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Both Pbx1 and E2A-Pbx1 Bind the DNA Motif ATCAATCAA Cooperatively with the Products of Multiple Murine *Hox* Genes, Some of Which Are Themselves Oncogenes

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***E2A-PBX1* is the oncogene produced at the t(1;19) chromosomal breakpoint of pediatric pre-B-cell leukemia. Expression of E2A-Pbx1 induces fibroblast transformation and myeloid and T-cell leukemia in mice and arrests differentiation of granulocyte macrophage colony-stimulating factor-dependent myeloblasts in cultured marrow. Recently, the *Drosophila melanogaster* protein Exd, which is highly related to Pbx1, was shown to bind DNA cooperatively with the *Drosophila* homeodomain proteins Ubx and Abd-A. Here, we demonstrate that the normal Pbx1 homeodomain protein, as well as its oncogenic derivative, E2A-Pbx1, binds the DNA sequence ATCAATCAA cooperatively with the murine Hox-A5, Hox-B7, Hox-B8, and Hox-C8 homeodomain proteins, which are themselves known oncoproteins, as well as with the Hox-D4 homeodomain protein. Cooperative binding to ATCAATCAA required the homeodomain-dependent DNA-binding activities of both Pbx1 and the Hox partner. In cotransfection assays, Hox-B8 suppressed transactivation by E2A-Pbx1. These results suggest that (i) Pbx1 may participate in the normal regulation of *Hox* target gene transcription in vivo and therein contribute to aspects of anterior-posterior patterning and structural development in vertebrates, (ii) that E2A-Pbx1 could abrogate normal differentiation by altering the transcriptional regulation of *Hox* target genes in conjunction with Hox proteins, and (iii) that the oncogenic mechanism of certain Hox proteins may require their physical interaction with Pbx1 as a cooperating, DNA-binding partner.**

The *PBX1* homeobox-containing gene was first identified as the chromosome 1 participant of the t(1;19) chromosomal translocation in pre-B-cell acute lymphoblastic leukemia (pre-B ALL [20, 38]). This translocation consistently creates the same chimeric gene, designated *E2A-PBX1* (12, 43), in which the majority of the *PBX1* gene, including the homeobox, is fused to the 5′ half of the *E2A* gene, which encodes two transactivation domains (11) but lacks sequences encoding the basic helix-loop-helix domain, which mediates DNA binding and dimerization (37 [Fig. 1A]). Differential splicing produces two forms of Pbx1 in normal cells (Pbx1a and Pbx1b) and two forms of E2A-Pbx1 in pre-B ALL cells (E2A-Pbx1a and E2A-Pbx1b [20, 38]). In animal models, expression of E2A-Pbx1a produces both myeloid and T-lymphoid leukemia (8, 18), and expression of either E2A-Pbx1a or E2A-Pbx1b in cultured marrow blocks differentiation of myeloid progenitors, resulting in the rapid outgrowth of factor-dependent myeloblast cell lines (21). Both E2A-Pbx1a and E2A-Pbx1b induce tumorigenic conversion of NIH 3T3 fibroblasts as assayed by tumor formation in nude mice; however, E2A-Pbx1b induces much larger foci in NIH 3T3 cells than does E2A-Pbx1a (19).

PBX1 is one member of a family of homeobox genes including *PBX2* and *PBX3* (34). Pbx1 exhibits 94% sequence identity with Pbx2 and Pbx3 between residues 40 and 315 but diverges substantially from Pbx2 and Pbx3 at its amino-terminal and carboxy-terminal ends. *PBX1* is expressed in all tissues except the B- and T-cell lineages, whereas *PBX2* and *PBX3* are expressed ubiquitously (34). The homeodomains of Pbx1, Pbx2, and Pbx3 bind the DNA motif ATCAATCAA, which we designated the PRS, for Pbx1-responsive sequence (24, 27, 49). E2A-Pbx1 strongly activates transcription through the PRS, while Pbx1 does not (27), suggesting that E2A-Pbx1 causes transformation by activating expression of genes containing a sequence similar to the PRS, which may normally be regulated by Pbx1, Pbx2, or Pbx3.

A variety of evidence suggests that Pbx1 regulates transcription through its interactions with other transcription factors on target gene promoters. Sequence homology between the Pbx1 homeodomain and the primitive homeodomain of the yeast repressor, α2, suggests that Pbx1 may share biochemical features of α2, such as cooperative binding to DNA with other transcription factors (26, 47, 51). In *Drosophila melanogaster*, expression of homeobox genes of the Antennapedia and Bithorax complexes (ANT-C and BX-C, respectively [Fig. 1B]), which together are designated the Homeotic complex (HOM-C), establish anterior-posterior identity of larval structures (33 [Fig. 1B]). Genetic evidence suggests that a *Drosophila* homolog of *PBX1*, termed *extradenticle* (*exd*), participates in regulating normal differentiation, in part, by functioning in concert with the products of certain HOM-C homeobox genes to activate or repress target gene expression (45). For instance, both Exd and Ultrabithorax (Ubx) are required to activate expression of the *decapentaplegic* (*dpp*) gene in parasegments posterior to number 7 (46). Exd has recently been found to bind DNA cooperatively with the Abdominal A (Abd-A) and Ubx homeodomain proteins, but not with Antennapedia (Ant) or Abdominal B (Abd-B [3, 48]), suggesting that Exd physically cooperates with specific homeodomain proteins to regulate target gene expression in vivo.

In mice and humans, four loci (*HoxA*, *HoxB*, *HoxC*, and *HoxD* [Fig. 1B]) contain homeobox genes that are similar to *Drosophila* HOM-C genes in their embryonic expression pat-

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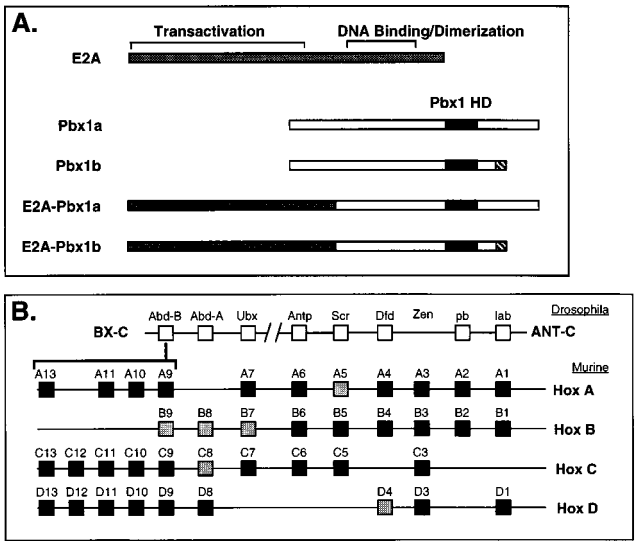


FIG. 1. (A) Structures of the Pbx1 and E2A-Pbx1 proteins used in this study. Shaded regions represent E2A sequences, and open regions represent Pbx1 sequences. The Pbx1 homeodomain, indicated by Pbx1 HD, is blackened, and the unique carboxy-terminal sequence of Pbx1b is designated by hatched regions. (B) Comparison of the homeobox genes contained in the *Drosophila* HOM-C with homologs contained in the four murine *Hox* gene clusters. The boxes representing *Hox* genes selected for this study are shaded.

