RBP-L AND RBP-J HAVE CRITICAL ROLES IN THE FUNCTION OF TWO FORMS OF THE PANCREAS TRANSCRIPTION FACTOR COMPLEX PTF1

APPROVED BY SUPERVISORY COMMITTEE

Raymond J. MacDonald, Ph.D.

Keith L. Parker, M.D., Ph.D.

Jane E. Johnson, Ph.D.

Deepak Srivastava, M.D.

Dedication

I would like to thank my advisor Dr. Ray MacDonald for his guidance and support. I am grateful that he allowed me to pursue a very intellectually interesting project. I am also grateful for the valuable scientific thinking and writing skills that he has taught me.

I thank my committee members Jane Johnson, Deepak Srivastava, and my chair, Keith Parker. All of them have always had helpful and interesting comments about my projects. I especially thank Jane for the collaboration.

I would like to thank the members of the MacDonald laboratory for all their help and support. Particularly, I would like to thank Galvin Swift, Toshihiko Masui, and Mike Hale for help with reagents, methods, and good scientific conversation. Past lab members also provided good help and friendship. Particularly, Ying Liu, Scott Rose, Mike Peyton, Ling Shi, Hideaki Kagami, Jaime Zoloty, and Andrew and Sonia Holland. I would like to thank Mike Henke and Stacey Glasgow for their invaluable help, ideas, collaboration.

I wish to express my gratitude to all my friends at UTSW. Among these friends, I would particularly like to thank Tara Davis, David Hawkins, Nancy Stallings, Tandi Collisson, Jon Moody, and many others for all their support. I also thank all the friends that I have made in Dallas.

Finally, I would like to thank my family for their unconditional love and support before and during my graduate school career. This dissertation is dedicated to my parents, Ken and Hazel Beres.

RBP-L AND RBP-J HAVE CRITICAL ROLES IN THE FUNCTION OF TWO FORMS OF THE PANCREAS TRANSCRIPTION FACTOR COMPLEX PTF1

by

THOMAS MATTHEW BERES

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2005

RBP-L AND RBP-J HAVE CRITICAL ROLES IN THE FUNCTION OF TWO FORMS OF THE PANCREAS TRANSCRIPTION FACTOR COMPLEX PTF1

Publication No. _____

Thomas Matthew Beres, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

Raymond J. MacDonald, Ph.D.

The decision of pancreatic precursor cells to differentiate into acinar or endocrine cells is regulated by a complex network of signaling and transcription factor pathways. P48 is a tissue-specific basic-helix-loop-helix (bHLH) transcription factor, which is essential for pancreas development and function. Mice lacking both *p48* alleles lack an exocrine pancreas and have a greatly reduced endocrine pancreas. The active form of P48 is the heterotrimeric complex, PTF1. This complex binds and regulates the transcription of genes encoding digestive enzymes within the exocrine pancreas. The PTF1 complex forms on the rat elastase 1 (*ELA1*) promoter by synergistically binding to a 21 base-pair site comprising an E-box (CANNTG) and a TC-box separated by one

helical turn. P48 binds the E-box as a heterodimer with class A bHLH proteins, while the third member of the complex contacts the TC-box, but cannot stably bind without the P48 heterodimer. PTF1 activates the genes encoding the digestive enzymes specifically in the acinar cells of the pancreas, but no developmentally relevent target genes for P48 have been identified. Human mutations that truncate P48 are associated with permanent neonatal diabetes mellitus (PNDM), a genetic disorder characterized by pancreatic and cerebellar agenesis. DNA binding and transcriptional activity of PTF1 is dependent on the interaction of P48 with either RBP-J, or its paralogue, RBP-L. The exclusive form of PTF1 in mature pancreatic acinar cells is a potent transcriptional activator containing RBP-L; however, RBP-J can form a similar, but low activity, complex. The P48-RBP interaction is primarily through two conserved peptides that resemble the RBP-J-interacting motif of the Notch intracellular domain (NotchIC). However, the NotchIC is excluded from PTF1 because it lacks affinity for RBP-L, and P48 occupies its docking site on RBP-J. PNDM associated mutations delete one or both critical peptides, indicating the requirement of a PTF1 complex for proper embryonic development. The inability of the NotchIC to integrate into PTF1 complexes demonstrates a Notch-independent role for mammalian Suppressor of Hairless (RBP-J) and its paralogue RBP-L.

Table of Contents

List of Figures

List of Tables

Prior Publications

1. Beres, T.M., Masui, T., Swift, G.H., Shi, L., Henke, R.M., and MacDonald, R.J. Mammalian Suppressor of Hairless (RBP-J) and its paralogue RBP-L participate in PTF1, an organ-specific and Notch-independent bHLH complex. Submitted.

2. Ten Hagen, K.G., Bedi, G.S., Tetaert, D., Kingsley, P.D., Hagen, F.K., Balys, M.M., Beres, T.M., Degand, P., Tabak, L.A. (2001). Cloning and characterization of a ninth member of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family, ppGaNTase-T9. J. Biol Chem. 276, 17395-404.

3. Ten Hagen, K.G., Tetaert, D., Hagen, F.K., Tichet, C., Beres, T.M., Gagnon, J., Balys, M.M., VanWuyckhuyse, B., Bedi, G.S., Degand, P., Tabak, L.A. (1999). Characterization of a UDP-GalNAc:polypeptide N-acetylgalactosaminlytransferase that displays glycopeptide N-acetylgalactosaminyltransferase activity. J. Biol Chem. 274, 27867-74.

4. Ten Hagen, K.G., Hagen, F.K., Balys, M.M., Beres, T.M., VanWuyckhuyse, B., Tabak, L.A. (1998). Cloning and expression of a novel, tissue specifically expressed member of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family. J. Biol Chem. 273, 27749-54.

5. Hagen, F.K., Ten Hagen, K.G., Beres, T.M., Balys, M.M., VanWuyckhuyse, B.C., Tabak, L.A. (1997). cDNA cloning and expression of a novel UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase. J. Biol Chem. 272, 13843-8.

6. Ten Hagen, K.G., Beres, T.M., Szpirer, J., Szpirer, C., Tabak, L.A. (1997). Chromosomal organization and expression analysis of two distinct genes encoding glutamine/glutamic acid-rich proteins. Biochem J. 324, 177-84.

Abbreviations

AMY:	amylase
bHLH:	basic Helix-loop-helix
BETA2:	Beta-cell E-box transactivator 2
BSA:	bovine serum albumin
CMV:	cytomegalovirus
CPA:	carboxypeptidase
CSL:	CBF-1, Suppressor of Hairless, LAG-1
CTRB:	chymotrypsin B
Dll1:	Delta-like gene 1
DMEM:	Dulbecco modified Eagle medium
EBNA:	Epstein Barr Virus Nuclear Antigen
ELA1:	elastase I
EMSA:	Electrophoretic mobility shift assay
FBS:	Fetal bovine serum
FGF:	Fibroblast growth factor
Hes1:	hairy/Enhancer-of-split-1
Hh:	Hedgehog
ISL:	Islet 1
IVT:	*in vitro* translation
MEIS:	myeloid ecotropic viral integration site
ngn3:	neurogenin 3

PBS: phosphate buffered saline

PBX: pre-B cell homeobox gene

PCR: polymerase chain reaction

PDX1: Pancreatic and Duodenal -homeobox 1

PTF1: Pancreas Transcriptiona Factor 1

RBP-J: Recombining binding protein

RBP-L: Recombining binding protein-like

RT: reverse transcription/Reverse Transcriptase

SHH: Sonic Hedgehog

Su(H) Suppressor of Hairless

TALE: Three amino acid loop extension

TRP: trypsin

UTR: untranslated region

Wg: Wingless

Chapter 1

General Introduction and Literature Review

The research in this dissertation is the characterization of PTF1, a trimeric protein complex containing three transcription factors. This complex consists of two basic helix-loop-helix (bHLH) proteins and a member of the CSL (CBF-1, Suppressor of Hairless, LAG-1) family of DNA binding transcription factors. PTF1 activates the tissue-specific expression of digestive enzyme genes in the acinar cells of the pancreas (and to a much lesser extent, the stomach and duodenum). One component of this complex, PTF1A/P48, is expressed in the pancreas, duodenum, stomach, and developing nervous system, and is critical for the embryonic development of the pancreas (Kawaguchi et al., 2002; Krapp et al., 1998). (P48/*p48* will be used to describe the protein/*gene* PTF1A/*Ptf1a* to avoid confusion when referring to PTF1, the complex and PTF1A, the subunit of PTF1.) This chapter will summarize background information related to the dissertation work. It will introduce the pancreas as model for the study of organogenesis, discuss the signaling pathways that induce pancreatic development from the endoderm, the resulting cascade of transcription factors that induce differentiation of the different cell types of the pancreas, and how transcription factors contribute to the function of terminally differentiated cells by activating target genes. Many of these transcription factors act in both the development and function of the pancreas, so it is essential to summarize the roles that transcription factors play in both of these processes.

The second part of the introduction will concentrate on the transcription factor P48, and its functional form as part of a three-protein complex. The two different classes of bHLH proteins will be discussed in reference to their basic structure, mechanism of

action, and function in gene activation. The basic structure and function of CSL and related proteins will be discussed in relation to gene activation within the context of signaling pathways and transcription.

The third part of the introduction will relate the first two sections to a discussion of digestive enzyme genes as a model for the study of tissue-specific gene expression, and how the results of these studies might be extrapolated to understand the function of PTF1 during development. Specifically, I will discuss studies of the DNA binding and transactivating properties of PTF1 as they relate to the activation and maintenance of acinar-specific gene expression. The rat elastase 1 gene (*ELA1*) will be the primary focus of these studies, as it is a well-established system in the study of acinar-specific gene expression.

Pancreas and Pancreogenesis

The pancreas is a multifunctional gland derived from a prepatterned region of the embryonic endoderm. It is composed of both endocrine and exocrine tissue. The endocrine tissue secretes blood sugar regulating hormones into the bloodstream to regulate glucose homeostasis. Exocrine tissue secretes digestive enzymes into the intestine. Defects in the endocrine pancreas, such as diabetes mellitus, represent a major threat to health. Diseases such as pancreatitis and pancreatic cancer represent nearly exclusively exocrine-derived defects in the pancreas. A great deal of research is dedicated to curing these afflictions. Research has focused on defining gene regulation necessary for the formation and function of terminally differentiated pancreatic cells, and outlining the signaling pathways and gene expression cascades that lead to the

differentiation of the different cell types from precursor cells. Understanding these programs may lead to *in vitro* based solutions for these major medical problems.

The first part of the literature review discusses the anatomy and function of the pancreas, formation of the pancreas during embryogenesis, and the signaling pathways and transcription factors involved in pancreas development and function. Many of the transcription factors are expressed during development as well as in terminally differentiated cells of the pancreas. Understanding how these genes function in the mature pancreas may also shed light on how they function during pancreatic development.

Anatomy and Function of the Pancreas

Endocrine pancreas

The endocrine pancreas makes up only about 1-2% of the mass of the mammalian adult pancreas. It consists of five cell types that secrete hormones and peptides that regulate protein and lipid metabolism, and most notably, blood glucose homeostasis. The five endocrine cell types are grouped in clusters known as the islets of Langerhans. These cell types are distinguished by the biosynthesis of specific hormones and differences in morphology. The β-cells, which comprise >75% of the cells of the islets, are located on the interior of the islets and secrete insulin to lower blood sugar. The α-cells secrete glucagon to raise blood sugar. The δ-cells secrete somatostatin, and the PP-cells secrete pancreatic polypeptide. These cells are all found in the periphery of the islets. Finally, the more recently identified ε-cells, also found in the periphery of the

islets, secrete the appetite-regulating peptide ghrelin (Kojima et al., 2001; Prado et al., 2004; Volante et al., 2002; Wierup et al., 2004). The islets secrete these hormones directly into the bloodstream via the extensive vasculature present in the pancreas.

Exocrine pancreas

The islets of Langerhans are embedded in the exocrine tissue of the pancreas. More than 85% of the pancreas is exocrine tissue, comprising acinar cells and ductal cells. The acinar cells produce and secrete about twenty different digestive enzymes. High-level production of digestive enzymes is essential for the digestive function of the pancreas, and the vast majority of mRNAs present in the pancreas encode these digestive enzymes (Harding et al., 1977; Harding and Rutter, 1978; Pictet et al., 1972). These enzymes digest different food types. Amylase digests carbohydrates. Pancreatic lipase, phospholipase, and cholesterol esterase digest fat. The pancreas also contains several proteolytic enzymes such as trypsin, chymotrypsin, carboxypeptidase, and elastase. The ductal cells secrete fluid to channel the digestive enzymes into the duodenum.

The acinar cells are organized in lobulated, branched structures known as acini. The basal side of acinar cells is surrounded by the basal lamina, which separates the epithelial cells from the adjacent mesenchyme. The apical sides of the acinar cells face the lumen. In contrast to endocrine cells, acinar cells are polarized in that substances only enter through the basal side and exit through the apical side. Digestive enzymes, initially secreted as inactive zymogens, are released from the acinar cells into the lumen.

They are then flushed through the ducts into the duodenum where the zymogens become active digestive enzymes.

Pancreas Development

The pancreas forms from a prepatterned part of the endodermal gut tube. The first signs of pancreatic development are the thickening of the epithelium of the dorsal and ventral pancreatic anlage, followed by the evagination of the dorsal and ventral pancreatic buds. This bud formation is asynchronous, as the dorsal pancreatic bud forms at embryonic day 9.5 (E9.5) in the mouse, whereas the ventral bud forms about a day later. These buds then branch out to form tree-like structures as they grow. Lobulations in the monolayer of epithelial cells likely form as they divide and proliferate within the confined prepatterned area. The monolayer of cells is maintained by the division of cells exclusively along the axis parallel to the apical (and basal) sides of the forming acinar cells. As cells branch and proliferate, they also cytodifferentiate as they are directed to become the specific cells of the exocrine and endocrine pancreas (Figure 1.1. modified from Pictet and Rutter 1972 and Kim and MacDonald 2002). The branching lobulated structures become the acini, while a limited number of cells divide along the axis perpendicular to the apical and basal sides of these acini. These cells later become endocrine cells. After the primary transition of prepatterned endoderm to the pancreatic buds, the next stage of development is dedicated to proliferation of precursor cells and

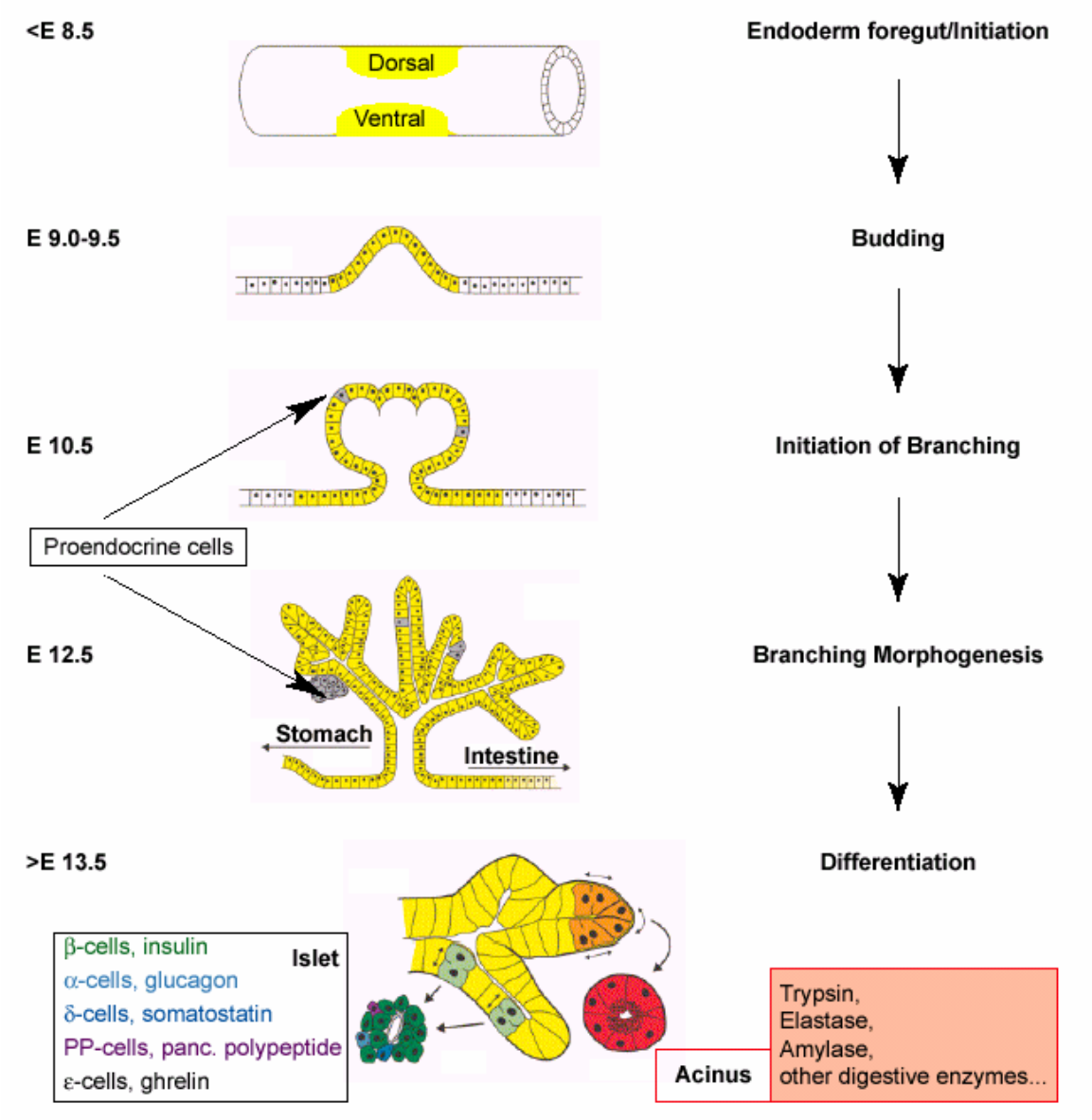


Figure 1.1 Overview of pancreas development.
The pancreas develops from a *Pdx1*-expressing region of the prepatterned foregut endoderm. Different stages of development are shown. *Pdx1*-expressing cells are shown in yellow. Preacinar cells are shown in orange, while a differentiated acinus is shown in red. Islet cell precursors are shown in grey and light green. Differentiated islets are indicated by the multi-color cluster of cells. Double-sided arrows indicate the axis of division for islet and acinar precursors. Important products of differentiated islet and acinar cells are indicated in the large boxes.

the formation of branched structures. Beginning at about E13.5, the pancreatic epithelial cells undergo a wave of differentiation characterized by the formation of endocrine and

exocrine cells, and a dramatic rise in the expression of digestive enzyme genes in acinar cells and genes encoding glucose regulating hormones in endocrine cell clusters. This wave is sometimes referred to as the secondary transition. The dorsal and ventral pancreatic glands merge at embryonic day 16-17, forming a single fused gland. Finally, the endocrine clusters form spheroidal structures of the islets of Langerhans close to birth.

The multiple steps of pancreatic development require a complex series of events involving the actions of signaling molecules and transcription factors. Studies of signaling pathways have focused on their effects on the pancreatic epithelium and the surrounding mesenchyme. The requirements for transcription factors in development have focused on the identification of specificity of transcription factor locations, as well as the effects of disrupting the expression of these factors. The effects of major signaling events and transcription factors on both the developing pancreas and terminally differentiated cell types of the mature pancreas are discussed in the following sections.

Signaling during pancreas development

The formation of the pancreas from the endoderm requires certain inductive signals from adjacent germ layers. Signals from the ectoderm and the mesoderm have profound effects on the patterning of the endoderm (Wells and Melton, 2000). Beginning at E7.5 the endoderm sheet around the outside of the embryo folds into the primitive gut tube. This endodermal gut tube contains anterior-posterior information that allows regions to receive later signals from the adjacent layers. These signals induce formation

of various organs such as the liver, lung, intestine, stomach, and the pancreas (Wells and Melton, 1999). Specifically, these adjacent germ layers are essential for the morphological changes of the pancreas from part of the prepatterned gut tube to proliferating branched structures, to terminally differentiated cells of the exocrine and endocrine pancreas (Golosow and Grobstein, 1962; Kim and MacDonald, 2002). Thus, signaling plays a major role in the development and function of the pancreas. One major role is the specification of the prepatterned endoderm as pancreatic tissue. Several signaling pathways are either directly or indirectly essential to permit the gut endoderm to form pancreatic tissue. Signaling through the Hedgehog (Hh), Tranforming Growth Factor (TGF-β), Wingless/WNT, and Fibroblast Growth Factor (FGF) pathways all play roles in the induction of initial budding and branching of the prepancreatic endoderm as well as induction of transcription factors essential for early development and later function of the pancreas, whereas FGF and Notch are essential for proliferation of precursor cells and cell fate determination.

Inductive signals

The dorsal and ventral pancreas form by a morphologically similar manner of budding and branching, but the programs of induction are different. The initiation of dorsal and ventral pancreatic budding is asynchronous and differs in requirements of signals and transcription factors. Hedgehog signaling specifies endodermal tissue of the gut as non-pancreatic during the early stages of embryonic development. Discussion of the Hh pathway and its role in pancreas development is important in understanding how

pancreatic development is localized to a specific section of the gut tube, while the effects of upstream signals on this pathway also warrant mention.

Originally identified in *Drosophila* as essential for embryonic development (Lee et al., 1992; Nusslein-Volhard and Wieschaus, 1980), *Hh* genes have been identified in a multitude of organisms. While *Drosophila* has only one *Hh* gene, mammals have three, Sonic (*Shh*), Indian (*Ihh*), and Desert (*Dhh*) (Chuang and Kornberg, 2000; Hebrok et al., 2000). These *Hh* genes encode diffusible secreted proteins that bind to target receptors on the surface of cell membranes. The receptor for Hh is one of two multipass transmembrane proteins in the Patched family. Normally, Patched (Ptc) binds to and represses the activity of a seven transmembrane protein, Smoothened (Smo), a protein with similarities to G protein-coupled receptors. Upon binding of the Hh ligand, Ptc releases Smo from its repression and allows for activation of target genes, possibly through the Gli family of proteins. The most widely studied Hh protein is SHH. Regulation of *Shh* expression and activity is modulated by other molecules and signaling pathways. Hedgehog-Interacting protein (Hip) attenuates SHH signaling by sequestering the active Shh peptides (Chuang and McMahon, 1999). The TGF-β family member Activin has been shown to repress transcription of *Shh* (Hebrok et al., 1998; Kim et al., 2000; Levin et al., 1997), while the WNT pathway has been shown to activate *Shh* transcription (Pinson et al., 2000; Tabata and Kornberg, 1994). Thus, induction or repression of SHH signaling would regulate downstream targets of the SHH pathway.

Repression of *Shh* expression allows for the expression of pancreatic markers in the gut endoderm during early development (Hebrok et al., 1998). During early pancreatic development, the notochord, a mesenchymal tissue, comes in contact with the

gut endoderm. Signals from the notochord repress *Shh* expression in chick prepancreatic endoderm and induce the expression of pancreatic markers including insulin, glucagon, and carboxypeptidase A (Kim et al., 1997). The notochord factors Activin β B and FGF2 can substitute for notochord to repress *Shh* expression and induce pancreatic markers in this portion of the endoderm. Expression of *Shh* or contact with SHH soaked beads represses pancreas development and expression of markers such as *Pdx1* and insulin. Conversely, contact with Activin-soaked beads induces expression of these pancreatic markers (Hebrok et al., 1998). Chemical inhibition of SHH signaling with cyclopamine also expands the expression region of pancreas development in embryonic chick (Kim and Melton, 1998). As pancreatic development continues, the dorsal pancreatic endoderm loses contact with the notochord due to the fusion of the dorsal aorta. The contact of the dorsal pancreatic endoderm with the dorsal aorta has the same effect of inhibiting *Shh* expression in the pancreatic epithelium (Kim and MacDonald, 2002). Thus, expression of *Shh* in the dorsal gut endoderm results in an intestinal phenotype, whereas continual repression by signals from the notochord and then the dorsal aorta suppresses expression of *Shh* and allows development of the dorsal pancreas.

Similar to the dorsal pancreas, *Shh* is not expressed in the ventral prepancreatic endoderm. However, the strategy for induction of the bud is different in that the area that becomes the ventral pancreas is biased toward pancreas development. This region does not contact the cardiac mesoderm, which produces local concentrations of FGF2 that induce *Shh*, but does receive signals from the blood vessel endothelia to promote pancreatic development (Lammert et al., 2001; Lammert et al., 2003). Ventral pancreatic endoderm cultured in the absence of SHH or FGF2 expresses pancreatic markers. Shh

signaling on either side of the developing ventral pancreatic endoderm inhibits formation of pancreatic buds in these locations. These signals, combined with BMP (TGF-β) signals from the septum transversum induce hepatic differentiation (Deutsch et al., 2001; Matsumoto et al., 2001; Rossi et al., 2001). Thus, both the ventral and dorsal pancreatic buds develop from areas of the endoderm that are free of SHH. However, the ventral gut endoderm is biased toward pancreas development, while only the areas that contact the FGF2 expressing cardiac mesoderm and the BMP expressing septum transversum become liver. Thus, *Shh* expression sets the boundaries of endoderm from which the pancreatic buds will develop (Summarized in Figure 1.2).

Signals during proliferation and cell fate determination of the pancreas

After the initiation of budding and branching of the pancreas from the gut endoderm, signaling continues to play an essential role in the proliferation of the epithelium, as well as the determination of the different cell fates in formation of the full functional pancreas. Recently, much study has been devoted to the roles of FGF and Notch Signaling in pancreas development. The results of these studies have indicated roles for Notch and FGF in the processes of proliferation and differentiation of pancreatic epithelial cells during embryonic development.

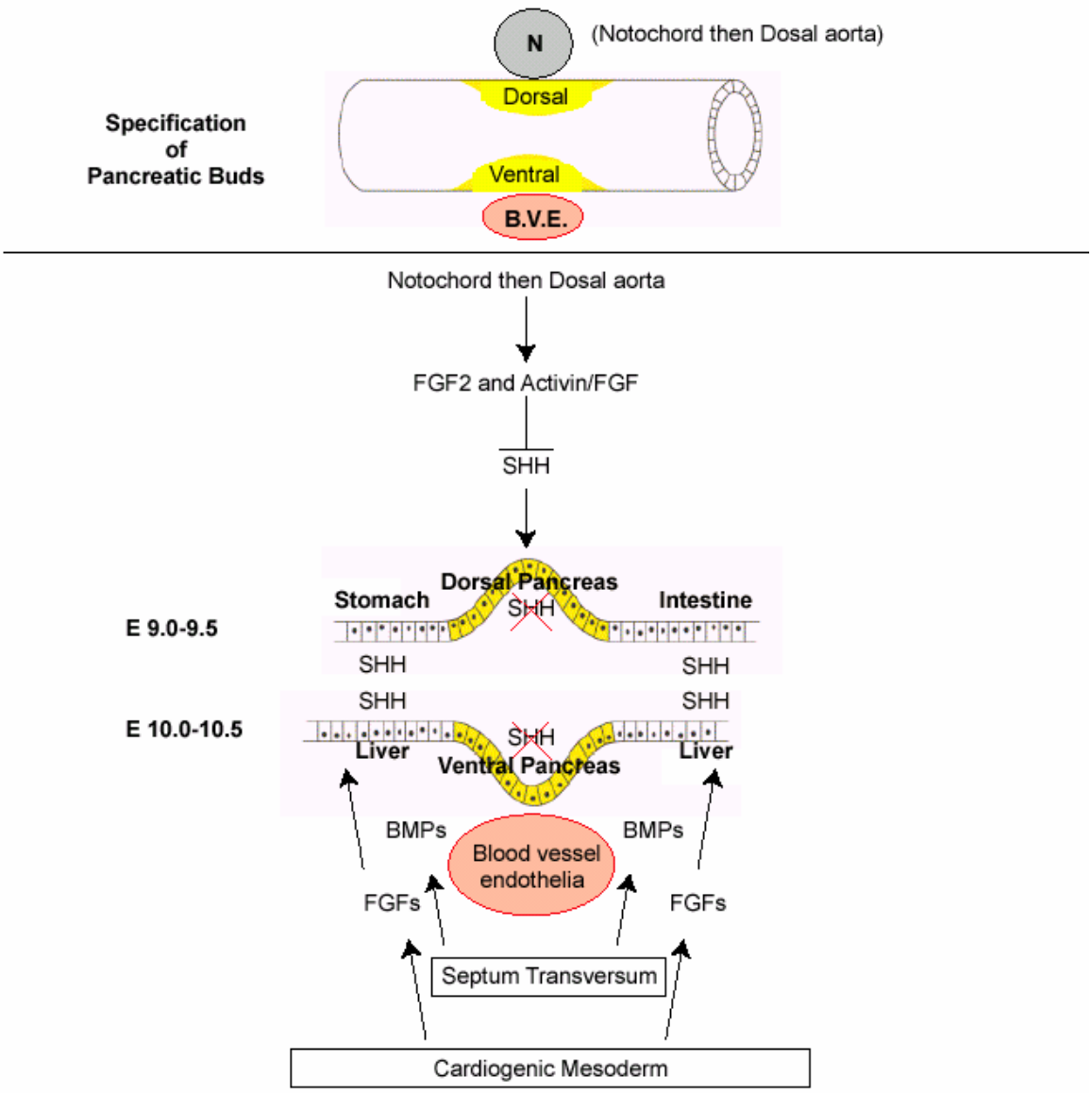


Figure 1.2 Inductive signaling mechanisms of the pancreatic buds.
The induction of dorsal and ventral pancreatic buds depends on the absence of SHH signaling in the prepancreatic foregut endoderm. Signals from the notochord, then the dorsal aorta block *Shh* expression in the dorsal foregut endoderm. Signals from blood vessel endothelia bias the ventral foregut endoderm toward a pancreatic fate.

Notch signaling dictates cell fate determination by lateral specification. Cells that express Notch ligands, such as Delta, signal to cells expressing the transmembrane receptor. Upon activation of this pathway, the Notch intracellular domain (NotchIC) is

cleaved, and interacts with a nuclear proteins, including Su(H) and Mastermind (MAM), to activate transcription of specific target genes (Artavanis-Tsakonas et al., 1999; Fryer et al., 2002). Hairy enhancer of split (Hes) genes are targets of the Notch pathway. The *Hes 1* gene product blocks myogenesis and neurogenesis by binding to the regulatory regions and directly repressing expression of genes necessary for the differentiation of these tissues (Ishibashi et al., 1994; Sasai et al., 1992). Indeed, *Hes1* deficient mice have a premature generation of neurons and an upregulation of the gene encoding the neurogenic factor MASH1 (Ishibashi et al., 1995). Thus, cells expressing a ligand of Notch (like Delta) differentiate, and differentiation is repressed in the adjacent cells that express the Notch receptor.

A similar, Notch-controlled process also occurs in the pancreas. Studies in which Notch pathway components are disrupted result in an increase in endocrine cells. Specifically, disruption of *Hes1*, *RBP-J*, and the Notch ligand Delta-like (*Dll*), or inhibition of Notch1 by overexpression of the inhibitory Notch3 intracellular domain result in an increase of cells expressing *Ngn3* (Apelqvist et al., 1999), a marker of endocrine precursor cells. This increase of endocrine precursors is at the expense of exocrine cells. Hence, the Notch pathway is implicated in the lateral specification of endocrine pancreas.

In addition to its lateral specification function in the pancreas, Notch may function in the proliferation of the expanding pancreatic epithelial cells by suppressing differentiation. This function of Notch is the result of FGF signaling. It was originally shown that mesenchymal FGF10 expression is necessary for the maintenance of the epithelial precursor population that expresses *Pdx1* (Bhushan et al., 2001). Ectopic

expression of *Fgf10*, under the control of the *Pdx1* promoter led to a hyperplastic pancreas with undifferentiated cells that expressed *Pdx1* and several markers associated with pancreatic epithelium prior to terminal differentiation. Specifically, Notch pathway components including Notch1 and Notch2, the ligands Jagged 1 and Jagged 2, and the target gene *Hes1*, a known repressor of differentiation, were all upregulated in the FGF10 positive cells (Norgaard et al., 2003). Transgenic expression of activated Notch1IC from the *Pdx1* promoter leads to the elimination of exocrine cells and a reduction of endocrine cells (Hald et al., 2003).

While the absence of SHH is required for early pancreatic development, the presence of Hh proteins is essential later in the development and function of the pancreas. The ligands and receptors of the Hh pathways are expressed later in the developing and the mature pancreas (Hebrok et al., 2000; Li et al., 1999). However, overexpression of *Shh* and *Ihh* during later development disrupts pancreatic development (Kawahira et al., 2005), so their expression and activity are likely regulated tightly. The exact nature of the requirement for Hedgehog proteins in later pancreatic development and function is still unclear.

The SHH, Notch, and some FGF pathways are the most thoroughly studied signaling pathways in pancreatic development to date (Summarized in Figure 1.3). Roles for other FGFs for branching morphogenesis have been shown in lung and salivary glands (Hoffman et al., 2002; Liu et al., 2003; Steinberg et al., 2005), but not studied in

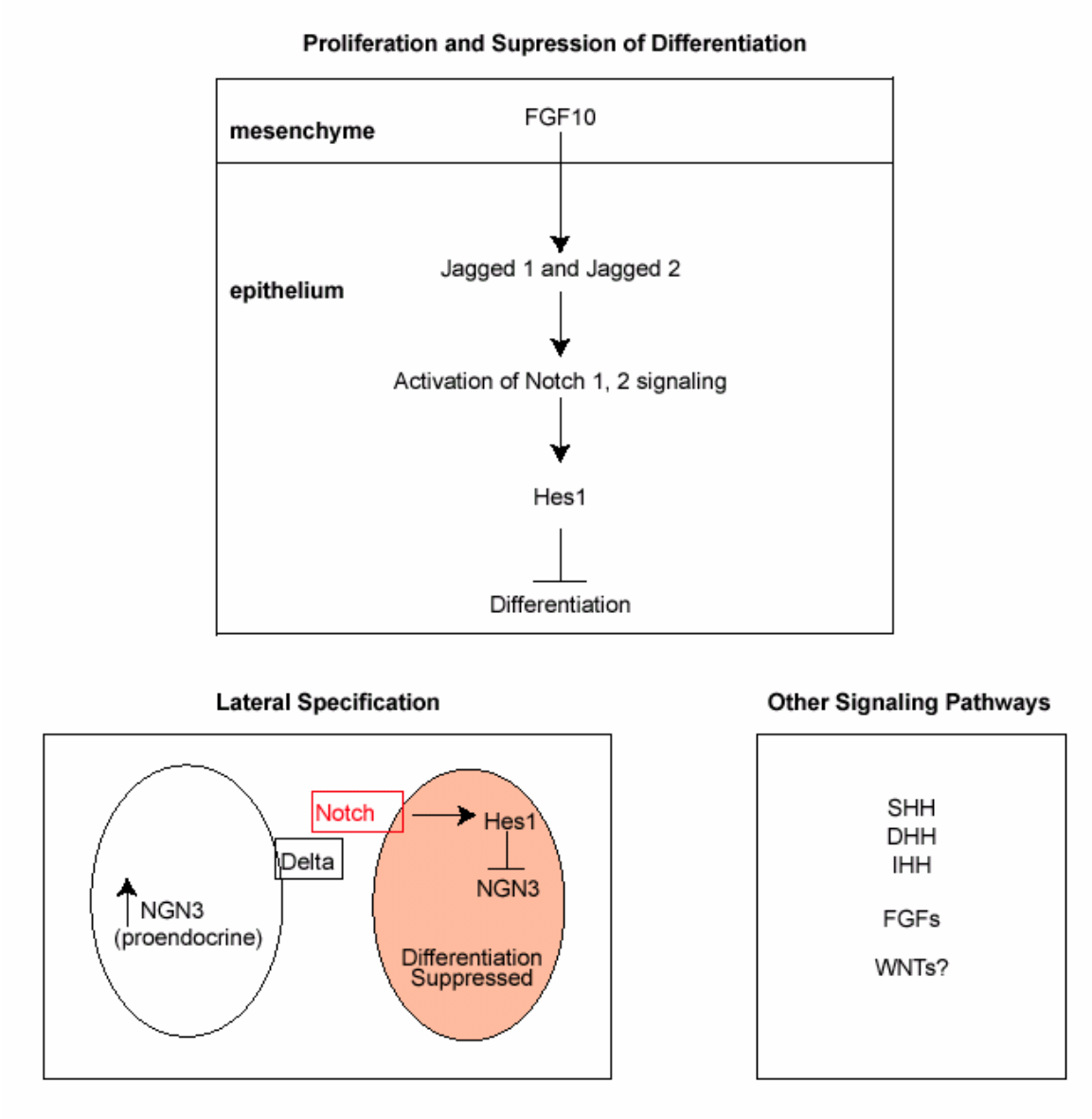


Figure 1.3 Models for signaling during the proliferation and differentiation of the pancreatic epithelium.
Top: FGF10 activates Notch signaling to allow for the proliferation of the pancreatic epithelium without differentiation. Bottom Left: During differentiation, pro-endocrine cells may use Notch signaling to repress the endocrine phenotype in adjacent cells. Bottom Right: A list of signaling molecules with undefined roles for the differentiation of pancreatic cell types.

pancreas. However, FGFs 1, 4, 7, and 10 expression patterns implicate a potential function in pancreas development (Hebrok et al., 2000). Further expression analysis and functional studies of FGF, WNT, and TGF-β family members and pathway components are ongoing and will likely lead to defined roles in pancreas development.

Transcription factor cascades during pancreatic development

The pancreatic developmental program requires the expression of transcription factors in addition to signaling molecules. Mesodermal signals induce the expression of many transcription factors. These proteins participate in the various steps of pancreatic development as well as the function of terminally differentiated endocrine and exocrine cells. Commitment of the pancreatic anlage requires the expression of several transcription factors. These factors work in a tightly regulated manner to induce the expression of genes encoding more factors. The tissue-specific expression of combinations of transcription factors is responsible for the specification of organ-specific developmental program as well as the function of the differentiated cells within the organ. Factors are often identified as gene activators in terminally differentiated cells of the pancreas. Targeted deletions of genes encoding proteins have often been used to investigate their importance, and have frequently revealed roles for these factors in pancreas development. Indeed, some of the transcription factors required for the initiation of pancreatic development also function in the terminally differentiated cells of the pancreas (Summarized in Figure 1.4).

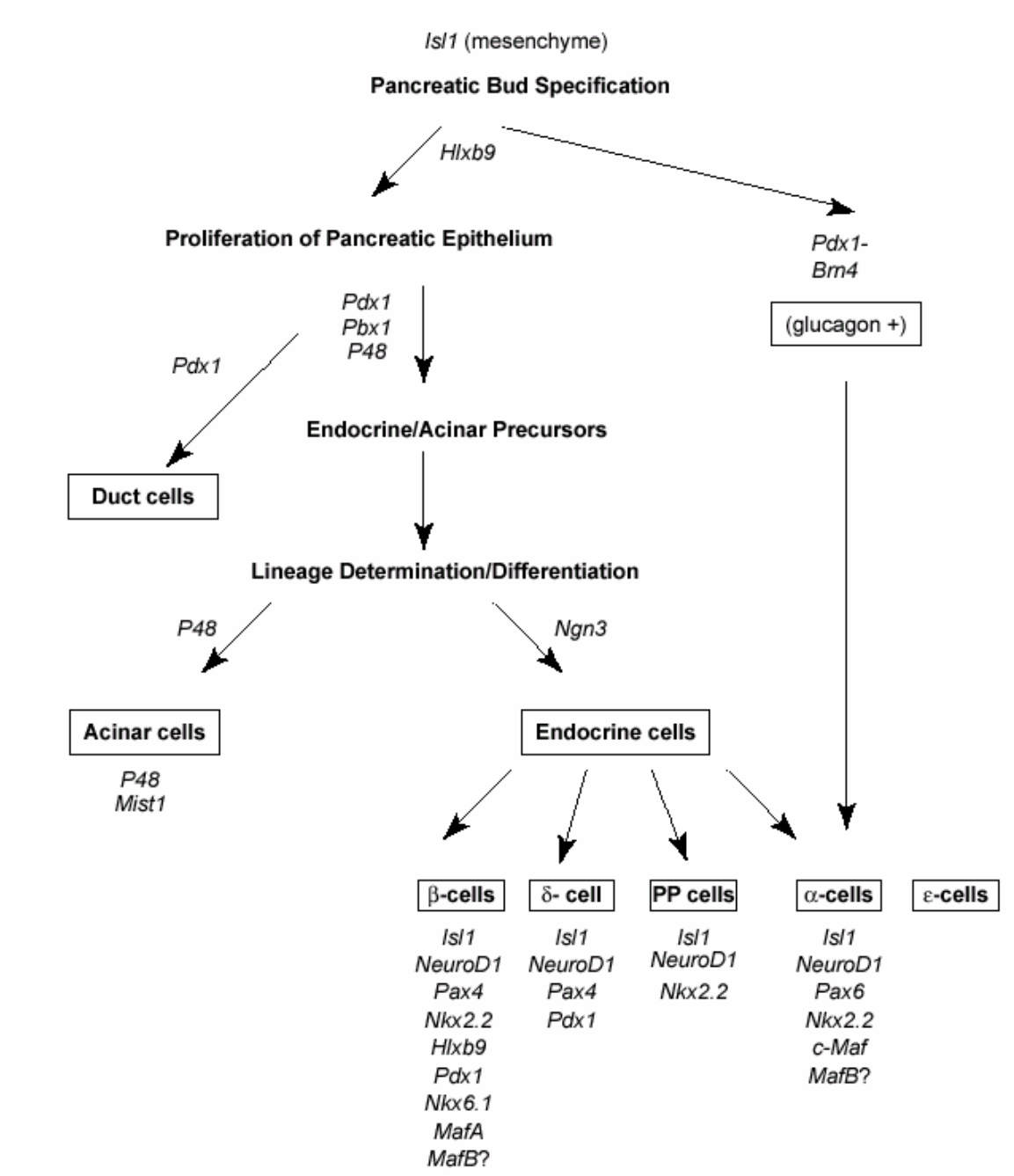


Figure 1.4 A representation of important transcription factors that are expressed at stages of pancreas development.

