Dlx Transcription Factors in Pancreas Development: Regulation of Glucagon

by

Andrew Ho

A Thesis submitted to the Faculty of Graduate Studies of

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in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biochemistry & Medical Genetics

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OF

MASTER OF SCIENCE

Table of Contents

Acknowledgements

If you're reading this acknowledgement page, then you're possibly my supervisor, on my graduate committee, or have a passing interest in Dlx and/or pancreas. But more than likely, you're another graduate student who's about to write-up their work and needs to see the format of another Thesis. Well congratulations: it was a long road and we both made it to the end. When I began my Masters studies in 2003, I had the expectation of a quick 2 years and then it would be on to something else. Here I am in 2006 (3 years later) and just finally finishing. I'm happy to say, though, that the time was worthwhile. As I prepare to begin Medical school, I have 3 solid years of personal growth behind me with events that have been truly unforgettable and will help me seize the future. From science conferences and seminars, to moving away from home (Vancouver), to the year I spent in the Army Reserve.

As with most research, when you're in the lab on those Fridays, Saturdays and Sundays, working alone and doing some tedious, repetitive task, you start to wonder if it's all worthwhile – for me there were plenty of those times. This page is dedicated to my family, friends and colleagues who kept me on track and helped me reach my goals.

First, to the Big Boss (Dr. David D. Eisenstat): You are one of the most outstanding scientists and clinicians that I know and I seek to emulate your work ethic everyday. Thanks for the opportunity to work and learn in your lab and for teaching me to play the game of Life. And for your continued support when things got rough or didn't work.

To my Family: Mom, Dad and my sister. Thanks for all the support. You're always there when it matters.

To all my friends: Scattered like the wind, seems like we all went our own way. Hopefully I'll see all of you back in Vancouver before the 2010 Olympics. And to my new friends in Winnipeg, thanks for all the good times and hopefully there will be many more to come.

To my committee and lab members: Thanks for all the advice and for teaching me new things in a way that didn't break my ego. Guoyan, Mario, Trung: good times you "suckas". Shunzhen, Fen, Qing Ping: you're the foundation that keeps everything balanced.

Thanks to the Manitoba Institute of Cell Biology, CancerCare Manitoba and the Manitoba Institute of Child Health for funding that made this work possible and I hope someday this work will contribute to a beneficial clinical application.

It's not the end my friend; The best is yet to come...

Andrew Frank Ho, in Winnipeg. June 26, 2006.

Abstract

Enhancing our understanding of the development and regulation of the pancreas will be critical to the success of treatments of pancreatic diseases such as diabetes and cancer. In the pancreas, small collections of endocrine cells, known as the islets of Langerhans, are found interspersed among exocrine cells. The islets are made up of α, β, δ and PP cells that secrete essential regulatory hormones into the bloodstream. These four cell types are all derived from a PDX-1 expressing progenitor cell. Several important transcription factors in the pancreatic developmental cascade are homeodomain proteins including: NKX2.2, NKX6.1, PAX4 and PAX6. The Distal-less homologs *Dlx*1 and *Dlx*2 are homeobox-containing transcription factors which play an important role in the development of the vertebrate striatum, neocortex, retina and craniofacial structures. Of particular interest, *Dlx*1 and *Dlx*2 are highly expressed in the developing pancreas and are co-expressed with important hormones and transcription factors. The *Dlx*1/*Dlx*2 double null mouse has reduced expression of both insulin and glucagon. We have performed chromatin immunoprecipitation with our previously characterized specific antibodies to DLX1 and DLX2 using pancreatic tissues obtained at birth and in the adult. We have demonstrated that both *Dlx* homeoproteins bind to the promoter regions of the mouse preproinsulin I and proglucagon genes *in vivo*. This binding has been confirmed by electrophoretic mobility shift assays *in vitro*. Luciferase gene reporter assays were carried out on the proglucagon gene promoter to assess the functional effect of DLX1 and DLX2 binding. Hence, *Dlx*1 and *Dlx*2 may play a role in pancreatic islet cell development by direct transcriptional regulation of both insulin and glucagon expression.

