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The Hox Cooperativity Motif of the Chimeric Oncoprotein E2a-Pbx1 Is Necessary and Sufficient for Oncogenesis

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E2a-Pbx1 chimeric oncoproteins result from fusion of the *E2A* and *PBX1* genes at the sites of t(1;19) chromosomal translocations in a subset acute lymphoblastic leukemias. Experimentally, E2a-Pbx1 transforms a variety of cell types, including fibroblasts, myeloid progenitors, and lymphoblasts. Structure-function studies have shown that contributions from both E2a and Pbx1 are necessary for oncogenesis, but the Pbx1 homeodomain is dispensable and the required portion of Pbx1 has not been delineated. In this study, we used deletional and site-directed mutagenesis to identify portions of Pbx1 necessary for oncogenic and transcriptional activities of E2a-Pbx1. These studies defined a motif (named the Hox cooperativity motif [HCM]) carboxy terminal to the Pbx homeodomain that is required for cooperative DNA binding, cellular transcriptional activity, and the oncogenic potential of E2a-Pbx1. The HCM is highly conserved throughout the Pbx/exd subfamily of divergent homeodomain proteins and functions in DNA-binding assays as a potential contact site for Hox dimerization. E2a-Pbx1 proteins with interstitial deletion or single-point mutations in the HCM could neither activate transcription in cellular assays nor transform NIH 3T3 cells. An E2a-Pbx1 mutant containing 50 amino acids of Pbx1b spanning the HCM but lacking the homeodomain was capable of inducing fibroblast transformation. Thus, the HCM is a necessary and sufficient contribution of Pbx1 for oncogenesis induced by E2a-Pbx1 and accounts for its homeodomain-independent transforming properties. Since subtle alterations of the Pbx HCM result in complete abrogation of transforming activity whereas the homeodomain is entirely dispensable, we conclude that interactions mediated by the HCM are more important for transformation by E2a-Pbx1 than interactions with cognate Pbx DNA sites.

The *PBX1* proto-oncogene was originally identified at the site of t(1;19) chromosomal translocations in acute pre-B-cell leukemias (14, 29). The *PBX1* gene codes for two isoforms of a highly conserved protein containing a homeodomain DNA-binding motif. Leukemic translocations result in fusion of Pbx1 with products of the *E2A* gene, which are members of the basic helix-loop-helix family of transcriptional proteins. The resultant E2a-Pbx1 fusion proteins have features of chimeric transcription factors consisting of the amino-terminal portions of E2a fused to the carboxy-terminal portions of Pbx1 containing its DNA-binding homeodomain. Protein fusion alters the transcriptional properties of Pbx1, converting it from a nonactivator of synthetic reporter genes to a potent activator in transient transcriptional assays (21, 23, 41).

Pbx proteins comprise a functionally and biochemically distinct subclass of homeodomain proteins. The *Drosophila* homolog of Pbx, extradenticle (exd), has been shown genetically to act in parallel with Hox proteins in the determination of segmental identity (33, 36). A similar role for Pbx is supported by its potential involvement in a highly conserved autoregulatory loop that controls HoxB1 expression in the mouse hindbrain (35). In vitro, Pbx/exd proteins modulate the DNA-binding affinity and specificity of Hox and non-Hox homeodomain proteins (4–6, 13, 20, 24, 28, 34, 38, 40). Cooperative DNA binding occurs through adjacent DNA half-sites (6) and requires the highly conserved hexapeptide motif upstream of the Hox homeodomain. The biochemical and genetic studies indicate that Pbx/exd proteins serve as important DNA-binding cofactors for Hox proteins and suggest that the resultant binding activities of Pbx-Hox complexes contribute to the position-specific activities of Hox genes.

The importance of Pbx proteins in controlling developmental decisions is underscored by recurrent mutation of Pbx1 in human leukemias and, experimentally, by the ability of E2a-Pbx1 to transform several cell types. When directed to the lymphoid compartment of transgenic mice, E2a-Pbx1 induces T-lineage lymphoblastic lymphomas (9). Retroviral gene transfer of E2a-Pbx1 into primary mouse bone marrow cells results in myeloid leukemias that are dependent on cytokines for survival but show impaired terminal differentiation (15). In NIH 3T3 fibroblasts, E2a-Pbx1 abrogates contact inhibition and induces anchorage-independent growth and tumorigenicity in nude mice (16, 26). In vivo, E2a-Pbx1 also renders lymphoid cells highly susceptible to apoptosis, resulting in profound lymphoid hypoplasias, particularly of the B-cell lineage (9). Since E2a-Pbx1 retains its ability to cooperatively bind DNA in vitro with Hox proteins, its oncogenic contribution may be to alter the transcriptional effector properties of Pbx-Hox complexes with consequent disruption of Hox regulatory pathways.

Oncogenic conversion of Pbx1 represents a gain-of-function mutation, since neither Pbx1 nor its highly related family members, Pbx2 and Pbx3, display oncogenic activity when hyperexpressed in fibroblasts (26). Rather, transformation by E2a-Pbx1 is critically dependent on fusion with E2a. Mutagenesis experiments have identified two essential E2a-derived domains, transcriptional activation domains AD1 and AD2, whose deletion result in loss of transformation potential by E2a-Pbx1 as measured in NIH 3T3 cells (26). The oncogenic activity of E2a-Pbx1 is also dependent on contributions from Pbx1, since deletion of all Pbx sequences abrogates transformation of NIH 3T3 cells (26). Therefore, simple loss of function by E2a lacking its basic helix-loop-helix motifs is not on-

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cogenic. These studies support an oncogenic role for E2a-Pbx1 as a chimeric transcriptional activator. However, potentially inconsistent with this model is the observation that the DNA-binding homeodomain of Pbx1 is not required for in vitro transformation of NIH 3T3 cells or for induction of lymphoblastic lymphomas in transgenic mice (26). Further resolution of the mechanistic role of E2a-Pbx1 in oncogenesis would be enhanced by a delineation of the portions of Pbx1 that are necessary for transformation.