Commitment to pancreatic formation

The most intensively studied transcription factor required for pancreatic development is PDX1. PDX1 was first identified as a protein that binds and activates the insulin and somatostatin genes (Ohlsson et al., 1993; Petersen et al., 1994). PDX1 is a homeodomain factor similar to the HOX proteins that are essential for proper patterning along the anterior-posterior axis in developing organisms. These proteins are characterized by the similar sixty amino acid homeodomain that binds AT-rich DNA sequences (Scott et al., 1989). Like many other homeodomain proteins, PDX1 contains a nuclear-localization signal and a transcriptional activating domain (Peshavaria et al., 2000). The deletion of *Pdx1* in mice revealed a function in pancreatic development in addition to its function in terminally differentiated endocrine cells (Ahlgren et al., 1998; Jonsson et al., 1995; Jonsson et al., 1994).

Pdx1 expression begins at E 8.5 in the ventral region of the prepatterned gut endoderm. Lower levels of expression are seen in the prospective duodenum and stomach. As previously mentioned, the area of high *Pdx1* expression in the developing pancreas lacks *Shh* (Hebrok et al., 1998; Kim and MacDonald, 2002). Expression of *Pdx1* is widespread in the proliferating and branching pancreatic epithelium. As the secondary wave of differentiation proceeds, expression of *Pdx1* is down-regulated in the ductal and acinar exocrine cells, although these cells continue to express low levels of PDX1. In differentiated islets, high-level *Pdx1* expression is only seen in β-cells and a subset of δ-cells (Ohlsson et al., 1993). The pancreatic buds form within the region of

Pdx1 expression, indicating a likely developmental function. Targeted deletion of *Pdx1* resulted in mice that lack a pancreas. PDX1 is not required for the evagination of the pancreatic buds, but pancreatic development is arrested prior to the proliferation and extensive branching of pancreatic epithelial cells and differentiation of endocrine and exocrine cells (Jonsson et al., 1995; Jonsson et al., 1994; Offield et al., 1996). Additionally, a human homozygous for a naturally occurring mutation that eliminates PDX1 function was also born without a pancreas (Stoffers et al., 1997).

Functions of PDX1, as related to both organogenesis and activity of terminally differentiated cells, rely on its interaction with other factors. PDX1 can interact with other transcription factors to function in activation of downstream gene targets. One specific important example is the interaction of PDX1 with the three amino acid loop extension (TALE) homeodomain protein PBX1 (Burglin, 1997; Swift et al., 1998). This interaction plays a role in pancreatic development and in the function of terminally differentiated cells. PBX1 interacts with a variety of transcription factors to alter the DNA binding specificity in the activation of target genes. DNA binding PDX1-PBX1 complexes have been shown to regulate both endocrine gene targets like somatostatin and exocrine gene targets like *ELA1* (Goudet et al., 1999; Liu et al., 2001; Peers et al., 1995; Swift et al., 1998). In addition to its activity in terminally differentiatied pancreatic cells, PDX1-PBX1 complexes have been implicated in pancreatic development (Dutta et al., 2001; Kim et al., 2002). *Pbx1* expression during the proliferation of the pancreatic epithelium occurs in a more defined region than that of PDX1. Hence, PBX1 may refine PDX1 activity. Rescue of *Pdx1* knockout mice with a transgene encoding a PDX1 protein that lacks the PBX1 interacting domain (amino acid sequence FPWMK) have all

the pancreatic cells types, but have a hypoplastic phenotype that leads to death three weeks after birth due to pancreatic insufficiency (Dutta et al., 2001). Ablation of Pbx1 resulted in pancreatic hypoplasia and defects in exocrine and endocrine differentiation, as exhibited by the severe reduction in the expression of regulators of pancreatic differentiation such as *Ngn3* and *Isl1*, followed by death between embryonic day 15 and 16 (Kim et al., 2002). The gross morphological phenotype of the *Pbx1* knockout mice was more severe than that of the rescue of *Pdx1* deficient mice with the mutant *Pdx1* transgene, indicating that the interaction of PBX1 with multiple proteins may be involved in pancreatic development.

HLXB9 is essential for the formation of the dorsal pancreatic bud. *Hlxb9* is expressed in the notochord, all along the entire dorsal endoderm, and in the prepancreatic ventral endoderm. Targeted deletion of *Hlxb9* resulted in neonatal lethality, likely due to neural defects. In the pancreas of *Hlxb9*-/- mice, development of the dorsal pancreas was arrested prior to the evagination of the dorsal bud (Li et al., 1999). Additionally, markers of pancreatic development such as PDX1, ISL1, NKX2.2, and glucagon were not detected in the presumptive dorsal pancreatic anlage. Thus, *Hlxb9* deficient mice fail to specify the dorsal pancreatic program of development. In contrast to the dorsal pancreas, the ventral bud of the pancreas did evaginate and continue to develop, but the remnant pancreas in mutant mice had small islets with reduced numbers of insulin-producing β-cells (Harrison et al., 1999). *Pdx1* is expressed in the ventral bud of *Hlxb9* mutant mice, and thus does not have the epistatic relationship implicated in the dorsal pancreatic phenotype. Additionally, expansion of *Hlxb9* expression using the *Pdx1* promoter severely disrupted endocrine and exocrine differentiation (Li and Edlund, 2001). This

agrees with the implication that HLXB9 can function as a transcriptional repressor as shown in developing neurons (Thaler et al., 1999). Hence, HLXB9 functions in the induction of pancreatic dorsal bud evagination and the functioning of mature β-cells, but is not necessary for ventral bud evagination or the specification of the endocrine and exocrine cell fates.

ISL1 is a LIM homeodomain protein first isolated from β-cells. It is expressed in the early pancreatic mesenchyme and is instrumental in the formation of the dorsal aorta. As previously mentioned, signals from the dorsal aorta likely maintain a *Shh*-free domain necessary for the formation of the dorsal pancreas. *Isl1* is not, however, expressed in the ventral mesenchyme. Mice with deletion of *Isl1* die at E10 due to lack of dorsal aorta formation (Ahlgren et al., 1997). Since, the dorsal aorta maintains the signals to suppress *Shh* expression in the prepancreatic endoderm, the early function of ISL1 is likely important for the regulation of *Shh* and the initiation of the dorsal pancreatic bud.

Terminal differentiation of pancreatic islet cells

Much attention has been focused on defining the transcription factors necessary for the formation of the terminally differentiated cells that compose the islets. The association of the loss of β-cells, or β-cell function, with severe health problems has focused studies on the processes involved in the formation of β-cells. Although mature islets do not form until relatively close to birth, endocrine cell markers are expressed throughout pancreatic development starting at E9.5. Glucagon-expressing cells are detected at this time in the developing pancreatic bud, while insulin expression is

detected as early as E10.5. However, the somatostatin-producing δ-cells do not arise until E15.5 and PP cells until near birth (Sander and German, 1997). As previously mentioned, endocrine and exocrine cells differentiate from pancreatic epithelial precursors that express PDX1. Other transcription factors such as HLXB9, NKX2.2, ISL1, PAX4 and PAX6, NKX6.1, NGN3 and NEUROD1/BETA2 regulate the differentiation and maintenance of the phenotypes of the various islet cell types. The exception is the glucagon-expressing cells that do not require expression of PDX1.

The determination of the endocrine cell lineages requires the activity of the bHLH transcription factor NGN3. *Ngn3* expression begins at E9.5. *Ngn3* expression is transient, peaking at E13.5 and decreasing after E15.5. In neonatal and adult mice, NGN3 is not detected. NGN3 is present only in the endocrine precursor cells. When the cells exit the cell cycle and undergo differentiation, *Ngn3* expression is shut off. At the secondary transition, a central core of precursor cells expresses *Ngn3*, while the differentiating cells do not (Gu et al., 2003; Gu et al., 2002). *Ngn3* deficient mice fail to develop islet cells, and die postnatally due to diabetes (Gradwohl et al., 2000). Gain-of-function studies also reveal a pro-endocrine function for NGN3. When the region of *Ngn*3 expression was expanded with a *Ngn3* transgene under the control of the *Pdx1* promoter, this causesd an increase in glucagon-expressing endocrine cells at the expense of exocrine cells (Apelqvist et al., 1999). Additionally, adenoviral-mediated expression of *Ngn3* in human pancreatic duct cells activates the endocrine program, leading to the expression of endocrine markers like NEUROD1 (Heremans et al., 2002). NGN3 heterodimerizes with E47, binds to the *NeuroD1* promoter and activates the *NeuroD1* expression (Huang et al., 2000). This is further evidence for a pro-endocrine function.

Thus, NGN3 is essential for the initiation of the pancreatic endocrine program, but is not necessary for its maintenance.

The bHLH protein NEUROD1 is present in all islet cell types and activates the expression of islet-specific genes such as *insulin*. *NeuroD1* expression is first detected in a subset of cells in the pancreatic epithelium at day E9.5 (Naya et al., 1997). Despite its important function in the function of islets, NEUROD1 is not required for endocrine cell specification. *NeuroD1* deficient mice form all endocrine cell types, as products of these terminally differentiated cells, as insulin, glucagon, and somatostatin, are detected in mutant mice (Naya et al., 1997). However, levels of apoptosis are dramatically increased, and the islet cell population fails to expand between E14.5 and E17.5. These mice failed to develop mature islets and had a dramatically reduced number of β-cells, resulting in death from diabetes by five days after birth. Consistent with its role in endocrine function, *NeuroD1* is a direct target of the pro-endocrine transcription factor NGN3 (Huang et al., 2000). Additionally, NEUROD1 directly binds to and activates the insulin promoter as a heterodimer with an E2A protein (Naya et al., 1995). It is thought that after activation by NGN3, NEUROD1 maintains the endocrine program after *Ngn3* is shut off. Thus, studies have shown examples of how NEUROD1 affects islet maturation and maintains its phenotype.

The NK family member Nkx2.2 is required for proper endocrine development and function. Initially expressed in most pancreatic epithelial cells at E9.5, *Nkx2.2* expression is later restricted to the α–, β–, PP, and ε- cells of the islets (Sussel et al., 1998; Wierup et al., 2004). Despite its broad early expression that overlaps with that of *Pdx1*, *Nkx2.2* is not required for the expansion of the pancreatic precursor cells. Mice with a targeted

deletion of *Nkx2.2* do not show a defect in this step of development (Sussel et al., 1998). *Nkx2.2-/-* mice develop all the different islet cell types but they have fewer α- and PP cells. Most strikingly, the β-cells fail to mature, and there is a loss of insulin activation. Thus, NKX2.2 is important for the maturation, but not the specification of β-cells.

ISL1 is important for the formation of the islets. In addition to its early expression in the dorsal pancreatic mesenchyme, *Isl1* is expressed in the endocrine cells of the ventral and dorsal pancreas. Although the *Isl1* knockout mice do not form a dorsal pancreatic bud, they do have a ventral pancreas. However, these mice lack all endocrine cells (Ahlgren et al., 1997), confirming a cell-autonomous function for ISL1 in endocrine cell development. Additionally, *Ngn3-/-* mice lack *Isl1* expression (Gradwohl et al., 2000). Thus, ISL1 is likely not involved in the selection of endocrine cell lineages, as it is likely downstream of both *Ngn3* and *NeuroD1* expression in endocrine cells, but it plays a role for the survival of endocrine cells shortly after the selection of the endocrine cell fate.

Nkx6.1, like *Nkx2.2*, is broadly expressed in the developing pancreatic epithelium. Later, *Nkx6.1* expression is restricted to β-cells. Early *Nkx6.1* expression is within the broad region of *Pdx1* expression in the gut, but only in the cells that will become pancreas and not adjacent tissues like stomach or duodenum (Oster et al., 1998a; Oster et al., 1998b; Watada et al., 2000). Expression of *Nkx6.1* may also rely on the presence of high levels of PDX1 (Grapin-Botton et al., 2001). However, unlike *Pdx1-/-* mice, *Nkx6.1* knockout mice do not exhibit a defect in proliferation of the pancreatic epithelium (Sander et al., 2000), showing that, like *Nkx2.2*, it is not required for this step of development. Mice with a targeted deletion of *Nkx6.1* initiate the endocrine program,

but lack mature β-cells; however, they have a normal development of α-cells (Sander et al., 2000). *Nkx6.1* is downstream of *Nkx2.2* in β-cell development, as mice mutant for *Nkx2.2* lack *Nkx6.1* expression. Based on the ability of NKX6.1 to work as a repressor during neural development (Vallstedt et al., 2001), it may suppress α-cell development, while the α-cell-specific NK family member NKX6.2 (Oster et al., 1998b) may repress the β-cell phenotype.

Pax genes are essential for the development of multiple organs, including, eye, kidney, brain, and pancreas. They contain a paired domain and a homeodomain. PAX 4 and PAX6 are expressed in the pancreatic endoderm, and subsequently only in the endocrine cell lineages of the pancreas (Dahl et al., 1997; Mansouri et al., 1996). Deletion of both these genes eliminates the formation of mature endocrine cell lineages, however PAX4 and PAX6 regulate the formation of different subsets of the mature islet cell population (Sosa-Pineda, 2004; Sosa-Pineda et al., 1997; St-Onge et al., 1997).

Studies of both of these genes revealed the expression patterns during pancreas development and the specific effects of the loss of either or both of these genes. The strategy for deleting these genes was the insertion of the lacZ gene into each Pax gene. Expression analysis from mice heterozygous for these mutations revealed that *Pax4* is expressed in a subset of pancreatic epithelial cells beginning at E9.5 and becomes restricted to the endocrine cells. Mice, homozygous for the *Pax4* deletion initiate the endocrine cell program but lack β- and δ-cells, and have larger than normal numbers of α-cells (Sosa-Pineda et al., 1997). Similarly, *Pax6* expression is present in a subset of the prepancreatic endodermal cells at E9.0, but also becomes restricted to the endocrine cells types. *Pax6-/-* mice lack α-cells but have all other islet cell types (St-Onge et al., 1997).

By way of comparison, mice with the naturally occurring *Sey*^{Neu} mutation of *Pax6* have all cell types of the islets, but lower numbers of each and abnormal morphology of islets (Sander et al., 1997). Because PAX6 can bind and activate genes in α-, δ-, and β- cells such as glucagon, somatostatin, and insulin respectively, this mutation likely interferes with expression of genes in each islet cell type. Indeed, this mutation still contains the homeodomain required for DNA binding, but deletes a region C-terminal to the domain. Thus it may bind targets but fail to activate them, acting as a repressor.

The MAF subset of the leucine zipper family of transcription factors is important for islet cell function. MAFA, MAFB, and c-MAF are all expressed in the islet cells (Olbrot et al., 2002). These factors are responsible for the tissue-specific expression of insulin. Specifically, MAFA is a component of the β-cell specific RIPE3b1 binding complex of the insulin promoter and is not present any other cell types of the islets (Matsuoka et al., 2004). Consistent with a β-cell function, MAFA is absent in the few insulin positive cells present in the pancreas of *Nkx6.1* knockout mice (Matsuoka et al., 2004). The cell-type-specific expression of the MAF family members in the islets, and the identified tissue-specific developmental function of MAFs (particularly MAFB) in myeloid and hindbrain differentiation (Kelly et al., 2000; Theil et al., 2002) implicates a function for MAFs in the specification of the different endocrine cell types. Currently these functions have not been identified.

PDX1 and HLXB9 were previously discussed for their roles in pancreatic cell specification and proliferation (Li et al., 1999; Offield et al., 1996). In addition to early roles, both genes are expressed in mature β-cells. In addition to the disruption of dorsal bud formation, *Hlxb9-/-* mice have disrupted islet organization, as well as a decrease in β-

and δ-cells (Harrison et al., 1999; Li et al., 1999). Although cells of the ventral pancreatic remnant express *Pdx1* and β-cell marker Nkx6.1, they do not undergo proper β-cell maturation. Investigation of the later function of PDX1 involved the expression of CRE under the control of the rat insulin promoter. This allowed for the β-cell-specific removal of the *Pdx1* gene flanked by lox P sites. Thus, PDX1 function during early pancreatic development is not disrupted. Mutant mice had a large reduction in the number of insulin expressing β-cells when compared to normal mice (Ahlgren et al., 1998). This is evidence that PDX1 is necessary for maintaining β-cell phenotype in differentiated cells.

Although most endocrine cells originate from the *Pdx1*-expressing pancreatic epithelium, a subset of endocrine cells is derived from a *Pdx1*-independent lineage. Early in pancreatic development, cells expressing glucagon (and sometimes insulin) appear. Unlike the majority of islet precursors, they do not express *Pdx1*, *Nkx6.1*, and *Glut2* (Herrera, 2000). This is more similar to intestinal cells. These cells still form if normal expression of *Pdx1* or *Hlxb9* is perturbed (Li et al., 1999; Offield et al., 1996) and also form upon genetic disruption of the exocrine pancreas (Krapp et al., 1998). The POU factor BRN4 has been indicated as an activator of glucagon expression, and is expressed in the *Pdx1*-independent lineage (Heller et al., 2004). Thus, there is evidence for a lineage of endocrine cells derived from precursors that are different from precursors of the majority of endocrine cells.

Differentiation of the exocrine pancreas

In contrast to the endocrine pancreas, much is less is known about which transcription factors program the differentiation of the exocrine pancreas. Only two transcription factors have been identified as having exocrine-specific expression patterns, P48 and MIST1. These factors are expressed in the exocrine pancreas as well as a limited number of other organs.

P48 has been intensely studied as a factor essential for the formation of the exocrine pancreas. Initially discovered as an activator of the pancreatic digestive enzyme genes, subsequent disruption of the *p48* gene revealed a complete loss of the exocrine pancreas with the formation of some displaced endocrine cells in the spleen (Krapp et al 1998). An additional loss-of-function study that disrupted the *p48* locus with the *Cre* gene showed several interesting things (Kawaguchi et al., 2002). First, activation of CRE recombinase in the normal region of *p48* expression allowed for expression of lacZ in ROSA 26 reporter mice. Mice homozygous for this disruption failed to form a pancreas, and lacZ positive cells were relegated to a duodenal fate. Mice heterozygous for this disruption allowed for lineage tracing of *p48* positive cells. Surprisingly, endocrine, ductal and acinar cells all stained for lacZ, implicating P48 in a role earlier than that of exocrine differentiation. Indeed *p48* expression is first seen at E9.5, substantially earlier than the timepoint of acinar cell differentiation. Rescue of *Pdx1* knockout mice with a *Pdx1* transgene expressed from the *p48* promoter restored pancreatic tissue to these mice (Kawaguchi et al., 2002). Thus, in addition to its function in the mature acini, P48, along with PDX1 is important for the proliferation of pancreatic precursors that differentiate to become both endocrine and exocrine cell types of the mature pancreas. A study to

examine temporal requirements for *p48* expression, with *p48* under the control of a tetracycline-regulated promoter has been initiated (M. Hale, personal communication). The results of these studies will help differentiate the early and late functions of P48. Additionally, P48 was shown to interact with RBP-J, a mediator of Notch signaling in yeast two-hybrid experiments (Obata et al., 2001), and overexpression of activated Notch1 causes the loss of exocrine pancreas (Hald et al., 2003). Taken together, these results suggest that the Notch pathway may also affect P48 function.

MIST1 has been identified as an exocrine-specific transcription factor. In addition to its expression the exocrine pancreas, MIST1 is also present in the exocrine cells of the salivary glands and the chief cells of the stomach (Lemercier et al., 1997; Pin et al., 2000; Yoshida et al., 2001). Mist1-lacZ heterozygotes exhibit expression in some pancreatic precursor at E10.5, but expression is relegated to the differentiating cells on the periphery of the epithelial region. *Mist1-/-* mice lose characteristics of differentiated exocrine cells, however exocrine transcription of digestive enzymes is not lost (Pin et al., 2001). Instead, cells in these mice express ductal and acinar markers, indicating a loss of stable exocrine cell identity. The role of MIST1 in exocrine differentiation and function is still unclear. It may have a function that is at least partially redundant with P48. The misexpression of *Mist1* in endocrine precursor cells might give further information about the function of MIST1 in the developing pancreas.

The Active form of P48 is the trimeric complex PTF1

Although P48 plays a role in acinar cell function in the mature pancreas, it is also implicated (along with PDX1) in the proliferation of pancreatic precursor cells. Despite its participation in these essential processes, the mechanism by which P48 works is not fully understood. P48 is a bHLH protein that is part of a three-protein complex, PTF1. This complex also contains ubiquitously expressed class A bHLH protein (E-protein) and another, previously unidentified, 64kDa protein (Rose et al., 2001; Roux et al., 1989). RBP-J, a member of the CSL family of DNA binding factor is another protein that can interact with P48, but had not been identified as a component of PTF1. P48 has not been isolated as part of any other naturally-occurring protein complex other than PTF1, and it is currently thought that P48 is functional only within the context of this complex. Hence, understanding the dynamics of this complex will shed light on its mechanism of action and potentially the identification of gene targets other than those encoding digestive enzymes.

P48 and other class B bHLH proteins

P48, a transcription factor essential for pancreatic development and function, is a class B bHLH protein (Krapp et al., 1996; Krapp et al., 1998; Rose et al., 2001). Class B bHLH proteins share several important characteristics. They are expressed in a tissue-specific manner, and heterodimerize with Class A bHLH proteins via the helix-loop-helix motif. Binding to DNA requires the basic region adjacent to the helix-loop-helix region

(Blackwell and Weintraub, 1990; Murre et al., 1989a). Class B bHLH proteins such as MASH1, MyoD, and P48 are essential for the development of particular tissues such as the neural, muscle, and pancreas respectively (Guillemot, 1995; Krapp et al., 1998; Valdez et al., 2000). These proteins all have a basic-helix-loop helix domain that is essential for heterodimerization with class A bHLH protein and for DNA binding (Murre et al., 1989a; Murre et al., 1989b). The involvement of class B bHLH proteins in the development and function of specific organs is dependent on highly localized expression of these transcription factors. These tissue-specific class B bHLH proteins interact with the more broadly expressed class A bHLH proteins, and other molecules. P48 has been shown to interact with several other proteins; these include the CSL family member RBP-J and a variety of Class A bHLH proteins (Obata et al., 2001; Rose et al., 2001).

Class A bHLH proteins

Class A bHLH proteins work by forming either homodimers or heterodimers. The Class A bHLH proteins are the so-called ubiquitously expressed bHLH proteins. The most studied members include the two E2A splice variants, E12 and E47 (Murre et al., 1989b). Class A bHLH proteins, such as E12, E47, E2.2, HEB, and others, have been shown to regulate transcription by forming homodimers, or by forming heterodimers with class B bHLH proteins, like P48 and MyoD. E12 and E47 have been shown to be important for regulation of the insulin gene (German et al., 1991). Indeed, E47 heterodimerizes with the class B bHLH protein NEUROD1 and binds specific gene promoters to activate transcription of targets such as insulin (Naya et al., 1995). HEB

also binds in complexes with class B bHLH or other class A bHLH proteins to activate gene expression (Rose et al., 2001; Sawada and Littman, 1993; Takeuchi et al., 2001). Thus A bHLH proteins represent a broadly expressed class of transcription factors that regulate gene expression by interacting with more tissue specifically expressed molecules.

Repressive bHLH proteins

In addition to an activation function, bHLH proteins can also have a repressive function. One example is the AbHLH protein HEB, which can function to repress gene expression as a homodimer (Chiaramello et al., 1995). This mechanism of repression is not well understood, but it may involve strong binding of homodimers to DNA to interfere with the binding of active complexes. Other non-DNA binding helix-loop-helix proteins, Inhibitors of Differentiation (IDs), can repress transcription by binding to A bHLH proteins and some class B bHLH proteins, and prevent their interaction with normal bHLH heterodimerization partners (Langlands et al., 1997). IDs lack the basic domain, and interfere with the DNA binding function when bound to partners. Interestingly, IDS can activate transcription by interfering with the formation of repressive HEB homodimers (Chiaramello et al., 1995).

Another class of repressive bHLH proteins actively represses transcription of gene targets. Specifically, these proteins form heterodimers with bHLH partners and bind DNA. However, proteins like MyoR and HES contains active repressor domains (Rose et al., 2001; Sawada and Littman, 1993; Takeuchi et al., 2001). HES proteins contain the repressive WRPW domain, which recruits active repressors like Groucho to keep

chromatin in closed structure and prevent transcription (Fisher and Caudy, 1998; Fisher et al., 1996). As previously mentioned, *Hes1* is activated by the Notch signaling pathway. Activation of *Hes1* by this pathway represses many genes necessary for differentiation.

The CSL family of Proteins

RBP proteins, RBP-J and RBP-L are mammalian orthologues of an essential mediator of the Notch signaling pathway, *Drosophila* Suppressor of Hairless. The crystal structure of Lag-1, the *C. elegans* orthologue of Su(H), has revealed that it contains two REL-homology domains separated by a β–trefoil domain (Kovall and Hendrickson, 2004) and (Figure 1.5). The N-terminal REL-homology region (RHR) and the β-trefoil domain make extensive, yet specific, contacts with DNA to mediate binding as a monomer to the TTCCCACG consensus sequence. The C-terminal RHR contains an IPT/TIG domain similar to one present in other proteins such as NF-κ B. This IPT/TIG domain is similar to an immunoglobulin fold, and it has been hypothesized to contribute to DNA binding of the protein. This has not been fully studied, and the residues of LAG-1 shown to contact DNA reside outside of this IPT/TIG domain. However, this domain is likely essential for maintaining structure of the protein that allows for DNA binding, as deletions of this domain eliminate DNA binding (Kovall and Hendrickson, 2004).

In addition to binding DNA, the CSL family of proteins interacts with a variety of proteins to regulate the expression of Notch activated target genes. Specifically, Su(H), and its mammalian orthologue RBP-J, can mediate both repression and activation of these targets. In the absence of Notch signaling, Su(H) is bound to its target sequence on

DNA. Su(H) interacts directly with a large corepressor complex that contains factors such as SMRT, CIR, and SKIP, and recruits histone deacetylases (HDACs) to keep these regions of the chromatin in a closed structure (Fryer et al., 2002; Hsieh and Hayward, 1995; Hsieh et al., 1999). Upon activation of Notch signaling, the membrane bound Notch receptor is cleaved, releasing its intracellular domain. This domain (NotchIC) interacts with Su(H) in the nucleus. It is during this process that the corepressor complex is displaced by a not fully understood mechanism. The NotchIC, bound to Su(H), then recruits molecules such as Mastermind and histone acetyltransferases (HATs) PCAF and GCN to open the local chromatin structure and allow for transcriptional activation of target genes (Artavanis-Tsakonas et al., 1999; Fryer et al., 2002).

Interaction of Su(H) with regulators of Notch signaling occurs largely through its β-trefoil domain. It is a hydrophobic pocket in this domain that interacts with the RAM domain of the Notch intracellular domain to activate transcription of targets. This region contains a peptide with a ΦWΦP motif (where Φ is a hydrophobic residue) that likely interacts with the hydrophobic pocket. A few other proteins, such as KYOT2 and EBNA-2 contain similar motifs (Kovall and Hendrickson, 2004). Indeed KYOT2 has been shown to inhibit Notch activation by interacting with RBP-J (Qin et al., 2004; Qin and Han, 2004; Taniguchi et al., 1998). P48 was also identified as an interacting partner of RBP-J using a yeast two-hybrid system with RBP-J as bait (Obata et al., 2001). It is possible that other proteins containing this motif regulate RBP-J targets. A region adjacent to this pocket interacts with CIR and SMRT to mediate repression of transcription. Interaction of the β-trefoil domain with corepressors and the NotchIC is mutually exclusive, and partially explains the displacement of the corepressor complex

by the NotchIC and activation complex (Hsieh et al., 1999; Kovall and Hendrickson, 2004).

In addition to its Notch-mediated activation of targets, Su(H) and a similar paralogue may act in Notch-independent pathways. Studies of the development of the *Drosophila* mechanosensory bristles have shown that Su(H) is important for this activity, but the mechanism is Notch-independent. However, it is not clear how Su(H) works in a Notch-independent manner (Barolo et al., 2000). A paralogue of RBP-J has been identified and named RBP-L (Minoguchi et al., 1997). Biochemical studies have revealed that RBP-L is likely an activator that works independent of the Notch pathway (Minoguchi et al., 1997; Tani et al., 2001). RBP-L shares 48% identity and 65% similarity with RBP-J (Figure 1.5). Initial expression studies revealed that unlike RBP-J, which seems to be ubiquitously expressed, RBP-L is expressed in only a few tissues. During the initial identification of RBP-L, lung was shown to have the highest levels of RBP-L transcript, with lower amounts in brain and possibly spleen (Minoguchi et al., 1999; Minoguchi et al., 1997; Tani et al., 2001). Although RBP-L has high conservation with the β-trefoil domain of RBP-J, coimmunoprecipitation experiments showed that RBP-L does not interact with any of the four NotchICs (Minoguchi et al., 1997). Thus, RBP-L is likely a constitutive activator. This activation domain likely lies in the N- or C-terminal region of RBP-L, since those regions have little similarity to those of RBP-J. Furthermore, although the DNA consensus binding sequence has been identified to be the same as RBP-J using CASTing, no gene targets of RBP-L have been identified (Minoguchi et al., 1997).

Mammalian Su(H), RBP-J and its vertebrate-specific paralogue, RBP-L, are potential candidates for the unidentified protein in the PTF1 complex. P48 has been identified as a protein that has the ability to interact with RBP-J (Obata et al., 2001). Since RBP-J is a broadly expressed protein, co-expression of P48 in the nervous system and pancreas during development is not surprising. More intriguing is the tissue-specific expression of RBP-L. Even though P48 can bind RBP-J in biochemical experiments, the existence of a complex bound to the regulatory regions of target genes has not been shown *in vitro* or *in vivo*. Despite these implications, RBP proteins had not been identified as part of the PTF1 complex.

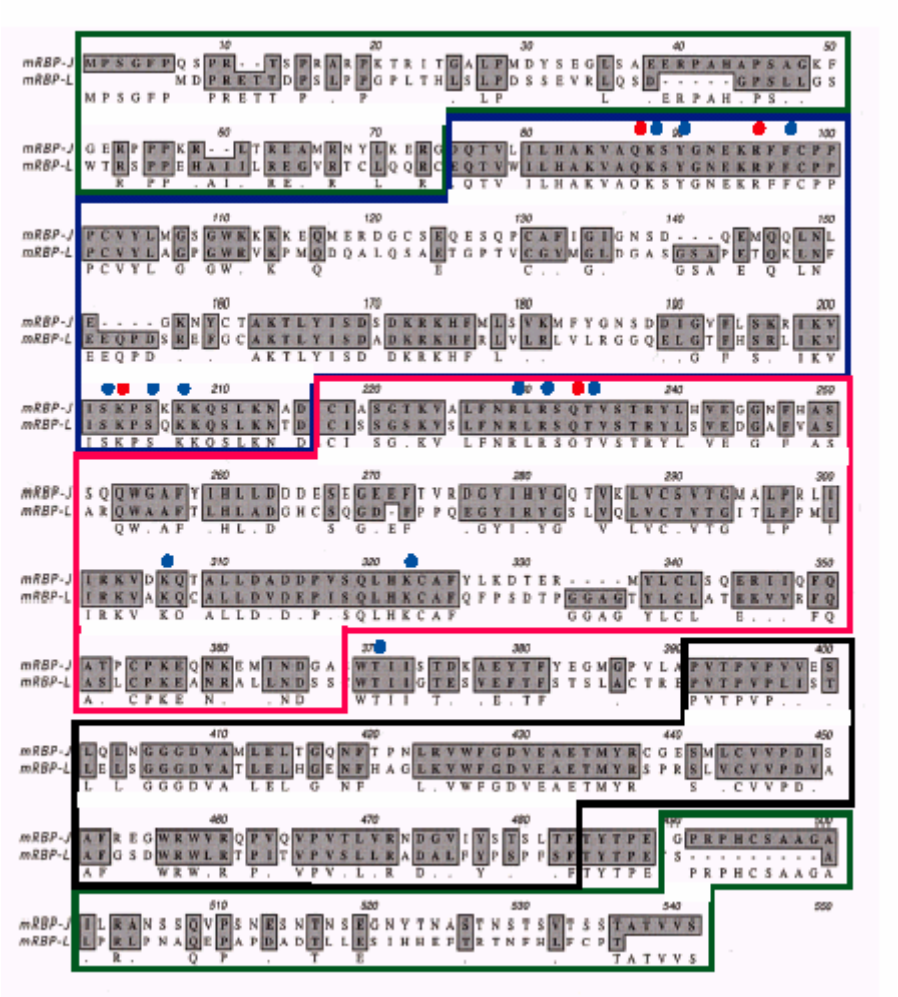


Figure 1.5 Sequence conservation between mouse RBP-J and mouse RBP-L.
A sequence alignment between mouse RBP-J and RBP-L proteins. Green boxes represent the highly divergent N- and C- termini. Other boxes represent the structural domains of RBP-J based on the crystal structure of LAG-1. blue, N-termina RHR; red, BTD; black C-terminal RHR. Colored dots denote amino acid contacts with DNA (also based on the LAG-1 crystal structure). red, direct contact of amino acid side chain; blue, contact of backbone.

Digestive Enzyme Genes as a model for PTF1 Study

Digestive enzyme genes represent a good model for studying the activity of P48 and the PTF1 complex. P48 is essential for pancreas development, but no developmentally important gene targets of P48 or PTF1 have been identified. Although P48 is a class B bHLH protein that heterodimerizes with AbHLH proteins on an E-box, the PTF1 complex contains three proteins. This three-protein PTF1 complex is the only known active form of P48, but the mechanism of its transcriptional activation is still unclear. The digestive enzymes genes, such as *ELA1* and *Ctrb*, are the only known targets of PTF1 and thus, the only known targets of P48. To completely understand how PTF1 activates transcription, it is important to identify the third member of the complex. The identification of this protein is essential to understanding the mechanism of DNA binding as well as what other molecules might interact with PTF1.

The promoters of the digestive enzymes each contain a conserved PTF1 binding site consisting of an E-box and a TC-box (Figure 1.6). The conservation of these enhancer elements suggests that the mechanism by which PTF1 activates these gene targets is the same.

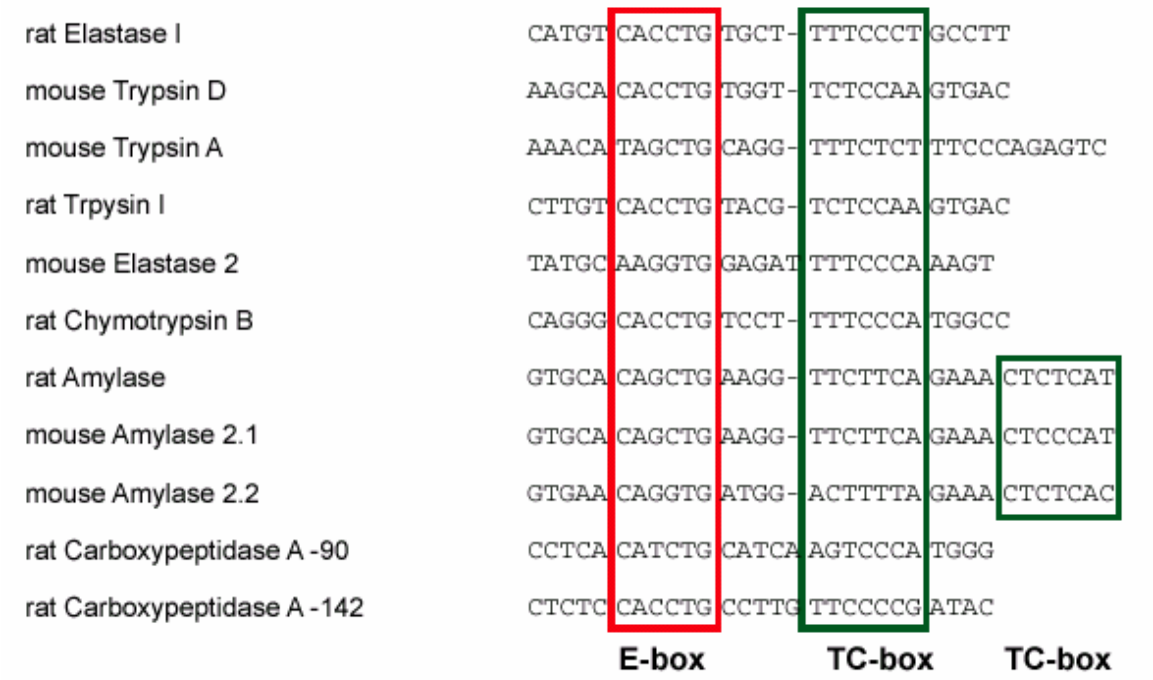


Figure 1.6 Conservation of PTF1 binding sites in the transcriptional regulatory regions of acinar-specific genes.
The PTF1 binding site consists of an E-box (denoted by the red rectangle) one or two turns away from a TC-box (denoted by a green rectangle)

Indeed, individual mutations of both the E-boxes and the TC-boxes show that these elements are each important for the PTF1 binding and activation of these gene targets (Rose and MacDonald, 1997; Rose et al., 2001). The unknown component of PTF1 interacts with the TC-box (Rose et al., 2001). Deciphering the mechanism by which PTF1 activates these known targets will likely provide insight into the mechanism by which P48 activates its target genes during pancreatic development.

Goals of dissertation research

The development and function of organs in eukaryotes involves a complex network of signal transduction and transcription factor cascades. Tissue-specific activation of particular genes, such as those encoding digestive enzymes, is directly regulated by transcription factors that cooperate in complexes to activate gene transcription. The activity of these complexes is often affected by the presence or absence of other tissue-specific factors or complexes.

Digestive enzyme genes, such as *ELA1*, are specifically expressed in the exocrine pancreas. High-level, tissue-specific expression of these genes is dependent on activation by the PTF1 complex via a bi-partite enhancer element. The transcriptional regulatory region of the *ELA1* gene contains this conserved PTF1 binding site, and has been well-studied as a good example of acinar-specific gene expression. P48 is also essential in both the development and function of the pancreas. However, its only known mechanism of activation is as a component of a three-protein complex, PTF1. To this point, only two of the three proteins in PTF1 had been identified. During my dissertation research, I have focused on the following specific goals.

1. Identification of the third component of the PTF1 complex. In addition to P48 and an AbHLH, what other factor is present in the complex? Is the factor an orthologue of Su(H) as some of the evidence suggests? (Figure 1.7)
2. Characterization of the organization of the complex. Which proteins bind to which DNA sequences of the A element? Which protein-protein interactions

are necessary for binding of the complex to DNA? Which proteins are essential for transcriptional activation of target genes?

3. Elucidating whether this complex interacts with other factors or signaling pathways. Specifically, if the third component is an RBP protein, is the activity of the complex enhanced or repressed by Notch signaling. Does the PTF1 complex itself affect Notch signaling?

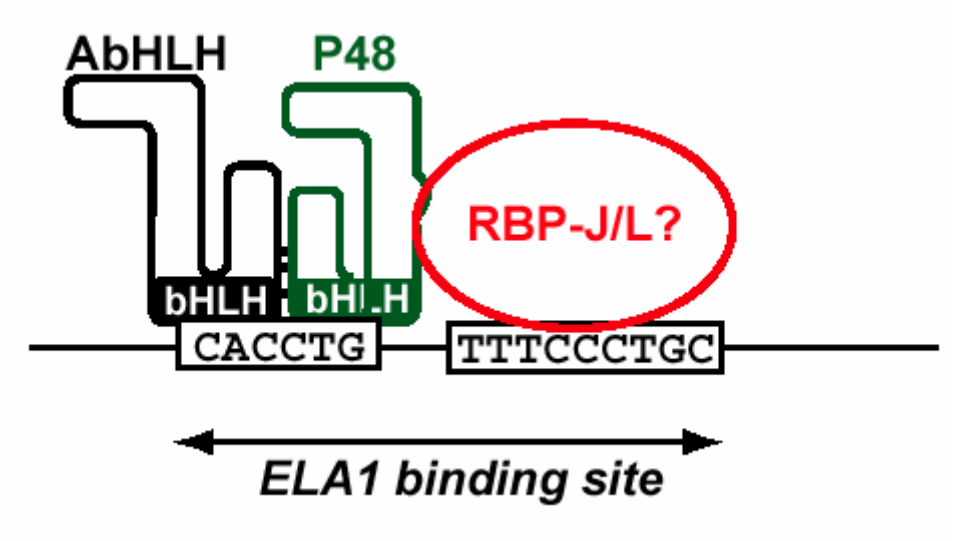


Figure 1.7 A proposed model for PTF1 binding to the *ELA1 A* element.
P48 and an AbHLH protein contact the E-box, while the unidentified 64 kDA protein contacts the TC-box. RBP-J or RBP-L are proposed as candidates for the 64 kDA protein.

Chapter 2

Material and Methods

Antibodies

P48 antibody

Rabbit polyclonal antiserum against the C-terminus of mouse P48 was described in (Rose et al., 2001) and affinity purified.

RBP-L antiserum

The rabbit polyclonal antiserum against RBP-L was raised for us by AnaSpec (San Jose, CA) against a synthetic amino acid peptide (C-PNAQEPAPDADTLLE) corresponding to a 15 amino acid sequence located near the C-terminus of mouse RBP-L.

RBP-J antibody

Rat monoclonal anti-mouse RBP-Jκ (catalog number 2ZK0043) was obtained from the Institute of Immunology Co., Ltd (Tokyo, Japan). The whole human RBP-J protein was used as the antigen, but the resultant monoclonal antibody does not recognize RBP-L in supershift EMSA experiments.

HEB, E2-2, E47, E2-2/E12 antibodies

Anti-HEB serum was a gift from Dr. S. Sawada and was previously described (Sawada and Littman, 1993). Mouse monoclonal anti- E2.2, anti-E12/E2-2, anti-E47 antibodies that recognize human and rodent proteins were obtained from BD Biosciences Pharmingen (San Diego, CA).

HA antibody

Rabbit polyclonal anti-HA antibody that recognizes the epitope tag from influenza hemaglutinin (HA) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA)

Cloning of cDNAs

The double-stranded cDNA encoding mouse RBP-L was derived by RT-PCR amplification from mouse pancreatic RNA. cDNAs encoding the Drosophila orthologues of P48, E12, and RBP-J (Fer1, daughterless (DA), and Su(H), respectively) were derived by RT-PCR using Drosophila melanogaster gastric caecae RNA, a gift from J. Zoloty (UT Southwestern, Dallas, TX). Plasmids encoding *Drosophila* cDNAs were prepared by Dr. Toshihiko Masui and Tracy Chow. The cDNA for human P48 was derived from adult human pancreas RNA by Dr. Toshihiko Masui, using RT-PCR. The cDNA encoding myc-tagged human RBP-J was derived by PCR amplification from the plasmid SG5-myc-CBF1 (Hsieh et al., 1996), a gift from Dr. S. D. Hayward (Johns Hopkins Medical Center, Baltimore, MD). The cDNA plasmids for the production of

HEB, E47 (PAN1), and E12 (PAN2) by coupled in vitro transcription and translation reactions (IVT) have been described (Rose et al., 2001).

