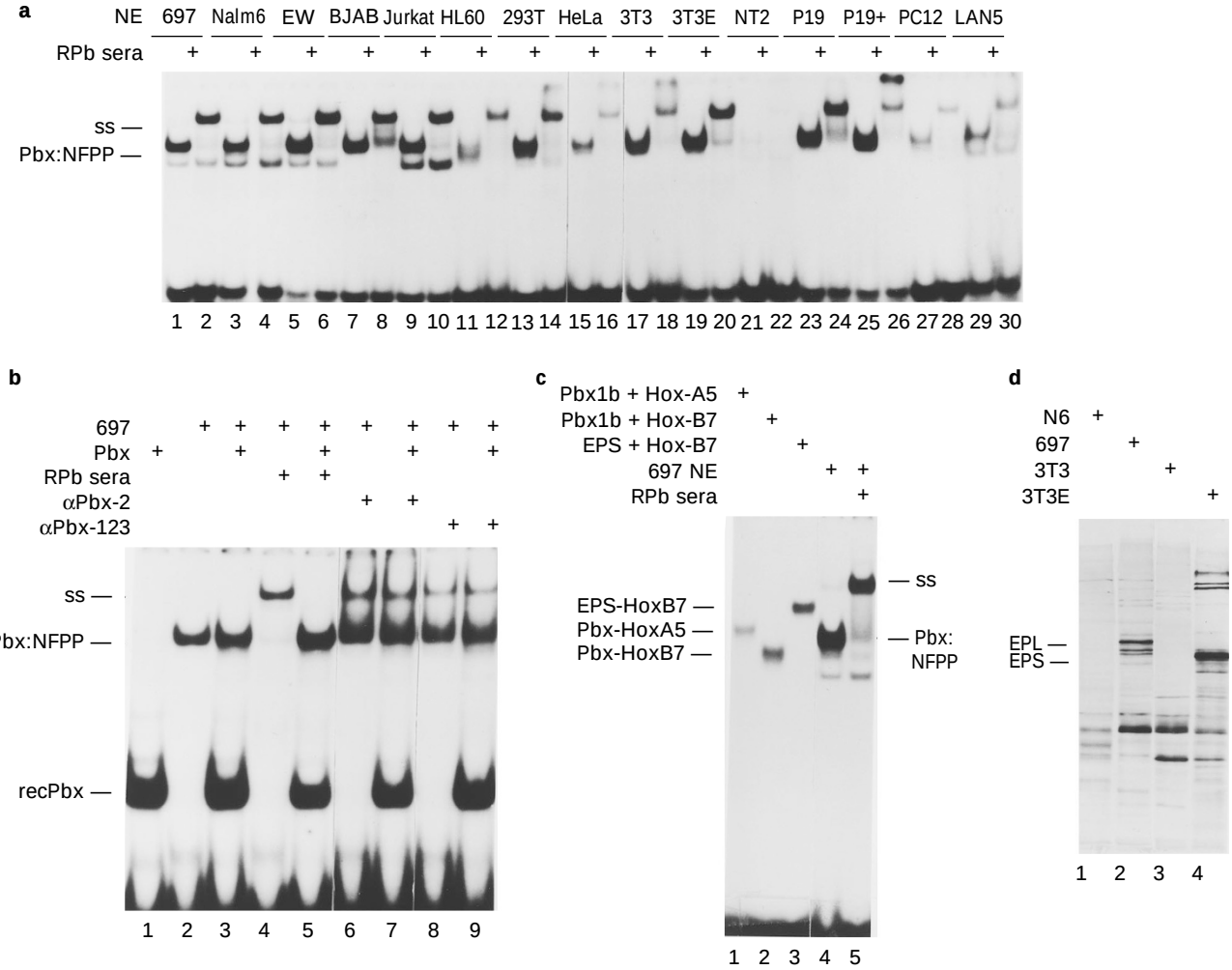


Figure 3 The PCE binds a Pbx-containing complex in 697 pre-B cells and in many other cell types. (**a**) Examination of the abundance of the PCE complex using EMSA and nuclear extracts from 15 human and mouse cell types designated above each lane. 3T3E designates NIH3T3 transformed by E2a-Pbx1 (lanes 19 and 20). Supershift analysis (even numbered lanes) was performed by addition of the cross-reactive anti-Pbx1 serum. (**b**) Analysis of the presence of Pbx proteins in the PCE complex. The ability of different anti-Pbx sera to supershift the PCE complex was tested in the absence (lanes 2, 4, 6, 8) or the presence (lanes 1, 3, 5, 7) of competing recombinant Pbx1b. The recombinant Pbx1b encodes a C-terminus that differs from that of Pbx1a, which is also contained in Pbx2 and Pbx3a, and which contains the peptide sequence used to generate the αPbx-123 serum. The antisera used for supershifting are cross-reactive anti-Pbx1 serum (RPb; lanes 4, 5), anti-peptide Pbx2-specific serum (αPbx-2; lanes 6, 7) and anti-peptide Pbx-cross-reactive serum (αPbx-123; lanes 8, 9). (**c**) The mobility of the Pbx : NFPP complex (lane 4) was compared to that of heterodimers of Hox proteins with either Pbx1 (lanes 1, 2) or E2a-Pbx1 (lane 3) formed on the PRS motif. (**d**) Abundance of E2a-Pbx1 in nuclear extracts from Nalm-6 (lane 1) 697 (lane 2), NIH3T3 (lane 3) and E2a-Pbx1-transformed NIH3T3 (lane 4) cells, analysed by immunoblotting with the cross-reactive RPb sera

Pbx1:Hox:DNA complexes (data not shown). Therefore, both antibodies could be used as reagents to purify simultaneously both endogenous partners and DNA motifs that complex with Pbx proteins or with E2a-Pbx1. Using t(1;19) cell extract and either anti-E2a-serum (lanes 1–3) or RPa serum (lanes 10–12), copurification of ^{35}S-labeled HoxA5 was dependent upon specific addition of the Pbx-Hox motif, TGATTGAT (PRS; lanes 2, 11), with only background levels isolated in the absence of DNA (lanes 1, 10) or in the presence of a mutant version of the PRS (lanes 3, 12). Using identical conditions, a cooperative DNA-binding mutant of HoxA5 (HoxA5W179A) was not copurified (lanes 4–6). Nuclear extracts from Nalm-6 cells, which lack the t(1;19) translocation, were incapable of supplying the factor required for isolation of HoxA5 by anti-E2a serum in either the presence or absence of the TGATTGAT oligonucleotide (lanes 7–9), but resulted in the efficient isolation of HoxA5 by RPa serum (not shown). Therefore, as formerly demonstrated for recombinant Pbx1 and E2a-Pbx1, endogenous E2a-Pbx1 and Pbx proteins in pre-B cells also heterodimerize with Hox proteins. This coimmunoprecipitation technique was therefore used to screen a random oligonucleotide library exposed to 697 cell nuclear extract in order to identify the optimal DNA motif bound by both Pbx proteins and E2a-Pbx1 in conjunction with endogenous cellular factors.

