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# Pbx-Hox Heterodimers Recruit Coactivator-Corepressor Complexes in an Isoform-Specific Manner

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**Homeobox (*hox*) proteins have been shown to regulate cell fate and segment identity by promoting the expression of specific genetic programs. In contrast to their restricted biological action in vivo, however, most homeodomain factors exhibit promiscuous DNA binding properties in vitro, suggesting a requirement for additional cofactors that enhance target site selectivity. In this regard, the *pbx* family of homeobox genes has been found to heterodimerize with and thereby augment the DNA binding activity of certain *hox* proteins on a subset of potential target sites. Here we examine the transcriptional properties of a forced *hox-pbx* heterodimer containing the pancreas-specific orphan homeobox factor *pdx* fused to *pbx-1a*. Compared to the *pdx* monomer, the forced *pdx-pbx1a* dimer, displayed 10- to 20-fold-higher affinity for a consensus *hox-pbx* binding site but was completely unable to bind a *hox* monomer recognition site. The *pdx-pbx* dimer stimulated target gene expression via an N-terminal *trans*-activation domain in *pdx* that interacts with the coactivator CREB binding protein. The *pdx-pbx* dimer was also found to repress transcription via a C-terminal domain in *pbx-1a* that associates with the corepressors SMRT and NCoR. The transcriptional properties of the *pdx-pbx1* complex appear to be regulated at the level of alternative splicing; a *pdx-pbx* polypeptide containing the *pbx1b* isoform, which lacks the C-terminal extension in *pbx1a*, was unable to repress target gene expression via NCoR-SMRT. Since *pbx1a* and *pbx1b* are differentially expressed in endocrine versus exocrine compartments of the adult pancreas, our results illustrate a novel mechanism by which *pbx* proteins may modulate the expression of specific genetic programs, either positively or negatively, during development.**

Homeobox proteins have been shown to regulate cell fate and segment identity by promoting the expression of specific genetic programs. Initially characterized as an endoderm-specific homeobox protein (35) and later as a transcription factor for the somatostatin (15, 17) and insulin genes (23), the orphan homeobox protein *pdx*, for example, performs a critical role in pancreatic development; targeted disruption of the *pdx* gene leads to a null pancreas phenotype with pancreatic morphogenesis arrested at the early bud stage (11, 21).

Predating the appearance of visible pancreatic rudiments, *pdx* expression is first detected histologically at embryonic day 8.5 (E8.5) (8). Although initially produced in both exocrine and endocrine compartments of the developing pancreas, *pdx* expression shifts to β cells, where it regulates insulin gene expression and functions importantly in glucose homeostasis (8, 17, 25, 28). Heterozygous $pdx7^{+/-}$ mice develop glucose intolerance in adulthood (7), and mutations in the human *pdx* gene are associated with maturity onset diabetes (33).

Like other Hox proteins, Pdx binds promiscuously to target promoters containing a consensus CTAATG recognition site, but its affinity for certain sites is strongly potentiated by heterodimerization with *pbx* (26). The importance of *pbx* in modulating hox activity is perhaps best illustrated by studies in *Drosophila melanogaster* showing that the *pbx* homologue *exd* strongly influences segmentation (27). Complex formation with *pbx* and *exd* requires a conserved YPWMK pentapeptide motif located upstream of the homeodomain in *pdx* and other *hox* proteins (3, 4, 6, 27, 30). The crystal structures of *ubx-exd* and *hox1b-pbx1* complexes reveal that the pentapeptide motif functions primarily in protein-protein interactions, articulating with a hydrophobic pocket in the homeodomains of both *pbx* and *exd* (24, 29).

Although *exd* potentiates *hox* activity in most cases, repressive effects have also been described. *pbx* has been found to inhibit target gene, for example, in transfected cells, although the mechanism underlying this function is unclear (16). To evaluate the mechanism by which *pbx-hox* complexes activate or repress target gene expression without potential interference from other nuclear factors, we have employed a forced *pdx-pbx* heterodimer in which *pdx* sequences are fused in frame to *pbx1*. Our results illustrate a novel mechanism by which *pbx* proteins may influence the expression of genetic programs during development.

