

A conserved motif N-terminal to the DNA-binding domains of myogenic bHLH transcription factors mediates cooperative DNA binding with Pbx–Meis1/Prep1

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ABSTRACT

The t(1;19) chromosomal translocation of pediatric pre-B cell leukemia produces chimeric oncoprotein E2a–Pbx1, which contains the N-terminal transactivation domain of the basic helix–loop–helix (bHLH) transcription factor, E2a, joined to the majority of the homeodomain protein, Pbx1. There are three Pbx family members, which bind DNA as heterodimers with both broadly expressed Meis/Prep1 homeodomain proteins and specifically expressed Hox homeodomain proteins. These Pbx heterodimers can augment the function of transcriptional activators bound to adjacent elements. In heterodimers, a conserved tryptophan motif in Hox proteins binds a pocket on the surface of the Pbx homeodomain, while Meis/Prep1 proteins bind an N-terminal Pbx domain, raising the possibility that the tryptophan–interaction pocket of the Pbx component of a Pbx–Meis/Prep1 complex is still available to bind tryptophan motifs of other transcription factors bound to flanking elements. Here, we report that Pbx–Meis1/Prep1 binds DNA cooperatively with heterodimers of E2a and MyoD, myogenin, Mrf-4 or Myf-5. As with Hox proteins, a highly conserved tryptophan motif N-terminal to the DNA-binding domains of each myogenic bHLH family protein is required for cooperative DNA binding with Pbx–Meis1/Prep1. *In vivo*, MyoD requires this tryptophan motif to evoke chromatin remodeling in the Myogenin promoter and to activate Myogenin transcription. Pbx–Meis/Prep1 complexes, therefore,

have the potential to cooperate with the myogenic bHLH proteins in regulating gene transcription.

INTRODUCTION

Homeodomain (HD) transcription factors specify positional and lineage information through regulation of target gene transcription during embryogenesis and in adult organisms (1–5). Class I Hox proteins bind TAAT core elements monomerically or bind TGATTNAT elements as heterodimers with Pbx1, Pbx2 and Pbx3 (6–12), a divergent family of HD proteins that contain an additional three amino acid loop in their HD and which also includes Meis1, Meis2, Meis3, TGIF and Prep1 (13–16). On TGATTNAT elements, Pbx binds the 5' TGAT core (17) and heterodimerization can change the specificity of the Hox protein for the N residue of its 3' TNAT core from an A to a T or G (18–20). In this heterodimer, a conserved tryptophan motif adjacent to the Hox HD binds the Pbx1 HD (17,21). Pbx–Hox heterodimers can activate transcription when the Hox protein contains a transcriptional activation domain [e.g. the HoxB1 R4 autoregulatory element (22), the EphA2 R4-specific enhancer (23) and the Somatostatin distal enhancer element (12)] or they can augment the function of other transcriptional activators while exhibiting little intrinsic transactivation potential [e.g. with AP1 on the *Drosophila* LAB-1 enhancer, (24) and with Ptf-1 in the eucaryotic elastase promoter (25)]. In this context, Pbx–Hox complexes function as both selectors and co-activators that restrict and augment the function of other transcriptional activators.

Meis1, Meis2, Meis3 and Prep1 also heterodimerize with Pbx proteins, binding canonical TGAC 3' half-sites in TGATTGAC elements (26–28) in which the C residue at position four of the Meis/Prep1 core precludes binding of Pbx–Hox complexes (29). This element was first identified by immunoselection

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with Pbx antisera using nuclear extracts and it was named the Pbx-cooperativity element (PCE) (29). A PCE that binds Pbx–Meis/Prep1 is essential for cAMP responsiveness of the bovine CYP17a promoter (30), and PCE elements can activate transcription of artificial promoter constructs in response to Pbx1 plus Prep1 expression (31). In other promoters, PCE elements function similar to that of Pbx–Hox elements in directing the specificity of and augmenting the potency of other transcriptional activators. In the uPA enhancer, activation through PEA3/AP1 and AP1 elements requires binding of Pbx–Prep1 to an intervening PCE, restricting uPA expression to cells that both contain nuclear Pbx–Prep1 and exhibit activation of signaling through AP1 (32,33). Likewise, a PCE in the Somatostatin promoter binds Pbx–Prep1 and augments transcription 10-fold through an adjacent motif that binds the HD transcriptional activator, Stf-1 (31). Because mutation of the *Drosophila* Pbx homolog, Exd, alters transcription of the target genes of homeotic complex genes (Hox homologs) and because the tryptophan-interaction pocket on the surface of the Pbx HD is unoccupied in Pbx–Meis/Prep1 complexes, another mechanism by which Hox proteins could cooperate with Pbx proteins is through binding a monomeric TAAT-like Hox element and contacting an adjacent Pbx–Meis/Prep1 complex using its tryptophan motif. Thus far, however, no one has described a physical interaction between Pbx–Meis/Prep1 complexes bound to PCE elements and Hox proteins or other transcriptional activators bound to their distinct motifs.

After identifying TGATTGAC as the optimal motif bound by Pbx–Meis/Prep1, we were surprised to discover that this same element had been immunoselected previously in a search for optimal E-box elements that bound Myogenin (34). In that study, using nuclear extract derived from C2C12 myotubes and antisera to Myogenin, selected E-box motifs (5'-CANNTG-3') were flanked on their 5' side by 5'-TGATTGAC-3' elements that bound an unknown cellular factor designated COMP. This coincidence prompted us (i) to test whether Myogenin-containing heterodimers could interact on DNA with Pbx–Meis/Prep1; (ii) to determine whether the remaining three members of the myogenic basic helix–loop–helix (bHLH) family of proteins (MyoD, Mrf-4 and Myf-5) also co-select TGATTGAC sites 5' to E-box motifs; (iii) to identify the molecular basis of these cooperative interactions. Here we report that heterodimers of all myogenic bHLH proteins with E2a bind DNA cooperatively with Pbx–Meis1/Prep1, and that cooperativity is mediated by an essential tryptophan residue within a highly conserved motif N-terminal to the bHLH domain of all MyoD family members. This is the first identification of a transcription factor complex that binds this motif in myogenic bHLH proteins and suggests that interaction with Pbx–Meis/Prep1 may regulate transcription of a subset of the genetic targets of myogenic bHLH proteins. In MyoD, this conserved motif is required for both transcriptional activation of the myogenin gene and for chromatin remodeling in the myogenin promoter. Finally, the fact that bHLH heterodimers containing E2a can physically interact and functionally synergize with HD heterodimers containing Pbx suggests that oncoprotein E2a–Pbx1 may combine independent transcription functions that normally cooperate *in vivo* in the context of independent bHLH and HD heterodimers.

