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The novel homeoprotein Prep1 modulates Pbx–Hox protein cooperativity

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The products of the mammalian *Pbx* and *Drosophila exd* genes are able to interact with Hox proteins specifically and to increase their DNA binding affinity and selectivity. In the accompanying paper we show that Pbx proteins exist as stable heterodimers with a novel homeodomain protein, Prep1. Here we show that Prep1–Pbx interaction presents novel structural features: it is independent of DNA binding and of the integrity of their respective homeodomains, and requires sequences in the N-terminal portions of both proteins. The Prep1–Pbx protein–protein interaction is essential for DNA-binding activity. Prep1–Pbx complexes are present in early mouse embryos at a time when Pbx is also interacting with Hox proteins. The use of different interaction surfaces could allow Pbx to interact with Prep1 and Hox proteins simultaneously. Indeed, we observe the formation of a ternary Prep1–Pbx1–HOXB1 complex on a HOXB1-responsive target *in vitro*. Interaction with Prep1 enhances the ability of the HOXB1–Pbx1 complex to activate transcription in a cooperative fashion from the same target. Our data suggest that Prep1 is an additional component in the transcriptional regulation by Hox proteins.

Keywords: homeobox/gene expression/transcription/ developmental regulation/protein–protein interaction

Introduction

The homeodomain protein Pbx1 was originally discovered in human pre-B acute lymphoid leukemia (preB-ALL) as a C-terminal fusion to the 483 N-terminal residues of the IgK enhancer-binding protein E2A-E12 (Kamps *et al.*, 1990; Nourse *et al.*, 1990). The E2A–Pbx fusion appears to produce a novel chimeric protein with Pbx DNA-binding specificity and E2A transactivation properties. Pbx itself does not appear to be a transactivator but the fusion to the E2A protein confers transactivating and oncogenic properties (Kamps *et al.*, 1991; Dedera *et al.*, 1993; Kamps and Baltimore, 1993; Uckun *et al.*, 1993; Van Dijk *et al.*, 1993; Lu *et al.*, 1994, 1995; Monica *et al.*, 1994; Hunger, 1996). The Pbx family includes two other members, Pbx2 and Pbx3 (Monica *et al.*, 1993). Pbx1 and Pbx3, in addition, exist in two alternatively spliced forms, Pbx1a and -1b, Pbx3a and -3b (Kamps *et al.*, 1990; Nourse *et al.*, 1990). The sequence of Pbx proteins is highly conserved and shares extensive homology with the *Caenorhabditis elegans* protein ceh-20 and *Drosophila* Exd; together they constitute the PBC class of homeodomain proteins (Burglin and Ruvkun, 1992; Monica *et al.*, 1993). Besides the homeodomain, PCB family proteins contain two highly homologous regions in their N-terminal part, termed PCB-A and PCB-B, of unknown function (Burglin and Ruvkun, 1992). Pbx1a, -2 and -3a are all 50 kDa proteins with major differences only in the first 43 amino acids and minor differences in the C-terminal portion. The alternative b forms of Pbx1 and Pbx3 are C-terminally truncated with an apparent molecular mass of 40 kDa (Monica *et al.*, 1993).

The *Drosophila* Pbx homologue extradenticle (Exd) (Rauskolb *et al.*, 1993) cooperatively interacts with homeobox proteins encoded by the Homeotic Complex selector genes, determining the expression pattern of homeotic target genes (Chan *et al.*, 1994; Rauskolb and Wieschaus, 1994; Popperl *et al.*, 1995). Exd acts as a co-factor directing different homeotic selector proteins to different target genes (Wilson and Desplan, 1995). Similarly, the Pbx proteins display cooperative binding with a subset of the Hox proteins (Chang *et al.*, 1995, 1996; Knoepfler and Kamps, 1995; Lu *et al.*, 1995; Phelan *et al.*, 1995; Van Dijk *et al.*, 1995; Chan *et al.*, 1996; Chan and Mann, 1996; Lu and Kamps, 1996; Peltenburg and Murre, 1996), cooperating in activating promoters containing a Pbx responsive element (PRS) (White, 1994; Lu *et al.*, 1995; Phelan *et al.*, 1995; Wilson and Desplan, 1995; Mann and Chan, 1996). Pbx can further direct different HOX proteins to slightly different target sequences (Chang *et al.*, 1996). The interaction between Pbx and Hox proteins requires both homeodomains, a stretch of 20 aa C-terminal to the Pbx homeodomain and the conserved pentapeptide sequence YPWMX or a similar ANW amino acid motif N-terminal to the Hox homeodomain (Chang *et al.*, 1995, 1996; Knoepfler and Kamps, 1995; Lu *et al.*, 1995; Phelan *et al.*, 1995; Van Dijk *et al.*, 1995; Chan *et al.*, 1996; Chan and Mann, 1996; Lu and Kamps, 1996; Mann and Chan, 1996; Peltenburg and Murre, 1996).

Intracellularly, Pbx is found complexed with a novel homeodomain protein Prep1 (Berthelsen *et al.*, 1998). The Prep1–Pbx complex forms a DNA-binding activity factor previously identified and purified as the Urokinase Enhancer Factor 3 (UEF3), a factor important for the regulation of the urokinase enhancer, as well as of several other AP-1 controlled promoters (Nerlov *et al.*, 1992; Berthelsen *et al.*, 1996; De Cesare *et al.*, 1996, 1997). By complexing, Prep1 and Pbx acquire a strong DNA-binding activity (Berthelsen *et al.*, 1998). Unlike the Pbx–Hox complex, Prep1 and Pbx dimerize efficiently in solution

Table I. Oligonucleotides used in this study

o-17	CAGCAATCAGCA**TGACAG**CCTCCAGC
o-17mI	............CTCG..........
o-1	CACCTGAGAG**TGACAG**AAGGAAGGCAGGGAG
o-1m	.........T.C..................
oHP	CGAA**TTGATTGAT**GCACTAATTGGAG
oHPm	....CC.....................
oPRS	CGAAA**TTGATTGAT**GCGCCCCGCGCT
oPRSm	....CC.....................
B1-R3	GATCCGGGGG**GTGATGGAT**GGGCGCTGGGA

Comparison of the o-17 and o-1 oligonucleotides containing the two different uPA promoter UEF3 binding sites and their mutated homologs. A dot indicates an identical base. The core binding site is shown in bold letters. Below, comparison of the three oligonucleotides containing binding sites for Pbx and Pbx–Hox, shown in bold.

independently of the presence of the DNA target site, and the complex is resistant to high salt concentrations and to chromatographic manipulations. Interestingly, Prep1 does not contain any YPWM or similar motif; in fact there is no W residue N-terminal to the homeodomain of Prep1. Prep1 and Pbx most likely exist as a stable complex in the nucleus, as stable Prep1–Pbx complexes can be isolated from nuclear extracts (Berthelsen *et al.*, 1998).

In this paper we explore the consequence of the Prep1–Pbx complex formation with respect to Hox protein activity. Prep1 and Pbx interaction broadens the DNA-target selectivity of both. Indeed, the Prep1–Pbx complex not only binds to the TGACAG sequence of the urokinase enhancer by which it was originally purified, but binds with equal affinity to Pbx and Pbx–Hox-responsive sequences (PRS). The interaction between Prep1 and Pbx requires sequences in the N-terminus of both proteins, but is independent of the integrity of the homeodomains, showing that the Prep1–Pbx interaction is clearly different from the Pbx–Hox interaction. On the other hand, both N-terminal sequences and intact homeodomains are required for DNA binding of Prep1–Pbx. We further find nuclear Prep1–Pbx complexes in 9.5 days post conception (d.p.c.) mouse embryos at a time when Pbx functionally interacts with Hox proteins. Thus the formation of a Prep1–Pbx complex may interfere with the formation and/or the function of a Hox–Pbx complex. In fact, Pbx interaction with Prep1 or Hoxb1 is not mutually exclusive; instead, in co-transfection experiments Prep1 is shown to stimulate the transactivating activity of HOXB1–Pbx. We also show that Prep1, Pbx and HOXB1 together can form a ternary complex on PRS-like DNA targets. Even though Prep1 and Pbx contain similar homeodomains, Prep1, unlike Pbx, does not bind directly to HOXB1. Overall these data suggest that Prep1 is an additional co-factor of Pbx–Hox regulated transcription.