List of Abbreviations

bHLH = basic-helix-loop-helix

ChIP = chromatin immunoprecipitation

DLX = DLX protein

Dlx = Dlx gene

DLX1/2 = DLX1 and DLX2

DNA = deoxyribonucleic acid

DNase = deoxyribonuclease

EMSA = electrophoretic mobility shift assay

E# = embryonic day # (i.e. E13 = embryonic day 13)

Islets = islets of Langerhans

Luciferase = luciferase reporter gene assay

MODY = maturity onset diabetes of the young

PP = pancreatic polypeptide

WT = wild-type

List of Tables

List of Figures

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Figures 6, 7, 8 (Jiang, 2003) Am J Physiol Endocrinol Metab.

Figure 10 (Kemp, 2003), Springer DE.

Chapter I: Introduction

The Pancreas

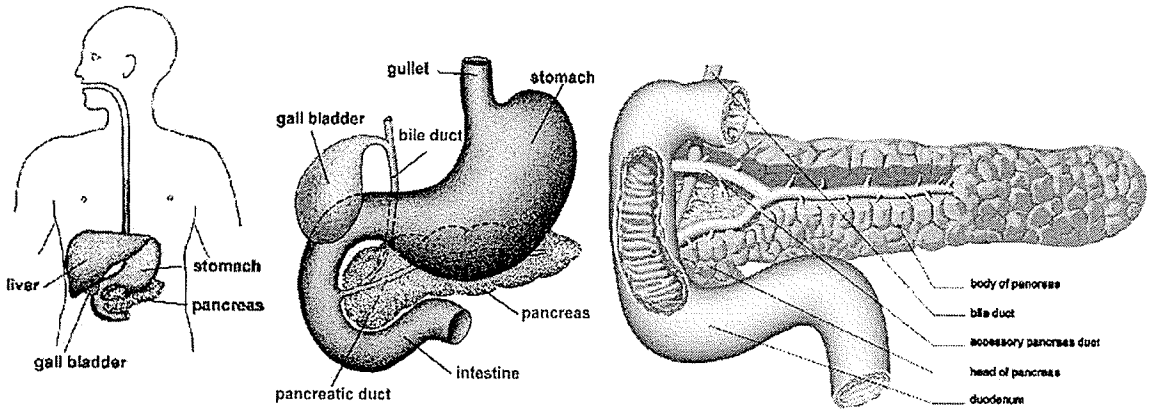

Figure 1: The pancreas is an organ located on the left side of the body posterior to the stomach. The pancreatic duct runs the length of the organ and secretes digestive enzymes into the duodenum. Image obtained from http://www.top5plus5.com/What%20is%20the%20Pancreas.html accessed August 14, 2006.

The pancreas is an organ located in the abdominal cavity, posterior to the stomach, and closely associated with the duodenum on the left side of the body (Figure 1). Light pink or tan in color, it is divided into lobules, which are mostly composed of grape-like clusters of exocrine cells called acini. Exocrine tissue comprises 95% of the pancreas. Embedded in a matrix of acini are endocrine tissues called the Islets of Langerhans ("islets", Figure 2). The right side of the organ (called the head) is the widest part of the organ and lies in the curve of the duodenum while the tapered left side (called the body of the pancreas) extends slightly upward and ends near the spleen (called the tail). The main pancreatic duct runs the length of the organ and receives exocrine secretions from interlobular ducts. Unlike most retroperitoneal organs, the pancreas does not have a well-defined capsule. Another unique

feature of the pancreas is that it is both an important exocrine and endocrine organ. The exocrine function is to secrete digestive enzymes into ducts, that drain like the branches of a tree into the main pancreatic duct, which then connects to the duodenum. Digestive enzymes such as pancreatic amylase, trypsin, chymotrypsin and pancreatic lipase help to digest carbohydrates, proteins and fats and therefore, the pancreas is important in regulating the metabolism of these nutrients. As well, several other digestive enzymes such as ribonuclease, deoxyribonuclease, gelatinase and elastase are also secreted. The endocrine function of the pancreas is contained within the cells of the islets, which are composed of four cell types: α (secretes glucagon, 15-20% of islet cells), β (insulin, 60-80%), δ (somatostatin, 5-10%) and PP (pancreatic polypeptide, <2%) (reviewed in Kim, 2001; Edlund, 2002). These cells secrete essential regulatory hormones into the bloodstream. β-cells comprise the majority of the islet and form a "core" while the other three cell types are arranged in a peripheral ring around this β-core. Somatostatin was first isolated from hypothalamic tissues and was found to be an inhibitor of pituitary growth hormone secretion. In pancreas, some studies have shown that it can inhibit gastrointestinal secretions (reviewed in Guillemin, 2005). Currently, pancreatic polypeptide does not have a well-defined function. In contrast, insulin and glucagon have well-defined roles in the regulation of blood glucose levels.

