The Role of PTF1-P48 in Pancreatic Acinar Gene Expression*

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The 100-base pair ELA1 transcriptional enhancer drives high level transcription to pancreatic acinar cells of transgenic mice and in transfected pancreatic acinar cells in culture. The A element within the enhancer is the sole positively acting element for acinar specificity. We show that the acinar cell-specific bHLH protein PTF1-P48 and the common bHLH cofactor HEB are part of the PTF1 complex that binds the A element and mediates its activity. Acinar-like activity of the enhancer can be reconstituted in HeLa cells by the introduction of P48, HEB, and the PDX1-containing trimeric homeodomain complex that binds the second pancreatic element of the enhancer. The 5′ region of the mouse Ptf1-p48 gene from −12.5 to +0.2 kilobase pairs contains the regulatory information to direct expression in transgenic mice to the pancreas and other organs of the gut that express the endogenous Ptf1-p48 gene. The 5′-flanking sequence contains two activating regions, one of which is specific for acinar cells, and a repressing domain active in non-pancreatic cells. Comparison of the 5′-gene flanking regions of the mouse, rat, and human genes identified conserved sequence blocks containing binding sites for known gut transcription factors within the acinar cell-specific control region.

The formation and function of the exocrine and endocrine tissues of the pancreas rely on two key transcription factors, PTF1-P48 and PDX1. PTF1-P48 is a class B bHLH1 protein that appears in differentiating epithelial cells of the embryonic pancreas and becomes restricted to the acinar cells of the mature pancreas (1). P48 is the acinar cell-specific component of the PTF1 complex that binds many, if not all, of the promoters of the genes that encode the pancreatic digestive enzymes (2). The PTF1 complex contains two additional bHLH proteins (3): P64, which binds DNA (4) and is an isofrom of HEB,2 and P75, which is required for importing the complex into the nucleus but does not contact DNA directly (5). P48 is required for the formation of pancreatic acinar and ductal cells; neonatal mice with both Ptf1-p48 alleles inactivated lack all exocrine tissue, although islet-like endocrine cell clusters still form (3).

PDX1 is a HOX-like homeoprotein present selectively in the pancreas and rostral duodenum (6–9). PDX1 binds and activates the promoter of several pancreatic β-cell-specific genes, including insulin (7), islet amyloid polypeptide (10, 11), and glucagon transporter type 2 (12). PDX1 is required in early pancreatic development for the formation of both endocrine and exocrine tissues (13) and later for the maintenance of islets (14, 15). In the pancreas of adult animals, PDX1 is present in mature β-cells (7, 16) and at a lower level in mature acinar cells (6, 17).

As a member of the Antennapedia class of homeodomain proteins, PDX1 contains the characteristic pentapeptide motif just amino-terminal to the homeodomain and forms heterodimers with PBX members of the TALE class of homeodomain proteins (18, 19). The interaction between PDX1 and PBX appears to be required for the normal expansion of the endocrine and exocrine compartments during pancreogenesis (20). In pancreatic acinar cells, PDX1 binds and participates in the activation of the elastase I gene (ELA1) enhancer as part of a trimeric complex with PBX1b and MEIS2, another TALE-class homeoprotein (19, 21).

The ELA1 enhancer is a simple model for the control of pancreatic acinar cell-specific transcription. The minimal enhancer resides 100–200 bp upstream of the 5′ end of ELA1 and comprises just three functional elements (A, B, and C). The acinar cell activity of the enhancer resides in the A element, which is the binding site for PTF1. A homomultimeric repeat of the A element directs transgene transcription to pancreatic acinar cells of mice (22). Within the context of the enhancer, however, the single A element requires the cooperation of either the B or the C element (23). In acinar cells the B element binds the trimeric complex of PDX1-PBX1b-MEIS2 (19, 21). The action of the B element within the context of the enhancer requires the homeoprotein complex, because mutations that eliminate binding of the complete trimer without affecting PDX1 binding block the activity of the B element.