Results

DNA binding specificity of the Prep1–Pbx complex

Pbx proteins alone bind preferentially to the Pbx responsive sequence (PRS) TTGATTGAT, identified by PCR-mediated binding-site selection (Lu *et al.*, 1994). This sequence is also a high affinity binding site for Pbx–Hox heterodimers (Chang *et al.*, 1995; Mann and Chan, 1996) and is present in the oligonucleotides oPRS (Lu *et al.*, 1994) and oHP used in this study (Table I). Similar sites binding Pbx–Hoxb1 complexes are present in the autoregulatory element (b1-ARE) of the Hoxb1 gene (Popperl *et al.*, 1995). In particular we have used an oligonucleotide containing the third repeat (R3) of the b1-ARE (B1-R3, see Table I for sequences). The PRS nonanucleotide sequence is quite different from the urokinase enhancer Prep1–Pbx target sequence, TGACAG, present in the o-1 oligonucleotide, which is efficiently bound by the Prep1/Pbx heterodimer (Berthelsen *et al.*, 1998). We have tested whether Prep1–Pbx can also bind the PRS motif. To this end we have performed EMSA analysis employing both crude HeLa nuclear extracts and *in vitro* translated proteins (Figure 1). Using crude nuclear extracts with labeled o-1 we observe that formation of the two complexes (UC and LC) can be competed specifically by excess unlabeled o-1 oligonucleotide and equally efficiently by the oHP oligonucleotide (Figure 1A). The mutated oHPm that is unable to bind Pbx and Pbx–HOX (Chang *et al.*, 1995) is also unable to compete for binding to o-1. Similarly, when using labeled oHP as a probe in EMSA with HeLa nuclear extracts we see formation of two complexes indistinguishable from those obtained with o-1 (Figure 1B). Formation of the two complexes is specifically competed by both the o-1 and oHP, with equal efficiency. The complexes formed by o-1 and oHP in fact seem to be identical as they are recognized by both anti-Prep1 and anti-Pbx antibodies (Figure 1A and B). Interestingly, the complexes bound to the oHP target contained both Pbx and Prep1, and not Pbx alone. This suggests that Prep1–Pbx binds equally well to the different core binding sites, TGACAG and TGATTGAT. To confirm this, we have tested the DNA binding specificity of Prep1–Pbx1a complex produced *in vitro*. Both o-1 (Figure 1C) and oHP (Figure 1D) produce a retarded complex of equal intensity that in both cases is sensitive to anti-Prep1 antibodies (Figure 1C and D, last lanes). In this case, all the PRS containing oligonucleotides that we employed, as well as o-1, competed with equal efficiency for binding of the Prep1–Pbx complex to either the o-1 or oHP target. None of the mutated oligonucleotides used were able to compete. On the basis of the above experiments we conclude that the Prep1–Pbx complex exhibits a high affinity, specific DNA binding towards both the TGACAG and the TGATTGAT motifs.

Prep1–Pbx1a interaction requires N-terminal sequences of both proteins

The interaction between Prep1 and Pbx has different properties than that between Pbx and Hox proteins (Berthelsen *et al.*, 1998). To characterize further the interaction between Prep1 and Pbx proteins we have employed mutations of both proteins and tested for their ability to form complexes and to bind DNA.

To address the importance of the Prep1 homeodomain we constructed two mutations within the Prep1 homeodomain (Figure 2A): Prep1HDI50Q contains a glutamine residue at position 50 in the homeodomain instead of an isoleucine, a position important in determining the DNA-binding specificity (Treismann *et al.*, 1989); the entire homeodomain (residues 259–318) is deleted in Prep1ΔHD. To test the role of the N-terminal conserved portion of Prep1, we further employed a Prep1 with the two Meis-homologous regions, HR1 and HR2 (residues 58–137) deleted (Berthelsen *et al.*, 1998). The constructs were

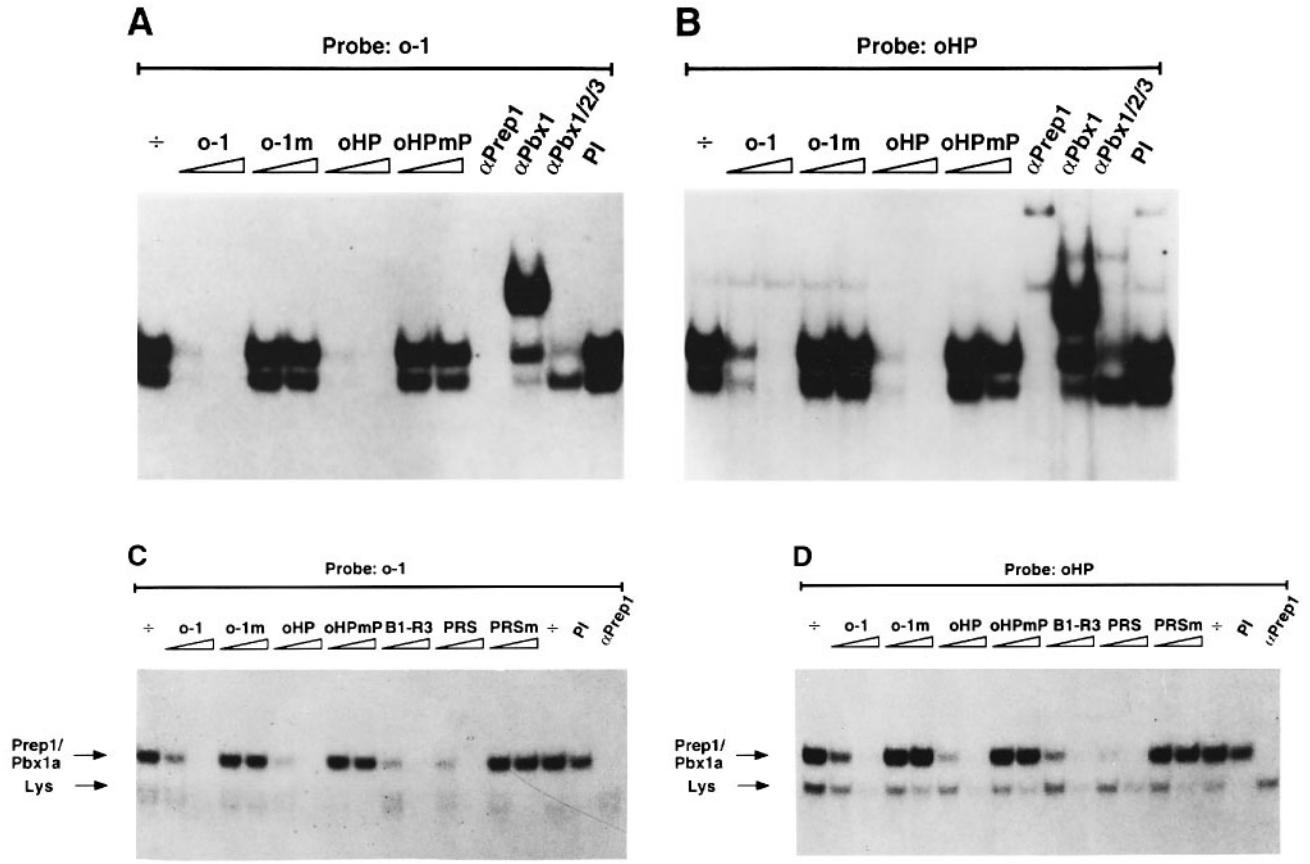

Fig. 1. DNA-binding specificity of the Prep1–Pbx heterodimers. Comparison of UEF3 DNA binding to either the o-1 or oHP oligonucleotides. (**A**) EMSA analysis with labeled o-1 oligonucleotide and HeLa nuclear extract (HeLa NE), showing formation of UC and LC complexes. Binding was competed by addition of excess unlabeled oligonucleotides, as indicated, using 50- and 500-fold molar excess. The nature of the retarded complexes were accessed by co-incubation with antibodies against Prep1 (αPrep1), Pbx1 (αPbx1), Pbx (αPbx1/2/3) or preimmune sera (PI). (**B**) Same conditions as in (A), but using labeled oHP oligonucleotide as probe. (**C**) DNA-binding specificity of *in vitro* produced Prep1–Pbx1 complex. EMSA analysis with o-1 oligonucleotide and *in vitro* co-translated Prep1–Pbx1a products. The binding specificity was assessed by competition with various oligonucleotides; the sequences of these are shown in Table I. Competition was performed by addition of 50- and 500-fold molar excess cold oligonucleotides. To verify that the observed band contained Prep1, we co-incubated with αPrep1 or PI. (**D**) Same conditions as in (C), but using labeled oHP oligonucleotide as probe. The reticulocyte lysate contains a non-specific endogenous activity that binds the o-1 and the oHP, oligonucleotides (marked by Lys).

translated *in vitro* together with Pbx1b (Figure 2B, left) and immunoprecipitated with anti-Pbx1 antibodies (Figure 2B, right). Both homeodomain mutants, Prep1HDI50Q and Prep1ΔHD, are co-precipitated as efficiently as wild-type Prep1, suggesting that the Prep1 homeodomain is not involved in the Pbx1 interaction in solution. In contrast, Prep1ΔHR1+2 does not co-precipitate with Pbx1b, indicating that the Prep1 HR1 and HR2 regions include the Pbx1 interaction domain. We further tested the DNA-binding ability of the co-translates by EMSA, using two different Prep1–Pbx DNA target sites, the o-1 and b1R3 oligonucleotides (Figure 2C). DNA binding of the Prep1HDI50Q–Pbx1b complex is impaired with respect to wild-type Prep1–Pbx1b on both target sites. No binding is observed when using co-translates of Pbx1b with Prep1ΔHD or Prep1ΔHR1+2. Overall, this shows that the Prep1 homeodomain, while not being required for Prep1–Pbx1 interaction, is important for DNA binding of the complex. In addition, since the homeodomain is still present in the Prep1ΔHR1+2 the loss of DNA-binding activity of this mutant protein when co-translated with Pbx1 suggests that pre-formation of a Prep1–Pbx complex is required for DNA binding.