## MATERIALS AND METHODS

**Plasmid and transfections.** Wild-type (SP) and mutant (SμP) *pbx-pdx* forced heterodimers were constructed by three-way ligation into a *Pst*I/*Xba*I-cut pBK cytomegalovirus (CMV) vector (Stratagene). The rat *pdx1* cDNAs (wild-type and mutant lacking the *pbx* interaction motif) were amplified by PCR by using the primers 5′-TTACTACTGCAGATTATGGTATACCCATACGATGTTCCAG ATTACGCTGGGCCCATGAATAGTGAGCAG-3′ (sense strand containing Kozak consensus and HA tag at the N terminus) and 5′-GGGCTCGAGCCGG GGTTCCTGCGG-3′ (antisense strand lacking stop codon). The amplified fragments were then digested with *Pst*I and *Xho*I. The human Pbx1 cDNA was amplified by PCR by using the primers 5′-GCACTCGAGGGCATGGACGAG CAGCCCAGG-3′ (sense strand) and 5′-CTTCTTTCTAGATCACTTGTCGT CGTCGTCTTTGTAGTCGTTGGAGGTATCAGAGTG-3′ (antisense strand, containing stop codon and FLAG tag). The PCR-amplified Pbx1 cDNA fragment was then digested with *Xho*I and *Xba*I. Pbx1 and Pdx1 fragments were then ligated into the pBK-CMV vector to generate the chimeric expression constructs that encoded polypeptides of 105 kDa. The *pbx* interaction-defective heterodimer, referred to as SμP, contains point mutations in the pentapeptide motif (FPWMK/AAGGQ) at amino acids (aa) 119 to 123. Somatostatin TSEI and TSEII constructs and NcoR-SMRT plasmids have been described elsewhere (25, 26). Transfection assays were performed in 293T cells as previously described

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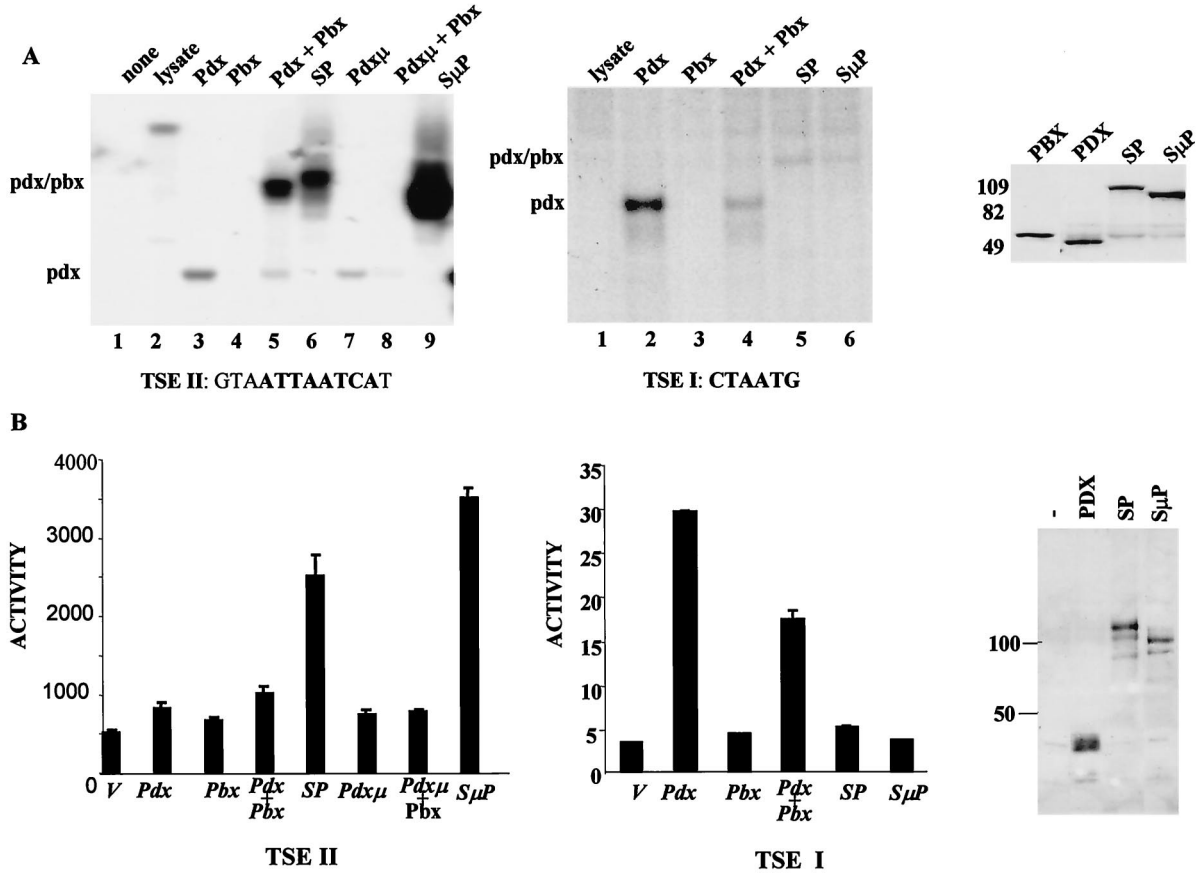