In this report we show that the actions of PTF1-P48 and PDX1 converge at the transcriptional enhancer of the acinar cell-specific ELA1 gene. The cooperative interaction of these two pancreatic factors can be recapitulated in a non-pancreatic cell line. Introduction of P48 and HEB into HeLa cells allows the formation of a bHLH complex that cooperates with the PDX1-PBX1b-MEIS2b complex to activate transcription through an ELA1 minienhancer. To begin understanding the acinar specificity of Ptf1-p48, we also demonstrate that the 5′-flanking region of the mouse gene contains much of the regulatory information for acinar cell-specific transcription.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY043285 and AF298816.

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1 The abbreviations used are: bHLH, basic helix-loop-helix; kb, kilobase(s); bp, base pair(s); EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; hGH, human growth hormone.

2 P. Wellauer, personal communication.

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EXPERIMENTAL PROCEDURES

RNA and Protein Assays—RNA was isolated from cell lines and mouse tissues using Trizol (Life Technologies, Inc.) following the manufacturer’s instructions, except that RNA from pancreas tissue was isolated by the procedure of Chirgwin et al. (24). Conservative dissection protocols were devised to prevent pancreatic contamination of other dissected organs. RNase protection assays were performed with an Ambion RPAIII kit according to the manufacturer’s instructions. Western blot analyses were performed as described previously (19). To help ensure the loading of equivalent amounts of protein, the protein concentrations of cell and tissue lysates were quantified by the Bradford procedure (Bio-Rad) and the relative amounts and quality were verified by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from cultured cells by a modification of Dignam et al. (25) as described (26). Nuclear extracts from murine organs were prepared as described by Rose et al. (22). EMSA was performed as described by Sawada and Littman (27) with 40 fm of a [32P]-labeled, 21-bp, double-stranded oligonucleotide (sense strand, 5'-GTCACCTGCTTTTTCCTGTC-3') spanning the A element of the ELA1 enhancer.

For antibody supershift EMSA, 1 or 2 ml of antisera or immunoglobulin solution, optimized for each antibody, was preincubated with the nuclear extract and the mixture then added to the binding reactions. The rabbit antisera against PTF1-P48 was raised for us by AnaSpec (San Jose, CA) against a synthetic amino acid peptide (C-KSFDNIE-NEPFPFEFS) corresponding to the carboxy-terminal 16 amino acids of mouse and rat P48. For immunoprecipitation the peptide was coupled to keyhole limpet hemocyanin through a cysteine included as the amino terminus of the peptide. Anti-HEB serum was the gracious gift of Dr. S. Sawada (27), anti-E2.2 was obtained from PharMingen (San Diego, CA), and anti-E12/47 was from Santa Cruz Biotechnology.

In vitro translated proteins were synthesized with the TNT kit from Promega, Inc. (Madison, WI). The p48 cDNA was constructed by gene splicing by overlap extension (28) of the two exons from the genomic DNA incorporating the Xenopus ß-globin 5'-untranslated leader (29). The p48 cDNA was inserted into pcDNA3.1 (Invitrogen Corp., Carlsbad, CA) containing the CMV enhancer/promoter. Dr. R. Baer provided the HEB cDNA (27). The translation in vitro of PDX1, PBX1b, and MEIS2b has been described (21).

Genomic Clones Encoding the Rat and Mouse Ptf1-p48 Genes—Clone GS8 12626 from a P1 phage library of rat Sprague-Dawley genomic DNA (30) bearing the rat p48 gene and clone GS8 12899 from a P1 phage library of mouse ES 129 genomic DNA bearing the mouse gene were identified by polymerase chain reaction screening and obtained from Genome Systems, Inc. (IncyteGenomics), St. Louis, MO. For cross-species comparisons we sequenced the mouse Ptf1-p48 gene region from −7524 to +6006 (GenBank accession no. AF286116) and the rat Ptf1-p48 gene from −5198 to +545 (GenBank accession no. AY043255). We then extended the sequence from Koelder et al. (31; GenBank accession no. X98446) upstream of −594.