In order to find the Prep1 interacting surfaces of Pbx1, we repeated the experiments with Pbx1a mutants (Figure 3A). Pbx1aΔ283–285 contains a small deletion in the homeodomain and is functionally unable to interact with HOXB1 (Di Rocco *et al.*, 1997). We took further advantage of the naturally occurring Pbx mutant, E2A–Pbx1. The oncogenic fusion product E2A–Pbx1 contains the trans-activating domain of the E2A protein, substituting the first 88 residues of Pbx1 including part of the PCB-A domain. In addition, we used a Pbx1a deleted of the first 140 residues including all of the PCB-A domain, but still having an intact PCB-B domain (Di Rocco *et al.*, 1997). These Pbx1a constructs were co-translated *in vitro* with Prep1 (Figure 3B, left) and analyzed by immunoprecipitation and EMSA experiments. Immunoprecipitation with an anti-Prep1 serum (Figure 3B, right) shows that both full length Pbx1a and Pbx1aΔ283–285 co-precipitate with Prep1. In contrast, bands corresponding to Pbx1aΔ1–140 or E2A–Pbx1a are not co-precipitated. Thus, as in the case of Prep1, sequences in the N-terminal part of Pbx and not in the homeodomain seem to be important for interaction with Prep1. When tested for DNA binding (Figure 3C), we observe that of the co-translates only

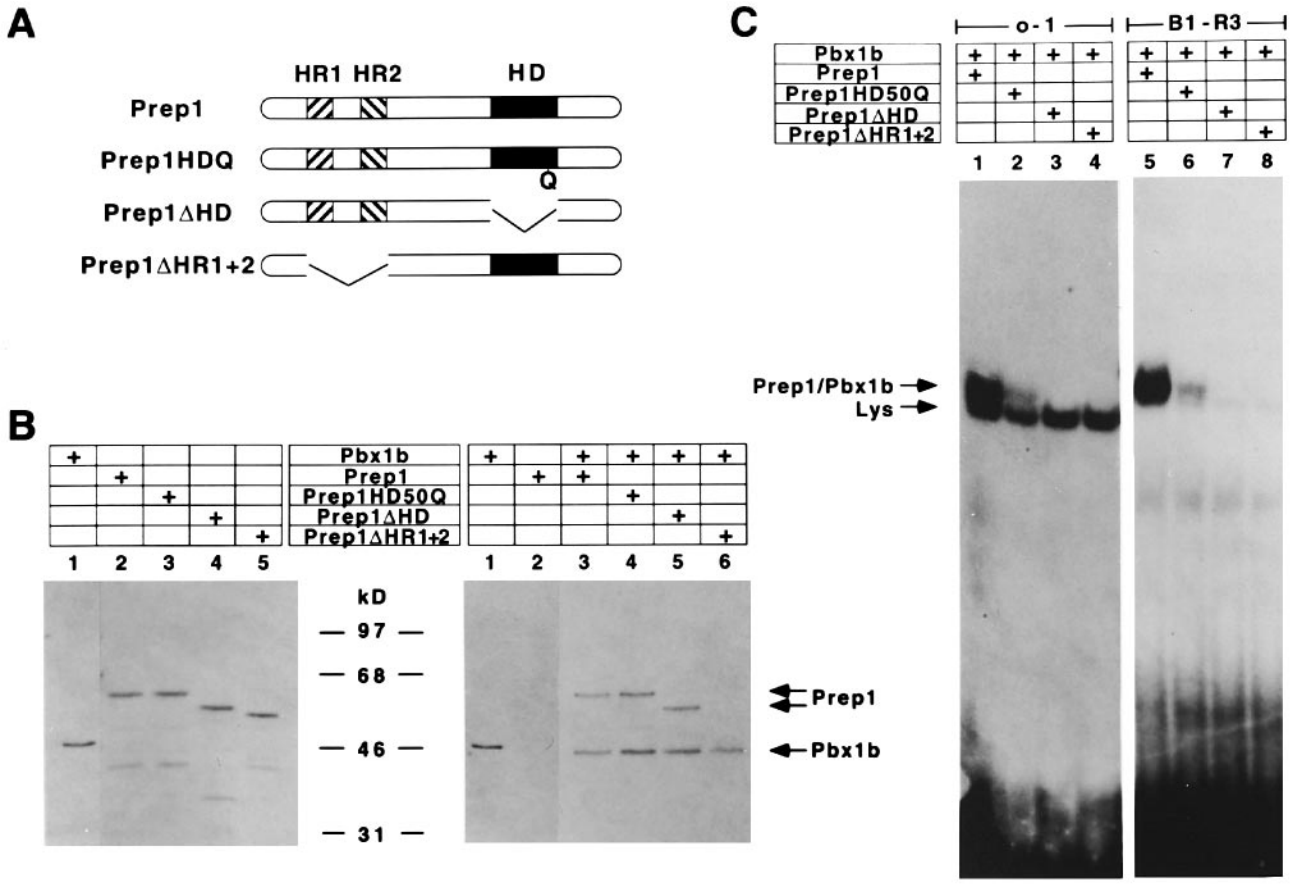

Fig. 2. Pbx-interacting domains of Prep1. (**A**) Schematic representation of Prep1 and Prep1 mutants, showing the position of the homeodomain (HD) and of the Meis-homologous regions, HR1 and HR2. Blank spaces represent deletions. (**B**) Left panel: SDS–PAGE of *in vitro* translated Pbx1b and Prep1 constructs, as indicated. As observed before (Berthelsen *et al.*, 1998), *in vitro* translated Prep1 display a major full-length product, and several minor truncated products. Right: immunoprecipitation of *in vitro* translated Pbx1b (lane 1), Prep1 (lane 2) and co-translation of Pbx1b with different Prep1 derivatives, as indicated (lanes 3–6). The translates were immunoprecipitated with anti-Pbx1 antibodies and Protein A–Sepharose, and the precipitates were resolved by SDS–PAGE. Bands corresponding to Pbx1b and Prep1 derivatives are indicated by arrows. Molecular weights are indicated. We used Pbx1b in this study to be able to discriminate between the migration of the PrepΔHR1+2 product (co-migrates with Pbx1a) and Pbx1. (**C**) EMSA analysis of cooperative DNA binding of Pbx1b and Prep1 mutants. The co-translates, as indicated, were incubated with the TGACAG-containing o-1 oligonucleotide (left panel) or with the Hoxb-1 promoter b1-ARE R3-site (right panel). Bands corresponding to the Pbx1b–Prep complex are indicated by arrows. An endogenous factor present in the lysate (marked by 'Lys') binding strongly to the o-1 oligonucleotide, and weakly to the R3-oligonucleotide, is observed.

Prep1–Pbx1a can bind the target sites o-1 or R3, while no DNA-binding activity is observed with the Pbx1a mutants, Pbx1aΔ283–285, Pbx1Δ1–140 or E2A–Pbx1a.

In conclusion, formation of the Prep1–Pbx complex prior to binding seems to be mandatory for DNA binding of the complex as mutations that interrupt complex formation also interrupt DNA binding. Further, the dimerization surfaces of Prep1 and Pbx1 include the N-terminus of both proteins but not the homeodomains. Hence the interaction involves novel dimerization domains.

Prep1–Pbx complexes are present during mouse embryogenesis

We have searched for the presence of DNA-binding Prep1–Pbx complexes in nuclear extracts isolated from E9.5 d.p.c. mouse embryos. EMSA shows specific DNA-binding activity, giving two retarded bands identical to those of HeLa cells (Figure 3). Incubation with specific antibodies further shows that both bands are inhibited by anti-Prep1 antibodies. Moreover, antibodies directed against Pbx1 or against all Pbxa forms (αPbx1/2/3) inhibit formation of, or supershift, either of the two complexes, UC and LC, and a combination of antibodies recognizing Pbx1 and Pbx2 inhibit formation of both complexes. These results show that the retarded bands observed in embryonic extracts are complexes of Prep1 with Pbx1a, Pbx1b or Pbx2. As in the case of HeLa cell extracts (Berthelsen *et al.*, 1998), we have not found Pbx3 associated with Prep1 in the 9.5 d.p.c. mouse embryonic extracts. In conclusion, Pbx proteins are found complexed with Prep1 at a time during development where Pbx is also known to functionally interact with Hox proteins. Thus the presence of the Prep1–Pbx complexes might interfere with the function of a Pbx–Hox complex.