MATERIALS AND METHODS

Mutagenesis

Site-directed mutations were introduced in cDNAs using the Muta-gene phagemid *in vitro* mutagenesis kit (Bio-Rad). Deletion mutations in Hox and Meis1 were created by introducing two *MluI* sites in the same reading frame on either side of the region to be deleted and excising the internal *MluI* fragment. As a result of this approach, an N-terminal *MluI* site is present in each of the deletion mutants, converting the second and third amino acid to threonine and arginine. All mutations were verified by sequence analysis.

Transcription–translation *in vitro*

Transcription–translation was performed *in vitro* using the Promega TNT Reticulocyte Lysate System according to the manufacturer's specifications. Comparable amounts of wild-type and mutant proteins were added to gel shifts by monitoring the incorporation of ³⁵S-methionine in parallel reactions and normalizing for differences in transcription–translation efficiencies.

Electrophoretic mobility shift assays (EMSA)

Double-stranded oligonucleotides were labeled with ³²P-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 extended using dNTPs and Klenow polymerase. Bound and free probe were separated by electrophoresis in 10% acrylamide gels formed in 0.5× TBE (27 mM Tris, 27 mM boric acid, 0.6 mM EDTA) and run in the same buffer. For EMSA, 20–40 000 c.p.m. of probe (30 Ci/μmol) was incubated with nuclear extract in the presence of 1 μg 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. EMSA gels were dried and visualized by autoradiography. Abundance of mutant and wild-type proteins was normalized by performing parallel transcription–translation reactions using ³⁵S-methionine followed by molecular quantitation. For oligonucleotide competition assays, double-stranded, unlabeled oligonucleotides were mixed with labeled probe before or after complex formation. Complex abundancies were measured using a phosphorimager. EMSA for Pbx–Meis1/Prep1 and tetrameric complexes was performed as described above, with the exception that formation of these complexes was performed using 20–40 000 c.p.m. of probe (30 Ci/μmol), 3–8 μl of *in vitro* translated proteins and 0.2 μg of poly(dI:dC) in a buffer containing 10 mM Tris (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 3 mM MgCl₂ and 12% glycerol for 30 min at room temperature. A version of E12 that lacks the first 493 residues of E12 and behaves identically to E12 in tetramer formation with Pbx–Meis1/Prep1 (E12N) was utilized in certain reactions (35). E12P, a second mutant form of E12 lacking the first 529 residues of E12, was generated by PCR. The consensus probe (TTGATTGACAGGAACAGGTG) was used for tetrameric complex formation.

Immunoselection assays

Immunoselection was conducted as described previously (34). Aliquots of 10 μg of the original double-stranded oligonucleotide containing 35 consecutive randomized positions were used. This represents approximately 10¹⁴ different sequences. Six

cycles of consecutive immunoselection followed by amplification by PCR were performed. Selection of DNA sequences bound by Myogenin was performed with the monoclonal anti-Myogenin antibody 1F5D7 as described (37). DNA sequences bound by MyoD, Mrf-4 and Myf-5 were selected using anti-HA antibodies and versions of MyoD, Mrf-4 and Myf-5 modified by addition of the HA tag. Aliquots of 100 μ l of anti-mouse-coated magnetic beads were incubated overnight with 10 ml of culture supernatant from the monoclonal 12CA5 anti-HA cell line. Samples of 4 μ l of each *in vitro* translated HA-cDNA were then mixed with 4 μ l of myotube nuclear extract and incubated at room temperature for 20 min with either 10 μ g of the degenerate library or 10 μ l of the PCR-amplified material from the previous cycle. Aliquots of 1 μ l of anti-HA-coated beads were then added and incubated on a vibrating platform to keep the beads in suspension for 1 h at room temperature. The beads were then washed three times with cold PBS containing 0.1% NP-40 and 0.1% BSA and amplified by PCR as described previously (34). Fifty-nine oligonucleotides selected by Myogenin, 113 oligonucleotides selected by Mrf-4 and 23 oligonucleotides selected by Myf-5 were sequenced. The TGATTGAC motif was present in five, seven and two non-identical oligonucleotides, respectively, within each of these pools.

Luciferase assays

Luciferase assays were conducted in NIH 3T3 cells using Superfect-mediated transfection of DNA expression constructs encoding Pbx1a, Meis1c, MyoD or Myogenin in conjunction with Luciferase reporters containing either a single E-box, a single Pbx–Meis/Prep1 site, a paired PCE–E-Box site or four repeated E-boxes and, in each case, CS2- β -Gal. Luciferase values were normalized to β -Gal using the MUG assay for β -Gal activity.

Endogenous gene assays

The effects of Pbx and Meis1 on endogenous genes were analyzed by transfection of 3T3 cells with expression vectors encoding wild-type or mutant forms of MyoD together with Pbx and/or Meis1 constructs. Co-transfection with 4R-CAT was performed to adjust for MyoD activity. Twenty-four hours after myogenic induction, cells were immunostained with rabbit polyclonal anti-CAT (5 Prime \rightarrow 3 Prime Inc.) and mouse monoclonal F5D anti-Myogenin or mouse monoclonal MF20 anti-Myosin Heavy Chain. CAT and Myogenin or Myosin was visualized after binding a secondary antibody of fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit antibodies.

cDNAs

Meis3 was a generous gift of Dr T. Nakamura. We also thank Dr C. Murre for his generous gift of Pbx2 and Pbx3 cDNAs.

RESULTS

All four MyoD family members in C2C12 myotube nuclear extract bind E-box elements in conjunction with a complex that binds TGATTGAC

To determine whether MyoD, Myf-5 and Mrf-4 also select a TGATTGAC motif and position it in the same orientation 5' to the E-box as did Myogenin, immunoselection was performed