Identification of the Human PTF1-p48 Gene—A search of the human genome data bases with the nucleotide sequence of the mouse p48 mRNA identified a single locus (GA x2HTBPJKG32 from Celera (Ref. 32) and NT 024073.2 from the International Human Genome Sequencing Consortium (Ref. 33)) containing the human p48 orthologue. The human gene maps to 10p12 at −22 Mb. An open reading frame encoding 328 amino acids has 92 and 89% sequence identity with the mouse and rat protein sequences, respectively (the mouse and rat proteins are 97% identical), and is divided into two exons at the same site as the mouse and rat genes. The Celera and NCBI sequences for the 10-kb proximal 5'-gene flanking region differ by only 21 bp; none of the differences occurred in the blocks conserved in the distal regulatory region among the rat, mouse, and human genes.

Nucleotide Sequence Analyses—Pairwise comparisons between the 5-kb proximal 5′ regions of the mouse, rat and human genes using the Bayesian Phylogenetic Footprint program (34) (www.wadsworth.org) identified three domains with high concentrations of conserved sequence blocks. Potential binding sites for known transcription factors were searched in the conserved domains using MatInspector Pro (35), accessible at genomatix.gsf.de.

Cultured Cells and Transfection—A variety of acinar and insulinoma pancreatic tumor cell lines were used to distinguish transcriptional regulation between pancreatic endocrine and exocrine cells. 266-6 (ATCC CRL-2151) is a differentiated acinar cell line derived from a pancreatic adenocarcinoma induced in a transgenic mouse line expressing an elastase-ß-antigen fusion transgene (36). C5-2E is a highly differentiated acinar line derived from a clonal adenocarcinoma induced by a trypsin-ß-antigen transgene. AR4-2J cells (ATCC CRL-1492) were derived from a rat acinar asasserine-induced tumor (37). All three lines express hydrolytic digestive enzymes characteristic of differentiated pancreatic acinar cells. RIN1046-38 (RIN3b) (58), βTC3 (39), and INS1(40) cell lines express insulin and retain properties of differentiated pancreatic β-cells.

266-6, C5-2E, and NIH3T3 cells were transfected by electroporation and treatment with sodium butyrate (41). Fragments of the 5′ flanking region were linked to a lacZ reporter gene by standard recombinant techniques. The common fusion site at +211 of the p48 gene is a TATA-containing TFI site at 13 nucleotides upstream of the p48 initiator codon. ß-Galactosidase activity was assayed with a Tropix Galacto-Light Plus kit.

HeLa cells were transfected with Fugene (Roche) according to the manufacturer’s protocol. The P48 expression plasmid is described above in the section detailing the EMSA protocols. The expression plasmids for PDX1, PBX1b, MEIS2b, and HEB have been described (21). All expression plasmids were based on pCDNA-3.1 or -1.1 (Invitrogen Corp., Carlsbad, CA) and driven by the CMV enhancer/promoter.

Transgenic Mice—The p48-hGH fusion transgene contained a 12.7-kb fragment of the p48 gene spanning from a NotI site at −12.5 kilobase pairs to the SfiI site at +211 bp fused to a 2.7-kb hGH reporter gene at +3. The 210 nucleotides of p48 transcribed region included in the transgenic contained only a single exon, and the hGH reporter gene contained all introns and the five exons plus 0.5 kilobase pair of 5′ flanking DNA. A fragment containing the fusion transgene devoid of plasmid vector sequences was isolated and injected into fertilized mouse eggs to create transgenic mice as described previously (42).

Immunocytochemistry—Pancreatic tissue was fixed in Carnoy’s fixative, embedded in paraffin and sectioned (5 µm) onto polylysine-coated slides for immunolocalization of hGH. Rabbit anti-hGH antibody (Dako) was diluted 1:500. Biotinylated anti-rabbit IgG, streptavidin-peroxidase, and AEC chromogen were from a Zymed Laboratories Inc. Histostain Plus kit and used according to the manufacturer’s instructions.