In this report, we demonstrate that the oncogenic and transcriptional properties of E2a-Pbx1 require a highly conserved motif flanking the Pbx1 homeodomain. This portion of Pbx1 is essential for oncogenesis in the presence or absence of the homeodomain and likely accounts for the homeodomain-independent transformation properties of E2a-Pbx1. The implicated region of Pbx was also shown to be required for cooperative DNA binding by E2a-Pbx1 and Hox proteins, suggesting that it functions as a contact site for Hox and possibly other proteins as well. These features are consistent with a cofactor role for Pbx proteins and indicate that the oncogenic activity of E2a-Pbx1 is critically dependent on its ability to interact with heterologous proteins.

MATERIALS AND METHODS

Expression constructs and mutagenesis. Several of the E2a-Pbx1 expression constructs used for these studies have been reported previously (26). Additional mutant forms of E2a-Pbx1 were constructed by PCR and standard cloning techniques. All DNA fragments generated by PCR were sequenced to rule out possible PCR-induced mutations. Mutant *E2A-PBX1* cDNAs were cloned into pSP64 or pSP65 vectors (Promega, Madison, Wis.) for in vitro transcription and translation, pCMV1 for transient transfections, or cos-MSV-tk-neo for retroviral gene transfer studies.

EMSA. Proteins for electrophoretic mobility shift assay (EMSA) were produced in vitro from SP6 expression plasmids by use of a coupled reticulocyte lysate system as described previously (6). To ensure that approximately equal amounts of various in vitro-translated E2a-Pbx1 proteins were added to the binding reactions, proteins were labeled in parallel with [^{35}S]methionine, subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, quantitated on a PhosphorImager (Molecular Dynamics), and normalized for the number of methionine residues in each protein. DNA-binding reactions were performed at 4°C for 30 min, using conditions reported earlier (6), and subjected to EMSA using 6% polyacrylamide gels (0.75-mm thickness) in 0.25× Tris-borate-EDTA buffer. DNA probes (50,000 cpm/binding reaction) consisted of gel-purified, end-labeled, double-stranded oligonucleotides with core sequences matching the consensus TGATTNATGG (5).

Transient transfections and transcriptional assays. E2a-Pbx1 cDNAs were expressed under control of the cytomegalovirus promoter in pCMV1. The reporter construct contained three copies of a Pbx-Hox binding site immediately upstream of a minimal promoter [liver/bone/kidney alkaline phosphatase, pLD44cat(X) (19)] and a chloramphenical acetyltransferase (CAT) reporter gene. The human B-precursor acute lymphoblastic leukemia cell line REH was transfected by electroporation with DEAE-dextran as described previously (21). A luciferase-expressing plasmid under control of the Rous sarcoma virus (RSV) long terminal repeat promoter was included in each transfection as an internal control. Forty-eight hours after transfections, luciferase and CAT assays were performed as described previously (21). For each experiment, transfections were transformed in duplicate on at least three separate occasions with similar results.

Retroviral stocks and generation of stable cell lines. The various mutant forms of *E2A-PBX1* cDNAs were cloned into the *Eco*RI site of the cos-MSV-tk-neo vector. Retroviral stocks were generated by transient transfection of Bosc cells (31), using a calcium phosphate transfection method. Culture supernatants containing viral stocks were collected 48 and 72 h after transfection. Following centrifugation and filtration, serial dilutions of the retroviral supernatants were used to infect low-passage-number NIH 3T3 cells obtained from the American Type Culture Collection. Diluted viral stocks and NIH 3T3 cells were incubated overnight with 6 μg of Polybrene per ml in Dulbecco's modified Eagle's medium (DMEM) containing 5% calf serum. The medium was replaced with fresh medium the next day. After 1 more day, the NIH 3T3 cells were placed under selection in the presence of 500 μg of G418 per ml. The medium was replaced with fresh medium every 3 to 4 days for 3 weeks. To determine the viral titer, G418-resistant cells were stained with methylene blue and the colonies were counted.

Cell culture and transformation assays. Low-passage-number NIH 3T3 cells were used in all transformation experiments. Cells were cultured in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and 5% calf serum. Infected cells were selected for 2 to 4 weeks in G418 (500 μg/ml). Selected cells were split into parallel cultures, which were then used for soft-agar assays and expression analyses, respectively. For soft-agar assays, 10^5 cells were resuspended in DMEM containing 5% calf serum and 0.35% Noble agar and then plated on a bottom layer of 0.5% Noble agar. After 21 days, colonies were scored and photographed. Colonies were defined as consisting of 21 or more cells. Results were based on an average of three independent experiments. E2a-Pbx1a yielded 95 colonies under these conditions. Constructs scored as negative in this assay displayed no clusters containing more than two cells at 21 days.