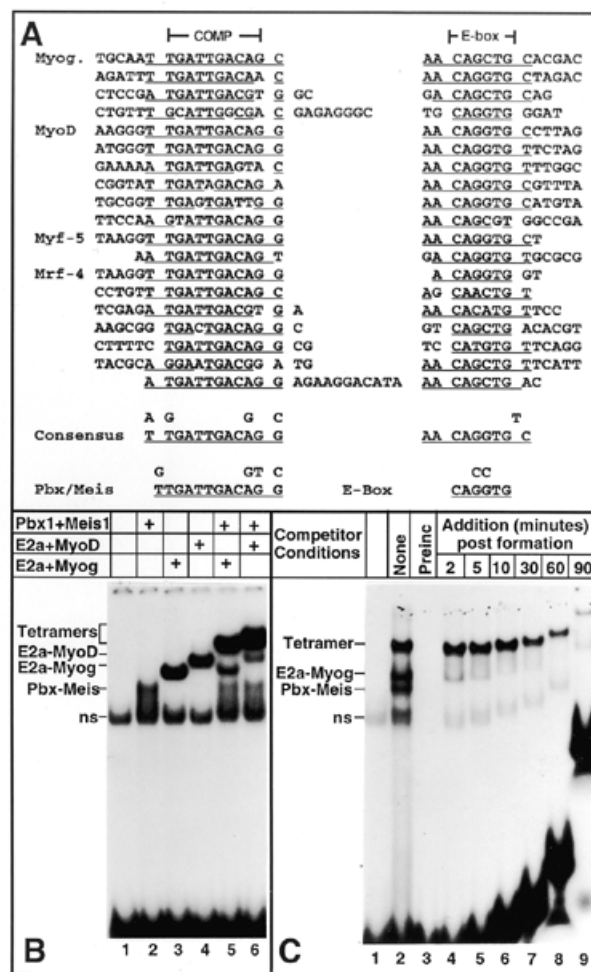


Figure 1. MyoD family members bind DNA cooperatively with Pbx–Meis1/Prep1 and interact with a nuclear factor that binds TGATTGAC, the Pbx–Meis1/Prep1 element. (A) Immunoselection of Pbx–Meis1/Prep1 binding sites from degenerate oligonucleotide libraries using nuclear extract from C2C12 myoblasts and antisera specific for MyoD, Myf-5 and Mrf-4. Sites are compared to those originally selected using antisera to Myogenin (34). (B) Heterodimers of Pbx1–Meis1 bind cooperatively to the consensus DNA sequence TTGATTGACAGGAACAGGTG with E2a–MyoD or E2a–Myogenin. EMSA analysis was performed in the presence of the recombinant proteins indicated above each lane. (C) Off-rate analysis of Pbx–Meis1, E2a–Myogenin and Pbx–Meis1–E2a–Myogenin complexes. All recombinant proteins were produced by coupled transcription–translation as described in Materials and Methods.

using nuclear extract from C2C12 myotubes, antisera specific for each factor and the same degenerate DNA library used in anti-Myogenin selections, which contains 35 sequential randomized positions. Fifteen E-box sequences, representing 6–10% of the total number of oligomers sequenced from each selection pool, contained TGATTGAC-like sites (Fig. 1A; see Materials and Methods). In each case the order and orientation of the E-box and the TGATTGAC sequence were the same and the consensus sequence containing both elements was 5'-TTGATTGACAGGAACAGGTG-3', in which both the TGATTGAC core

sequence, as well as the 5'-flanking nucleotide (T) and 3'-flanking nucleotides (AG) were identical to the PCE motif immunoselected by an anti-Pbx serum (29).

Pbx–Meis1 and Pbx–Prep1 bind DNA cooperatively with MyoD–E2a and Myogenin–E2a

Thus far, Pbx–Meis1/Prep1 heterodimers are the constituents of all nuclear extract complexes that bind TGATTGAC (29,31,32). Therefore, the ability of Pbx1–Meis1 to bind DNA cooperatively with MyoD–E2a and Myogenin–E2a was tested by EMSA, using proteins produced by coupled transcription–translation *in vitro* and a DNA probe containing the consensus sequence (TTGATTGACAGGAACAGGTG; Fig. 1B). While heterodimers of Pbx1–Meis1 (lane 2), Myogenin–E2a (lane 3) and MyoD–E2a (lane 4) bound the probe, addition of Pbx plus Meis1 to binding reactions containing E2a plus either MyoD or Myogenin resulted in the formation of a new higher order complex and a 50–80% reduction in each dimeric complex (lanes 5 and 6). In isolation, Pbx1, Meis1, E2a or MyoD failed to bind the probe (data not shown). The mobility difference of the putative tetrameric complex containing MyoD or Myogenin paralleled the mobility difference between MyoD–E2a and Myogenin–E2a heterodimers, indicating that the higher order complex contains MyoD or Myogenin. Antisera supershift experiments and mutational analysis demonstrated that all four factors are in the higher order complex (data presented below) and we will refer to this higher order complex as 'tetrameric'. The abundance of the tetrameric complex was greater than either of its heterodimer constituents, indicating a cooperative form of DNA binding in which a surface of Pbx1–Meis1 binds a surface of E2a–MyoD/Myogenin or in which the binding of one complex to DNA alters the structure of DNA such that binding of the second complex is stabilized. Off-rate analysis using competition with excess cold oligonucleotide confirmed that the tetrameric complex was more stable than either of its heterodimer constituents. While half the Pbx–Meis1 or E2a–Myogenin dimers dissociated from DNA within 30 s or 1 min, respectively, 30 min was required to dissociate half of the tetrameric complex (Fig. 1C).

Multiple criteria were used to demonstrate that Pbx proteins, Meis1/Prep1 proteins, E2a and MyoD/Myogenin were contained in the tetrameric complex. In the presence of E2a plus Myogenin, addition of Pbx1 or Pbx3 plus either Meis1 or Prep1 induced formation of tetrameric complexes (Fig. 2A, lanes 5, 6, 9 and 10) whose differential mobilities paralleled the difference in mobility of Pbx1 or Pbx3 with Meis1 (lanes 3 and 4) or with Prep1 (lanes 7 and 8). Therefore, both Pbx proteins and Meis1/Prep1 proteins are in the tetrameric complex. This conclusion was confirmed by demonstrating: (i) that both spliced forms of Pbx1 (Pbx1a and Pbx1b), which differ by 7 kDa at their C-termini, also produced higher order complexes whose differential mobilities paralleled those of their heterodimers with Meis1; (ii) that Pbx1 antisera supershifted and partially disrupted the tetrameric complex; (iii) that inclusion of a C-terminal deletion of Meis1 increases the mobility of both Meis1–Pbx1 and of the tetrameric complex (data not shown). Homothorax, the *Drosophila* homolog of Meis1/Prep1, also formed heterodimers with Pbx1 and Pbx3 (lanes 11 and 12) as well as tetrameric complexes with E2a–Myogenin (lanes 13 and 14). E2a must also be in the complex because different truncations of E2a (E12P or E12N; see Materials and Methods) yielded tetrameric complexes of vastly different mobilities (Fig. 4B, lane 4 versus 8). An anti-E2a