RESULTS

The A Element Binding Activity Contains the Acinar Cell-specific P48 and Common bHLH Proteins—The 21-bp, acinar cell-specific A element of the ELA1 enhancer comprises an E-box characteristic of bHLH-binding proteins and a TC-box (Fig. 1A). Nuclear extracts from either rat pancreas or pancreatic acinar cell lines form a major acinar cell-specific complex on the A element (a double labeled PTF1, Fig. 1B) in electrophoretic mobility shift assays. Mutations in the TC-box or the E-box that disrupt this binding (Fig. 1B) concomitantly abolish the transcriptional activity of the A element in transfected acinar cell lines (22, 43). Therefore, both the E- and TC-boxes are required for binding the A-element complex and for the activity of the A-element in vivo.

Wellsauer and colleagues (2) originally described the acinar cell-specific binding activity termed PTF1, which binds the ELA1 A element and related elements containing E- and TC-boxes in the promoter regions of amylase and other pancreatic acinar cell-specific genes. PTF1 is a multisubunit bHLH complex containing a 48-kDa, pancreatic, acinar cell-specific bHLH protein called P48 (1). To verify that the A-element gel shift complex is PTF1, we prepared an antisera against a 16-residue peptide derived from the carboxy-terminal sequence identical in rat and mouse P48s. The P48 antisera supershifted the PTF1 doublet (Fig. 1C). To identify the bHLH partner(s) of P48 in the rat PTF1 complex, we examined the effect of antisera against the common (type A) bHLH proteins on formation of the complex. Antiserum against IEB/REB1 or PAN2/E124 supershifted a fraction of the total rat PTF1 com-
plex, whereas anti-E2.2 had no effect. In other experiments with a different preparation of pancreatic nuclear extract, virtually all of the PTF1 was shifted by the anti-HEB antisera (data not shown); therefore, REB/HEB is the major type A bHLH protein in the rat PTF1 complex, with a small fraction of the complex containing PAN2/E12.

In vitro translated P48 together with either HEB/REB or PAN2/E12 formed a complex that bound the A element (Fig. 1D). The electrophoretic mobilities of the P48-HEB and P48-PAN2/E12 complexes, however, were slightly greater than that of the PTF1 complex from pancreatic nuclear extracts (Fig. 1D, middle panel). Binding reactions containing all three in vitro translated bHLH proteins formed the same two bands (one containing the HEB dimer and the other P48-HEB plus P48-PAN2) but no higher order complex (Fig. 1D, right panel). Therefore, under these conditions P48 and either HEB or E12 do not reconstitute a complex with the same DNA binding specificity or mobility as PTF1. Consequently, it appears that the natural PTF1 may contain an additional protein (or modified form of HEB or E12) that not only mediates binding to the TC-box but also imposes the need for a TC-box for PTF1 binding to DNA.

The Distribution of P48 Corresponds to the Sites of Action of the A Element—The A element is active in pancreatic acinar cells in situ and acinar cell lines in culture, but it is inactive in pancreatic endocrine cells and non-pancreatic cells (22, 23). p48 mRNA is present in all three independently derived pancreatic acinar cell lines examined and absent from both pancreatic β-cell lines (Fig. 2A). Moreover, the P48 protein has a similar distribution; it is present in acinar lines and absent from β-cell lines, a pancreatic ductal line (AR4-IP), and non-pancreatic cell lines such as Rat2 fibroblasts (Fig. 2B).

The endogenous ELA1 genes as well as transgenes driven by multimers of the elastase A element are expressed in the stomach, parts of the intestine as well as the pancreas (22). To determine whether the activity of the A element might be mediated by a P48-bHLH complex in each of these organs, we examined the distribution of p48 mRNA and protein. p48 mRNA was detected at moderate levels in total RNA preparations of mouse pancreas, which expresses high levels of elastase and the other acinar digestive enzymes, and at much lower levels in stomach and duodenum (Fig. 2C), which express ~100-fold lower levels of the acinar enzymes (22, 44). p48 mRNA was absent from all other organs tested. The presence of P48 protein in pancreas and stomach was confirmed by Western analysis of nuclear extracts of these tissues (Fig. 2D). These results are consistent with the role of P48 in the high level expression of elastase I, amylase, and other secretory enzymes in the pancreas and lower expression of these enzymes in the stomach and intestine (22, 44, 45).
Reconstitution of Acinar Cell-specific Activity in Non-pancreatic Cells—The A and B elements act cooperatively in the ELA1 enhancer to direct high levels of transcription in pancreatic acinar cells (23). The trimeric homeodomain complex of PDX1, PBX1b, and MEIS2b mediates the activity of the B element in acinar cells (19, 21). To test whether the P48/H18528 HEB complex can cooperate with the PDX1 homeodomain complex, we have examined the ability of exogenously expressed P48 and PDX1 to activate a transfected reporter gene controlled by a tandem combination of the A and B elements in HeLa cells.