Prep1 enhances Pbx–HOX-dependent transactivation

To investigate whether Prep1 could interfere with the formation and functioning of a Hox–Pbx complex we chose the transcriptional activation of the Hoxb-1 autoregulatory element (b1-ARE) by the HOXB1–Pbx1 complex as a model system. We have previously shown that a Pbx1–HOXB1 complex cooperatively binds to, and activates transcription from, the R3 site contained in the b1-ARE

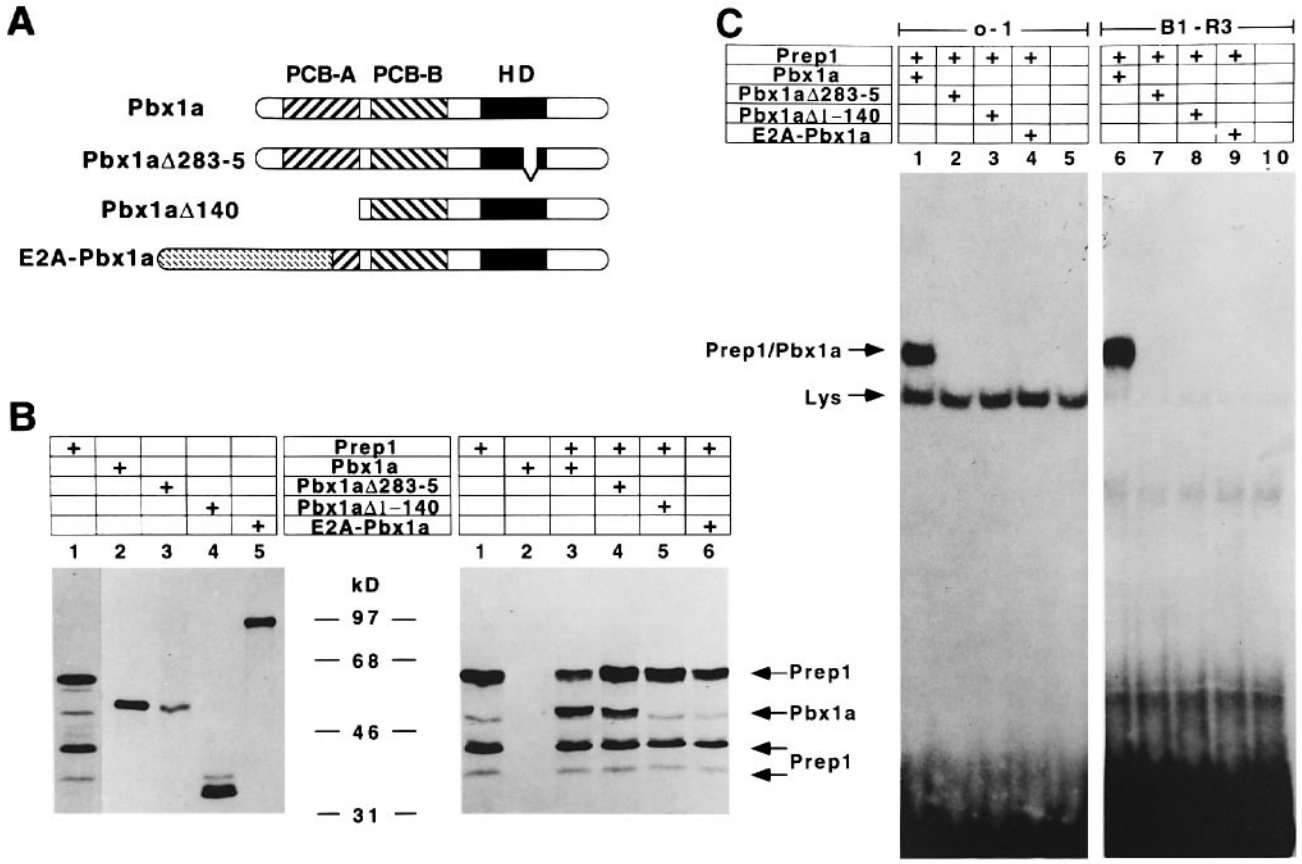

Fig. 3. Prep1-interacting domains of Pbx1. (**A**) Schematic representation of Pbx1a and the Pbx1a mutants, showing the position of the homeodomain (HD) and of the conserved PBC-A and PBC-B regions. Blank spaces represent deletions. (**B**) Left panel: SDS–PAGE of *in vitro* translated Prep1, Pbx1a constructs or E2A–Pbx1a, as indicated. As observed before (Berthelsen *et al.*, 1998), *in vitro* translated Prep1 display a major full-length product, and several minor truncated products. Right: immunoprecipitation of *in vitro* translated Prep1 (lane 1), Pbx1b (lane 2) and co-translates of Prep1 with different Pbx1a derivatives or E2A–Pbx1a, as indicated (lanes 3–5). The translates were immunoprecipitated with anti-Prep1 antibodies and Protein A–Sepharose and the precipitates were resolved by SDS–PAGE. Bands corresponding to Prep1 and Pbx1a derivatives are indicated by arrows. Molecular weights are indicated. (**C**) EMSA analysis of cooperative DNA binding of Prep1 and Pbx1a mutants. The Prep1–Pbx1a-derivative co-translates, as indicated, were incubated with the TGACAG-containing o-1 oligonucleotide (left panel) or with the Hoxb-1 promoter b1-ARE R3-site (right panel). Bands corresponding to the Prep–Pbx1a complex are indicated by arrows. An endogenous factor present in the lysate (marked by 'Lys') binding strongly to the o-1 oligonucleotide, and weakly to the R3-oligonucleotide, is observed.

(Di Rocco *et al.*, 1997). We have also shown above that a Prep1–Pbx1 complex can bind to the R3 site of the b1-ARE. We analyzed the effect of co-expression of Prep1 on the Pbx1–HOXB1-dependent transactivation through the b1-ARE enhancer in transient transfections of COS cells. The b1-ARE basal activity is not stimulated by either Pbx1a, HOXB1 or Prep1 alone (data not shown). Co-transfection of HOXB1 and Pbx1a induces a 4 to 5-fold activation of transcription, as previously reported (Di Rocco *et al.*, 1997). Co-expression of Prep1 with either Pbx1 or HOXB1 does not significantly stimulate the b1-ARE reporter activity (Figure 5C) even though transfected cells displayed increased DNA-binding activity, as shown by EMSA (data not shown). However, co-expression of Prep1 with both Pbx1 and HOXB1, instead of antagonizing the HOXB1–Pbx1 complex transcriptional activation, causes an additional increase of the reporter activity (9-fold over basal level). In order to understand whether the stimulation of the HOXB1–Pbx1 complex activity requires the DNA binding function of Prep1, we employed the Prep1ΔHD and the Prep1HD50Q mutants in the cotransfection experiments, which have impaired DNA-binding functions. Both mutants are still able to enhance the transcriptional activity of the HOXB1–Pbx1 complex activity, the Prep1ΔHD mutant causing an even higher stimulation of the complex activity. Thus, the DNA-binding activity by Prep1 is not required for enhancing the HOXB1–Pbx1-mediated transcriptional activation. Next we tested Prep1ΔHR1+2, which is unable to form a stable complex with Pbx proteins *in vitro*. Prep1ΔHR1+2 has lost the ability to enhance HOXB1–Pbx1 complex activity. We also tested a HOXB1 mutant lacking the N-terminal activation domain (HOXB1HD, Figure 5A and B). This mutant can still complex with Pbx1 and causes a low level activation of the reporter (Di Rocco *et al.*, 1997). Co-expression of Prep1 with HOXB1HD and Pbx1 leads to an enhancement of the activation displayed by HOXB1HD and Pbx1 (Figure 5C). Finally, we tested a Pbx1 mutant representing a deletion of the first 140 amino acids, which comprises the conserved PBC-A domain. The Pbx1Δ1–140 mutant, while being unable to interact stably with Prep1 *in vitro*, is able to form a transcriptionally active complex with HOXB1 on the b1-ARE region. The activity of the HOXB1–Pbx1Δ1–140 complex cannot be further enhanced by co-expression with Prep1 (Figure 5C).