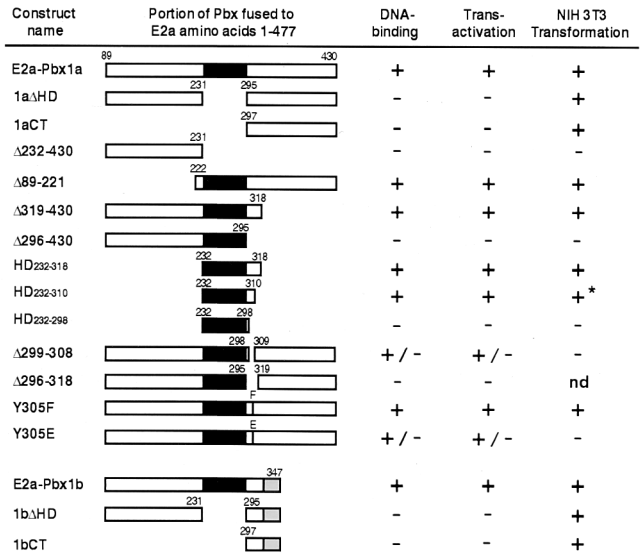

FIG. 1. Schematic illustrations of E2a-Pbx1 mutants and summary of their functional properties. The portions of Pbx1 fused to amino acids 1 to 477 of E2a are shown schematically on the left. Amino acid numbering is based on the wild-type Pbx sequence (25). Filled boxes indicate the Pbx homeodomain; gaps denote deletions. Results of DNA-binding and transcriptional assays are indicated by + (activity at least 90% of intact E2a-Pbx1 activity), +/− (activity less than 50% of intact E2a-Pbx1 activity), and − (less than 5% of E2a-Pbx1 activity). Results of NIH 3T3 transformation assays are displayed as + (induction of soft-agar growth or foci) and − (no foci or growth of colonies containing more than two cells in agar at 21 days following seeding). The positive result indicated by an asterisk denotes induction of colonies comprised exclusively of fewer than 20 cells, indicative of transforming activity weaker than that of intact E2a-Pbx1. nd, not determined.

Immunoblotting. G418-selected NIH 3T3 cells expressing E2a-Pbx proteins were lysed in 2× SDS buffer (4% SDS, 10% 2-mercaptoethanol, 100 mM Tris [pH 6.8], 20% glycerol, 0.2% bromophenol blue) containing protease inhibitors. Proteins were separated by SDS-polyacrylamide (10%) gel electrophoresis and immobilized on nitrocellulose filters by electrophoretic transfer. E2a-Pbx chimeric proteins were detected by using an E2a-specific monoclonal antibody (11).

RESULTS

E2a-Pbx1 requires sequences carboxy terminal to the Pbx homeodomain for cooperative DNA binding with Hox proteins. To determine which portions of the chimeric E2a-Pbx1 oncoprotein are required for cooperative DNA binding with Hox proteins, different mutant forms of E2a-Pbx1 (Fig. 1) were added to DNA-binding reactions and analyzed by EMSA. The binding site used for these studies consisted of a Pbx-Hox consensus sequence (TGATTGATGG) that we showed in previous studies is cooperatively bound by wild-type Pbx1 and HoxB7 (5). DNA binding results are shown in Fig. 2 and summarized in Fig. 1. In this assay, cooperative binding with HoxB7 required the Pbx homeodomain since the 1aΔHD mutant of E2a-Pbx1 did not form a shifted complex in EMSA (Fig. 2, lane 5). Homeodomain alone, however, was not sufficient because a construct containing the Pbx1 homeodomain fused to E2a amino acids 1 to 477 (construct $HD_{232-298}$) was incapable of cooperative DNA binding (Fig. 2, lane 11), sug-

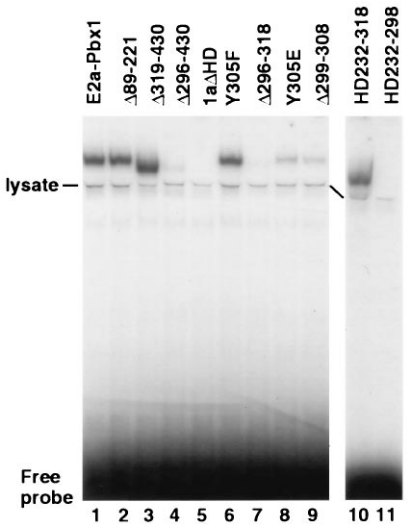

FIG. 2. EMSA of cooperative DNA binding displayed by various mutant E2a-Pbx1 proteins in the presence of HoxB7. In vitro-translated E2a-Pbx1 proteins (as indicated above the gel lanes) and HoxB7 were incubated in DNA-binding reaction mixtures in the presence of radiolabeled probe and then subjected to EMSA. Binding reaction mixtures contained 2 μl of each specifically programmed lysate. The DNA probe consisted of an oligonucleotide containing the Pbx-Hox binding site TGATTGATGG (5).

gesting that additional regions of the chimeric protein were necessary to mediate cooperativity.

The results from several additional constructs implicated a small region of Pbx1 downstream of the homeodomain to be essential for cooperativity. First, cooperative DNA binding was abrogated following deletion of all Pbx amino acids downstream (Δ296-430) but not those upstream (Δ89-221) of the homeodomain (Fig. 2, lanes 2 and 4). Second, restoration of 24 downstream amino acids to Δ296-430 (construct Δ319-430) restored full cooperative DNA binding to the chimeric protein (Fig. 2; compare lanes 3 and 4), thereby localizing the required sequences to highly conserved amino acids immediately flanking the Pbx homeodomain. Third, interstitial deletion of 10 or 23 amino acids from this implicated region (Δ299-308 or Δ296-318, respectively) in an otherwise intact E2a-Pbx1 protein impaired or eliminated cooperative DNA binding (Fig. 2, lanes 7 and 9). Fourth, a single nonconservative amino acid mutation within this region (Y305E) compromised the ability of E2a-Pbx1 to bind DNA cooperatively with HoxB7 (Fig. 2, lane 8), whereas a more conservative change from tyrosine to phenylalanine (Y305F) did not detectably alter cooperative DNA binding (Fig. 2, lane 6). Finally, a minimal construct containing the Pbx1 homeodomain plus 24 downstream amino acids fused to $E2a^{1\text{-}477}$ ($HD_{232\text{-}318}$) was capable of cooperative DNA binding with HoxB7, in contrast to no cooperativity observed with the homeodomain alone ($HD_{232\text{-}298}$) (Fig. 2, lanes 10 and 11).