Plasmid construction for expression of cDNAs

Plasmid xPBX1a-MCS/SP73 was previously prepared by Dr. Ying Liu (Swift et al., 1998). This plasmid contains the *Xenopus laevis* β-globin 5' untranslated region for enhanced translational efficiency as well as an insert of the double stranded cDNA encoding the PBX1a protein. All cDNAs used for IVT were cloned into this construct after removal of the PBX1a cDNA insert. cDNAs used for expression in cell lines via transient transfection were cloned into pcDNA3.1/V5/His-TOPO vector (Invitrogen Corporation, Carlsbad, CA).

P48 expression plasmids

The plasmid SP73-P48 was previously created by cloning the mouse P48 cDNA into the Nco1/Xho1 sites of xPBX1a-MCS/SP73 (Rose et al 2001). Mutations and deletions of this construct were generated using the Quikchange (Stratagene, San Diego, CA) protocol or PCR-SOEing (Horton et al., 1990). For expression in mammalian cell lines, pcDNA3.1-P48 and the associated mutant forms were created by cloning the HindIII/Xho1 fragment containing the *Xenopus laevis* β-globin 5' untranslated region and wild-type or mutant mouse P48 cDNA from SP73-P48 into the HindIII/XhoI sites of the

pcDNA3.1 vector. Similar constructs were generated using human P48, and mutations were created by Dr. Toshihiko Masui using the Quikchange protocol.

HEB expression plasmids

The HEB construct used for IVT was a gift from Richard Baer (Columbia University, New York, NY). The plasmid pcDNA1.1amp-HEB, used for expression of HEB in mammalian cell lines, was previously prepared by Dr. Scott Rose (Rose et al., 2001).

E47and E12 expression plasmids

Plasmids for IVT of cDNAs encoding E47 and E12 were a previous gift from Dr. Michael German (University of California at San Francisco, San Francisco, CA). They contain the Syrian hamster Pan1 and Pan2 cDNAs (E47 and E12 respectively) (German et al., 1991).

RBP-J expression plasmids

SG5-myc-human-CBF1 (RBP-J) was a generous gift from S. D. Hayward. An improved IVT construct, pSP73-RBP-J, was created by amplifying the cDNA from the SG5-mycCBF1 plasmid by PCR, and cloning the product into the MscI/XhoI sites of the xPBX1a-MCS/SP73 vector. For the expression of myc-tagged human RBP-J in

mammalian cell lines a DNA plasmid was created by cloning the HindIII/XhoI fragment from pSP73-RBP-J into the HindIII/XhoI site of pcDNA3.1. Unfortunately, the myc-tag at the 5' end of the construct also contained a HindIII site and so was deleted. The construct was repaired by cloning EcoRI fragment from pSP73-RBP-J into the first pcDNA3.1-RBP-J. The EcoRI sites were located just 5' of the HindIII site and in the middle of the RBP-J cDNA. This repair was done with the help of Dr. Toshihiko Masui.

RBP-L expression plasmids

pSP73-RBP-L was created by cloning the NcoI/StuI fragment containing the mouse RBP-L cDNA coding and 3'untranslated region into the NcoI/SmaI sites of xPBX1a-MCS/SP73. N-terminal deletions of RBP-L were created using PCR to generate truncations from the 5' end of RBP-L cDNA with an NcoI site at the start of the coding sequence and a 3' primer downstream of a unique MluI site. The NcoI/MluI sites were cloned into the NcoI/MluI sites of pSP73-RBP-L. Internal N-terminal deletions of RBP-L were created using PCR-SOEing. C-terminal deletions were created using the Quikchange protocol. For expression in mammalian cell lines, pcDNA3.1-RBP-L plasmids were created by cloning the HindIII/NotI fragment bearing the cDNA from pSP73-RBP-L (or the mutant forms) into the HindIII/NotI sites of pcDNA3.1.

Notch expression plasmids

Plasmids containing cDNAs encoding amino acids 1751-2295 of the mouse Notch Intracellular Domain alone, or fused to the activation domain of the Epstein Barr Virus Nuclear Anitgen 2, (SG5-HA-mNotch and SG5-HAmNotchEBNA, respectively) were generous gifts from Dr. S. D. Hayward. Improved IVT constructs, pSP73-NICD and pSP73NICDE, were created by amplifying the cDNAs from the SG5 plasmids by PCR and cloning them into the xPBX1a-MCS/SP73 vector. Plasmids for expressing the NotchIC or the NotchIC-EBNA fusion in mammalian cell lines were made by cloning the fragments from the pSP73-NICD or pSP73-NICDE vectors into pcDNA3.1.V5HIS-TOPO.

***In vitro* transcription and translation**

Coupled *in vitro* transcription and translation (IVT) was performed with the TNT coupled reticulocyte lysate system according to the manufacturer's instructions (Promega. Madison, WI). Typical *in vitro* translation reactions were 25 μl, containing 12.5 μl of rabbit reticulocyte lysate, 1X TNT reaction buffer, 0.5 μl Sp6 or T7 RNA polymerase, 1mM amino acid mixture, 0.5 μl (20 units) of recombinant RNasin ribonuclease inhibitor, and 1 μg of DNA template. Parallel reactions were prepared with ^{35}S-methionine. The radioactive reactions contained 2 μl ^{35}S-methionine (>1,000 Ci/mmol at 10 mCi/mol; NEN). Reactions were incubated at 30^{o}C for 90 minutes. The translation products were stored at –80^{o}C.

The quality of IVT proteins was evaluated using the ^{35}S-methionine labeled proteins. Incorporation of the ^{35}S-methionine was calculated by the Glass Filter C/F wash assay. In this assay, 1 ul of the translation product was spotted on the GF/C glass microfiber filter (Whatman, Maidstone, England) in duplicate. One filter was boiled in 3% trichloroacetic acid (TCA) for 5 minutes, rinsed twice with room temperature TCA, and dried under a heat lamp. Both the washed and unwashed filter were counted in a liquid scintillation counter. Percent incorporation was calculated by cpm from the washed filter divided by cpm from the unwashed filter and multiplied by 100. 1 to 2 μl of the ^{35}S-labeled product was resolved by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). A ^{14}C-labeled high range protein molecular weight ladder was used to compare and calculate the molecular weight of IVT products. The gel was then fixed in 20% methanol and 10% acetic acid for 5 minutes, rinsed in water, and vacuum dried onto Whatman paper at 80^{o}C. The dried gel was exposed under a Phosphorimager screen overnight, and the gel image was visualized by scanning the screen with a Storm PhosphorImager and analyzed using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). IVT products from the nonradioactive reactions were used for EMSA.

EMSA and the antibody supershift assay

EMSA Oligonucleotide Probes

The sequences of the oligonucleotides used as probes in EMSA are listed in Table 2.1 and 2.2. The A element represents the PTF1 binding site encompassing nucleotides -115 to -96 of the rat elastase I (*ELA1*) enhancer. It is the primary site used to study the PTF1 complex. Mutants of this element provided useful tools for studying PTF1 binding and transcriptional activity. One mutant of the A element, AmE (C to T), has a one base change in the E-box that eliminates PTF1 binding completely. Another mutant, AmT, changes two C's to A's in the TC-box, and eliminates binding of the trimer, but not the heterodimer. This mutation is based on previous mutation used to eliminate transcriptional activation of the RBP-J binding site (Hsieh et al., 1996). The third mutant combines the mutations from AmE and AmT. To create an oligonucleotide probe with an RBP-J consensus binding site (AmEpT), the E-box was completely changed as previously described to prevent bHLH protein binding (Rose et al 2001) and the TC-box was changed to the consensus RBP-J binding site (Hsieh et al., 1996). Other binding sites used in this study come from enhancers from genes encoding mouse elastase II (*Ela2*), rat carboxypeptidase A1 (*Cpa1*), rat chymotrypsin B (*Ctrb*), and mouse trypsin D (*Trpd*). All these sites were used to analyze the PTF1 complex by EMSA. Additionally,

an oligonucleotide containing two copies of the RBP-J binding site, O_{54} (Minoguchi et al., 1997), was also used for EMSA analysis.

NAME	Sequence
A/top	GTCACCTGTGCTTTTCCCTGC
A/bot	GCAGGGAAAAGCACAGGTGAC
AmE/top	GTCACTTGTGCTTTTCCCTGC
AmE/bot	GCAGGGAAAAGCACAAGTGAC
AmT/top	GTCACCTGTGCTTTTCAATGC
AmT/bot	GCATTGAAAAGCACAAGTGAC
AmET/top	GTCACTTGTGCTTTTCAATGC
AmET/bot	GCATTGAAAAGCACAAGTGAC
ApTC/top	GTCACCTGTGCTTTTCCCACG
ApTC/bot	CGTGGGAAAAGCACAGGTGAC
AmEpTC/top	GTAGTAGTTGCTTTTCCCACG
AmEpTC/bot	CGTGGGAAAAGCAACTACTAC

Table 2.1 Summary of the EMSA oligonucliotide probes related to the *ELA1* A element.
The sequences of the top and bottom strands of each oligonucleotide are shown 5' to 3'.

Probe labeling

Oligonucleotide probes for EMSA were phosphorylated with ^{32}P-γATP as the substrate. The reaction mix contained 1X kinase buffer (New England Biolabs, Inc., Beverly, MA), 3 µl of ^{32}P-γATP (30 µCi), 6 pmol of the top oligonucleotide strand, 0.75 µl of T4 polynucleotide kinase (7.5 units) in 15 µl. The reaction was incubated at 37^{o}C for 30 minutes. 30 µl of TE2N (10 mM Tris pH 7.5, 1 mM EDTA, 200 mM NaCl) was added to stop the reaction at the end of the incubation. Five-fold excess (15 µl or 30 pmol) of non-radioactive bottom strand oligonucleotide was added to make the labeled probe double stranded. The mixture was heated to 65^{o}C and cooled slowly to room

temperature. Incorporation of ^{32}P was measured by DE81 filter binding assay. 1 µl of labeled probe was spotted on duplicate DE81 ion exchange filters (Whatman). One filter was washed with 0.4 M Na_2HPO_4 four times for 10 minutes each. The filter was then rinsed with water, then with 95% ethanol and dried. Both the washed and unwashed filters were counted by scintillation counter. Incorporation was calculated as the ratio between the cpm from the washed versus the unwashed filter. The probes were precipitated using 150 µl of ethanol and incubating at –20°C for at least an hour (overnight if possible). Precipitated probes were recovered by centrifugation at 14,000 rpm for 15 minutes at 4°C. The ethanol was removed and the pellet allowed to air dry. The pellet was then resuspended in 60 µl of TEN for use in binding reactions.

NAME	Sequence
mELA2/top	GCAAGGTGGAGATTTTCCCAAA
mELA2/bot	TTTGGGAAAATCTCCACCTTGC
rCPA-90/top	CACATCTGCATCAAGTCCCATG
rCPA-90/bot	CATGGGACTTGATGCAGATGTG
rCPA-142/top	TCCACCTGCCTTGTTCCCCGAT
rCPA-142/bot	ATCGGGGAACAAGGCAGGTGGA
rChymB/top	TCCACCTGCCTTGTTTCCCATG
rChymB/bot	CATGGGAAACAAGGCAGGTGGA
mTrpD/top	CACACCTGTGGTTTTCTCGAT
mTrpD/bot	GTGTGGACACCAAAAGAGCTA
O54/top	CCTGGAACTATTTTCCCACGGTGCCCTTCCGCCCATTTTCCCACGAGTCGCGAG
O54/bot	CTCGCGACTCGTGGGAAAATGGGCGGAAGGGCACCGTGGGAAAATAGTTCCAGG

Table 2.2 Summary of the EMSA oligonucliotide probes from other promoters.
The sequences of top and bottom strands of each oligonucleotide are shown 5' to 3'. Oligonucleotides derived from the PTF1 binding sites of digestive enzyme genes except O_{54}.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described by (Rose et al., 2001). Nuclear extracts from pancreas were previously prepared by Dr. Scott Rose. Mobility shift binding reactions were typically 20 μl in volume and contained: 10 mM HEPES pH 7.9, 90 mM NaCl, 4 mM Tris pH 7.9, 1 mM EDTA, 100 mM DTT, 40 fmole of labeled probe, and nuclear extract or IVT protein. Reactions containing nuclear extract also contained 6 μg of Bovine Serum Albumin (BSA) and 2 μg poly dIdC as a non-specific competitor. Reactions containing IVT proteins had 0.2 μg of poly dIdC and no BSA and were preincubated for 10 minutes at 37^{o}C prior to the addition of the probe. Upon addition of the probe, the reactions were incubated for 15 minutes at 30^{o}C. 15 μl of each reaction was loaded and resolved on a 4.0% non-denaturing polyacrylamide gel in 1X Littman Running Buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.0). Gels were pre-run for about 30 minutes at 25 mA at 4^{o}C and run at 30 mA for about 1 hour and 45 minutes. Upon completion of electrophoresis, the gels were fixed for at least 5 minutes in 10% methanol and 10% acetic acid, rinsed with water, and vacuum dried at 80^{o}C onto Hoefer blotter paper. The dried gels were exposed under a phosphorimager screen overnight. The gels were visualized by scanning the screens on a Storm Phosphorimager and analyzed with ImageQuant software. Typically 1.5 to 3 μg of nuclear extract or 0.5 to 3 μl of rabbit reticulocyte lysates were used for the EMSA reactions. A 20:1 molar ratio of P48: AbHLH was used to see the formation of the P48: AbHLH heterodimer in the absence of RBP proteins. A 20:1:8 molar ratio of P48: AbHLH: RBP protein was used in order to compare the electrophoretic mobilities of the trimers to the mobilities of the heterodimers.

To test whether the Notch Intracellular Domain (NotchIC) interferes with the formation of the PTF1 complex on the *ELA1* A element oligonucleotide, increasing amounts of IVT NotchIC were added to EMSA reactions containing 1:1:1 molar ratios of IVT P48, E12, and RBP proteins. To test whether P48 inhibits formation of an RBP-J/NotchIC complex on an RBP-J site, increasing amounts of IVT P48 or P48-W298A (which does not interact with RBP-J) were added to EMSA reactions containing a 1:1 molar ratio of IVT RBP-J and NotchIC proteins.

EMSA supershift

Antibody supershift EMSA was performed by mixing 1 to 2 μl of antibody or antiserum with nuclear extracts or IVT proteins and incubating at room temperature (22^{o}C) for 10 minutes prior to the addition of oligonucleotides in EMSA binding reactions.

Cell culture and transient transfection

Reporter plasmids

The activities of the A element and associated mutant elements, AmE, AmT, AmET, ApT, and AmEpT were tested by transient transfection of HEK 293 cells with the expression of exogenous transcription factors. All of the binding sites tested were based on the 26 base pair A element of the *Ela1* gene (Rose et al., 1994). Six copies of the A element (6A) were previously cloned into the EcoRV and SalI sites of a pUC119 plasmid containing a minimal promoter from the -92 to +8 of the rat elastase I gene linked to the hGH (human growth hormone) reporter gene (Rose et al., 1994). A minimal promoter construct (EIp.luc) was created by placing the rat elastase 1 (*Ela1*) basal promoter (-92 to

+8) at the 5' end of the luciferase reporter gene of PGL3-basic (Promega, Madison, WI) by cloning the BamHI/XbaI fragment from -92to+8-hGH into the BglII/XbaI site of PGL3basic. To create a luciferase reporter with the six tandem copies of the A element (6A.EIp.luc), the hGH construct was digested with HindIII, ends were filled in with the Klenow fragment of DNA polmerase, and digested with BamHI. The fragment was then cloned into the SmaI/BglII site of PGL3basic (Promega. Madison, WI). Mutants of 6A were created using three oligonucleotides each for the top strand and the bottom strand. Oligonucleotides were designed to anneal in a staggered manner such that the 5' end had an NheI overhang and the 3' end was blunt. The oligonucleotides were purified using native polyacrylamide gel electrophoresis (PAGE). The four internal oligonucleotides were phosphorylated using T4 polynucleotide kinase. The six oligonucleotides were then annealed and ligated into the NheI/EcoRV sites of PGL3-92+8. The sequences of one of the six tandem copies for 6A and each associated mutant sequence are summarized in Table 2.3.

Reporter Name	EMSA Name	(Sequence) x 6
6A.EIpluc	A	TCATGTCACCTGTGCTTTTCCCTGCC
6AmE.EIp.luc	AmE	TCATGTCACTTGTGCTTTTCCCTGCC
6AmT.EIp.luc	AmT	TCATGTCACCTGTGCTTTTCAATGCC
6AmET.EIp.luc	AmET	TCATGTCACTTGTGCTTTTCAATGCC
6ARBP.EIp.luc	ApTC	TCATGTCACCTGTGCTTTTCCCACGC
6R.EIp.luc	AmEpTC	TCATGTAGTAGTTGCTTTTCCCACGC

Table 2.3 Summary of reporter constructs used for transient transfections.
Sequences from 5' to 3', represent one copy of six tandem repeats driving the luciferase reporter gene.

Culture and transfection of HEK 293 cells

Transfection of 293 human embryonic kidney cells (ATCC CRL-1573) was performed with Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. 293 cells were cultured on 100 mm culture dishes (Falcon) at 37^{o}C in DMEM (Dulbecco's Modified Eagle Medium) containing 4.5 g/L of glucose and L-glutamine, but no sodium pyruvate (Mediatech, Herndon, VA). Medium was supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and Amphotericin B and a penicillin/streptomycin mixture (Mediatech, Herndon, VA). Cells were passaged every two to three days on 100 mm or 150 mm culture dishes. For transfections, 50,000 cells were plated on each well of 6 well plates (BD Biosciences Discovery Labware, Bedford, MA).

For transient transfection, cells were transfected two days after plating. All introduced transcription factors were under the control of a CMV promoter, as was the β-galactosidase internal control (pCMV-β; Clontech, San Francisco, CA). The total amount of CMV enhancer/promoter and plasmid DNA was adjusted to be constant in each transfection, with insertless pcDNA3.1 added as needed. Wild-type and mutant PTF1 binding site were each tested by placing a tandem repeat of six sites upstream of the *Ela1* basal promoter in EIp.luc (e.g., 6A.EIp.luc). To test whether P48 inhibits transcriptional activity of an RBP-J/NotchIC complex on RBP-J sites, six tandem copies of the consensus RBP-J site were placed upstream of the *Ela1* basal promoter in Elp.luc (6R.EIp.luc). Typical mixtures of expression plasmids contained 0.2 μg of each plasmid encoding a transcription factor, 0.8 μg the appropriate reporter plasmid, 0.2 μg of pCMV-

β, and insertless pcDNA3.1 plasmid for a total of 2 μg of plasmid DNA. DNA was mixed with 6 μl Fugene 6 in 194 μl unsupplemented DMEM. DNA and Fugene 6 reagent were mixed and incubated at 22°C for 15 minutes. For each mixture, 100 μl was added dropwise to each of two wells containing 50,000 293 cells and swirled gently. Cells were incubated for 48 hours at 37°C in 5% CO_2.

Transcriptional activity was assayed 48 hours posttransfection. Cells were lysed with 100 μl of cell lysis buffer from the Galacto-light Plus kit (Tropix, Inc., Bedford, Mass). Lysed cells were scraped and transferred to 1.5 ml centrifuges tubes. Cellular debris was removed by a short centrifugation, and the transfer of supernatant to a new tube. Reporter activity was quantified using 10 μl of lysate from transfected cells in a luciferase activity assay (Luciferase Assay System, Promega, Madison, WI). All transfection results were corrected for varying transfection efficiencies according to the β-galactosidase activity from the cotransfected pCMV-β using 5 μl of cell lysate assayed with Galacto-light Plus (Tropix, Inc., Bedford, Mass). Each construct was assayed in duplicate in two or more independent experiments.

Expression analysis

RT-PCR

RNAs from 19 adult mouse tissues, various stages of mouse embryonic pancreas, various stages (e57-59, e72-74, adult) of human pancreas, human purified islets, and human embryonic kidney 293 cells were analyzed for the presence of message from the *p48*, *Rbp-L*, *Rbp-J,* and *Actin* genes. Total RNA was prepared from dissected organs from adult mice using the guanidine thiocyanate technique (Chirgwin et al., 1979;

MacDonald et al., 1987). RNAs were stored as ethanol precipitates. cDNAs were produced using 1 μg RNA. The RNA samples were treated with DNaseI in 1X DNaseI buffer (Invitrogen) in a total volume of 10 μl at room temperature for 20 minutes. The reaction was stopped with 1 μl 25 mM EDTA and heat inactivation at 65^{o}C for 10 minutes. cDNA synthesis was carried out in 1X first strand synthesis buffer (Invitrogen), 40mM DTT, 40 units RNase inhibitor, 10 mM dNTPs, random primers, and DEPC water in a total volume of 40 μl. The reaction was split in half: 1 μl reverse transcriptase (SSII enzyme from Invitrogen) was added to one half for the reverse transcriptase reaction; the other half was carried out in the absence of SSII enzyme to confirm the absence of genomic DNA contamination. The reactions were incubated at 42^{o}C for 90 minutes. Reactions were diluted 1:3 and stored at –20^{o}C. Aliquots of each cDNA preparation were then subjected to PCR amplification with gene-specific primers. (Table 2.4) Amplification of cDNAs was carried out for 30 cycles under the following conditions: Denature for 1 minute at 94^{o}C, anneal for 1 minute at 60^{o}C, and extend for 1 minute at 72^{o}C. The exception was the amplification of *actin*, which was amplified for 25 cycles. Reactions used 25 ng of the RT reaction (3 μl of diluted product), 1.5 μl 25 mM $MgCl_2$, 0.25 μl 10mM dNTP mix, 9.6 μl 0.75 μM gene specific primer mix, and 0.5 μl Taq polymerase in a total volume of 40 μl.

Name	Sequence
mP48/top	CGCGTCTTTGTGCATATTGT
mP48/bot	CGGAGTTTCCTGGACAGAGT
mRbp-L/top	GGAGCTGCACGGAGAAAA
mRbp-L/bot	GTGTGAACTCGTGGTGGATG
mRbp-J/top	GAATTTCCACGCCAGTTCAC
mRbp-J/bot	ATACAGGGTCGTCTGCATCC
mActin/top	AGCCATGTACGTAGCCATCC
mActin/bot	ACATCTGCTGGAAGGTGGAC
hP48/top	CCCAGACTCGGCTGAAGAT
hP48/bot	TAAGCAGGACGTTTTCTGGC
hRbp-L/top	TCCACGGAGAGAAACTTCCAC
hRbp-L/bot	GCGTGAACTCCTGATGATG
hRbp-J/bot	GAATTTCCACGCCAGTTCAC
hRbp-J/bot	ATACAGGGTCGTCTGCATCC

Table 2.4 Summary of PCR primers used to screen for gene-specific transcripts.
The sequence of each primer is shown from 5' to 3'.

Chapter 3

Identification of the Components of the Acinar PTF1 Complex and Examination of their Expression Patterns

Introduction

PTF1 activates the expression of the digestive enzyme genes in the acinar pancreas. The rat elastase I (*ELA1*) gene is expressed at high levels in the acinar cells of the pancreas and has been extensively studied as a model of pancreas-specific gene expression. Lower amounts of transcript have been in detected in other digestive organs such as the stomach and duodenum (Rose et al., 2001). A 500 bp regulatory region, located immediately upstream of the transcriptional start site of *ELA1*, contains a non-specific promoter region, a positive regulatory region containing three enhancer elements (A, B, and C), and a region that represses activity in islet cells (Figure 3.1) (Kruse et al., 1993; Kruse et al., 1995; Rose et al., 1994; Swift et al., 1998; Swift et al., 1994). The organ specific enhancer region directs expression to the acinar and islet cells of the pancreas through the activity of the A, B, and C elements. PTF1 binds the A element to direct acinar specfic expression. The B element has a dual role. A PDX1:PBX1:MEIS complex binds the B element and cooperates with PTF1 in the activation of *ELA1* in acinar cells. In β-cells, the B element is activated by PDX1, however a repressor region does not allow its activation in β-cells within the context of the 500 bp *ELA1* regulatory region (Viswanath et al., 2000). The A element is the essential acinar-specific element in the enhancer, while the B element has been shown to cooperate with the A element to

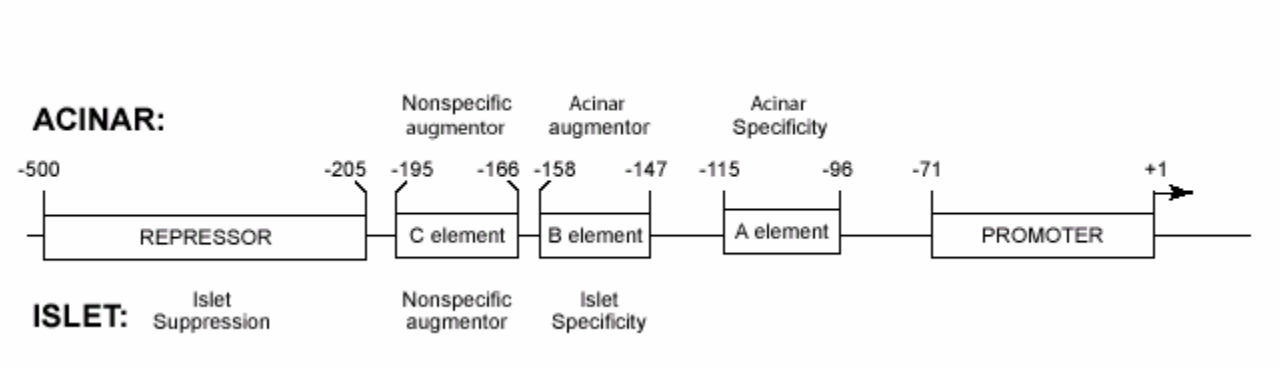


Figure 3.1 Schematic representation of the transcriptional regulatory region of the rat *ELA1* gene.
The 500 bp regulatory sequence contains a promoter, an enhancer that contains the A, B, and C elements, and a repressor region. The location relative to the transcriptional start site, and the function of each element in the acinar and islet tissues of the pancreas are indicated.

augment transcriptional activity in pancreatic acinar cells. The C element is non-specific augmentor of transcriptional activity that strengthens the enhancer activity in acinar cells.

PTF1 binding activity was initially identified in EMSA and DNase I footprinting assays using pancreatic nuclear extracts (Cockell et al., 1989). This complex was shown to comprise three proteins, two of which were identified (Krapp et al., 1996; Rose et al., 2001; Sommer et al., 1991). One is P48, a class B bHLH protein that is specifically expressed at high levels in the pancreas. The other is any one of several ubiquitously expressed class A bHLH proteins (AbHLH). Attempts to reconstitute a complex of the same mobility as PTF1 using P48 and any of the AbHLH proteins yielded a complex of faster mobility than the authentic PTF1 complex. Furthermore, although a transgene driven by six copies of the A element recapitulates high levels of expression in acinar cells of the mouse pancreas, the same reporter is not highly active in cell culture even in the presence of exogenous P48 and HEB (Rose et al., 2001). Thus, it is likely that the third component of PTF1 is necessary for high-level transcriptional activation. This chapter describes the identification of the third component of the acinar PTF1 complex, its transcriptional activity within the context of PTF1, and its tissue distribution.

Suppressor of Hairless, and its mammalian orthologue, RBP-Jκ (RBP-J) are DNA-binding transcription factors that have been extensively studied. These proteins are the known mediators of Notch signaling (Artavanis-Tsakonas et al., 1999). In the absence of Notch signaling, Su(H) and RBP-J repress transcription of target genes, but upon activation of Notch signaling, they activate the same genes. Additionally, the auto-activation function of Su(H) identified in mechanosensory organ development in

Drosophila melanogaster (Barolo et al., 2000) does not require Notch signaling. However, the mechanism of this newly identified Su(H) function is not known.

The vertebrate-specific paralogue of Su(H), RBP-L, has been identified and partially characterized (Minoguchi et al., 1999; Minoguchi et al., 1997). SU(H), RBP-J, and RBP-L have identical DNA binding preferences to sequences that are similar to the TC-box in the PTF1 binding site (Minoguchi et al., 1997). The P48 subunit of PTF1 has been shown to interact with RBP-J in yeast two hybrid assays (Obata et al., 2001), but has not been been identified as a component of PTF1.

In this chapter, I tested the possibility that an RBP protein is the third, and previously unknown, subunit of the PTF1 complex. The results revealed that RBP-L is indeed present in the PTF1 complex isolated from the adult pancreas, and interestingly, that RBP-J can also form complexes of the same mobility as PTF1 with the P48 and AbHLH subunits. Antiserum that specifically recognizes RBP-L and not RBP-J was used in EMSA supershift assays to identify RBP-L as the third component of authentic PTF1, the A element-binding complex present in the nuclear extracts from adult rat pancreas. I also reconstituted the trimeric complex with recombinant P48, one of three AbHLH proteins, and either RBP-L or RBP-J proteins in EMSA experiments. The recombinant trimeric complexes have the same mobility as PTF1 in native acrylamide gels regardless of which AbHLH or RBP protein was used, indicating a probable heterogeneity of the complex, and confirming the presence of only three proteins in a single complex. Using transient transfection assays, I confirmed that RBP-L is necessary for high-level activation of the *ELA1* A element by PTF1. Finally, I examined the tissue distribution of P48, RBP-L, and RBP-J transcripts using RT-PCR on cDNAs from a

panel of mouse organs. While RBP-J message was present in all organs analyzed, P48 and RBP-L messages were highly restricted, with extremely high levels of both in the pancreas. The specific co-expression of high levels of transcripts encoding components of PTF1 is consistent with the tissue specific presence of PTF1 and the high-level activation of digestive enzyme genes in the pancreas.

Results

The TC-box of digestive enzyme genes is similar to the RBP-J consensus binding site

The first indications that an RBP protein might be a subunit of the PTF1 complex came from the results of yeast two-hybrid experiments (Obata et al., 2001) and careful re-evaluation of the TC-box of PTF1 binding sites. The PTF1 binding site is a bipartite element consisting of an E-box and a TC-box separated by one or two helical turns of DNA. These binding sites are highly conserved and occur in the regulatory regions of most, if not all, genes encoding pancreatic digestive enzymes (Cockell et al., 1989). The PTF1 complex is a trimeric complex consisting of the pancreas-specific class B bHLH protein (p48), a class A bHLH protein (p75), and an unidentified protein (p64). P48 and a class A bHLH protein heterodimerize on the E-box, while the unknown component (p64) contacts the TC-box (Cockell et al., 1989; Krapp et al., 1996; Rose et al., 2001; Roux et al., 1989; Sommer et al., 1991). A yeast two-hybrid study, using the broadly expressed mediator of Notch signaling RBP-J as bait, captured P48 from an embryonic day 9.5 mouse cDNA library, indicating that P48 can interact with RBP-J (Obata et al., 2001).

The TC-box present in each PTF1 binding site is similar to the consensus binding site for RBP-J (Table 3.1 and Tun et al., 1994). The TC-box of the *ELA1* PTF1 binding site matches six of nine base pairs of the RBP-J consensus binding site, but the *Ctrb* site TC-box is nearly an exact match, with eight matching base pairs. Other PTF1 binding sites match fewer base pairs of the RBP-J consensus binding site, but have another TC- box

one helical turn away from the TC-box located proximal to the E-box (e.g.*rAMY* in Figure 1.6 and Table 3.1). However, RBP-J had not been identified as a component of the authentic PTF1 complex. RBP-L, a protein with high similarity to RBP-J, is a tissue-specific factor shown not to have an interaction with the intracellular domains of Notch1, 2, 3, or 4. Additional experiments used CASTing to show that the preferred RBP-L binding site is the same as the RBP-J consensus binding site (Minoguchi et al., 1997). Thus, RBP-J and RBP-L were good candidates for the unknown component of the PTF1 complex.

Gene	TC-box Sequence	bp Match
SUH/RBP-J consensus	TTTCCCACG	9
rTrpI	TCTCCAAGT	5
mTrpd	TTTCTCCTT	5
mTrpa*	TTTCTCTTT	5
rELA1	TTTCCCTGC	6
mELA2	TTTCCCAAA	7
rCtrb	TTTCCCATG	8
rAMY*	TTCTTCAGA	4
mAMY2.2*	ACTTTTAGA	2
rCPA-90	AGTCCCATG	6
rCPA-142	TTCCCCGAT	5

Table 3.1 The TC-boxes from PTF1 binding sites are similar to the RBP-J consensus binding site. Sequences are 5' to 3'. The asterisk represents TC-boxes that have another TC-box one helical turn downstream of the identified sequence.

Specificity of the anti-RBP antibodies in supershift EMSA

To test whether the antisera from rabbits immunized with the C-terminal RBP-L peptide antigen recognized the PTF1 complex, antisera from two different rabbits were tested in supershift EMSA reactions (Figure 3.2A). 1 μl anti-RBP-L serum from either rabbit A2165 or rabbit A2166 was added to separate binding reactions and compared to those with no antiserum or those with 1 μl preimmune serum. Antiserum from rabbit A2166 generated a strong, slower migrating band when added to binding reactions containing adult pancreas nuclear extract, whereas preimmune serum or no serum did not. The serum from rabbit A2165 generated a very faint supershifted band. The serum from A2166 was therefore the best choice for examining RBP-L within the context of the PTF1 complex.

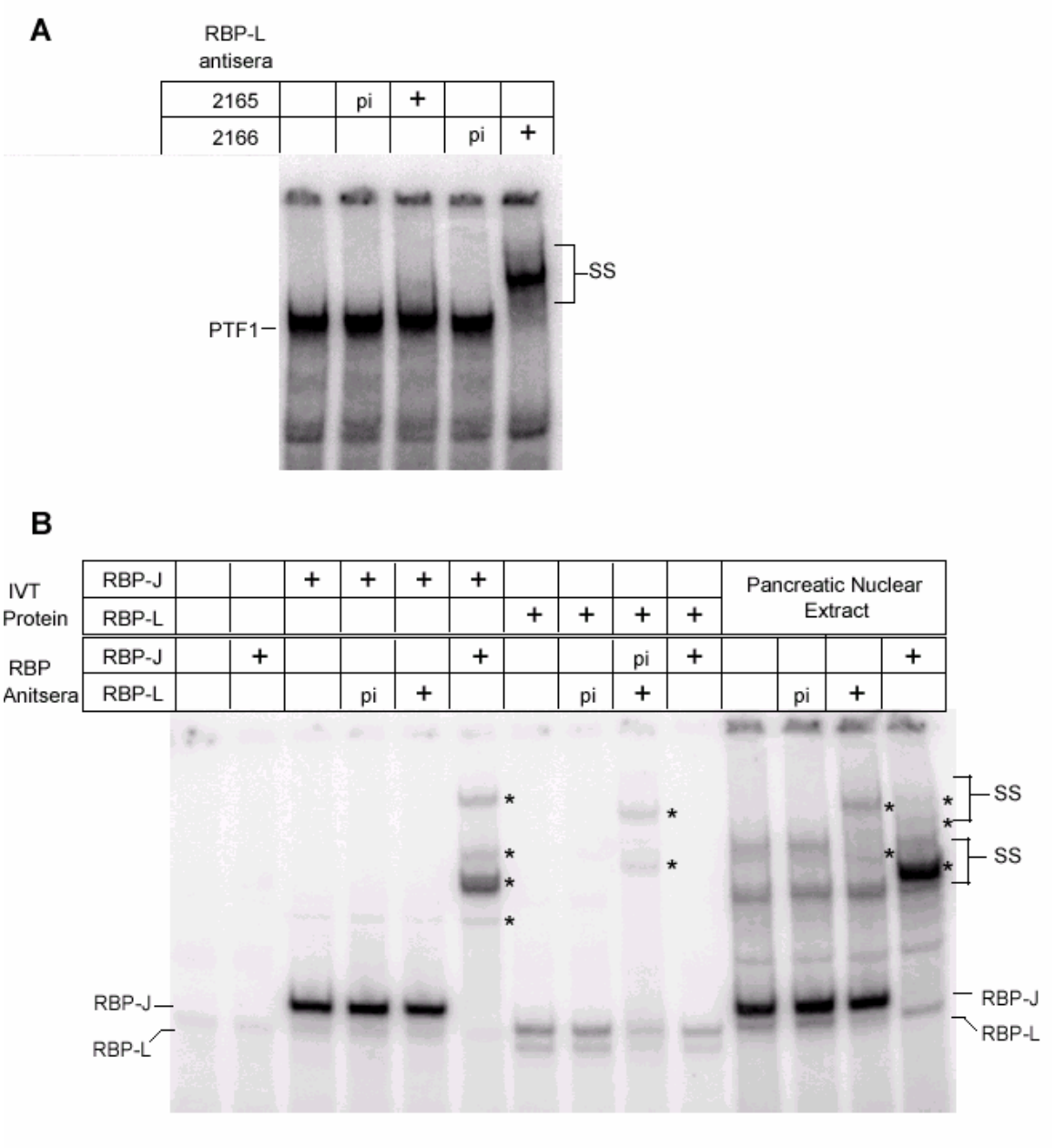


Figure 3.2 Antiserum specific for RBP-L and antibody specific for RBP-J do not cross-react.
A. Antisera from two rabbits immunized with an RBP-L peptide as an antigen were tested for their ability to supershift the PTF1 complex from adult pancreatic nuclear extracts by EMSA using the *ELA1* A element.
B. Anti-RBP-L antisera (2166) recognizes IVT and endogenous RBP-L, but not IVT or endogenous RBP-J. Anti-RBP-J antibody recognizes IVT and endogenous RBP-J, but not RBP-L. Analysis was by EMSA supershift using the O_{54} oligonucleotide. pi, pre-immune;; asterisks denote supershifted complexes (also indicated by SS). Each EMSA supershift reaction used 1 µl of antibody/antisera .

The specificity of the polyclonal anti-RBP-L antiserum and monoclonal anti-RBP-J antibody was tested using the recombinant RBP-J and RBP-L proteins in supershift EMSA experiments. To identify which RBP protein is in the PTF1 complex, it is essential to use individual antibodies or antisera that only interact with the specified protein and not both RBP proteins. The RBP-L antiserum is likely specific for RBP-L and not RBP-J because it was generated using a peptide from the C-terminus of RBP-L that is absent in RBP-J. However, available antibodies against RBP-J were generated either from the whole RBP-J protein or domains of RBP-J that are partially conserved with RBP-L. IVT RBP-L and RBP-J were bound in separate reactions to the double stranded oligonucleotide O_{54} (Figure 3.2B) which contains two RBP-J/RBP-L consensus binding sites (Minoguchi et al., 1997). The anti-RBP-L antiserum shifted the RBP-L-DNA complex, but not the RBP-J-DNA complex, while the anti-RBP-J antibody shifted the RBP-J-DNA complex, but not the RBP-L-DNA complex. In similar experiments using pancreatic nuclear extracts from adult rats, complexes with mobilities identical to those of recombinant RBP-L and RBP-J formed. The band with the mobility identical to the recombinant RBP-J complex was supershifted by the addition of anti-RBP-J antibody, while the band with the mobility identical to the recombinant RBP-L complex was supershifted by the addition of anti-RBP-L antibody. A slower migrating band was also supershifted with the addition of anti-RBP-L antibody. This is likely a multi-protein complex that contains RBP-L. These results show that antibodies against RBP-L and RBP-J do not cross-react in EMSA and that RBP-L and RBP-J proteins are indeed present in rat pancreatic nuclear extracts.

RBP-L is in the authentic PTF1 complex

In EMSA experiments with the *ELA1* PTF1-binding site, the authentic PTF1 complex migrated as a broad band. This suggested that PTF1 is a heterogeneous complex. To confirm the presence of P48 and an AbHLH as the known components of PTF1, I mixed antibodies specific for each of these proteins with separate binding reactions that contained pancreatic nuclear extract and the A element double-stranded oligonucleotide probe. Addition of an antibody that recognizes P48 to the binding reaction eliminated the PTF1 complex band completely and generated a much slower migrating band. The addition of antibodies that recognize specific individual AbHLH proteins to separate binding reactions supershifted a fraction of the PTF1 complex. The antibody that recognizes E12/E2.2, eliminated a fraction of the complex without giving a supershifted band. Addition of a mixture of all the antibodies that recognize specific AbHLH proteins to the same binding reaction eliminated nearly all of the complex, and generated bands of much slower mobility than PTF1 (Figure 3.3). These results indicate that authentic PTF1 is a population of similar complexes, each containing P48 and one AbHLH protein that can be HEB, E2-2, E12, or E47.

To determine whether RBP-J or RBP-L is part of the PTF1 complex, I tested whether antibodies against either protein could recognize the complex in nuclear extracts prepared from adult rat pancreas. The addition of antiserum, specific for RBP-L that does not recognize RBP-J, supershifted the entire PTF1 complex. (Figure 3.3) A monoclonal antibody that recognizes RBP-J but not RBP-L had no effect of the PTF1 band. Although previous experiments have shown that P48 can interact with RBP-J

(Obata et al., 2001), these results indicate that PTF1 from adult pancreas contains RBP-L in addition to P48 and one of the AbHLH proteins, but not RBP-J.

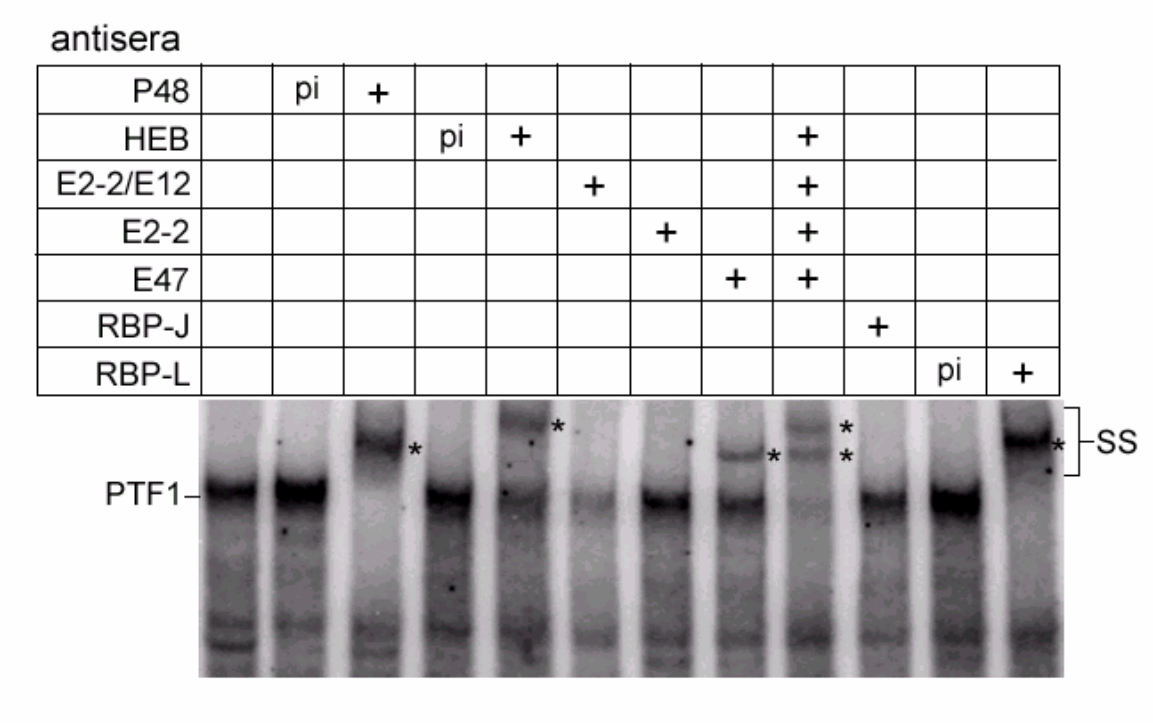


Figure 3.3 RBP-L is a subunit of the PTF1 complex.
EMSA supershift analyses of complexes with nuclear extract from adult rat pancreas using 1 μl of antisera or antibody to P48, HEB, E12, E47, E2.2/E12, RBP-J, or RBP-L. pi, pre-immune; asterisks represent supershifted complexes (also indicated by SS).