FIG. 1. A forced *pdx-pbx* heterodimer, referred to as SP, binds selectively to and stimulates target gene expression from a consensus *hox-pbx* (TSEII) but not a monomeric *hox* (TSEI) binding site on the somatostatin promoter. (A) Gel mobility shift assay of SP binding activity compared with monomeric *pdx* on somatostatin TSEII (left) and TSEI (middle) elements. *pbx* interaction-defective *pdx* polypeptides containing (FPWMK/AAGGQ) substitution in the *pbx* interaction motif (aa 119 to 123) were examined either in the context of the *pdx* monomer (*pdx*μ) or a forced heterodimer (SμP). Gel mobility shift assays were performed with $^{32}$P-labeled somatostatin oligonucleotides plus in vitro-translated *pdx* and *pbx* polypeptides. (Right) Western blot assay of in vitro-translated *pbx*, *pbx*, SP, and SμP constructs show equivalent levels of expression. (B) Transient assay of *pdx* and SP polypeptides after cotransfection with somatostatin TSEII (left) and TSEI (middle) reporter constructs. In this and all subsequent assays, reporter activity is shown after normalizing to β-Gal activity from co-transfected RSV–β-Gal construct. (Right) Western blot assay of *pdx*, SP, and SμP polypeptides in nuclear extracts of transfected 293T cells with *pdx*-specific antiserum.

(20), and reporter activities were normalized to activity from cotransfected Rous sarcoma virus–β-galactosidase (RSV–β-Gal) expression plasmid.

**DNA-binding studies.** Gel mobility shift assays were performed with $^{32}$P-labeled double-stranded somatostatin TSEII or TSEI oligonucleotides plus in vitro-translated *pdx* and *pbx* polypeptides as described previously (25, 26).

**Western blotting and pull-down assays.** Western blot and co-immunoprecipitation assays were performed as previously described (20). Pbx1-specific antiserum was obtained from Santa Cruz Biotech. Pdx1 antiserum has been reported elsewhere (8, 15). For glutathione *S*-transferase (GST) pull-down assays, $^{35}$S-labeled polypeptides were incubated with GST resins in binding buffer (20 mM HEPES, pH 7.0; 2 mM $MgCl_2$; 20% glycerol; 0.2 mM EDTA; 0.05% NP-40; 1 mM β mercaptoethanol) for 30 min at room temperature. Binding reactions were then washed four times with binding buffer, and the bound fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

## RESULTS

**A forced *pdx-pbx* heterodimer displays selective DNA binding and *trans*-activation properties.** To characterize *pdx-pbx* activity, we constructed a forced heterodimer, referred to as SP, which contains the full-length *pdx* polypeptide fused at its C terminus to *pbx1a*. Compared with monomeric *pdx*, the SP dimer displayed 10 to 20 times higher affinity for a *pbx-hox* binding site on the somatostatin promoter (TSEII) (26) in gel mobility shift assays (Fig. 1A, left, compare lanes 3, 5, and 6). Mutagenesis of the *pbx* interaction motif in *pdx* (FPWMK/AAGGQ) actually enhanced binding of the mutant SμP construct to the TSEII site, suggesting that the pentapeptide motif in *pdx* functions exclusively in protein-protein interactions and that fusion of *pdx* with *pbx* is sufficient to promote cooperative DNA binding (Fig. 1A, left, compare lanes 6 and 9).

By contrast with their activities on the somatostatin TSEII element, SP and SμP polypeptides showed far lower affinity for a consensus hox monomer binding site on the somatostatin promoter (TSEI) compared to *pdx* alone (Fig. 1A, middle, compare lanes 2, 5, and 6). Consistent with the notion that *pbx* may destabilize the binding of heterodimerized hox proteins to certain sites, the addition of *pbx* also inhibited binding of monomeric *pdx* to the TSEI site in vitro (Fig. 1A, middle, compare lanes 2 and 4). These results indicate that two-site binding (*pbx* and *pdx*) is required to stabilize interaction of the *pbx-pdx* dimer with DNA and that single-site interaction with DNA (*pdx*) in this context is not sufficient for stable occupancy.

To evaluate the transcriptional properties of the *pdx-pbx* dimer, we performed transfection assays in 293T cells. *pdx* alone induced a TSEII reporter construct 1.5-fold, and co-transfection with *pbx* potentiated *pdx* activity somewhat in 293T cells (Fig. 1B, bottom left). Reflecting their enhanced DNA binding activities relative to *pdx* in gel shift assays, wild-