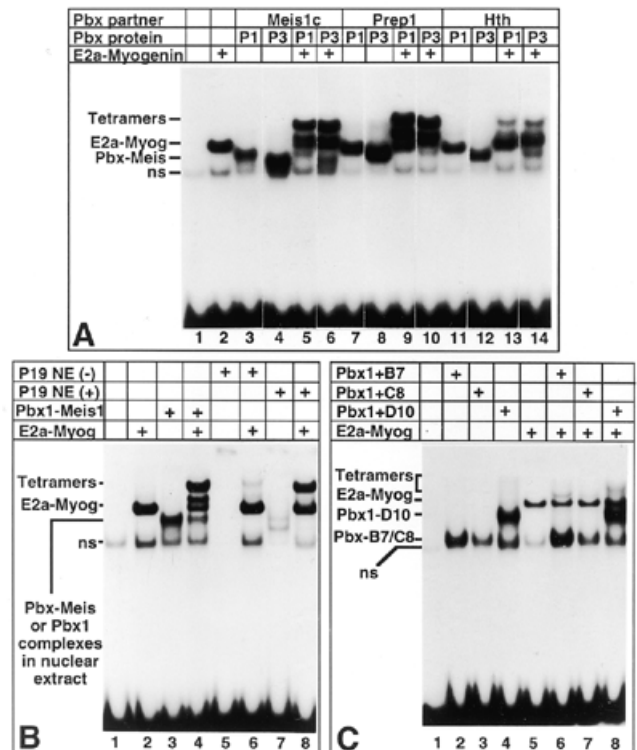


Figure 2. E2a–Myogenin binds DNA cooperatively with heterodimers containing different members of the Meis and Pbx families, with Pbx complexes from nuclear extract, but not with Pbx–Hox complexes. (A) Cooperative tetramer formation using various Pbx and Meis1/Prep1 family proteins. Binding reactions contain equivalent amounts of Pbx1b (P1), Pbx3b (P3), Meis1c, Prep1 and Hth, as indicated above lanes, and were analyzed by EMSA. Addition of Myogenin plus E2a is designated by a + sign. (B) E2a–Myogenin heterodimers bind DNA cooperatively with Pbx-containing complexes from nuclear extract. Binding reactions contained nuclear extract from untreated P19 cells [P19 NE (-)] or cells treated for 7 days with RA [P19 NE (+)] and either no additions or addition of E2a plus Myogenin, as indicated above each lane. (C) Pbx–Hox dimers fail to interact with E2a–Myogenin on DNA. A binary probe in which the PCE element was replaced with a Pbx–Hox element (bold in **TTGATT-TATGA-GAACAGGTG**) was used for EMSA. Binding reactions contain the recombinant proteins indicated above each lane. All recombinant proteins were produced by coupled transcription–translation as described in Materials and Methods.

serum also supershifted and partially disrupted formation of the tetrameric complex (data not shown). Collectively, these data demonstrate (i) that the higher order complex contains Pbx, Meis, MyoD and E2a, strongly suggesting that the complex is tetrameric, and (ii) that the mechanism of cooperative binding between various heterodimers of Pbx1/3–Meis1/Prep1 and heterodimers of MyoD–E2a or Myogenin–E2a is conserved.

PCE-binding complexes in nuclear extracts bind DNA cooperatively with E2a–MyoD/Myogenin

In P19 embryonal carcinoma cells, which can exhibit muscle differentiation, the abundance of PCE-binding complexes, like that of Pbx and Meis proteins, can be up-regulated upon treatment with retinoic acid (RA) (29,36,37). Formerly, we demonstrated

that 100% of the P19 cell nuclear extract complex that binds the PCE is supershifted by anti-Pbx sera (29). To test whether such endogenous Pbx complexes can exhibit cooperative DNA binding with E2a-MyoD, nuclear extracts from uninduced and RA-induced P19 cells were examined for complexes that bound 5'-TTGATTGACAGGAACAGGTG-3' in the presence or absence of recombinant E2a and Myogenin (Fig. 2B). Nuclear extract from uninduced P19 cells, which contains low amounts of PCE-binding complex (lane 5) (36), produced a small, but detectable, higher order complex upon addition of E2a and Myogenin (lane 6 versus 2) that co-migrated with the tetrameric complex formed with recombinant factors (lane 4). In contrast, nuclear extract from P19 cells treated with RA for 7 days contained higher levels of the endogenous PCE-binding complexes (lane 7) and, upon addition of E2a and Myogenin, produced high levels of the higher order complex (lane 8). The addition of E2a plus Myogenin also eliminates formation of the PCE-binding complexes (lane 8 versus 7), indicating that endogenous Pbx-Meis/Prep1 complexes are strongly stabilized on DNA through their interaction with E2a-Myogenin. These results also indicate that the interactions underlying tetramer formation in our EMSA assays are not an artifact of either protein overexpression or production of partially denatured 'sticky' forms of Pbx or Meis1/Prep1, which could bind Myogenin-E2a or MyoD-E2a through non-specific hydrophobic surfaces.

Pbx-Hox dimers cannot substitute for Pbx-Meis1/Prep1 dimers in cooperation with Myogenin-E2a

The ability of Pbx-Hox dimers to cooperate with E2a-Myogenin dimers in DNA binding was tested by substituting TGATTTAT, the optimal motif for binding Pbx-HoxB7, Pbx-HoxC8 or Pbx-HoxD10 heterodimers, for the PCE motif in conjunction with a 3' E-box (probe TTAGTTTATAGGAACAGGTG). The spacing, order and orientation of sites was maintained. Heterodimers of Pbx1 plus HoxB7, HoxC8 or HoxD10 bound this probe efficiently (Fig. 2C, lanes 2-4). However, in the presence of E2a plus Myogenin, heterodimers of Pbx plus HoxB7, HoxC8 or HoxD10 formed only faint higher order complexes that co-migrated with the tetrameric complex but whose very weak abundance, in comparison with that of each individual heterodimer, indicated tetramer formation by a non-cooperative mechanism. This observation suggested that the surface of Pbx-Meis1/Prep1 that binds MyoD-E2a is either not present or not available for interaction in the context of Pbx-Hox heterodimers.

Formation of the tetrameric complex requires specific orientation and spacing of heterodimer elements

Based on the orientation of each heterodimer on its separate element, the predicted order of the tetrameric complex on the tandem element is 5'-Pbx1, Meis1, MyoD, E2a-3' (28,38). Inversion of the PCE abolished tetramer formation (Fig. 3A, lanes 7-12 versus 1-6). Inversion of the E-box resulted in weaker binding of E2a-MyoD and E2a-Myogenin dimers (lanes 15 and 16) with retention of cooperative tetramer formation (lanes 17 and 18); however, in this case, cooperative interactions of E2a-MyoD or E2a-Myogenin with Pbx-Meis1 could result from enforced binding of E2a-MyoD to a sub-optimal CACCTG E-box relative to the adjacent PCE site (38). Transposition of the two motifs while maintaining their same orientation (lanes 19-24) also abolished tetramer formation. Decreasing

the separation of the PCE and E-box from five to three bases abrogated tetramer formation (Fig. 3B, lanes 7-12) while increasing separation to 10 nt retained efficient tetramer formation (lanes 13-18). Increasing separation to 15 nt strongly suppressed tetramer formation in conjunction with Meis1c (lanes 19-24) but did not significantly affect tetramer formation in conjunction with full-length Meis1 (data not shown).