TGATTGAC is the motif specifically selected by Pbx-containing nuclear complexes from the t(1;19) pre-B cell line, 697

Immunoprecipitation with *RPa serum* was used to affinity purify oligonucleotides sequences from a pool of random oligonucleotides mixed with 697 cell nuclear extract and selected oligonucleotides were amplified by PCR. This selection-amplification procedure was repeated nine times. Sequence analysis of 48 selected oligonucleotides revealed that 22 oligonucleotides or 46% shared the same TGATTGAC motif with 0–3 mismatches (Figure 2). None of the selected oligonucleotides contained any previously-characterized Pbx-Hox motifs (TGATTGAT, TGATTAAT, or TGATTTAT). Seven oligonucleotides (15%) contained the exact TGATTGAC consensus. This site was termed the *P*bx *c*onsensus *e*lement (PCE). The PCE was similar to all Pbx elements in its content of a 5′ TGAT half-site, which is predicted to bind a Pbx protein, and was most related to a TGATTGAT Pbx-Hox motif in its 3′ half-site, differing only in its content of cytosine at the fourth base (TGA*C*). No consensus motif for E2a-Pbx1 was identified by using an anti-E2a serum in conjunction with this same selection protocol, or by using an anti-epitope serum in conjunction with a protocol in which *in vitro* translated, epitope-tagged, E2a-Pbx1 was added exogenously to nuclear extracts, suggesting that the binding properties of E2a-Pbx1 with the PCE partner differ substantially from those of Pbx proteins.

The PCE binds a Pbx-containing complex in 697 pre-B cells as well as in many other cell types

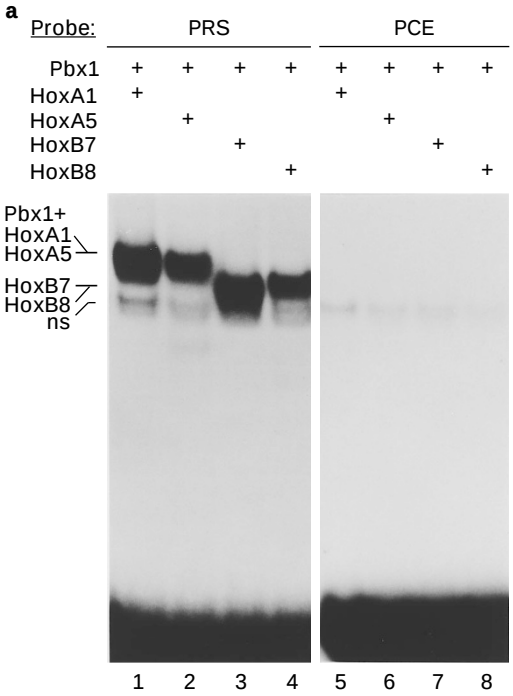
The PCE bound an abundant complex in both human cell lines (697 and Nalm-6 pre-B cells, EW and BJAB B-cells, Jurkat T cells, HL60 myelomonocytic cells, 293T and HeLa epithelial cells, NT2 embryonic carcinoma cells and LA-N-5 neuroblastoma cells) and in murine cell lines (both undifferentiated and differentiated P19 cells, NIH3T3 fibroblasts, PC12 neuronal pheochromocytoma) that was quantitatively supershifted by RBb serum (see Materials and methods for description; Figure 3a). 697 and Nalm-6-pre-B cells, BJAB and EW B cell, and Jurkat T cell nuclear extracts contained one band representing one or possibly multiple types of complexes, while nuclear extracts from most other cell lines (e.g. 293T) contained a second smaller complex. The fact that RPa serum was used to isolate the PCE while RPb serum was used to verify the presence of Pbx proteins bound to the PCE by supershift analysis strongly suggested that the PCE was selected as a Pbx-specific consensus binding motif. To address the unlikely possibility that antibodies in this serum might bind a totally unrelated transcription factor that recognized the PCE and therefore also supershifted this complex in EMSA, the ability of recombinant Pbx1 protein to block the ability of RPb sera to supershift the complex was examined. While recombinant Pbx1b did not block formation of the PCE complex (Figure 3b, lane 3), it eliminated the ability of the RPb serum to supershift the PCE complex (lane 4). To demonstrate that the RPa serum bound bona fide Pbx family members rather than a factor related to but distinct from Pbx proteins, an anti-peptide serum that bound specifically to Pbx2 (designated αPbx-2), and a second anti-peptide serum that bound a conserved sequence in the carboxyl terminus of Pbx1a, Pbx2, and Pbx3a (designated αPbx-123) were used in supershift analysis. Both antisera supershifted approximately 20% of the PCE complex (lanes 6 and 8). In neither case, was this supershift blocked by preincubation with recombinant Pbx1b protein (lanes 7 and 9), consistent with the facts that recombinant Pbx1b contains neither the unique Pbx2 peptide nor the 'b' type carboxylterminal sequence. Addition of fivefold more anti-Pbx2 serum did not induce the formation of a second shifted complex, suggesting that the complex is not a Pbx2 homodimer (data not shown).

The mobility of the PCE complex was similar to that of Pbx-Hox heterodimers, suggesting it is comprised of a dimers of a Pbx protein and a heretofore uncharacterized partner (Figure 3c, lanes 1, 2, and 4). The mobility of the PCE complex was also surprisingly homogenous in light of a much broader range of mobilities of different Pbx-Hox complexes in this region of the gel (Lu *et al*., 1995). For the purpose of its initial characterization, we designate this activity that heterodimerizes with Pbx proteins as NFPP, for 'nuclear factor Pbx partner'. This NE screen suggested that NFPP was not a Class I Hox protein because undifferentiated P19 cells generally do not expresss Class I Hox genes. The PCE failed to bind a larger E2a-Pbx1-containing complex when mixed with nuclear extracts from 697 pre-B cells or from E2a-Pbx1-transformed NIH3T3 fibroblasts (Figure 3a, lanes 1, 2, 19, 20), suggesting that E2a-Pbx1 fails to heterodimerize with NFPP. Both lines contained E2a-Pbx1 in their nuclear extracts, and the E2a-Pbx1-transformed NIH3T3 fibroblasts contained approximately five times as much E2a-Pbx1 as did the 697 cells (Figure 3d, lanes

1–4). Failure to heterodimerize with E2a-Pbx1 distinguished NFPP from Class I Hox proteins, which heterodimerize as efficiently with E2a-Pbx1 as they do with Pbx proteins (Lu *et al*., 1995).

