DNA Binding and Transcriptional Activation by a PDX1-PBX1b-MEIS2b Trimer and Cooperation with a Pancreas-specific Basic Helix-Loop-Helix Complex

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In pancreatic acinar cells, the HOX-like factor PDX1 acts as part of a trimeric complex with two TALE class homeodomain factors, PBX1b and MEIS2b. The complex binds to overlapping half-sites for PDX1 and PBX. The trimeric complex activates transcription in cells to a level about an order of magnitude greater than PDX1 alone. The N-terminal PDX1 activation domain is required for detectable transcriptional activity of the complex, even though PDX1 truncations bearing only the PDX1 C-terminal homeodomain and pentapeptide motifs can still participate in forming the trimeric complex. The conserved N-terminal PBC-B domain of PBX, as well as its homeodomain, is required for both complex formation and transcriptional activity. Only the N-terminal region of MEIS2, including the conserved MEIS domains, is required for formation of a trimer on DNA and transcriptional activity: the MEIS homeodomain is dispensable. The activity of the pancreas-specific ELA1 enhancer requires the cooperation of the trimer-binding element and a nearby element that binds the pancreatic transcription factor PTF1. We show that the PDX1-PBX1b-MEIS2b complex cooperates with the PTF1 basic helix-loop-helix complex to activate an ELA1 minihancer in HeLa cells and that this cooperation requires all three homeoprotein subunits, including the PDX1 activation domain.

The HOX transcription factors are key mediators for establishing the embryonic body plan and guiding organogenesis (1–5). The many members of this large gene family have distinct roles in development, despite having very similar DNA binding specificities and overlapping patterns of expression. At least part of the increased binding specificity required to distinguish the roles of the various HOX proteins comes from their interaction with members of the TALE class of homeodomain proteins (for review see Ref. 4). The TALE proteins are characterized by the presence of a 3-amino acid loop extension between α-helices 1 and 2 of the homeodomain (5) and include both the PBC class (mammalian PBX proteins, Drosophila Extradenticle, and Caenorhabditis elegans Ceh-20 (6)) and the MEIS-like TALE factors (mammalian MEIS and PREP1 proteins, Drosophila Homothorax (HTH); C. elegans Ceh-25 (5, 7)).

Other homeodomain proteins important in development also form complexes with the TALE proteins, including the pancreas-duodenal homeobox-1 protein (PDX1) (8–10).

Pdx1 is a member of the PARAHOX cluster of homeobox genes implicated in the development of endoderm-derived organs (11). Pdx1 is essential to the development of both the endocrine and exocrine compartments of the pancreas (12–14). The interaction of PDX1 with PBX is required for the expansion of the various endocrine and exocrine cell types during development (15). Although Pdx1 is expressed throughout the pancreas during embryonic development, its expression is predominantly localized to the β-cells of the islets of Langerhans of adult mammals (16, 17). PDX1 has been implicated in the transcriptional control of a number of β-cell-specific genes, including insulin (16, 18), glucokinase (19), islet amyloid polypeptide (20, 21), and glucose transporter type 2 (22). PDX1 also participates in the activation of an acinar cell-specific gene, elastase 1 (ELA1); in this instance it acts as part of a trimeric complex with PBX1b and MEIS2 (10).

Our previous studies identified the PDX1-PBX1b-MEIS2 trimer as one of two pancreatic transcription factor complexes that cooperate in the transcriptional activation of the ELA1 enhancer in acinar cells (23). The ELA1 enhancer comprises three functional elements (A, B, and C) within 100 bp (24). The proper acinar activity of the enhancer requires the synergistic cooperation between the A and B elements (25, 26). The PDX1-PBX1b-MEIS2b complex binds and mediates the acinar activity of the B element (10). The other factor, PTF1, is a bHLH acinar cell-specific complex that binds and mediates the activity of the A element (24, 27). PTF1 is essential for the formation of the exocrine pancreas (28) as well as for the transcription of the digestive enzyme genes (24, 29).

In this report, we show which domains of PDX1, PBX1b, and MEIS2b are essential for the formation of the trimeric complex, for binding of the complex to DNA, for transcriptional activity, and for cooperative activation with PTF1. PDX1 contributes the only transcriptional activation domain, even though the activity of the trimer is an order of magnitude greater than that of PDX1 alone. PDX1 recruits PBX1b, and they bind in tandem to adjacent PDX1 and PBX half-sites. PBX1b recruits MEIS2b to the DNA-bound complex. MEIS2b is required for both the transcriptional activity of the PDX1-PBX1b-MEIS2 trimer and its cooperation with PTF1.