In vitro reconstitution of the PTF1 complex

P48 cannot bind the PTF1-site alone, but it can bind as a part of a heterodimer with any of several broadly expressed class A bHLH proteins (Figure 3.4B). The AbHLH proteins E47 and HEB form homodimers on the E-box in the absence of P48. Formation of complexes in EMSA by HEB-HEB and E47-E47 is due the production of

full length and truncated products in IVT reactions (Figure 3.4A). Addition of P48 to binding reactions containing HEB or E47 reduces or eliminates the formation of these homodimers and causes the formation of P48-HEB or P48-E47 heterodimers. E12 does not readily form homodimers (Figure 3.4B and Sun and Baltimore, 1991), but does form a heterodimer with P48. Mutations in the E-box eliminate heterodimer binding, but disruption of the TC-box has no effect on the formation of this complex (Rose and MacDonald, 1997; Rose et al., 2001 and Figure 3.7). These results demonstrate that P48-AbHLH binds to the E-box.

A complex with the same electrophoretic mobility as PTF1 could be reconstituted with *in vitro* synthesized P48, RBP-L, and any one of the AbHLH proteins, HEB, E12, or E47. All three of the possible P48 heterodimers have a faster electrophoretic mobility than PTF1 (Rose et al., 2001 and Figure 3.4B). Mixing P48 with E47 or HEB in EMSA experiments generated a heterodimer with a faster mobility than that of authentic PTF1 and a homodimer complex with a slower mobility than that of PTF1 (Rose et al., 2001 and Figure 3.4B). Addition of RBP-L to binding reactions containing P48 and an AbHLH protein generated a slower migrating band with the same mobility as the authentic PTF1 complex. Thus the formation of PTF1 requires the addition of RBP-L to the binding reaction with P48 and an AbHLH protein. The compositions of these reconstituted complexes were confirmed by the addition of antibodies against each of the components, in separate reactions.

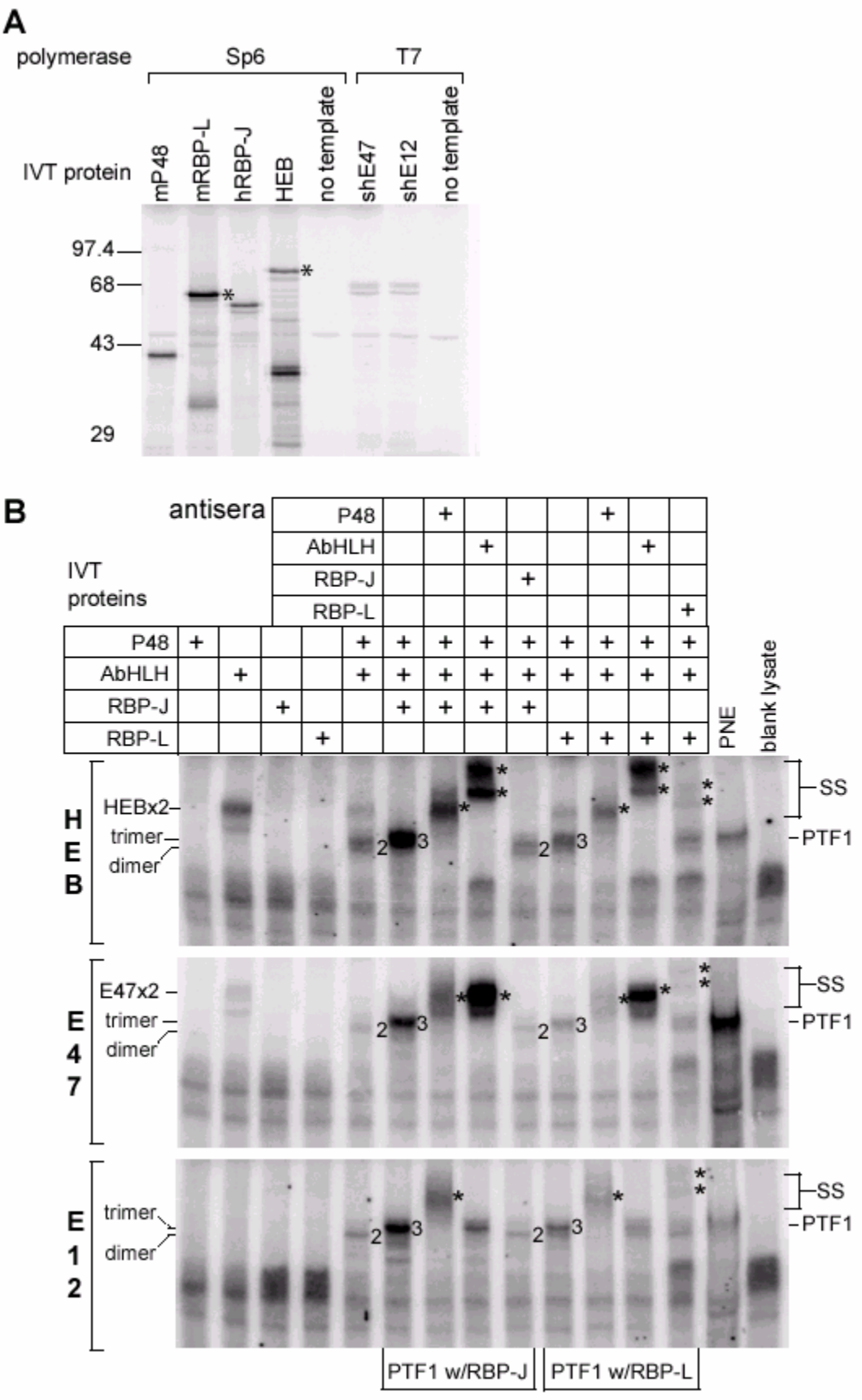

A
polymerase
Sp6
T7
IVT protein
mP48
mRBP-L
hRBP-J
HEB
no template
shE47
shE12
no template
97.4
68
43
29
B
antisera
P48
AbHLH
RBP-J
RBP-L
IVT proteins
PNE
blank lysate
HEB
HEBx2
trimer
dimer
E47
E47x2
trimer
dimer
E12
trimer
dimer
SS
PTF1
PTF1 w/RBP-J
PTF1 w/RBP-L

Figure 3.4 Reconstitution of complexes with the same mobility as authentic PTF1 using IVT proteins.
A. ^{35}S-Methionine IVT proteins separated on a 10% SDS-polyacrylamide gel. The primary full length IVT product is usually the most intense band with the larger size. The asterisks represents the full-length products of HEB and RBP-L.
B. IVT P48 forms heterodimeric complexes with HEB, E47, or E12, and trimeric complexes with the addition of RBP-L or RBP-J. Antibodies that recognize each component confirm their presence in the complex. 2, dimer; 3, trimer; asterisks represent supershifted complexes (also indicated by SS)

Although, only RBP-L was detected in the authentic PTF1 complex, a PTF1 complex with RBP-J can also form. Addition of IVT RBP-J to binding reactions containing P48 and an AbHLH generated a band with the same mobility as that of the authentic PTF1 complex. Each band representing the heterotrimeric complex was completely or partially eliminated by the addition of each antibody. Addition of each antibody also generated a slower migrating (supershifted band) except the E12/E2.2 and RBP-J antibodies where addition of the antibodies eliminated, or partially eliminated, the trimeric complex; likely by interfering with its formation.

RBP-J is excluded from the adult PTF1 complex

Even though a complex with the same mobility as PTF1 can be reconstituted with either RBP-L or RBP-J (Figure 3.4B), only RBP-L was detected in the PTF1 complex from pancreatic nuclei (Figure 3.3). I tested the relative abilities of RBP-J and RBP-L to form a PTF1 complex on the *ELA1* PTF1 binding site by reconstituting the complex in EMSA experiments with a mixture of both proteins and P48 and E12. In separate binding reactions, I mixed equimolar amounts of P48, E12, and one RBP protein to confirm the ability to form the trimer. To compare the relative abilities of the RBP proteins to integrate into the complex, I mixed equimolar amounts of IVT P48, E12, RBP-J, and RBP-L in the same binding reaction (Figure 3.5A). The heterodimer migrated as a single band, and each reconstituted trimer migrated with a slightly slower mobility than the heterodimer as expected. A mixture of all four proteins generated a complex with the same mobility as the three-protein mixture. The addition of the

antibody that recognizes P48 confirmed its presence in the complex. The monoclonal antibody that recognizes RBP-J eliminated nearly all of the trimeric complex, while addition of the antibody that recognizes RBP-L very little of the complex. Thus, it would seem that a PTF1-like complex containing RBP-J forms more effectively than one containing RBP-L, in apparent contrast to PTF1 from adult rat pancreatic nuclei.

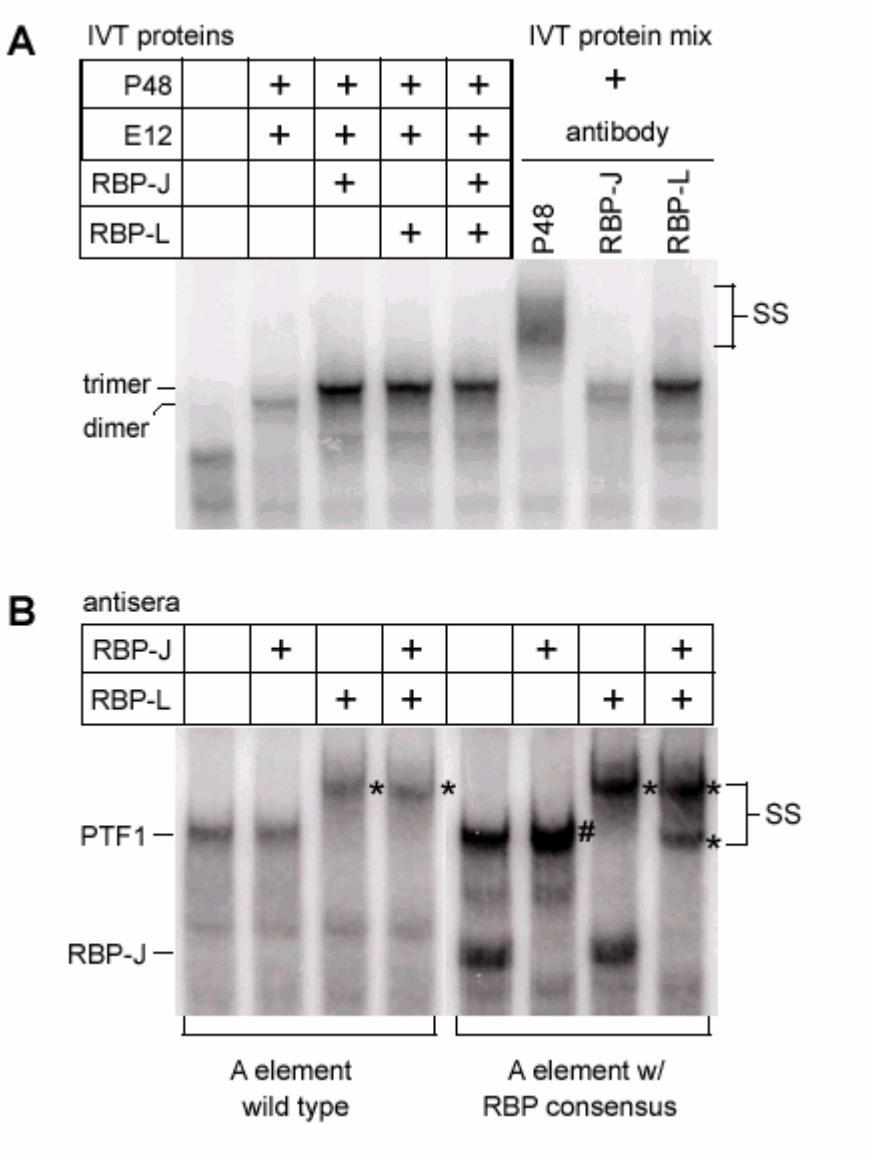


Figure 3.5 RBP-J is excluded from the PTF1 complex in adult pancreatic nuclear extracts.
A. IVT RBP-J forms a reconstituted PTF1 complex with P48 and E12 more effectively than IVT RBP-L does. PTF1 trimers were assembled by mixing equimolar amounts of IVT P48, E12, RBP-J and RBP-L. The relative amounts of trimer with P48 and RBP-J or RBP-L were estimated from the amount of band depletion with the subunit-specific antibodies.
B. RBP-J is in adult pancreatic nuclear extract, but not as part of a PTF1 complex. Complexes from nuclear extracts, bound to either the wild-type ELA1 PTF1-binding site or a site in which the TC-box was changed to the full consensus sequence for RBP-J, were incubated with antibody to either RBP-J or RBP-L. asterisks represent supershifted complexes (also indicated by SS); # indicates supershifted RBP-J monomer migrating with a similar mobility to the authentic PTF1 complex (compare with adjacent lanes).

As a more sensitive test for the presence of an RBP-J form of PTF1 (PTF1-J) in pancreatic nuclear extracts, I altered the TC-box and adjacent nucleotides of the *ELA1* PTF1 site to create the consensus binding sequence shared by both RBP-J and RBP-L (Hsieh et al., 1996; Minoguchi et al., 1997). Even though this sequence was shown to be the consensus binding sequence for both RBP-J and RBP-L, observations from EMSA experiments have indicated that IVT RBP-J binds this sequence as a monomer with a slightly higher affinity than that of RBP-L (Minoguchi et al., 1997). In duplicate EMSA experiments using the A element from the *ELA1* PTF1 binding site and the altered element with the optimized RBP-J binding site with pancreatic nuclear extracts, only RBP-L was detected as part of the authentic PTF1 complex (Figure 3.5B). The mutant with the RBP-J consensus binding site appeared to have a higher affinity for the PTF1 complex. In addition to the PTF1 band, experiments using the *ELA1* PTF1 with the optimal RBP-J binding site generated a much faster migrating band. Addition of the antibody that recognizes RBP-J did not have an effect on the PTF1 complex on the wild-type or mutant *ELA1* PTF1 binding site, but eliminated the fast migrating complex on present on the mutant binding site. Since the anti-RBP-J antibody has already been shown to recognize IVT RBP-J, this confirmed the presence of RBP-J in the nuclear extract. The addition of the antiserum that recognizes RBP-L supershifted all of the PTF1 complex on both probes, but it did not have an effect on the fast migrating RBP-J complex associated with the mutant binding site. The addition of a mixture of both antibodies supershifted both bands associated with the mutant probe and generated a band with approximately the same mobility as the PTF1 complex. Thus, the supershifted

RBP-J monomer had a sightly slower mobility than the authentic PTF1 band. Even though RBP-J is present in the nuclear extract, and the recombinant RBP-J binds its consensus site much better than recombinant RBP-L does, only the RBP-L containing form of PTF1 was detected.

Because the reconstituted PTF1-J complex binds more effectively than PTF1-L complex, the presence of only PTF1-L in nuclear extracts suggests that a mechanism exists in cells to exclude RBP-J from the PTF1 complex *in vivo* prior to DNA binding. Indeed, the *ELA1* promoter is enriched by immunoprecipitation of chromatin from adult rat pancreas with antibodies that recognize P48 and RBP-L, but not with an antibody that recognizes RBP-J (T. Masui, personal communication). This *in vivo* binding of P48 and RBP-L, but not RBP-J, to the *ELA1* enhancer gives further evidence for the exclusion of RBP-J from endogenous complex. Because RBP-J is endogenous to most tissues and cell lines, it is likely that it is excluded from PTF1 in favor of RBP-L for high-level transcriptional activity of PTF1. Although only RBP-L is detected in the PTF1 complex, there remains the possibility that a similar complex with RBP-J also exists either in organs expressing P48, but lacking RBP-L or during development of the pancreas at a stage before RBP-L is expressed.

RBP-L is essential for the high transcriptional activity of PTF1

A reporter driven by six copies of the A element is highly active in acinar pancreas of transgenic mice (Rose et al., 1994; Rose et al., 2001), but is only active at low levels in cell culture in the presence of exogenous P48 and a common AbHLH.

Forced expression of P48 and a common AbHLH such as HEB or E47 cannot activate to high levels a cotransfected reporter driven by the tandem repeats of a PTF1 binding site from the *ELA1* (6A.EIp.hGH) or *Ctrb* promoters (Obata et al., 2001; Rose et al., 2001). I tested whether expression of exogenous RBP-L could supply the missing transcriptional activation within the context of the PTF1 complex (Figure 3.6B). The human embryonic kidney cell line 293 has endogenous RBP-J, and the common AbHLH proteins, but lacks P48 and RBP-L (Figure 3.6A)**.** Expression of P48 by transient transfection only activates the 6A.EIp.luc reporter three-fold more than in the absence of P48. Co-expression of P48 and HEB in 293 cells activated the luciferase reporter driven by six copies of the *ELA1* PTF1 binding site 15-to 20-fold higher than without the exogenous transcription factors. However, co-expression of RBP-L with P48 and HEB increased this activation another 25-fold, to a total of 450-fold. In contrast, expression of exogenous RBP-J with P48 and HEB by cotransfection did not substantially change the extent of the activation by P48 and HEB alone. The addition of P48 and RBP-L without exogenous HEB was nearly as effective (320-fold activation) as the addition of exogenous P48, HEB and RBP-L, likely due to the presence of endogenous AbHLH proteins. Expression of HEB, RBP-L, or both in the absence of P48 did not increase the activity of the reporter to the levels present in the absence of exogenous transcription factors. Hence, RBP-L but not RBP-J provides the high transcriptional activation by the PTF1 complex.

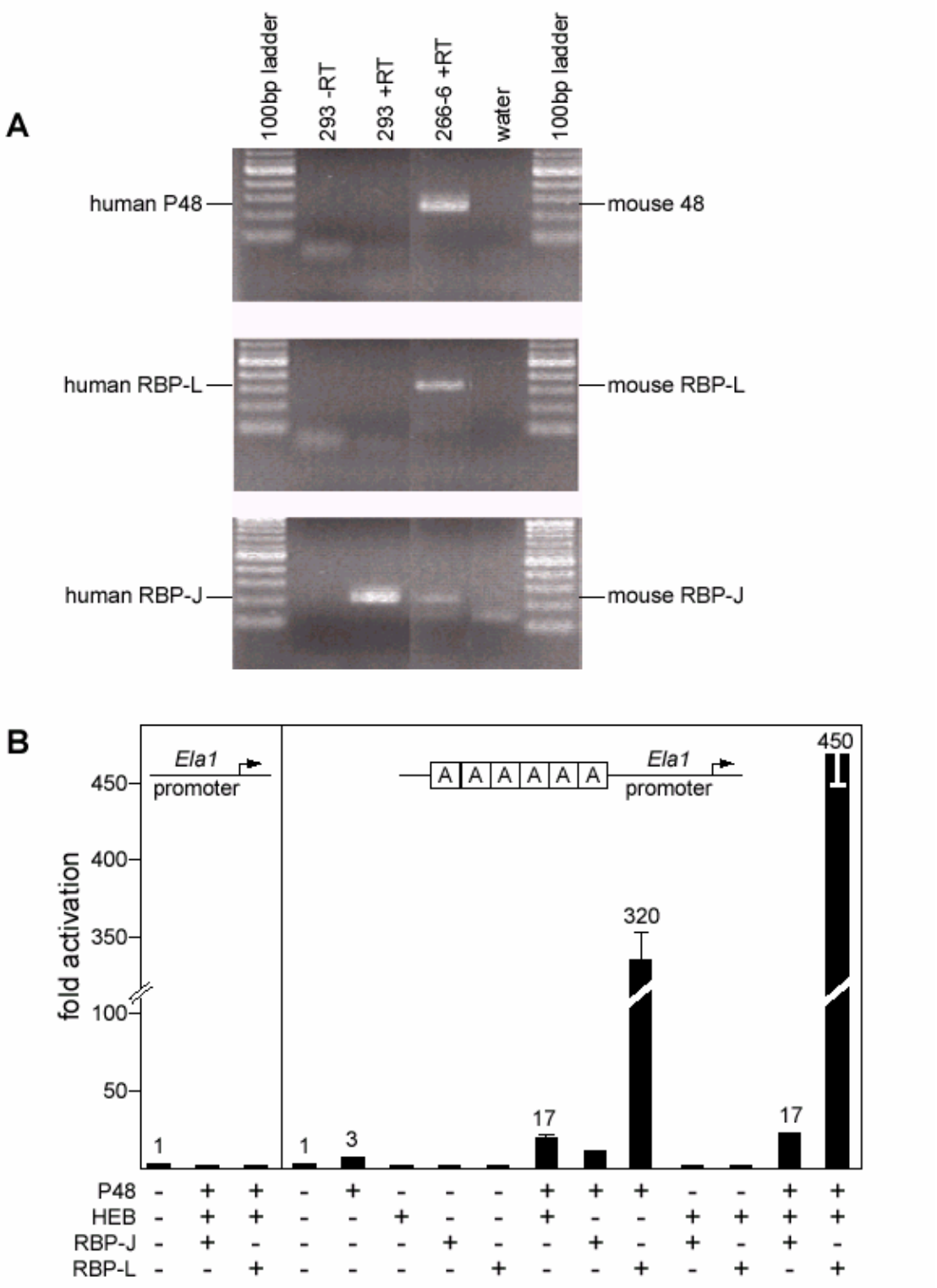
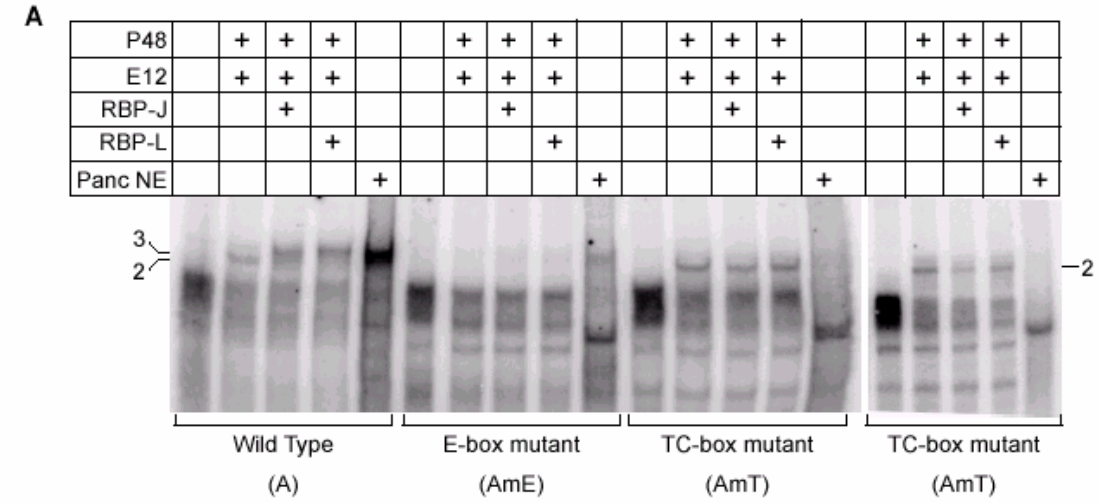

A
100bp ladder
293 -RT
293 +RT
266-6 +RT
water
100bp ladder
human P48
mouse 48
human RBP-L
mouse RBP-L
human RBP-J
mouse RBP-J
B
Ela1
promoter
A A A A A A
Ela1
promoter
fold activation
450
400
350
100
50
1
1
3
17
320
17
450
P48 - + + - + - - - + + + - - + +
HEB - + + - - + - - + - - + + + +
RBP-J - + - - - - + - - + - + - + -
RBP-L - - + - - - - + - - + - + - +

Figure 3.6 RBP-L is the subunit of PTF1 critical for high-level transcriptional activation of the *ELA1* A element.
A. RT-PCR analysis shows that 293 cells express *Rbp-J* but do not express *p48* or *Rbp*-L, while a mouse acinar cell line (266-6) expresses all three.
B. The relative ability of the 6A-EIp.luc reporter gene in 293 cells was assayed in the presence or absence of co-transfected P48, HEB, RBP-J, or RBP-L, individually or in various combinations as indicated. Transcription from the EIp.luc plasmid (containing only the *ELA1* minimal promoter from –92 to +8 inserted upstream of the luciferase gene) was unaffected by the addition of transcription factors via co-transfection. All values are the mean of at least four transfections. Error bars represent standard errors of the means.

The E-box and TC-box are essential for PTF1 binding and transcriptional activity

Mutation of each component of the *ELA1* PTF1 binding site disrupts binding of the authentic and reconstituted PTF1 complexes. Previous studies have shown that mutating the E-box to a non-E-box sequence eliminates binding of PTF1 from pancreatic nuclear extracts, as well as binding of a reconstituted P48-AbHLH heterodimer (Rose et al., 2001). Interestingly, mutating the E-box from CACCTG to CACTTG also has the same effect on authentic PTF1 binding to the *ELA1* PTF1 site. This E-box mutant is part of the PTF1 site from the human *ELA1* enhancer, which does not bind a PTF1 complex (Rose and MacDonald, 1997; Rose et al., 2001). Although the mutant sequence is an E-box, it is apparently not one that is preferred by P48. As an additional criterion, to establish the congruence of the authentic and reconstituted complexes, I performed EMSA reactions using IVT PTF1 component proteins or pancreatic nuclear extracts using the wild-type *ELA1* binding site or E-box mutant (Figure 3.7A). The single base pair mutation eliminated binding of the authentic PTF1 complex to this oligonucleotide probe. The same mutation also eliminated formation of a P48-AbHLH heterodimer as well as the reconstituted PTF1 complexes containing either RBP-L or RBP-J. Thus the E-box is essential for binding of both the heterodimer and the PTF1 complex.


A
P48
E12
RBP-J
RBP-L
Panc NE
3
2
2
Wild Type
(A)
E-box mutant
(AmE)
TC-box mutant
(AmT)
TC-box mutant
(AmT)

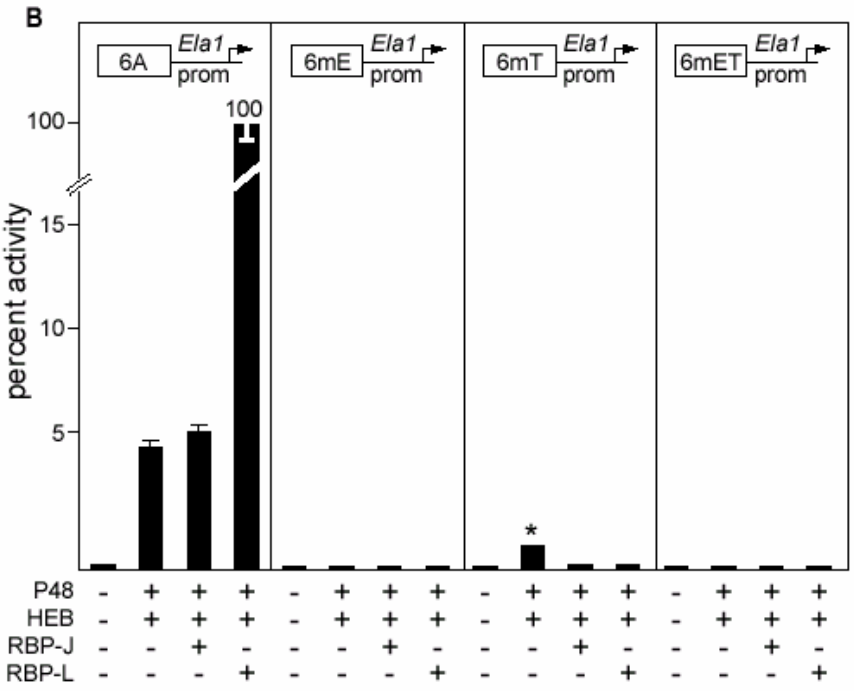

B
6A
Ela1
prom
6mE
Ela1
prom
6mT
Ela1
prom
6mET
Ela1
prom
100
15
10
5
percent activity
100
*
P48
HEB
RBP-J
RBP-L

Figure 3.7 The E-box and TC-box are necessary for PTF1 binding and transcriptional activity on the *ELA1* A element.
A. The binding of reconstituted and authentic PTF1 complexes to wild-type and mutant *ELA1* PTF1 binding sites. Addition of RBP-J or RBP-L in equimolar amounts with P48 and E12 inhibits binding of the heterodimer in the absence of a TC-box (far right panel). B. Activation of the 6A reporter is dependent on both the E-box and the TC-box. Activation of the wild-type A element (6A) was compared to activation of the E-box mutant (6AmE), the TC-box mutant (6AmT), or a mutant with both the E-box and the TC-box mutated (6AmET). The asterisk in the 6AmT panel highlights the reduced ability of exogenous P48 and HEB (with endogenous RBP-J) to activate the reporter in the absence of a TC-box. Addition of exogenous RBP proteins inhibits this activation (next two bars) due to the formation of trimer, which cannot bind an E-box alone. Values represent percent of wild-type activation, and are the mean of at least 3 transfections; error bars are the standard errors of the means.

Although RBP-L and RBP-J do not bind to the TC-box of the *ELA1* PTF1 binding site in the absence of an E-box, the TC-box is essential for PTF1 binding to this site. The TC-box of the *ELA1* PTF1 binding is similar to the consensus binding site shared by RBP-J and RBP-L. To test whether the TC-box is essential for binding of the reconstituted PTF1-L or PTF-J complexes, I performed EMSA experiments using IVT P48, E12, and RBP-L or RBP-J. Binding of the monomer of RBP-J to its consensus site can be eliminated by the mutation of two base pairs within this sequence (Hsieh and Hayward, 1995; Hsieh et al., 1996). Additionally, DNaseI footprinting showed that specific residues, particularly the C's, on the TC-box are contacted by the PTF1 complex (Cockell et al., 1989). The *ELA1* PTF1 binding site is already a departure from an RBP-J consensus site. Thus, I mutated the last two of the three adjacent C residues in the TC-box to disrupt the binding of PTF1. Only the P48-E12 heterodimer was formed on the mutant site. These results show that critical residues within the TC-box, but not necessarily a full consensus RBP-J binding site, are required for PTF1 binding to the *ELA1* PTF1 site.

To verify that the complete trimeric PTF1 complex is responsible for the activation of PTF1-target genes, I tested whether the transcriptional activation of the 6A.EIp.luc reporter in transfected cells had the same DNA sequence requirements that the complex has for binding DNA in EMSA experiments (Figure 3.7B). Indeed, altering the *ELA1* PTF1 binding site in the same manner that eliminated PTF1 binding in EMSA also eliminated transcriptional activation by transfected PTF1 components. The mutation of the TC-box that still allows the binding of a P48-AbHLH heterodimer, but eliminates binding of PTF1 in EMSA experiments retained detectable, but very low, activation.

This level of activation is 4-fold less than the activity induced by exogenous cotransfected P48 and HEB on a wild-type PTF1 binding site. This suggests that the endogenous RBP-J contributes to the binding of P48-HEB when an effective TC-box is present. This is likely because the PTF1 complex forms in solution, and is precluded from binding to an E-box without a paired TC-box. Consistent with this interpretation, RBP-L can be coimmunoprecipitated from pancreatic nuclear extracts with an antibody that recognized P48 (T. Masui, personal communication). The addition of exogenous RBP-J or RBP-L further reduced activation of the TC-box mutant. These results indicate that the association of RBP-J with P48 and HEB enhances binding but does not greatly enhance the activation potential of the complex, while RBP-L contributes both to complex binding and high-level activation of PTF1 targets.

Authentic and reconstituted PTF1 complexes have unique but flexible DNA binding requirements

The DNA-binding properties of PTF1 are unique compared to other bHLH factor complexes. Canonical bHLH complexes of class B bHLH-AbHLH heterodimers or AbHLH-AbHLH homodimers require a six base pair E-box plus a bias for contributions from perhaps one or two flanking base pairs (Blackwell and Weintraub, 1990). In contrast, the trimeric PTF1 complex requires a TC-box positioned one or two base-pairs away from an E-box (Cockell et al., 1989; Rose et al., 2001). A single base-pair change in the E-box or a two base-pair mutation that disrupts the near RBP-consensus present in the TC-box can prevent binding of the trimeric PTF1 complex (Rose et al., 2001 and Figure 3.7A). These unusual DNA-binding properties of the trimeric complex are

independent of the identities of the class A bHLH partner and the RBP protein. The binding of a P48-AbHLH heterodimer alone is unaffected by changes in the TC-box, but a full PTF1 complex does not bind a lone E-box. Thus, the association with RBP-L or RBP-J must alter the DNA binding properties of a P48-HLH heterodimer such that the binding of the trimer now requires the TC-box as well.

To investigate the binding properties of PTF1 and its subunits to various PTF1 target sites, I performed EMSA using IVT PTF1 component proteins or pancreatic nuclear extracts. I used oligonucleotide probes containing six different PTF1 binding sites from the promoters of various digestive enzyme genes: rat elastase I (r*ELA1*), mouse elastase 2 (m*ELA2*), rat carboxypeptidase A1 (r*Cpa1*), rat chymotrypsin B (r*Ctrb*), and mouse trypsin D (m*Trpd*). Although all of these sites bound the authentic and reconstituted PTF1 complex, they showed variable affinities for the individual subunits of PTF1 (Figure 3.8). The results of these EMSA experiments revealed four categories of functional PTF1 binding sites. The first category is PTF1 binding sites that bind P48-AbHLH heterodimers, but not RBP proteins as separate monomers. These sites, including r*ELA1*, r*CPA*-142, and m*Trpd*, generated a P48-heterodimer band in the absence of RBP proteins, but not an RBP protein band in the absence of the heterodimer. The second category is one in which the PTF1 site binds an RBP protein in the absence of the P48 heterodimer, but does not bind the P48 heterodimer in the absence of an RBP protein. An example of this site is m*ELA2*.

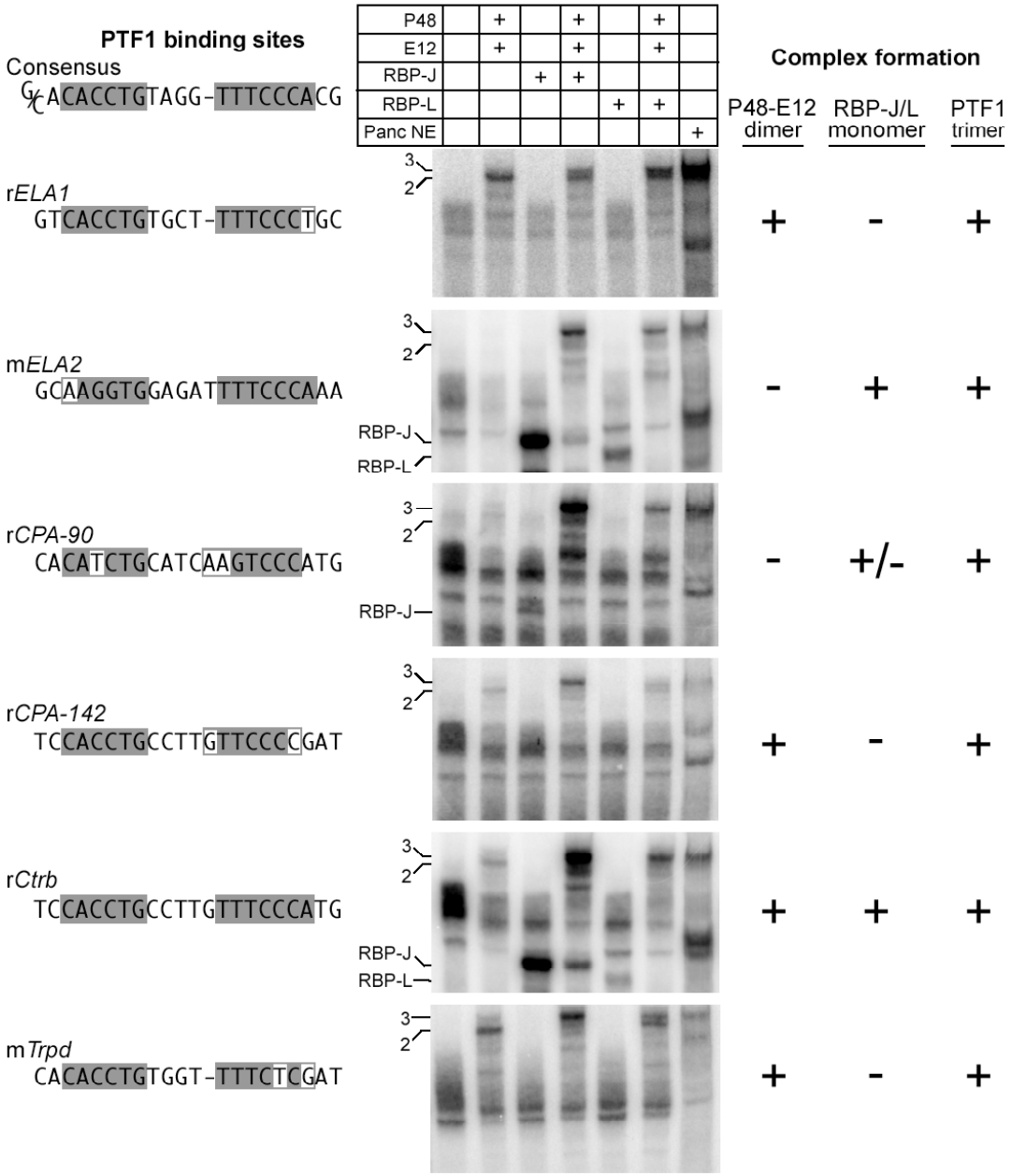


Figure 3.8 DNA binding of the PTF1 subunits is cooperative.
Reconstituted and authentic PTF1 binds to PTF1 sites from the promoter regions of several digestive enzyme genes. The amount of complex formation depends on the particular sequence of the E-box or the TC-box, but all bind the trimer. 3, PTF1 trimer; 2, P48-E12 dimer

The third category is one that binds PTF1 but does not efficiently bind either individual subunit separately, as shown by the r*CPA*-90 site. In EMSA experiments, this site did not bind the P48 heterodimer, and binding of an RBP protein was very weak, but the trimer binding is strong. The final category is one in which the PTF1 site binds both the full PTF1 complex as well as either subunit separately, such as r*Ctrb*. Sequence analysis and footprinting experiments showed that there are multiple digestive enzyme genes that have PTF1 binding sites, containing both an E-box and a TC-box one or two (like amylase) helical turns apart (Cockell et al., 1989). The sequences of the E-boxes and TC-boxes are often not optimal consensus binding sites for individual subunits of the PTF1 subunit. Thus binding of the three subunits to DNA is highly cooperative.

P48 and RBP-L are selectively expressed at high levels in the pancreas

Both P48 and RBP-L have organ-restricted patterns of expression (Cockell et al., 1989; Minoguchi et al., 1999; Minoguchi et al., 1997; Rose et al., 2001). Additionally, high-level expression of the genes for hydrolytic enzymes such as *ELA1* is limited largely to the acinar pancreas (Harding and Rutter, 1978; MacDonald et al., 1986; Rose et al., 1994; Rose et al., 2001). To examine whether the expression of P48 and RBP-L is spatially coincident, I used RT-PCR to survey for the presence of mRNA for *p48*, *Rbp-L*, and *Rbp-J* in RNA from nineteen adult mouse organs (Figure 3.9A). *p48* and *Rbp-L* are co-expressed at high levels in the pancreas and at low levels in the duodenum. Additionally, *p48* mRNA was also detectable in stomach and *Rbp-L* mRNA in brain and lung. Unlike a previous RT-PCR survey (Minoguchi et al., 1997), *Rbp-L* message was

not detected in the spleen. *Rbp-J* message was detected in all tissues screened. The high-level co-expression of *p48* and *Rbp-L* transcripts in the pancreas implies an opportunity for complex formation that is highly specific. Furthermore, I screened for the presence of *Rbp-L* message in RNA from human pancreas at various stages of development, as well as purified human islets by RT-PCR (Figure 3.9B). *Rbp-L* message was detected in pancreatic tissue from each stage and as well as in RNA from purified human islets.