The synthetic BA enhancer is inactive when transfected into HeLa cells (Fig. 3), as expected because HeLa has neither P48 nor PDX1. Co-expression of P48 and its HEB partner had no discernible effect on the activity of the BA construct (Fig. 3). PDX1, PBX1b, and MEIS2b together increased the expression of the reporter gene 4-fold, consistent with the ability of the trimer to activate a homomultimeric repeat of the B element (21). Co-expression of both the P48-HEB and the PDX1-PBX1b-MEIS2 complexes increased expression of the BA construct 7-fold. The intermediate level of activity with the PDX1 trimer plus P48 but without exogenous HEB is likely the result of the presence of endogenous HEB in HeLa cells (46). The cooperative activation required the presence of the bHLH binding site of the A element (data not shown). These results demonstrate cooperation between the P48-bHLH and the PDX1-homeodomain complexes on a control region that contains binding sites for both.

**Fig. 2.** p48 mRNA and protein are present selectively in pancreatic acinar cells and cell lines. **A**, p48 mRNA is present in mouse (266-6 and C5-2E) and rat (AR4–2J) acinar cell lines (designated a), but absent from mouse (βTC-3) and rat (Ins1) β-cell lines (designated b). **Bottom**, schematic of the RNase protection assay that detects the 5′ end of the p48 mRNA. The protected fragment is 253 nucleotides for the homologous mouse mRNA and a collection of shorter fragments for the heterologous rat mRNA (from AR4–2J cells) due to sequence divergence. These results show that the mouse Ptf1-p48 gene has a single predominant transcriptional start site 224 bp upstream of the initiator AUG codon. **B**, Western detection of P48 protein in extracts from the pancreatic acinar cell lines (a), but not from the pancreatic ductal (d), β-cell (b), or non-pancreatic (n) cell lines. The position of the 43-kDa protein size standard is indicated. **C**, p48 mRNA is largely restricted to the gastrointestinal tract in mice. Shown is one of three experiments with similar results. The relative amounts of p48 mRNA (indicated at bottom) were quantified with ImageQuant software from a Molecular Dynamics PhosphorImager scan. The signal for the other organs was not above the background level. **D**, Western detection of P48 in nuclear extracts from mouse pancreas and two separate stomach isolates confirm the expression of P48 protein.

**Fig. 3.** The PTF-1 bHLH complex cooperates in vivo with the pancreatic homeodomain protein complex containing PDX-1. Cooperative activation of an ELA1 mini-enhancer by P48-HEB and the PDX1 HD trimeric complex containing PBX1b and MEIS2b was examined in a co-transfection experiment with HeLa cells. The BA.EIp.hGH reporter (top) has three tandem repeats of the B and A elements fused together and linked upstream of the minimal promoter (−92 to +8) of the ELA1 gene driving the human growth hormone (hGH) reporter gene (+3 to +2700) (23). Activation of the BA minienhancer is compared with that of the minimal promoter (−92). **PDX1 trimer**, cotransfection with CMV-driven expression vectors for mouse PDX1, PBX1b, and MEIS2b. **P48 or HEB**, cotransfection with CMV-driven expression vectors for mouse P48 or human HEB, respectively. Total amount of CMV expression plasmid was constant in all transfections, adjusted as necessary by the addition of empty CMV vector. Transfections were normalized according to the expression levels of a cotransfected CMV-lacZ expression plasmid. The error bars represent the standard errors of the mean of four or more determinations.