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Oligonucleotides—The ELA1 B element has been described previously (10); the top strand sequences of the B element and its derivatives B2 and B7 are shown in Fig. 1A. The CRS1 element from the bovine CYP17 gene has the top strand sequence of CTCGAGGAGCCGTTATGAGCAGCTGAGGAAG, and the bottom strand sequence of TCACGCTTGCGTAGAAGCTGGTAGCCTGGAGCGT, the adjoining PBX and MEIS binding sites are underlined (30).

Antibodies—Polyclonal rabbit antibodies against PDX1, PBX, PBX1, and MEIS2 have been described previously (10). The polyclonal rabbit antisera specific for the A and B isoforms of MEIS2 proteins were raised against an 18-amino acid peptide, PMSGMGMGMMGMGQWYHM, corresponding to amino acids 377–394 of MEIS2b. Similarly, a 16-amino acid peptide, SVDPNVQGMVQDIHAQ, spanning amino acids 455–470 of MEIS2d, was used to produce the anti-MEIS2d/a. A 7-amino acid insert, GFLDDFS, corresponding to amino acids 346–352 of MEIS2a and MEIS2e, is specifically present in these two isoforms. The antibody oMEIS2a/c was raised against a 14-amino acid peptide containing a tandem repeat of the insert. All of the MEIS isoform-specific antibodies were raised against peptide sequences common to mouse and human orthologs.

DNA Constructs—The B element reporter construct (5B.EIp.hGH) has five head-to-tail copies of the B element (Fig. 1A) linked to the Ela1 minimal promoter from −92 to +8 and fused to an hGH reporter gene (10). In the B2 and B7 multimer constructs, sense strand and antisense strand oligonucleotides containing five copies of the B2 or B7 element (Fig. 1A) were annealed and cloned into the Ela1 minimal promoter/hGH reporter plasmid. The mini-enhancer construct 3(BA).EIp.hGH was inserted into pcDNA1.1/Amp. The B element reporter construct (5B.EIp.hGH) was transferred into pcDNA1.1/Amp for transfection experiments.

Polymerase chain reaction amplification of the corresponding coding sequences from the cDNA for HEB (gift of Dr. Richard Baer, New York, NY) were transcribed in vitro (IVT) proteins were incubated at 37 °C for 40 min. 5 μl of poly(A) polymerase containing 10 μM HEPES (pH 7.6), 25 μM NaCl, 0.1% Nonidet P-40, and 5 μM EDTA. After gentle mixing by rotation at 4 °C for 1 h, 20 μl of protein A beads (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the mixing was continued overnight. Following the incubation, the beads were washed then resuspended in SDS-gel loading buffer. Samples were separated by SDS-10% polyacrylamide gel electrophoresis, and the IVT proteins were transferred onto a PhosphorImager (Molecular Dynamics, Santa Clara, CA).

Cell Transfection—Transfections of the RIN1046-38 β-cell line (RIN-38) (36) and HeLa (ATCC CCL-2) were performed with FuGene (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. All of the plasmids for the expression of transcription factors, as well as the β-galactosidase internal control plasmid (pCMVβ, CLONTECH, Palo Alto, CA) utilized the CMV enhancer/promoter. All transfections contained the same total amount of CMV enhancer/promoter, balanced as necessary by the addition of insertless CMV vector (e.g. pcDNA1.1/Amp). The activity of the hGH reporter gene was assayed by radioimmunoprecipitation (Nichols Institute, San Juan Capistrano, CA). Transfections of HeLa cells were corrected for relative efficiency based on the activity of the cotransfected β-galactosidase reporter assayed with a Galacto-Light Plus kit (Tropix Inc., Bedford, MA). Transfections of RIN1046-38 cells used IVT proteins in a cotransfection with a representative efficiency based on the activity of a cotransfected Pdx1 Luciferase reporter, in which the luciferase reporter gene was under the transcriptional control of the Pdx1 gene promoter sequence. Luciferase activity was assayed with a kit from Promega (Madison, WI). Transfection results reported are the mean and standard error of a minimum of four transfections, except as noted. For those deletion plasmids of PDX1, PBX1b, and MEIS2b that were significantly less than wild type activity in transfections, we confirmed the production of protein and its nuclear localization by immunohistochemical staining using monoclonal antibodies recognizing their peptide tags (PDX1 with anti-V5, Invitrogen, Carlsbad, CA; PBX1b with anti-FLAG, Sigma Chemical Co., St Louis, MO; MEIS2b with anti-T7, Novagen, Madison, WI). All were expressed and localized to the nucleus at approximately wild type levels, except for PD116–206, which was found only in the cytoplasm (data not shown).