Class I Hox proteins and NFPP exhibit different DNA-binding specificities

To compare the DNA-binding specificities of NFPP with that of Class I Hox proteins, the ability of recombinant Hox proteins to heterodimerize on the PCE was determined. All Class I Hox proteins tested (HoxA1, HoxA5, HoxB7, or HoxB8) failed to form heterodimers with Pbx1 on the PCE, while all formed heterodimers with Pbx1 on a TGATTGAT (PRS) element (Figure 4a). To test the function of complexes that form on the PCE *in vivo*, CAT assays were performed in Nalm-6 pre-B cells using reporters driven by a 4X PCE element. The PCE did not activate basal transcription, was very poorly activated by E2a-Pbx1



c

Probes		Pre-B ALL complexation		Pbx1:Hox complexation	
12345678910 11	Form	PCE	PRS	PCE	PRS
TTGATTGA C/T AG	WT	100	87	2	100
C	T5C	64	46	2	5
G	T6G	95	62	4	36
A	G7A	34	7	2	56
G	A8G	61	25	1	0
TA	AG10/11TA	88	95	1	147
GA	AG10/11GA	43	78	19	188
CC	AG10/11CC	81	58	0	17
AC	AG10/11AC	103	46	1	35

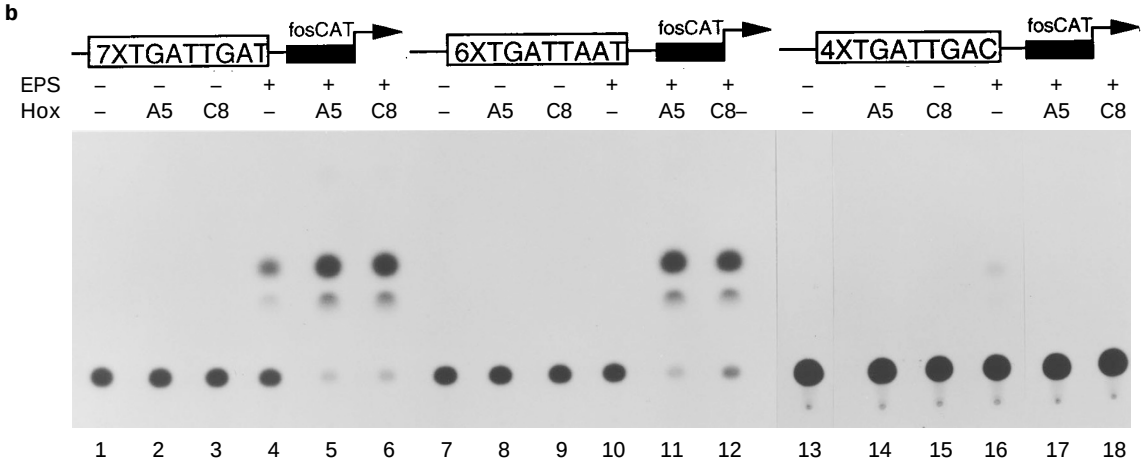


Figure 4 The DNA binding specificity of the Pbx-NFPP complex suggests that NFPP is not a Class I Hox protein. (**a**) Heterodimers of Pbx1 and Hox proteins bind the PRS but not the PCE. The ability of the PRS (lanes 1–4) and the PCE (lanes 5–8) to bind heterodimers of Pbx1 and HoxA1 (lanes 1, 5), HoxA5 (lanes 2, 6), HoxB7 (lanes 3,7) and HoxB8 (lanes 4, 8) was examined by EMSA. (**b**) The PRS, but not the PCE, orchestrates transactivation by E2a-Pbx1 plus Hox proteins. CAT reporter constructs indicated above each panel were contransfected in Nalm-6 pre-B cells together with vectors expressing E2a-Pbx1 (EPS) or the indicated Hox proteins. (**c**) Effects of base substitutions on binding of Pbx-NFPP to the PCE and on binding of Pbx and Hox proteins to the PRS. Binding is expressed as a percentage relative to 100% for the abundance of the pre-B ALL complex formed on the PCE and 100% for the abundance of the Pbx : Hox complexes formed on the PRS. The composite motif indicated at left is identical to the PCE when the base at position 9 is a C, and identical to the PRS when the base at position 9 is T. All data for Pbx : Hox complex abundance are the average of Pbx complexation with HoxA1, HoxA5, and HoxB7 and in each case 2–3 separate experiments were conducted, except for probes T6G, Ag-10/11CC and AG-10/11AC, which represent single data points. All data for *in vivo* complex abundances are the averages of 3–5 separate experiments, except for probes T6G, AG-10/11CC, AG-10/11AC, and specifically PCE-A8G and PCE-AG-10/11-GA which represent single data points. The wild type probes of the PRS and PCE were as follows, with the first base in case representing base number one for reference for base variants: PRS(TTGATTGATAG) and PCE(TTGATTGACAG)

(Figure 4b, lane 16), and orchestrated no cooperative transactivation by E2a-Pbx1 plus Hox proteins (lanes 17 and 18). In contrast, Pbx-Hox heterodimer elements orchestrated efficient cooperative transactivation by E2a-Pbx1 plus Hox proteins (lanes 5, 6, 11, 12). Because of the similarity of the PCE to a cAMP-responsive element (TGATGGACAG) within the CYP17 promoter, the ability of the PCE to mediate protein kinase A-inducible transcriptional activation was tested; however, transcription from the 4xPCE-CAT construct was less than twofold inducible by cotransfection with a protein kinase A expression vector (data not shown).

To further address the issue of the DNA binding specificity of Pbx:NFPP and Pbx:Hox complexes, identical mutations were introduced into PRS and PCE elements and the effect of these mutations on complexation of Pbx and Hox proteins to the PRS *in vitro* were compared with their effects on binding of the nuclear complex. Seven of eight variants of the PCE exhibited no complex formation by Pbx and Hox proteins, while a GA 3′ dinucleotide variant yield a low abundance complex (Figure 4c). Each of these mutants exhibited unimpaired or strong binding to the Pbx-NFPP nuclear extract complex. To further contrast NFPP binding specificity to that of Class I Hox proteins, the effects of nucleotide substitutions on the binding of Pbx-NFPP to a PCE was compared with the effect of the same mutations on binding of Pbx-Hox heterodimers to a PRS (Figure 4c). Conversion of T5 to C, which creates a TGA*C* Pbx half-site, reduced Pbx-Hox heterodimer formation on the PRS by 95% while reducing the abundance of the Pbx-NFPP complex on the PCE by only 36%. Altering the A8 to G, the position predicted to contact the ubiquitous Asparagine 51 of the HD, extinguished Pbx-Hox binding to the PRS based element, but reduced binding of the cellular complex to the PCE by only 39%. Similarly, conversion of the dinucleotide at the 3′ edge (positions 10/11) of the Hox core sequence to combinations known to destabilize Pbx-Hox binding (CC or AC) reduced Pbx-Hox complexation by 83% and 65% respectively, while having little effect on the binding of the endogenous complex. Conversion of this dinucleotide to GA increased binding of Pbx-Hox complexes to the PRS-based probe by 80%, but reduced binding of the cellular complex to the PCE probe by almost 60%. The same GA dinucleotide conversion mediated a small degree of Pbx:Hox complexation on the PCE, suggesting that Pbx:Hox complex may weakly associate with PCE elements if provided with optimal 3′ dinucleotides, but providing further evidence that NFPP is not a Class I Hox protein because GA is clearly not optimal for NFPP.