terns and in the amino acid sequences of their DNA-binding homeodomains, but not in sequences outside the homeodomain (33). In mice, aberrant expression of these *Hox* genes produces homeotic transformations of structures along the anterior-posterior axis, indicating that *Hox* genes, like their *Drosophila* cognates, play a role in establishing differentiation of anterior-posterior structures (3–7, 9, 13, 14, 25, 28, 29, 36, 42, 44). On the basis of the physical interaction of Exd with Abd-A and Ubx in *Drosophila* species and the similar functions of HOM-C genes and *Hox* genes, we have investigated whether Pbx1 and E2A-Pbx1 exhibit cooperative DNA binding with specific homeodomain proteins encoded by genes of the four *Hox* loci. We selected three *Hox* genes that are positional homologs of Abd-A and Ubx (*Hox-B7*, *Hox-B8*, and *Hox-C8*); two that are more closely related to the *Drosophila* homeobox genes *Sex-combs reduced* (*Scr*) and *Deformed* (*Dfd*) (*Hox-A5* and *Hox-D4*, respectively); and one homeobox gene (*Engrailed-2*) that is not located in the *HoxA*, *-B*, *-C*, or *-D* loci in humans and is unrelated to any of the homeobox genes of the *ANT-C* or *BX-C* in *Drosophila* species. Hox-B7, Hox-B8, Hox-C8, and Hox-A5 are also dominant oncoproteins in fibroblasts (32), and Hox-B8, like E2A-Pbx1, arrests myeloid differentiation in murine marrow cultures (39, 40). We find that Pbx1a and Pbx1b, as well as E2A-Pbx1a and E2A-Pbx1b, cooperate with Hox-A5, Hox-B7, Hox-B8, Hox-C8, and Hox-D4 to form a tight complex on the PRS itself. No combinations of Hox proteins alone elicited cooperative DNA binding. In the absence of DNA, the interaction between Pbx1 and Hox proteins was detectable but very weak. These data suggest that Pbx1 may cooperate with Hox proteins in vivo to regulate target gene expression and therein contribute to normal differentiation in vertebrates. Cooperative binding with Hox proteins suggests that E2A-Pbx1 may cause pre-B ALL, at least in part, by altering transcription of homeobox target genes. Finally, these data suggest the possibility that the transcriptional mechanism by which *Hox-B7*, *Hox-B8*, *Hox-C8*, and *Hox-A5* mediate their oncogenic properties may involve cooperative DNA binding with Pbx proteins.

MATERIALS AND METHODS

Construction of recombinant plasmids. Epitope-tagged Hox-B8 and Hox-C8 proteins were constructed by introducing an oligonucleotide encoding MGYPY DVPDYAGSPGLQGG at the amino terminus. This oligonucleotide contained the first methionine in the correct translational initiation context. The underlined region represents the sequence of the flu epitope tag (10). The glutathione *S*-transferase (GST)–Pbx1b fusion protein contains the full-length Pbx1b coding sequence. Construction of the Pbx1bN51S mutant was performed as described previously (27). All cDNAs were cloned in the vector pGEM3zf−, pGEM4z, or pGEM3z (Promega) and expressed by coupled transcription and translation with SP6 polymerase. Purified Pbx1 and Hox-B8 proteins used for DNase I protection assays were derived from GST-Pbx1 (27) or GST-Hox-B8 fusion proteins, isolated after cleavage from GST sequences with thrombin, as described previously (27). The GST-Hox-B8 fusion was constructed by introducing an *Mlu*I site just after the initiating methionine residue in Hox-B8 and using an *Mlu*I-*Eco*RI adapter to clone Hox-B8 sequences downstream of the *Eco*RI site in the vector pGEX-2T.

EMSAs. Double-stranded oligonucleotides were labeled with [^{32}P]ATP to the same specific activities by phosphorylating a short oligonucleotide that annealed to the 3′ portion of each binding-site oligonucleotide and then synthesizing the complementary strand by using deoxynucleoside triphosphates and Klenow fragment. For the electrophoretic mobility shift assay (EMSA), 20,000 cpm of probe was incubated with 3 μl of in vitro-translated proteins in the presence of 1 μg of poly(dI-dC) in a buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 5% glycerol for 30 min at room temperature. Bound probe and free probe were separated by electrophoresis in 5.0% acrylamide gels, formed in 0.6× TBE (27 mM Tris, 27 mM boric acid, 0.6 mM EDTA), and run in the same buffer. After drying of the gel, the protein-DNA complexes were visualized by autoradiography. On the basis of the number of methionine and cysteine residues, approximately equal amounts of Hox proteins were added, with the exception of Hox-D4, in which case 1/10th the normal amount was added.

Methylation interference and DNase I protection assays. Methylation interference analysis was performed with end-labeled DNA fragments containing the cloned BS2 oligonucleotide, as described previously (1). DNase I protection assays were also performed as described previously (1).

Transfections and CAT assays. COS cells were plated onto six-well plates 24 h prior to transfections and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. A mixture of reporter and expression plasmids was introduced into COS cells by calcium phosphate coprecipitation (27). Cells were harvested 40 to 48 h posttransfection. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (27).

Western blotting (immunoblotting). Expression plasmids were transfected into COS cells in parallel with the transfections for CAT assays. Cells were harvested 40 h posttransfection. Transfected cells from one well were lysed in 90 μl of 1× protein sample buffer, boiled for 5 min, and loaded (60 μl of cell lysate) onto a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel. After being transferred to nitrocellulose, proteins were detected either by specific antiserum to Pbx1 or by the 12CA5 monoclonal antibody.

In vitro transcription and translation. In vitro transcription and translation were performed with the Promega TNT Coupled Reticulocyte Lysate System in accordance with the manufacturer's protocol and employing SP6 polymerase.

Expression of GST-fusion proteins and preparation of the affinity resin. B121 cells (10 ml) containing the expression vector of GST or GST-Pbx1b were grown at 37°C to an A_{260} of 0.5. Expression of GST proteins was induced by addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. Cells were collected and lysed at 4°C by three short bursts (15 s per burst) of sonication in 1 ml of 20 mM Tris (pH 7.5)–150 mM NaCl–1 mM EDTA–1 mM dithiothreitol–phenylmethylsulfonyl fluoride (A buffer). The cell lysate was adjusted to a final concentration of 600 mM NaCl and incubated on ice for 10 min. Soluble proteins were collected after centrifugation in a microcentrifuge for 10 min at 4°C and were mixed with 25 μl of prewashed glutathione agarose in a 1.5-ml Eppendorf tube at 4°C for 1 h. The affinity resin was washed four times with 1 ml of A buffer containing 600 mM NaCl, washed twice with regular A buffer, and then used immediately for the in vitro protein-protein interaction assay.

In vitro protein-protein interaction assay. Fifteen to 30 μl of ^{35}S-labeled Hox-B8, Hox-C8, or Hox-B7 protein was precleared by incubation with 25 μl of GST-affinity beads in a total volume of 300 μl supplemented with 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 50 μM ethidium bromide (B buffer) at 4°C for 1 h. The precleared sample was then incubated at 4°C for 2 h with either GST or GST-Pbx1b-affinity beads. After a brief centrifugation, the supernatant containing unbound proteins was transferred to a clean tube and saved for analysis with SDS-polyacrylamide gel electrophoresis (PAGE). The resin was washed six times with 1 ml of B buffer at room temperature. The bound proteins were eluted with 1 M NaCl. The eluates were precipitated with trichloroacetic acid, and the pellets were resuspended in

1× protein sample buffer and analyzed by SDS-PAGE. Proteins were detected by autoradiography or silver staining.