In the presence of Pbx-Prep1, Myogenin can bind the E-box in the absence of E2a

Mixing Pbx1, Meis1, Myogenin and E2a produced a second higher order complex that migrated faster than the tetrameric complex but slower than either the E2-Myogenin or Pbx-Meis complexes (e.g. Fig. 2A, lane 5, and B, lane 4), suggesting that Myogenin or E2a alone might interact with Pbx-Meis1 or that Pbx or Meis1 alone might interact with Myogenin-E2a. To determine whether E2a or Myogenin was capable of binding Pbx-Prep1 on DNA, twice the normal amount of E2a or Myogenin was added to binding reactions containing Pbx plus Prep1 (Fig. 4). Neither E12 nor Myogenin bound the probe significantly (lanes 5 and 6) (39); however, while addition of E12 to Pbx1-Prep1 heterodimers failed to form an additional complex (lane 7 versus 2), addition of Myogenin shifted approximately one-third of the Pbx1-Prep1 complex to a slower mobility (lane 8), demonstrating that Myogenin will bind DNA cooperatively with Pbx-Prep1 in the absence of E2a. The middle band in the tetramer assay in lane 4 that co-migrates with Myogenin-E12 is therefore likely to be comprised predominantly of Pbx-Prep-Myogenin complexes. Myogenin did not associate with Pbx-Prep1 complexes in the absence of an E-box; rather, its ability to interact with Pbx-Prep1 required the presence of a correctly positioned E-box, as the probe containing an inverted copy of the Pbx-Prep1 site precluded formation of the higher order complex in the presence of Myogenin (Fig. 3, lanes 7-12). These results suggested the unanticipated prediction that the myogenic bHLH component of the E2a heterodimer binds Pbx-Meis1/Prep1. Deletion analysis of E12 also supported this prediction. A version of E12 (E12N) that lacks the first 493 residues behaved identically to wild-type E12 in tetramer formation with Pbx-Meis1/Prep1 (Fig. 4B, lanes 1-4). Failure of full-length E12 to interact with Pbx-Prep1 was not due to its intrinsic failure to form homodimers on DNA because a version of E12 that eliminates a sequence that blocks homodimerization (produced by deletion of the first 529 residues; E12P) (35) homodimerized readily on the E-box (Fig. 4B, lane 5), but failed to form a higher order complex in the presence of Pbx1-Meis1 (lane 7 versus 3), while an E12P-MyoD heterodimer, which also bound the E-box (lane 6), mediated tetramerization that was as efficient as that of E12N (lane 8 versus 4) or wild-type E12 (compare with Fig. 4A, lane 4). These results support the hypothesis that MyoD is the component of E2a-MyoD that binds Pbx-Meis1/Prep1 and that some conserved structure present in all four myogenic bHLH proteins contacts Pbx-Meis/Prep1.

A tryptophan motif conserved in all myogenic bHLH proteins mediates interaction with Pbx-Meis1/Prep1

Sequences conserved in MyoD, Myogenin, Mrf4 and Myf5 are restricted to their bHLH domains and a tryptophan-containing motif juxtaposed to the bHLH domain that is required for the

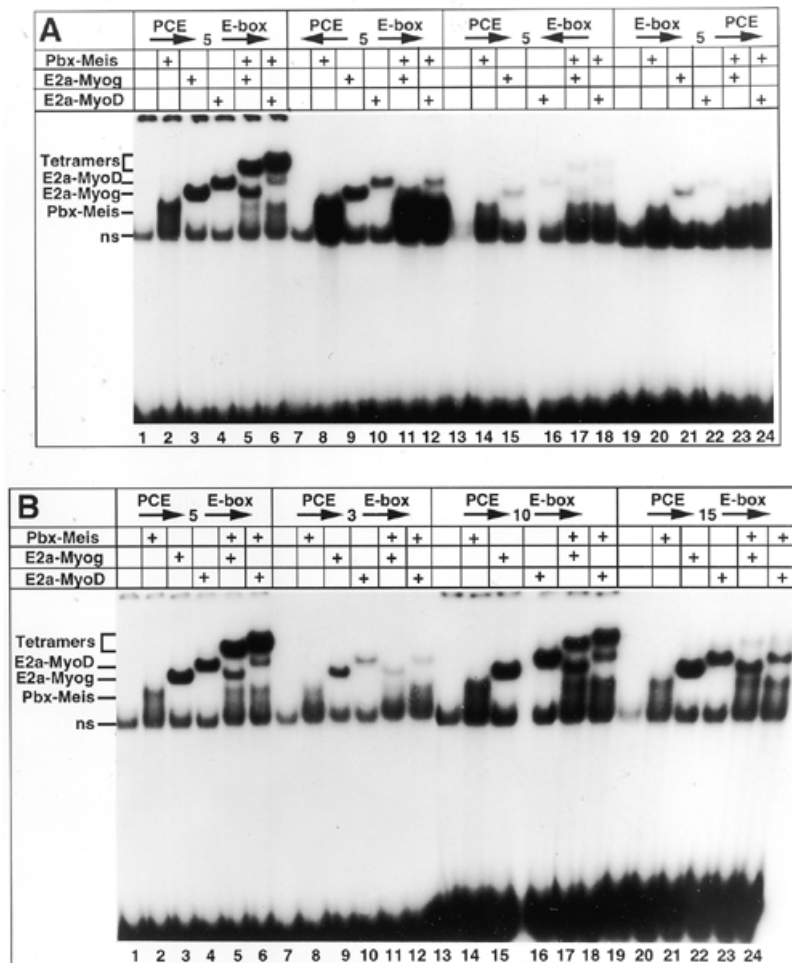


Figure 3. Cooperative interaction between heterodimers requires a specific orientation, order and spacing of conjugate elements. (A) Effects of orientation and order. Recombinant proteins indicated above each lane were analyzed for complex formation by EMSA using the probes indicated above each lane. Wild-type probe (TTGATTGACAGGAACAGGTG; lanes 1–6), a probe in which the Pbx–Meis element was inverted (CCTGTCAATCAAGGAACAGGTG; lanes 7–12), a probe containing an inverted E-box (TTGATTGACAGGAACACCTG; lanes 13–18) and a probe with the PCE and E-box sites transposed (CAGGTGCAGTTGATTGACAGG; lanes 19–24). (B) Effects of spacing. Recombinant proteins indicated above each lane were analyzed for complex formation by EMSA using the wild-type probe, which contains 5 nt separating the core elements (lanes 1–6), a probe with 3 nt separating the elements (TTGATTGACAGACAGGTG; lanes 7–12), a probe with 10 nt separating the elements (TTGATTGACAGGAAAGGAACAGGTG; lanes 13–18) and a probe with 15 nt separating the elements (TTGATTGACAGGAAAGGAAGGAACAGGTG, lanes 19–24). Core elements in each probe are underlined and intervening sequences are printed in bold.