RESULTS

Trimeric Complex Binds Only to Sites Containing Both PDX1 and PBX Half-sites—We previously identified a trimeric homeoprotein complex that binds to and mediates the activity of the B element of the elastase I (ELA1) enhancer in pancreatic acinar cell lines (10). The complex consists of a pancreatic HOX-like protein, PDX1, and two TALE-class homeodomain proteins, PBX1b and an isoform of MEIS2/MRG1. The trimeric complex binds an 11-bp region of the B element containing overlapping half-sites for PDX1 and PBX but no recognizable site for MEIS (Fig. 1A). Mutations in either the PDX1 or PBX half-site eliminate trimer binding (10). The central role of PDX1 in trimer binding was demonstrated by mutation of a single nucleotide in the PDX1 half-site of the B element (the A at position 7 of the PBX-HOX consensus); neither PDX1 nor the PDX1-containing trimeric complex from 266-6 acinar cell nuclear extracts bound detectably to the oligonucleotide containing this A to C transversion (B7, Fig. 1B) and this B7 oligonucleotide was unable to compete for trimer binding in EMSA competition assays (Fig. 1C). These results confirmed the binding of PDX1 to its half-site and demonstrated that the formation of the trimeric complex is dependent on an intact PDX1 half-site.

To be able to study the structure of the trimeric complex, it was necessary to increase the PBX binding affinity of the B element without altering the qualitative nature of the activity of the element in cells. The binding affinity is low, because nucleotides at positions 1, 2, and 6 differ from the consensus...
binding sequence for PBX/HOX heterodimers (37, 38). Changing the A at position 2 to T matches the consensus PBX half-site (B2, Fig. 1A) and increased the binding of the trimeric complex from acinar cell nuclear extracts without affecting the binding of the PDX1 monomer (Fig. 1B). In competition assays the B2 element was 5-fold more effective than the unmodified B element (Fig. 1C). Antiseras specific for PDX1, PBX, and MEIS2 eliminated the B and B2 complexes with equal effectiveness (Fig. 1D), confirming that the homeodomain protein composition is the same for the two complexes. The transcriptional activities of B and B2 in transfected cells were also qualitatively the same (see Fig. 4, below). We subsequently used the more effective B2 element to test the ability of various forms of the three transcription factors to form complexes on DNA and activate transcription.

The Trimeric Complex Is Composed of PDX1, PBX1b, and MEIS2b/MEIS2d—To determine which of four MEIS2 isoforms are present in the trimeric complex, we developed antisera against distinguishing peptide regions of the isoforms. Four alternatively spliced variants of Meis2 mRNA encode four distinct protein products: 2a, 2b, 2c, and 2d (39). MEIS2a and 2b have a different C-terminal tail (18 amino acids) than MEIS2c and 2d (94 amino acids); MEIS2a and 2c share a common seven amino acid insert, GFLLDPS, which MEIS2b and 2d lack. The antiserum specific for MEIS2a/b supershifted most of the trimeric complex from acinar cell extracts, leaving behind a residual complex of slightly slower electrophoretic mobility (Fig. 2A, lane 3). The antiserum specific for MEIS2c/d decreased the amount of the trimeric complex slightly (lane 5 versus lane 4), although a supershifted band was not observed. Antiserum against the MEIS2a/c-specific heptapeptide did not reduce the amount of the trimeric complex more than the preimmune serum (lanes 6 and 7). These results indicate that MEIS2b and possibly MEIS2d are present in the complex, but MEIS2a and 2c are not. To determine whether the residual slower mobility complex remaining after treatment with aMEIS2a/b antiserum contained MEIS2d, nuclear extract was treated with a mixture of aMEIS2a/b and aMEIS2c/d antisera (Fig. 2B, lane 4). The combination eliminated all of the trimeric complex. In contrast, combining aMEIS2a/b and aMEIS2a/c did not eliminate the slower migrating complex (lane 6), further showing that MEIS2c is not in the trimeric complex.