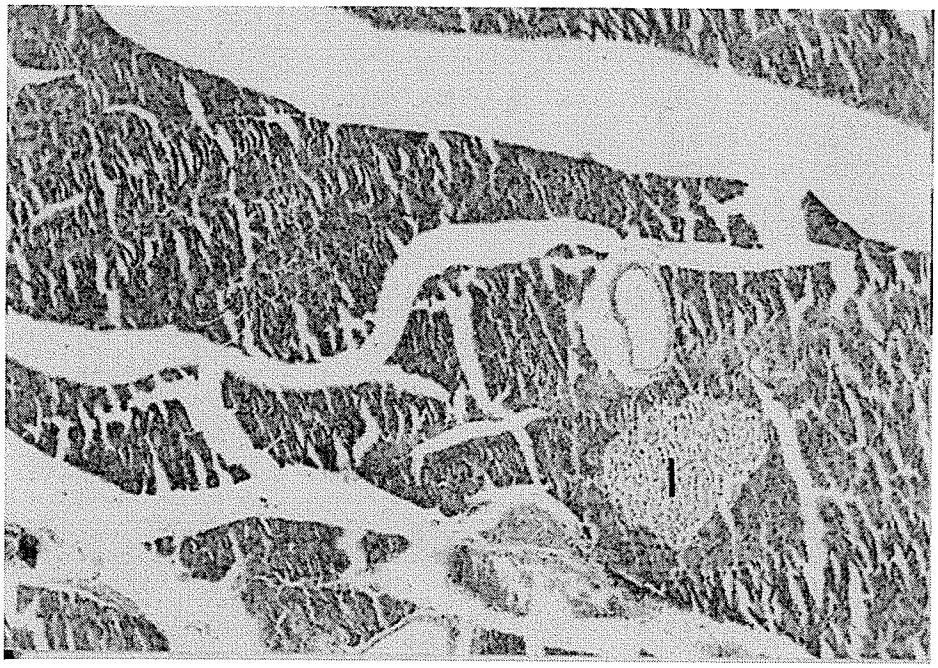

Figure 2: Hematoxylin & Eosin stained rat pancreas section. The pale staining region (I) is the endocrine portion of the pancreas, an Islet of Langerhans. Islets are found throughout the pancreas and are surrounded by exocrine tissue. Image obtained from www.ams.ac.ir/AIM/0261/Image130.gif accessed January 8, 2006.

Medically, there are two prominent and deadly pancreatic diseases. One is pancreatic cancer, with roughly 216,000 new cases every year (World Health Organization, 2003). It is the fourth leading cause of cancer deaths in most industrialized countries, with 10% of the North American population diagnosed each year (~30,000/year USA, ~3,000/year Canada). Pancreatic cancer is difficult to diagnose and treat, and death results rapidly from a small tumor burden due to disruption of pancreatic functions which are essential to life. The predominant form of pancreatic malignancy is ductal adenocarcinoma which is thought to arise from ductal cells (Hruban, 2000). Another disease affecting the pancreas is *diabetes mellitus*, which is a disorder primarily affecting the regulation of blood glucose.

Diabetes

The most famous disorder of the pancreas is *diabetes mellitus* (diabetes) which roughly translates from Greek as "honey-sweet urine" and aptly describes the diagnostic method of ancient times. Since then, we have seen great improvements in our understanding of this disease, such as the discovery of insulin in 1921 by Banting and Best and improvements in islet cell transplantations following the Edmonton Protocol in 2000. There are currently 2 million Canadians (roughly 7% of the population) with diabetes. A similar percentage of Americans also have diabetes. Globally, the World Health Organization estimates a 2.8% prevalence of diabetes worldwide in 2000, with this number expected to reach 4.4% in 2030 (Wild, 2004). There are two forms of diabetes: insulin-dependent diabetes mellitus (IDDM or Type I) and non-insulin-dependent diabetes mellitus (NIDDM or Type II). Secondary classification of these forms takes into account gestational or additional medical complications. The general phenotype of diabetes is hyperglycemia-induced osmotic diuresis (excessive thirst and urination due to high blood glucose levels). Over time, diabetes often leads to other complications such as problems with blood vessels (cardiovascular disease, retinopathies), neuropathies affecting both the somatic and autonomic systems (loss of function or sensation in parts of the body such as limbs or internal organs) and kidney failure (Canadian Diabetes Association, 2006).