Reconstitution of Acinar Cell-specific Activity in Non-pancreatic Cells—The A and B elements act cooperatively in the ELA1 enhancer to direct high levels of transcription in pancreatic acinar cells (23). The trimeric homeodomain complex of PDX1, PBX1b, and MEIS2b mediates the activity of the B element in acinar cells (19, 21). To test whether the P48-HEB complex can cooperate with the PDX1 homeodomain complex, we have examined the ability of exogenously expressed P48 and PDX1 to activate a transfected reporter gene controlled by a tandem combination of the A and B elements in HeLa cells.

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p48 Gene Control Sequences Direct Expression to the Mouse Gut—Because the restricted expression of P48 protein appears to be responsible, in turn, for the cell-type and stage-restricted transcription of ELA1 and other acinar cell-specific genes, we analyzed the transcriptional regulatory regions required for pancreatic acinar cell transcription of the Ptf1-p48 gene in mice. A 12.7-kb fragment spanning from /H11002 to /H11001 of the mouse Ptf1-p48 gene (Fig. 4 A) activated transcription of a hGH reporter transgene in the pancreas of mice (Fig. 4 B). This Ptf1-p48 fragment contains the transcriptional start and 211 nucleotides of the 5′ untranslated region of Ptf1-p48. The 5′ end of the pancreatic transgenic transcript was mapped with the RNA probe used to identify the 5′ end of the mouse p48 mRNA (see Fig. 2 A). The 253-nucleotide protected fragment indicative of the endogenous mRNA is detected for the pancreatic RNA samples from all three transgenic mice tested as well as the mouse 266-6 acinar cell line. A 211-nucleotide protected fragment is indicative of the presence of the p48-hGH fusion transcript at levels characteristic of the three founder animals (compare with panel B).

FIG. 4. A fusion transgene of the 12.5-kb upstream flanking region of the p48 gene with the hGH reporter gene is expressed in mouse pancreas. A, the organization of the P1 genomic clone containing the mouse p48 gene and flanking sequences. The gene fragment from the NotI site at −12.5 kb to the SfiI site at +211 of p48 was fused to the hGH reporter gene. The two exons of p48 are shown as rectangles containing untranslated (open) and coding (filled) regions. The restriction endonuclease sites are abbreviated as follows: N, NotI; S, SfiI; X, XhoI; E, EcoRI; P, PstI. B, expression of the transgene was monitored by an RNase protection assay with pancreatic RNA isolated from 12 independent founder mice identified above the lanes. The RNA probe (225 nucleotides) was derived from the 3′-untranslated region of hGH and leaves a protected fragment of 155 nucleotides. The estimated number of transgene copies and the level of transgenic hGH mRNA are indicated above and below the scan, respectively. The lane marked 10k/cell contains the signal for pancreatic RNA from a transgenic mouse expressing 10,000 hGH mRNA molecules/cell. The levels of transgenic hGH mRNA were estimated by quantification of the hybridization signals using ImageQuant software from a Molecular Dynamics PhosphorImager scan. C, the p48-hGH transgene used the single, correct transcriptional start site PTF1-p48 gene. The 5′ end of the pancreatic transgenic transcript was mapped with the RNA probe used to identify the 5′ end of the mouse p48 mRNA (see Fig. 2 A). The 253-nucleotide protected fragment indicative of the endogenous mRNA is detected for the pancreatic RNA samples from all three transgenic mice tested as well as the mouse 266-6 acinar cell line. A 211-nucleotide protected fragment is indicative of the presence of the p48-hGH fusion transcript at levels characteristic of the three founder animals (compare with panel B).
independently derived mice were analyzed for transgenic specific expression pattern of the endogenous transgene copies (see Fig. 3).

Mouse 926-3 was an F1 male derived from the 926-1 founder with 10 mRNA. Mouse 939-4 was an female founder with 16 transgene copies.