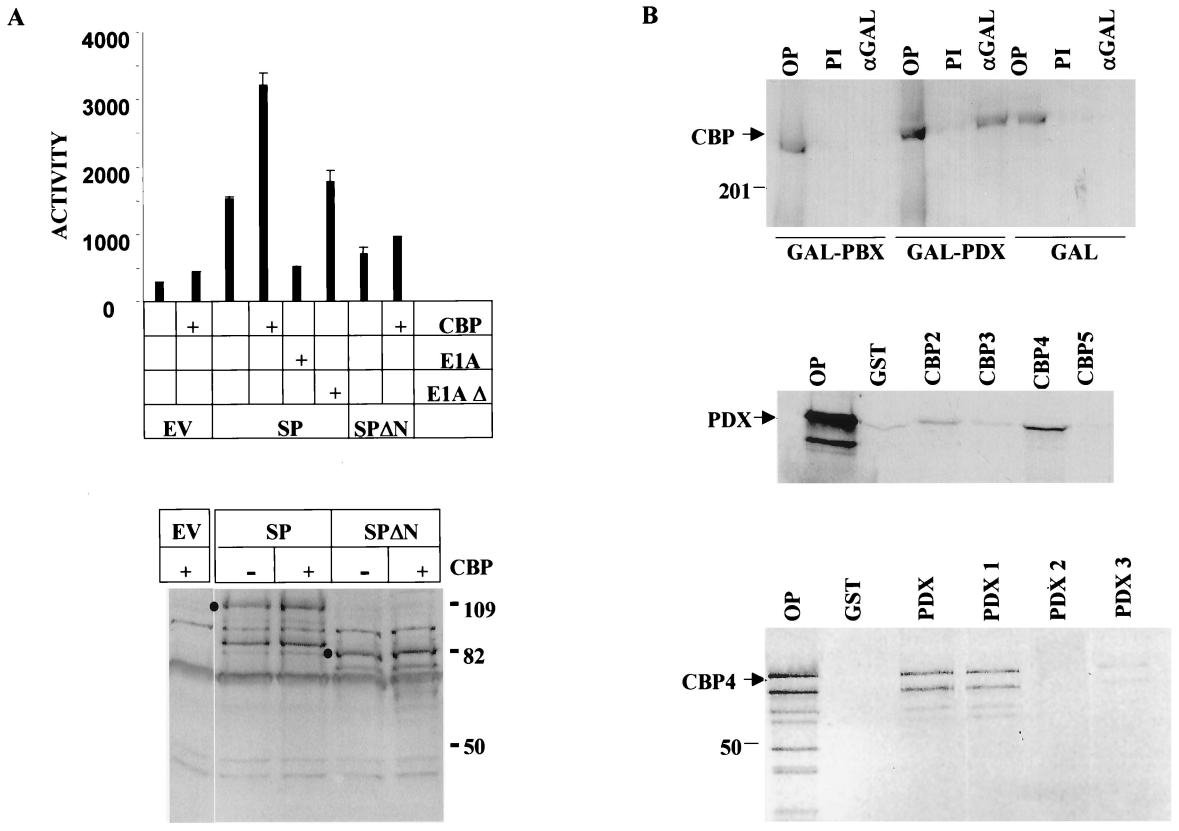


FIG. 2. The *pdx-pbx* heterodimer stimulates target gene expression via an N-terminal *trans*-activation domain in *pdx* that associates with the coactivator CBP. (A) The top panel shows the transcriptional activity of wild-type (SP) or mutant (SPΔN) *pdx-pbx* forced heterodimer lacking N-terminal *Pdx trans*-activation domain (aa 1 to 135) in 293T cells. SP and SPΔN activities were evaluated on a somatostatin TSEII luciferase reporter plasmid. Cells cotransfected with CBP and E1A expression vectors (wild type and CBP/P300 interaction defective [E1AΔ]) indicated. In the bottom panel, the expression levels of SP and SPΔN in transfected 293T cells by Western blot assay with anti-*pdx* antiserum are shown. Cells co-transfected with CBP expression vector or empty vector indicated over each lane. (B) CBP associates with the N-terminal *trans*-activation domain of *pdx* in vivo. (Top) Western blot assay of CBP recovered from immunoprecipitates of GAL4 (αGAL) in 293T cells cotransfected with expression plasmids for CBP plus either GAL4 DNA-binding domain (aa 1 to 147), GAL4-*PDX*, or GAL4-*PBX*, as shown. OP, 10% of total input extract; PI, preimmune serum. (Middle) GST pull-down assay of $^{35}$S-labeled CBP fragments with the following amino acid endpoints: CBP2 (1 to 737), CBP3 (737 to 1626), CBP4 (1626 to 2260), and CBP5 (2260 to 2389). Assays were performed with GST or GST-*pdx* polypeptides bound to glutathione-Sepharose beads as indicated. OP, 10% of total input. (Bottom) GST pull-down assay of $^{35}$S-labeled CBP4 (aa 1626 to 2260) with GST-*pdx* polypeptides with the following amino acid endpoints: *PDX*, full-length *pdx* (1 to 283). *PDX*1 (1 to 140), *PDX*2 (141 to 215), and *PDX*3 (210 to 283).

type SP and mutant (SμP) dimers stimulated TSEII reporter activity five- and sevenfold, respectively (Fig. 1B, left).

Confirming the ability of *pbx* to block recruitment of *pdx* to promoters containing monomer binding sites, SP and SμP constructs were completely inactive on a TSEI reporter construct (Fig. 1B, middle). The *pdx* monomer stimulated TSEI reporter activity 15-fold, but overexpression of *pbx* inhibited TSEI-dependent reporter expression by ca. 50% (Fig. 1B, middle). These results suggest that, in addition to augmenting binding to consensus *hox-pbx* recognition sites, *pbx* family members may repress transcription from promoters containing monomer binding sites, depending on their affinities for *hox* partners in solution.