ability of MyoD to activate Myogenin gene transcription *in vivo* and to remodel chromatin at the Myogenin promoter (40). Conservation of this element suggests it performs a conserved transcriptional function, yet no nuclear factor has yet been found that binds this motif. The consensus of this tryptophan motif is CL-hydrophobic residue-WACK-hydrophobic residue-CKRK (Fig. 5C). To identify MyoD surfaces that bind Pbx–Meis1/Prep1, a panel of MyoD deletion mutants previously analyzed for their ability to induce chromatin remodeling and to activate Myogenin gene transcription was examined for their ability to mediate tetramerization (Fig. 5A) (40). Former studies revealed that elimination of residues 63–99 (MyoDΔ63–99) and 218–269 (MyoDΔ218–269) reduced the

ability of MyoD to activate transcription of the Myogenin gene and to induce chromatin remodeling of the Myogenin promoter by 99.5 and 78%, respectively (40). Each of the four MyoD deletion mutants dimerized efficiently with E2a (Fig. 5A, lanes 3–7) and MyoDΔ170–209 and MyoDΔ218–269 formed tetramers as abundant as those formed by wild-type MyoD (when normalized to dimer formation with E2a; lanes 8, 10 and 11 versus 3, 5 and 6). MyoDΔ63–99, however, exhibited only 5% and MyoDΔ270–318 only 25% of the tetramerization potential of wild-type MyoD (lanes 9 and 12 versus 8). Therefore, the ability of MyoD–E2a to interact with Pbx1–Meis1 parallels the ability of MyoD to activate transcription of the Myogenin gene.

Within sequences encompassed by residues 63–99 of MyoD, the only residues that are conserved with each of the other

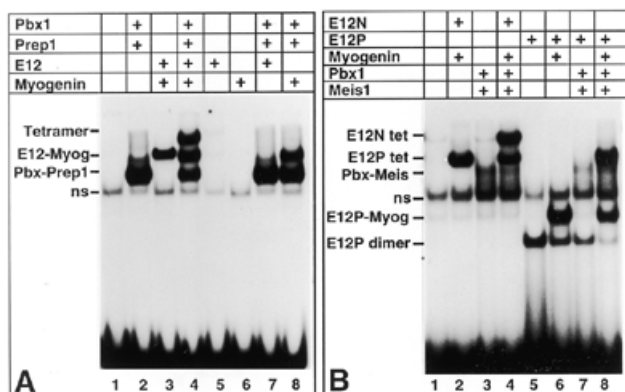


Figure 4. Myogenin alone, but not E2a alone, binds DNA cooperatively with Pbx–Meis1/Prep1. (A) Myogenin, but not E2a, binds DNA cooperatively with Pbx–Prep1. Binding reactions contained the wild-type binary DNA probe plus the recombinant proteins indicated above each lane and were resolved by EMSA. (B) Sequences N-terminal to the bHLH domain of E12 are dispensable for interaction with Pbx–Meis1/Prep1, and a form of E2a that homodimerizes on DNA efficiently fails to bind DNA cooperatively with Pbx–Meis1. E12N contains a deletion of the first 493 residues of E2a. E12P lacks the first 529 residues of E12 and can homodimerize efficiently. In all cases, the wild-type consensus DNA probe was used. Recombinant proteins added to binding reactions are indicated above each lane and were produced by coupled transcription–translation as described in Materials and Methods.

myogenic bHLH proteins are 93–99, part of the tryptophan motif that juxtaposes the bHLH domain (Fig. 5C). Analysis of site-directed mutants within this conserved motif of myogenin demonstrated that mutation W68A disrupted >90% of the tetramerization potential of the Myogenin–E2a complex while not altering its dimerization potential (Fig. 5B, lanes 10 versus 6; controls in lanes 7 and 3). Mutation CL→AA at residues 65 and 66 reduced tetramerization by 40% while not altering dimerization potential (lane 8 versus 4) and mutation KRK→AAA encompassing residues 74–76 had no effect on either dimerization or tetramer formation (lane 9 versus 5). These data indicate that MyoD, Myogenin, Mrf-4 and Myf-5 utilize the N-terminal half of this conserved tryptophan motif to bind Pbx–Meis1/Prep1. Interestingly, MyoR, a bHLH protein highly related to MyoD that antagonizes activation of muscle cell target genes by MyoD contains the cognate sequence **GSLGAAGGCKRK** just upstream of its bHLH domain, retaining the CKRK sequence that is dispensable for interaction with Pbx–Meis1/Prep1, but lacking the essential CL-X-W sequence (41).

The Pbx–Meis1/Prep1-interaction motif of MyoD is not essential for cooperative transactivation, but is essential for activation of the Myogenin gene *in vivo*

Originally, COMP and E-boxes were shown to synergize >30-fold in activating reporter gene transcription coincident with muscle differentiation induced in C2C12 myoblasts by serum withdrawal (34). In NIH 3T3 fibroblasts, reporter constructs driven by either a single E-box or a single PCE failed to respond to co-

transfection with a Myogenin or MyoD expression vector while a reporter driven by the PCE–E-box consensus was activated 3- and 14-fold by co-expression of Myogenin or MyoD, respectively (Fig. 6A). Physical interaction with Pbx–Meis1/Prep1 was not, however, essential for the cooperative effects of the PCE, as MyoD Δ 92–99, which eliminates the CL and W residues required for interaction with Pbx–Meis1/Prep1, retained 60% of the wild-type level of transcriptional activation on reporter constructs driven by tandem elements. Nonetheless, MyoD Δ 92–99 was only 2% as active as wild-type MyoD in inducing endogenous Myogenin gene expression in NIH 3T3 fibroblasts (Fig. 6B). Mutation of W96 and C98 to alanine resulted in only 1% of wild-type values of endogenous Myogenin gene activation. MyoD Δ 92–99 and MyoD W96A/C98A activated transcription of the endogenous Myosin Heavy Chain gene at efficient levels, 57 and 52% that of wild-type MyoD, respectively. Therefore, the interaction of MyoD complexes with Pbx–Meis1/Prep1 *in vitro* correlates with its ability to activate endogenous Myogenin gene transcription *in vivo*. Co-transfection of vectors expressing Pbx1 or Meis1c had no specific effects on either transactivation of reporter constructs by MyoD or on activation of the endogenous Myogenin gene *in vivo*, suggesting that an excess of Pbx–Meis/Prep1 complexes are present in NIH 3T3 cells or that a Meis1/Prep family member different from Meis1c may mediate *in vivo* function. Indeed, NIH 3T3 fibroblasts contain large amounts of Pbx complexes that bind TGATTGAC (29).