RESULTS

Pbx1 and E2A-Pbx1 bind DNA cooperatively with Hox-B7, Hox-B8, Hox-C8, Hox-A5, and Hox-D4. The ability of Pbx1 to bind DNA cooperatively with other *Hox* gene products was examined by EMSA (Fig. 2A) with full-length homeodomain proteins synthesized in vitro with rabbit reticulocyte extracts (Fig. 2C) and a DNA probe (BS1 [see Fig. 4A]) containing both a canonical Hox binding sequence (TCAATTAA) and the PRS (ATCAATCAA). The same oligonucleotide was used to examine cooperative DNA binding between Exd and Abd-A or Ubx (48). In the absence of Hox proteins, neither Pbx1b (lane 2) nor Pbx1a (lane 3) bound the probe, suggesting that Pbx1 does not bind tightly to the PRS in the presence of competing poly(dI-dC). In the absence of Pbx1 proteins, addition of Hox-B7 (lane 4), Hox-B8 (lane 8), Hox-A5 (lane 16), or Hox-D4 (lane 20) caused the formation of minor bands, indicative of weak binding between the Hox proteins and the BS1 probe. Combinatorial addition of either Pbx1b or Pbx1a with Hox-B7 (lanes 5 to 7), Hox-B8 (lanes 9 to 11), Hox-C8 (lanes 13 to 15), Hox-A5 (lanes 17 to 19), or Hox-D4 (lanes 21 to 23) resulted in the formation of a larger complex whose abundance was directly proportional to the amount of Pbx1 added (lanes 5 and 6, 9 and 10, 13 and 14, and 17 and 18). The amount of Hox-D4 added to the reaction mixtures was 1/10th that of the other Hox proteins, because of its inefficient production in transcription-translation reactions. With this method of analysis, En-2 did not exhibit cooperative binding (data not shown). Importantly, no larger complexes were observed by addition of combinations of Hox proteins (lanes 24 to 29). With the same oligonucleotide probe, E2A-Pbx1b and E2A-Pbx1a also exhibited cooperative binding (Fig. 2B) with Hox-B7 (lanes 7 and 8), Hox-B8 (lanes 11 and 12), Hox-C8 (lanes 15 and 16), Hox-A5 (lanes 19 and 20), and Hox-D4 (lanes 23 and 24), forming complexes larger than those formed with Pbx1b (lanes 6, 10, 14, 18, and 22). These results suggested that both Pbx1 and E2A-Pbx1 bound DNA cooperatively with Hox proteins.

The cooperative binding complex contains both Pbx1 and Hox proteins, requires the DNA-binding function of and by both partners, and contains a single Pbx1 molecule. To prove that both Pbx1 and Hox proteins are components of the apparent Pbx1-Hox complex, the hemagglutinin (HA) nonapeptide epitope, which binds monoclonal antibody 12CA5, was fused to Hox-B8 (creating HA-Hox-B8) and Hox-C8 (creating HA-Hox-C8). These epitope-tagged Hox proteins were used to form the cooperative binding complex, and the content of both Hox and Pbx1 proteins was examined with 12CA5 or anti-Pbx1 antibodies (Fig. 3A). In the absence of Pbx1, HA-Hox-B8 formed a slightly larger complex with probe BS1 (lane 3) than did Hox-B8 itself (lane 2). DNA binding by HA-Hox-B8 was abolished by preaddition of 12CA5 antibody (lane 6), while DNA binding by the wild-type Hox-B8 protein was not (lane 5), indicating that the binding of 12CA5 to the epitope tag disrupts the interaction between HA-Hox-B8 and DNA because it binds exclusively to the HA epitope. HA-Hox-B8 also formed the cooperative binding complex with Pbx1 (lane 8). Formation of this complex was likewise disrupted by addition of 12CA5 (lanes 12 and 13); however, 12CA5 did not have any effect on the complex formed between untagged Hox-B8 and Pbx1 (lane 14), proving that 12CA5 does not disrupt complex formation by binding either Pbx1 or Hox-B8 sequences but rather by binding the HA tag. An anti-Pbx1 serum was used to demonstrate the presence of Pbx1 in the complex. Addition of this antiserum did not alter the binding of HA-Hox-B8 to the probe (lane 7), nor could it immunoprecipitate radiolabeled Hox-B8 (not shown); however, it induced the quantitative supershift of the complex formed by addition of HA-Hox-B8 plus Pbx1b (lanes 9 and 10), demonstrating that Pbx1 is present in the complex. The same analysis was performed with HA-Hox-C8 to prove that both Pbx1 and Hox-C8 are likewise contained in the cooperative binding complex (lanes 15 to 27).

To determine whether the DNA-binding activity of both Pbx1 and Hox proteins is essential for complex formation, DNA-binding mutants of Pbx1b or Hox-B8 were tested for their abilities to participate in complex formation. In each mutant, the invariant asparagine residue at position 51 in helix 3 of the homeodomain was changed to serine (mutation designated N51S). Earlier, this mutation was found to abrogate DNA binding by the Pbx1 homeodomain and to produce a stable E2A-Pbx1 protein that was unable to activate transcription of a CAT reporter gene driven by the PRS (27). In contrast to wild-type Hox-B8 (lane 31), Hox-B8-N51S failed to bind the BS1 probe (lane 32). Likewise Hox-B8-N51S failed to form the cooperative binding complex in the presence of wild-type Pbx1b (lane 35). Similarly, Pbx1b-N51S failed to produce this complex in conjunction with wild-type Hox-B8 (lane 34). Thus, both Pbx1 and Hox-B8 require their DNA-binding ability for complex formation.

To determine whether the complex contained one molecule of Pbx1 or whether two molecules of Pbx1 were present, each perhaps binding one of the ATCAA repeats of the PRS, we mixed Hox-B7 with both Pbx1 and E2A-Pbx1 in the same binding reaction mixtures (lanes 36 to 40). If a single Pbx protein cooperates with Hox-B7, then only two complexes should form with Hox-B7, one containing Pbx1 and a second containing E2A-Pbx1. However, if two molecules of Pbx1 are present, three complexes should form with Hox-B7, one containing two molecules of Pbx1, a second containing two molecules of E2A-Pbx1, and a third containing both Pbx1 and E2A-Pbx1. In separate binding reactions, Pbx1a or Pbx1b was matched with each E2A-Pbx1a or E2A-Pbx1b in the presence of Hox-B7. In all cases, only two complexes were formed (lanes 37 to 40), suggesting that one Pbx1 protein is present in each complex.

The PRS comprises the core sequence required for cooperative binding. Sequences responsible for cooperative binding to BS1 were first examined by mutational analysis. Conversion of 5′ sequences representing the canonical Hox binding site (TCAATTAA) to a GC-rich sequence (probe BS2 [Fig. 4A]) did not alter cooperative binding of Pbx1 with Hox-A5, Hox-B7, Hox-B8, and Hox-C8 (compare lanes 2 to 5 with 7 to 10). Thus, similar to what was observed for Exd and Ubx, the Hox binding sequence was dispensable for cooperativity (48). Within the context of this upstream mutation, further mutation of residues 3′ to the PRS from TTT to GGC reduced the abundance of the Pbx1-Hox complex somewhat (probe BS3, lanes 11 to 15), suggesting that these residues contribute to the stability of the complex. Next, each TC sequence (underlined) within the PRS (ATCAATCAA) was mutagenized to GG in probes BS4 and BS5, respectively. In both cases, this resulted in a substantial reduction of Hox protein binding and the complete elimination of the Pbx1-Hox complex (lanes 16 to 25). This demonstrated that Hox proteins bound specifically to the PRS. Because Hox proteins bind optimally to sequences containing an ATTA core while Pbx1 invariantly selects C at the third position of this sequence, we mutated each C residue in the PRS to T, creating two new ATTA sequences (probes BS6 and BS7). This mutation should result in stronger Hox protein binding and increased abundance of the Pbx1-Hox