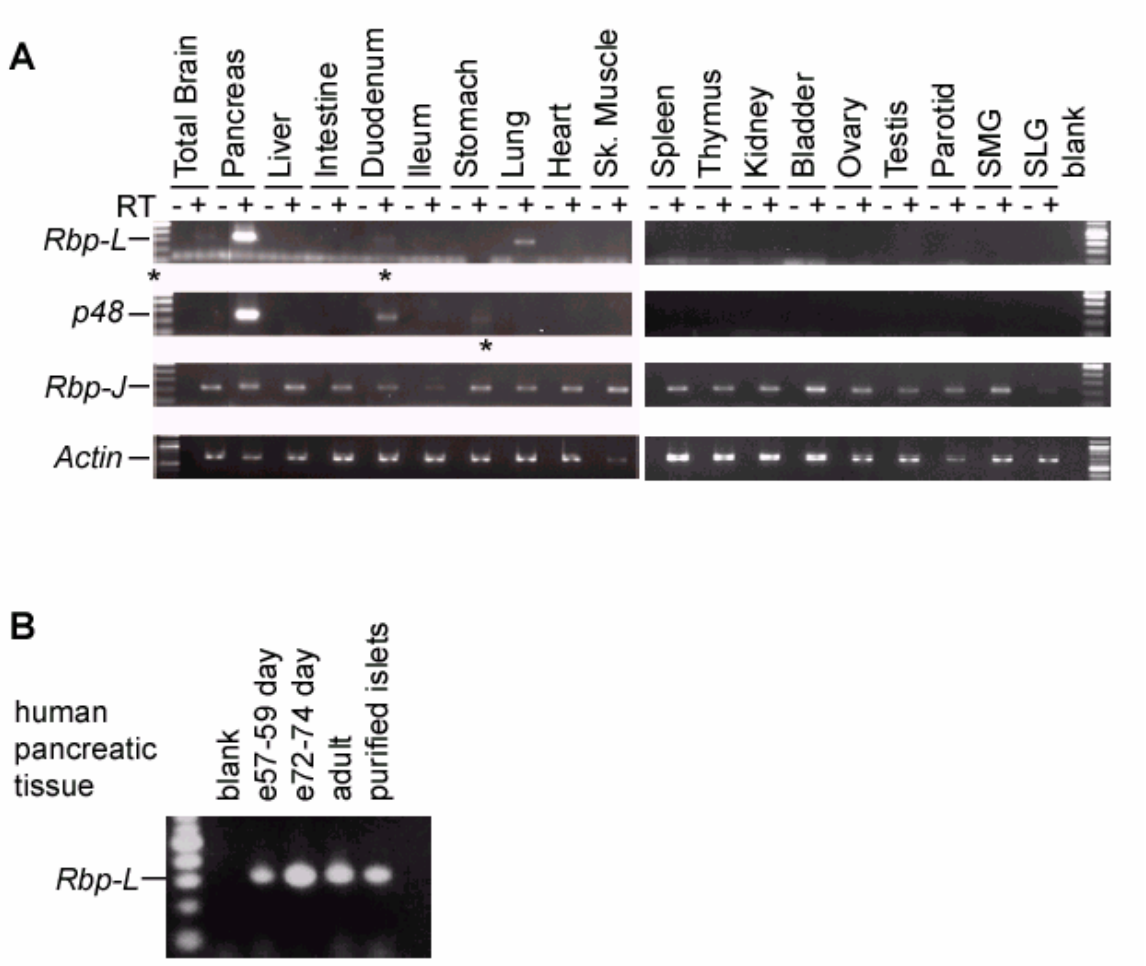


Figure 3.9 Distribution of *Rbp-L*, *P48* and *Rbp-J* expression.
A. *Rbp-L* and *p48* transcripts are present at high levels selectively in the pancreas. Amplified products from RT-PCR assays using RNAs isolated from nineteen adult mouse organs shows that *Rbp-L* transcripts, like *p48*, transcripts are present at high levels in the pancreas. Asterisks: low level of *Rbp-L* mRNA in the duodenum and brain and *p48* mRNA in the stomach. *Rbp-J* was detected in all organs tested. B. *Rbp-L* transcript is detected in human embryonic and adult pancreas. Amplified products from RT-PCR assays using RNA isolated from human embryonic or adult whole pancreas or purified islets.

Summary

PTF1 is a heterogeneous complex that contains two tissue-specific components. Previous studies have indeed shown that PTF1 can contain the AbHLH proteins E47 or E12 or HEB (Rose et al., 2001), but it was thought that two AbHLH proteins might be components of the complex, E47 or E12 and a pancreas-specific splice variant of HEB (Rose et al., 2001 and P.K. Wellauer unpublished). I have shown that HEB can be part of the PTF1 complex, but only as a single AbHLH in the trimer. Instead of a bHLH protein, the third PTF1 subunit, RBP-L, is a protein with high similarity to the ubiquitously expressed DNA-binding protein, RBP-J. Interestingly, although both RBP-J and RBP-L are present in pancreatic nuclear extracts, and a complex with the same mobility as authentic PTF1 can include P48, AbHLH, and RBP-J, only RBP-L is part of the PTF1 complex. This implies the presence of a mechanism of excluding the low activity RBP-J so the complex can integrate the high activity RBP-L. This complex defines a mechanism for employing RBP proteins for transcriptional activation in the absence of Notch signaling. This activation is important for expressing the digestive enzymes to the high levels necessary for the digestive function of the pancreas.

Binding and transcriptional activation of PTF1 target sequences by reconstituted and authentic PTF1 complexes requires both an E-box and a TC-box. Mutations to the E-box that eliminate the binding of the P48 heterodimer to the *ELA1* PTF1 binding site also eliminate binding and activation by the full PTF1 complex. Although, the TC-box present in the *ELA1* PTF1 binding site does not bind RBP-proteins in the absence of the

E-box, specific base pairs are conserved within the TC-box that allow for the cooperative binding of RBP-proteins in context of the other PTF1 subunits. Activation of the *ELA1* luciferase reporter by the exogenous expression of PTF1 components in cell culture has the same requirement for a TC-box as binding of PTF1 does in EMSA. Indeed, mutation of the TC-box in this reporter greatly reduces already low-level activity by the cotransfected P48 and HEB, showing that the integration of an RBP-protein is essential for effective binding of the trimer. The additional expression of an RBP-protein actually inhibits the activity of the reporter. This and co-immunoprecipitation experiments (T. Masui, personal communication) show that the PTF1 complex forms in solution and this conformation inhibits binding to lone E-boxes. Interestingly, the high variability of target PTF1 sites in digestive enzyme gene promoters shows that PTF1 binding is highly cooperative and does not require the ability of individual subunits to bind their cognate sites independently.

The formation of PTF1 is restricted by the availability of its subunits. High levels of *p48* and *Rbp-L* transcript are coincident exclusively in the pancreas. While transcripts from both genes are also present individually in other tissues, high-level of co-expression is limited to the pancreas, with low-levels of both in the duodenum. Immunofluorescence experiments using pancreatic sections from adult mouse showed that the P48 and RBP-L proteins are co-expressed in the nuclei of pancreatic acinar cells. These results also showed localization of RBP-L in the nuclei of islet cells, but no P48 (Ling Shi, personal communication). Finally, chromatin immunoprecipitation (ChIP) experiments have shown that both P48 and RBP-L are bound to several of these digestive enzyme gene promoters in pancreatic chromatin, but not liver chromatin (T. Masui, personal

communication)**.** Logically, PTF1 can only bind its target genes in tissues where all PTF1 subunits are expressed. Therefore both P48 and RBP-L might function through different mechanisms in other tissues, but PTF1 containing RBP-L is only present and functional in the acinar pancreas.

Chapter 4

Structure and Organization of the Pancreatic PTF1 complex

Introduction

Both RBP-L and RBP-J can be part of the PTF1 complex, but only RBP-L was identified as part of the authentic complex from adult pancreas. The results of tranfections experiments revealed that the RBP-L form of PTF1 is a complex with high transcriptional activity, while RBP-J has much lower transcriptional activity (Figure 4.1). Both forms of the PTF1 complex require the E-box and TC-box of the bipartite PTF1 site for binding and transcriptional activity by this complex, but the domains necessary for interaction between protein components have not been elucidated.

To fully understand how this trimeric complex mediates the activity of the A element in acinar cells, it is essential to first understand how the PTF1 complex is organized, particularly when bound to DNA. Important goals include defining which proteins in the complex interact, which domains are essential for these interactions, which domains are essential for transcriptional activity, and whether the complex interacts with other proteins. Some of the protein-protein interactions within PTF1, such as the P48-AbHLH interaction are already known (Rose et al., 2001), but the interactions between P48 and RBP-L or RBP-J have not been determined.

P48 has a basic helix hoop helix (bHLH) domain, similar to those of other class B bHLH proteins, that is essential for heterodimerization with AbHLH proteins and binding to E-boxes (Krapp et al., 1996; Murre et al., 1989a; Rose et al., 2001). Class A bHLH proteins also contain a bHLH domain that is used to dimerize with other AbHLH proteins

or with tissue-specifically expressed class B bHLH proteins (Rose et al., 2001; Sawada and Littman, 1993; Takeuchi et al., 2001). No other functional domain of P48 has been explicitly defined. More recently, the C-terminal region was shown to be necessary for P48 activity in pancreatic and cerebellar development, (Sellick et al., 2004), but the function of this domain is not clear.

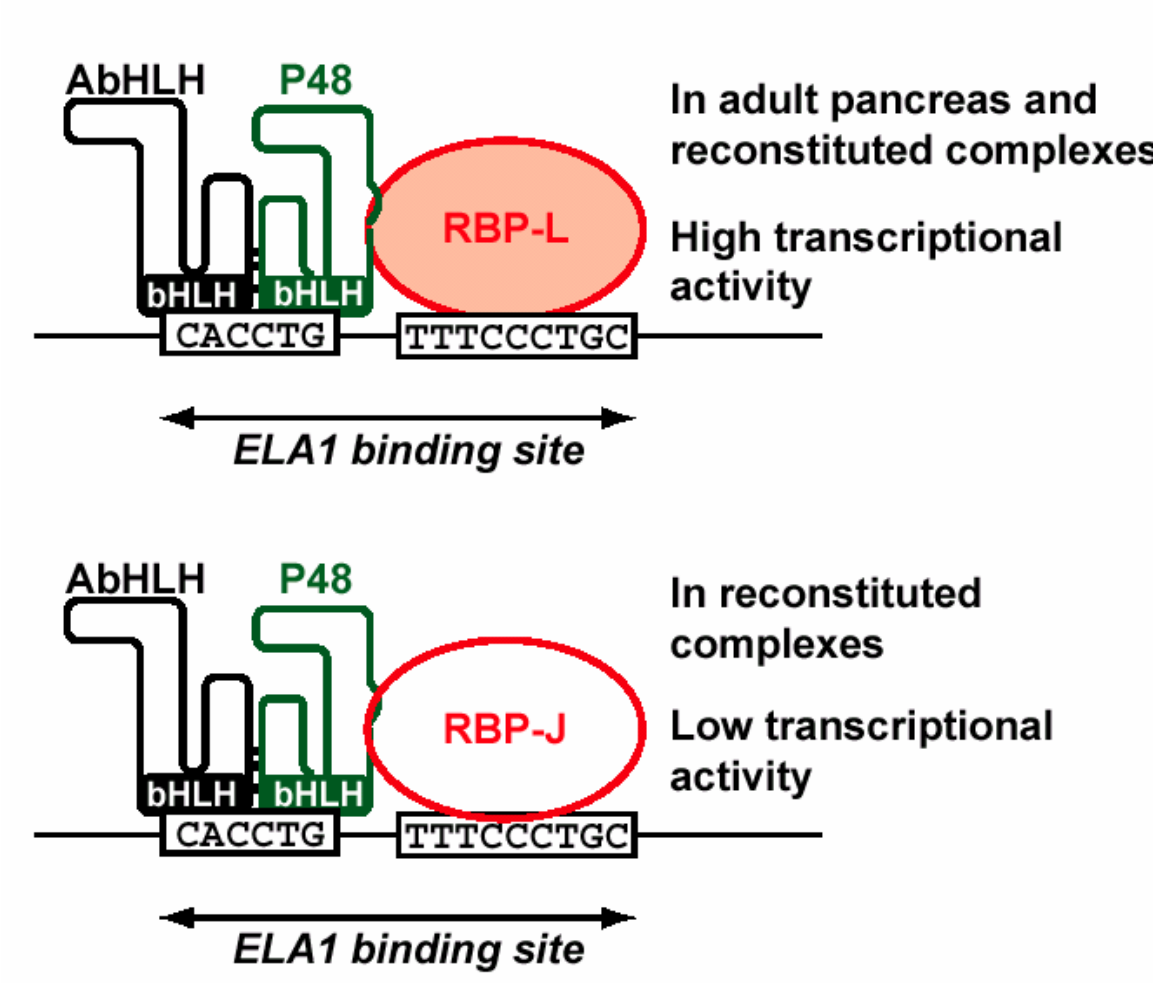


Figure 4.1 A model for two forms of PTF1 that have different transcriptional activity.
The PTF1 complex can be reconstituted with P48, an AbHLH protein, and either RBP-L or RBP-J. The RBP-L complex (present in adult pancreatic nuclear extracts) has high transcriptional activity, while the RBP-J complex has lower transcriptional activity.

To define the interactions between P48 and RBP-L, I created mutants of mouse P48 and RBP-L, and assayed their ability to bind DNA using EMSA experiments. Furthermore, I tested the ability of mutant P48 and RBP-L to activate the 6A.EIp.luc reporter. Because mutations in human *p48* that cause truncations of the C-terminus of the P48 protein are associated with loss of pancreatic and cerebellar development (Sellick et al., 2004), I tested whether these mutant human proteins, in a complex with HEB and an RBP protein, are able to activate the 6A.EIp.luc reporter.

RBP-J and RBP-L are highly similar DNA-binding proteins. Both proteins contain an N-terminal Rel-Homology region (RHR), a central beta trefoil region (BTF), and a C-terminal RHR. Specific regions within the N-terminal RHR and the BTF are essential for DNA binding (Kovall and Hendrickson, 2004). These domains are highly conserved between RBP-L and RBP-J. RBP-J has been shown to interact with the NotchIC and mediate activation of genes targeted by the Notch signaling pathway (Artavanis-Tsakonas et al., 1999; Fryer et al., 2002). In contrast, RBP-L does not interact with the NotchIC and has been implied to be a constitutive activator (Minoguchi et al., 1997; Tani et al., 2001). Both the N- and C-termini of these proteins are highly divergent. This may account for the inability of RBP-L to bind the NotchIC and for its ability to activate transcription in the absence of Notch signaling. The N- and C- termini of RBP-L may contain an activation domain not present in RBP-J. RBP-J interactions with the NotchIC as well as other molecules like SKIP, SMRT, MAM1 have been extensively studied (Fryer et al., 2002; Hsieh et al., 1996; Hsieh et al., 1999), and further studies to define the domains that interact with other proteins are ongoing. RBP-L, however, has not been extensively studied. Because a PTF1 complex can be

reconstituted with RBP-J as well as RBP-L, and RBP-J has the ability to interact with the NotchIC, it is possible that the NotchIC may associate with the PTF1-J complex and modify its activity. I tested the ability of the NotchIC to bind to and modify the transcriptional activity of the RBP-L and RBP-J forms of PTF1. Additionally, I tested the ability of the NotchIC to modify PTF1-J transcriptional activity. I also tested the ability of P48 to bind to an RBP-J-NotchIC complex on DNA and affect RBP-J mediated Notch activation. The results of these competition experiments confirmed that the NotchIC can have a direct effect on the activity of the PTF1 complex and P48 can have a direct effect on the RBP-J-NotchIC complex *in vitro*.

Results

Two Peptides in the C-terminal domain of P48 are conserved.

Examining which domains are conserved in orthologues from phylogenetically different organisms offered insight into which domains of P48 might be important for its interaction with RBP proteins. Comparison of the amino acid sequences of mouse and zebrafish P48 and *Drosophila* Fer1 revealed previously unidentified conserved domains (R.J. MacDonald, personal communication summarized in Figure 4.2). The basic-helix-loop helix domains are highly conserved in all three organisms. Mouse and zebrafish share a broad level of conservation in the C-terminal region of the protein. Other than the bHLH region, the only domains that share amino acid sequence conservation between all three organisms are two short peptides located near the C-terminus (C1, HSLSW and C2, WTPEDPR). Both the sequence and spacing between these peptides are highly conserved. In mouse and zebrafish, these domains are within the broadly conserved domain of the C-terminal region of P48. These peptide sequences are within an important functional domain of P48 (Sellick et al., 2004) and are candidates for the RBP-interaction domains.

Because *Drosophila* Su(H) shares high sequence conservation with its mammalian orthologue RBP-J, and *Drosophila* does not have an RBP-L protein, it is likely that Su(H) might substitute for the function of RBP-J or RBP-L in the context of a PTF1-like complex. To determine whether *Drosophila* Su(H) might also be functional in a PTF1-like complex, I tested whether Su(H) could activate transcription of the *ELA1*

reporter when cotransfected with P48 and HEB into 293 cells (Figure 4.3). EMSA experiments had shown that the *Drosophila* orthologues of P48, E12, and RBP-J (Fer1, DA, and Su(H) respectively) form a trimeric complex on the *ELA1* PTF1 binding site (T. Masui, personal communication). Additionally, like mammalian PTF1, the *Drosophila* trimeric complex did not bind the mutant versions of the E-box and TC-box mutant *ELA1* PTF1 site. Mixtures of IVT mammalian and *Drosophila* orthologues could also form complexes on the *ELA1* PTF1 binding site, further showing the conservation of PTF1 components (T. Masui, personal communication). Indeed, co-expression of Su(H) with P48 and HEB in 293 cells activated the *ELA1* PTF1 reporter to more than 70-fold over levels in the absence of exogenous transcription factors. The extent of this activation was substantially greater than the activation induced by the addition of exogenous mammalian RBP-J, but less than that induced by the addition of RBP-L. The phylogenetic conservation of composition and function of PTF1 indicates that PTF1 is likely an important regulator in invertebrates as well as mammals.

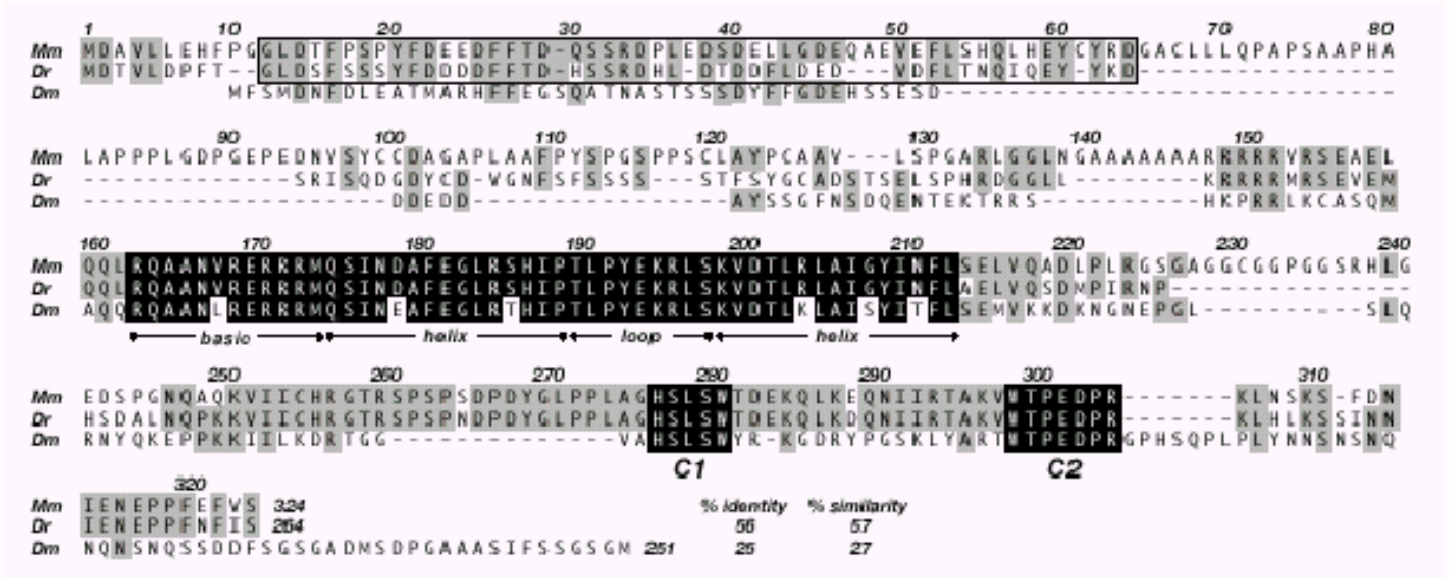


Figure 4.2 Two peptide regions near the C-termuns of P48 are conserved from mammals to fruit flies.
Alignment of the amino acid sequences from mouse P48, zebrafish P48, and *Drosophila* Fer 1. The black shading represents the bHLH domain (required for heterodimerization with class A bHLH proteins and DNA binding) and peptides C1 and C2 (which are conserved among all three organisms). Gray shading represents lower conservation. Note the broad area of gray shading at the C-terminus of of P48 from mouse and zebrafish.

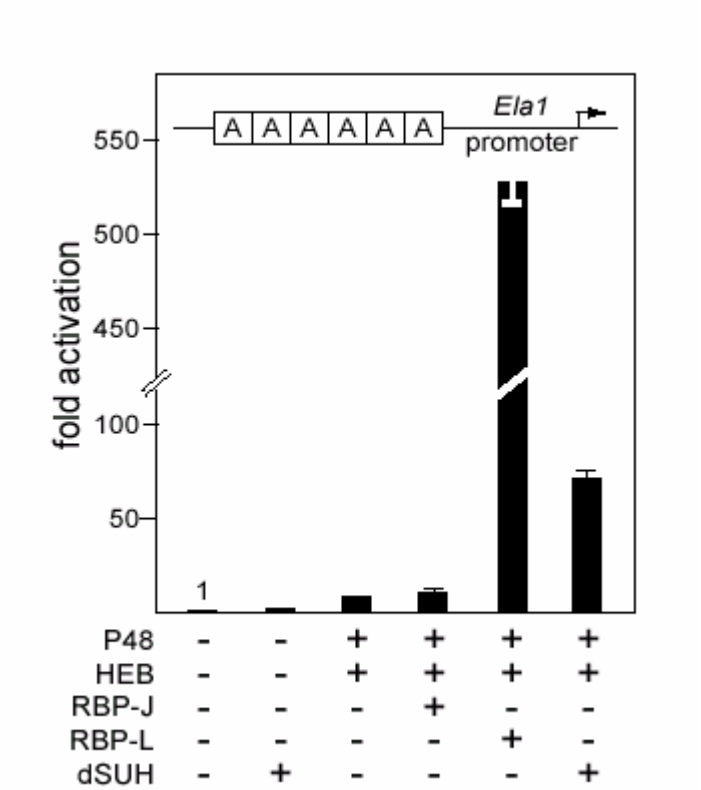


Figure 4.3 The function of PTF1 is conserved between mammals and flies.
The relative activity of the 6A.EIp.luc reporter gene in 293 cells was assayed in the presence or absence of cotransfected P48, HEB, RBP-J, RBP-L, or Su(H) in the combinations indicated. All values are the means of 4 transfections. Error bars represent standard errors of the means.

Two Peptides in the C-terminal domain of P48 are essential for interaction with RBPJ/L.

To determine whether the conserved domains from the C-terminus of P48 are essential for its interaction with RBP-proteins and formation of the PTF1 complex, I tested whether mutations in these domains disrupted the formation of a complete, DNA binding, PTF1 complex. I created deletions and point mutations in the C1 and C2 domains, as well in the region N-terminal to the C1 peptide (Figure 4.4). I mixed the wild-type or mutant IVT P48 proteins with IVT E12 and RBP-J or RBP-L in EMSA experiments (Figure 4.5). The deletion of either C-terminal peptide had no effect on the formation of DNA-binding heterodimers with E12, but these mutations did have effects on the formation of the reconstituted PTF1 trimers. The deletion of the C2-peptide region prevented the association of RBP-J, but had little or no effect on the association of RBP-L. Deletion of the C1 peptide completely eliminated the recruitment of RBP-L to the complex, but also partially disrupted the incorporation of RBP-J. Because tryptophan residues often play key roles in the formation of other transcription factor complexes (Knoepfler et al., 1999; Liu et al., 2001) I tested whether the tryptophan residues in each of the peptides were necessary for P48 recruitment of RBP-J or RBP-L to the reconstituted PTF1 complexes. Similar to the C2 peptide deletion, an alanine substitution for tryptophan 298 eliminated the recruitment of RBP-J but not RBP-L to the complex. Substitution of an alanine for tryptophan 280 partially disrupted recruitment of RBP-J but had no effect on the recruitment of RBP-L. Thus, the incorporation of RBP- proteins into the PTF1 complex requires the conserved C-terminal peptides of P48. However, the C2

peptide is more important for the recruitment of RBP-J and the C1 is more important for the recruitment of RBP-L.

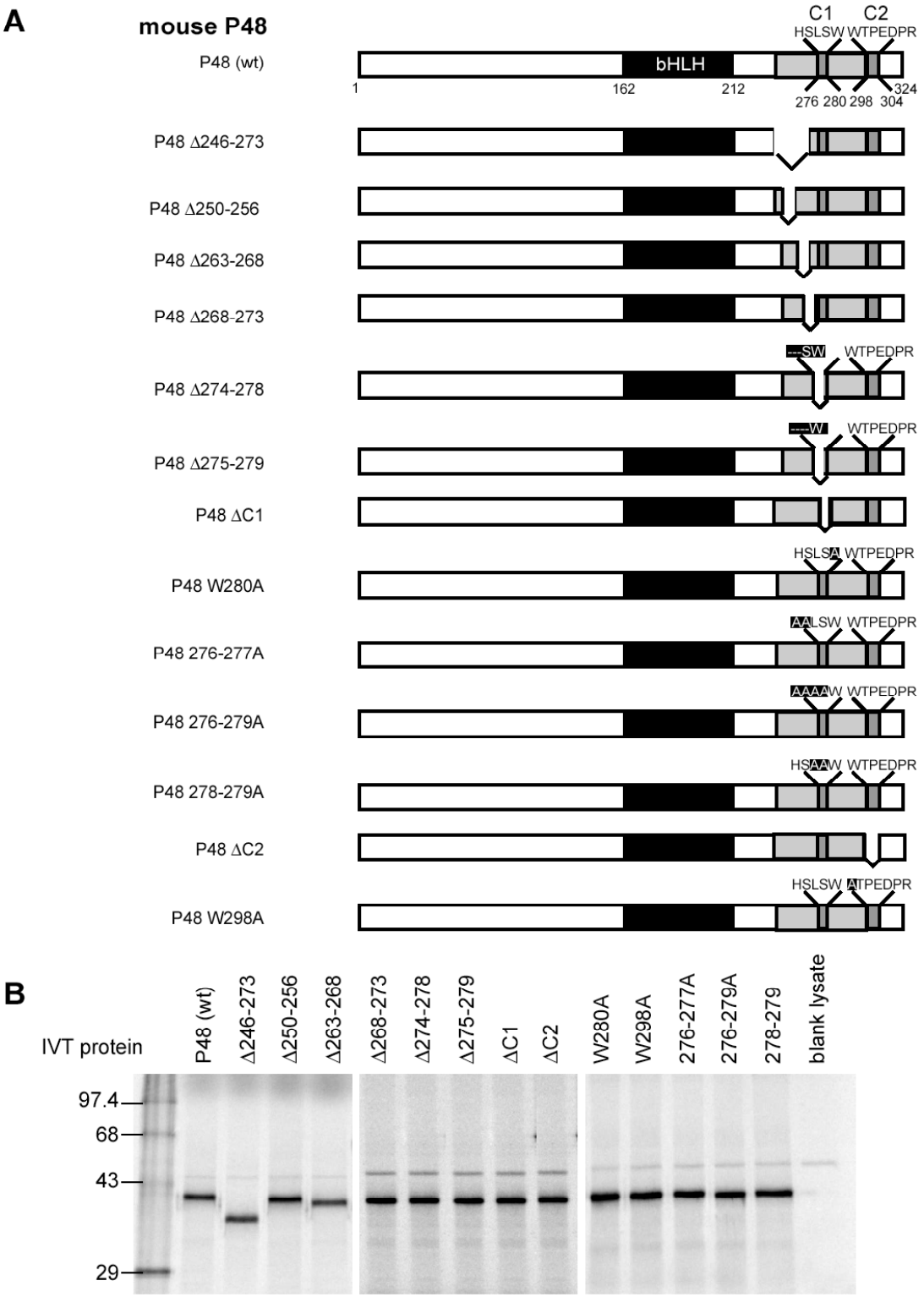

A
mouse P48
C1
C2
HSLSW WTPEDPR
P48 (wt)
bHLH
1
162
212
276 280 298 304
324
P48 Δ246-273
P48 Δ250-256
P48 Δ263-268
P48 Δ268-273
---SW WTPEDPR
P48 Δ274-278
----W WTPEDPR
P48 Δ275-279
P48 ΔC1
HSLSA WTPEDPR
P48 W280A
AALSW WTPEDPR
P48 276-277A
AAAAW WTPEDPR
P48 276-279A
HSAAW WTPEDPR
P48 278-279A
P48 ΔC2
HSLSW ATPEDPR
P48 W298A
B
IVT protein
P48 (wt)
Δ246-273
Δ250-256
Δ263-268
Δ268-273
Δ274-278
Δ275-279
ΔC1
ΔC2
W280A
W298A
276-277A
276-279A
278-279
blank lysate
97.4
68
43
29

Figure 4.4 The mouse P48 deletion series. A. Wild-type and mutant mouse P48 proteins are shown schematically. Black, bHLH; dark grey, C1 and C2 peptides with sequence denoted above the boxes; light grey, C-terminal domain conserved in vertebrates. **B.** Wild-type and mutant mouse P48 synthesized by *in vitro* translation. 35S-Met labeled RBP-L translation products were resolved by 10% SDS PAGE.

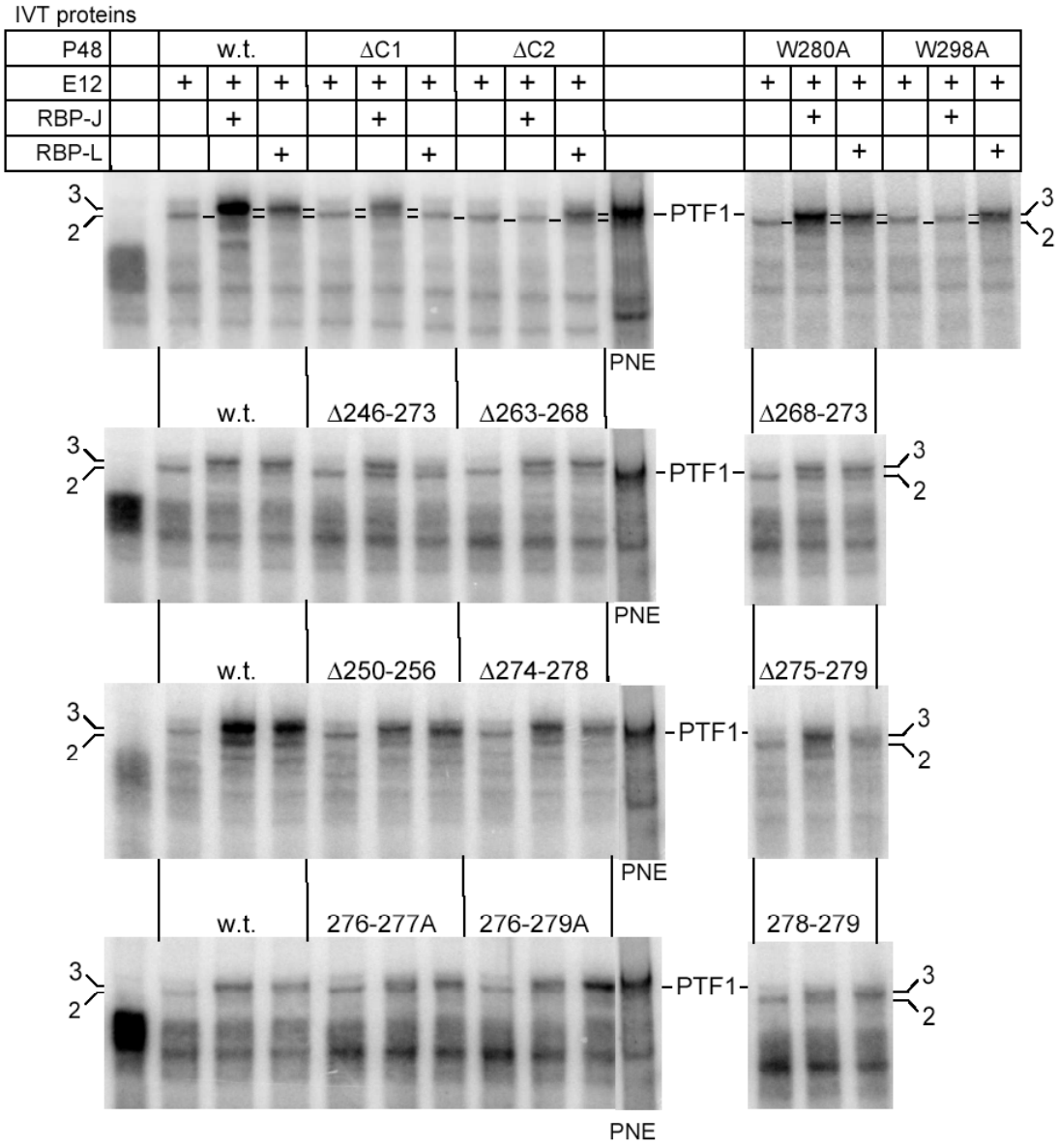


Figure 4.5 The C1 and C2 peptides mediate the interaction between P48 and RBP proteins.
EMSA analyses of the abilities of IVT wild type and mutant mouse P48 proteins to form DNA-binding heterodimers with IVT E12 or DNA-binding trimers with IVT E12 plus RBP-L or RBP-J. All P48 mutants formed the heterodimer as effectively as wild type. Upper panel: mutants of the C1 and C2 peptides. Lower three panels: partial mutants of the C1 peptide and mutants of the region conserved in vertebrates. 3, trimer; 2, dimer; PNE, pancreas nuclear extract

In addition to the conservation of peptides C1 and C2, mouse and zebrafish P48 share broader conservation in the C-terminal region (Figure 4.2). Because RBP-L is vertebrate-specific, I tested whether this region of P48 that is specifically conserved in vertebrates contributes to the interaction with RBP-L. Deletion of amino acids 246 to 273, which are conserved among vertebrates but not in Drosophila, severely affected the recruitment of RBP-L, but much less so RBP-J. A similar result was obtained using a P48 protein with a deletion of amino acids 250 to 256. Deletions of amino acids 263 to 268 or 268 to 273 had a small effect of the recruitment of RBP-L, but little or no effect on the recruitment of RBP-J. Finally, since the alanine substitution for tryptophan 280 had no effect on the recruitment of RBP-L in EMSA experiments, but deletion of C1 eliminated this recruitment, I tested whether specific residues of the conserved peptide other than tryptophan contribute to the interaction with RBP-L. Deletions of five amino acids immediately N-terminal to tryptophan 280 greatly reduced, but did not completely eliminate, the recruitment of RBP-L. A similar result was obtained by using P48 with a deletion of amino acids 274-278. These deletions had a more modest effect on the recruitment of RBP-J than the recruitment of RBP-L. Surprisingly, the substitution of alanine for two or four of the non-tryptophan amino acids of C1 had little or no effect on the recruitment of either RBP-L or RBP-J. These contrasting effects suggest that the two RBP proteins emphasize different features in overlapping domains of P48, with the region conserved in vertebrates making a greater contribution to the recruitment of RBP-L.

The interaction between P48 and an RBP-protein is essential for PTF1 transcriptional activity

To verify that the strong transcriptional activity of PTF1 is dependent on the interaction of P48 and RBP-L, I tested whether the mutations of the C1 and C2 peptides that disrupted the interaction with RBP-J or RBP-L in EMSA experiments also inhibited transcriptional activation in transfected 293 cells (Figure 4.6). In chapter one, I confirmed that RBP-L is a strong transcriptional activator in the context of the PTF1 complex (Figure 3.6), so its contribution to the activation of the 6A.EIp.luc-reporter gene can be readily monitored. However, the transcriptional activity of a PTF1-J complex is low by comparison, and addition of exogenous RBP-J does not substantially increase this activation. To monitor the activity of exogenous RBP-J as related to its recruitment to the PTF1-J complex, I used a strong, constitutively active form created by fusing the VP16 activation domain to the N-terminus of RBP-J (Toshiko Masui, unpublished). P48W298A, which could recruit RBP-L but not RBP-J, lost nearly all transcriptional activity in combination with VP16-RBP-J, but retained much of its activity with RBP-L. The remaining activity of the 6A.EIp.luc reporter with transfected P48, HEB, and VP16-RBP-J is likely due to the residual activity of the P48W298A-HEB heterodimer. The effects of the W280A substitution were similar the W298A substitution for RBP-L and slightly more modest for VP16-RBP-J. The mutant P48 lacking the C1 peptide region had little or no transcriptional activity with the cotransfected HEB and RBP-L and limited activity with cotransfected HEB, and VP16-RBP-J. This is consistent with the

relative ability of P48 to interact with each RBP protein in EMSA experiments. Thus, the binding and transcriptional activity of the PTF1 complex requires the recruitment of RBP proteins by P48 (Summarized in Figure 4.7).

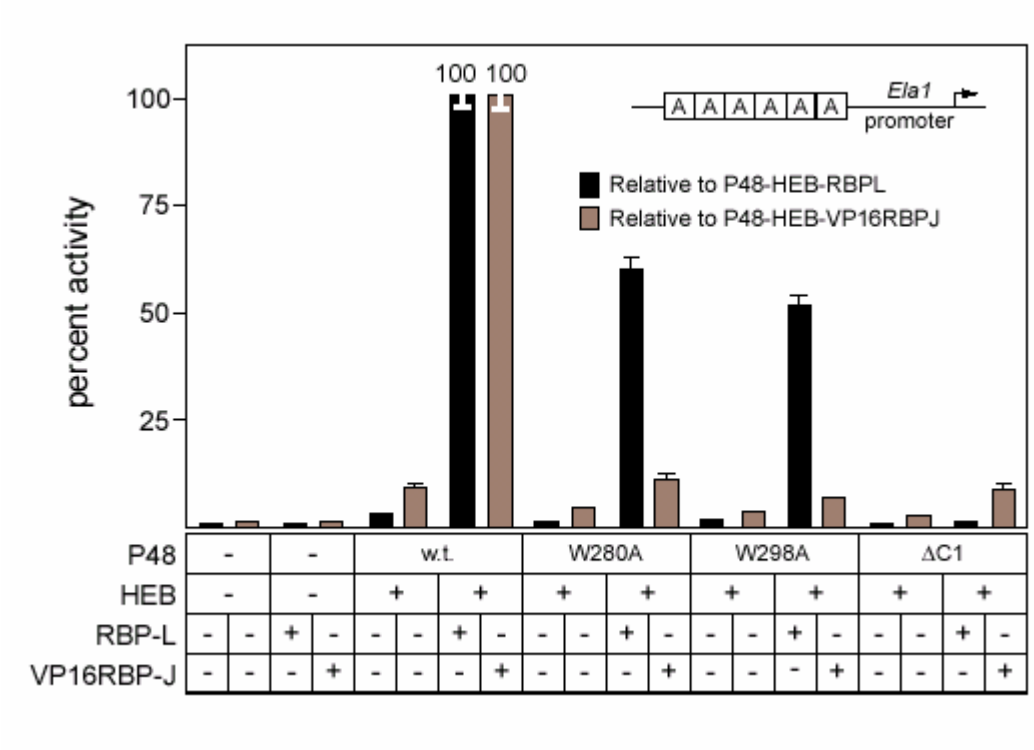


Figure 4.6 Transcriptional activation by PTF1 requires the interaction of P48 and RBP-L or RBP-J. A VP16-RBP-J fusion protein was used as an indicator of RBP-J function. The relative activity of the 6A.EIp.luc reporter construct in 293 cells was assayed in the presence or absence of cotransfected HEB, RBP-J, VP16-RBP-J, or RBP-L and wild-type or mutant P48, individually or in various combinations as indicated. All values are the means of at least three transfections. Error bars represent standard errors of the means.

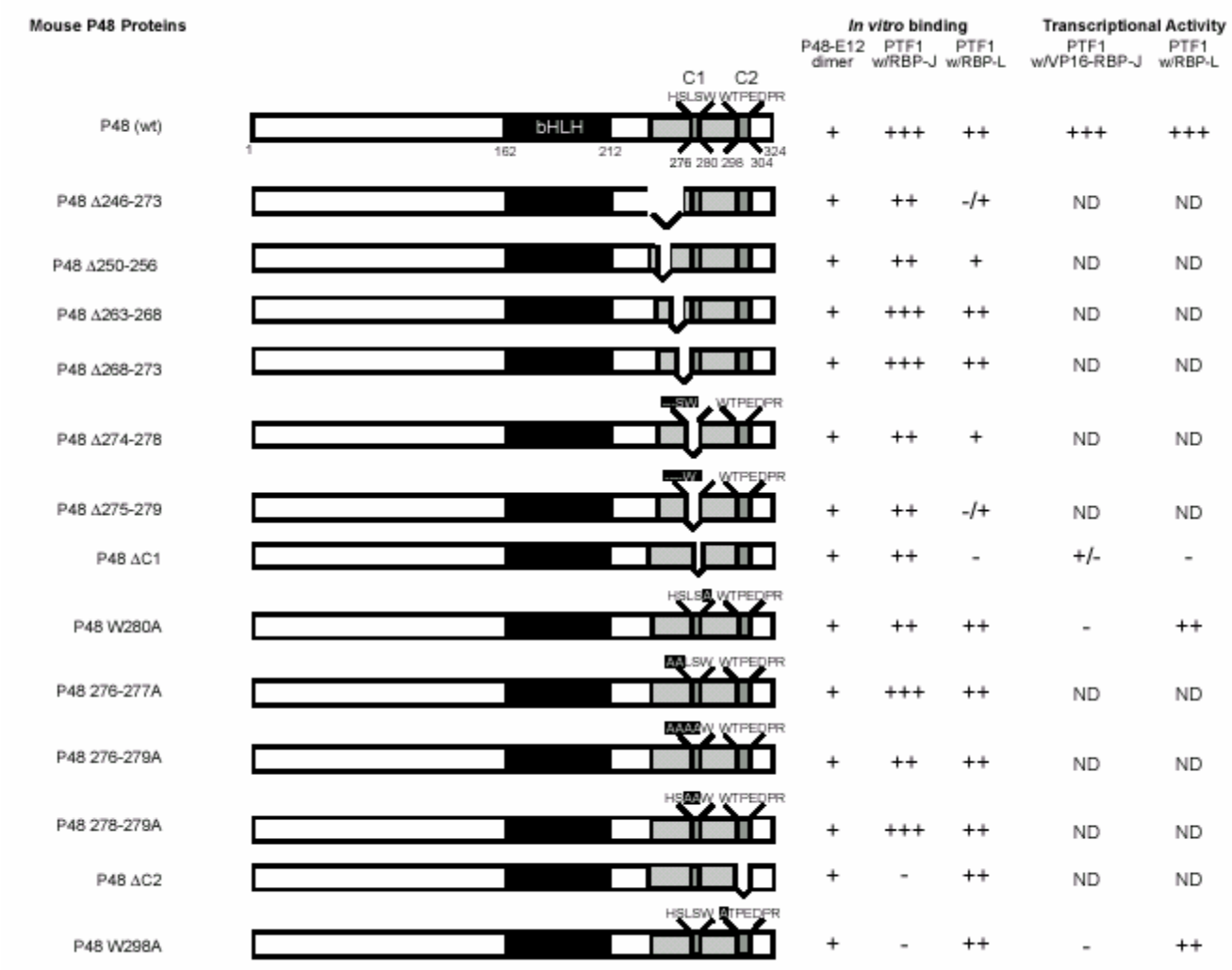


Figure 4.7 Summary of P48 domains required for interaction with RBP-L and RBP-J for the binding and transcriptional activity of PTF1.
Wild-type and mutant mouse P48 proteins are shown schematically. Level of heterodimer and trimer formation with indicated proteins in EMSA experiments, and level of transcriptional activity in transient transfection assays is shown. + or – indicates the presence or absence of activity, with strength of activity indicated by the number of symbols. ND, not done.