In islets (i) and in surrounding two venules and arteriole. Pancreatic tissue sections were stained for hGH. Staining of a very few cells showing variegated immunostaining (red-brown) of the hGH reporter protein in acinar cells at high and low levels, staining of a very few cells in islets (i), and the absence of staining in cells of the duct (d) and surrounding two venules and arteriole. Pancreatic tissue sections were derived from mouse 926-3 (Fig. 4). Panel B, higher magnification of the islet in panel A showing hGH staining of two cells (arrow). Panel C, higher magnification of an acinar region showing accumulation of hGH in apical regions of typical acini (one is outlined) characteristic of sorting to zymogen granules.

Further analysis of the 5′-gene flanking region by transfection into acinar and non-pancreatic cell lines revealed the presence of two activating regions, one of which is specific for the acinar cell lines, and a repressing domain active in NIH3T3 fibroblast cells. Stepwise 5′ truncations of the Ptf1-p48 flanking region decreased activity in the acinar cell lines, indicating the presence of a distal positive control region between −7 and −2.4 kb and a second, proximal one between −571 and −63 bp (Fig. 7A). Deletion of the distal region (construct f) diminished activity about 10-fold. Further truncations (constructs d and e) had no additional effect until the proximal region was removed, leaving only the minimal promoter (construct i), which decreased expression another 30-fold to near background levels. Whereas the proximal region (construct e) was active in both acinar and NIH3T3 cell lines, the enhancing activity of the distal region was detected only in the acinar lines and required the proximal region (compare constructs g and h). Most of the activity of the distal region was contained within a 1.7-kb fragment (construct i). Further truncation of the distal region eliminated activity (data not shown), suggesting that controlling elements are distributed throughout the −4.1 to −2.4 kb fragment.

Differential effects of stepwise 5′ deletions on activity in the acinar versus fibroblast cell lines were evident (Fig. 7B). Whereas acinar activity decreased with progressive 5′ deletions (indicating removal of activating sequences), activity in NIH3T3 fibroblast cells increased with the deletions (indicating removal of repressing sequences). Deletion of the region from −7 to −2.4 kb increased activity 2-fold in NIH3T3 cells, with a further 2-fold increase upon deletion of the region from −2.4 to −1.1 kb. The positive activity of the proximal region in NIH3T3 cells was virtually the same as for the acinar cell lines, conferring a 30-fold increase in expression over the minimal promoter (compare constructs e and f).

These results indicate that the acinar cell specificity of the Ptf1-p48 gene in vivo is derived from both positive and negative control elements. The inhibitory elements manifested in NIH3T3 cells are either quiescent in the acinar cell lines or are overridden by acinar cell-specific activating elements.

Properties of the Acinar Specific Distal Control Region—Comparison of the 5′-flanking sequences of the mouse, rat, and human PTF1-p48 genes identified three regions of high conservation: −3.1 to −2.3, −1.0 to −0.6, and 0.12 kb to the start site of transcription (Fig. 8, top). The −3.1 to −2.3 region, which is contained within the minimal active region (Fig. 7), contains 14 short sequence elements conserved among the genes from all three species. Several of these elements contain consensus binding sites for known gut endoderm transcription factors. Despite the concentration of conserved elements, the −3.1 to −2.3 kb subregion was not sufficient to enhance the activity of the −0.6 kb proximal region in the acinar cell line (data not shown).
E12 can bind cooperatively with PBX/Pdx1 to and mediate the acinar activity of the A element of the pancreas-specific bHLH factors. Although the acinar digestive enzymes had been thought to be pancreas-specific, their mRNAs and proteins can be detected in other regions of the distal foregut including the pyloric stomach and the intestine. Expression of acinar enzyme mRNAs in non-pancreatic sites in the gut, we have been unable to detect PTF1 binding activity in nuclear extracts from stomach and intestine, and Krupp et al. (1) could not detect p48 mRNA by Northern hybridization. However, low levels of p48 mRNA were detectable by an RNase protection assay and P48 protein by Western analysis (Fig. 2). Thus, the distribution of P48 does indeed coincide with the pattern of expression of the digestive enzymes and therefore is likely responsible for the activation of the digestive enzymes genes in extra-pancreatic sites in the gut.