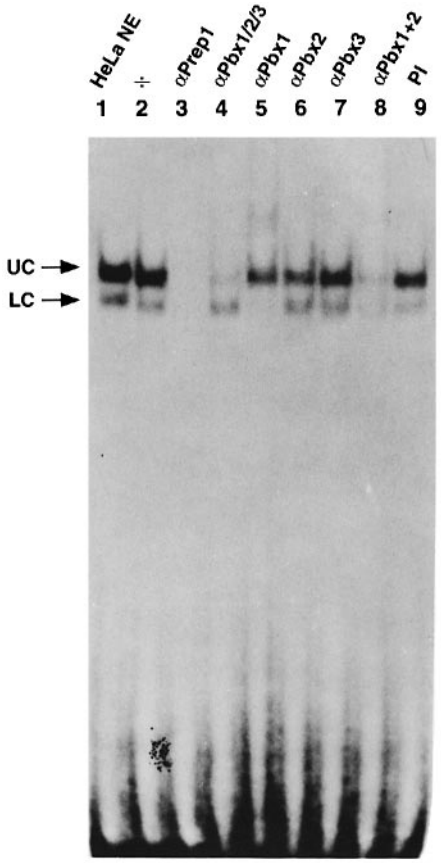

Fig. 4. Prep1–Pbx complexes are present in mouse embryos. EMSA analysis of nuclear extracts of 9.5 d.p.c. mouse embryos (lanes 2–9) and of HeLa nuclear extracts (HeLa NE, lane 1) using the TGACAG motif (o-1 oligonucleotide). The subunit composition of the complexes was investigated by co-incubating the binding reaction with specific antibodies as indicated. PI: preimmune serum. A combination of αPbx1 and αPbx2 antibodies inhibits all binding.

In conclusion, these results indicate that Prep1, instead of preventing the formation of the Pbx1–HOXB1 complex or sequestering Pbx1 in an inactive complex on the b1-ARE, is able to further enhance transcriptional activity of the HOXB1–Pbx1 complex on the b1-ARE. While the DNA-binding function of Prep1 is not required for stimulation of the HOXB1–Pbx1 complex activity, the interaction surfaces between Prep1 and Pbx1 are necessary for stimulation. This suggests that Prep1 can directly interact with and stimulate a DNA-bound HOXB1–Pbx1 complex through Pbx.

Prep1, Pbx and HOXB1 form a ternary complex on DNA

We have analyzed by EMSA whether Prep1 can affect the formation of a Pbx–Hox complex. Complexes of Prep1–Pbx1a and Pbx1a–HOXB1 can bind to the b1-ARE R3 element, and can be distinguished by their different mobility (Figure 6A, lanes 3 and 6). No complexes are observed with Prep1 and HOXB1 (Figure 6, lane 7). When all three proteins are present (Figure 6, lane 8) the Pbx–HOXB1 band disappears, substituted by a slower migrating band. This slow migrating band, which might represent a ternary Prep1–Pbx1a–HOXB1 complex on DNA, is not observed when we use a homeodomain-less HOXB1 mutant (HOXB1ΔHD, see Figure 5A and B), indicating that formation of this complex requires HOXB1 DNA-binding function (Figure 6A, lanes 13–15). Similarly, no slow migrating retarded band is observed by using the Prep1ΔHR1+2 mutant that is unable to complex with Pbx (Figure 6B, lanes 1–2). Further proof that the slower migrating band represents a ternary Prep1–Pbx1a–HOXB1 complex is obtained by the use of specific anti-Prep1 and anti-Pbx1 antibodies which inhibit the formation of both the slower migrating band as well as the Prep1–Pbx complex (Figure 6B). Most interestingly, the Prep1 homeodomain is not required for the assembly of the ternary complex on DNA. In fact, mutation or deletion of the Prep1 homeodomain, that reduces or abolishes DNA binding of the Prep1–Pbx complex (Prep1ΔHD and Prep1HD50Q), increases the intensity of the ternary complex (Figure 5A, lanes 4 and 5). These results are in agreement with our transfection experiments (Figure 5C) where we observe enhancement of the HOXB1–Pbx1 complex activity by Prep1 mutated in the homeodomain.

To verify that the interaction between Prep1 and HOXB1–Pbx1 occurs via Pbx1 and to rule out the possibility that Prep1 could also interact with HOXB1, we have performed a GST-pulldown assay. A GST–HOXB1 homeodomain (GST–B1HD) fusion protein was incubated with *in vitro* translated Pbx1a or Prep1, precipitated with glutathione–Sepharose and the precipitates resolved by SDS–PAGE (Figure 7). We observe no interaction between GST–B1HD and Prep1, while a specific interaction can be observed with Pbx1a. No interactions are detected with the GST control resin.This result is in agreement with the observation that, in EMSA, HOXB1 can form a complex with Pbx1a, but not with Prep1 (Figure 6A). Taken together these data show that Prep1 is able to directly interact with a DNA-bound Hox–Pbx complex through the Pbx moiety, forming a transcriptionally active ternary complex.

Discussion

It is clearly established that Pbx/Exd interact with Hox–HOM-C proteins and that this is an important requirement for Hox–HOM-C function (Mann, 1995; Mann and Chan, 1996). Nevertheless, the properties of the Pbx–Hox or Exd–HOM-C protein complexes do not explain all of the functions of the Hox–HOM-C proteins (Wilson and Desplan, 1995). The findings reported in this paper may begin to shed light in this direction.

Prep1 and HOX interact with Pbx through different surfaces

Specific amino acid residues have been identified in HOX proteins that mediate the interaction with Pbx/Exd proteins. Hox proteins known to interact with Pbx contain a conserved pentapeptide sequence YPWMX or a similar ANW amino acid motif located N-terminally to the homeodomain (Chang *et al.*, 1995, 1996; Johnson *et al.*, 1995). Together with the homeodomain, this motif is essential for the heterodimerization of HOX–HOM-C proteins with Pbx/Exd (Chang *et al.*, 1995, 1996; Johnson *et al.*, 1995; Knoepfler and Kamps, 1995; Lu *et al.*, 1995; Neuteboom *et al.*, 1995; Phelan *et al.*, 1995; Van Dijk *et al.*, 1995; Peltenburg and Murre, 1996; Chan and Mann, 1996; Chan *et al.*, 1996; Lu and Kamps, 1996). Interaction of Pbx