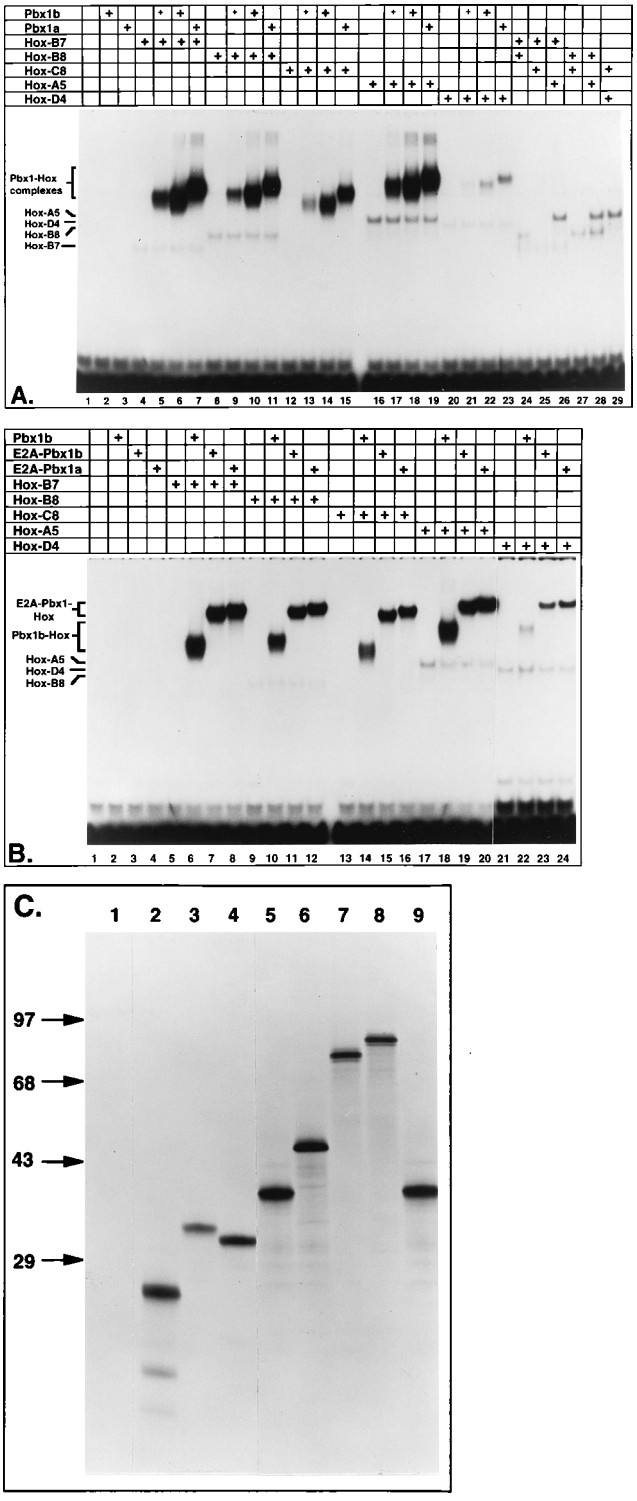


FIG. 2. Addition of Pbx1 plus Hox-B7, Hox-B8, Hox-C8, Hox-A5, or Hox-D4 forms a tight complex with DNA. (A) Combinations of in vitro-translated Pbx1a or Pbx1b and Hox proteins (as designated above each lane) were incubated with probe BS1 (see Fig. 4), and protein-DNA complexes were analyzed by native gel electrophoresis. A total of 3 μl of rabbit reticulocyte translation mix was added in all cases. When required, translation mix expressing the pGEM3zf− vector only was added to bring the total mix to 3 μl. A plus sign represents the addition of 1.5 μl of translation mix. The small plus signs in the Pbx1b row represent addition of 0.5 μl of translation mix. The amounts of Hox-B7, Hox-B8, Hox-C8, and Hox-A5 added to parallel reaction mixtures were equivalent; however, 1/10th the amount of Hox-D4 was added because of inefficient expression of Hox-D4 from the vector. (B) Comparison of cooperative binding to Hox proteins with either Pbx1b or E2A-Pbx1 protein. The homeodomain protein added to each of the binding reactions is indicated above each lane. (C) Analysis of transcription-translation products of a subset of the homeodomain proteins used in this study. Lanes 1 to 9: empty vector, Hox-B7, Hox-B8, Hox-C8, Pbx1b, Pbx1a, E2A-Pbx1b, E2A-Pbx1a, and Pbx1bN51S, respectively.

complex if the Hox protein binds at this position and decreased abundance if Pbx1 binds at this position. In the case of BS6, this mutation increased Hox binding but did not substantially alter the abundance of Pbx1-Hox complexes (lanes 26 to 30); however, mutation of the second C to T in BS7 eliminated formation of the Pbx1-Hox complexes while increasing formation of the complex containing the Hox protein alone (lanes 31 to 35). This suggests that Pbx1 binding requires the 3′ but not the 5′ C residue and that within the complex the Hox protein is positioned 5′ to Pbx1 on the PRS. Although BS7 provided a much better binding site for Hox proteins because it contains the extended binding motif TCAATTAATT, it was not a suitable sequence for cooperative binding with Pbx1, demonstrating that more than just an excellent Hox binding sequence is required for formation of the Hox-Pbx1 complex. Finally, changing the CGC sequence immediately 5′ of the PRS to TCA (probe BS8) had no effect on the abundance of formation of either Hox or the Pbx1-Hox complex (data not shown). Thus, the PRS itself contains all of the essential elements required for the cooperative binding of Pbx1 and Hox proteins. Oligonucleotide competition experiments further supported this interpretation, because an oligonucleotide containing the PRS flanked by dissimilar 5′ and 3′ sequences competed efficiently for complex formation between Pbx1b and Hox-A5, Hox-B7, Hox-B8, and Hox-C8 (Fig. 4B, lanes 3, 6, 9, and 12, respectively), while a nonspecific oligonucleotide did not (lanes 4, 7, 10, and 13).

DNA base contacts within the Pbx1-Hox complexes were next analyzed by methylation interference with a probe containing the BS2 oligonucleotide (Fig. 5A). Contacts were examined in a complex containing E2A-Pbx1 and Hox-A5. In the sense strand, methylation of the two AA (underlined) sequences (GC-ATCAATCAA-TTT) interfered with complex formation, while methylation of the G residue at position −2 had no effect. The low abundance of the band arising from methylation of the first A in the PRS on strand 1 precluded determination of whether it was a site of protein contact. Likewise, in the opposing strand, methylation of the three A residues at the 3′ boundary of the PRS or of the G residue at the 5′ boundary did not interfere with complex formation, while methylation of either the A or G residue in the two internal AG sequences did interfere with complex formation.

The positioning of the Pbx1 and Hox proteins on the PRS was examined by DNase I footprint analysis on probes containing the BS2 oligonucleotide (Fig. 5B). Increasing concentrations of recombinant Pbx1 (1, 4, and 16 ng in lanes 3 to 5, respectively) caused protection of a region whose boundaries were defined by positions indicated to the right of the panel. In contrast, increasing concentrations of recombinant Hox-B8 (1, 4, and 16 ng in lanes 7 to 9) protected the same region but extended both the 5′ and 3′ protected boundaries to the positions indicated. At protein concentrations insufficient to produce a footprint by addition of Pbx1 or Hox-B8 alone (1 ng each [lanes 3 and 6]), a mixture of each protein formed a strong footprint whose 5′ boundary matched that of Hox-B8 and whose 3′ boundary matched that of Pbx1.