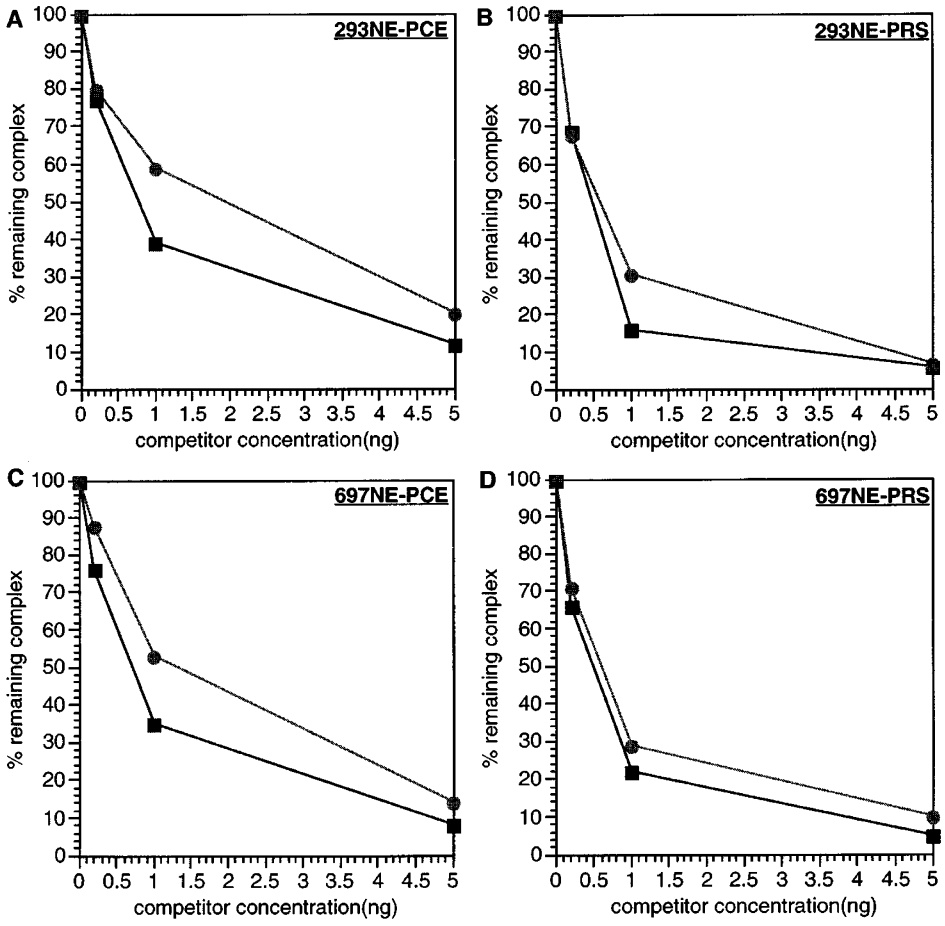


Figure 5 Competition analysis suggests that Pbx-NFPP is the major activity in nuclear extracts that binds canonical Pbx-Hox motifs. (**a**) Competition analysis was conducted using a PCE (**a**) or PRS (**b**) probe, 293 cell NE, and the PRS and PCE as competitors. (**c**) Competition analysis was conducted using the PCE (**c**) or PRS (**d**) probes, 697 cell NE, and the PRS and PCE as competitors. Data points represent the averages of two experiments. (Squares represent the PCE competitor and circles represent the PRS competitor).

Collectively, these data suggest that Class I Hox proteins and NFPP exhibit different DNA-binding specificities.

Competition analysis suggests that Pbx-NFPP is the major cellular activity that binds a subset of Pbx-Hox motifs

The possibility that NFPP was the nuclear extract activity that bound the TGATTGAT Pbx-Hox motif was further tested by competition analysis. As expected, TGATTGAC competed more effectively than did TGATTGAT in disruption of a Pbx-NFPP complex from 697 pre-B and 293 epithelial cells formed on the PCE (Figure 5a and c). Likewise, the cellular complex binding TGATTGAT was also competed more effectively by the PCE than by TGATTGAT (Figure 5b and d), despite the fact that Pbx-Hox complexes fail to bind the PCE, suggesting again that the cellular complex that binds the PRS is not comprised of Pbx-Hox heterodimers but rather of Pbx-NFPP heterodimers.

Pbx-binding motifs in cellular promoters bind a partner similar to NFPP during gel shift analysis

Promoter elements known to bind Pbx proteins were used as gel-shift probes in conjunction with nuclear extract from undifferentiated and neuronally-differentiated P19 embryonal carcinoma cells, and the affinities