These data convincingly demonstrate that the major determinants for E2a-Pbx1 to bind DNA cooperatively with Hox proteins reside in the Pbx homeodomain and 24 adjacent, downstream amino acids. This downstream region is highly conserved in the Pbx/exd/ceh20 subfamily of homeodomain proteins (see Fig. 8) and, in part, has a predicted alpha-helical secondary structure. We shall refer to this region as the Hox cooperativity motif (HCM) based on its in vitro requirement for Hox cooperative DNA binding. Our results also demonstrate that the presence of heterologous E2a sequences in the chimeric oncoprotein do not detectably alter cooperative DNA binding, as measured by our in vitro assay, compared to wild-type Pbx1 reported previously (6).

The transcriptional activity of E2a-Pbx1 chimeric oncoproteins requires the Pbx homeodomain and HCM. Chimeric E2a-Pbx1 oncoproteins display transcription activation properties in transient transcriptional assays, but the role of the HCM in this capacity has never been assessed. To address this issue, we determined the transcriptional properties of various E2a-Pbx1 mutant proteins in transiently transfected REH cells (Fig. 3), using a reporter gene containing the Pbx-Hox site employed for the DNA-binding studies described above. Deletion of either the homeodomain (construct 1aΔHD) or the HCM (constructs Δ296-430 and Δ299-308) from an otherwise intact E2a-Pbx1 completely abrogated activation of the reporter gene. A conservative point mutation in the HCM (Y305F) preserved transcriptional activity, whereas a nonconservative point mutation (Y305E) destroyed the ability of E2a-Pbx1 to function in this assay. The minimal portion of Pbx required for transcriptional activity was contained in construct $HD_{232\text{-}318}$, containing both the homeodomain and HCM. Deletion of the HCM from this minimal construct eliminated reporter gene activation (Fig. 3). Therefore, the homeodomain plus HCM were not only necessary but also sufficient contributions from Pbx to confer transcriptional competency on E2a-Pbx1 under these experimental conditions.

The oncogenic activity of E2a-Pbx1 requires the HCM of Pbx. E2a-Pbx1 is capable of transforming NIH 3T3 cells and in this capacity requires the transactivation domains of the E2a moiety (26). However, previous studies have not delineated which portions of Pbx are essential, although the homeodomain is clearly dispensable (26). To further investigate this, we performed soft-agar assays to measure the anchorage-independent growth of NIH 3T3 cells expressing different mutated versions of E2a-Pbx1. These cells were generated by retroviral gene transfer, and expression of the constructs was determined by Western blot analysis using an anti-E2a antibody (Fig. 4).

The dispensability of the homeodomain was confirmed by showing that ΔHD mutants of both E2a-Pbx1a and E2a-Pbx1b were capable of inducing anchorage-independent growth (constructs 1aΔHD and 1bΔHD [Fig. 1]), thus extending previous studies demonstrating no requirement for the Pbx homeodomain for focus formation by NIH 3T3 cells and lymphomagenesis in transgenic mice (26). To define a region of Pbx that may be essential for transformation, additional Pbx sequences were

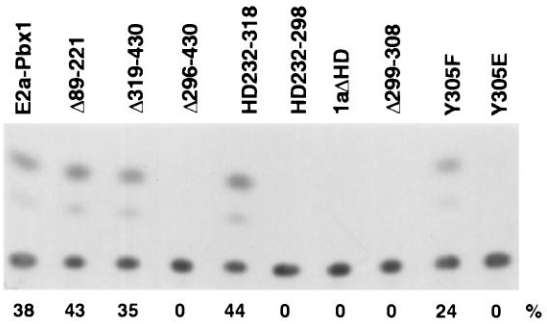

FIG. 3. Transcriptional properties of wild-type and mutant E2a-Pbx1 proteins in transiently transfected REH cells. Representative results are shown for CAT assays assessing relative transcriptional properties of E2a-Pbx1 proteins on a reporter gene containing three copies of the Pbx-Hox binding site TGATTG ATGG. The percentages of CAT conversion are indicated at the bottom. The results shown are representative of those obtained in at least three independent experiments. Transfection efficiencies were standardized by using a cotransfected RSV-luciferase construct.

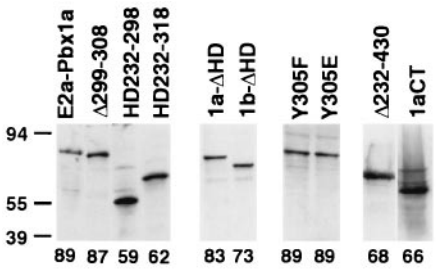

FIG. 4. Western blot analysis of wild-type and mutant E2a-Pbx1 proteins expressed in stably transfected NIH 3T3 cells. Whole-cell extracts from NIH 3T3 cells stably transfected with various E2a-Pbx1 expression constructs were analyzed by Western blotting using an anti-E2a monoclonal antibody (11). Transfected constructs are indicated above the lanes. Molecular weights (in thousands) of the expressed proteins and of markers are given at the bottom and at the left, respectively.

deleted from the homeodomain-lacking chimera. This finding implicated Pbx amino acids downstream of the homeodomain as critical for transformation, since construct 1aCT maintained its oncogenic effects whereas construct Δ232-430, which contained only Pbx amino acids 89 to 231 upstream of the homeodomain, did not (Fig. 1 and 5). Previous studies of ours (26) showing no transformation by 1aCT resulted from poor expression of this unstable protein. The required portion of Pbx was further narrowed to an even smaller region overlapping the HCM by construct 1bCT, consisting of the C-terminal 50 amino acids of Pbx1b fused to $E2a^{1-477}$, which induced anchorage-independent growth of NIH 3T3 cells.