**The *pdx-pbx* dimer stimulates transcription by associating with the C/H3 domain of the coactivator CBP.** *pdx* has been shown to regulate both somatostatin and insulin gene expression via an N-terminal *trans*-activation domain (aa 1 to 140) (25). The ability of E1A to block insulin gene expression (32), potentially by sequestering the coactivator CREB binding protein (CBP) and its paralog P300, prompted us to examine whether *pdx* stimulates target gene expression, in the context of the *pdx-pbx* dimer, by associating with this coactivator. In transient-transfection assays, wild-type but not CBP interaction-defective E1A oncoprotein (E1AΔ) potently inhibited activation of the TSEII reporter via the SP polypeptide in 293T cells (Fig. 2A, top). Conversely, overexpression of CBP stimulated target gene activation two- to threefold by SP (Fig. 2A, left). CBP potentiation required the *pdx trans*-activation domain; CBP had no effect on TSEII reporter induction via a mutant SP dimer (SPΔN) lacking that region (Fig. 2A, left).

To determine whether *pdx* interacts physically with CBP in vivo, we performed coimmunoprecipitation studies. CBP was efficiently recovered from immunoprecipitates of GAL4 *pdx* in transfected 293T cells (Fig. 2B, top). By contrast, no CBP was detected in anti-GAL4 immunoprecipitates from cells transfected with GAL4-*pbx* or GAL4 DNA binding domain expression vectors, indicating that the SP dimer associates with CBP via residues in *pdx* (Fig. 2B, top). To assign relevant interaction domains that mediate complex formation between *pdx* and CBP, we performed affinity interaction assays. Using fragments of CBP fused to GST, we observed selective binding of $^{35}$S-labeled *pdx* to the C/H3 region, which coincides with the E1A binding domain of CBP, but not to other regions (Fig. 2B, middle). In reciprocal pull-down assays with a series of GST *pdx* polypeptides, the $^{35}$S-labeled C/H3 region of CBP was found to bind specifically to the N-terminal *trans*-activation