PDX1, PBX1b, and MEIS2 proteins synthesized by translation in vitro can form trimeric complexes on DNA (Fig. 2B, lanes 7 and 8). The complex formed with recombinant PDX1-PBX1b-MEIS2b comigrates with the predominant trimer from 266-6 acinar cells (Fig. 2B, lane 7), whereas the PDX1-PBX1b-MEIS2d complex (lane 8) comigrates with the slower mobility complex that remains after aMEIS2b treatment. Thus, we conclude that the major molecular form of the acinar trimeric complex comprises PDX1, PBX1b, and MEIS2b and that MEIS2d replaces MEIS2b in a small fraction of the complexes.

The presence of MEIS2b and 2d in acinar cells was confirmed by Western blot analysis (Fig. 3). Unlike PBX1b, which is present in pancreatic acinar but not β-cell lines (10), the MEIS2 proteins were detected in nuclear extracts from both. The levels of the MEIS2 proteins were lower in β-cells than in acinar cells.

The Trimeric Complex Is More Transcriptionally Activate Than PDX1—Multimers of the B element are transcriptionally active in the β-cell line RIN1046-38 (RIN-38), due to the presence of endogenous PDX1 in these cells (10). PDX1 is absent in these β-cells (10), and MEIS2 is present at extremely low levels (Fig. 3). Adding either PBX1b or MEIS2b to the β-cell line by transfection had little or no effect on the activity of the B multimer or on the B element with the augmented PBX1b binding site (B2) (Fig. 4A). Co-expression of PBX1b and MEIS2b in RIN-38 β-cells increased the activity of either the B or B2 multimer severalfold over the activity directed by the endogenous PDX1 (7-fold for the B element; 4-fold for the B2 element). These results indicate that the trimer complex is more transcriptionally active in RIN-38 cells than PDX1 alone. The B7 mutation which eliminated PDX1 and trimer binding in EMSA had no activity in the β-cell line, whether in the presence or absence of cotransfected PBX1b and MEIS2b (Fig. 4A), confirming the central importance of PDX1 binding to the activity of this element.

Domains of PDX1 Required for Trimer Binding and Activity—PDX1 contains three important functional domains (Fig. 5A): an activation domain (AD) at the N terminus (amino acid residues 1-79), a pentapeptide motif (PP; residues 121-125) that directly contacts PBX, and the homeodomain (HD) (residues 145-206) (9, 40, 41). A cryptic C-terminal activation domain also functions in the transcriptional activation of the somatostatin gene by PDX1 (40). We constructed a series of PDX1 deletion mutants to test the function of each domain in the context of the trimer (Fig. 5A). The mutant proteins were synthesized by in vitro translation (IVT), and their ability to bind DNA was assayed by EMSA. Full-length PDX1 binds to the B2 element as a monomer, a heterodimer with PBX1b, and a heterotrimer with PBX1b and
MEIS2b Complex

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MEIS2b (Fig. 5B, lane 2). PDX1 mutants missing the regions C-terminal to the HD or N-terminal to the PP motif had similar DNA binding activities as the wild type PDX1 (lanes 3, 4, and 6). When the PP motif was also deleted, however, the protein was unable to form heterodimeric and heterotrimeric complexes on DNA (lane 5). Therefore, only the DNA-binding domain and the PBX-interaction motif are required for PDX1 to participate fully in forming the trimeric complex on DNA. These are the same domains required for dimer formation with PBX1 (Fig. 5B and Ref. 9).

To investigate which PDX1 domains are essential for trimer function, we tested the activity of the PDX1 deletion series by cotransfection with PBX1b and MEIS2b into a non-pancreatic cell line (HeLa). HeLa has no endogenous PDX1, although it contains both PDX1b (42) and MEIS2 (data not shown). Multimers of the B2 element were inactive in HeLa in the absence of cotransfected transcription factors (Fig. 4B). Addition of PDX1 activated the B2 element at least 50-fold, whereas addition of either PBX1b or MEIS2b alone or together had a barely detectable activating effect. Addition of either PBX1b or MEIS2b in cotransfections with PDX1 augmented the PDX1 activity 2- to 4-fold, whereas cotransfection of all three transcription factors increased activity 9-fold. The synergistic activity of the three proteins is consistent with the cooperative binding of PBX1b and MEIS2b with PDX1. As seen for the RIN-38 β cell line, the activity of the B2 element in the presence of the various combinations of transcription factors paralleled the activity of the natural B element (data not shown). The increased PDX1 activity by cotransfection of MEIS2b, which was not observed with transfected RIN-38 cells, is likely due to the presence of endogenous PBX1b, which is absent in RIN-38 cells.