Mutations of human p48 associated with pancreatic and cerebellar agenesis disrupt RBP-mediated activation by PTF1.

The P48 subunit of PTF1 is essential for pancreas development (Kawaguchi et al., 2002; Krapp et al., 1998). Recently, naturally occurring mutations have been identified in the gene encoding human P48 that disrupt the function of this protein. These mutations are correlated with permanent neonatal diabetes mellitus (PNDM), a genetic disorder associated with pancreatic and cerebellar agenesis (Sellick et al., 2004). One mutation causes the deletion of the conserved C2 peptide. The other mutation deletes both peptides. Deletion of the thirty-two C-terminal amino acids of mouse P48 renders it unable to activate a reporter gene regulated by tandem repeats of the *Ctrb* PTF1 binding site (Sellick et al., 2004), but the mechanism by which P48 activates transcription and participates in embryonic development has not been defined. EMSA experiments using recombinant versions of these mutant human P48 proteins have shown that they efficiently form heterodimers with E12, but they do not efficiently form a trimeric complex with RBP-proteins. The mutant that deleted the C2 domain formed extremely small amounts of the trimer, while the mutant that deleted both the C1 and C2 domains failed to form any of the trimeric complex (T. Masui, personal communication summarized in Figure 4.8A). I expressed the mutants of human P48 in 293 cells along with HEB and RBP-L or VP16-RBP-J to test whether the inability of P48 to activate transcription is due to a disruption of its interaction with RBP proteins (Figure 4.8B). In contrast to wild-type human P48, which was able to activate the 6A.EIp.luc reporter to the expected high levels, transfection of the mutant human P48 did not activate

transcription with the addition of exogenous RBP-L or VP16-RBP-J. Thus, naturally occurring human mutations of *p48* render its encoded protein inactive by disrupting the interactions of P48 with RBP-proteins, and likely lead to the severe developmental abnormality associated with PNDM.

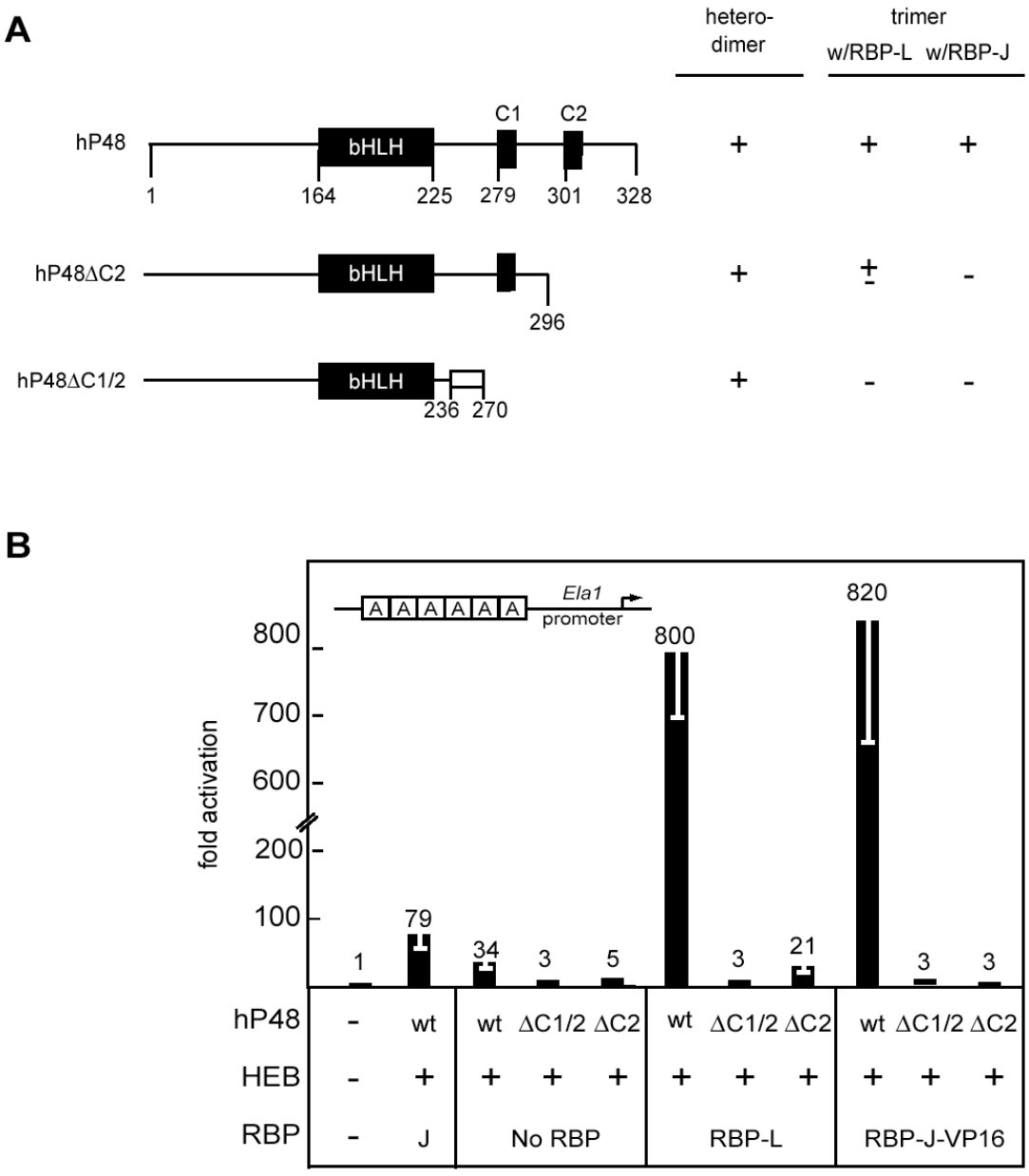


Figure 4.8 Human mutants of P48 are ineffective because they do not interact with RBP proteins. A. A Schematic representation of wild-type and mutant human P48 proteins, and their binding activities on the *ELA1* A element with E12, or E12 and RBP-J or RBP-L. Binding activityis indicated by a + or -. Summary of binding activity provided by T. Masui. B. Mutant human P48s do not provide transcriptional activation of the *ELA1* A element. The relative activity of the 6A.EIp.luc reporter construct in 293 cells was assayed in the presence or absence of cotransfected HEB, VP16-RBP-J, or RBP-L, and wild-type or mutant human P48, individually or in various combinations as indicated. All values are the means of at least three transfections. Error bars represent standard errors of the means.

The binding of P48 and the NotchIC to RBP-J is mutually exclusive

Interaction of the NotchIC with RBP-J/SUH is mediated by the interaction of a small peptide in the NotchIC with a hydrophobic pocket in the BTF Domain of Su(H), and peptides similar to this motif occur in several other proteins, such as EBNA2 (Kovall and Hendrickson, 2004). The C-terminal peptides in P48 are strikingly similar to the Su(H)-interacting peptide of the NotchIC (R.M Henke, personal communication). The similarities of the C1 and C2 peptides of P48 to this motif imply that P48 and the NotchIC might interact with RBP-J via the same site, and thus each may have antagonistic effects on the binding of the other to RBP-J. To test whether the NotchIC can inhibit P48 interaction with RBP-J in a PTF1-J complex, I added IVT NotchIC to a mixture of IVT P48, E12, and RBP-J in EMSA reactions with the *ELA1* PTF1 binding site oligonucleotide (Figure 4.9A). IVT P48, E12, and RBP-J readily formed a trimeric complex. Addition of increasing concentrations of IVT NotchIC disrupted formation of this trimeric complex, and caused the disappearance of the trimer band in a concentration dependent manner. A reconstituted trimeric complex containing P48, E12, and RBP-L, which does not interact with the NotchIC was affected much less by the addition of IVT NotchIC. Additionally, I tested whether the NotchIC antagonizes activation of the 6A.EIp.luc by cotransfected P48, HEB, and VP16-RBP-J in a concentration dependent manner (Figure 4.9B). Indeed, cotransfection of increasing amounts of the NotchIC inhibited transcriptional activation by P48, HEB, and endogenous or cotransfected RBP-J. Cotransfection of the NotchIC also had the same effect on the high level of reporter

activation by cotransfected P48, HEB, and VP16-RBP-J. Cotransfection of 293 cells with the NotchIC only inhibited transcriptional activity of the P48, HEB, RBP-L complex a small amount, and in a manner that was not dependent on the concentration of the transfected NotchIC plasmid. Therefore, the NotchIC inhibits formation of the PTF1 complex containing RBP-J and its activation of PTF1 target site by direct interference with the P48-RBP-J interaction, while the inability of NotchIC to interact with RBP-L precludes its interference with the DNA binding and transcriptional activity of PTF1containing RBP-L complex.

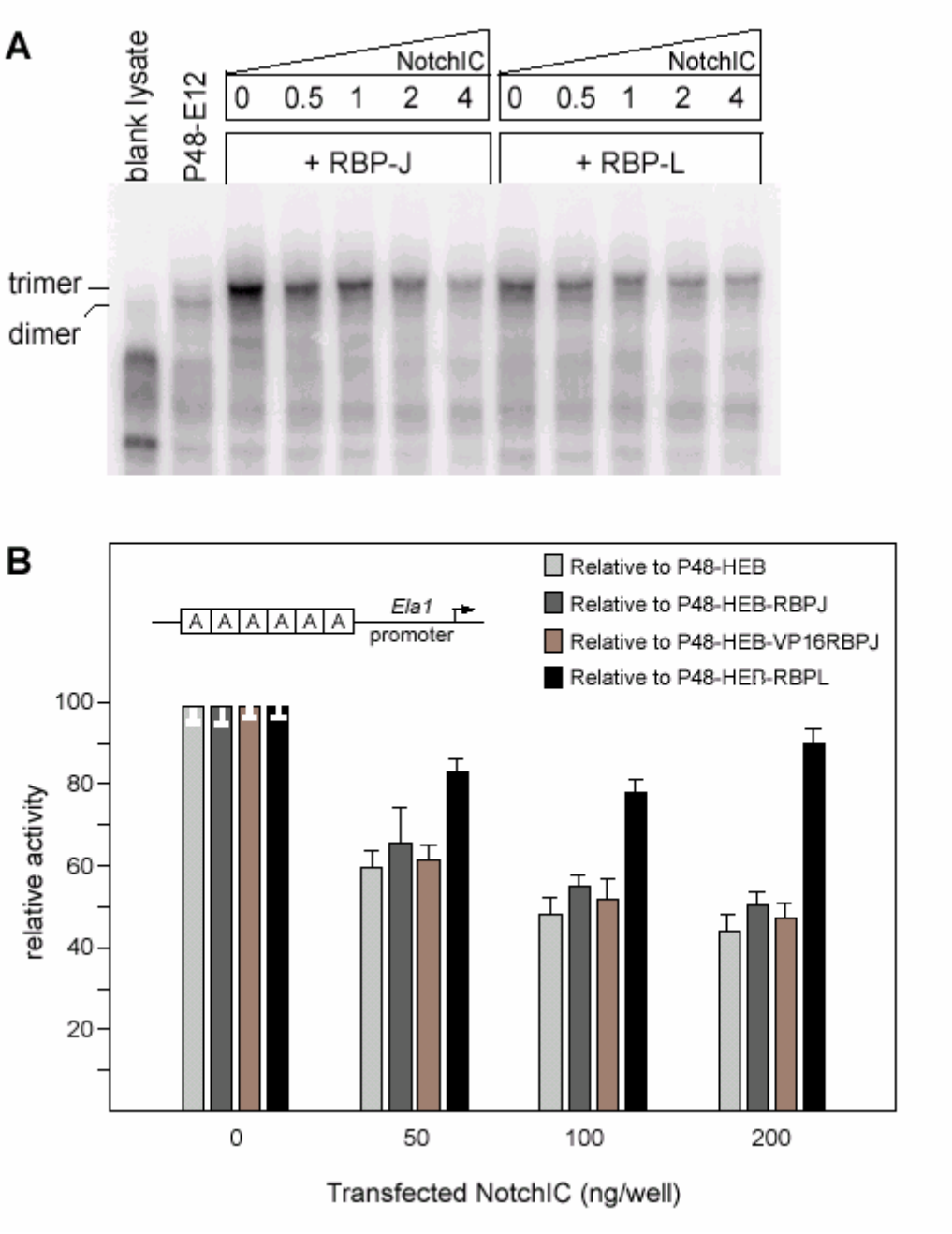


Figure 4.9 The NotchIC interferes with the binding and transcriptional activity of a PTF1-J complex. A. EMSA analyses of PTF1 complexes containing either RBP-J or RBP-L in the absence or presence of various amounts of the NotchIC. Amounts of NotchIC are molar ratios relative to P48. B. The relative activity of the 6A.EIp.luc reporter in 293 cells was assayed in the presence or absence of cotransfected P48, HEB and RBP-J, VP16-RBP-J, or RBP-L, with varying amounts of the NotchIC expressing construct as indicated. Amounts of transfected NotchIC plasmid are indicated. All values are the means of at least three transfections. Error bars represent standard errors of the means.

Because Notch activation of gene targets is mediated by the interaction of the NotchIC with RBP-J (Artavanis-Tsakonas et al., 1999; Hsieh et al., 1996), and P48 can also interact with RBP-J (Obata et al., 2001 and Figure 3.4B), it is possible that P48 can disrupt this NotchIC-RBP-J activity. RBP-J, but not RBP-L, can interact with the NotchIC (Minoguchi et al., 1997). I used EMSA experiments to confirm that an RBP-J NotchIC complex, but not an RBP-L-NotchIC complex, can bind the RBP-J consensus site although both RBP proteins can bind the site as monomers (Figure 4.10A left). However, RBP-L, but not RBP-J, can form a complex with P48 on the RBP-J consensus site (Figure 4.10A right; last lane). To test whether P48 can inhibit formation of a NotchIC-RBP-J complex, I added increasing amounts of IVT P48 to binding reactions containing IVT NotchIC and RBP-J mixed with an oligonucleotide containing an RBP-J consensus binding site. Antibody supershifts of binding reactions containing IVT RBP-J, NotchIC, or both confirmed that the RBP-J NotchIC complex formed on the RBP-J consensus binding site (Figure 4.10B, left). Addition of IVT P48 interfered with the formation of the RBP-J-NotchIC-DNA complex in a concentration dependent manner as shown by the progressive disappearance of the NotchIC-RBP-J band (Figure 4.10B, right). This interference is a direct result of P48 binding to RBP-J and blocking the docking site for the NotchIC, because the ability of a non-RBP-J binding mutant of P48 to disrupt the RBP-J-NotchIC complex is greatly reduced. Similarly, P48 can interfere with RBP-J mediated activation by the NotchIC. Adding increasing amounts of P48 to 293 cells expressing the NotchIC inhibits the ability of the NotchIC to activate a luciferase reporter under the control of six tandem repeats of the consensus RBP-J

binding site (6R.EIp.luc) (Figure 4.10C). This inhibition is attenuated when the non-RBP-J binding mutant of P48 is expressed instead of wild-type P48. Thus, P48 can inhibit activation of a Notch target gene by directly interfering with the formation of the NotchIC –RBP-J-DNA complex.

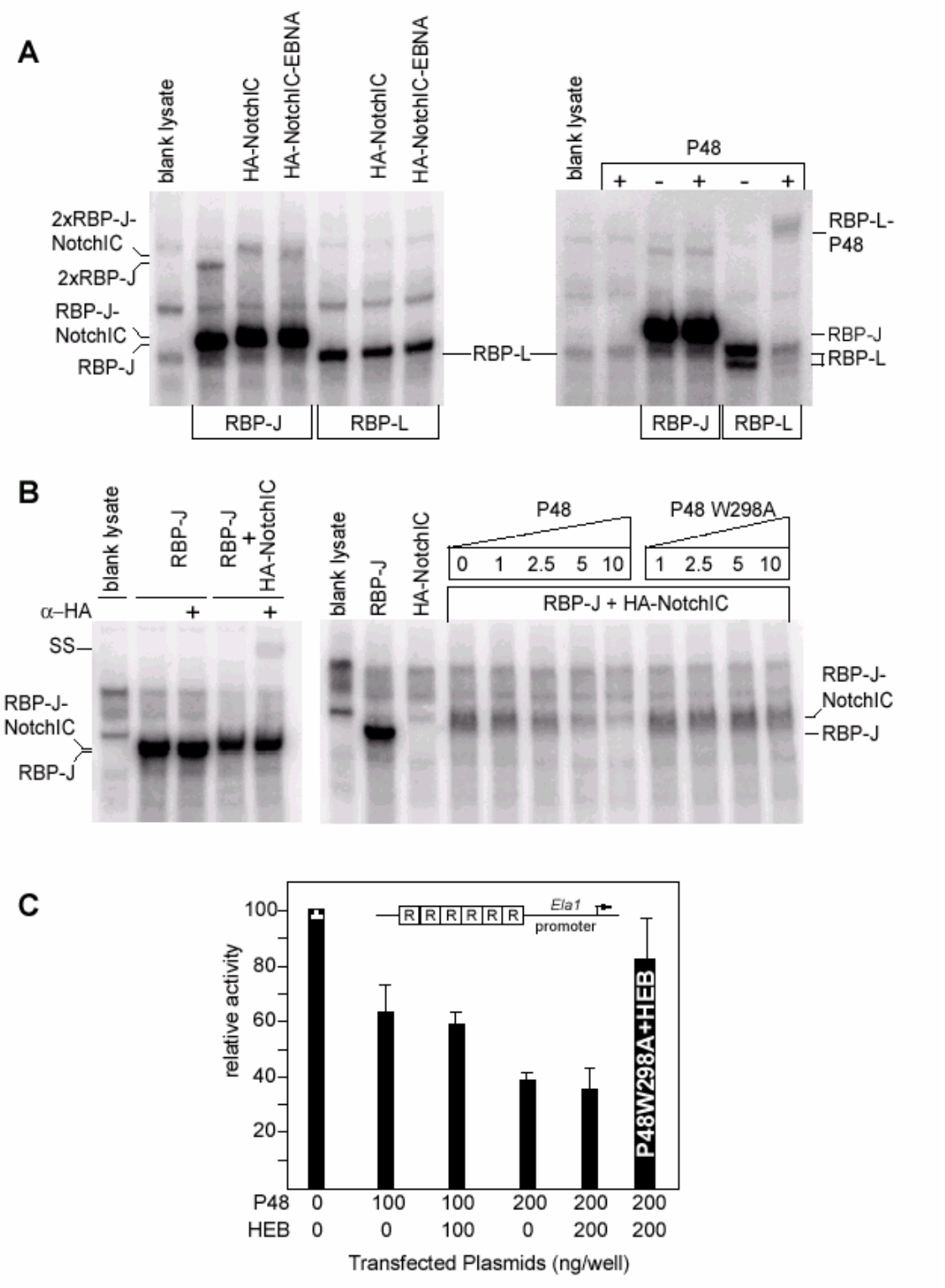

A
blank lysate
HA-NotchIC
HA-NotchIC-EBNA
HA-NotchIC
HA-NotchIC-EBNA
blank lysate
P48
+ - + - +
2xRBP-J-NotchIC
2xRBP-J
RBP-J-NotchIC
RBP-J
RBP-L
RBP-L-P48
RBP-J
RBP-L
RBP-J
RBP-L
RBP-J
RBP-L
B
blank lysate
RBP-J
RBP-J + HA-NotchIC
α–HA
+
+
blank lysate
RBP-J
HA-NotchIC
P48
0 1 2.5 5 10
P48 W298A
1 2.5 5 10
RBP-J + HA-NotchIC
SS
RBP-J-NotchIC
RBP-J
RBP-J-NotchIC
RBP-J
C
relative activity
100
80
60
40
20
R R R R R R
Ela1
promoter
P48W298A+HEB
P48 0 100 100 200 200 200
HEB 0 0 100 0 200 200
Transfected Plasmids (ng/well)

Figure 4.10 P48 interferes with NotchIC enhanced binding and transcriptional activation by RBP-J. A. EMSA analyses of RBP-J protein interactions with the NotchIC (left) or P48 (right) on the O_{54} double-stranded olgonucleotide (containing 2 copies of the RBP-J site). B. EMSA analyses of the binding of an RBP-J-NotchIC complex (left) to a single RBP-J binding site. The components of the complex were confirmed by the addition of 1 μl an antibody against the HA tag on the IVT NotchIC protein. SS indicates the supershifted complex. EMSA analyses indicates interference by P48 with the binding of the NotchIC-RBP-J complex to a single RBP-J site (right). IVT RBP-J and NotchIC were mixed in 1:1 molar ratios. IVT mouse P48 or the P48W298A mutant was added in increasing amounts. Numbers indicate molar ratios of P48 to RBP-J-NotchIC. C. The relative activity of the 6R.EIp.luc reporter construct in 293 cells was assayed in the absence or presence of cotransfected NotchIC and varying amounts of P48, P48W298A, and HEB as indicated. Amounts of cotransfected plasmid are indicated. All values are the means of at least three transfections. Error bars represent standard errors of the means.

Neither the N-terminus nor the extreme C-terminus of RBP-L defines a domain necessary for interaction with P48 and high-level transcriptional activation.

Although RBP-L and RBP-J have largely similar amino acid sequences, they vary in their abilities to bind the NotchIC (Minoguchi et al., 1997), and in their ability to activate transcription within the context of a PTF1 complex (Figure 3.6). Previous studies showed that RBP-J is the important DNA-binding mediator of Notch signaling (Artavanis-Tsakonas et al., 1999; Fryer et al., 2002; Hsieh et al., 1996). Its activity is due to the ability to directly and concurrently interact with DNA and the NotchIC. By comparison, although RBP-L can bind the same target sequence as RBP-J *in vitro*, it does not interact with the NotchIC and is not a mediator of Notch signaling (Minoguchi et al., 1997). Unlike RBP-J, which can be recruited to form a PTF1-like complex with low transcriptional activity, RBP-L is part of a highly active PTF1 complex (Figure 3.6). Both RBP-proteins can interact with P48 in the context of the PTF1 complex, however RBP-J more readily forms a PTF1 complex *in vitro* (Figure 3.4B and Figure 3.5A).
To look for the domains of RBP-L that interact with P48 or the domains that are necessary for high-level transcriptional activity, I created mutants of RBP-L that deleted domains at the N- and C-terminus of RBP-L (Figure 4.11). I compared the ability of these mutants to form a PTF1 complex, bind DNA as a monomer, and activate transcription in transient transfection assays with that of wild-type RBP-L. Using EMSA experiments, I tested to see which of these RBP-L deletion mutants could still bind its target sequence, but not integrate into the PTF1 complex (Figure 4.12). I expressed these

mutant proteins in transfection experiments along with HEB and P48 to see which of these deletions could still activate the 6A.EIp.luc reporter (Figure 4.13). Deletion of the divergent N-terminal sixty-three amino acids or the extreme C-terminal amino acids of RBP-L had no effect on the ability of RBP-L to activate high-level transcriptional of the reporter in 293 cells. The N-terminal deletion mutant of RBP-L did form a trimeric complex with the other PTF1 components in EMSA experiments (Figure 4.12). The C-terminal deletion has not yet been tested in this assay, but since it retains the high- level transcriptional activity, it must still integrate into the PTF1 complex (Figure 4.13). Mutants deleting further into the N-terminus of RBP-L lost the ability to integrate into the complex, but also could not bind a target sequence in EMSA experiments. Two internal deletions in this region that left the N-terminus intact also lost both of these binding abilities. The domain of RBP-L that interacts with P48 may reside in a region common to both RBP-J and RBP-L, but this may be within a region that is also important for DNA binding. The domain necessary for high-level transcriptional activation in PTF1 may reside in a region further in from the extreme C-terminus of RBP-L (Summarized in Figure 4.14).

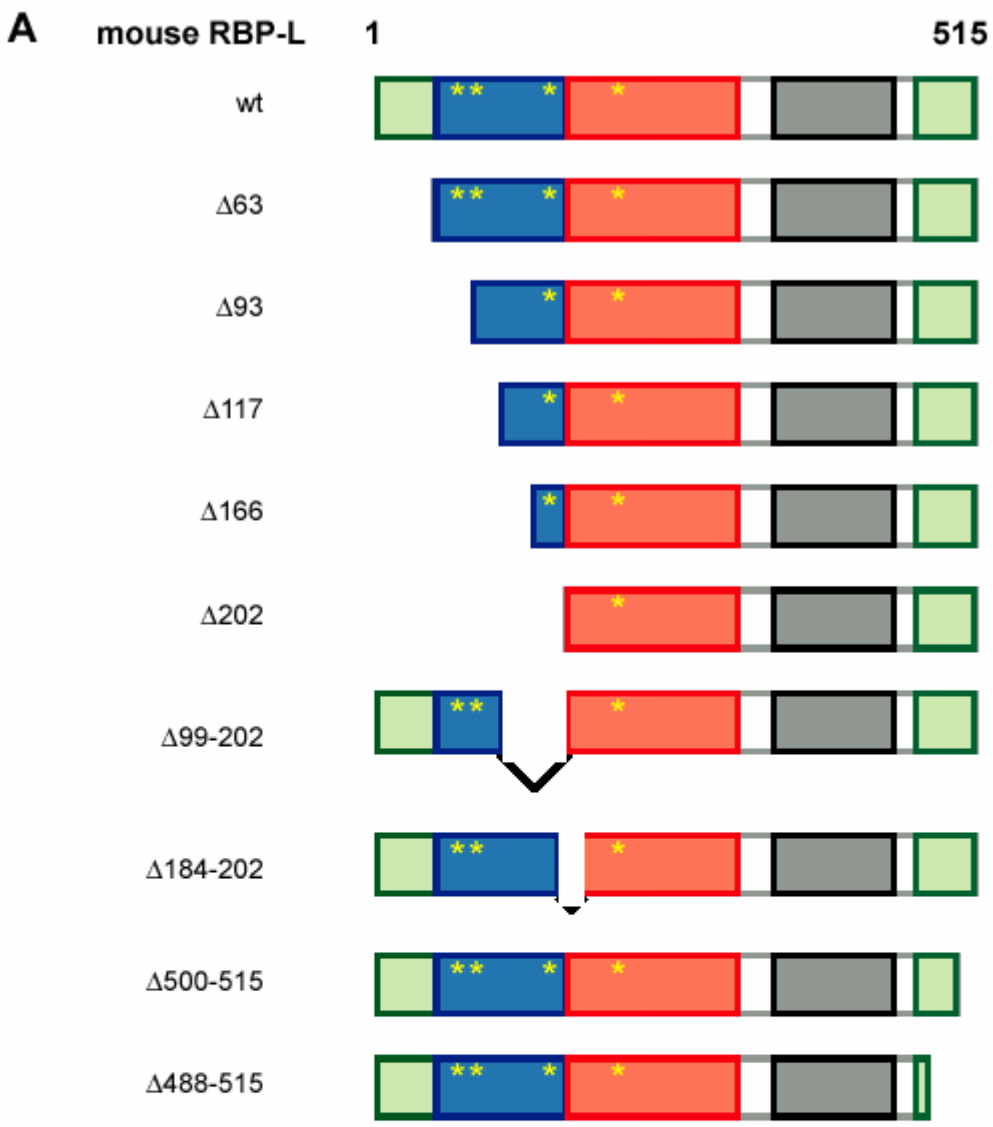

A
mouse RBP-L
1
515
wt
Δ63
Δ93
Δ117
Δ166
Δ202
Δ99-202
Δ184-202
Δ500-515
Δ488-515
B
IVT protein
wt
Δ63
Δ93
Δ117
Δ166
Δ202
blank lysate
Δ99-202
Δ184-202
97.4
68
43
29

Figure 4.11 The deletion series for RBP-L.
A. Wild-type and mutant mouse RBP-L proteins are shown schematically. red, Beta trefoil domain; blue, N-terminal RHR; black, C-terminal RHR;, green, divergent regions; yellow asterisks, critical DNA binding amino acid residues. B. Wild-type and mutant RBP-L synthesized by *in vitro* translation. ^{35}S-Methionine labeled RBP-L translation products were resolved by 10% SDS PAGE. IVT Δ500-515 and Δ488-515, not done.

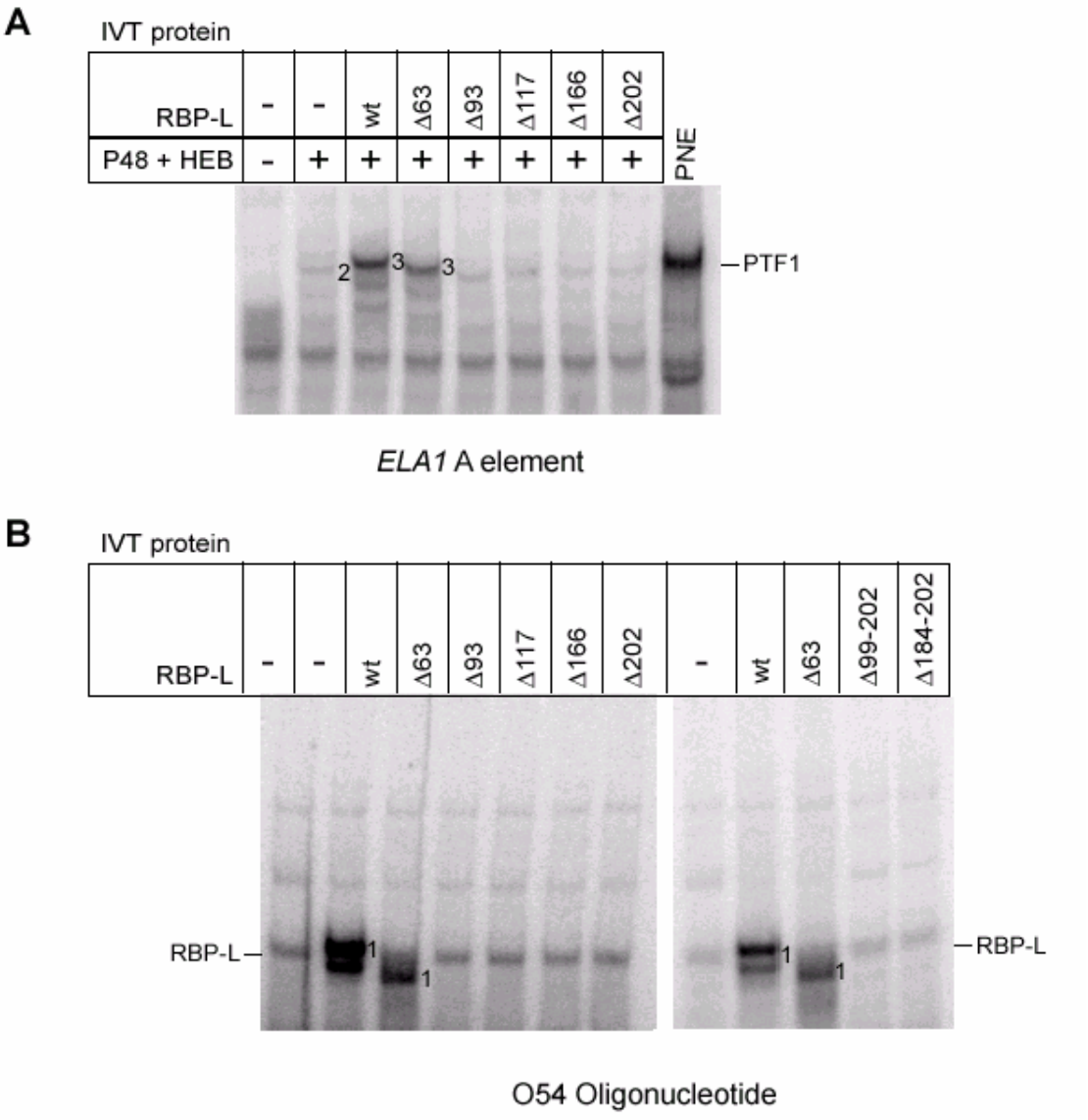


Figure 4.12 Effects of RBP-L deletions on PTF1 binding activity.
A. EMSA analyses of the ability of IVT wild-type or mutant RBP-L to bind the ELA1 A element as part of the PTF1 complex with IVT P48 and E12. 2, dimer; 3, trimer; PNE, pancreas nuclear extract. B. EMSA analyses of the abilities of IVT wild-type and mutant RBP-L to bind an RBP-J site as a monomer. 1, monomer.

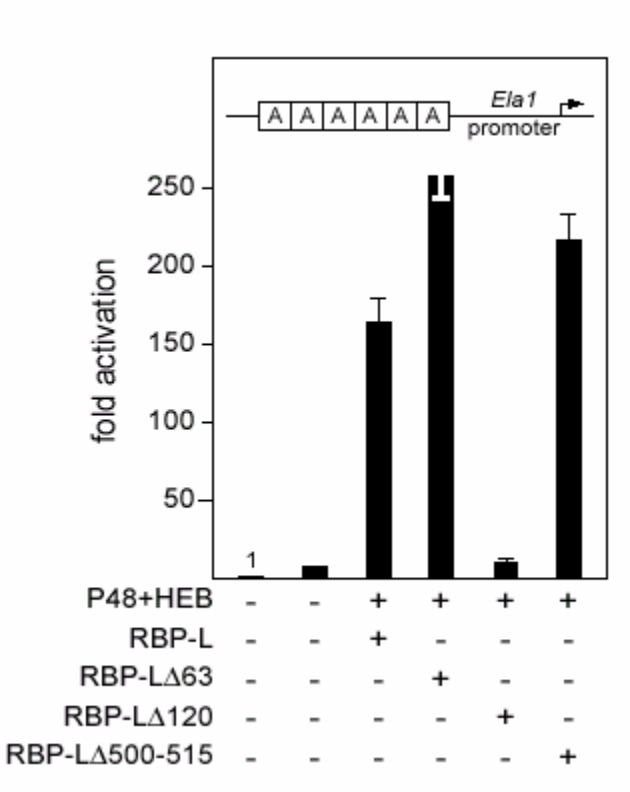


Figure 4.13 Effects of RBP-L deletion on the transcriptional activity of PTF1.
The relative activity of the 6A.EIp.luc reporter construct in 293 cells was assayed in the presence or absence of cotransfected HEB, P48, and wild-type or mutant RBP-L, in various combinations as indicated. All values are the means of at least four transfections. Error bars represent standard errors of the means.

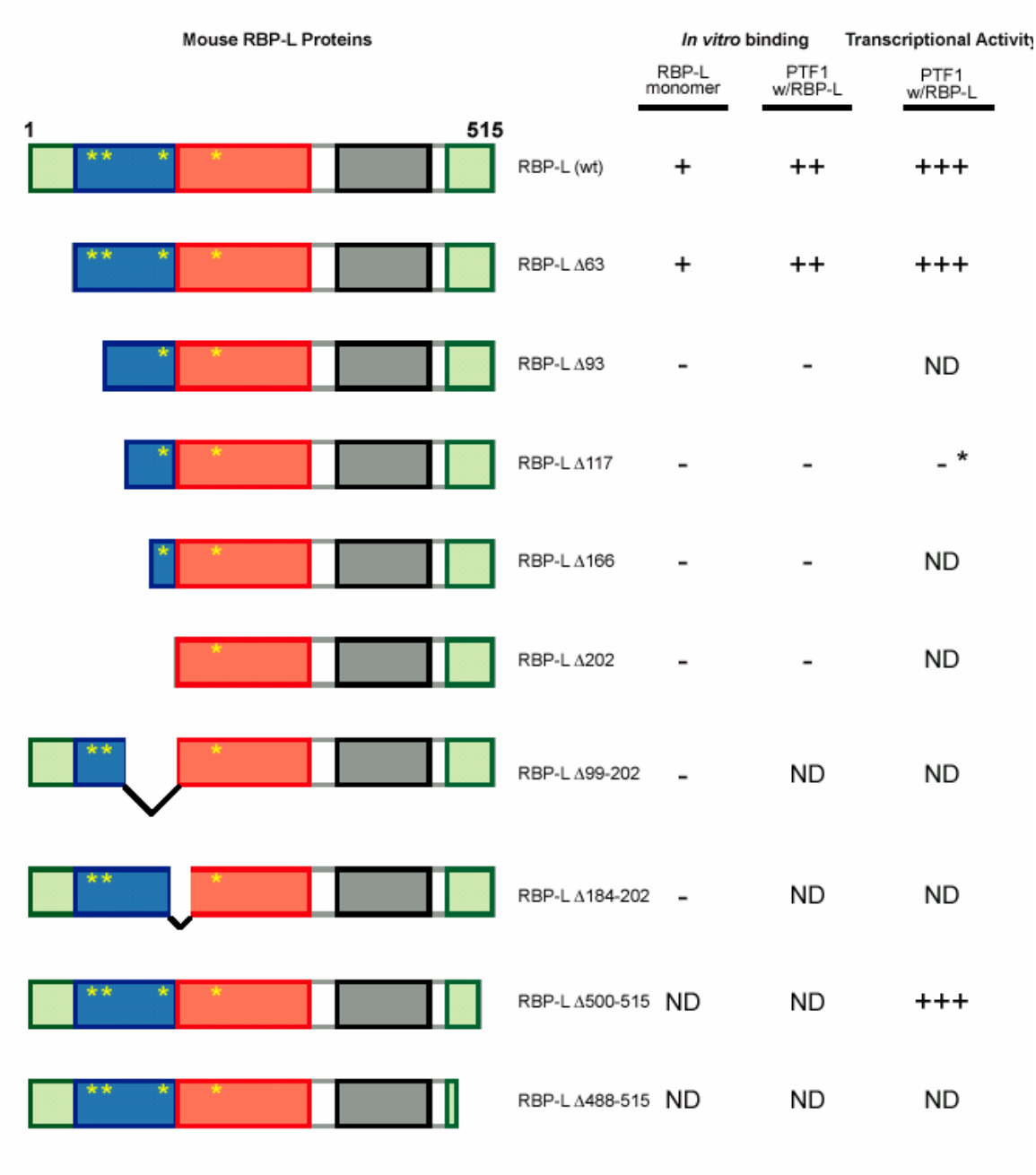


Figure 4.14 Summary of RBP-L domains required for PTF1 binding and activation.
Wild-type and mutant mouse RBP-L proteins are shown schematically. red, Beta trefoil domain; blue, N-terminal RHR; black, C-terminal RHR;, green, divergent regions; yellow asterisks, critical DNA binding amino acid residues. Level of trimer formation on the *ELA 1* A element with indicated proteins, or level of monomer formation on O_{54} in EMSA experiments, and level of transcriptional activity in transient transfection assays is shown. + or – indicates presence or absence of activity, with strength of activity indicated by the number of + symbols. ND, not done; asterisk indicates that this transfection was actually done using the similar construct RBP-LΔ120 created by T. Masui.

Summary

Experiments to define the domains necessary for the formation of a fully functional PTF1 complex revealed that the phylogenetically conserved peptides from the C-terminus of P48 are similar in amino acid sequence and in function to the RBP-J-interacting peptide in the NotchIC. The P48 C-terminal peptides are essential for the interaction of P48 with RBP proteins. The interaction of P48 with RBP-J blocks the docking site of the NotchIC, and precludes the integration of the NotchIC into the complex. Consequently, the addition of Notch does not enhance PTF1 activity. Indeed, excess NotchIC inhibits formation and transcriptional activity of PTF1 by blocking the P48-interaction site of RBP-J. P48 requires both the tryptophan-containing peptides in its C-terminus for full interaction with RBP-J, but peptide C2 is not essential for interaction with RBP-L. Both of these peptides are essential for full transcriptional activity of PTF1-J and –L complexes. The requirement for the C-terminal peptides for the formation of the PTF1 complexes is consistent with the requirement for these same peptides for transcriptional activation by the PTF1 complexes. The domains of RBP-L necessary for interaction with P48 have not been defined, but are not in the N terminus or C-terminus of the protein. Similarly, although I have not defined the region of RBP-L necessary for its high transcriptional activation ability, this domain likely resides in the C-terminal region.

Although the conserved C-terminal peptides of P48 mediate its interaction with RBP proteins, different peptides are emphasized in the interaction with RBP-J vs. RBP-L (Figure 4.15). Specifically, the tryptophan within the C1 peptide contributes to the recruitment of RBP-J into a PTF1 complex and transcriptional activity of this complex,

while the tryptophan of the C2 peptide is absolutely essential for these activities. The full C1 peptide is essential for the recruitment of RBP-L to a PTF1 complex and the transcriptional activity of this complex. The region of broader conservation in mammals and zebrafish but not in *Drosophila* also contributes to the recruitment of RBP-L into a reconstituted PTF1 complex but not the recruitment of RBP-J. Hence, RBP-L likely has a more extensive interaction with P48 than RBP-J. Furthermore, naturally occurring mutations that cause C-terminal truncations of human P48 delete one or both of the C1 and C2 peptides and eliminate the ability of RBP-J or RBP-L to form an active PTF1 complex with P48 and an AbHLH (Sellick et al., 2004) and Figure 4.8). Thus, the transcriptional activity and developmentally important function of P48 are dependent on its ability to interact with RBP proteins.

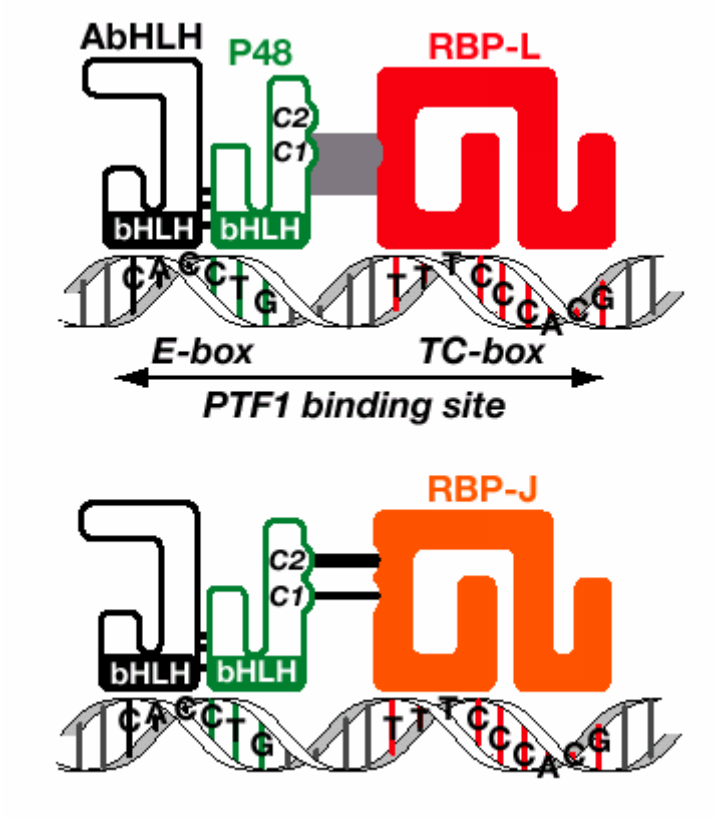


Figure 4.15 A model of the interactions between P48 and RBP-L or RBP-J in two different forms of the PTF1 complex.