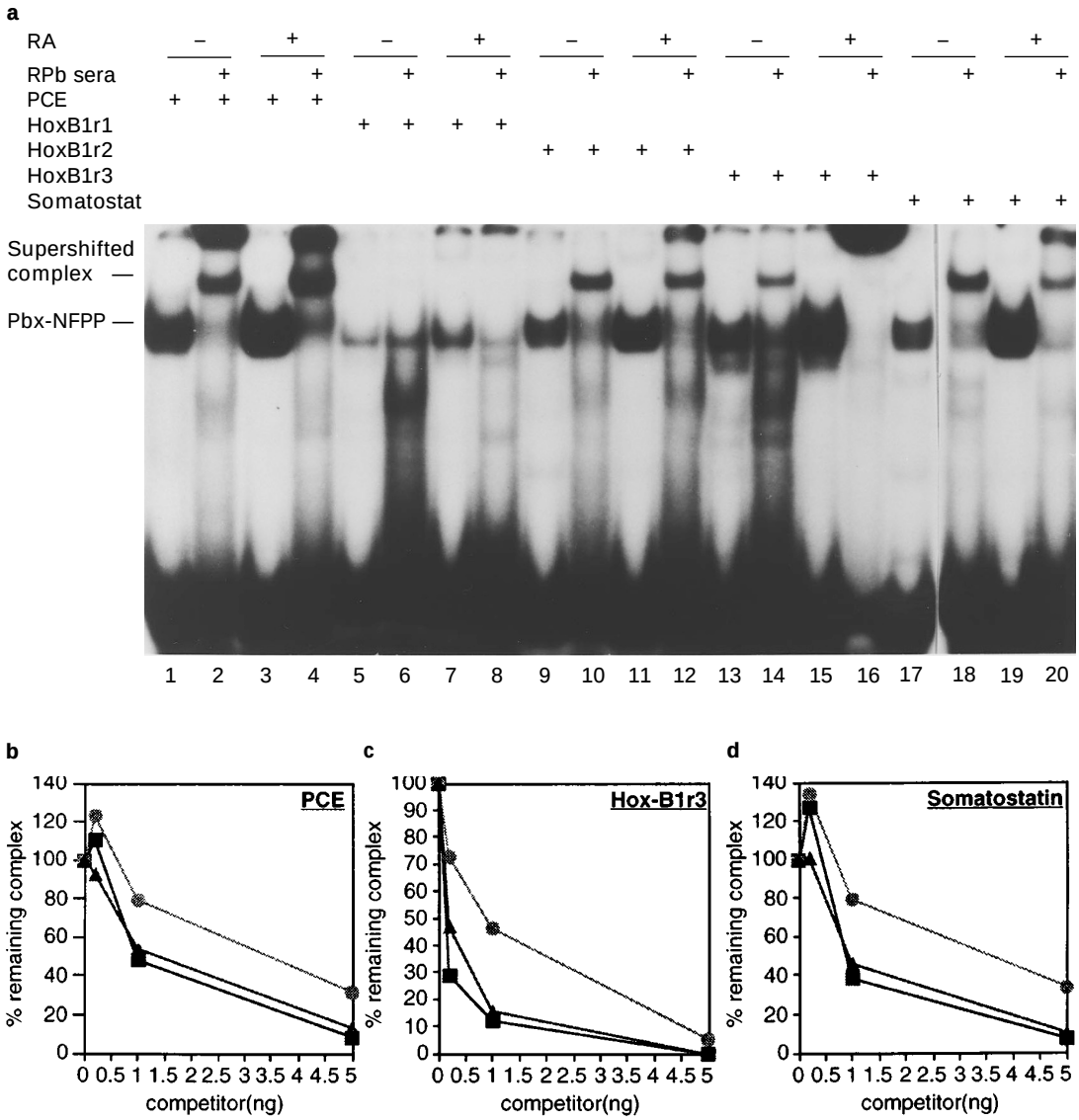


Figure 6 Pbx1-binding motifs in cellular promoters bind a partner similar to NFPP. (**a**) EMSA was conducted using NE from uninduced P19 cells (lanes 1–2, 5–6, 9–10, 13–14, and 17–18) and P19 cells induced to undergo neuronal differentiation with RA (lanes 3–4, 7–8, 11–12, 15–16, and 19–20) and probes containing the specified elements whose sequences are reported in Results. Probes used include the PCE (lanes 1–4), repeat one of the Hox-B1 promoter (HoxB1r1:lanes 5–8), repeat two of the Hox-B1 promoter (HoxB1r2: lanes 9–12), repeat three of the Hox-B1 promoter (HoxB1r3: lanes 13–16), and a PRS-like element from the Somatostatin promoter (lanes 17–20). Complexes formed were tested for their ability to be supershifted by the cross-reactive RBb sera (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20). (**b**) Complexes formed on the PCE (**b**), the Hox-B1r3 (**c**), and the Somatostatin element (**d**) were tested for their ability to be disrupted by oligonucleotide competitors including the PCE, the Hox-B1r3, and Somatostatin element. For (**b**)–(**d**), squares represent the PCE competitor, circles the HoxB1r3 competitor, and triangles the somatostatin element competitor

of nuclear factors for these sites were subjected to competition with the PCE and with the promoter element itself. The human HoxB1 promoter contains three repeats that contribute to normal expression and bind a Pbx-containing complex (repeat 1; AGATGGATGG, repeat 2; TGATTGAAG, and repeat 3; TGATGGATGG). Undifferentiated P19 cells do not express Class I Hox genes, while P19 cells induced to undergo neuronal differentiation by incubation in retinoic acid (RA) for 7 days expression Class I Hox genes. While the HoxB1 repeat 1 failed to form a complex that supershifted with the cross-reactive RPb serum (lanes 5–8), repeats 2 (lanes 9–12) and 3 (lanes 13–16) bound Pbx-containing complexes from NE derived from cells both before and after RA-induced differentiation, and this complex comigrated with those bound to the PCE (lanes 1–4). The somatostatin promoter contains the Pbx-binding motif, TTGATTGATTT, which cooperates with an adjacent element that binds the STF1 homeodomain protein to form a minimal pancreatic beta cell-specific enhancer (Vallejo *et al*., 1992). This sequence also bound a Pbx-containing complex that comigrated with the complex bound to the PCE. Competition experiments demonstrated that the PCE was a more efficient competitor for disruption of complexes formed on both the HoxB1 and somatostatin promoter elements than were these elements themselves (Figure 6b–d), suggesting that they also bind Pbx-NFPP dimers *in vitro*.

Endogenous E2a-Pbx1 actively heterodimerizes with Hox proteins, but not with NFPP, suggesting that E2a-Pbx1 targets Pbx-Hox motifs, but not PCEs

The fact that NE from neither 697 cells nor NIH3T3 fibroblasts transformed by E2a-Pbx1 forms an E2a-Pbx1-containing gel shift complex on either the PRS or the PCE suggests that NFPP fails to dimerize with

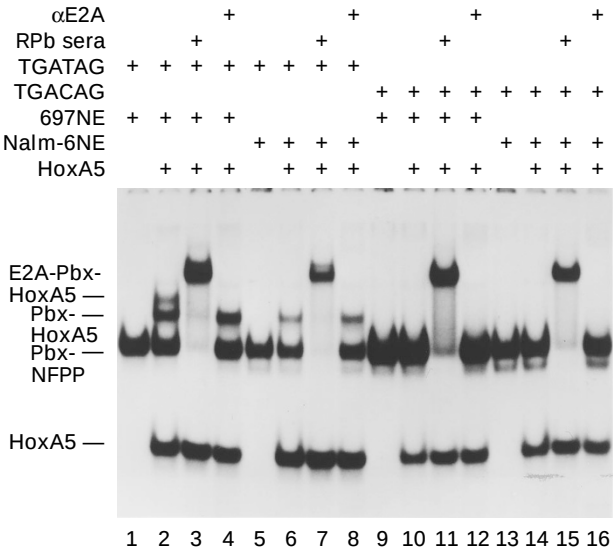


Figure 7 Endogenous E2a-Pbx1 heterodimerizes with a subset of Pbs partners. Exogenous Hox-A5 was added (lanes 2–4, 6–8, 10–12, 14–16) to gel-shift reactions formed on the PRS (lanes 1–8) or PCE (lanes 9–16) probes and using nuclear extracts from 697 (lanes 1–4, 9–12) or Nalm-6 (lanes 5–8, 13–16) cells. The cross-reactive RPb rabbit serum was added to lanes 3, 7, 11, and 15 and an anti-E2a serum to lanes 4, 8, 12, and 16. The free probe was run off the bottom of the gel

E2a-Pbx1, that NFPP is the Pbx partner on both elements, and that neither Pbx-Hox nor E2a-Pbx1-Hox complexes form detectable complexes on bona fide *in vitro* Pbx-Hox motifs. This observation suggests that E2a-Pbx1 only substitutes for Pbx proteins in heterodimer complexes containing a permissive partner, the identity of which is dictated by the sequence of the 3′ half-site. This hypothesis was affirmed by demonstrating that addition of recombinant HoxA5 was capable of producing a new E2a-Pbx1-containing complex bound to the PRS probe, but not to the PCE probe (Figure 7). This complex, which was supershifted by addition of the cross-reactive anti-Pbx serum (lane 3) and was disrupted by addition of anti-E2a serum (lane 4) was formed exclusively on the PRS in nuclear extracts from 697 cells and was not formed on the PCE. Likewise, Pbx-HoxA5 complexes were also restricted in their formation to the PRS. HoxA5 formed monomeric complexes on the 5′ TGAT portion of both probes as observed previously. (Lu *et al*., 1995). Thus, as indicated in Figure 1, E2a-Pbx1 retains active DNA-binding function in t(1;19)-containing cell lines, but it does not substitute for Pbx proteins in Pbx-NFPP complexes, and it is not observed in gel-shift experiments because permissive partners, such as Hox proteins, are either not abundant enough or lack the ability to function as heterodimer partners due to post-translational modification.