Reporter gene activation by the trimer, as well as by PDX1 alone, required the N-terminal activation domain of PDX1 (Fig. 5C). Both the PDX1116–284 and PDX126–284 mutants missing the activation domain were inactive, either in the presence or absence of cotransfected PBX1b and MEIS2, even though the PDX1116–284 could form DNA-binding trimers (Fig. 5B). These results imply that neither PBX1b nor MEIS2b contribute an activation domain to the trimer.

Domains Important for the Function of PBX1b—Three polypeptide domains are uniquely conserved among the PBC class of homeodomain proteins (mammalian PBX proteins, Drosophila Extradenticle, and C. elegans Ceh-20 (6)). The PBC-A domain near the N terminus of the proteins (Fig. 6A) mediates the interaction of PBX with MEIS and the closely related PREP1 protein (7, 43, 44). The function of the PBC-B domain, located between the PBC-A and the HD, has not been delineated. In addition, the C terminus of the HD is extended by a fourth α-helix, which forms part of the hydrophobic pocket that binds the pentapeptide motif of HOX-like proteins (45, 46). We tested a series of PBX1b deletion mutants to determine which of the PBX1b-conserved domains are critical for trimer formation, DNA binding, and transcriptional activation (Fig. 6).

In vitro synthesized, full-length PBX1b does not bind the B2 element, but does bind as a heterodimer with PDX1 and as a trimeric complex with PDX1 and MEIS2b (Fig. 6B, lane 2). Illustrating the critical role that helix 4 plays in complex formation, deletion of sequences C-terminal to it had no effect (PBX1b1–318, lane 3), whereas further deletion to remove helix 4 abolished both dimer and trimer formation (PBX1b1–295, lane 4). Deletion of both PBC domains abolished the formation of the trimer on DNA without affecting the interaction between PBX1b and PDX1 (PBX1b233–347, lane 5). However, the PBC-A domain alone is not sufficient to bring MEIS into the trimeric complex, because deletion of just the PBC-B domain also abolished the trimer formation (PBX1bΔB, lane 6). Thus, formation of the trimer on DNA requires helix 4 of the PBX1b HD to recruit PDX1 and the PBC domains to recruit MEIS2b.

PBX and MEIS can form stable dimers, even in the absence of DNA (42, 44), and the PBX-MEIS heterodimer binds a consensus sequence of adjacent PBX and MEIS half-sites (7, 44). To confirm that the failure of PBX1b233–347 and PBX1bΔB to form trimeric complexes was due to their inability to interact with MEIS2b, we examined the formation of PBX1b-MEIS2b dimers by coimmunoprecipitation and their ability to bind a...
composite PBX-MEIS site (Fig. 6C). In the absence of DNA, deletion of both PBC domains (PBX1b233–347) eliminated dimerization with MEIS2b, whereas deletion of PBC-B alone (PBX1b233–347) reduced, but did not eliminate, dimer formation. To analyze the binding of PBX-MEIS dimers on DNA, we used the CRS1 element from the bovine CYP17 gene, which contains adjacent PBX and MEIS half-sites (30). PBX1b233–347 and MEIS2b formed a very small amount of bound complex, but PBX1b233–347 did not, paralleling the results in solution. The dimer of PBX1b1–295 and MEIS2b, although fairly stable in solution, did not bind DNA efficiently (lane 4). These results agree with previous findings that the region immediately C-terminal to the HD (i.e. helix 4) is important for stabilizing PBX binding to DNA and for interacting with HOX-like proteins (44, 45). Deletion of the entire region N-terminal to the HD (PBX1b233–347) or just the PBC-B domain (PBX1b1–295) permits binding to both the B2 and CRS1 elements as a monomer (Fig. 6B, lanes 5 and 6; Fig. 6C, lanes 5 and 6). Relief of inhibition of DNA binding by deletion of the PBX N-terminal region has also been observed by Neuteboom and Murre (47).