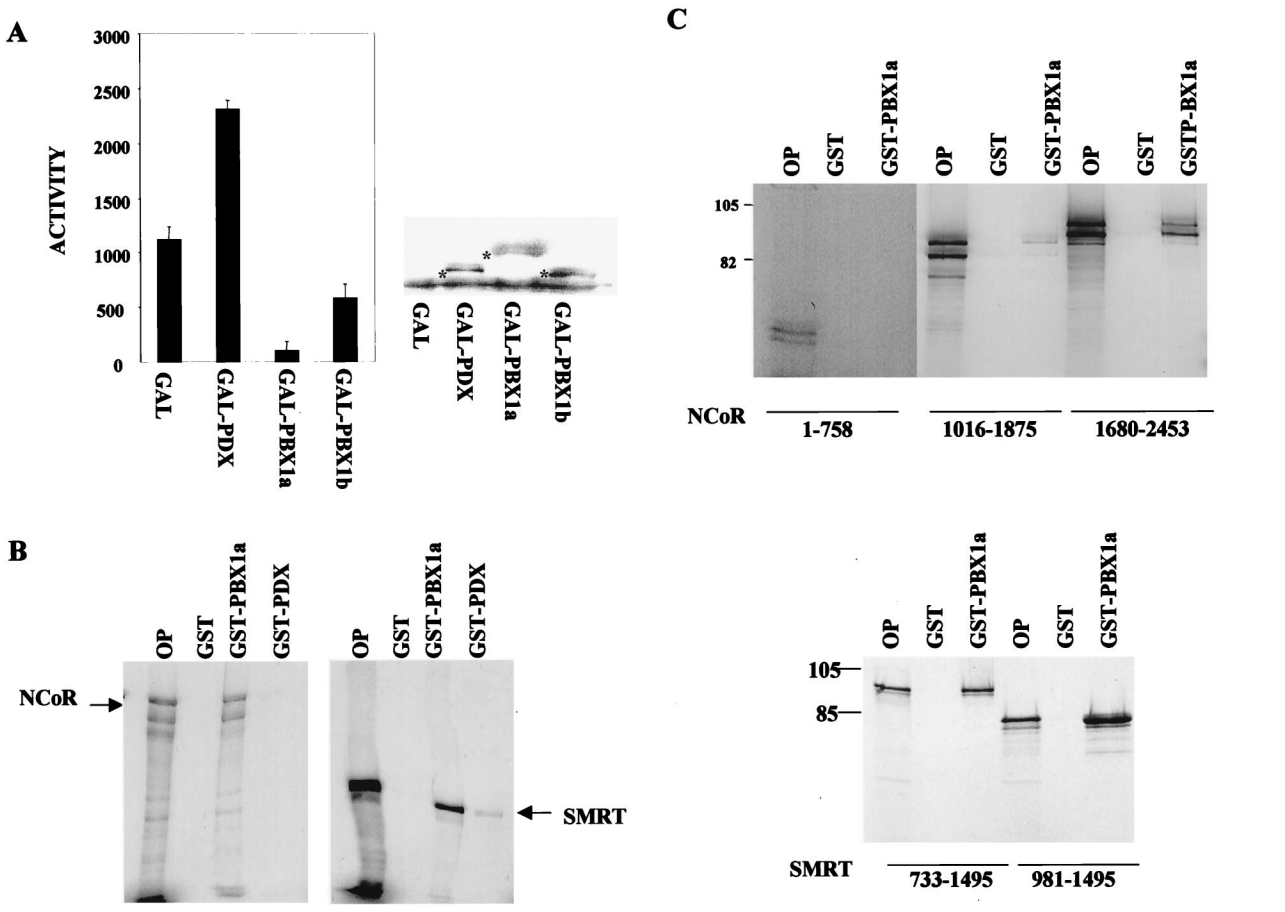


FIG. 3. *pbx1a* associates with the corepressors NCoR and SMRT. (A) Transient-transfection assay of GAL4-*pbx1a* and GAL4-*pbx1b* polypeptides on a GAL4 thymidine kinase luciferase reporter plasmid in 293T cells. The activities of GAL4 DNA-binding domain and GAL4-*pdx* polypeptides are shown for comparison. A Western blot assay of nuclear extracts from transfected 293T cells with αGAL4 antiserum was done to show comparable expression levels of each GAL4 fusion construct. Asterisks indicate immunoreactive bands corresponding to each polypeptide. (B) GST pull-down assays of $^{35}$S-labeled NCoR and SMRT after incubation with GST, GST-*pbx1a*, or GST-*pdx* polypeptides in vitro. OP, 10% of input. (C) Carboxy-terminal domains of NCoR and SMRT associate with *pbx1a*. GST pull-down assay of $^{35}$S-labeled NCoR (top) and SMRT (bottom) polypeptides with GST or GST-*pbx1a* glutathione-Sepharose resins. Inclusive amino acid endpoints for each NCoR and SMRT polypeptide are shown.

domain (aa 1 to 140) but not to the homeodomain (aa 140 to 215) or the carboxy-terminal region (aa 210 to 283) of *pdx* (Fig. 2B, bottom).

***Pbx1* represses transcription via a C-terminal domain that interacts with the corepressors NCoR and SMRT.** To evaluate regulatory contributions from *pbx* towards target gene expression, we fused *pbx1a* to the GAL4 DNA binding domain. After transfection into 293T cells, GAL4-*pbx1a* potently repressed transcription from a GAL4 thymidine kinase luciferase reporter (Fig. 3A). Remarkably, a GAL4 fusion construct containing the alternatively spliced *pbx1b* polypeptide, which lacks the carboxy-terminal 83 aa in *pbx1a*, was fivefold less active in repressing target gene expression (Fig. 3A). Expression levels of GAL4 *pbx1a* and GAL4 *pbx1b* were comparable in transfected cells, however, suggesting that the carboxy-terminal region in *pbx1a* is required for target gene repression in 293T cells (Fig. 3A, right).

Previous studies, demonstrating that the corepressors NCoR and SMRT (5, 10) interact functionally with the homeodomain proteins Pit-1 and Rpx-1 (36), prompted us to examine whether *pbx* inhibits transcription via a similar mechanism. In GST pull-down experiments, $^{35}$S-labeled NCoR and SMRT polypeptides were found to bind efficiently to glutathione-Sepharose beads containing GST-*pbx1a* but not GST-*pdx* (Fig. 3B). The carboxy-terminal region corresponding to the nuclear hormone receptor binding domain of each corepressor (3, 7) appeared to mediate interaction with *pbx1a*; other regions did not bind detectably to GST-*pbx1a* resin in affinity interaction assays (Fig. 3C).

In reciprocal binding assays, SMRT (Fig. 4A) and NCoR (not shown) were found to bind efficiently to the alternatively spliced carboxy-terminal region of *pbx1* (Fig. 4A, lane 6). A weaker secondary interaction of SMRT with the N-terminal 240 aa of *pbx1a* was also noted, however (Fig. 4A, compare lanes 2 and 4). In agreement with these truncation studies, *pbx1b*, which lacks the C-terminal domain in *pbx1a*, did not interact detectably with either NCoR or SMRT (Fig. 4B, compare lanes 2 and 3). The ability of the alternatively spliced carboxy-terminal region in *pbx1* to associate with NCoR and SMRT prompted us to test whether *pbx1a* and *pbx1b* display different regulatory properties in the context of a *pbx-pdx* heterodimer. In transient-transfection assays, forced dimers containing either *pbx1a* (SPa) or *pbx1b* (SPb) induced TSEII reporter activity seven- to eightfold in 293T cells (Fig. 4C). Overexpression of either NCoR or SMRT strongly inhibited SPa activity on the TSEII reporter without altering the expres-