Type I diabetes is a disorder of glucose homeostasis that has a heterogeneous phenotype, resulting from β-cell destruction. It exhibits 30 to 50% concordance in monozygotic twins with a 6% risk to sibs (Todd, 1990). It is therefore suggested that the disease is dependent

on environmental factors in addition to dependence on genetic susceptibility. Many different loci have been studied and Type I diabetes has been found to be extremely polygenic. Current clinical management includes dietary alterations and daily insulin injections to maintain blood glucose within an acceptable range. The emergence of symptoms of Type I diabetes is often spontaneous. However, the onset of the disease is typically preceded by anti-insulin antibodies, and numerous studies of non-obese diabetic mice (NOD) show pancreatic enrichment of T-lymphocytes directed against insulin or β-cells, demonstrating that Type I diabetes is an autoimmune disease (reviewed in Tisch and McDevitt 1996). The onset of Type I diabetes is usually in childhood and symptoms usually only manifest after the majority of islets have already been destroyed. Currently, there is intense interest in islet transplantation as a possible cure for Type I diabetes. A major breakthrough in treatment was achieved with the development of the Edmonton Protocol (Shapiro, 2000) in the summer of 2000, involving a novel transplantation procedure given to seven diabetic patients. Unlike earlier transplants, this procedure used three new anti-rejection drugs instead of corticosteroids, and donor islets from multiple pancreata were given to patients. This protocol was the first to successfully transplant islet cells into patients without rejection. A 5-year follow up was recently conducted (Ryan, 2005) in which it was found that many patients did not retain full insulin independence. As well, benefits from the procedure must be weighed against risks/dependence on immunosuppressive drugs. In the Edmonton protocol, patients received between 1-4 transplantations of approximately 500,000 islets each time. A human pancreas contains approximately 1 million islets, but current preservation and harvesting techniques required

that 2 donors be pooled together to yield 500,000 islets. The need for ongoing immunosuppressive therapy and the scarcity of donor islets have precluded the widespread adoption of islet transplantation as a mainstream therapy. However, research is currently aimed toward utilizing stem cells or possibly pancreatic progenitor cells to generate islets (Narushima, 2005; Dor, 2004; Seaberg, 2004). This approach would have the advantage of generating an unlimited number of islets and additionally, might overcome issues with rejection of foreign islets, but much work needs to be done to better understand the nature of pancreas development and the identity of pancreas and/or β-cell progenitors.

Type II diabetes is also a glucose homeostasis disorder, but is the result of either impaired insulin secretion or decreased insulin sensitivity (or sometimes both). The disease is polygenic, and characterized by gene-gene and gene-environment interactions. Onset of Type II diabetes is usually in late adulthood between ages 40-60 and patients are generally obese and display symptoms which are termed the "metabolic syndrome" - insulin resistance, hypertension and hypertriglyceridemia (Malecki, 2005). By contrast, another form of Type II diabetes, Maturity Onset Diabetes of the Young (MODY or autosomal dominant type II diabetes) has onset less than 25 years of age. Patients are usually non-obese and do not display the metabolic syndrome. As well, MODY has a heavy monogenetic basis with 6 known loci which greatly increase disease susceptibility (Winter, 2003).

MODY1	HNF4α
MODY2	Glucokinase
MODY3	HNF1α
MODY4	PDX1
MODY5	HNF1β
MODY6	NeuroD1

Table 1: Six known forms of MODY and their associated loci. One locus, MODY2, involves glucokinase which is the first enzyme in the glycolysis pathway. The other five loci involve transcription factors which are known to be important in regulation of pancreas development.

Of the 6 forms, 5 involve important pancreas development transcription factors. The other, MODY2, involves glucokinase, which is the first enzyme in the glycolysis pathway responsible for glucose metabolism and catalyzes the phosphorylation of glucose to glucose-6-phosphate. Type II diabetes is often controlled by exercise and careful meal-planning, and medication to enhance insulin secretion and/or tissue sensitivity, but may sometimes require insulin injections.