Off rate analysis suggests that Hox complexes containing E2A-Pbx1 are as stable as those containing Pbx1. The off rate

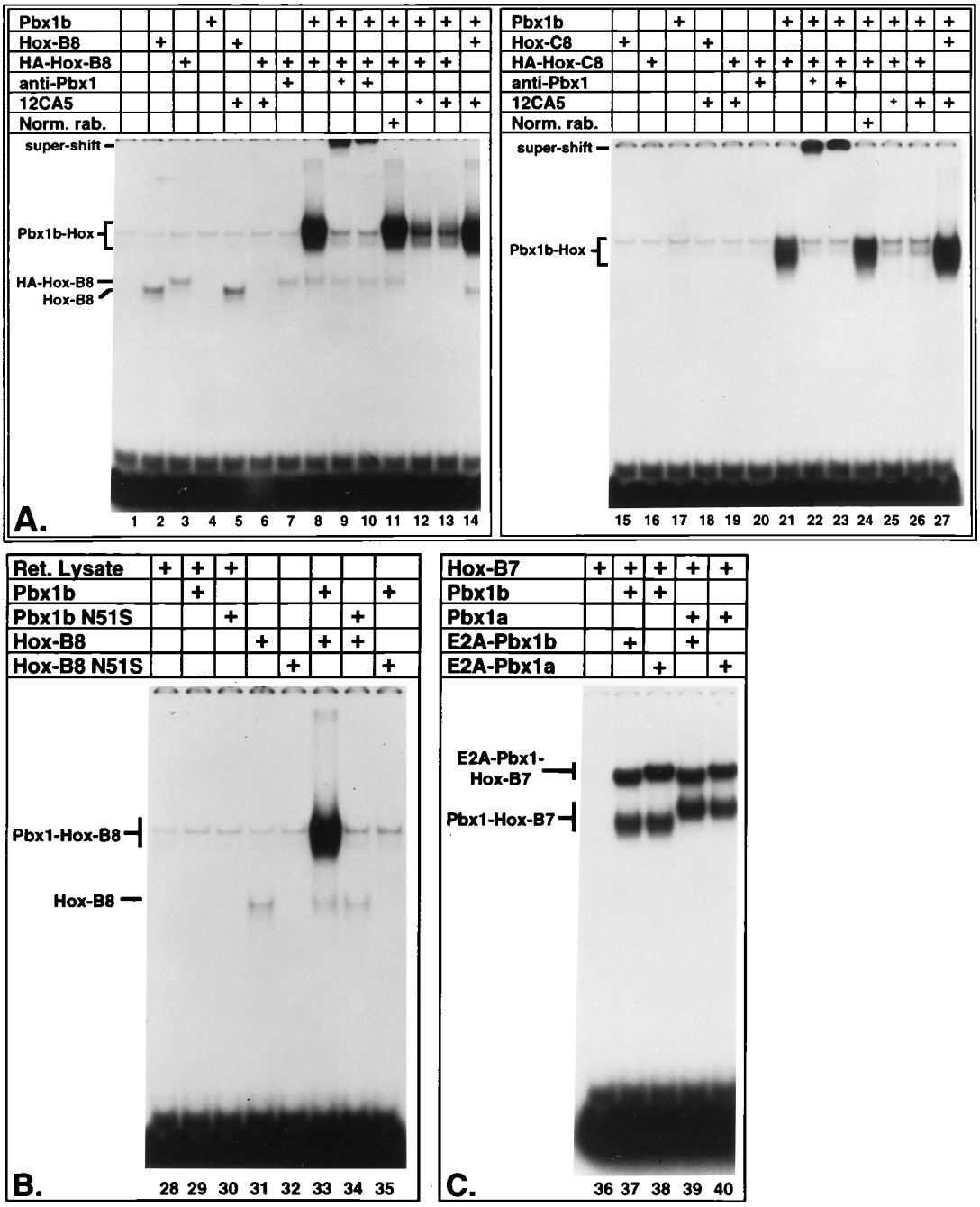
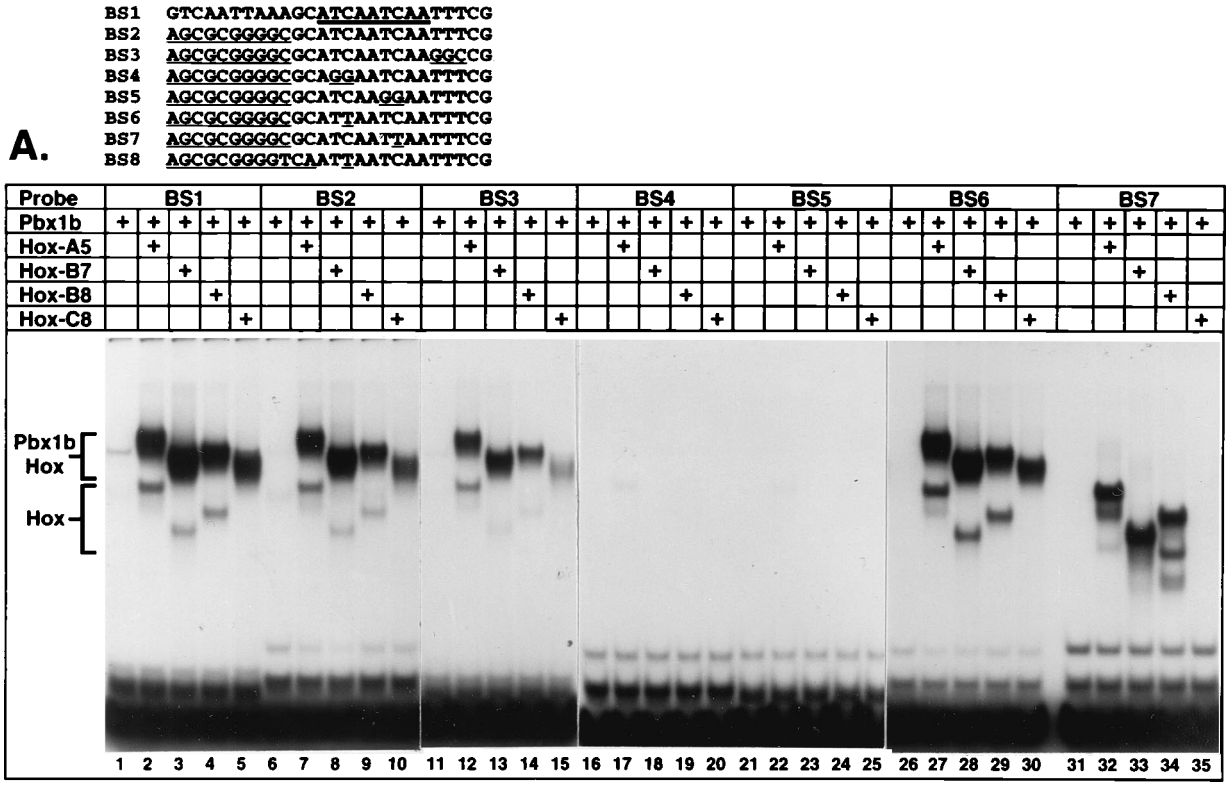


FIG. 3. Both Pbx1 and Hox proteins are present in the cooperative binding complex, and both require homeodomain-dependent DNA-binding activity. Probe BS1 was incubated with various homeodomain proteins in the presence or absence of specific antisera and analyzed by EMSA. (A) Both Pbx1 and Hox-B8 or Hox-C8 are present in the cooperative binding complex. A plus sign represents addition of 1.5 μl of reticulocyte lysate translation mix containing the homeodomain protein. For additions of normal rabbit (Norm. rab.) and anti-Pbx1 serum, the full-size plus sign indicates addition of 0.5 μl of serum to binding reaction mixtures. For addition of 12CA5 monoclonal antibody, the small plus sign represents addition of 100 ng and the large plus sign represents addition of 1.0 μg. (B) Both Pbx1 and Hox-B8 require homeodomain-dependent DNA binding for complex formation. A plus sign represents addition of 1.5 μl of reticulocyte lysate (Ret. Lysate) translation mix containing the indicated homeodomain protein. Constructs encoding protein mutants are described in the text. (C) A single molecule of Pbx1 or E2A-Pbx1 is contained in complexes with Hox-B7. A plus sign represents addition of 1.0 μl of reticulocyte lysate translation mix containing the indicated homeodomain proteins. All samples contained 3.0 μl of total reticulocyte lysate translation mix.