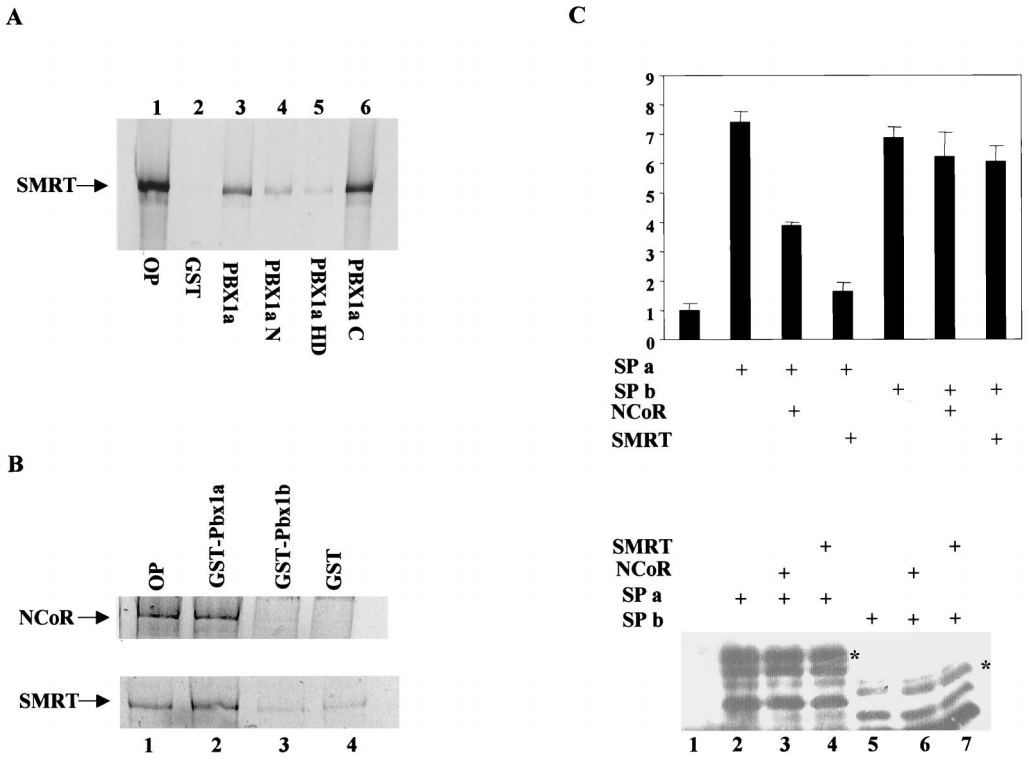


FIG. 4. NCoR and SMRT are selectively recruited to *pdx-pbx* complexes containing *pbx1a* but not the alternatively spliced *pbx1b* polypeptide, which lacks the carboxy-terminal NcoR-SMRT interaction domain. (A) GST pull-down assay of $^{35}$S-labeled NCoR and SMRT polypeptides to GST-*pdx1a* and GST-*pdx1b* resins or GST only. OP, 10% of total input. (B) Pull-down assay of $^{35}$S-labeled SMRT with GST-*pbx* polypeptides. OP, 10% of total SMRT input; pull-downs were performed with glutathione-Sepharose resins containing either GST alone (GST), full-length GST-*pbx1a* (Pbx1a), or *pbx* fragments including N-terminal aa 1 to 240 (Pbx1aN), residues 233 to 320 containing the homeodomain (PBX1aHD), or carboxy-terminal residues 287 to 430 (PBX1aC). (C) In the upper panel, the results of a transient-transfection assay of SPa and SPb expression constructs containing full-length *pdx* fused to *pbx1a* and *pbx1b*, respectively, are shown. SPa and SPb activities were examined on a somatostatin TSEII luciferase reporter. Cotransfection with NCoR or SMRT expression vectors is as indicated. The lower panel shows a Western blot assay of nuclear extracts from transfected 293T cells with anti-*pdx* antiserum to show comparable expression of SPa (lanes 2 to 4) and SPb (lanes 5 to 7). Lane 1 shows extracts from cells transfected with empty vector.

sion levels of the SPa polypeptide in transfected cells, suggesting that the repression we observed with these constructs reflects functional recruitment of NCoR and SMRT to the promoter (Fig. 4C). By contrast with SPa, NCoR and SMRT had no repressive effect on target gene induction via the SPb dimer, which lacks the NCoR-SMRT interaction domain (Fig. 4C). Taken together, these results suggest that *pbx-hox* dimers may either repress or activate target gene expression depending on relative cellular levels of *pbx1a* and *pbx1b* isoforms.