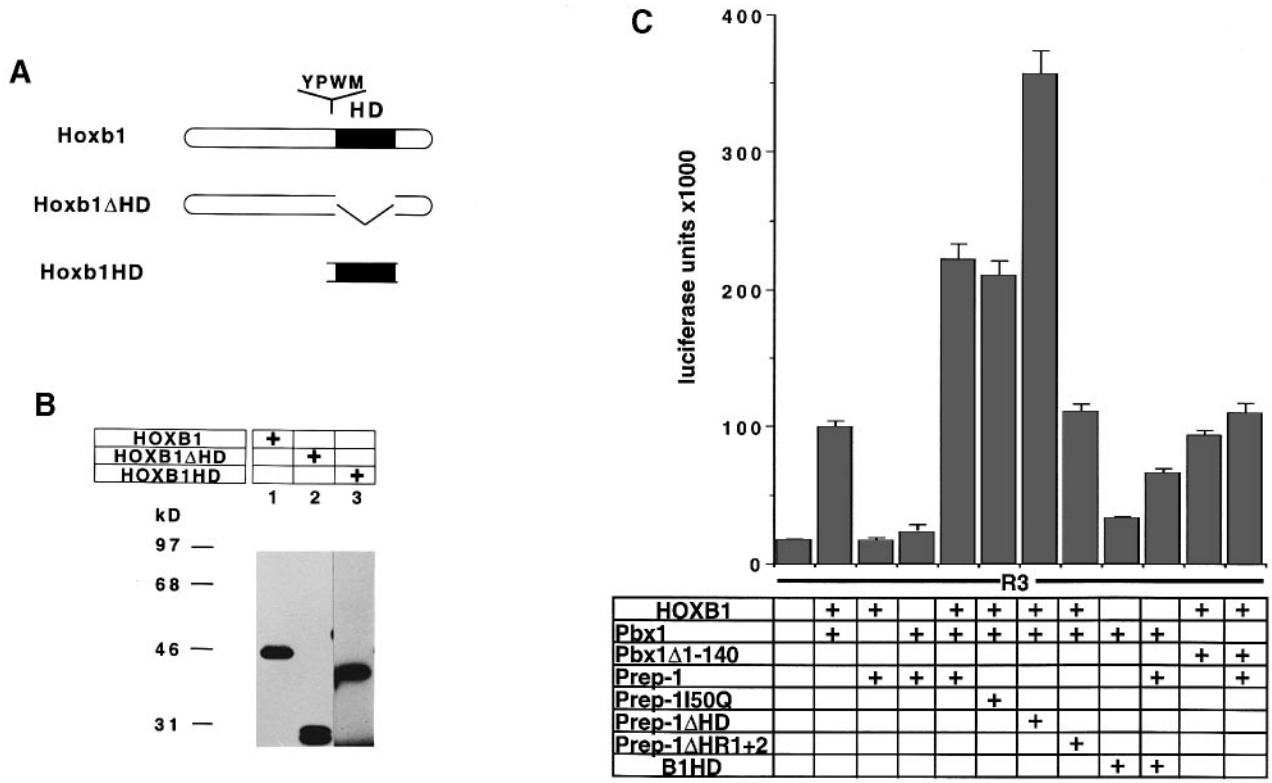

Fig. 5. Prep1 enhances Pbx1–HOXB1 transcriptional activity. (**A**) Schematic representation of HOXB1 and HOXB1 mutants used in this study. The homeodomain (HD) and the conserved pentapeptide motif, YPWMX, are shown. Blank spaces represent deletions. (**B**) SDS–PAGE analysis of *in vitro* translated HOXB1 derivatives. (**C**) Luciferase activity assayed from extracts of transiently transfected COS cells. Cells were transfected with 8 μg of the pAdMLR3 reporter construct, with 4 μg of the pSGHOXB1 or the pSGB1HD expression constructs, together with 8 μg of the pSGPbx1a or PSGPbx1Δ1–140 expressors, where indicated. Cells were also cotransfected with 8 μg of pSGPrep1 or of the pSGPrep1 mutant derivatives as indicated. 0.2 μg of the pCMVb-gal plasmid were co-transfected as an internal standard. Bars represent the mean luciferase activity ±SEM of at least four independent experiments.

with various Hox proteins was reported to occur only in the presence of the DNA target (Chang *et al.*, 1995), even if Hox proteins can interact with Exd/Pbx proteins in a yeast two-hybrid assay (Johnson *et al.*, 1995) and *in vitro* in GST pulldown assays (this work). Thus, it is not clear whether *in vivo* Pbx/Exd interacts with the HOM-C–HOX proteins in solution, or whether the interaction only occurs on specific DNA sequences. *In vitro* data, however, at least suggest that DNA binding promotes complex formation.

Prep1 on the other hand forms a strong complex with Pbx in solution independently of DNA binding (Berthelsen *et al.*, 1998). Indeed, complex formation precedes DNA binding as uncomplexed Prep1 and Pbx bind DNA poorly. Unlike Hox proteins, Prep1 does not contain any YPWMX or similar motif. In fact, Prep1 does not contain any W residues N-terminally to the homeodomain. Together with the strength of the Prep1–Pbx complex, the DNA-independence classifies the Prep1–Pbx interaction as being different from Pbx–HOX, probably involving structurally different dimerization motives.

In this paper we have further investigated this point. Unlike Hox–Pbx, we have found that the interaction between Prep1 and Pbx1 does not depend on the integrity of the two homeodomains. Deletion or disruption of the homeodomain of either Prep1 or Pbx1 does not impair the ability of the proteins to complex with each other, even though the resulting complex is unable to bind DNA. Instead we find that deletion of the N-terminal, conserved PCB-A domain (Burglin and Ruvkun, 1992) of Pbx1 (Pbx1aΔ1–140) prevents its interaction with Prep1 in solution, as well as its ability to cooperatively bind DNA with Prep1. The PCB-A domain is a conserved region between the PCB family proteins. Similarly, the PCB-A domain is required for Meis-1 interaction (Chang *et al.*, 1997). In contrast, the deletion of the PBC-A domain does not affect the ability of Pbx to interact with the Hox proteins and to cooperatively activate transcription (Di Rocco *et al.*, 1997). In addition, we have identified two novel regions in the N-terminus of the Prep1 protein, that are important for the Prep1–Pbx1 interaction. These two regions, that we call HR1 and HR2, display strong homology between Prep1 and the Meis family of homeodomain proteins (Berthelsen *et al.*, 1988). Deletion of the HR1 and HR2 domains not only prevents Prep1–Pbx interaction in solution but also the cooperative DNA-binding activity of the Prep1–Pbx complex, despite the presence of an intact DNA-binding domain.

Thus reciprocal interaction surfaces are located in the N-terminus of both Prep1 and Pbx1. Our data further suggest that dimerization and DNA binding occur through separate domains and that dimerization is required for DNA binding. This interaction represents a novel way by which homeodomain proteins are able to dimerize.

Our observation that the N-terminal PCB-A domain of Pbx1 is important for the Prep1–Pbx1 interaction is interesting since in the naturally occurring E2A–Pbx1

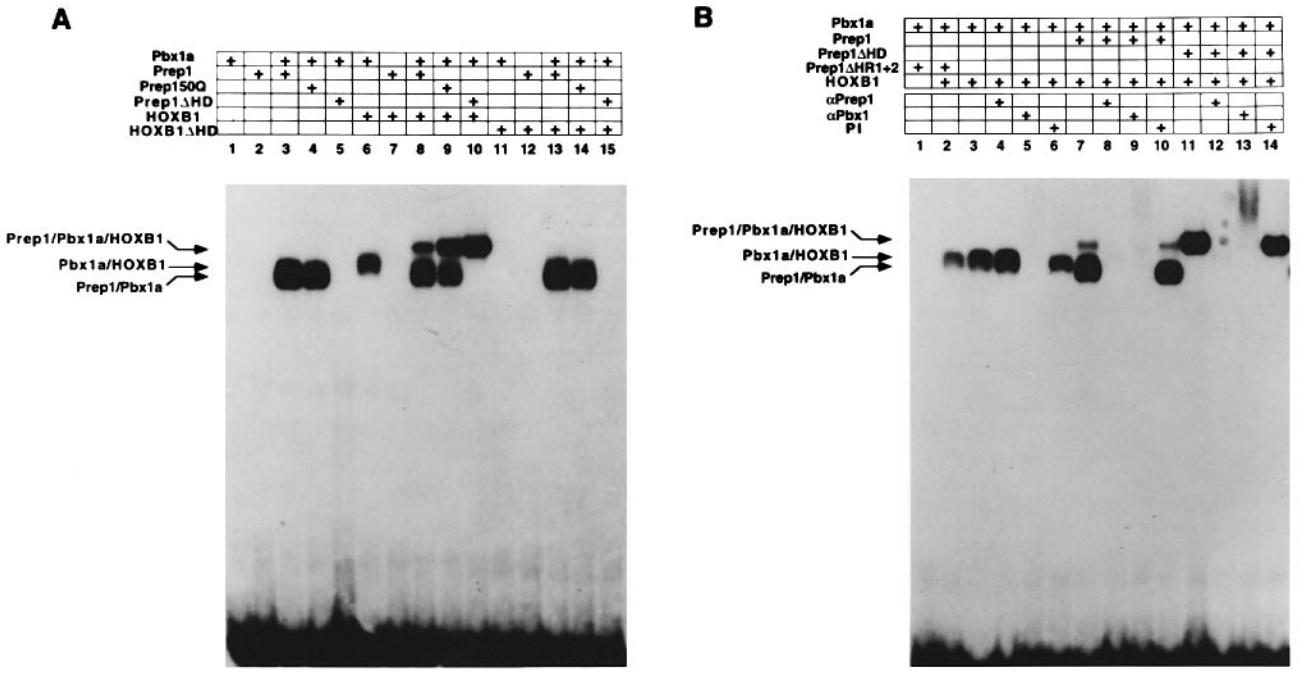

Fig. 6. Prep1 interacts with a HOX–Pbx complex. (**A**) EMSA analysis of combinations of the Prep1, Pbx1a and HOXB1 *in vitro* translated proteins, and mutated derivatives thereof, as indicated, using an oligonucleotide representing the R3 sequence of the b1-ARE enhancer. Migration of the various complexes are indicated with arrows. The EMSA was performed with 0.5 μg poly-dIdC per reaction and 3 mM spermidine. With this lower amount of non-specific competitor, the Prep1HD50Q–Pbx1a complex binds stronger than observed before (Figure 2C). Binding is, however, still impaired compared with the Prep1–Pbx1a complex. (**B**) EMSA analysis of the Prep1–Pbx1a–HOXB1 complex. The HOXB1–Pbx1a (lanes 3–6), the Prep1–HOXB1–Pbx1a (lanes 7–10), and the Prep1ΔHD–HOXB1–Pbx1a (lanes11–14) complexes were challenged with anti-Prep1 and anti-Pbx1 antibodies. PI: preimmune serum. The EMSA was performed under similar conditions as (A).

oncogenic fusion protein, the ~90 most N-terminal residues of Pbx1, including part of the PCB-A domain, have been replaced by the activation domain of the E2A protein. Indeed, this fusion protein is incapable of interacting and cooperative DNA binding with Prep1, thus behaving like the Pbx1 PBC-A-deletion mutant. E2A–Pbx1 would therefore be predicted to have a more restricted spectrum of possible DNA targets and to not be subjected to a Prep1-regulatory effect. In addition, the E2A–Pbx1 oncogene could also be free to interact with other partners, and bind and transactivate through target sites not bound by Prep1–Pbx, as proposed (Knoepfler and Kamps, 1997), although the observation that the homeodomain of the E2A–Pbx1 proteins is partly dispensable for transformation (Monica *et al.*, 1994) would suggest that E2A–Pbx1 could also exert its oncogenic function by different mechanisms.