We analyzed the ability of each PBX1b mutant to participate in the transcriptionally active trimeric complex with PDX1 and MEIS2b in transfected cells (Fig. 6D). The PBX1b1–318 mutant is only slightly less active than the full-length PBX1b, indicating that no crucial domain is present C-terminal to the HD helix 4. Surprisingly, the PBX1b1–295 protein, although lacking helix 4 and unable to form a trimer with PDX1 and MEIS2b in EMSA, is active in transfections. The two PBC domain mutants, PBX1b233–347 and PBX1b1–295, had no detectable activity in transfections, indicating that a principal role for PBX1b in trimer activity is the recruitment of MEIS2b.

**Trimer Formation and Activity Requires the M1 and M2 Domains but Not the HD of MEIS2b**—The HD and two N-terminal MEIS domains (M1 and M2) are conserved among mammalian MEIS proteins, Drosophila Homothorax, C. elegans Ceh-25, and a closely related protein pKNOX/PREP1 (5, 7). The functional significance of these regions in DNA binding, protein interactions, and transcriptional activation was evaluated with a series of deletion mutants (Fig. 7).

MEIS2b does not bind the B2 element as a monomer or as a heterodimer with either PDX1 or PBX1b (data not shown). Deletion of regions C-terminal to the HD and N-terminal to the M1 domain had no impact on formation of a trimer (MEIS2b1–338 and MEIS2b237–394, Fig. 7B, lanes 3 and 4). Deletion of the M1 and M2 domains in MEIS2b192–294 eliminated the formation of a trimeric complex (lane 5). The MEIS2b HD has an inhibitory effect on trimer formation: removing the HD in MEIS2bΔHD, and MEIS2b1–275 enhanced the formation of the trimers (lanes 6 and 7). These results showed that the MEIS2b HD is dispensable for the formation of a heterotrimer on DNA. This is in agreement with the absence of a MEIS consensus site in the B element and with the absence of any sequence requirements beyond the PBX-PDX1 binding site for trimer binding and transcriptional activity (10).

Consistent with its inability to form a trimer, MEIS2b192–294 failed to dimerize with PBX1b both in solution and on CRS1 (Fig. 7C, lane 5). The MEIS2b HD was not required for the PBX-MEIS dimers to form in solution, but was essential for these dimers to bind DNA (MEIS2bΔHD and MEIS2b1–275, lanes 6 and 7). PREP1 can also dimerize with PBX in the absence of its HD, but, as for MEIS, the HD is required for DNA binding (43). Therefore, the N-terminal region containing the M1 and M2 domains is sufficient for trimer formation. The M2 domain is known to interact with PBX (7). Our deletion analysis did not unveil any direct interaction between MEIS2b and PDX1.

The region containing M1 and M2 was also sufficient for a fully active trimer in cotransfection experiments (Fig. 7D). All of the deletion mutants that contained the MEIS domains, including the minimal construct MEIS2b1–275, had approximately the same activity as the full-length MEIS2b. The MEIS2b192–294, which lacks both MEIS domains, had no activity.

**The PDX1-PBX1b-MEIS2b Trimer Cooperates with a bHLH Complex to Activate Transcription**—The synergistic interaction between the A and B elements of the ELA1 enhancer (26) suggests that the DNA-binding factor complexes that mediate their activities cooperate within the context of the ELA1 enhancer. PTF1, which binds the A element, comprises a bHLH heteromultimer that minimally contains the acinar cell-specific p48 protein (29) and a ubiquitous E-box binding protein (REB in rats, HEB in humans, ALF1 in mice).3,4 A mini-enhancer construct bearing three head-to-tail copies of tandem A and B elements (Fig. 8A) was used to test possible cooperativity between the PDX1-PBX1b-MEIS2b trimer and the p48-HEB dimer.

The PDX1-PBX1b-MEIS2b trimer activated the mini-enhancer nearly 4-fold in HeLa cells (Fig. 8B). Even though p48-HEB alone had no detectable effect on the mini-enhancer, it increased the activation by the trimer 2-fold. Addition of either PBX1b or MEIS2b to PDX1 in the presence of p48-HEB had little if any stimulatory effect, whereas adding PBX1b and MEIS2b together increased the activity of PDX1 with p48-HEB

3 P. Wellauer, personal communication.
4 S. Rose and R. J. MacDonald, unpublished.
Because the PDX116–206 does not accumulate in the nucleus of trans-dogenous to the reticulocyte lysate.
PDX1 has approximately the same mobility as the major complex expressed relative to the activity of wild type PDX1 (PDX1–284) alone.