To determine whether *pbx1a* and *pbx1b* levels are differentially regulated in the pancreas, we performed Western blot assays with *pbx1*-specific antiserum. In agreement with a previous report (34), only *pbx1b* was detected in the acinar cell line 266-6 as well as in extracts from whole pancreas, which consist predominantly of exocrine tissue (Fig. 5, lanes 2 and 3). *Pbx1b* was not present in extracts from freshly isolated pancreatic islets, but a prominent *pbx1a* immunoreactive band was readily apparent (Fig. 5, lane 1). These results indicate that *pbx1* isoforms are produced at different levels in exocrine versus endocrine cells of the pancreas where they may contribute to alternate activation of pancreatic genes via *pdx*.

## DISCUSSION

The *pbx* family of homeobox genes has been shown to modulate *hox* target gene activity by forming heterodimeric complexes that have high binding activity on a subset of potential DNA recognition sites. Our results demonstrate that *pbx* proteins may additionally inhibit *hox* target gene expression via two distinct mechanisms: by blocking binding of *hox* partners to monomer binding sites and by recruiting corepressor complexes to *hox-pbx* dimer binding sites.

When evaluated in the context of a forced *pdx-pbx* dimer, *pbx* strongly potentiated binding of *pdx* to a consensus *hox-pbx*

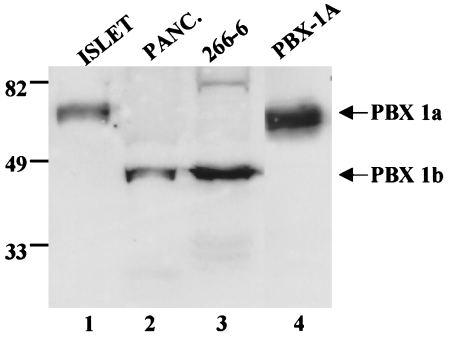


FIG. 5. *pbx1a* and *pbx1b* isoforms are differentially expressed in pancreatic islets and acinar cells. A Western blot assay of whole-cell extracts from freshly isolated islets (ISLET), whole pancreas (PANC.), and the acinar cell line 266-6 was done with α *pbx-1*-specific antiserum. The migration of in vitro-translated *pbx1a* polypeptide is shown. The relative positions of *pbx1b* and *pbx1a* polypeptides are indicated.

binding site (TSEII) on the somatostatin promoter, but it also repressed binding of *pdx* to a monomer site (TSEI). Consistently, the addition of *pbx* to gel mobility shift reactions reduced binding of *pdx* to the TSEI site. *pbx* has been found to heterodimerize with *hox* partners in solution (31), suggesting that *pbx* may repress transcription from promoters containing *hox* monomer binding sites, depending on the nuclear levels of *pbx*.

Using a forced heterodimer approach to evaluate the activity of the *pdx-pbx* complex, we observed that target gene activation via *pdx-pbx* relies on an N-terminal *trans*-activation domain that interacts functionally with the coactivator CBP. CBP, in turn, has been proposed to mediate target gene expression, in part, via an association with general factors in the transcriptional apparatus (14, 19) and by intrinsic histone acetyltransferase activities that may disrupt promoter bound nucleosomes (2, 22). The ability of CBP to mediate target gene activation via *pdx* thus suggests a chromatin-dependent mechanism by which the pancreas-specific genetic program is activated during development.

By contrast with *pdx*, *pbx* was found to repress target gene expression via a C-terminal domain that associates with the corepressors NCoR and SMRT. Remarkably, no recruitment of NCoR or SMRT to the promoter was observed in the context of a *pdx-pbx1* heterodimer containing the alternatively spliced *pbx1b* polypeptide that lacks the C-terminal *trans*-repression domain. These observations point to a potential function for alternative splice products of *pbx*: target gene expression may be alternately inhibited or activated by *pbx-hox* heterodimers depending on the relative expression levels of the two splice products.

The mechanism by which NCoR and SMRT repress target gene expression in this context is unclear. Previously shown to associate with histone deacetylases via Sin3 complexes (1, 9, 18), NCoR and SMRT may consequently block *pdx* activity by opposing CBP-mediated nucleosome acetylation. In this regard, *pbx* has been found to induce leukemic transformation in pre-B cells after fusion to the helix-loop-helix protein E2A (13). Importantly, the *pbx1b* alternative splice product lacking the C-terminal SMRT-NCoR interaction domain displays higher transforming activity compared to E2A-*pbx1a* fusion (12). Our results suggest that recruitment of SMRT-NCoR to the promoter via *pbx1a* may interfere with leukemogenesis, to some extent, by interfering with E2A-mediated target gene activation. In this regard, it will be of interest to determine whether B-cell transformation via E2a-*pbx1a* is potentiated by histone deacetylase inhibitors.

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H.A. and S.D. contributed equally to this work.

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