Proglucagon Regulation

The proglucagon gene is expressed in a tissue and species-specific manner in mammalian brain, pancreas and intestine (Tucker, 1996). There are 3 main mechanisms of regulating proglucagon function: regulation of proglucagon expression via its 5'-upstream promoter; regulation of proteolytic cleavage into proglucagon-derived peptides, which regulates the final protein products in a tissue-specific manner; and control of expression of receptors for the different proglucagon-derived peptides which regulates their biological activity. The discovery of proglucagon was accidental and resulted from the cross-reactivity of antibodies generated against glucagon to other proglucagon-derived peptides in the gut. This gut product of proglucagon was initially (erroneously) named "enteroglucagon". Subsequently, it has been found that "enteroglucagon" is in fact several different peptides which arise from the proteolytic cleavage of proglucagon. It is now known that glucagon and the various other peptides are the product of the proteolytic cleavage of proglucagon.

Proglucagon Promoter Regulation

In addition to regulation by proteolytic cleavage and receptor expression, regulation of the function of the proglucagon gene occurs at the transcription level via its 5'-upstream promoter. The promoter consists of an approximately (depending on species) 300 bp 5'-flanking region which is adjacent to the proglucagon gene. This region has been extremely

well characterized in rats. Although the human and mouse promoters share a high similarity with the rat (Figure 3), they have been much less studied.