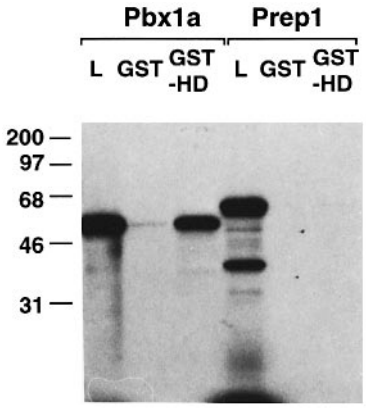

Fig. 7. Prep1 does not interact with HOXB1. *In vitro* translates of either Pbx1a (lanes 1–3) or Prep1 (lanes 4–6) were incubated with either glutathione–Sepharose-bound GST or GST–HOXB1 homeodomain fusion protein (GST–HD), precipitated and resolved by SDS–PAGE. L: load (*in vitro* translate).

DNA-binding specificities of Pbx-containing complexes

This work and other recent reports (Chang *et al.*, 1997; Di Rocco *et al.*, 1997; Knoepfler and Kamps, 1997; Berthelsen *et al.*, 1998), show that Pbx-containing complexes have selective DNA-binding specificity depending on the nature of the interacting partner (summarized in Table II). Pbx proteins complexed with Prep1 exhibit strong affinity and specificity for rather different kinds of binding sites, the TGACAG sequence of the urokinase enhancer, the Pbx responsive sequence (PRS, TGATTGAT) and the b1-ARE R3 site (GTGATGGAT) (Table II). Similarly, the Meis1–Pbx1 complex binds preferentially to a combined Pbx–Meis binding site TGAT-TGACAG, in which the Meis halfsite is identical to the TGACAG sequence of the urokinase enhancer bound by Prep1–Pbx, and the Pbx halfsite (TGAT) is identical to that of the PRS-sequence (Table II). Like the Prep1–Pbx complex, Meis1–Pbx is further able to bind to several Pbx–Hox responsive elements (Chang *et al.*, 1997). Also recently, a DNA binding activity of an yet unidentified factor, termed NFPP (nuclear factor Pbx partner), that interacts and binds DNA with Pbx has been described (Knoepfler and Kamps, 1997). NFPP–Pbx binds preferentially to a TGATTGAC motif (termed PCE; Table II), a sequence also bound by Prep1–Pbx (data not shown), but also to

Table II. DNA-binding specificities for different Pbx-interacting proteins

Site	Sequence	Prep1	Meis1	Pbx	Prep1–Pbx	NFPP–Pbx	Meis1–Pbx	Pbx–Hox[a]	Prep1–Pbx–Hox
UEF3 site	TGACAG	+	+	n.d.	+	n.d.	n.d.	–	–
PRS	TTGATTGAT	n.d.	n.d.	+	+	+	+	+	+
b1-ARE R3	GTGATGGAT	n.d.	n.d.	n.d.	+	n.d.	+	+	+
PCE	TGATTGAC	n.d.	n.d.	n.d.	+	+	n.d.	–	–
Pbx-Meis	TGATTGACAG	+	+	n.d.	n.d.	n.d.	+	–	–

'+' indicates binding, '–' indicates no observed binding. n.d.: not determined. A '+' signifies specific binding, and does not reflect differential DNA-binding affinities.

[a]DNA-binding specificity of Hox–Pbx depends on the Hox protein used. The PRS site binds a large number of different Hox proteins with Pbx, while cooperative binding to the b1-ARE R3 is restricted to a limit set of Hox proteins, including HOXB1. DNA-binding specificities of E2A–Pbx–Hox are identical to Pbx–Hox.

the PRS site (Knoepfler and Kamps, 1997). Our observations that Prep1 is expressed ubiquitously, and is associated with Pbx which together bind the PRS-like target sequences, are compatible with NFPP being Prep1.

The Pbx–Hox complexes, on the other hand, display high affinity and specificity for PRS-like sequences (Chang *et al.*, 1996), but do not bind the TGACAG, PCE and Pbx–Meis-consensus sites (Table II). In Pbx–Hox complexes, Pbx contacts the constant TGAT half of the PRS-site, with the last T residue being important for binding of both Pbx and HOX (Chan and Mann, 1996; Knoepfler *et al.*, 1996). In the Prep1–Pbx complex, both subunits contact DNA on the TGACAG motif, as seen by UV-cross linking with purified UEF3 (Berthelsen *et al.*, 1996). The TGACAG motif shares with PRS the TGA sequence, but lacks the adjacent conserved T residue important for Pbx binding to the PRS. Thus, the two binding sites are contacted by the Prep1–Pbx complex with comparable affinities, yet the contacts seem different.

Prep1 can form a transcriptionally active ternary complex with HOX and Pbx

Since Prep1 appears to be ubiquitously expressed in adult tissues (Ferretti *et al.*, manuscript in preparation) and Prep1–Pbx complexes can be observed in extracts from mouse embryos at a time when Hox proteins are expressed, we tested whether Prep1 or Prep1–Pbx complexes could affect the functions of Hox–Pbx complexes. For this, we exploited a well characterized Pbx-dependent autoregulatory element from the Hoxb-1 promoter (b1-ARE) (Popperl *et al.*, 1995). We have previously shown that a Pbx–HOXB1 complex cooperatively binds to, and activates transcription from, the R3 site contained in the b1-ARE (Di Rocco *et al.*, 1997). We have further shown that a Prep1–Pbx1 complex can efficiently bind with similar affinity to both the urokinase promoter o-1 site and to the b1-ARE R3 site (Berthelsen *et al.*, 1998). The coexpression of Prep1 and Pbx1 does not lead to activation of the b1-ARE driven reporter. Interestingly, co-expression of Prep1, HOXB1 and Pbx1 leads to an enhancement of the transcriptional activity with respect to HOXB1–Pbx1, indicating neither that the latter complex is antagonized by the formation of an inactive Prep1–Pbx1 complex on the b1-ARE, nor that Pbx is titrated from Hox–Pbx complexes, as might have been expected. Although a 2-fold increase in transactivation potential by Prep1 on the Pbx–HOXB1 complex is a relatively modest effect, the significance of these data is that they indicate a direct functional interaction between HOXB1, Pbx1 and Prep1. This possibility has been predicted, but so far not demonstrated, for Hox, Pbx and Meis (Chang *et al.*, 1997; Rieckhof *et al.*, 1997).

The enhancement of transcriptional activity by Prep1 requires the presence of the HR1 and HR2 conserved domains within the Prep1 N-terminal portion which represent the Pbx-interaction surface. The enhancement also requires the N-terminal portion of Pbx1, containing the conserved PBC-A region which proved necessary for the interaction with Prep1 *in vitro*. Conversely, DNA-binding by Prep1 is apparently not required since a homeodomain deletion mutant is capable of stimulating the HOXB1–Pbx1 complex activity even to a larger extent. *In vitro* DNA-binding experiments indicate that the observed enhancement could rely on the formation of a ternary complex between Prep1, HOXB1 and Pbx1 on the R3 site of the b1-ARE region. In agreement with the transfection results, ternary complex formation depends on the presence of the HR1 and HR2 regions in Prep1 and on the PBC-A domain within the N-terminus of Pbx1, both of which are necessary for the formation of a Prep–Pbx complex both in solution and on DNA. Conversely, ternary complex formation does not require the DNA-binding function of Prep1, again in accordance with the transfection experiment. The DNA-target specificity of the Prep1–Pbx–HOXB1 ternary complex is restricted to the PRS-like sequences as it binds to both the PRS and b1-ARE R3 sites but not to the TGACAG, PCE or Pbx–Meis sites (Table II), displaying a DNA-binding selectivity identical to that of the Pbx–HOXB1 complex. These results indicate that Prep1 or a preassembled Prep1–Pbx complex can functionally interact with a Hox–Pbx, or a Hox protein respectively, to form a transcriptionally active ternary complex on a Hox–Pbx target site. Prep1, Pbx and Hox genes are co-expressed at least in some tissues during development, therefore Prep1 might represent an additional component of transcriptionally active Hox–Pbx complexes. It is conceivable that other proteins characterized by the conservation of HR1 and HR2 domains, such as the Meis, are able to interact with Pbx proteins, and to modulate Pbx–Hox functions.