DISCUSSION

The differentiation of muscle cells and the transcription of muscle cell-specific genes is orchestrated by four myogenic regulatory genes encoding the bHLH proteins MyoD, Myogenin, Myf-5 and Mrf-4 (42). Typically, muscle cell-specific promoters activated by these factors contain multiple E-box elements and many also contain elements that bind Mef-2, a cofactor that lacks intrinsic transcriptional activation potential but strongly augments transcriptional activation by adjacent muscle bHLH–E2a complexes (43,44). Mef-2 binds a conserved sequence in the bHLH domain, and DNA elements that bind Mef-2 were also co-selected with E-box elements in the same anti-Myogenin immunoselection experiments that co-selected TGATTGAC and its associated nuclear factor, COMP (34). Here, we demonstrate that TGATTGAC elements are also co-selected with E-boxes during immunoselection of optimal sequences bound by the remaining three myogenic bHLH proteins, MyoD, Myf-5 and Mrf-4, and that recombinant Pbx–Meis1/Prep1 heterodimers, which are the constituents of all TGATTGAC-binding complexes isolated from nuclear extracts to date (29,31,32), function like COMP, exhibiting cooperative DNA binding with MyoD–E2a or Myogenin–E2a. This is the first example of a physical interaction between Pbx–Meis/Prep1 and a transcriptional activator and it is interesting to note that Pbx–Meis/Prep1 complexes function similarly to Mef-2, augmenting the function of other transcriptional activators while themselves not containing a strong activation domain (24,25,31–33).

Pbx–Meis1/Prep1 bound a CL-X-W motif that is a portion of a 12-residue conserved sequence residing directly N-terminal to the bHLH domains of MyoD, Myogenin, Myf-5 and Mrf-4, and which is the only sequence outside the bHLH domain that

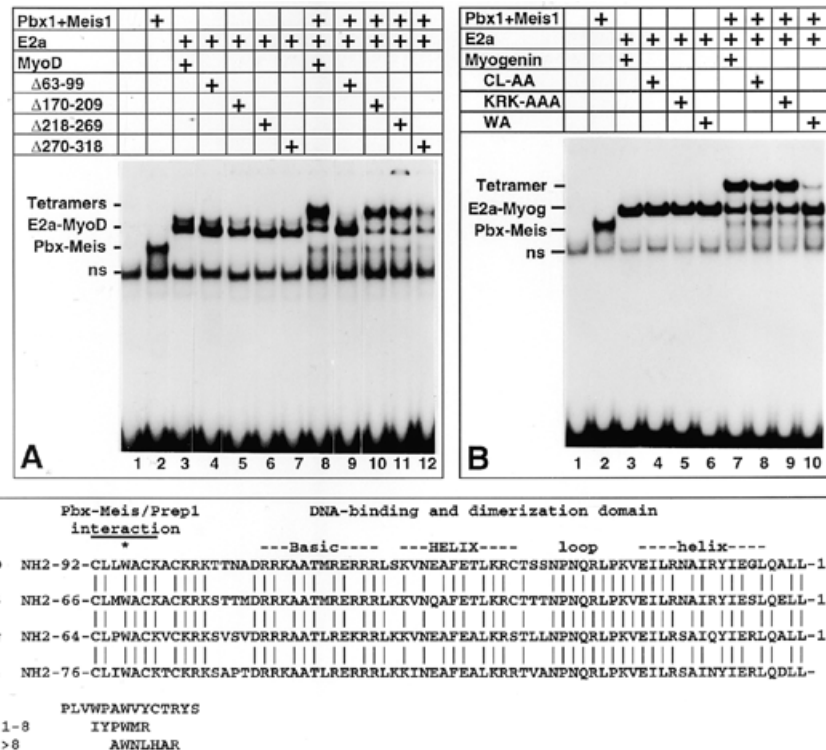


Figure 5. An essential tryptophan within a conserved motif N-terminal to the bHLH domain of MyoD is required for interaction with Pbx–Meis1/Prep1. (A) Deletion of residues 63–99 strongly compromises interaction with Pbx1–Meis1c. Wild-type E2a, Pbx1b and Meis1c were added to binding reactions as indicated above each lane in the presence of deletion mutants of MyoD and analyzed by EMSA. (B) Point mutations at a single W residue and an adjacent CL dipeptide in Myogenin suppress interaction with Pbx1–Meis1c. Myogenin point mutants indicated above each lane were evaluated for their ability to dimerize with E2a (lanes 3–6) and form tetramers with Pbx1b and Meis1c (lanes 7–10), using the wild-type consensus DNA probe and analysis by EMSA. (C) Sequence of the conserved tryptophan motif and its proximity to the bHLH domain within MyoD, Myf-5, Myogenin (Myog) and Mrf-4. The tryptophan motifs of Class I Hox proteins and Engrailed HD proteins that interact with the Pbx1 HD are listed below the tryptophan motif of the myogenic bHLH proteins.

is conserved in each factor. Pbx–Hox heterodimers themselves interact by a similar mechanism, in which an essential tryptophan within a highly conserved motif N-terminal to the DNA-binding domain of the Hox protein binds a pocket in the Pbx HD (17,21). Because Meis and Prep1 do not bind the tryptophan-interaction pocket on the Pbx HD, but rather interact with sequences N-terminal to the HD, the HD tryptophan-binding pocket may be available for interaction with other tryptophan-containing motifs, and it is possible that it binds the tryptophan residue in the CL-X-WACK-X-CKRK motif of the myogenic bHLH proteins in our *in vitro* assays. The context of the tryptophan residue within CL-X-WACK-X-CKRK is also consistent with its potential ability to bind the Pbx HD. The tryptophan motifs of Hox proteins are conserved within subfamilies but diverge significantly between subfamilies, having the consensus IYPWMR for paralogs 1–8 of Class I Hox proteins, ANWLHAR for paralogs 9–13 and WPAWVYCTRY for the eucaryotic homologs of *Drosophila* Engrailed (EN1 and EN2). These diverse motifs are similar to the CL-X-WACK sequence of the myogenic bHLH family in the sense that all contain the tryptophan residue within the context of hydrophobic residues and are flanked by a lysine or arginine residue two to five amino

acids to the C-terminus. Binding of the tryptophan in the CL-X-WACK-X-CKRK motif to a pocket in the Pbx1 HD would also account for failure of Pbx–Hox heterodimers to bind DNA cooperatively with Myogenin–E2a, as this pocket would already be occupied by the Hox tryptophan motif. Mutually exclusive interaction with multiple tryptophan-containing motifs of adjacent transcription factor complexes would provide a novel mechanism for permitting interaction of Pbx–Meis/Prep1 with multiple classes of transcription factors while restricting such interactions to the highest affinity factor at any one moment. Depending on the function of the interacting factor, such a system could permit Pbx–Meis/Prep1 complexes to cooperate in gene activation or repression, a function predicted for Pbx proteins based on studies of EXD null mutations (45).