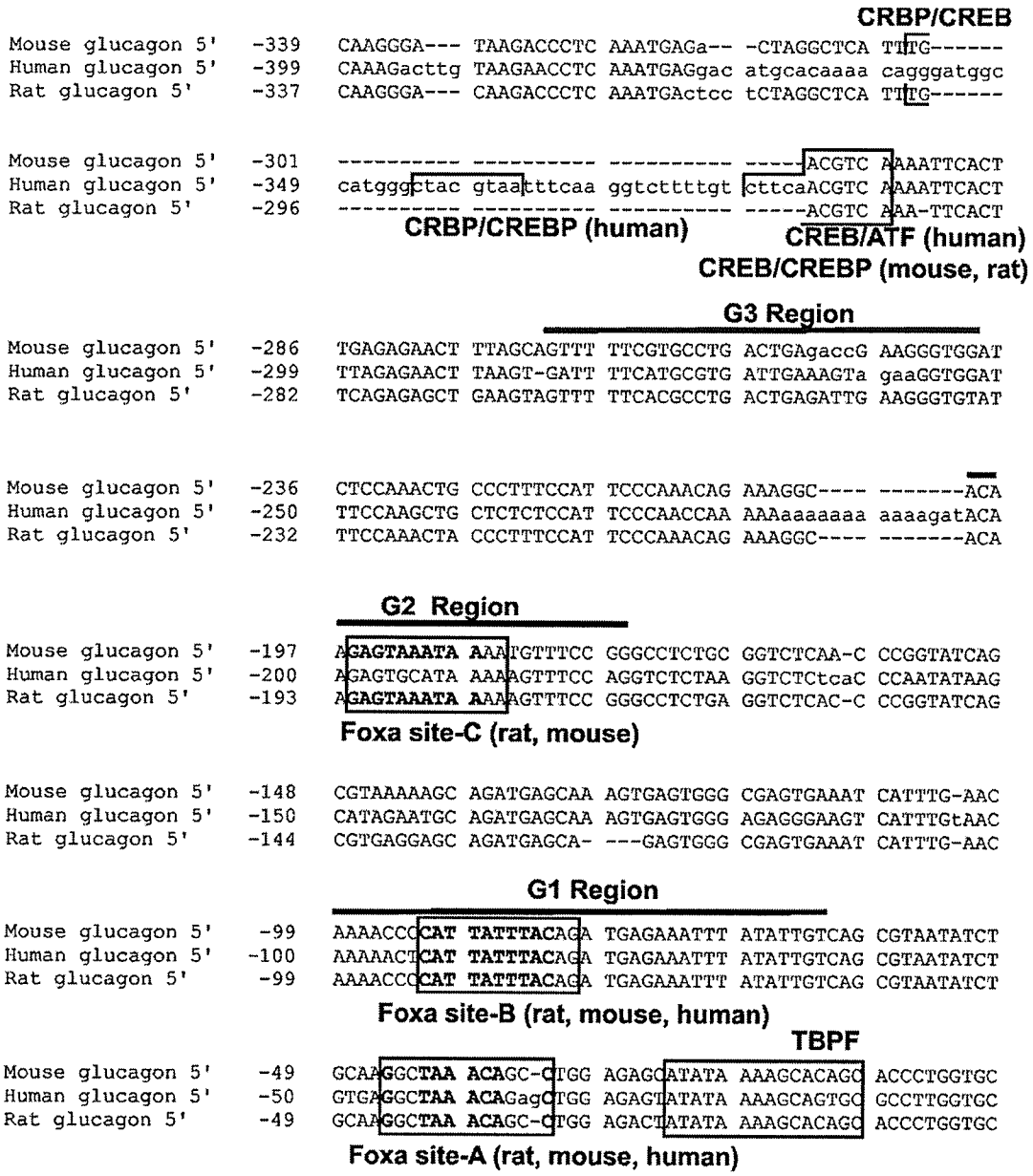

Figure 3: Proglucagon promoter homology among rat, human and mouse. The three species share a high degree of homology including known regulatory elements (G1, G2, G3) and regions of DNA (in bold) which are bound by transcription factors. Reproduced with permission from Sharma (2005).

Rat, mouse and human proglucagon promoters share four common sequence control elements, called G1, G2, G3 and G4, which are regions of DNA identified based on their ability to bind transcription factors or confer tissue or cell-specific expression. The studies carried out to determine these regions consist mostly of electrophoretic mobility shift assays (EMSA), DNase footprinting, and luciferase/chloramphenicol acetyltransferase (CAT) assays. In the majority of experiments, the DNA sequences have been defined by using various promoter fragments or deletions. Thus the exact sequence of the G1-G4 elements differs slightly from one study to another. The proglucagon promoter also contains several palindromic CANNTG motifs or "E boxes", to which bHLH transcription factors can bind. It is interesting to note that the proglucagon and pre-proinsulin promoters share many of these features (G elements, E boxes) and therefore may also share common regulatory transcription factors.

Despite its importance in diabetes, there have been very few studies into the regulation of the human proglucagon promoter. The majority of knowledge so far is derived from experiments in rats (Figure 4).

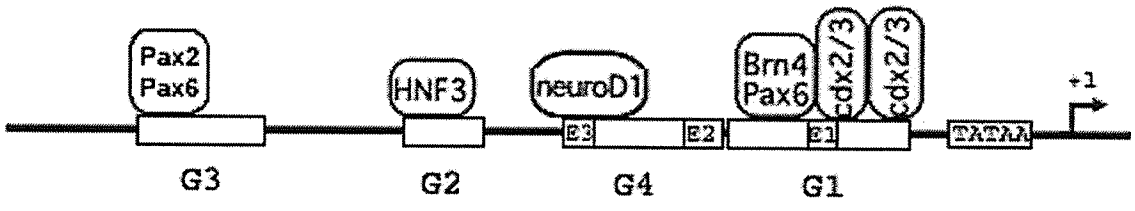

Figure 4: The rat proglucagon promoter contains four DNA binding elements (G1-G4). Each of these elements contains one or more DNA motifs which have been shown to be bound by transcription factors. Modified from Wilson, 2003.

There are numerous transcription factors that have been shown to bind to the proglucagon promoter (reviewed in Wilson, 2003), many of which are also extremely important in the early development of the pancreas. These include Pax6 (Sander, 1997; Andersen, 1999), HNF3 (also called Foxa; Kaestner, 1999), Brn-4 (Hussain, 1997), Cdx2/3 (Andersen, 1999; Hussain, 1999) and NeuroD1 (also called BETA2, Dumonteil, 1998). Therefore, pancreas development and control of glucagon expression are related processes.

The G1 element is a proximal promoter element of the proglucagon promoter and is arguably the most important as it confers islet α cell-specific expression (Morel, 1995). In addition to this, the G1 element also contains most of the known binding sites for transcription factors including three AT-rich regions and two TAAT motifs (homeobox proteins bind to these sites) and a paired-domain consensus sequence (paired-domain proteins bind to these sites) which is known to be bound by Brn-4, Pax6 and Cdx2/3. Pax6 is known to form a functional complex with Cdx2/3 (Andersen, 1999).

The G4 element is slightly upstream of G1 and contains two E boxes. The complete G4 element acts as an enhancer in glucagon-producing cells (Cordier-Bussat, 1995). The pancreas development transcription factor NeuroD1 is known to bind G4 and activate expression of proglucagon (Dumonteil, 1998).

The distal G2 and G3 elements contain cell-specific enhancer-like properties which increase proglucagon expression in islet α-cells. However, there are also two known inhibitor binding sites in these regions. Insulin can inhibit proglucagon expression. This

inhibition is proposed to be mediated by islet-specific proteins which bind to the G3 element (Philippe, 1995). The transcription factor HNF3 can inhibit by binding to G2.