of complexes containing Hox-A5 and Pbx1 was compared with those of complexes containing Hox-A5 and E2A-Pbx1 to determine whether fusion with E2A alters the stability of the complex formed with the PRS. A typical analysis is shown in Fig. 6. Inclusion of a 200-fold molar excess of unlabeled competitor BS2 eliminated complex formation with the radioactive BS2 probe (lanes 2 and 12). Addition of unlabeled BS2 oligonucleotide after complex formation with the labeled BS2 probe resulted in time-dependent dissociation from the labeled probe. Duplicate initial rate measurements indicated that complexes containing E2A-Pbx1 were somewhat more stable than those containing Pbx1. The dissociation half-life rate of



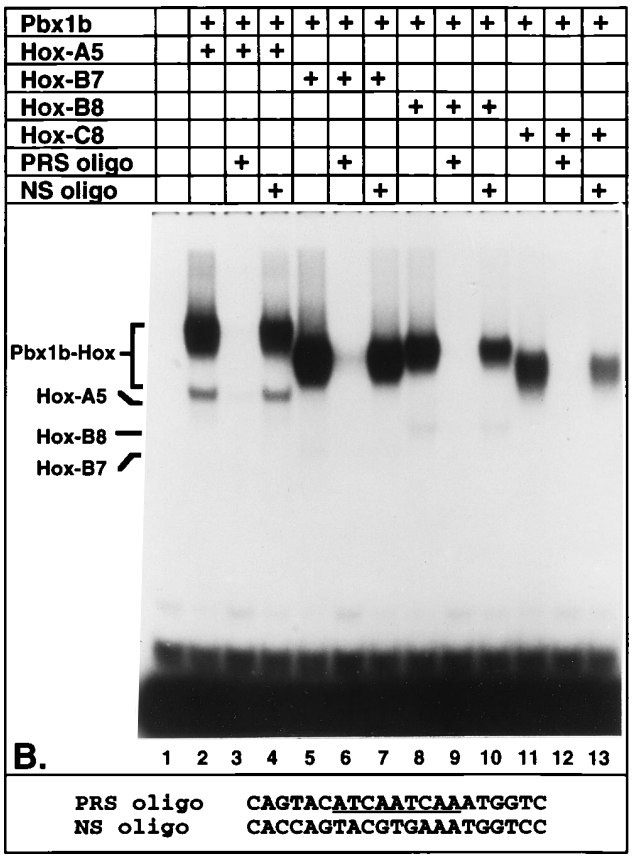


FIG. 4. Cooperative binding occurs on the PRS element. (A) Mutational analysis of the DNA-binding site. Complex formation between Pbx1b and Hox-A5, Hox-B7, Hox-B8, or Hox-C8 was examined by EMSA with binding sites contained in probes BS1 to BS8 (sequences specified above gel shift panel). A plus sign represents addition of 1.5 μl of reticulocyte lysate translation mix encoding the indicated proteins. (B) Competition analysis with oligonucleotides containing the PRS or unrelated sequences. A total of 1.5 μl of the indicated homeodomain proteins was added to each binding reaction mixture. The sequences of oligonucleotide (oligo) competitors are indicated below the panel. NS, nonspecific.

Hox-A5 complexes containing E2A-Pbx1 was 35 ± 2 min, while that of those containing Pbx1 was 25 ± 1.5 minutes. Likewise, the dissociation half-life rate of Hox-B7 complexes containing E2A-Pbx1 was 9.2 ± 0.5 min, while that of complexes containing Pbx1 was 6.8 ± 0.3 min.

Transactivation by E2A-Pbx1 is repressed by Hox-B8 but not by Hox-C8. Functional interactions between Hox proteins have demonstrated that Hox-B8 can repress Hox-B9-induced transcriptional activation of the N-CAM promoter (16) and that both Hox-C8 and Hox-D8 can repress Hox-D9-induced transcriptional activation of the Hox-D9 promoter (52). In other contexts, however, Hox-C8 and Hox-D8 can induce transcriptional activation (52). Therefore, we investigated whether Hox-B8 or Hox-C8 could induce transcription of a PRS-reporter construct in conjunction with Pbx1 proteins or repress transcriptional activation of this construct by E2A-Pbx1. Expression of Pbx1, HA-Hox-B8, or HA-Hox-C8 did not activate transcription of a 7xPRS-CAT reporter construct in COS monkey kidney cells (Fig. 7A, lanes 2 to 4). Likewise, coexpression of Pbx1b with either HA-Hox-B8 or HA-Hox-C8 did not induce transcriptional activation (lanes 5 and 6). However, while expression of E2A-Pbx1 induced efficient activation of 7xPRS-

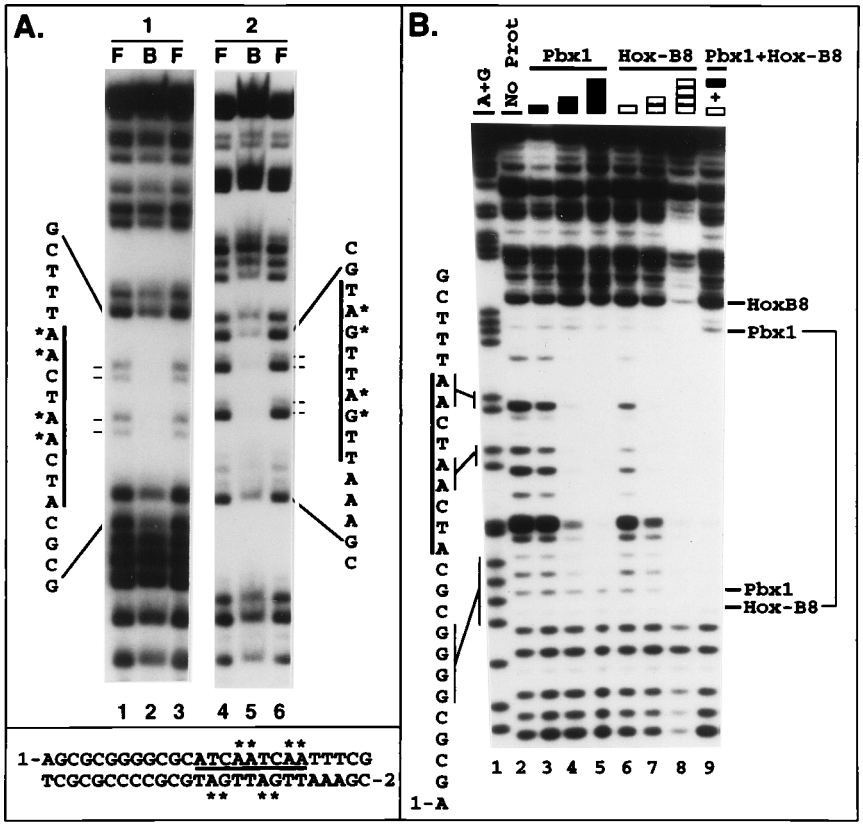


FIG. 5. Pbx1 and Hox proteins contact the PRS in an apparent 5′-Hox–3′-Pbx1 order. (A) Methylation interference analysis of the DNA sequences contacted in the cooperative binding complex containing E2A-Pbx1 and Hox-A5. The sequence of the BS2 oligonucleotide contained in the probe is shown below panel A, and sites of contact are indicated by asterisks. Contacts formed with strand 1 are analyzed in lanes 1 to 3, and those formed with strand 2 are analyzed in lanes 4 to 6. The sequences of strands 1 and 2 are indicated vertically adjacent to the labeled fragments. B represents DNA cleavage products derived from the complex (bound); F represents DNA cleavage products derived from the probe (free). (B) Footprint analysis of the cooperative binding complex suggests that the Hox protein binds 5′ to Pbx1. Lanes: 1, A+G sequencing reaction; 2, DNase I digestion in the absence of added homeodomain proteins; 3 to 5, digestion in the presence of 1, 4, and 16 ng of recombinant Pbx1 protein; 6 to 8, digestion in the presence of 1, 4, and 16 ng of recombinant Hox-B8 protein; 9, digestion in the presence of 1 ng of Pbx1 plus 1 ng of Hox-B8. The boundaries protected against digestion by DNAse I by Pbx1 or Hox-B8 are indicated to the right.