It could be envisaged that the b1-ARE region could be bound by three different complexes: Prep1–Pbx, Hox–Pbx, or the ternary Prep1–Pbx–Hox complexes, respectively (Figure 8). Competition for binding between the three complexes would allow a more flexible regulation of the responsive promoter element. In this model, a transcrip-

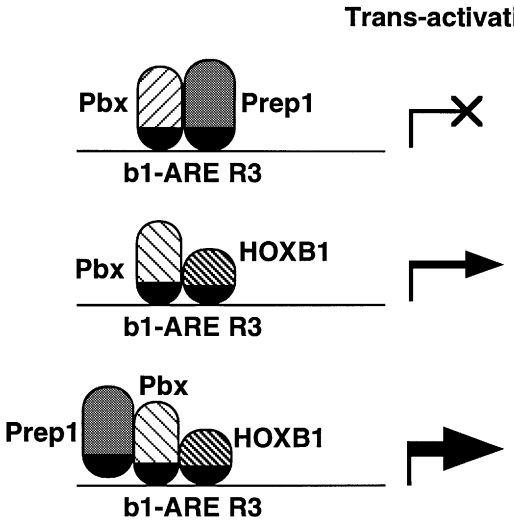

Fig. 8. Model of complexes binding to the b1-ARE enhancer R3 site. (Top) Prep1–Pbx complex binding to the R3 site does not lead to transcriptional activation. (Middle) Pbx–HOXB1 complex activates transcription through the R3 site. (Bottom) A ternary Prep1–Pbx–HOXB1 complex displays stronger transactivating potential. In the ternary complex, Prep1 DNA binding is not necessarily required, since a deletion of its DNA-binding domain does not impair complex formation and stimulation of the HOXB1/Pbx1 activity.

tionally inactive Prep1–Pbx complex would compete for binding to the same site bound by active Hox–Pbx or Prep1–Pbx–Hox complexes. Indeed, when deleting the homeodomain from Prep1, which prevents binding of the inactive Prep1–Pbx complex, we observe an increased activity of the Prep1–Pbx–Hox complex, whose assembly does not depend upon the Prep1 homeodomain. Alternatively, the possibility exists that the b1-ARE R3 site is not the optimal binding site for the ternary complex, therefore explaining the negative interference by the Prep1 homeodomain. In this view, one could imagine the existence of a consensus sequence allowing binding of three proteins at the same time.

In the Prep1-stimulation of Pbx–Hox activation, Prep1 apparently does not contribute with a transactivation domain to the ternary complex as Gal4-DNA-binding domain fusion proteins of either Prep1 or the N-terminal part of Prep1 (residues 1–246) fail to activate a Gal4 reporter construct in co-transfection experiments, indicating that Prep1 lacks a conventional transactivation domain (data not shown). This is in agreement with the observed lack of transactivation by Prep1–Pbx complexes and with the inability of multimerized urokinase UEF3 binding sites to mediate transactivation (De Cesare *et al*., 1996). Thus Prep1 might modulate the activity of Pbx–Hox complexes either by adding DNA-binding specificity to the complex on some target-sites, even if DNA binding by Prep1 is not required on the b1-ARE R3 site, by stabilizing the Pbx–Hox complex on DNA, or by synergistically recruiting basal transcriptional machinery or additional co-factors. Additional experiments are required to further characterize the interactions between Prep1 and Pbx proteins, and to assess the role of Prep1 as a partner for Pbx proteins function *in vivo*.

Materials and methods

Plasmid constructs

All expression constructs used in this study were cloned in the pSG5 vector (Stratagene), with exception of pBSE2A–Pbx1a. The constructs pSGPbx1, pSGPbx1Δ1–140, pSGPbx1Δ283–285, pSGHOXB1, pSGB1HD and pSGHOXB1ΔHD are previously described (Di Rocco *et al.*, 1997). pSGPrep1 was constructed by insertion of the Prep1 cDNA into the *Bam*H1 site of pSG5. The pSGPrepHD50Q, pSGPrep1ΔHD and pSGPrep1ΔHR1+2 mutant plasmids were all constructed by PCR-mediated mutagenesis using the High Fidelity Polymerase system (Boehringer Mannheim), and cloned into pSGPrep1. All constructs were confirmed by DNA sequencing. pBSE2A–Pbx1a was constructed by isolating the E2A–Pbx1a cDNA *Eco*R1–*Bgl*II fragment from the pGEM3-EPL plasmid (a kind gift from P.Knoepfler), and placing the fragment in pBluescript (Stratagene).

Protein production and immunoprecipitation

All pSG5-derived expression vectors were co-translated *in vitro* using the coupled TNT transcription/translation system (Promega), as described previously (Berthelsen *et al.*, 1998).

In vitro produced Prep1-derived proteins were co-translated with Pbx1b and precipitated with 3 μl αPbx1 (Santa Cruz Biotechnology). Pbx1a-derived proteins were co-translated with Prep1 and immunoprecipitated with anti-Prep1 antibodies, as described previously (Berthelsen *et al*., 1998).

EMSAs

Electrophoretic Mobility Shift Assays (EMSAs) of Prep1–Pbx complexes were done as described (Berthelsen *et al*., 1998). EMSAs of Pbx1–HOXB1 complexes and ternary Prep1–Pbx1–HOXB1 complexes were performed using similar binding conditions as previously described for Pbx–Hox (Chang *et al.*, 1995). 2 μl of reticulocyte lysates containing combinations of *in vitro* co-translated Prep1, Pbx1 and HOXB1 were mixed on ice with 18 μl PPH binding buffer (10 mM Tris–Cl pH 7.5, 75 mM NaCl, 1 mM EDTA, 6% glycerol, 3 mM spermidine, 1 mM DTT and 0.5 mM PMSF) containing 0.5 μg poly-dIdC, 20 000 c.p.m. ^{32}P-labeled and eventual antibody. After 10 min on ice, the samples were incubated at room temperature for 20 min. The reactions were separated by a 5% PAGE in 0.5× TBE. A low amount of non-specific competitor (0.5 μg poly-dIdC per reaction) but with addition of 3 mM spermidine were used in order to decrease non-specific DNA absorbance.

Transfections and cell extracts

COS cells were grown in DMEM supplemented with 10% newborn calf serum (Gibco-BRL) and 100 IU/ml of penicillin and 100 μg/ml streptomycin. Cells were transfected by calcium-phosphate precipitation in 10 cm dishes. In a transfection experiment, cells were transfected with 8 μg of the pAdMLR3 reporter construct, with 4 μg of the pSGHOXB1 or the pSGB1HD expression constructs, together with 8 μg of the pSGPbx1a or the PSGPbx1Δ1–140 expressors, where indicated. Cells were also co-transfected with 8 μg of pSGPrep1 or of the pSGPrep1 mutant derivatives where indicated. 0.2 μg of the pCMVb-gal plasmid were cotransfected as an internal standard. Cells were harvested 48 h after transfection, lysed and assayed for luciferase and β-galactosidase expression as previously described (Zappavigna *et al.*, 1994).

GST-retention assay

Glutathione *S*-transferase (GST) fusion proteins were produced using the pGEX bacterial expression vector system (Pharmacia, Uppsala, Sweden). pGEXB1HD was constructed ligating a *Bam*HI fragment, containing the HOXB1 homeodomain, into the *Bam*HI site of pGEX. pGEXPbx141–430 was constructed ligating a PCR amplified Pbx1 deletion mutant into the *Bam*HI site of the pGEX2T vector. The fusion proteins were expressed in *Escherichia coli* according to established methods (Smith and Johnson, 1992), with minor modifications. Briefly, bacteria transformed with pGEX constructs were grown in 100 ml of LB to an O.D. of 0.3–0.5, induced with 0.5–1.0 mM IPTG and harvested after 2 h. Bacterial pellets were resuspended in 500 μl of PBS and sonicated (5 strokes of 15 s each at low energy with a Branson sonifier). Insoluble material was removed by centrifugation and the supernatant incubated with glutathione–Sepharose 4B resin (Pharmacia) for 30 min at 4°C on a rotating wheel. Beads were washed four times with PBS and once with HND buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 0.1% NP-40, 5 mM DTT, 10 mg/ml BSA). 10 μl (packed volume) of the resin bearing equal amounts of either GST or the fusion proteins were batch-

adsorbed for 1 h at +4°C on a rotating wheel to ^{35}S-labeled proteins in 200 µl of HND buffer. The resin was washed four times in a 100-fold (v/v) excess of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.1% NP-40, 200 µg/ml), resuspended in Laemmli sample buffer and analyzed by SDS–PAGE. An aliquot of the ^{35}S-labeled proteins, produced in rabbit reticulocyte lysate (Promega) according to the manufacturer's recommendations from *in vitro* T7 polymerase-transcribed RNA (Melton *et al.*, 1984), was loaded as a control.

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