Further analyses implicated the HCM as essential for transformation. Its mutation by either interstitial deletion (Δ299-308) or point mutation (Y305E) completely abrogated growth in soft agar induced by E2a-Pbx1 (Fig. 1). In addition, construct $HD_{232-318}$, containing both the Pbx homeodomain and HCM, induced anchorage-independent growth, whereas $HD_{232-298}$, containing only the homeodomain, did not (Fig. 1 and 5). A minimal construct containing the homeodomain and partial HCM ($HD_{232-310}$) displayed weak oncogenic activity (Fig. 1), defined by induction of cell clusters of less than 21 cells but not colonies in soft agar. We conclude from these analyses that the HCM is both necessary and sufficient for transformation mediated by the chimeric E2a-Pbx1 oncoprotein. This accounts for transformation observed in fibroblasts and lymphoblasts in the absence of the homeodomain, which, by itself, is not a sufficient piece of Pbx for transformation and, in fact, is completely dispensable.

The HCM of Pbx mediates interactions with Hox proteins. The preceding analyses indicated that both transactivation and transformation by E2a-Pbx1 are dependent on the HCM. Two possibilities may account for the role of the HCM: it may mediate interactions with heterologous proteins, including class I Hox proteins such as those used for our DNA-binding assays, or alternatively, given its proximity to the Pbx homeodomain, the HCM may be essential for the Pbx homeodomain to bind appropriately to DNA. To distinguish between these possibilities, we performed additional DNA-binding assays to assess the effect of the HCM on the Pbx homeodomain. For these studies, we replaced the E2a portion of E2a-Pbx1 with glutathione *S*-transferase (GST), creating fusion proteins containing the Pbx homeodomain alone or in combination with the HCM (GST-HB and GST-HB_{hcm}, respectively).

EMSA performed in the absence of added Hox proteins showed that GST-HB and GST-HB_{hcm} bound weakly but specifically to the DNA sequence ATGATTGAT with comparable affinities (Fig. 6A, lanes 1 and 3). Binding was highly unstable, with the two proteins displaying equally rapid dissociation kinetics (Fig. 6B). Neither protein bound to related DNA sites (Fig. 6A, lanes 5, 7, 9, 11, 13, and 15) that differed in composition at nucleotide position 7 (N_7 position) (ATGATTTAT, ATGATTAAT, or ATGATTCAT). This nucleotide position has been shown previously to also play an important role in the ability of various Pbx-Hox complexes to bind cooperatively to the same panel of consensus sites (5). The current studies indicate that the Pbx homeodomain itself displays specificity for a consensus site containing G at the N_7 position and, importantly, that the HCM does not contribute to this observed binding specificity.

In contrast, the GST-Pbx proteins displayed marked differences in their abilities to cooperatively bind DNA with Hox proteins. GST-HB_{hcm} showed robust cooperative binding with HoxB7 on three of the sites (Fig. 6A, lanes 2, 6, and 10). The GST-HB_{hcm}–HoxB7 complex displayed a dissociation half-life of about 5 min, significantly greater than that of GST-HB_{hcm} alone (Fig. 6B). The inability of GST-HB_{hcm} to cooperatively bind on the fourth site ATGATTCAT (Fig. 6A, lane 14) is consistent with previous studies showing that C is not tolerated by Pbx-Hox heterodimeric complexes at the N_7 position of the consensus (5). GST-HB, on the other hand, was incapable of binding cooperatively with HoxB7 on any of the sites (Fig. 6, lanes 4, 8, 12, and 16). Therefore, the presence of the HCM did not change the DNA-binding affinity or specificity of the Pbx homeodomain. Rather, it imparted on the homeodomain an ability to bind DNA cooperatively with HoxB7 (and other class I Hox proteins [data not shown]). Given that the cooperative binding between GST-HB_{hcm} and HoxB7 did not require the Pbx homeodomain to prebind monomerically to DNA (Fig. 6, lanes 5, 6, 9, and 10) and that the HCM did not affect the intrinsic DNA-binding properties of the Pbx homeodomain, the HCM most likely mediates protein-protein contact with the Hox component of the heterodimeric complex.

The relative contributions of the Pbx homeodomain and HCM were also evaluated in transient transcriptional assays. Constructs $HD_{232-298}$ and $HD_{232-318}$, containing fragments of

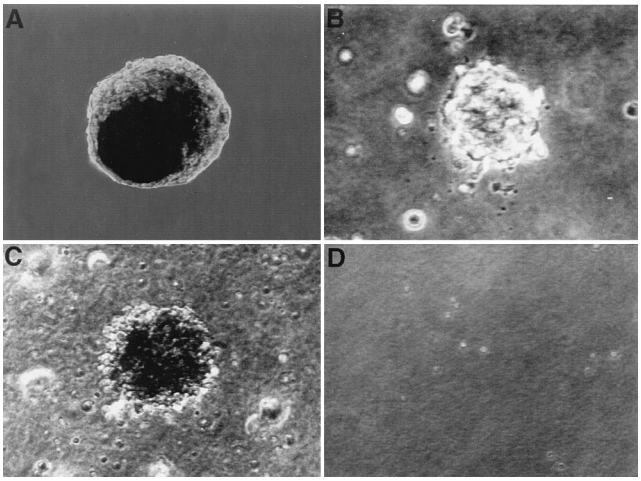

FIG. 5. Representative results of soft-agar assays of NIH 3T3 cells expressing E2a-Pbx1 constructs. (A) Representative colonies formed by E2a-Pbx1a-expressing NIH 3T3 cells after 21 days in soft agar. (B) NIH 3T3 cells expressing the 1aCT construct formed colonies similar to those formed by cells expressing full-length E2a-Pbx1. (C and D) The $HD_{232-318}$ construct, containing both the homeodomain and HCM, induced anchorage-independent growth in soft agar, whereas construct $HD_{232-298}$, containing only the homeodomain from Pbx1, did not.