CAT transcription (lane 7), coexpression with HA-Hox-B8 strongly repressed E2A-Pbx1-induced activation (lane 9), while coexpression with HA-Hox-C8 did not (lane 8). Cells cotransfected with vectors expressing HA-Hox-B8 and E2A-Pbx1 contained as much E2A-Pbx1 as cells cotransfected with HA-Hox-C8 and E2A-Pbx1 (Fig. 7B, lanes 8 and 9), indicating that HA-Hox-B8 did not inhibit expression of E2A-Pbx1. Likewise, expression of HA-Hox-B8 was equivalent to that of HA-Hox-C8 in cells cotransfected with the E2A-Pbx1 expression vector (Fig. 7C, lanes 8 and 9), indicating that lack of repression by Hox-C8 did not arise from its inefficient expression. Thus, expression of Hox proteins in vivo can influence the transcriptional function of E2A-Pbx1.

DISCUSSION

In this study, we used probe mutagenesis, methylation interference, and competition analysis to establish that murine Hox proteins exhibit cooperative binding to ATCAATCAA with Pbx1a and Pbx1b, as well as with their oncogenic derivatives E2A-Pbx1a and E2A-Pbx1b. Pbx1 sequences required for cooperativity with Hox proteins must therefore be located between residues 89 and 315, which are contained in both forms of Pbx1 and E2A-Pbx1 and which are 94% identical to homologous regions of Pbx2 and Pbx3. Representative homeodomain proteins (Hox-A5, Hox-B7, Hox-B8, Hox-C8, and Hox-D4) encoded by genes of each of the four *Hox* gene clusters (*HoxA*, *HoxB*, *HoxC*, and *HoxD*) exhibited cooperative binding to the PRS with Pbx1 proteins, suggesting that cooperativity with Pbx1 is not restricted to the members of a single locus. Cooperative binding was not restricted to Hox proteins that were positional homologs of Abd-A or Ubx, because Hox-A5 and Hox-D4, whose homeodomains are more similar to those of the *Drosophila* proteins Scr and Dfd, respectively, also demonstrated cooperative binding.

Cooperative binding of Pbx1 and Hox proteins to the PRS suggests that each protein binds a DNA sequence within the PRS in a specific manner. Experimentally, this interpretation is supported by the facts that both proteins must retain homeodomain-dependent DNA binding to exhibit cooperativity and that probe mutagenesis and DNA footprinting suggest that Pbx1 is positioned 3′ to the Hox protein. We have also observed that 1.0% of in vitro-translated Hox-A5 or Hox-B7 binds a GST-Pbx1 fusion protein in the presence of 50 μM ethidium bromide (23), while less than 1/20 of this amount binds GST alone (data not shown), suggesting that weak interactions occur between Pbx1 and Hox proteins.

It is not clear how two different homeodomain proteins geometrically fit on a DNA element as short as the PRS. In crystal structures of DNA complexes containing the homeodomain of yeast α2 (50), the third alpha helix (recognition helix)

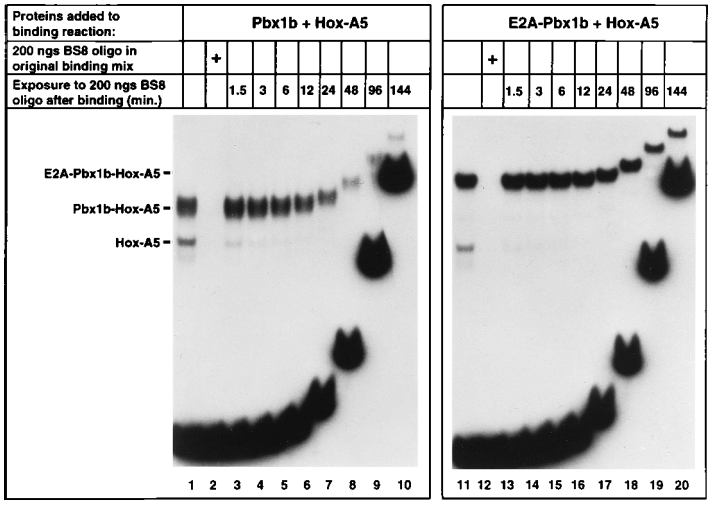


FIG. 6. Cooperative binding complex containing E2A-Pbx1 is as stable as that containing Pbx1. Off rate analysis of cooperative binding complexes containing Hox-A5 and either Pbx1 (lanes 1 to 10) or E2A-Pbx1 (lanes 11 to 20). Both the radioactive probe and the cold competitor were oligonucleotide (oligo) BS2. Two hundred nanograms of BS2 was added either prior to addition of homeodomain proteins (lanes 2 and 12) or after formation of the complex for the indicated times (lanes 3 to 10 and 13 to 20).

of the homeodomain contacts nucleotides 1 to 3 in the major groove and extends contacts to nucleotides 4 to 7 on the minor groove. Thus, one possibility is that the recognition helices of adjacent homeodomains are bound in the same orientation adjacent to each other. In this configuration, the upstream homeodomain would contact the minor groove just opposite the recognition helix of the downstream homeodomain. This overlapping configuration could be constrained within 11 nucleotides. Alternatively, the homeodomains could be positioned in opposite orientations, occupying both faces of the DNA. In this case, only nine nucleotides would be required to form a binding site. Determination of the specific sequences contacted by both Pbx1 and Hox proteins may become more evident upon identification of an optimum site for cooperative binding. Distinguishing the exact geometry of Pbx and Hox proteins bound to the PRS will certainly yield new insights into the mechanisms of interaction between DNA and homeodomain proteins.

To investigate whether Pbx1 and Hox proteins can cooperate in vivo to influence transcription, we performed cotransfection assays with expression constructs encoding Pbx1 proteins and Hox-B8 or Hox-C8. Hox-B8, Hox-C8, and Hox-D8 can all suppress transcription activated by other Hox proteins (16, 52). Mutational analysis of the ability of Hox-D8 to repress transcriptional activation by Hox-D9 led to the conclusion that repression by Hox-D8 is DNA-binding independent and requires only an amino-terminal effector domain and helix I of the homeodomain (52). Because helix I of the Hox-D8 homeodomain was also essential for strong binding of in vitro-translated Hox-D9, it was suggested that repression by Hox-D8 is mediated by direct interaction with Hox-D9 and that paralogs of D8, such as Hox-B8, will also repress through a similar mechanism (52). In our assays, Hox-B8 suppressed transactivation by E2A-Pbx1 but did not suppress general transcription, because the level of E2A-Pbx1 (Fig. 7B, lane 9) or Pbx1 (lane 6) expressed from cotransfected vectors remained strong. While we cannot conclude that Hox-B8 and E2A-Pbx1 physically interact in cells, these experiments provide the first evidence that expression of Hox proteins can impact transcriptional regulation by a Pbx1 protein and suggest the possibility that they may interact in vivo, as well as in vitro.