The essential tryptophan residue is embedded within a highly conserved 12-residue sequence and is essential for activation of the Myogenin gene *in vivo* and for chromatin remodeling of the Myogenin promoter (40). Point mutations and deletions, including W96, not only strongly suppressed interaction of MyoD–E2a with Pbx1–Meis1/Prep1, but also virtually eliminated the ability of MyoD to activate transcription of the Myogenin gene and remodel chromatin in the Myogenin promoter (40).

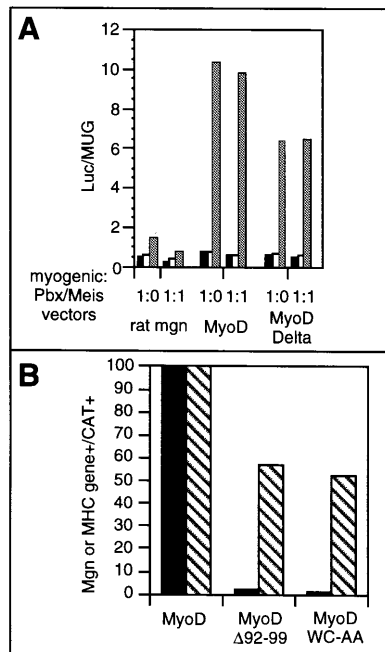


Figure 6. The conserved tryptophan motif of MyoD is not essential for the cooperative transcriptional properties of the E-box and PCE elements, but is essential for transcriptional activation of the Myogenin promoter. (A) Transcriptional activity of reporters driven by either a single E-box (hatched), a single PCE (open) or an E-box paired with a PCE (filled). Reporters were co-transfected into NIH 3T3 cells with vectors expressing Myogenin, MyoD or MyoD Δ 92-99 alone or in the presence of vectors expressing Pbx1b plus Meis1c. Luciferase activity was normalized to an internal β -Gal control (MUG value). (B) MyoD mutants that fail to interact with Pbx–Meis1/Prep1 fail to activate transcription of the Myogenin gene *in vivo*. The abilities of wild-type and mutant versions of MyoD to activate expression of the Myogenin (filled bars) and Myosin Heavy Chain genes (MHC; hatched bars) *in vivo* were determined by immunofluorescence in transfected 3T3 cells using antisera specific for Myogenin (monoclonal antibody F5D) or MHC (mouse monoclonal Ab MF20). The efficiency of gene activation by wild-type MyoD is defined as 100% and transfection efficiency is controlled by co-transfection of vectors expressing CAT, and quantitating CAT-positive cells by immunofluorescence, using rabbit polyclonal anti-CAT (5 Prime \rightarrow 3 Prime Inc.).

Whether or not this means that MyoD or other myogenic bHLH proteins require interaction with Pbx–Meis/Prep1 complexes for activation of Myogenin or other target genes *in vivo* remains to be demonstrated. It is possible that the conserved tryptophan motif of muscle bHLH proteins, while essential for chromatin remodeling and selective promoter activation *in vivo* through interaction with a non-Pbx factor, nonetheless binds Pbx–Meis/Prep1 in EMSA analysis simply because Pbx–Meis/Prep1 contains a high affinity interaction pocket. If this was the case, then failure to co-select the element that binds the actual interacting factor could be due to a lower affinity of the factor for DNA, its lower abundance or its more stringent DNA-binding specificity. While this is possible, the binding site requirements for Pbx–Meis/Prep1 are complex (10 consecutive nucleotides) and one would expect that the bona fide factor designed to bind this 12-residue motif

would have an affinity greater than that of Pbx–Meis/Prep1 and would have been isolated in our assay. Nonetheless, while the expression of Pbx complexes in muscle progenitors [C2C12 (34) and P19 EC cells (36)] suggests they play some role in gene expression in muscle cells, their transcriptional cooperation with muscle bHLH proteins *in vivo* remains unproven. It will be important to determine whether elimination of Pbx transcripts using antisense mRNA or elimination of functional Pbx proteins using dominant negative Meis/Prep1 in C2C12 myoblasts alters muscle differentiation or precludes expression of muscle-specific genes.

If the interaction between Pbx–Meis/Prep1 and muscle bHLH complexes promotes specific gene expression, the fact that deletion of the interaction motif on MyoD does not significantly alter synergy between MyoD and Pbx–Meis/Prep1 in co-transfection assays suggests that this interaction may be designed to juxtapose distant Pbx–Meis/Prep1 complexes next to bHLH–E2a complexes rather than to stabilize DNA binding by or activate a transcriptional function of either heterodimer. Such long distance interactions could mediate chromatin remodeling and could explain the fact that we do not find sites similar to TGATTGACAG flanking E-boxes in muscle-specific promoters. Synergy between bHLH and HD transcription complexes in transcriptional activation with and without accompanying chromatin remodeling has already been reported, both in synergistic transcriptional activation of Pho5 by the Pho2 HD protein and the Pho4 bHLH protein (46) and in activation of the elastase promoter, where a Pbx–Meis/Prep1 element promotes transcriptional activation by the Ptf-1 bHLH protein (25).

Finally, the juxtaposition of normal Pbx and E2a proteins through cooperative DNA binding of their heterodimer complexes is particularly provocative in light of the fact that E2a is fused to Pbx1 as a consequence of the t(1;19) translocation of pediatric pre-B cell leukemia. If Pbx proteins have novel biochemical functions that synergize with those of E2a in transcriptional activation or if the interaction of Pbx and E2a proteins in tetrameric complexes produces a new conformation in either protein that augments its ability to regulate transcription, then fusion of E2a to Pbx1 could reconstitute such synergistic effects persistently, forming a more potent transcriptional activator.

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