Discussion

In this study we utilize endogenous Pbx proteins in nuclear extracts to identify TGATTGAC (the PCE) as the highest-affinity motif for Pbx-containing complexes, and we define the biochemical properties of the complex that forms upon it. In a manner identical to that of DNA motifs selected by recombinant Pbx and Class I Hox proteins, the PCE contains two unspaced half-sites in which the Pbx binding site (TGAT) comprises the 5′ half of the motif, suggesting that the orientation and juxtaposition of Pbx and a cellular factor (here designated NFPP) are similar to that of Pbx and Hox proteins. Numerous observations, however, suggested that NFPP is not a Class I Hox protein. Unlike Pbx-Hox motifs, the PCE contains a 3′ TGAC motif, creating an overall sequence that is not recognized by Pbx-Hox heterodimers, and suggesting that while NFPP is a high-affinity partner for endogenous Pbx proteins, it differs fundamentally from previously-characterized Class I Hox proteins. The Pbx-NFPP complex was further distinct in its recognition of a variant motif in which the third A in the NFPP binding core, which is essential for Pbx-Hox binding to TGATTGAT and which binds the invariant Asn 51 in the Hox HD, could be altered to G without appreciable loss of complex formation. The fact that a hydrogen bond between Asp51 of Hox HDs and this adenine is essential for binding of Hox proteins to their DNA targets suggests that NFPP may not be a HD protein (Desplan *et al*., 1988; Kissinger *et al*., 1990; Laughon, 1991; Gehring *et al*., 1994). NFPP was also distinct from Class I Hox proteins in its failure to form heterodimers with endogenous E2a-Pbx1, even though E2a-Pbx1 was active as demonstrated by its ability to heterodimerize with exogenous HoxA5 on a PRS.

Finally, the fact that NFPP was present in NE from uninduced P19 cells, which generally do not express Class I Hox proteins suggests that NFPP is not a Class I Hox protein (Simeone *et al*., 1990, 1991; Zwartkruis *et al*., 1993), and failure of NFPP to heterodimerize on elements containing 3′ TAAT or TTAT core half-sites indicates NFPP is not a Hox protein from paralogues 3–8, which have been shown to prefer binding to such 3′ cores in conjunction with a 5′ TGAT half-site (Chang *et al*., 1996; Lu and Kamps, 1996).

Failure of E2A-Pbx1 to heterodimerize with NFPP is important because it suggests a model of leukemogenesis in which E2A-Pbx1 and Pbx proteins diverge in function at the level of heterodimeric, DNA-binding specificity. Three types of binding sites would be proposed in such a model. The first, a canonical PCE would be insensitive to the activity of E2a-Pbx1 because its partner, NFPP, fails to heterodimerize strongly with E2a-Pbx1. The second class of elements, represented by TGATTGAT, could bind either Pbx-Hox or Pbx-NFPP heterodimers *in vivo*, and introduction of large amounts of E2a-Pbx1 as a consequence of the t(1;19) translocation would introduce a new E2a-Pbx1-Hox heterodimer that could bind such sites and compete with both Pbx-NFPP and Pbx-Hox heterodimers for occupancy. The third category of site is represented by TGATTTAT or TGATTAAT, two Pbx-Hox motifs that fail to bind appreciable Pbx-NFPP complexes. In this case, E2a-Pbx1 would compete with Pbx proteins for the same set of partners, Hox proteins, to bind this type of recognition motif. *In vivo*, therefore, genes containing TGATTAAT or TGATTTAT elements would be predicted to be most sensitive to transcriptional activation by E2a-Pbx1, while those containing only the PCE should be more refractile. The inability to detect E2a-Pbx1 gel shift complexes on TGATTAAT, TGATTTAT, and TGATTGAT site, as well as on the PCE in nuclear extracts from t(1;19) cells, suggests that if E2a-Pbx1 heterodimerizes with endogenous Hox proteins, these complexes are undetectable in gel shift assays, possibly because of their low abundance or rapid off-rates.

What is NFPP? Several transcription factors bind half-sites containing TGAC, including members of the AP-1 and CREB/ATF families (Kerppola and Curran, 1993). While NFPP could be a member of either of these families, AP1 and CREB/ATF oligonucleotides failed to disrupt Pbx-NFPP complexes and the PCE oligonucleotide had little effect on the abundance of AP1 or CRE/ATF complexes bound to their recognition motifs, while addition of AP1 or CREB/ATF oligonucleotides eliminated such binding (data not shown). These experiments indicate that NFPP is not a components of AP1 or CREB/ATF. The possibility that NFPP is a HD protein but not a Class I Hox proteins is supported by studies of the DNA binding specificity of Hox11 (Tcl-3), a human oncoprotein linked with T cell actue lymphocytic leukemia. A HD fragment of Hox11 binds TAAC and to a lesser extent TAAT (Dear *et al*., 1993), while full-length Hox11 exhibited a preference for binding TAAG (Tang and Breitman, 1995). The ability of Hox11 to recognize bases other than T at the fourth position of its binding site is due to the presence of a unique threonine residue at position 47 of its HD, which when mutated to isoleucine interconverts DNA-binding specificity from TAAC to TAAT (Dear *et al*., 1993). A HD protein preferring a TAAC core could easily alter its second-position DNA-binding specificity to TTAC or TGAC in conjunction with Pbx1, as observed for Class I Hox proteins, and thus account for the activity that cooperates with Pbx proteins on the PCE. In contrast, NFPP might not be a HD protein. An example of such interaction between a HD and non-HD protein is illustrated by the ability of the MADS box transcription factor, serum response factor (SRF), to dimerize with members of the prd class of HD proteins on specific elements (Grueneberg *et al*., 1992). The fact that NFPP may not be a Hox protein is significant because Hox and Hox-related proteins are the only known partners of the Pbx family of proteins and have been presumed to be the dominant coregulators with both Pbx proteins and E2A-Pbx1.