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The trimeric complex of PDX1–PBX1b–MEIS2b binds a bipartite PBX-PDX1 site and is an order of magnitude more transcriptionally active in HeLa cells than PDX1 alone. The transcriptional activity of the complex is dependent on the activation domain of PDX1, as well as domains that mediate the interaction between PDX1 and PBX1b and between PBX1b and MEIS2b. Cooperative transcriptional activation with the pancreas-specific bHLH complex PTF1 requires the same structural domains and mimics the activity of the trimer within the context of the ELA1 enhancer in pancreatic acinar cells.

Organization of the DNA-bound Trimer—Based on the behavior of the deletion mutants of the three proteins, we propose a model for the organization of the PDX1–PBX1b–MEIS2b complex bound to DNA (Fig. 9). The trimer binds DNA through the HDs of PDX1 and PBX1b. The HD of MEIS2b forms no sequence-specific DNA interactions and is dispensable for DNA binding; indeed, DNA binding of the trimer is enhanced by the absence of the MEIS HD. PBX1b is the centerpiece of the complex; PBX1b interacts with PDX1 and MEIS2b through separate polypeptide domains. PBX1b and PDX1 bind adjacent DNA half-sites and interact directly through helix 4 of the PBX1b homeodomain and the pentapeptide motif just N-terminal to the PDX1 homeodomain. PBX1b recruits MEIS2b into the complex through interactions involving the N-terminal halves of the two proteins. Our analyses did not reveal any direct interaction between PDX1 and MEIS2b.

Our results are consistent with previous biochemical and structural analyses of HOX-PBX and PBX-MEIS heterodimers. These analyses showed that when PBX and HOX proteins are bound at adjacent sites, the HOX partner reaches across the DNA duplex and inserts the tryptophan of the pentapeptide motif into a hydrophobic pocket formed by the PBX homeodomain and helix 4 (45, 46). The PBC-A domain (7, 44) and the M2 domain of MEIS (7) are of primary importance in PBX-MEIS complex formation; however, some interaction is maintained even in the absence of the PBC-A domain (7).

Indeed, we have found that deletion of the PBC-B domain also disrupts PBX-MEIS interactions and eliminates formation of the DNA bound trimer (Fig. 6).

Recently, trimeric complexes containing PBX-like, PBX-like, and MEIS-like subunits have been reported with different modes of binding to DNA. Similar to the PDX1–PBX1b–MEIS2b complex, the PBX-MEIS complexes maintain DNA binding activity in the absence of the PBX homeodomain and helix 4. In addition, our studies indicate that the pentapeptide motifs of PBX1b and MEIS2b interact directly with DNA through helix 4, as reported for HOX-PBX and HOX-MEIS heterodimers (45, 46).

5 M. J. Peyton and R. J. MacDonald, unpublished data.
complex, a HOXB1-PBX1-PREP1 complex also binds DNA through the HOX and PBX HDs, and DNA binding does not require the homeodomain of PREP1 (43). Other trimeric HOX-PBX-like complexes require sequence-specific DNA binding of all three HDs (48–50), whereas at least one trimeric complex requires only an HOX binding site (51). Thus, in different contexts the trimeric complexes of HOX and HOX-like proteins with PBX-like and MEIS-like cofactors can require specific binding sites for only the HOX, for HOX and PBX, or for all three proteins.
Fig. 8. The PDX1-PBX1b-MEIS2b trimer and the p48-HEB dimer of PTF1 cooperate in activating transcription from an ELA1 mini-enhancer in HeLa cells. A, schematic of the 3(BA).Elp.hGH mini-enhancer. B, the relative activity of the 3(BA).Elp.hGH reporter gene in HeLa cells was assayed with or without cotransfected PDX1, PBX1b, MEIS2b, and p48-HEB individually or in various combinations as indicated. The gray line indicates the activity of the 3(BA).Elp.hGH in the absence of added transcription factors. This level of background activity was the same as that of a promoter-only Elp.hGH construct (data not shown), which was unaffected by added transcription factors in any combination. All values are the mean of six or more transfections, except for the pairwise combinations of PDX1 and PBX1b, PDX1 and MEIS2b, PBX1b and MEIS2b, and p48 and HEB, which represent the mean of two transfections. Error bars represent standard errors of the mean. C, the relative activity of PDX1, PBX1b, and MEIS2b deletion proteins in the cooperative activation of the BA mini-enhancer by the homeodomain heterotrimer and PTF1. The presence of full-length PDX1, PBX1b, and MEIS2b is indicated by +; the presence of particular PDX1, PBX1b, and MEIS2b deletion mutants is indicated by their identifying numbers from Figs. 5, 6, and 7. Values are the mean, with standard error, of four or more transfections.