The cooperative DNA binding exhibited by Pbx1 and Hox proteins suggests that developmental regulation by *Hox* genes in vertebrates may rely, in part, on the cooperative function of Pbx1 proteins in much the same way that appropriate axial differentiation in *Drosophila* species requires the combined functions of Exd and certain homeobox genes of the HOM-C. Mutations of *Hox* genes in mice that generate either gain of function (2, 14, 29, 36, 42) or loss of function (5–7, 9, 13, 25, 28, 44) reveal that *Hox* genes, like their *Drosophila* counterparts, direct regional embryonic development and are involved in anterior-posterior axial pattern formation. For instance, the ectopic expression of Hox-A7 induces conversion from the normal seven cervical vertebrae to eight cervical vertebrae and is accompanied by variations in the most anterior vertebrae that suggest a posterior-to-anterior transformation (22). In addition, ectopic expression of Hox-B8 causes duplication of forelimb structures and homeotic transformation of axial structures (4), and Hox-A5 is also essential for appropriate axial differentiation (13). Although a direct mouse target gene for coregulation by Pbx1 and a Hox protein has not yet been identified, the ability of Pbx1 to associate with Hox proteins, including both Hox-B8 and Hox-A5, suggests that Pbx1 and Hox proteins will likely cooperate to regulate normal transcription of certain *Hox* target genes. Thus far, the bovine *CYP17* gene is the only known gene to be transcriptionally regulated through a site that binds Pbx1 (17, 30), and Pbx1 does not bind this sequence cooperatively with Hox proteins (our unpublished observations). Thus, cooperative binding with Hox proteins may not represent the only mechanism for Pbx1 function in vivo.

In cultured marrow, E2A-Pbx1 blocks myeloid differentiation. When marrow-derived myeloblasts are cultured in granulocyte-macrophage colony-stimulating factor (GM-CSF), they exhibit concurrent growth and differentiation and ultimately differentiate into nonmitotic macrophages and neutrophils within 4 weeks (21). However, when these cultures are infected with E2A-Pbx1 virus, their terminal differentiation is blocked, and they proliferate continuously as GM-CSF-dependent progenitors (21). This ability of E2A-Pbx1 to block differentiation probably also mediates a block in differentiation at the pre-B-cell stage in human pre-B ALL cells expressing E2A-

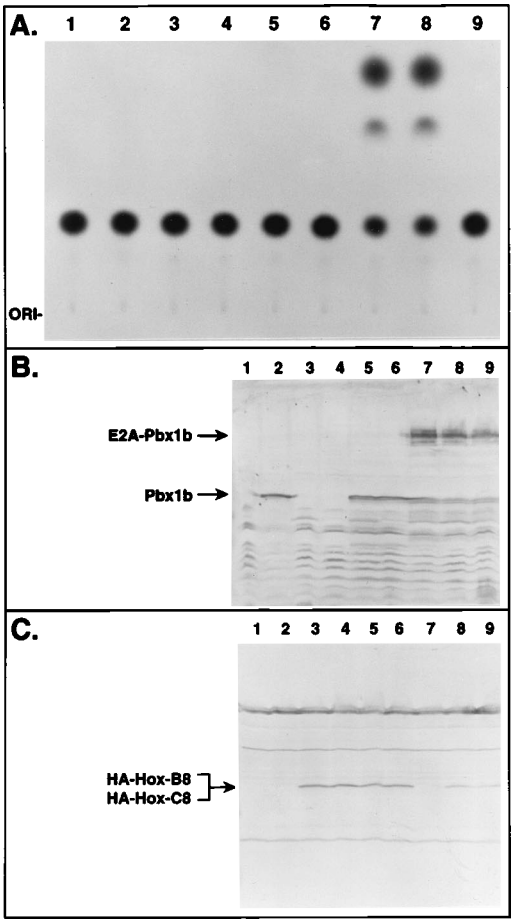


FIG. 7. E2A-Pbx1-activated transcription is suppressed by Hox-B8 but not by Hox-C8. The reporter construct 7×PRS-CAT was transfected into COS cells with combinations of vectors expressing Pbx1, E2A-Pbx1, HA-Hox-B8, or HA-Hox-C8. (A) CAT activity in cytosolic extracts from transfected cells. ORI, origin. (B) E2A-Pbx1 protein expression in the same whole-cell extracts with Western blotting and a polyclonal rabbit anti-Pbx1 serum. (C) HA-Hox protein expression in total cellular extracts from parallel plates of transfected cells, with Western blotting and monoclonal antibody 12CA5. All transfections contained the 7xPRS-CAT reporter. Cytomegalovirus expression vectors encoding the following proteins were added as follows: lanes 1, empty vector; lanes 2, Pbx1b; lanes 3, HA-Hox-C8; lanes 4, HA-Hox-B8; lanes 5, Pbx1b plus Hox-C8; lanes 6, Pbx1 plus Hox-B8; lanes 7, E2A-Pbx1b; lanes 8, E2A-Pbx1b plus Hox-C8; lanes 9, E2A-Pbx1b plus Hox-B8.

Pbx1. Because E2A-Pbx1 exhibits cooperative binding with Hox proteins, E2A-Pbx1 could abrogate normal differentiation by replacing Pbx1 or other Pbx proteins in Pbx-Hox regulatory complexes, thus inducing constitutive transcription of specific *Hox* target genes. This model would be consistent with the facts that pre-B ALL cells express members of the *HoxB* locus, including *Hox-B7* (31, 41) and that transcriptional activation by E2A correlates with the ability of E2A-Pbx1 to block myeloid differentiation (unpublished observation).

Some *Hox* genes are also oncogenes, and thus the association between Pbx1 and Hox proteins is an association between proto-oncoproteins. This observation may lead to the elucidation of a common mechanism through which both E2A-Pbx1 and Hox proteins induce transformation. Overexpression of Hox-A1, Hox-A5, Hox-A7, Hox-B7, and Hox-C8 induces malignant conversion of NIH 3T3 cells (32). Expression of Hox-B8 in mouse marrow produces factor-dependent outgrowths of myeloid progenitors (39) and contributes to myeloid leukemia in mouse bone marrow reconstitution experiments (40). Thus, overexpression of E2A-Pbx1 induces a phenotype similar to that observed for Hox *gene* overexpression. While E2A-Pbx1 might physically replace a normal Pbx protein in a Hox complex, leading to transcriptional activation of a target gene, overexpression of a transforming Hox protein could substitute for multiple Hox proteins that regulate gene transcription in conjunction with Pbx1. In this manner, both E2A-Pbx1 and oncogenic Hox proteins could alter transcription of the same or overlapping subsets of target genes and produce transformation by a similar mechanism. Examination of this model must await identification of the target genes of E2A-Pbx1 and oncogenic Hox proteins.

Cooperative interactions between Pbx1 and Hox proteins represent a form of interaction that was first predicted by sequence analysis of the Pbx1 homeodomain. When initially cloned, Pbx1 was found to contain a divergent homeodomain that was most homologous to that of the distantly related homeodomains of the yeast **a**1 and α2 proteins (20). α2 binds promoter elements cooperatively with **a**1 in diploid cells, therein repressing haploid-specific genes (15). In haploid cells, α2 binds DNA cooperatively with MCM1, repressing transcription of **a**-specific genes (15). Thus the **a**1 and α2 proteins may represent early progenitors of a class of homeodomain proteins whose interactions with both DNA and other transcription factors control simple switches in differentiation programs. The Pbx proteins of vertebrates may have evolved from such progenitors, maintaining both DNA-binding and protein-protein interactions with other transcription factors as key elements that target their transcriptional activities to specific genes.

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