The results of EMSA and transfection experiments have narrowed, but not explicitly defined the domain(s) of RBP-L that interact with P48, and the domain(s) that supply high transcriptional activity. Deletions of RBP-L that eliminated its N-terminal region sixty-three amino acids, a sequence divergent from that of RBP-J, were used in EMSA experiments, and confirmed that this region is not necessary for RBP-L interaction with P48. More extensive deletions of the N-terminus of RBP-L disrupted formation of PTF1 on the A element, but these deletions also eliminated the ability of RBP-L to bind to a consensus RBP-J/L site. The crystal structure of the *C. elegans*

orthologue of *Drosophila* Su(H), LAG-1 (Kovall and Hendrickson, 2004), and previous mutational studies of RBP-J (Chung et al., 1994; Kovall and Hendrickson, 2004) indicate that Su(H) contacts DNA via three regions N-terminal to the NotchIC-interacting domain. Deletions within the conserved N-terminal RHR of RBP-L each deleted at least one of these DNA-binding regions. Further analysis of these sequences in the N-RHR and BTF domains of RBP-J and RBP-L will likely narrow the candidate domain(s), disruptions in this region may have a general effect of disrupting the protein structure and DNA binding. Deletions of the divergent N-terminal region or part of the C-terminus still allow for high transcriptional activity of the PTF1 complex. Further deletion analyses of the C-terminus of RBP-L may yield a defined activation domain.

The presence of RBP-J, a protein that mediates Notch signaling by interacting with the NotchIC in a PTF1 complex, suggests that Notch may be able to activate transcription of target genes within the context of this complex. However, recent data imply that Notch signaling may antagonize PTF1 activity (Esni et al., 2004). Indeed, EMSA experiments demonstrate that addition of IVT NotchIC to binding reactions containing IVT PTF1 components inhibits formation of a PTF1 complex containing RBP-J, but has much less of an effect on a PTF1 complex containing RBP-L (Figure 4.9A). Similarly, the NotchIC inhibits activation of an *ELA1* PTF1 reporter (6AEIp.luc) by a P48-HEB-VP16-J complex but does not substantially inhibit activation by a P48-HEB-RBP-L complex (Figure 4.9B). Additionally, P48 inhibits formation of an RBP-J-NotchIC complex on an RBP-J consensus binding sequence (Figure 4.10B). P48 also inhibits Notch-enhanced activation by RBP-J in transfections (Figure 4.10C). Thus, high amounts of Notch signaling may interfere with PTF1 activity and embryonic

development by blocking the P48 docking site of RBP-J, while high amounts of P48 inhibit activation of traditional targets of Notch signaling by sequestering RBP-J.

Chapter 5

Discussion and Future Directions

PTF1 is a unique complex that is the functional form of P48

The functional form of the bHLH transcription factor P48 is the three-protein complex, PTF1 (Krapp et al., 1996; Rose et al., 2001; Roux et al., 1989; Sommer et al., 1991). Like other critical transcription factors such as PDX1, P48 is critical to both the development and the function of the pancreas (Kawaguchi et al., 2002; Krapp et al., 1998; Offield et al., 1996; Ohlsson et al., 1993). Specifically, in addition to its role in development, PTF1 is active in the tissue-specific activation of digestive enzyme genes in mature pancreatic acinar cells (Krapp et al., 1996; Rose et al., 2001). However, PDX1 can activate target genes either as a monomer (Ohlsson et al., 1993; Swift et al., 1998) or as part of a heterotrimeric homeodomain complex (Liu et al., 2001; Swift et al., 1998), while P48 is only known to function as part of a trimer. Although, P48 is required for pancreatic development, the only identified gene targets are those encoding the highly expressed pancreatic digestive enzymes, such as *ELA1,* markers of terminally differentiated acini.

Acinar-specific activation of the digestive enzyme genes is mediated via a bipartite enhancer element consisting of an E-Box and a TC-box. Previous studies had shown that PTF1 consists of at least two bHLH proteins, the tissue specifically expressed P48, and a ubiquitously expressed class A bHLH such as E47, E12, or HEB. Supershift EMSA experiments indeed confirmed that PTF1 is a molecularly heterogeneous complex

that is actually a mixture of complexes containing P48 and one of the AbHLH proteins (Rose et al., 2001 and Figure 3.3 and Figure 3.4B). Yeast two-hybrid experiments revealed that P48 is able to interact with the ubiquitously expressed protein, RBP-J (Obata et al., 2001). However RBP-J had not been identified as a part of PTF1. Examination of the RBP-J literature revealed the existence of a tissue-specifically expressed paralogue, RBP-L (R.M. Henke, personal communication and Minoguchi et al., 1997). Although Rbp-L was initially described as limited to the lung and brain (Minoguchi et al., 1997), RT-PCR analysis of nineteen adult tissues revealed high levels of *Rbp-L* and *p48* transcript co-expressed only in the pancreas. Although RBP-J and RBP-L both represented good candidates for a component of PTF1, RBP-L was revealed to be the third component in adult pancreas.

The PTF1 complex represents the first defined Notch-independent use of the Su(H) family of proteins. Su(H) and its mammalian orthologue RBP-J have long been studied as mediators of the Notch signaling pathway, which regulates cell fate decisions. Although studies have shown that Su(H) can act in a Notch-independent manner for *Drosophila* mechanosensory organ development and function (Barolo et al., 2000), no mechanism of Notch-independent activation by Su(H) had been defined. Additionally, the related RBP-L protein has a DNA target sequence identical to that of RBP-J, but is not responsive Notch (Minoguchi et al., 1997). Target genes for RBP-L had not been previously identified. Hence, the identification of RBP-L as the third component of PTF1 also identifies the acinar-specific digestive enzyme genes as the first known targets. Chromatin immunoprecipitation experiments confirm that RBP-L binds to these targets *in vivo* in adult pancreas (T. Masui, personal communication). Furthermore, the

reconstitution of a transcriptionally active PTF1 complex containing RBP-J identifies the first known mechanism for the Notch-independent use of RBP-J for transcriptional activation. The likelihood that RBP-J and RBP-L are essential for the development and maintenance of the pancreatic acini also identifies a Notch-independent function for mammalian Su(H) and its related vertebrate-specific paralogue in organogenesis. The function of RBP proteins in PTF1 is due to their ability to dramatically alter the DNA binding specificity of an otherwise traditional bHLH protein complex, and not their ability to interact with Notch.

The novel structure of the PTF1 complex specifies targets that are not just lone E-boxes or lone RBP-J binding sites (Figure 5.1). P48 can bind to a lone E-box as a heterodimer with an AbHLH, but PTF1 cannot bind to this same site. Mutation of the TC-box precludes the binding of PTF1 to the *ELA1* A-element, but still allows for binding of the heterodimer. However, addition of excess amounts of RBP-L or RBP-J can reduce the amount of bound heterodimer to the mutant site (Figure 3.7A). Additionally, deletion of the TC-box eliminates PTF1 binding activity and nearly all PTF1 transcriptional activity, providing evidence that the TC-box binding protein is essential for these activities (Figure 3.7B). The expression of exogenous RBP proteins eliminates the small amount of activity from the TC-box mutant of the *ELA1* PTF1 reporter provided by the P48 heterodimer because overexpression of the RBP proteins forces P48 into the PTF1 complex, which cannot bind to an E-box. Furthermore, P48 cannot form a complex with RBP-J on an RBP-J consensus site, and can actually inhibit RBP-J binding to a lone RBP-J site. Thus, RBP proteins alter the DNA binding

specificity of the P48-AbHLH heterodimer, and P48 alters the DNA binding specificity of RBP-J.

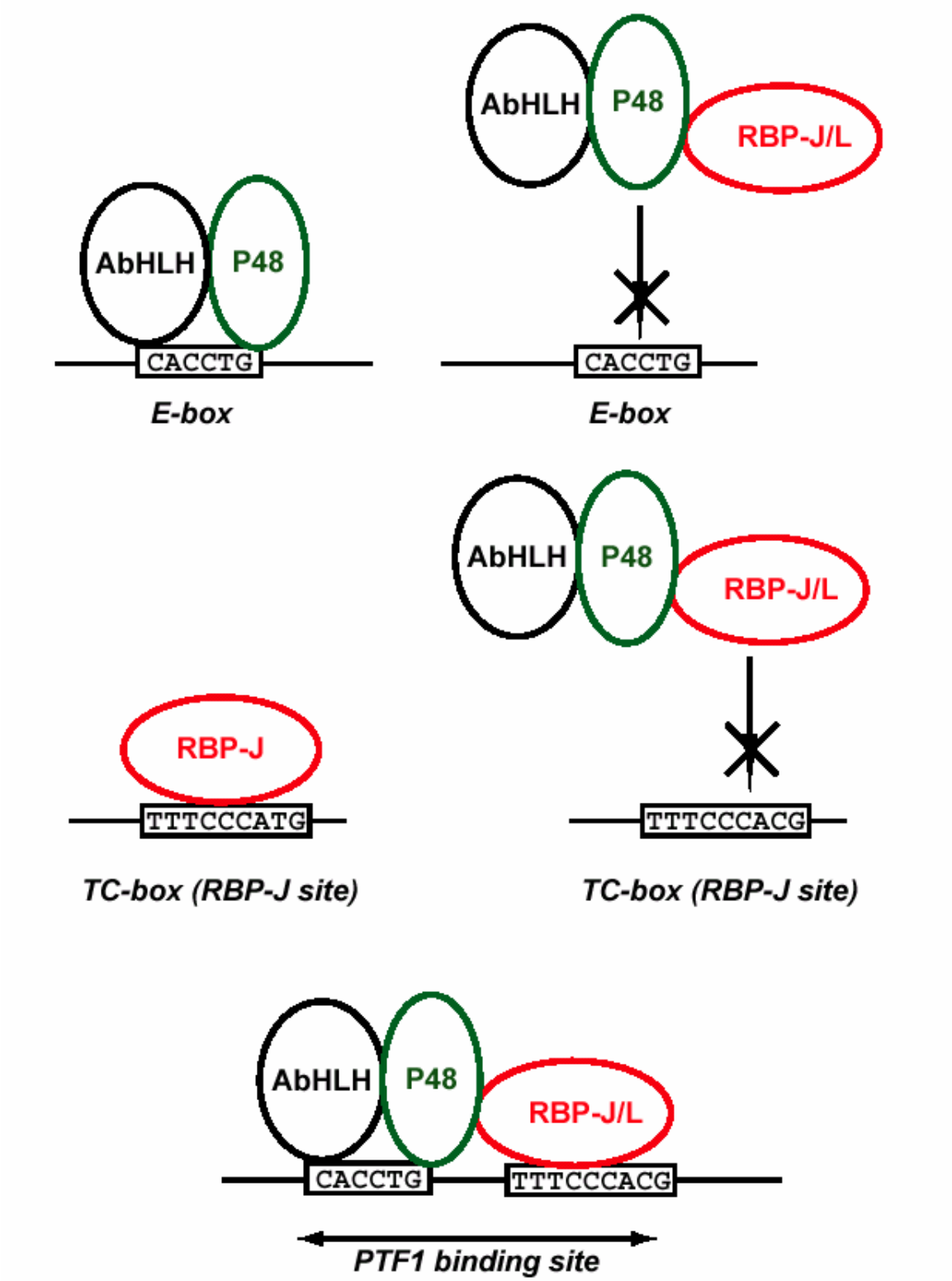

AbHLH
P48
CACCTG
E-box
AbHLH
P48
RBP-J/L
CACCTG
E-box
RBP-J
TTTCCCATG
TC-box (RBP-J site)
AbHLH
P48
RBP-J/L
TTTCCCACG
TC-box (RBP-J site)
AbHLH
P48
RBP-J/L
CACCTG
TTTCCCACG
PTF1 binding site

Figure 5.1 The PTF1 complex has unique binding requirements.
Top: E-boxes bHLH heterodimers in the absence of PTF1, but PTF1 does not bind lone E-boxes. Middle: RBP-J binds its consensus site, but PTF1 does not bind lone RBP-J sites. Bottom: PTF1 only binds sites containing both an E-box and a TC-box.

The Sequences of PTF1 binding sites control multiple mechanisms of gene regulation

The unusual binding preference of PTF1 does not require the exact consensus binding sites of the DNA-binding subunits of PTF1. *In vitro* reconstitution of PTF1 complexes on six different enhancer elements from digestive enzyme genes shows that a high amount of cooperativity is required for the ability of PTF1 to bind to a variety of target sequences (Figure 3.8). Like the *ELA1* PTF1 binding sequence, mouse *TRPd* and rat *CPA*-142 lack a RBP-J consensus binding sequence, but have a consensus E-box sequence. Hence, these sequences can be bound by the P48 heterodimer and PTF1, but not by RBP monomers. Conversely, the PTF1 binding site in mouse *Ela2* has a good RBP-J consensus site, so monomers of RBP proteins readily bind to this site. However, this site lacks a consensus E-box, so the P48 heterodimer does not bind, even though the full PTF1 complex does. Some sites, such as rat *Ctrb* have a good E-box and a good RBP consensus binding site. Hence, the lack of a TC-box near an E-box would preclude P48 activation of the E-box, and lack of an E-box near a TC-box would predictably inhibit activation of an RBP-J site in the presence of P48. Suboptimal consensus E-boxes and TC-boxes in close proximity allows for the binding of this unique complex. This flexibility allows for an expanded array of available targets, but extensive studies have not been done to examine which individual base pairs are required for the binding of PTF1.

E-box and TC-box sequences regulate transcriptional activity by DNA binding of the PTF1 complex, but the E-box may regulate transcriptional activity by repression as well as activation. PTF1 target sequences, such as *Ctrb*, containing TC-boxes that can bind RBP-J monomers, may be activated by Notch signaling in the absence of P48. However, the presence of the E-box may block Notch-induced activation of these RBP-J sites. A *Ctrb* PTF1 reporter was not activated in COS cells by transfection of a VP16-RBP-J fusion (Obata et al., 2001) . Additionally, changing the TC-box of the *ELA1* PTF1 site to a good RBP-J binding site does not allow for activation in 293 cells by a VP16-RBP-J fusion, the NotchIC, or a NotchIC-EBNA2 fusion (my preliminary results). Ablation of the E-box allows activation of the mutant *ELA1* PTF1 site reporter by the addition of these activators. These preliminary results concur with the published data that strongly imply that a repressor may be bound to the E-box in the absence of P48. A possible candidate repressor might be an HEB homodimer. HEB homodimers have been shown to have repressor activity (Chiaramello et al., 1995) and HEB readily forms a strong homodimer on the E-box of the *ELA1* and *Ctrb* PTF1 site. Additionally, a molar ratio of 10:1 P48:HEB is required to see the P48 heterodimer in EMSA experiments instead of the HEB homodimer, while the reconstituted PTF1 trimer can be seen using a 1:1:1: ratio of P48:HEB:RBP proteins (Figure 3.4B). This suggests that the cooperativity of the PTF1 trimer is required to efficiently displace an HEB homodimer, or possibly another repressor complex *in vivo* (Figure 5.2). In the absence of P48, a repressor bound to the E-box would prevent activation of genes such as *Ctrb* in the inappropriate context such as by Notch signaling or in other organs.

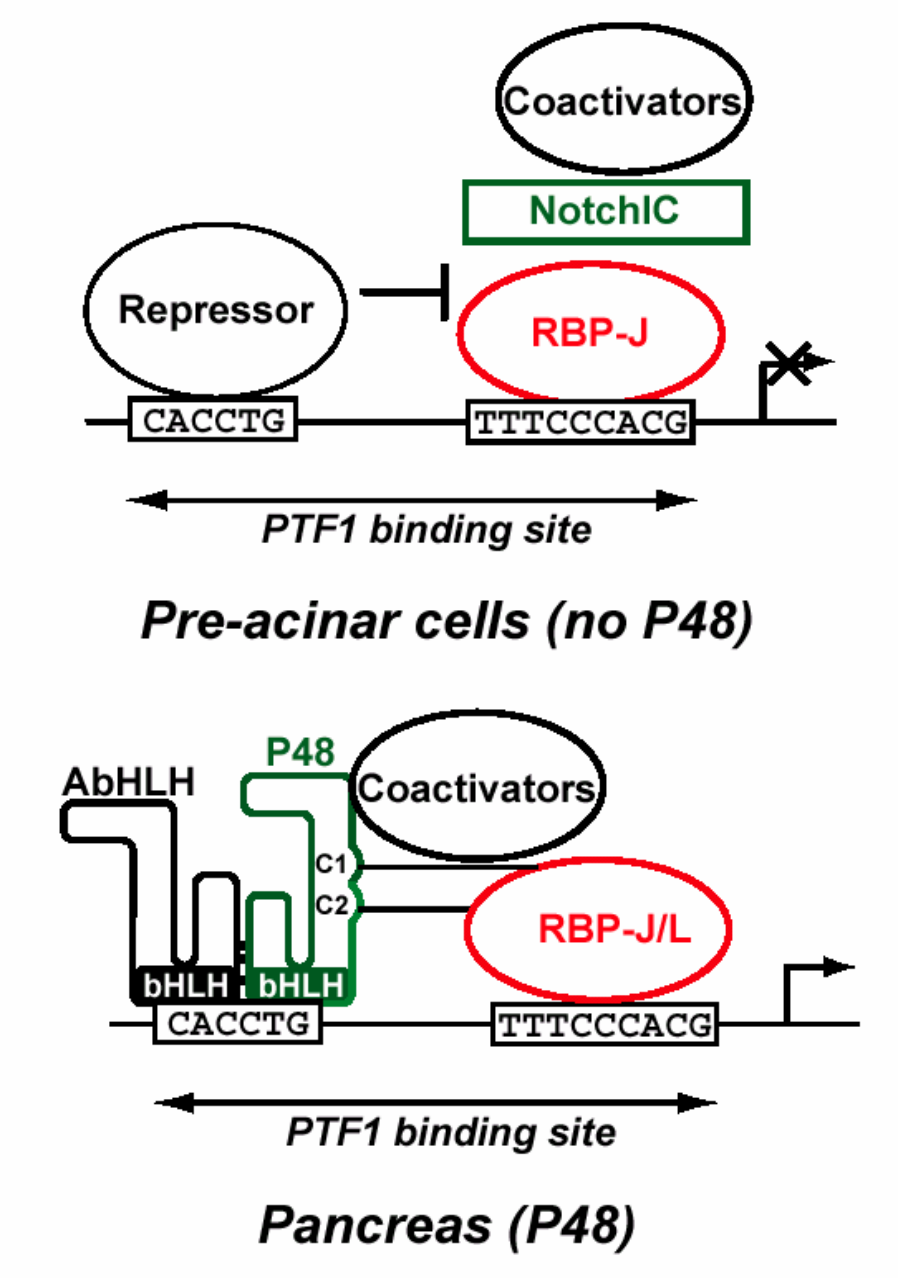


Figure 5.2 A model for the activation of digestive enzyme genes in the pancreas.
In preacinar cells where P48 is absent, digestive enzyme genes are turned off, possibly by a repressor bound to the E-box of the PTF1 binding site. In the pancreas, P48 forms the PTF1 complex, which activates the expression of digestive enzyme genes.

Potential PTF1 targets

Gene targets of PTF1 should contain the unique binding sequence of an E-box and a TC-box in their transcriptional regulatory regions. Indeed, the presence of E-box and TC-box sequences one or two helical turns away from each other can be used as the basis to search for targets other than the digestive enzyme genes. A search of the database using these criteria (CANYTGN {X} NNTCYC, X= spacing 4, 5, 14, 15, 16, 17, 18 against the human genome and restricting the query to 10 Kb upstream of known CDS's using RSA TOOLS) initially identified over 20,000 potential PTF1 targets, but a more stringent analysis reduced this number to less than 5,000, and further analysis allowed for the identification of some likely candidates (R.M.Henke, personal communication sample summarized in Figure 5.3). Such targets include important transcriptional regulators like *Math6*, *Sox2*, and even *Rbp-L* itself. These putative targets were ranked as some of the better candidates because their E-boxes and TC-boxes are good matches to consensus sites for the individual subunits of PTF1. PTF1 targets, such as carboxypeptidase A1 have at least one part of the sequence that diverges from the consensus, but still bind PTF1. The role of PTF1 in the activation of newly identified putative targets has not yet been studied.

One target of particular interest is *Rbp-L* itself. The promoter region of *Rbp-L* contains two potential PTF1 binding sites, both upstream of the transcriptional start site. Thus, *Rbp-L* expression may be autoregulated. Recent transient transfection studies have

shown that the proximal, but not the distal site is required for *Rbp-L* expression (T. Masui, personal communication). There are two observations of particular interest. First, the proximal site contains two E-boxes, one and two helical turns away from the TC-box. Second, the TC-box is a near consensus RBP-J binding site. This consensus RBP-J binding site is conserved from mammals to chickens (T. Masui, personal communication), thus implying a possible important function for a consensus RBP-J binding site. Transient transfections reveal that the *Rbp-L* PTF1 binding has higher transcriptional activity in the presence of a PTF1-J complex than that of *ELA1* (T. Masui, personal communication). However, mutating the TC-box of *ELA1* to the RBP-J consensus site raises the activity in the presence of transfected PTF1-J components (T. Masui and T. Beres, unpublished) The activity of the *Rbp-L* PTF1 binding site and the mutant *ELA1* binding site is still higher with PTF1 complex containing RBP-L than with a PTF1 complex containing RBP-J. Thus, slight variances in the sequence of PTF1 binding sites might be one more way or regulating levels of transcription. The presence of an active PTF1 binding site in the *Rbp-L* promoter makes it likely that RBP-L, in the context of the PTF1 complex, regulates its own expression.

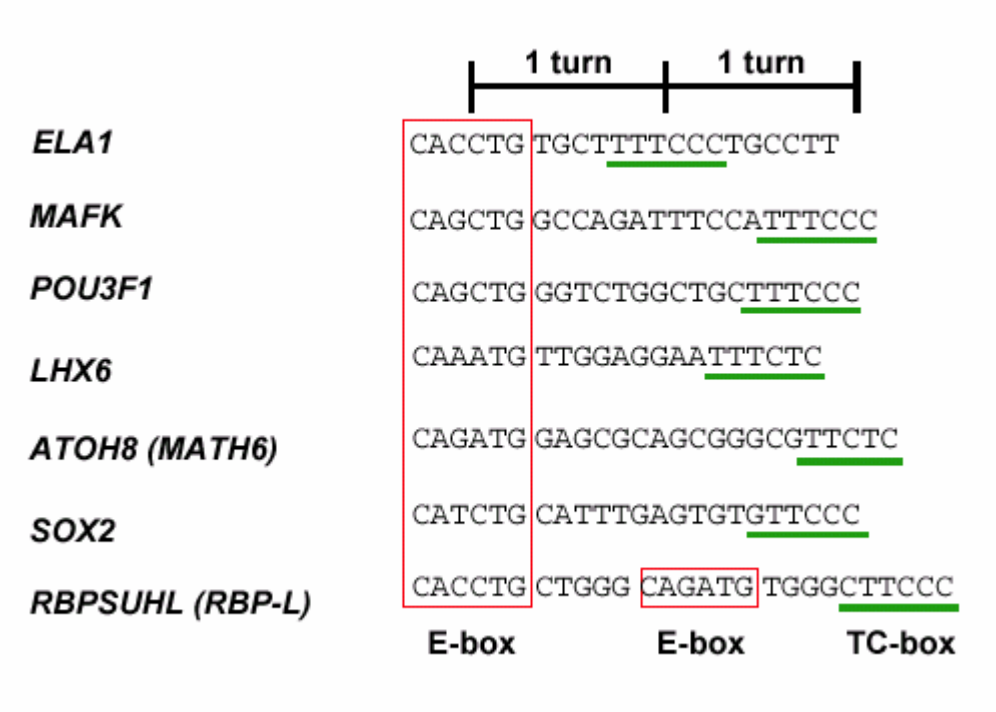


Figure 5.3 Potential gene targets of PTF1 with binding sites in their regulatory regions.
A partial list of potential PTF1 targets. E-boxes are denoted by red rectangles. TC-boxes are underlined in green.

Similarities and Differences of RBP-J and RBP-L forms and functions

The identification of RBP-L as the third component of PTF1 highlights one of several of the differences between RBP-L and RBP-J. RBP-J has been extensively studied as a mediator of Notch signaling. In the absence of Notch signaling RBP-J represses genes by recruiting corepressors that make chromatin structure inaccessible to activating transcription factors. Upon the activation of Notch signaling by a ligand such as Delta, the corepressor complex is removed, and a coactivator complex is recruited. Conversely, RBP-L is not responsive to Notch signaling because it does not interact with the NotchIC (Minoguchi et al., 1997 and Figure 4.10A). Although RBP-L has been the

target of limited study, it was discussed as a potential constitutive activator (Tani et al., 2001). Indeed, transfection studies in 293 cells showed that integration of RBP-L into PTF1 is required for high-level transcriptional activity by the PTF1 complex (Figure 3.6). Finally, RBP-J can also act as a transcriptional activator (though not as strong as RBP-L) in the context of a PTF1 complex (Summarized in Figure 5.4).

RBP-J is able to regulate transcription by multiple mechanisms, both the activation and repression of target genes. To repress target genes, RBP-J recruits a complex of corepressors and histone deacetylases to keep the local chromatin structure inaccessible to transactivating factors. The activation of the Notch pathway causes the binding of the NotchIC to RBP-J and the recruitment of coactivators and histone acetyltransferases to unravel the local chromatin structure and allow for the activation of target genes (Fryer et al., 2002; Hsieh et al., 1996; Hsieh et al., 1999). The PTF1 complex represents another mechanism of gene regulation by RBP-J. Integration of RBP-J into a PTF1 complex can induce low-level transcriptional activation of the PTF1 targets in a Notch-independent manner, possibly by a de-repression mechanism in which PTF1 replaces a repressor from the E-box that is present in the absence of P48. Thus, although RBP-J is ubiquitously expressed, use in the PTF1 complex requires the tissue-specific expression of P48, and targets containing the bipartite target sequences in the their regulatory regions.

In contrast to RBP-J, RBP-L is not responsive to the Notch pathway. Although, RBP-L prefers the same consensus site as RBP-J *in vitro*, RBP-J has a stronger affinity for these sites (Minoguchi et al., 1997 and Figure 3.4B). Additionally, RBP-L has not been detected as a monomer on DNA in experiments using nuclear extracts (Figure

3.5B). Transfecting large amounts of plasmid expressing RBP-L cannot activate a reporter driven by six copies of an RBP-J site in the absence or presence of an E-box one helical turn upstream (Figure 3.6 and T. Beres and T. Masui, preliminary data). The inability of RBP-L to activate lone RBP-J sites is likely because of the lower binding affinity of RBP-L. Because RBP-L is a constitutive activator and has no known repressor function, binding to RBP-J sites as a monomer would activate many genes normally only activated by Notch signaling. The binding of RBP-J to these sites as a monomer allows for tighter regulation. Thus, it is likely that RBP-L may only function in the context of PTF1 or a similar complex. The main function of RBP-L appears to be high-level activation of specific target genes as part of the PTF1 complex. This serves an important function by allowing for necessarily high expression of the digestive enzymes in the acinar pancreas, the first known gene targets of RBP-L.

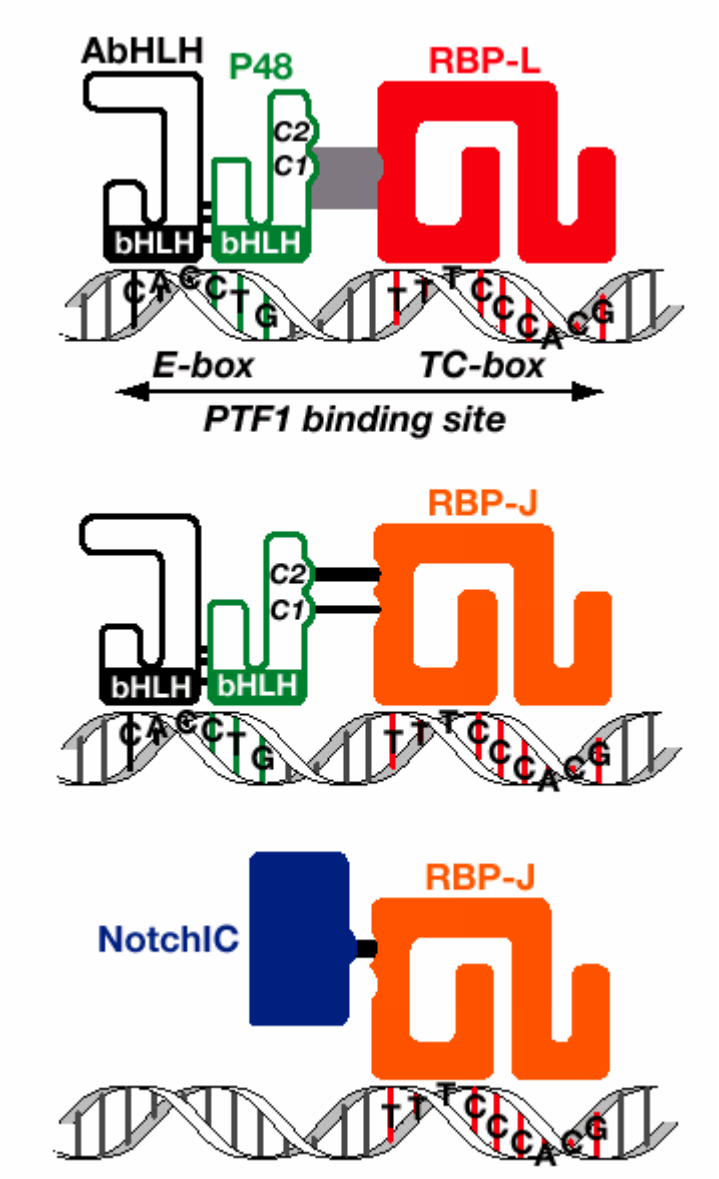


Figure 5.4 A model for activation of target genes by RBP-J and RBP-L.
RBP-L activation of target genes occurs only in the context of the PTF1 complex (top). RBP-J activation of target genes occurs within the context of the PTF1 complex (middle) or in a manner independent of PTF1, but dependent on the Notch pathway (bottom). Interaction domains between RBP proteins and P48 differ slightly and are indicated by black lines or grey bars connecting P48 and RBP.

The *Drosophila* Su(H) shares characteristics with both RBP-J and RBP-L. In addition to its conserved DNA binding preferences, Su(H), like RBP-J, interacts with the NotchIC. Also, like RBP-J, Su(H) can regulate transcription by multiple mechanisms, including Notch-dependent and Notch-independent ones. *Drosophila* mechanosensory bristles require Notch signaling for early development, but not for later development and physiological function. This later function relies on a Notch-independent autoactivation by Su(H) that is necessary for the development of the socket cells and subsequent function in mechanoreception (Barolo et al., 2000). Indeed the Su(H) binding sites in the Su(H) autoactivation enhancer region contain full or partial E-boxes in close proximity to the Su(H) binding sites (R.J. MacDonald, personal communication). Whether this autoactivation requires the interaction of Su(H) with the P48 orthologue Fer1 or another similar protein remains to be tested. The N- and C-termini of RBP-J have been shown to assist the BTD with the recruitment of the NotchIC domain (Kovall and Hendrickson, 2004; Tani et al., 2001). These domains are highly divergent in RBP-J, RBP-L, and Su(H). However, RBP-L has a candidate acidic activation domain in the C-terminus, while Su(H) has a candidate polyglutamine-rich region in its N- and C-terminal domains (R.M. Henke, personal communication). This may explain why both RBP-L and Su(H) have a higher transcriptional activation than RBP-J in the context of that PTF1 complex. The N- and C- termini of RBP-J may retain the ability to help recruit the NotchIC, but not the ability for high-level Notch-independent transcriptional activation. Conversely, differences in the N- and C-termini of RBP-L may be responsible for high-level transcriptional activation, but may also be partially responsible for the inability of RBP-L

to recruit the NotchIC. Defining the activation domain of RBP-L by deletion analyses will give further insight into both of these questions. It will be interesting to see if chimeras of RBP-J containing the potential RBP-L activation domain will convert RBP-J to a stronger activator, alone or in the context of a PTF1 complex, and to see if this change in the C-terminus of RBP-J has a negative effect on its ability to recruit the NotchIC.

Antagonistic effects of PTF1 and Notch signaling

The PTF1 complex represents the first identified Notch-independent mechanism for activation by RBP-J. The NotchIC cannot be detected as a component of the PTF1 complex, but it has been shown inhibit PTF1 activity in transient transfection experiments using pancreatic acinar cell line AR42J (Esni et al., 2004). Infection of AR42J cells with adenoviral vectors expressing the NotchIC or HES1 inhibited PTF1 binding. This effect was not a result of lowering P48 levels in the cells, but these experiments did not conclusively demonstrate that the NotchIC or HES1 directly interfered with PTF1 binding activity by interaction with the component proteins of PTF1 (Esni et al., 2004). Inhibition of PTF1 binding and transcriptional activity by the NotchIC is likely due to direct interference of the NotchIC with the P48-RBP-J interaction. Although PTF1 in adult pancreas contains only RBP-L, and RBP-L is expressed in the acinar cell line 266-6, AR42J cells have not been analyzed for levels of RBP-L and RBP-J. Thus the interference of the NotchIC with this activity may be due to the presence of a PTF1 complex containing RBP-J in AR42J cells instead of the PTF1

complex containing RBP-L. The studies outlined in this dissertation clearly show that adding IVT NotchIC to EMSA binding reactions containing P48, E12, and RBP-J inhibits formation of the reconstituted PTF1-J complex. This effect is concentration-dependent, and much greater than the effect on the reconstituted PTF1 complex containing RBP-L. This was expected since RBP-L does not interact with the NotchIC. This inhibition is further observed in transient transfection experiments in which expression of the NotchIC reduces activation of the *ELA1* A element reporter by P48, HEB, and RBP-J or a VP16-RBP-J fusion in a concentration-dependent manner. This effect on activation is not seen in 293 cells transfected with P48, HEB, and RBP-L. Hence, high-levels of Notch signaling may inhibit PTF1-J mediated gene activation, and a threshold level of P48 may be necessary to compete with the NotchIC for RBP-J binding.

P48 activates genes that are important for acinar cell function, but it also may inhibit targets of Notch signaling to keep cells in a differentiated state. EMSA experiments show that binding of an RBP-J-NotchIC complex to a lone RBP-J binding site is inhibited by mixing increasing amounts of IVT P48 with these proteins. This binding inhibition was significantly reduced when a mutant P48 that does not interact with RBP-J was used in duplicate EMSA experiments instead of wild-type P48. NotchIC mediated activation of a reporter by RBP-J is reduced by expression of P48. The effect is less when the mutant P48 is cotransfected instead of wild-type P48. It is likely that P48 interaction with RBP-J precludes NotchIC interaction with RBP-J. It is also likely that this P48-RBP-J complex cannot bind a lone RBP-J consensus binding site, because a heterodimer of P48-RBP-L, but not P48-RBP-J can be reconstituted on the optimal RBP-J/L binding sites present on the O_{54} probe in EMSA (Figure 4.10A). One way to confirm

that P48 blocks the interaction of the NotchIC with RBP-J instead of just converting the NotchIC-RBP-J complex to a NotchIC-RBP-J-P48 complex that does not bind DNA is by coimmunoprecipitation. In mixtures of P48, NotchIC, and RBP-J, coimmunopreciptations using anti-P48 antibodies should only yield P48 and RBP-J and not the NotchIC. Conversely, coimmunoprecipitation of NotchIC should only yield the NotchIC and RBP-J and not P48. These confirmatory experiments have not yet been done. It seems that formation of a PTF1 complex containing RBP-J can inhibit activation of Notch target genes by sequestering RBP-J. Hence high levels of P48 may inhibit transcription of Notch target genes, such as *Hes1*, whose activation inhibits differentiation. Experiments examining the effects of P48 on the activation of the *Hes1* promoter have not been done yet to test this idea. Inhibition of the suppressive maintenance function of Notch signaling may aid in the promotion of a differentiated state during the secondary transition, when RBP-L levels are low. However, in the mature pancreas, high-levels of RBP-L may bind the vast majority of P48, allowing RBP-J binding to lone RBP-J target sites. Although PTF1-J and NotchIC-RBP-J complexes have antagonistic effects on each other, inhibition in transient transfections is not complete. The NotchIC inhibition of PTF1 activity and the P48 inhibition of NotchIC-RBP-J activity reduced activation to only about 40% the activity in the absence of the NotchIC. A wider range of concentrations in transient transfections will be required to further examine whether this type of inhibition is feasible *in vivo*. If these complexes do truly inhibit each other, P48 might have a two-fold function to inhibit the precursor state in the absence of high levels of RBP-L and activate the initiation of target genes such as

RBP-L with a PTF1- J complex, and then likely maintains acinar cell function in the mature pancreas as part of the PTF1 complex.

A role for PTF1 component switiching in pancreas development and maintenance

The high amount of the PTF1 complex containing RBP-L in exocrine pancreas suggests that RBP-L is an important molecule in determining and maintaining acinar cell fate. Expression of *p48* and *Rbp-L* is coincident in maturing acini just after the secondary transition (G.H. Swift and V. Barbera, personal communication). At embryonic day 14.5, *p48* and *Rbp-L* are exclusively expressed in the pre-acini and excluded from the *Ngn3* expressing endocrine cells. Certainly, the high transcriptional activity of RBP-L within the context of the PTF1 complex is required to keep the very high expression levels of pancreatic enzymes necessary for digestion. At the very least, this implicates an important role for RBP-L in the maintenance of the acinar cell phenotype. Targeted deletions of *Rbp-L* have previously been carried out by inserting a lacZ coding region to disrupt the gene while using β-gal staining to examine the expression pattern of *Rbp-L* (Minoguchi et al., 1999). However, these, and previous studies of *Rbp-L* had only revealed expression in the lung and, to a lesser extent, the brain (Minoguchi et al., 1997). Analysis of the *Rbp-L* knockout mouse revealed no obvious brain defect, and, to our knowledge no pancreas defect. However, a study of the pancreatic phenotype was not reported, possibly because it was not done. Further studies of the pancreatic phenotype of *Rbp-L* knockout mice (T. Masui, under construction) will likely reveal whether RBP-L has an essential acinar function. It is likely that, at the very least, expression of digestive

enzyme genes will be substantially reduced in these mutant mice. It is also possible that acinar development may be inhibited or delayed. Also of interest will be to study mice in which a mutant P48 that cannot interact with RBP-L is knocked into the P48 locus. This would distinguish which processes during pancreatic development and function require a PTF1-L complex rather than a PTF1-J complex. However, the exact minimal domain of P48 required for its interaction with RBP-L has yet to be defined.

A PTF1 complex containing RBP-J may be of functional importance in the absence of RBP-L. Reconstitution of a PTF1 complex can be accomplished using either RBP-L or RBP-J, however only RBP-L is present in PTF1 from the mature pancreas. The presence of an active PTF1 site in the promoter of *Rbp-L* may indicate a function for a PTF1 complex that employs RBP-J during early in development. Furthermore, initial RT-PCR analysis of pancreas from various stages of mouse embryos indicates very low levels of *Rbp-L* transcript at embryonic day 11.5, while the level of *Rbp-J* transcript at this stage appears very high (Figure 5.5A). Interestingly, as development progresses, the level of *Rbp-J* transcript appears to decrease, while levels of *Rbp-L* transcript appear to dramatically increase. Re-evaluation of transcript levels using quantitative PCR indeed indicate that level of *Rbp-J* transcript decreases 10-fold from embryonic day 11.5 until birth, while the transcript level of *Rbp-L* increases about 100-fold. This is also the time in which the level of *p48* transcript dramatically increases (T. Masui, personal communication). These observations may indicate that the initial activation of target genes is by a PTF1-J complex, while high-level activation of target genes by the PTF1 complex with RBP-L may be important to the differentiation and function of acinar cells (Figure 5.5B).

It is likely that an RBP-J-containing PTF1 complex is important for embryonic development. As previously mentioned, mutations that eliminate P48 interactions with RBP-J and RBP-L lead to an apancreatic and acerebellar phenotype (Sellick et al., 2004). Several other lines of evidence imply an essential function for a PTF1 complex that contains RBP-J as opposed to one that contains RBP-L. First, RBP-J is present during cerebellar development, while RBP-L is not (Gray et al., 2004, T. Masui and R.M. Henke, personal communication). It is possible that interaction with a protein other than RBP-J is important for P48 function in cerebellar development, but no other protein-interacting domains have been identified, and no other proteins similar to RBP-J or RBP-L that interact with P48 have been described. Also, it seems that a PTF1-J complex might be essential for activation of *Rbp-L*. Critical and specific information will be gained by knocking the non-RBP-J-interacting mutant of P48 (W298A) into the wild-type locus. This will likely disrupt pancreas and cerebellum development, as well as the expression of certain putative target genes, like *Rbp-L*. The single amino acid mutation in mouse P48 disrupts its interaction with RBP-J, while the identified human mutations truncate the entire C-terminus and disrupt P48 interaction with RBP-J and RBP-L (Sellick et al., 2004). However, based on the phenotype of infants homozygous for deletions of the RBP-interacting domains of P48 (Sellick et al., 2004), it is likely that the formation of a PTF1-J complex is essential for the development of the pancreas and the cerebellum.