Proglucagon Expression

In mammalian pancreatic α-cells, proglucagon is cleaved to produce glicentin related pancreatic peptide (GRPP), glucagon, intervening peptide (IP-1) and major proglucagon fragment (Figure 5). Whereas in the intestine, proteolytic cleavage of proglucagon gives rise to glicentin, GRPP, oxyntomodulin, IP-2 and glucagon-like peptides 1 and 2 (GLP-1 and GLP-2). An intermediate profile of peptides is found in the brain including glucagon, GLP-1 and GLP-2 but the roles of proglucagon-derived peptides in this organ is less studied than in pancreas or intestine. Non-mammalian species such as fish (*Lophus americanus, Oncorhynchus myis*), chicken (*Gallus gallus*) and frog (*Xenopus Laevis*) have different expression patterns of proglucagon due to their different gene exon/intron sequences or alternative mRNA splicing (Irwin, 2001). As well, some species have duplications or deletions of the DNA sequences encoding the various mammalian-equivalent proglucagon-derived peptides. An additional level of diversity arises in the fact the analogous peptide may have different functions in different species. For example, GLP-1 is secreted in intestinal cells in mammals and has an insulinotropic effect whereas in fish, it is secreted in both pancreas and intestine, and has activity similar to that of glucagon (Irwin, 2001). Recently, a subset of glucagon expressing neurons within the avian retina have been found to regulate proliferation (Fischer, 2005).

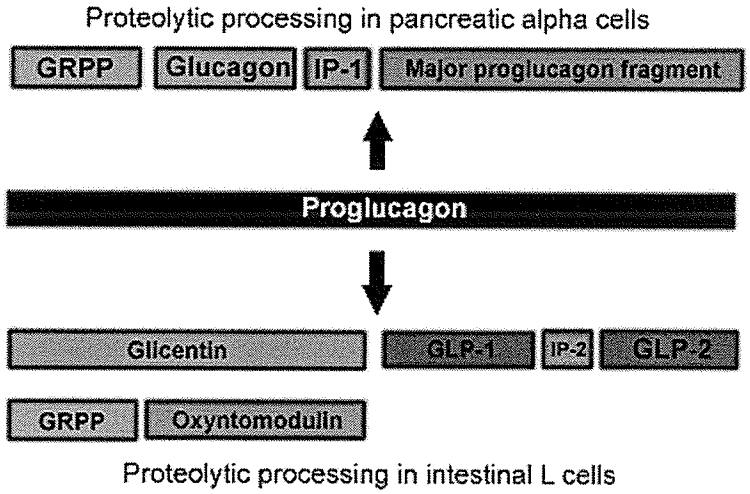

Figure 5: Proglucagon processing in pancreas produces the peptides GRPP, glucagon, IP-1 and major proglucagon fragment, whereas in the intestine, glicentin, GLP-1, IP-2 and GLP-2 are produced. An intermediate profile of these peptides is found in the brain. Image modified from http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/gi/eglucagon.gif accessed August 14, 2006.

Currently, only glucagon, GLP-1, GLP-2 and oxyntomodulin have any known biological activity, with only glucagon and GLP-1 having been studied in detail.

GLP-1 is a 30 aa peptide hormone which is a potent enhancer of insulin secretion in response to a glucose stimulus (Shalev, 1997). The mechanism of GLP-1 action is that it binds a trimeric G-protein coupled receptor (class B, secretin-like) which signals via modulating cAMP levels. Such GLP-1 receptors are found on α and β-cells in pancreas as well as in adipose tissue, lung and hypothalamus. The presence of GLP-1 receptors in the hypothalamus suggests that it may have a role in appetite regulation. Indeed, injections of GLP-1 into rats and mice result in decreased food intake in a dose-dependent manner (Shalev, 1997). Injection of GLP-1 can also enhance β-cell function in humans with Type

II diabetes. The GLP-1-receptor knock-out mouse is glucose intolerant and has elevated plasma glucose after fasting. It does not display any obvious phenotype with respect to body weight or food intake, suggesting that there may be redundant signaling systems or adaptive responses. These features of GLP-1 make it an attractive target for clinical application both in Type I and Type II diabetes, and it is likely that a GLP-1 knock-out mouse will be made in the future.