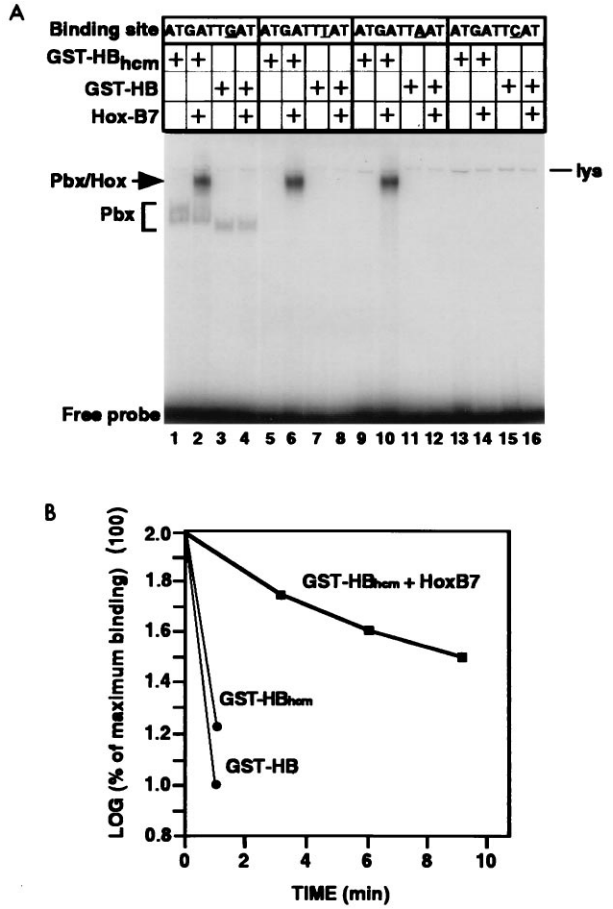

FIG. 6. The Pbx homeodomain requires a carboxy-terminal helical extension for cooperative DNA binding with Hox proteins in vitro. (A) GST-Pbx fusion proteins were incubated in DNA-binding reaction mixtures with radiolabeled probe in the presence or absence of in vitro-translated HoxB7 and then subjected to EMSA. GST-Pbx proteins differed by the inclusion (GST-HB_{hcm}) or exclusion (GST-HB) of the Pbx HCM. Proteins present in each binding reaction are indicated above the lanes. The identities of consensus binding sites present in the oligonucleotide probes are indicated at the top and differ only at the N_7 position of the consensus Pbx-Hox site (5). Where indicated, 2 μl of a HoxB7-programmed lysate (lys) was added to the binding reactions. (B) Kinetic analysis of dissociation rates, determined by using preformed DNA-protein complexes that were either subjected to immediate gel electrophoresis (time zero) or incubated in the presence of a 100-fold excess of unlabeled DNA for the times indicated prior to electrophoresis. Densitometric measurements of each complex were used to calculate the percentage of originally bound probe remaining in the complex at different time points. Dissociation was assessed on a Pbx-HoxB7 consensus site (ATGATTGATGG).

Pbx analogous to GST-HB and GST-HB_{hcm}, respectively, were cotransfected into REH cells with reporter genes containing Pbx-Hox consensus binding sites used above for EMSA. Construct $HD_{232-318}$ was capable of transactivating reporter genes containing G, A, or T at the N_7 position, in agreement with binding to these sites in EMSA studies. Transactivation of the A_7 and T_7 reporters was dependent on a cotransfected Hox protein, whereas that of the G_7 reporter was not (Fig. 7). In contrast, construct $HD_{232-298}$ was incapable of activating expression of any of the reporter genes even in the presence of exogenous Hox proteins (Fig. 7). Thus, although both GST-HB and GST-HB_{hcm} could both bind weakly and unstably to the G_7 site in vitro, only $HB_{232-318}$ was capable of transactivating a reporter containing this site. Furthermore, the ability of $HB_{232-318}$ to activate the G_7 reporter, but not the A_7 and T_7 reporters, in the absence of a cotransfected Hox partner strongly suggested the presence of endogenous partners that facilitate DNA binding through interactions with the HCM. These unidentified endogenous partners appear highly specific for G at position 7 of the consensus Pbx-Hox site, a feature characteristic of a subset of Hox proteins as shown previously (5). The alternative possibility that activation of the G_7 reporter resulted from binding of $HB_{232-318}$ alone without a cofactor is unlikely since no comparable activation was seen with $HB_{232-296}$, which, as a GST fusion protein in vitro, displayed binding properties similar to those of GST-HB_{hcm} on the G_7 site in the absence of Hox partners. The distinguishing feature between these proteins in vitro was the ability of GST-HB_{hcm} but not GST-HB to bind the G_7 site cooperatively with Hox proteins, and we therefore attribute their disparate activation properties to an ability to participate in heterologous interactions as well. The transactivation studies provide additional evidence that the HCM mediates heterologous interactions important for DNA binding and transactivation both with exogenously provided Hox partners and with unidentified endogenous partners.

DISCUSSION

This report extends the intriguing and unexpected observation, reported previously (26), that the DNA-binding motif of chimeric oncoprotein E2a-Pbx1 is not required for its oncogenic activity in fibroblasts or lymphoid progenitors. Although prior studies indicated that contributions from both E2a and Pbx1 were necessary for oncogenesis, they did not clearly delineate which portion of Pbx1 was required. Our current studies provide compelling evidence that a highly conserved region (named the HCM) flanking the Pbx1 homeodomain is the essential Pbx-derived element necessary for transformation in the presence or absence of the homeodomain and likely accounts for the homeodomain-independent transformation properties of E2a-Pbx1. These results have important implications for the mechanisms by which E2a-Pbx1 may recognize subordinate targets through which it effects transformation.