**Transcriptional Activity of the Trimer**—The polypeptide domains required for the increased transcriptional activity of the PDX1-PBX1b-MEIS2b trimer, in comparison to PDX1 alone, include those required for trimer binding to DNA (the PDX1 and PBX1b HDs) and for protein-protein interactions necessary for formation of a stable heterotrimer (the PDX1 pentapeptide, the PBX helix 4 and PBC domains, and MEIS M1 and M2 domains). The N-terminal activation domain of PDX1 is essential for transcriptional activity of the trimer, because a trimeric complex missing only this activation domain (PDX116–284) has no transcriptional activity (Fig. 5C).

The absence of transactivation potential in the trimer lacking the PDX1 activation domain indicates that PBX1b and MEIS2b do not have activation domains or that their activation domains do not function without the PDX1 activation domain. Co-expression of PBX1 and MEIS1 fails to activate a reporter gene driven by a multimer of PBX-MEIS binding sites (7). Other experiments also have failed to detect an activation domain in PBX1b (52, 53). The presence of PBX1b and MEIS2b does not appreciably increase the amount of PDX1 binding in gel shift assays, so it is unlikely that the effect of PBX1b and MEIS2b is to increase binding site occupancy by PDX1. Therefore, PBX1b and MEIS2b do not appear to contribute either activation domains to the trimeric complex or stabilization of PDX1 binding to DNA. Consequently, the basis of the increased transcriptional activity of the trimer, in comparison to PDX1 alone, is unclear.

**Cooperative Transcriptional Activation with the p48-HEB Complex**—The ELA1 enhancer comprises the A and B elements, which bind the bHLH complex PTF1 and the PDX1-PBX1b-MEIS2b trimer, respectively, as well as a non-cell-specific element that binds unknown factors (10, 24, 26, 27). The pancreatic activity of the enhancer is dependent on synergy between the A and B elements in transgenic animals and transfected pancreatic acinar cell lines (25, 26). Although PDX1 bears the only activation domain within the PDX1-PBX1b-MEIS2b trimer, enhancer mutations that eliminate the PBX1 half-site and trimer binding without eliminating PDX1 binding are transcriptionally inactive (10). Thus, the requirement for PBX and MEIS, as well as PDX1, for the cooperative activation of an ELA1 mini-enhancer in HeLa (Fig. 6) is consistent with previous results with the complete ELA1 enhancer.

The ELA1 mini-enhancer, comprising three copies of adjacent PBX-PDX1 and PTF1 binding sites, directs high level expression in the pancreas and gut of transgenic mice (26). The pancreatic expression of this mini-enhancer is more than an order of magnitude greater than that seen with a multimer of the PBX-PDX1 binding site or a multimer of the PTF1 binding site. The activity of the mini-enhancer in HeLa cells is dependent on the activation domain of PDX1, within the context of the
DNA-bound PDX1-PBX1b-MEIS2b complex (Fig. 8). The cooperative activation of the mini-enhancer in HeLa cells upon co-transfection with PDX1-PBX1b-MEIS2b and p48-HIB likely reflects the natural interaction of these two complexes in the acinar pancreas to activate the transcription of the transgenic mini-enhancer and the endogenous ELA1 gene as well.

Cooperation between homeoproteins and bHLH complexes have been shown to occur in two other instances. In the insulin gene enhancer, PDX1 contributes to synergistic activation in a manner different than its action in the ELA1 enhancer. PDX1 binds to the A3/A region of the rat insulin I enhancer in the absence of PBX and MEIS proteins and activates transcription by cooperating with the bHLH protein E47, which binds to the adjacent E2 site (54). In this case, PDX1 contributes protein-protein interaction domains to recruit multiple proteins, including E47, BETα2/NeuroD, and high mobility group protein I(Y), to an activation complex on the E2A3/4 mini-enhancer and the endogenous ELA1 gene as well.

ELA1

MLELA1

Requirement for an Active PDX1

MEIS2b Complex

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