Formally, NFPP could be another Pbx protein, the complex being comprised of Pbx homodimers, such as Pbx2/Pbx2, or Pbx heterodimers, such as Pbx3/Pbx2 heterodimers. However, if Pbx proteins dimerize, it is unlikely that they form homodimers, because recombinant Pbx1 does not dimerize on the PCE, and endogenous Pbx2 does not appear to dimerize on the PCE, evidenced by the fact that addition of fivefold more anti-Pbx2 N-terminal peptide sera did not produce a second supershift band that would be indicative of the binding of two antibody molecules. If Pbx heterodimers do form *in vivo*, the fact that the 3′ half-site of TGAC is paired with the 5′ half-site of TGAT would suggest that dimerization alters the DNA binding specificity of the Pbx protein bound to the 3′ half-site such that it now prefers a TGAC site. Interestingly, TGACTGAT probes can mediate weak Pbx1 : Hox dimerization suggesting that Pbx2 can bind a 5′ TGAC half-site (Knoepfler, unpublished observation) and indicating that dimerization of Pbx proteins on the PCE is at least theoretically possible in terms of DNA-binding specificity, though it is not directly supported by any data at this point.

A screening of the eukaryotic promoter data base of genbank reveals several promoters that contain perfect core PCE sequences, TGATTGAC (Altschul *et al*., 1990). The PCE is highly conserved in the promoters of the chorion A and B late gene families of B. morai. Analysis of the A/B.L12 chorion bi-directional promoter in B. morai determined that a mutation encompassing the PCE reduces promoter activity more than 100-fold (Spoerel *et al*., 1993) and the PCE is in a promoter region required for *in vivo* expression. Homologs of Pbx and NFPP could form a complex on the chorion promoter, however, transcription factors important for regulation of chorion gene expression are only beginning to be characterized and there is no direct evidence for a role for a Pbx homolog. The promoters of several other genes also contain perfect PCE sequences (human beta-2 tubulin, mouse interferon α5, frog albumin, rat PEPCK, hepatic cs, adeno-associated virus 2, and duck hepatitis B virus); however, their relevance in transcriptional regulation is unknown.

What is the activity of Pbx : NFPP complexes? This

question remains unanswered as of yet, but the data presented herein suggests that Pbx : NFPP may be important for regulating many of the same genes that have been proposed to be regulated by Pbx : Hox dimers. Pbx : NFPP complexes certainly do not function as basal transcription activators. Other assays suggest that Pbx : NFPP may be important for regulation of muscle specific genes during myogenesis (Funk and Wright, 1992). During a screen of a random oligonucleotide library for high affinity myogenenin heterodimer binding sites using a myogenin antibody, a strong selection of E-box motifs juxtaposed to PCE motifs were observed. This interaction was demonstrated to be important for increasing the stability of the myogenin complexes formed on E-boxes. Furthermore, while neither the PCE nor the E-box alone mediated significant transcriptional activation, together they yielded robust transcriptional activation. This suggests that Pbx : NFPP complexes can potentiate transcriptional activation by myogenin heterodimers. Understanding the basis of this form of physical and functional cooperation is clearly essential to understanding the transcriptional role of Pbx proteins. Importantly, the fact that the TGATTGAT site in the somatostatin promoter, which efficiently binds Pbx-NFPP, also synergizes with an adjacent site that binds the STF-1 homeodomain protein to create a minimal tissue-specific enhancer element reiterates a model in which heterodimers containing Pbx may cooperate with cell-type specific factors, such as STF-1 or myogenin, to orchestrate tissue-specific gene transcription. Further detailed analysis of these activities must await the cloning of NFPP and analysis of its function in conjuction with Pbx proteins.

Materials and methods

Selection of in vivo *binding sites by immunoprecipitation of Pbx-containing heterodimers*

A random double-stranded oligonucleotide library was prepared using the 3′ reverse primer, TGAACAGCTCTAGAGCATGC (no.1), and the 5′ forward primer, TACTGTCTGGATATCCTAGC (no.2) to amplify the degenerate oligonucleotide, TACTGTCTGGATATCCTAGC-25N-GCATGCTCTAGAGCTGTTCA, using two cycles of PCR. The double-stranded library was subjected to 9 rounds of selection/PCR amplification, using RPa serum. For selection, 10% of the PCR product was incubated with 1 μl of anti-Pbx1 serum, 2 μl of dI : dC (1 mg/ml), 25 μl of 697 NE (1.0 mg/ml), and 162 μl of IP buffer (10 mM Tris, pH 7.5, 75 mM NaCl, 0.05% NP40, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% BSA) for 1 h at 4°C followed by addition of 20 μl of protein G sepharose for 20 min. The sepharose was washed five times using 500 μl of IP buffer followed by one wash in IP buffer without BSA. Bound DNA was eluted in 100 μl of water by boiling for 5 minutes, followed by phenol/chloroform extraction precipitation, and resuspension in 10 μl of water. Amplification was for 20 cycles of PCR unless double stranded product was detected earlier as monitored every 5–10 cycles. The PCR was conducted as follows: 10 μl of selected DNA, 5 μl of PCR buffer, 2.5 μl of dNTPs (5 mM), 4 μl of primers no.1 and no.2 (10 pmol/μl), 27.5 μl water, 1 μl of Taq polymerase. Cycles consisted of 94°C, 1 min, 52°C, 2 min and 72°C, 2 min. Selected inserts were cloned in pBSK – cut with XbaI and EcoRV and sequenced.

Pbx antisera

Two anti-Pbx rabbit sera generated against a GST-fusion protein containing the portion of Pbx1 in E2a-Pbx1 (Kamps *et al*., 1991). These two antisera are designated, RPa and RPb, for rabbit Pbx1 antiserum A and B. RPa serum was used for the selection process while RPb was utilized for gel-shift analysis. Two commercially available antisera (Santa Cruz Biotechnologies) were also used: one specific for Pbx2, designated αPbx2 and one that cross-reacts between Pbx1, Pbx2, and Pbx3, designated αPbx123.

CAT assays

These assays were conducted in Nalm-6 cells as previously described (Lu *et al*., 1994) using pL1fos CAT vectors containing concatomerized elements constructed from annealed complementary oligonucleotides, except lipofectamine was used for transfections rather than calcium phosphate.

Immunoprecipitation

^{32}S-labeled *in vitro* transcribed and translated proteins were immunoprecipitated using RPa serum in the presence or absence of 1 μg of double-stranded DNA, separated by SDS–PAGE, fixed, enhanced, dried and subjected to autoradiography.

Mutagenesis

Hox A5 cDNA was mutated using the Muta-gene phagemid *in vitro* mutagenesis kit (Biorad). Point mutations were created by annealing oligos with the appropriate mismatches to single-stranded phagemid DNA, followed by synthesis of double-stranded DNA and transformation.

In vitro *transcription/translation*

In vitro transcription/translation was performed using the Promega TNT Coupled Reticulocyte Lysate System according to the manufacturers protocol. All cDNAs were transcribed using SP6 polymerase from pGEM 3Z or pGEM4Zf vectors.