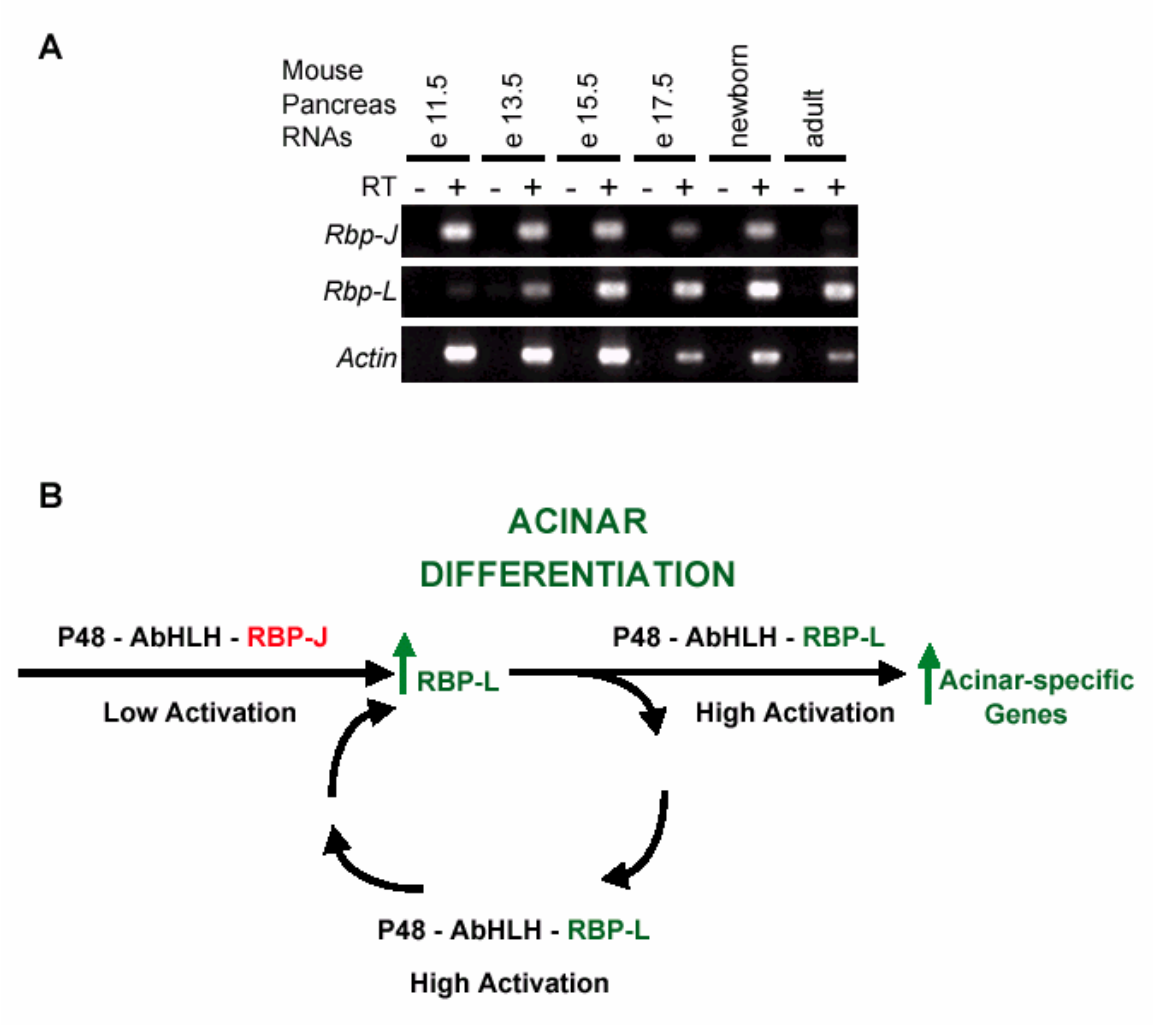


Figure 5.5 Component switching in the PTF1 complex during pancreatic acinar development. A. RT-PCR analyses of *Rbp-L* and *Rbp-J* transcripts in mouse pancreas using RNAs from various developmental stages. Results were compared to RT-PCR of Actin mRNA. B. A model for component switching of PTF1 during development. Low level activation of Rbp-L is activated by a PTF1 complex containing RBP-L. The presence of RBP-L results in the subsequent high-level activation of *Rbp-L* and acinar-specific genes.

PTF1 and PTF1-like complexes in the development and function of multiple organs

In addition to PTF1, other complexes that incorporate RBP proteins may form and regulate the development and function of tissues. Recent studies of SU(H), including the crystal structure of the *C. elegans* orthologue LAG-1, have yielded important insights into its interaction with the NotchIC. The sequence of the NotchIC that interacts with the hydrophobic pocket of Lag-1 is similar to sequences in other proteins. In addition to P48, EBNA and KYOT2 possess tryptophan-containing motifs, and interact with Su(H) and its mammalian orthologue, RBP-J. Indeed KYOT2 has been shown to inhibit Notch activity by competing for binding with RBP-J (Qin et al., 2004; Qin and Han, 2004; Taniguchi et al., 1998). More recently, it has been shown that the short peptide in KYOT2 that is similar to the RBP-J-interacting motif of the NotchIC can inhibit formation of a PTF1 complex containing RBP-J (T. Masui, personal communication). A further examination of the protein database revealed more proteins that contain similar motifs (R.M. Henke and T. Beres, summarized in Figure 5.6). Further examination will likely identify other proteins with this motif. Identification of other proteins that can interact with RBP-J in a manner similar to NotchIC or P48, and analysis of the RBP-J interacting peptides will refine the consensus sequence for these RBP-J-interacting peptides. Until now KYOT2 was the only protein containing this motif that had been shown to block RBP-J-NotchIC complex formation. Assuming other transcription factors do interact with RBP-J or RBP-L, RBP proteins may be involved in the development and function of multiple tissues in a manner that is independent of Notch signaling.

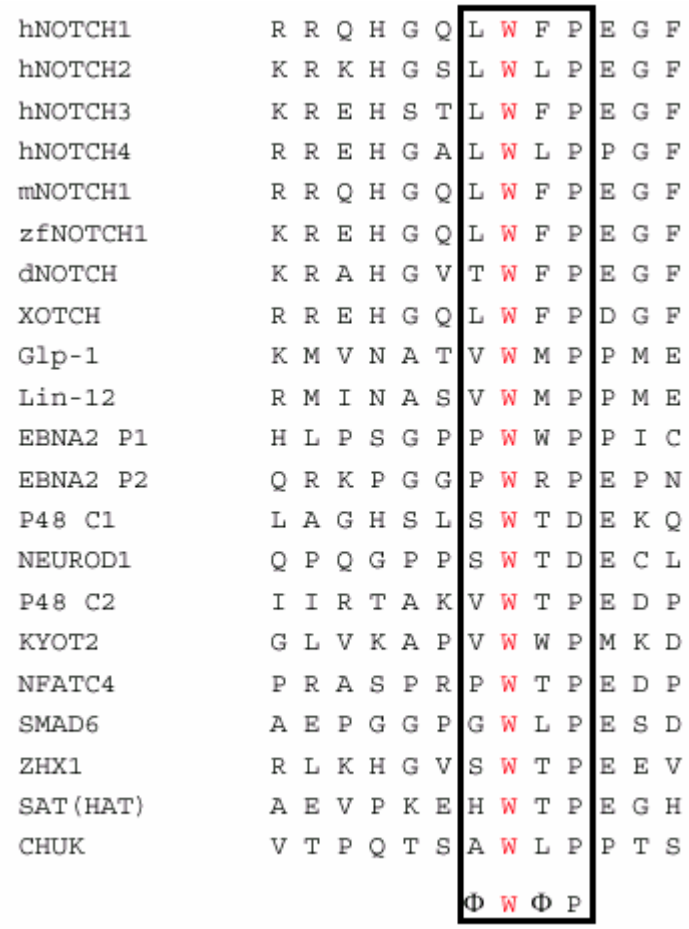


Figure 5.6 Sequence conservation of the NotchIC RAM domains and other proteins that interact with SUH family proteins.
Notch proteins from multiple organisms share a similar sequence in the region that interacts with Su(H) to EBNA2 P1, P48, and KYOT2. Other proteins shown share a similar sequence, but have not been shown to interact with Su(H) family proteins.

The formation of PTF1-like complexes is likely determined by tissue distribution of factors containing RBP protein-interacting motif, as well as levels of RBP-J and RBP-L. P48 activity during development of the cerebellum, as well as the pancreas, is dependent on its interaction with RBP proteins. Thus, the formation of the PTF1 complex containing RBP-J is determined by the tissue-specific expression of P48, not the ubiquitously expressed AbHLH or RBP-J proteins. Similarly, another transcription factor containing the RBP protein-binding motif may activate a different organ-specific developmental program in regions of the embryo that lack P48. As previously implied, binding of PTF1-like complexes to gene targets may be modulated by the E-box in the target site. Differences in the canonical CANNTG E-box sequence and its immediate flanking sequences create preferences for heterodimers or homodimers of bHLH proteins. Complete ablation of the E-box eliminates binding of the P48 heterodimer and the PTF1 complex (Rose et al. 2001), while the mutation of the CACCTG sequence to CACTTG, also eliminates binding of the P48 heterodimers and the PTF1 complex (Rose and MacDonald, 1997; Rose et al., 2001 and Figure 3.7A). Although the mutant sequence is still a canonical E-box, it is not one that is preferred by P48 or PTF1. As previously noted, some PTF1 binding sites (such as mouse *Ela2)* lack a canonical E-box, but still bind PTF1, while the C to T mutation in the *ELA1* PTF1 site still has a canonical E-box, but cannot bind PTF1. Variations in the E-box may modulate the specificity of PTF1-like complexes containing tissue-specifically expressed bHLH proteins other than P48.

Although PTF1 is essential for development and function of the acinar pancreas, the endocrine pancreas may also use a complex with RBP-L. The mature endocrine

pancreas in mouse lacks P48, a key component of the PTF1 complex. However, RT-PCR of human islet RNA (Figure 3.9), and immunofluorescence of mouse pancreas sections (L. Shi, personal communication) reveal a strikingly high amount of RBP-L transcript and protein in the mature islets. Like P48 and RBP-L protein in the acinar cells, RBP-L is localized in the nucleus in islets. Since P48 is not expressed in mouse islets, and RBP-L has not been shown to activate genes in the absence of the other PTF1 components, it is likely that another protein substitutes for P48 as an RBP-L interacting partner in an endocrine PTF1-like complex. Lineage tracing experiments show that some cells that initially express P48 become endocrine as well as exocrine cells (Kawaguchi et al., 2002). Thus, like the exocrine pancreas, initial development of the islets may rely on an RBP-J-containing PTF1 complex. When P48 expression is lost in cells that become islets, another protein may substitute for P48 to interact with RBP proteins. Initial scanning for proteins containing a Notch-IC like peptide that interacts with RBP proteins has identified some candidates. NFAT-c4 and NEUROD1 each have a motif that closely resembles the RBP-interacting peptides of P48. *NeuroD1* is activated by the class B bHLH protein NGN3, and maintains the expression of islet-specific genes (Heremans et al., 2002; Huang et al., 2000). A series of biochemical experiments, including EMSA and coimmunoprecipitation, will reveal if interactions between RBP proteins and a non-P48 protein occur in the pancreatic islets.

The proximal PTF1 binding site of *Rbp-L* is a good candidate for biochemical studies to identify an islet PTF1-like complex. RBP-L is expressed in islets, and *Rbp-L* is likely autoregulated by PTF1 in exocrine pancreas. It is possible that a PTF1 complex that substitutes another protein, such as NEUROD1, for P48 may be responsible for

autoregulation of *Rbp-L* in islets. Although the distal E-box of the *Rbp-L* PTF1 binding site is a good binding site for a P48-AbHLH heterodimer, the proximal E-box is not. This E-box might be one that favors the binding of an islet-specific bHLH protein like NeuroD1. However, initial tests to examine whether NEUROD1 can form a PTF1-like complex on the *Rbp-L* PTF1 binding site have suggested that this is not the P48 like protein in an islet complex (T. Masui, personal communication). An initial screen for islet-specific factors that bind the *Rbp-L* promoter should involve examining islet-specific factors for the NotchIC-like motif. The ability of a candidate factor to bind this site as part of a PTF1-like complex can be easily examined with IVT versions of these proteins to reassemble a complex for EMSA experiments. If these binding proteins are identified, it will be interesting to examine their ability to activate this promoter region in transient transfection experiments and to test whether the NotchIC-like peptide motif is essential for this activity. Identification of other candidates by coimmunoprecipitation with an anti-RBP-L antibody, or pull-downs with a biotinylated *Rbp-L* PTF1 binding site oligonucleotides using extracts from islets, or initially islet cell lines, may lead to the identification of RBP-L-interacting partner(s) in islets. Screening of islet cell lines will need to be done initially to identify which ones express *Rbp-L*, so those cells lines can be used in biochemical assays. Partners could be identified by mass spectrometry of pulled-down proteins. Success in the initial biochemical experiments would lead to *in vivo* experiments.

In addition to the development and function of the brain and endocrine and exocrine pancreas, it is likely that PTF1-like complexes may be at work in other organs. Because lung exhibits high levels of RBP-L transcript (Minoguchi et al., 1997) and is not

known to express P48, it is a good candidate for the study of PTF1-like complexes. Studies of RBP-L transcript levels by RT-PCR have revealed that the lung is the only other tissue besides pancreas that expresses high levels of RBP-L. *In situ* hybridization studies have revealed that *Rbp-L* expression is limited to a small tissue in the lung known as tracheal glands (Minoguchi et al., 1999; Minoguchi et al., 1997). Although a lung phenotype was not mentioned in the initial knockout studies of the *Rbp-L* gene in mice (Minoguchi et al 1999), further investigation of RBP-L function in lung has not been done. Expression studies using RNase protection (Rose et al., 2001) and RT-PCR (Figure 3.9A**)** have not detected *p48* in lung. Furthermore, P48 lineage tracing and knockout studies have not revealed expression of *p48* in the lung or a lung phenotype. However, since investigators were not specifically looking at lung, and its potential partner RBP-L is only expressed in a small cell population, a phenotype might not have been obvious. If the absence of P48 in the lung is confirmed, then RBP-L would likely bind to another partner in a PTF1-like complex. Again, the promoter of the *Rbp-L* gene itself would be the only known target of a PTF1-like complex in the lung. Thus it would be an ideal initial target for biochemical studies. Experiments similar to those mentioned for the islet pancreas using lung extracts, and searches for lung-specific proteins containing the RBP protein-interacting motifs will likely lead to the identification of RBP-interacting partners in lung. Like the digestive enzyme genes of the acinar pancreas, expression of RBP-L in the tracheal gland of the lung may be necessary for high-level expression of particular genes in this specific area.

PTF1 represents the first identification of a Notch-independent use of SU(H) orthologues. Additionally, digestive enzyme genes represent the first known targets for

the vertebrate-specific paralogue of SUH, RBP-L. In contrast to RBP-J, RBP-L might only activate transcription in the context of PTF1 or PTF1-like complexes. PTF1 activates genes important to the function of terminally differentiated acinar cells, but a PTF1-J complex may be essential for initial activation of the *Rbp-L* gene and other, as of yet unidentified, targets. A general mechanism for the activation of programs critical to the development and function of multiple organs may require the formation and activity of PTF1 and other similar tissue-specific complexes (Figure 5.7).

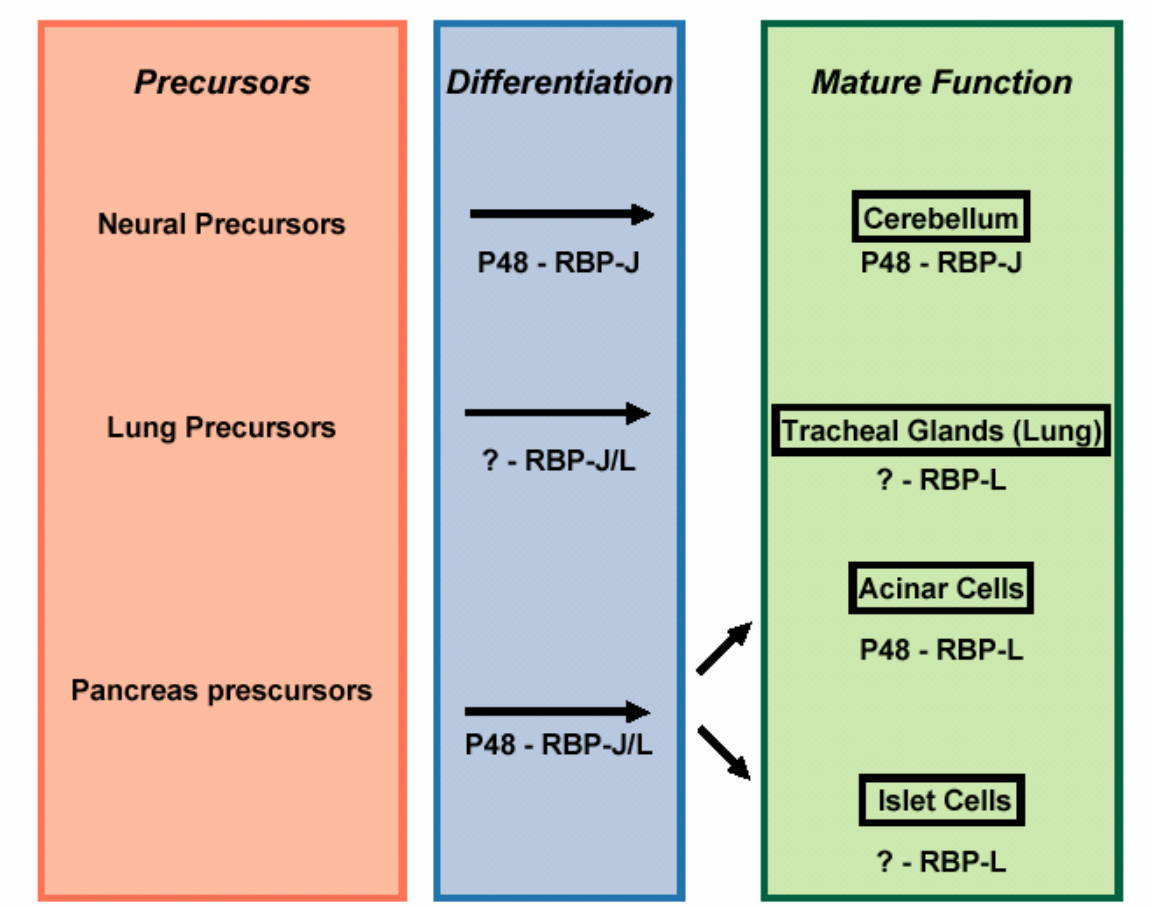


Figure 5.7 A model for Notch-independent use of RBP-J/L during the development and function of multiple organs.

Bibliography

Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K., and Edlund, H. (1998). beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev 12, 1763-1768.

Ahlgren, U., Pfaff, S. L., Jessell, T. M., Edlund, T., and Edlund, H. (1997). Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature 385, 257-260.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U., and Edlund, H. (1999). Notch signalling controls pancreatic cell differentiation. Nature 400, 877-881.

Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. Science 284, 770-776.

Barolo, S., Walker, R. G., Polyanovsky, A. D., Freschi, G., Keil, T., and Posakony, J. W. (2000). A notch-independent activity of suppressor of hairless is required for normal mechanoreceptor physiology. Cell 103, 957-969.

Bhushan, A., Itoh, N., Kato, S., Thiery, J. P., Czernichow, P., Bellusci, S., and Scharfmann, R. (2001). Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development 128, 5109-5117.

Blackwell, T. K., and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250, 1104-1110.

Burglin, T. R. (1997). Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. Nucleic Acids Res 25, 4173-4180.

Chiaramello, A., Neuman, K., Palm, K., Metsis, M., and Neuman, T. (1995). Helix-loop-helix transcription factors mediate activation and repression of the p75LNGFR gene. Mol Cell Biol 15, 6036-6044.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.

Chuang, P. T., and Kornberg, T. B. (2000). On the range of hedgehog signaling. Curr Opin Genet Dev 10, 515-522.

Chuang, P. T., and McMahon, A. P. (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature 397, 617-621.

Chung, C. N., Hamaguchi, Y., Honjo, T., and Kawaichi, M. (1994). Site-directed mutagenesis study on DNA binding regions of the mouse homologue of Suppressor of Hairless, RBP-J kappa. Nucleic Acids Res 22, 2938-2944.

Cockell, M., Stevenson, B. J., Strubin, M., Hagenbuchle, O., and Wellauer, P. K. (1989). Identification of a cell-specific DNA-binding activity that interacts with a transcriptional activator of genes expressed in the acinar pancreas. Mol Cell Biol 9, 2464-2476.

Dahl, E., Koseki, H., and Balling, R. (1997). Pax genes and organogenesis. Bioessays 19, 755-765.

Deutsch, G., Jung, J., Zheng, M., Lora, J., and Zaret, K. S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128, 871-881.

Dutta, S., Gannon, M., Peers, B., Wright, C., Bonner-Weir, S., and Montminy, M. (2001). PDX:PBX complexes are required for normal proliferation of pancreatic cells during development. Proc Natl Acad Sci U S A 98, 1065-1070.

Esni, F., Ghosh, B., Biankin, A. V., Lin, J. W., Albert, M. A., Yu, X., MacDonald, R. J., Civin, C. I., Real, F. X., Pack, M. A., et al. (2004). Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. Development 131, 4213-4224.

Fisher, A. L., and Caudy, M. (1998). Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. Genes Dev 12, 1931-1940.

Fisher, A. L., Ohsako, S., and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. Mol Cell Biol 16, 2670-2677.

Fryer, C. J., Lamar, E., Turbachova, I., Kintner, C., and Jones, K. A. (2002). Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes Dev 16, 1397-1411.

German, M. S., Blanar, M. A., Nelson, C., Moss, L. G., and Rutter, W. J. (1991). Two related helix-loop-helix proteins participate in separate cell-specific complexes that bind the insulin enhancer. Mol Endocrinol 5, 292-299.

Golosow, N., and Grobstein, C. (1962). Epitheliomesenchymal interaction in pancreatic morphogenesis. Dev Biol 4, 242-255.

Goudet, G., Delhalle, S., Biemar, F., Martial, J. A., and Peers, B. (1999). Functional and cooperative interactions between the homeodomain PDX1, Pbx, and Prep1 factors on the somatostatin promoter. J Biol Chem 274, 4067-4073.

Gradwohl, G., Dierich, A., LeMeur, M., and Guillemot, F. (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 97, 1607-1611.

Grapin-Botton, A., Majithia, A. R., and Melton, D. A. (2001). Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. Genes Dev 15, 444-454.

Gray, P. A., Fu, H., Luo, P., Zhao, Q., Yu, J., Ferrari, A., Tenzen, T., Yuk, D. I., Tsung, E. F., Cai, Z., et al. (2004). Mouse brain organization revealed through direct genome-scale TF expression analysis. Science 306, 2255-2257.

Gu, G., Brown, J. R., and Melton, D. A. (2003). Direct lineage tracing reveals the ontogeny of pancreatic cell fates during mouse embryogenesis. Mech Dev 120, 35-43.

Gu, G., Dubauskaite, J., and Melton, D. A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129, 2447-2457.

Guillemot, F. (1995). Analysis of the role of basic-helix-loop-helix transcription factors in the development of neural lineages in the mouse. Biol Cell 84, 3-6.

Hald, J., Hjorth, J. P., German, M. S., Madsen, O. D., Serup, P., and Jensen, J. (2003). Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. Dev Biol 260, 426-437.

Harding, J. D., MacDonald, R. J., Przybyla, A. E., Chirgwin, J. M., Pictet, R. L., and Rutter, W. J. (1977). Changes in the frequency of specific transcripts during development of the pancreas. J Biol Chem 252, 7391-7397.

Harding, J. D., and Rutter, W. J. (1978). Rat pancreatic amylase mRNA. Tissue specificity and accumulation during embryonic development. J Biol Chem 253, 8736-8740.

Harrison, K. A., Thaler, J., Pfaff, S. L., Gu, H., and Kehrl, J. H. (1999). Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. Nat Genet 23, 71-75.

Hebrok, M., Kim, S. K., and Melton, D. A. (1998). Notochord repression of endodermal Sonic hedgehog permits pancreas development. Genes Dev 12, 1705-1713.

Hebrok, M., Kim, S. K., St Jacques, B., McMahon, A. P., and Melton, D. A. (2000). Regulation of pancreas development by hedgehog signaling. Development 127, 4905-4913.

Heller, R. S., Stoffers, D. A., Liu, A., Schedl, A., Crenshaw, E. B., 3rd, Madsen, O. D., and Serup, P. (2004). The role of Brn4/Pou3f4 and Pax6 in forming the pancreatic glucagon cell identity. Dev Biol 268, 123-134.

Heremans, Y., Van De Casteele, M., in't Veld, P., Gradwohl, G., Serup, P., Madsen, O., Pipeleers, D., and Heimberg, H. (2002). Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3. J Cell Biol 159, 303-312.

Herrera, P. L. (2000). Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127, 2317-2322.

Hoffman, M. P., Kidder, B. L., Steinberg, Z. L., Lakhani, S., Ho, S., Kleinman, H. K., and Larsen, M. (2002). Gene expression profiles of mouse submandibular gland development: FGFR1 regulates branching morphogenesis in vitro through BMP- and FGF-dependent mechanisms. Development 129, 5767-5778.

Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1990). Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques 8, 528-535.

Hsieh, J. J., and Hayward, S. D. (1995). Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. Science 268, 560-563.

Hsieh, J. J., Henkel, T., Salmon, P., Robey, E., Peterson, M. G., and Hayward, S. D. (1996). Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. Mol Cell Biol 16, 952-959.

Hsieh, J. J., Zhou, S., Chen, L., Young, D. B., and Hayward, S. D. (1999). CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. Proc Natl Acad Sci U S A 96, 23-28.

Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M., and Tsai, M. J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol Cell Biol 20, 3292-3307.

Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev 9, 3136-3148.

Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S., and Kageyama, R. (1994). Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. Embo J 13, 1799-1805.

Jonsson, J., Ahlgren, U., Edlund, T., and Edlund, H. (1995). IPF1, a homeodomain protein with a dual function in pancreas development. Int J Dev Biol 39, 789-798.
Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371, 606-609.

Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R. J., and Wright, C. V. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 32, 128-134.

Kawahira, H., Scheel, D. W., Smith, S. B., German, M. S., and Hebrok, M. (2005). Hedgehog signaling regulates expansion of pancreatic epithelial cells. Dev Biol 280, 111-121.

Kelly, L. M., Englmeier, U., Lafon, I., Sieweke, M. H., and Graf, T. (2000). MafB is an inducer of monocytic differentiation. Embo J 19, 1987-1997.

Kim, S. K., Hebrok, M., Li, E., Oh, S. P., Schrewe, H., Harmon, E. B., Lee, J. S., and Melton, D. A. (2000). Activin receptor patterning of foregut organogenesis. Genes Dev 14, 1866-1871.

Kim, S. K., Hebrok, M., and Melton, D. A. (1997). Notochord to endoderm signaling is required for pancreas development. Development 124, 4243-4252.

Kim, S. K., and MacDonald, R. J. (2002). Signaling and transcriptional control of pancreatic organogenesis. Curr Opin Genet Dev 12, 540-547.

Kim, S. K., and Melton, D. A. (1998). Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. Proc Natl Acad Sci U S A 95, 13036-13041.

Kim, S. K., Selleri, L., Lee, J. S., Zhang, A. Y., Gu, X., Jacobs, Y., and Cleary, M. L. (2002). Pbx1 inactivation disrupts pancreas development and in Ipf1-deficient mice promotes diabetes mellitus. Nat Genet 30, 430-435.

Knoepfler, P. S., Bergstrom, D. A., Uetsuki, T., Dac-Korytko, I., Sun, Y. H., Wright, W. E., Tapscott, S. J., and Kamps, M. P. (1999). A conserved motif N-terminal to the DNA-binding domains of myogenic bHLH transcription factors mediates cooperative DNA binding with pbx-Meis1/Prep1. Nucleic Acids Res 27, 3752-3761.

Kojima, M., Hosoda, H., and Kangawa, K. (2001). Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. Horm Res 56 Suppl 1, 93-97.

Kovall, R. A., and Hendrickson, W. A. (2004). Crystal structure of the nuclear effector of Notch signaling, CSL, bound to DNA. Embo J 23, 3441-3451.

Krapp, A., Knofler, M., Frutiger, S., Hughes, G. J., Hagenbuchle, O., and Wellauer, P. K. (1996). The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. Embo J 15, 4317-4329.

Krapp, A., Knofler, M., Ledermann, B., Burki, K., Berney, C., Zoerkler, N., Hagenbuchle, O., and Wellauer, P. K. (1998). The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev 12, 3752-3763.

Kruse, F., Rose, S. D., Swift, G. H., Hammer, R. E., and MacDonald, R. J. (1993). An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer. Genes Dev 7, 774-786.

Kruse, F., Rose, S. D., Swift, G. H., Hammer, R. E., and MacDonald, R. J. (1995). Cooperation between elements of an organ-specific transcriptional enhancer in animals. Mol Cell Biol 15, 4385-4394.

Lammert, E., Cleaver, O., and Melton, D. (2001). Induction of pancreatic differentiation by signals from blood vessels. Science 294, 564-567.

Lammert, E., Cleaver, O., and Melton, D. (2003). Role of endothelial cells in early pancreas and liver development. Mech Dev 120, 59-64.

Langlands, K., Yin, X., Anand, G., and Prochownik, E. V. (1997). Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. J Biol Chem 272, 19785-19793.

Lee, J. J., von Kessler, D. P., Parks, S., and Beachy, P. A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog. Cell 71, 33-50.

Lemercier, C., To, R. Q., Swanson, B. J., Lyons, G. E., and Konieczny, S. F. (1997). Mist1: a novel basic helix-loop-helix transcription factor exhibits a developmentally regulated expression pattern. Dev Biol 182, 101-113.

Levin, M., Pagan, S., Roberts, D. J., Cooke, J., Kuehn, M. R., and Tabin, C. J. (1997). Left/right patterning signals and the independent regulation of different aspects of situs in the chick embryo. Dev Biol 189, 57-67.

Li, H., Arber, S., Jessell, T. M., and Edlund, H. (1999). Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. Nat Genet 23, 67-70.

Li, H., and Edlund, H. (2001). Persistent expression of Hlxb9 in the pancreatic epithelium impairs pancreatic development. Dev Biol 240, 247-253.

Liu, Y., Jiang, H., Crawford, H. C., and Hogan, B. L. (2003). Role for ETS domain transcription factors Pea3/Erm in mouse lung development. Dev Biol 261, 10-24.

Liu, Y., MacDonald, R. J., and Swift, G. H. (2001). DNA binding and transcriptional activation by a PDX1.PBX1b.MEIS2b trimer and cooperation with a pancreas-specific basic helix-loop-helix complex. J Biol Chem 276, 17985-17993.

MacDonald, R. J., Hammer, R. E., Swift, G. H., Ornitz, D. M., Davis, B. P., Palmiter, R. D., and Brinster, R. L. (1986). Tissue-specific expression of pancreatic genes in transgenic mice. Ann N Y Acad Sci 478, 131-146.

MacDonald, R. J., Swift, G. H., Przybyla, A. E., and Chirgwin, J. M. (1987). Isolation of RNA using guanidinium salts. Methods Enzymol 152, 219-227.

Mansouri, A., Hallonet, M., and Gruss, P. (1996). Pax genes and their roles in cell differentiation and development. Curr Opin Cell Biol 8, 851-857.

Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function. Science 294, 559-563.

Matsuoka, T. A., Artner, I., Henderson, E., Means, A., Sander, M., and Stein, R. (2004). The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. Proc Natl Acad Sci U S A 101, 2930-2933.

Minoguchi, S., Ikeda, T., Itohara, S., Kaneko, T., Okaichi, H., and Honjo, T. (1999). Studies on the cell-type specific expression of RBP-L, a RBP-J family member, by replacement insertion of beta-galactosidase. J Biochem (Tokyo) 126, 738-747.

Minoguchi, S., Taniguchi, Y., Kato, H., Okazaki, T., Strobl, L. J., Zimber-Strobl, U., Bornkamm, G. W., and Honjo, T. (1997). RBP-L, a transcription factor related to RBP-Jkappa. Mol Cell Biol 17, 2679-2687.

Murre, C., McCaw, P. S., and Baltimore, D. (1989a). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56, 777-783.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., and et al. (1989b). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58, 537-544.

Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B., and Tsai, M. J. (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev 11, 2323-2334.

Naya, F. J., Stellrecht, C. M., and Tsai, M. J. (1995). Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev 9, 1009-1019.

Norgaard, G. A., Jensen, J. N., and Jensen, J. (2003). FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. Dev Biol 264, 323-338.

Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature 287, 795-801.

Obata, J., Yano, M., Mimura, H., Goto, T., Nakayama, R., Mibu, Y., Oka, C., and Kawaichi, M. (2001). p48 subunit of mouse PTF1 binds to RBP-Jkappa/CBF-1, the intracellular mediator of Notch signalling, and is expressed in the neural tube of early stage embryos. Genes Cells 6, 345-360.

Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L., and Wright, C. V. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 122, 983-995.

Ohlsson, H., Karlsson, K., and Edlund, T. (1993). IPF1, a homeodomain-containing transactivator of the insulin gene. Embo J 12, 4251-4259.

Olbrot, M., Rud, J., Moss, L. G., and Sharma, A. (2002). Identification of beta-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. Proc Natl Acad Sci U S A 99, 6737-6742.

Oster, A., Jensen, J., Edlund, H., and Larsson, L. I. (1998a). Homeobox gene product Nkx 6.1 immunoreactivity in nuclei of endocrine cells of rat and mouse stomach. J Histochem Cytochem 46, 717-721.

Oster, A., Jensen, J., Serup, P., Galante, P., Madsen, O. D., and Larsson, L. I. (1998b). Rat endocrine pancreatic development in relation to two homeobox gene products (Pdx-1 and Nkx 6.1). J Histochem Cytochem 46, 707-715.

Peers, B., Sharma, S., Johnson, T., Kamps, M., and Montminy, M. (1995). The pancreatic islet factor STF-1 binds cooperatively with Pbx to a regulatory element in the somatostatin promoter: importance of the FPWMK motif and of the homeodomain. Mol Cell Biol 15, 7091-7097.

Peshavaria, M., Cissell, M. A., Henderson, E., Petersen, H. V., and Stein, R. (2000). The PDX-1 activation domain provides specific functions necessary for transcriptional stimulation in pancreatic beta-cells. Mol Endocrinol 14, 1907-1917.

Petersen, H. V., Serup, P., Leonard, J., Michelsen, B. K., and Madsen, O. D. (1994). Transcriptional regulation of the human insulin gene is dependent on the homeodomain protein STF1/IPF1 acting through the CT boxes. Proc Natl Acad Sci U S A 91, 10465-10469.

Pictet, R. L., Clark, W. R., Williams, R. H., and Rutter, W. J. (1972). An ultrastructural analysis of the developing embryonic pancreas. Dev Biol 29, 436-467.

Pin, C. L., Bonvissuto, A. C., and Konieczny, S. F. (2000). Mist1 expression is a common link among serous exocrine cells exhibiting regulated exocytosis. Anat Rec 259, 157-167.

Pin, C. L., Rukstalis, J. M., Johnson, C., and Konieczny, S. F. (2001). The bHLH transcription factor Mist1 is required to maintain exocrine pancreas cell organization and acinar cell identity. J Cell Biol 155, 519-530.

Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J., and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature 407, 535-538.

Prado, C. L., Pugh-Bernard, A. E., Elghazi, L., Sosa-Pineda, B., and Sussel, L. (2004). Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proc Natl Acad Sci U S A 101, 2924-2929.

Qin, H., Wang, J., Liang, Y., Taniguchi, Y., Tanigaki, K., and Han, H. (2004). RING1 inhibits transactivation of RBP-J by Notch through interaction with LIM protein KyoT2. Nucleic Acids Res 32, 1492-1501.

Qin, H. Y., and Han, H. (2004). [Effect of KyoT2 binding protein KBP1 on RBP-J-mediated transcriptional activity]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 20, 544-547.

Rose, S. D., Kruse, F., Swift, G. H., MacDonald, R. J., and Hammer, R. E. (1994). A single element of the elastase I enhancer is sufficient to direct transcription selectively to the pancreas and gut. Mol Cell Biol 14, 2048-2057.

Rose, S. D., and MacDonald, R. J. (1997). Evolutionary silencing of the human elastase I gene (ELA1). Hum Mol Genet 6, 897-903.

Rose, S. D., Swift, G. H., Peyton, M. J., Hammer, R. E., and MacDonald, R. J. (2001). The role of PTF1-P48 in pancreatic acinar gene expression. J Biol Chem 276, 44018-44026.

Rossi, J. M., Dunn, N. R., Hogan, B. L., and Zaret, K. S. (2001). Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 15, 1998-2009.

Roux, E., Strubin, M., Hagenbuchle, O., and Wellauer, P. K. (1989). The cell-specific transcription factor PTF1 contains two different subunits that interact with the DNA. Genes Dev 3, 1613-1624.

Sander, M., and German, M. S. (1997). The beta cell transcription factors and development of the pancreas. J Mol Med 75, 327-340.

Sander, M., Neubuser, A., Kalamaras, J., Ee, H. C., Martin, G. R., and German, M. S. (1997). Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. Genes Dev 11, 1662-1673.

Sander, M., Sussel, L., Conners, J., Scheel, D., Kalamaras, J., Dela Cruz, F., Schwitzgebel, V., Hayes-Jordan, A., and German, M. (2000). Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development 127, 5533-5540.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes Dev 6, 2620-2634.

Sawada, S., and Littman, D. R. (1993). A heterodimer of HEB and an E12-related protein interacts with the CD4 enhancer and regulates its activity in T-cell lines. Mol Cell Biol 13, 5620-5628.

Scott, M. P., Tamkun, J. W., and Hartzell, G. W., 3rd (1989). The structure and function of the homeodomain. Biochim Biophys Acta 989, 25-48.

Sellick, G. S., Barker, K. T., Stolte-Dijkstra, I., Fleischmann, C., R, J. C., Garrett, C., Gloyn, A. L., Edghill, E. L., Hattersley, A. T., Wellauer, P. K., et al. (2004). Mutations in PTF1A cause pancreatic and cerebellar agenesis. Nat Genet.

Sommer, L., Hagenbuchle, O., Wellauer, P. K., and Strubin, M. (1991). Nuclear targeting of the transcription factor PTF1 is mediated by a protein subunit that does not bind to the PTF1 cognate sequence. Cell 67, 987-994.

Sosa-Pineda, B. (2004). The gene Pax4 is an essential regulator of pancreatic beta-cell development. Mol Cells 18, 289-294.

Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G., and Gruss, P. (1997). The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature 386, 399-402.

Steinberg, Z., Myers, C., Heim, V. M., Lathrop, C. A., Rebustini, I. T., Stewart, J. S., Larsen, M., and Hoffman, M. P. (2005). FGFR2b signaling regulates ex vivo submandibular gland epithelial cell proliferation and branching morphogenesis. Development 132, 1223-1234.

Stoffers, D. A., Zinkin, N. T., Stanojevic, V., Clarke, W. L., and Habener, J. F. (1997). Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet 15, 106-110.

St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A., and Gruss, P. (1997). Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. Nature 387, 406-409.

Sun, X. H., and Baltimore, D. (1991). An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. Cell 64, 459-470.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L., and German, M. S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development 125, 2213-2221.

Swift, G. H., Liu, Y., Rose, S. D., Bischof, L. J., Steelman, S., Buchberg, A. M., Wright, C. V., and MacDonald, R. J. (1998). An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through formation of a trimeric complex with PBX1b and MRG1 (MEIS2). Mol Cell Biol 18, 5109-5120.

Swift, G. H., Rose, S. D., and MacDonald, R. J. (1994). An element of the elastase I enhancer is an overlapping bipartite binding site activated by a heteromeric factor. J Biol Chem 269, 12809-12815.

Tabata, T., and Kornberg, T. B. (1994). Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. Cell 76, 89-102.

Takeuchi, A., Yamasaki, S., Takase, K., Nakatsu, F., Arase, H., Onodera, M., and Saito, T. (2001). E2A and HEB activate the pre-TCR alpha promoter during immature T cell development. J Immunol 167, 2157-2163.

Tani, S., Kurooka, H., Aoki, T., Hashimoto, N., and Honjo, T. (2001). The N- and C-terminal regions of RBP-J interact with the ankyrin repeats of Notch1 RAMIC to activate transcription. Nucleic Acids Res 29, 1373-1380.

Taniguchi, Y., Furukawa, T., Tun, T., Han, H., and Honjo, T. (1998). LIM protein KyoT2 negatively regulates transcription by association with the RBP-J DNA-binding protein. Mol Cell Biol 18, 644-654.

Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J., and Pfaff, S. L. (1999). Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron 23, 675-687.

Theil, T., Ariza-McNaughton, L., Manzanares, M., Brodie, J., Krumlauf, R., and Wilkinson, D. G. (2002). Requirement for downregulation of kreisler during late patterning of the hindbrain. Development 129, 1477-1485.

Tun, T., Hamaguchi, Y., Matsunami, N., Furukawa, T., Honjo, T., and Kawaichi, M. (1994). Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. Nucleic Acids Res 22, 965-971.

Valdez, M. R., Richardson, J. A., Klein, W. H., and Olson, E. N. (2000). Failure of Myf5 to support myogenic differentiation without myogenin, MyoD, and MRF4. Dev Biol 219, 287-298.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M., and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. Neuron 31, 743-755.

Viswanath, R. L., Rose, S. D., Swift, G. H., and MacDonald, R. J. (2000). A binary mechanism for the selective action of a pancreatic beta -cell transcriptional silencer. J Biol Chem 275, 40273-40281.

Volante, M., Allia, E., Gugliotta, P., Funaro, A., Broglio, F., Deghenghi, R., Muccioli, G., Ghigo, E., and Papotti, M. (2002). Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors. J Clin Endocrinol Metab 87, 1300-1308.

Watada, H., Mirmira, R. G., Leung, J., and German, M. S. (2000). Transcriptional and translational regulation of beta-cell differentiation factor Nkx6.1. J Biol Chem 275, 34224-34230.

Wells, J. M., and Melton, D. A. (1999). Vertebrate endoderm development. Annu Rev Cell Dev Biol 15, 393-410.

Wells, J. M., and Melton, D. A. (2000). Early mouse endoderm is patterned by soluble factors from adjacent germ layers. Development 127, 1563-1572.

Wierup, N., Yang, S., McEvilly, R. J., Mulder, H., and Sundler, F. (2004). Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. J Histochem Cytochem 52, 301-310.

Yoshida, S., Ohbo, K., Takakura, A., Takebayashi, H., Okada, T., Abe, K., and Nabeshima, Y. (2001). Sgn1, a basic helix-loop-helix transcription factor delineates the salivary gland duct cell lineage in mice. Dev Biol 240, 517-530.

Vita

Tom Beres was born in Columbus, Ohio on August 7th, 1973. He is the son of Kenneth and Hazel Beres. He graduated from Athens High School in Troy, Michigan in 1991 and attended the University of Rochester in Rochester, New York. While there he worked in for Dr. Lawrence Tabak in the Department of Dental Research as an undergraduate from 1993-1995. He received his Bachelor of Science degree in Microbiology in 1995. He continued working as a technician for Dr. Tabak until the summer of 1997. In August of 1997 he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas, and joined the laboratory of Dr. Raymond MacDonald in June of 1998. He was awarded the degree of Doctor of Philosophy in 2005.

Permanent address: 2375 Cumberland Dr.

Troy, MI 48085

This dissertation was typed by Thomas Beres.