Data from several constructs consistently demonstrated the importance of the HCM both as a minimal portion of Pbx1 sufficient for E2a-Pbx1-mediated oncogenesis and as a necessary motif that must remain intact within the context of an otherwise unaltered E2a-Pbx1 protein. In our studies, no other

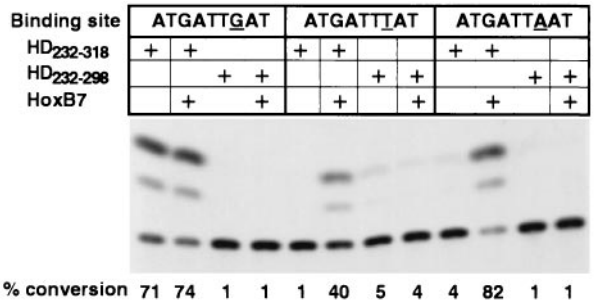

FIG. 7. The Pbx homeodomain and adjacent HCM are both required for transcriptional activity of E2a-Pbx1 on Pbx-Hox sites. Representative results are shown for CAT assays assessing relative transcriptional properties of mutant E2a-Pbx1 proteins containing the Pbx homeodomain with ($HD_{232-318}$) or without ($HD_{232-298}$) the flanking HCM. The binding sites present in each reporter gene are indicated at the top and differ only at the N_7 position of the consensus Pbx-Hox site (5). Proteins coexpressed transiently in transfected cells are indicated above the lanes. The results shown are representative of those obtained in at least three independent experiments. Transfection efficiencies were standardized by using a cotransfected RSV-luciferase construct.

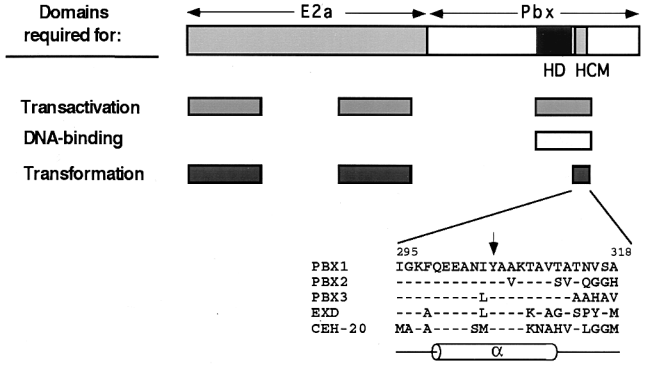

FIG. 8. Schematic depiction of functionally significant portions of E2a-Pbx1. Shaded bars beneath the schematic indicate portions of E2a and Pbx1 required for activity in assays measuring transactivation, cooperative DNA binding, and transformation. At the bottom, an alignment of the HCM is shown for homeodomain proteins in the Pbx/exd subfamily. The arrow indicates the conserved tyrosine that was mutated in this study. Amino acid numbering is based on that of Monica et al. (25).

portion of Pbx1 displayed similar characteristics. This contrasts with recent results of others implicating a region of Pbx1 upstream of the homeodomain (amino acids 89 to 232) in E2a-Pbx1 as responsible for homeodomain-independent transformation of NIH 3T3 cells (17). A mutant construct (T-627) lacking both the homeodomain and HCM induced foci in fibroblast monolayers but was unable to block differentiation of myeloid cells in vitro (17). We cannot account for this divergence from our studies, in which a comparable but not identical construct (Δ232-430) lacked oncogenic activity in NIH 3T3 fibroblasts. Furthermore, the absence of Pbx amino acids 89 to 232 in several transformation-competent constructs in our studies demonstrated that this region of Pbx is completely dispensable for induction of foci (26) and anchorage-independent growth (this study) of NIH 3T3 cells. These Pbx amino acids are also apparently dispensable for induction of human leukemias, as evidenced by a rare t(1;19) translocation resulting in expression of transcripts encoding variant E2a-Pbx1 fusion proteins lacking the N-terminal 233 amino acids of Pbx1 due to fusion of E2a with Pbx at amino acid 234 just upstream of the Pbx1 homeodomain (30).

The HCM is also notable for the fact that it is important for the transcriptional activity of E2a-Pbx1. The HCM is required by both wild-type (6) and chimeric Pbx proteins to cooperatively bind DNA with Hox partners, indicating that protein fusion with E2a does not alter the structural requirements of Pbx to form heterodimeric complexes with class I Hox proteins under these experimental conditions. In our studies, the HCM did not detectably alter the DNA-binding specificity or affinity of the Pbx homeodomain. Other investigators (22) have measured a modest (approximate fivefold) effect of the HCM on binding affinity by monomeric Pbx homeodomain, a potential effect that we may not have detected due to differences in binding conditions or in protein preparations used. In contrast, the effect of the HCM on cooperative DNA binding is substantially (about 2 orders of magnitude) greater. The HCM lies immediately carboxy terminal to the Pbx homeodomain and, in part, has a predicted helical secondary structure but otherwise shares no similarity with previously characterized motifs (Fig. 8). It is conserved in sequence and secondary structure throughout the small subfamily of homeodomain proteins that includes the mammalian Pbx proteins, *Drosophila* exd, and *Caenorhabditis elegans* ceh-20 (3, 25, 36). We have proposed, based on molecular modeling and mutagenesis studies, that the HCM may serve as a critical site of contact between Pbx and Hox proteins (5). In a model of the Pbx-Hox-DNA complex, the HCM can be positioned in vicinity of the Hox hexapeptide which is required for cooperative DNA binding with Pbx (5). Covalent fusion of Pbx1 to HoxB7 at the HCM and hexapeptide, respectively, creates a forced heterodimeric protein with DNA-binding properties indistinguishable from those displayed by cooperative complexes of the unfused proteins (7). These observations are consistent with the possibility that the HCM and hexapeptide interact noncovalently in Pbx-Hox complexes; however, alternative contacts have been proposed (22), and their ultimate resolution will require crystallographic studies.