Electrophoretic mobility shift assays (EMSA)

Double-stranded oligonucleotides were labeled with [^{32}P]-ATP to the same specific activities by phosphorylation of a common reverse oligonucleotide that was annealed to the 3′ portion of oligonucleotides containing different DNA-binding motifs, and then filled in using dNTP's and Klenow polymerase. Bound and free probe was separated by electrophoresis in 15% acrylamide gels formed in 0.5X TBE (27 mM Tris; 27 mM boric acid; 0.6 mM EDTA) and run in the same buffer. 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 μg of poly (dI : dC) in a buffer containing 10 mM Tris (pH 7.5), 1 mM DTT, 0.1% NP-40, and 5% glycerol for 30 min at room temperature. 6% EMSA gels were dried and visualized by autoradiography. Abundance of mutant and wild-type Hox-A5 proteins was normalized by performing parallel transcription-translation reactions in the presence of [^{35}S]-methionine. For oligonucleotide competition assays, double-stranded, unlabeled oligonucleotides were mixed with labeled probe prior to initiating gel shift assays. Complex abundances were measured using a phosphoimager.

Nuclear extraction

Cells were washed with cold PBS and resuspended in 5 × cell volumes of buffer A, swollen 10 min, and dounced 20

× with the A pestal to free nuclei. Nuclei were spun down, washed in buffer A (10 mM Hepes(pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.0 mM PMSF, and 0.1% aprotinin) and extracted with three nuclear volumes of buffer C (20 mM Hepes(pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1.0 mM PMSF, and 0.1% aprotinin) for 1 h. Following centrifugation, the supernatant was dialyzed against 100 volumes of buffer D (20 mM Hepes(pH 7.9), 20% glycerol. 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 1.0 mM PMSF, and 0.1% aprotinin) overnight at 4°C, respun, and the last supernatant was assayed for protein concentration before storing at −80°C. 1 μg of nuclear extract was used for EMSA.

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References

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ. (1990). *J. Mol. Biol.*, **215,** 403–410.
Chang CP, Shen WF, Rozenfeld S, Lawrence HJ, Largman C and Cleary ML. (1995). *Genes & Dev.* **9,** 663–674.
Chang CP, Brocchieri L, Shen WF, Largman C and Cleary ML. (1996). *Mol. Cell Biol.*, **16,** 1734–1745.
Dear TN, Sanchez-Garcia I and Rabbitts TH. (1993). *Proc. Natl. Acad. Sci.*, **90,** 4431–4435.
Dedera DA, Waller EK, LeBrun DP, Sen-Majumdar A, Stevens ME, Barsh GS and Cleary ML. (1993). **Cell**, 833–843.
Desplan C, Theis J and O'Farrell PH. (1988). *Cell*, **54,** 1081–1090.
Funk WD and Wright WE. (1992). *Proc. Natl. Acad. Sci.*, **89,** 9484–9488.
Gehring W, Affolter JM and Bürglin T. (1994). *Annu. Rev. Biochem.*, **63,** 487–526.
Grueneberg DA, Natesan S, Alexandre C and Gilman MZ. (1992). *Science*, **257,** 1089–1095.
Johnson FB, Parker E and Krasnow MA. (1995). *Proc. Natl. Acad. Sci.*, **92,** 739–743.
Kagawa N, Ogo A, Takahashi Y, Iwamatsu A and Waterman MR. (1994). *Journal of Biol. Chem.*, **269,** 18716–18719.
Kamps MP and Baltimore D. (1993). *Mol. Cell. Biol.*, **13,** 351–357.
Kamps MP, Look T and Baltimore D. (1991). *Genes & Dev.*, **5,** 353–368.
Kamps MP, Murre C, Sun X and Baltimore D. (1990). *Cell*, **60,** 547–555.
Kamps MP, Wright DD and Lu Q. (1996). *Oncogene*, **12,** 19–30.
Kerppola TK and Curran T. (1993). *Mol. Cell. Biol.*, **13,** 5479–5489.
Kissinger CR, Liu BS, Martin-Blabco E, Kornberg TB and Pabo CO. (1990). *Cell*, **63,** 579–590.
Knoepfler PS and Kamps MP. (1995). *Mol. Cell. Biol.*, **15,** 5811–5819.
Knoepfler PS and Kamps MP. (1997). *Mech. Dev.*, In press.
Knoepfler PS, Lu Q and Kamps MP. (1996). *Nucleic Acids Research.*, **24,** 2288–2294.
Laughon A. (1991). *Biochemistry*, **30,** 11357–11367.
Lebrun DP and Cleary ML. (1994). *Oncogene*, **9,** 1641–1647.
Lu Q and Kamps MP. (1996). *Mol. Cell. Biol.*, **16,** 1632–1640.
Lu Q and Kamps MP. (1997). *Oncogene*, **17,** 75–83.
Lu Q, Wright DD and Kamps MP. (1994). *Mol. Cell. Biol.*, **6,** 3938–3948.
Lu Q, Knoepfler PS, Scheele J, Wright DD and Kamps MP. (1995). *Mol. Cell. Biol.*, **15,** 3786–3795.
Monica K, Galili N, Nourse J, Saltman D and Cleary ML. (1991). *Mol. Cell. Biol.*, **11,** 6149–6157.
Monica K, LeBrun DP, Dedera DA, Brown R and Cleary ML. (1994). *Mol. Cell. Biol.*, **14,** 8304–8314.
Neuteboom STC, Peltenburg LTC, van Dijk MA and Murre C. (1995). *Proc. Natl. Acad. Sci USA*, **92,** 9166–9170.
Nourse J, Mellentin JD, Galili N, Wilkinson J, Stanbridge E, Smith SD and Cleary ML. (1990). *Cell*, **60,** 535–545.
Peers B, Sharma S, Johnson T, Kamps M and Montminy M. (1995). *Mol. Cell. Biol.*, **15,** 7091–7097.
Phelan ML, Ranbaldi I and Featherstone MS. (1995). *Mol. Cell. Biol.*, **15,** 3989–3997.
Pöpperl H, Bienz M, Studer M, Chan SK, Aparicio S, Brenner S, Mann RS and Krumlauf R. (1995). *Cell*, **81,** 1031–1042.
Rauskolb C, Peifer M and Wieschaus E. (1993). *Cell*, **74,** 1101–1112.
Rauskolb C and Wieschaus E. (1994). *EMBO J.*, **13,** 3561–3569.
Roberts VJ, van Dijk MA and Murre C. (1995). *Mechanisms of Development*, **51,** 193–198.
Simeone AD, Acampora D, Arcioni L, Andrews PW, Boncinelli E and Mavilio F. (1990). *Nature*, **346,** 763–766.
Simeone AD, Avantaggiato V, Moroni MC, Mavilio F, Arra C, Cotelli F, Nigro V and Acampora D. (1991). *Mech. of Devel.*, **33,** 215–228.
Spoerel NA, Nguyen HT, Towne S and Kafatos FC. (1993). *J. Mol. Biol.*, **230,** 151–160.
Tang S and Breitman ML. (1995). *Nucleic Acids Research*, **23,** 1928–1935.
Vallejo M, Miller CP and Habener JF. (1992). *J. Biol. Chem.*, **267,** 12868–12875.
Van Dijk MA and Murre C. (1994). *Cell*, **78,** 617–624.
Van Dijk MA, Voorhoeve P and Murre C. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 6061–6065.
Zwartkruis F, Kruyt F, van der Saag PT and Meijlink F. (1993). *Exper. and Cell Res.*, **205,** 422–425.