Because P48 appears to be a pleiotropic regulator of acinar cell differentiation and function (3), it is important to understand the controls that specify the stage- and cell-specific activation of the Ptf1-p48 gene. We have shown that 12.5 kb of the adjacent 5' -flanking region of the mouse Ptf1-p48 gene largely recapitulates the expression pattern of the endogenous gene in mature animals. This region directs expression appropriately to pancreatic acinar cells and the stomach and duodenum, and not to most other organs examined. However, the transgene has an expanded expression domain in the gut (including the jejunum and colon), as well as some inappropriate expression sites (spleen, testes, and uterus). It is possible that some of the misexpression is due to position effects at the integration sites of the transgenes. However, because the inappropriate expression was present in two independent transgenic lines, it is more likely that additional regulatory sequences that normally suppress expression outside the foregut are missing from the 12.5-kb region of transgene. For example, the E1a1 gene itself requires an 80-bp silencer, normally present adjacent to but separate from the transcriptional enhancer, to suppress transcription in cells other than acinar cells (49).

Although the 5' -flanking DNA of the mouse and rat Ptf1-p48 genes have extensive regions of sequence conservation, Knöpfel and colleagues (47) did not detect acinar enriched expression using the rat flanking region in cell transfection assays. The acinar activity we observed in transfected mouse cell lines is verified by the activity in transgenic animals. The basis of the
Disparity may lie with the nature of the different acinar tumor cell lines used, the seemingly minor sequence differences between the rat and mouse genes, or details of the test constructs.

The mouse 5'-gene flanking sequence contains three broad transcriptional control regions: a gene-proximal activating region between +500 and +63 that is non-cell-specific, an acinar cell-specific region between +7 and +2.4 kb, and an overlapping negative-control area between +7 and −1.1 kb that suppresses transcription in non-pancreatic cells. The gene-proximal end of the acinar cell-specific region contains many

**Fig. 8.** Sequence elements within the distal control region of the mouse gene conserved among the mouse, rat, and human PTF1-p48 genes. **Upper panel,** the Bayesian Phylogenetic Footprint prediction program (34) identified three broad regions of sequence conservation between the mouse and human 5'-flanking regions. **Middle panel,** a graphical representation of the conserved region spanning −3.1 to −2.4, which is contained within the experimentally defined distal control region (Fig. 7), containing 14 short elements (7–62 nucleotides in length and designated A through N) that are conserved among the three PTF1-p48 orthologues. The gray regions for the human gene represent repetitive sequences that have been inserted or expanded relative to the mouse and rat genes. **Lower panel,** the sequence of each element conserved in order as well as sequence. Potential binding sites for gut endoderm transcription factors were predicted by MatInspector Pro (35) for several of the conserved elements; the numbers between the boxes indicate the number of nucleotides between the elements for each gene. Only those binding sites with a core similarity $\geq 0.75$ and a matrix similarity $>0.8$ for more than two genes are shown; the number associated with each factor site is the expectation value for the number of sites per 1000 base pairs of random DNA sequence (35). Consensus sequences derived from the matrices of the binding sites are shown in bold.

disparity may lie with the nature of the different acinar tumor cell lines used, the seemingly minor sequence differences between the rat and mouse genes, or details of the test constructs. The mouse 5'-gene flanking sequence contains three broad transcriptional control regions: a gene-proximal activating region between −500 and −63 that is non-cell-specific, an acinar cell-specific region between −7 and −2.4 kb, and an overlapping negative-control area between −7 and −1.1 kb that suppresses transcription in non-pancreatic cells. The gene-proximal end of the acinar cell-specific region contains many
sequence elements conserved among the mouse, rat, and human PTF1-p48 genes within a 700-bp region. Several of the conserved elements are potential binding sites for gut transcription factors, including ETS, HNF4, and GATA factors. No PTF1 binding sites are conserved. Further analyses will identify the binding sites and binding factors critical to acinar cell-specific transcription.

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