Although the HCM is the most critical portion of Pbx1 that must remain intact for functional competence of E2a-Pbx1 in assays that measure DNA binding, transcriptional activation, or oncogenicity, there was variability in the magnitudes of the effects that various HCM mutations had on E2a-Pbx1 activity in each of these assays. For instance, the Y305E mutant displayed partial impairment of its ability to bind DNA cooperatively with Hox partners, whereas its transforming capabilities were completely abrogated. Furthermore, this mutant displayed differences in its ability to activate transcription of synthetic reporter genes in transiently transfected cells. Decreased activity was observed on DNA sites requiring cotransfected Hox partners (data not shown), whereas complete inability to activate was observed on a site requiring interaction with endogenous partners (Fig. 3). A small deletion within the HCM (Δ299-308) yielded similar variability, whereas mutants with larger deletions (Δ296-318, Δ296-430, and $HD_{232-298}$) displayed more consistent loss of activity in all assays. These and other mutational analyses delineated a minimal region of 24 amino acids required for optimal HCM function.

We attribute the variations observed in functional consequences of specific mutations to their subtotal disruption of HCM structure and suggest that individual Pbx-interacting proteins have different requirements for contact with the HCM. Interactions with the unidentified factors required for transactivation (on the ATGATTGAT site) or transformation appear highly sensitive to even minor alterations of the HCM. Interestingly, some of these same endogenous partners (i.e., those required for transformation) can maintain a functionally competent interaction with the HCM in the absence of a homeodomain which would be expected to normally play a role in stabilizing a ternary complex with DNA. We have also observed discordant effects of some HCM mutations on transformation compared to their effects on the ability of E2a-Pbx1 to induce apoptosis of lymphoid progenitors (39). Thus, the HCM is likely to mediate interactions with a range of potential DNA-binding partners involved in various cellular processes, and these partners display differing affinities and structural requirements for HCM interaction.

Functionally defined motifs of E2a-Pbx1 (Fig. 8) include the E2a transactivation domains AD1 and AD2 and the Pbx homeodomain and HCM. Earlier studies have addressed the requirements for AD1 and AD2 in E2a-Pbx1-mediated transcription and transformation (17, 26). The studies presented here establish important roles for the HCM in these same processes but show disparate results regarding the need for the Pbx homeodomain. These differences result from limitations of our transcriptional assays which do not completely delineate the functional properties of the HCM, which in our cellular transformation assays appears to be responsible for the homeodomain-independent transforming capability of E2a-Pbx1. Several possible models could account for the observed dominant role of the HCM versus the homeodomain for oncogenic

activity of E2a-Pbx1. One possibility is that the HCM interacts directly with DNA either specifically or nonspecifically. Although this possibility cannot be formally ruled out, the HCM does not share homology with known DNA-binding motifs, nor does it display charge characteristics favorable for interaction with DNA. Furthermore, our data show that the HCM does not contribute to DNA-binding specificity of the Pbx homeodomain. It is more likely, as discussed above, that the HCM mediates protein interactions. In this capacity, interactions could occur with partners that are themselves bound or unbound to DNA. The latter could lead to dominant-negative effects by Pbx fusion proteins through sequestration of heterologous partners such as class I Hox proteins. A similar mechanism may underlie the oncogenic properties of chimeric Hox proteins such as NUP98-HoxA9 in myeloid leukemias through sequestration of Hox cofactors such as Pbx at or near the nuclear pore complex (2, 27). In support of these potential dominant-negative scenarios, we have shown that Pbx and Hox proteins can interact, albeit weakly, in the absence of DNA (6) and that endogenous E2a-Pbx1 proteins in t(1;19) cells do not bind Pbx-Hox sites in EMSA (12).

We favor a model in which the HCM mediates interactions with DNA-bound partners, and these interactions are sufficient to tether the homeodomain-lacking fusion protein to relevant promoters. Precedent for such interactions is provided by *Drosophila* homeodomain proteins ftz and PRD, which in the absence of a ftz homeodomain maintain their ability to synergistically activate transcription of an engrailed-derived promoter (1). To date, we have been unable to reconstruct such an interaction in vitro or in transiently transfected cells in assays using ΔHD mutants of E2a-Pbx1 and class I Hox proteins on consensus Pbx-Hox DNA sites. This inability suggests one of several possibilities: the Hox proteins tested do not correspond to the endogenous partners involved in transformation events; Pbx-Hox sites require the correct promoter context, which is not provided in our experiments; and/or unidentified factors stabilize Pbx-Hox interactions in vivo. Since subtle alterations of the Pbx HCM result in complete abrogation of transforming activity whereas the homeodomain is entirely dispensable, we conclude that interactions mediated by the HCM are considerably more important for transformation by E2a-Pbx1 than interactions with cognate Pbx DNA sites. This scenario dictates a dominant role for unidentified heterologous Pbx-interacting proteins in the recognition of response elements in genes whose expression is altered by E2a-Pbx1. Potential candidates are class I Hox proteins, some of which are expressed in NIH 3T3 cells (8, 10, 18, 37), or possibly non-Hox homeodomain proteins with the capacity to cooperatively bind DNA with Pbx (32, 38). Additional efforts to identify transformation partners will help elucidate the subordinate genes through which E2a-Pbx1 effects cellular transformation.

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