GLP-2 is a 30 aa peptide hormone which is released in the intestine and brainstem in response to nutrient ingestion. It binds to the GLP-2 receptor, a G-protein coupled receptor (class B, secretin-like) which signals via cAMP. Little is known about which cell types express the receptor within the brain and intestine. In rodents, it has been shown that GLP-2 can stimulate proliferation of intestinal crypt cells (Estall, 2003). As well, administration of exogenous GLP-2 attenuates intestinal injury in mouse models. These results suggest that GLP-2 has a role in controlling intestinal proliferation and apoptosis.

Oxyntomodulin is a 37 aa peptide which is identical to glucagon with an additional 9 aa at its C-terminus. A few studies have suggested that it can reduce body weight in both rodents and humans (Wynne, 2005).

Glucagon is a 29 aa peptide hormone which is released by the brain and pancreatic α-cells where proglucagon is processed by prohormone convertase enzymes (Furuta, 2001). It signals through its receptor on the cell surface, which is a 485 aa, 7-transmembrane G protein-coupled receptor belonging to the Class A family (rhodopsin-like). Thus far,

expression of the glucagon receptor has been identified in multiple tissues including liver, brain, pancreas, kidney, intestine and adipose tissues (Christophe, 1996). Quantitative studies of mRNA levels have demonstrated that receptor expression is stimulated by glucose and inhibited by cAMP in liver (Abrahamsen, 1995). Binding of glucagon to its receptor leads to change in receptor conformation and activation of two known associated G proteins: G_q and $G_s\alpha$. Activation of $G_s\alpha$ leads to activation of adenylate cyclase, increases in intracellular cAMP levels and the subsequent activation of protein kinase A (PKA). Activation of G_q leads to activation of phospholipase C, production of inositol 1,4,5-triphosphate ($INSP_3$), opening of $INSP_3$-gated calcium channels and increased intracellular calcium (Figure 6). Increases in PKA and calcium levels lead to increased blood glucose. PKA directly phosphorylates and activates phosphorylase kinase, while calcium acts indirectly through binding to calmodulin. Calcium can bind either to an endogenous calmodulin subunit contained within phosphorylase kinase, or through exogenous calcium/calmodulin complexes (Nadeau, 1997).

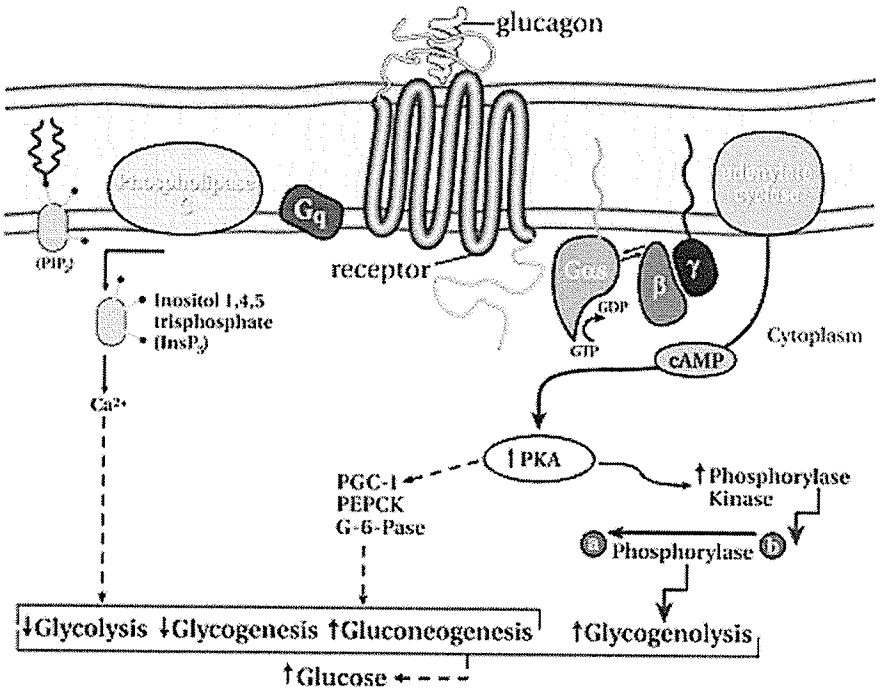

Figure 6: Glucagon receptor signaling. Glucagon binds to its G-protein coupled receptor leading to activation of adenylate cyclase and phospholipase C which increase intracellular cAMP and inositol-1,4,5-triphosphate ($INSP_3$) respectively. These chemicals activate their signaling pathways and result in increased blood glucose levels. Reproduced with permission from Jiang (2003).

Together with insulin, glucagon is the major hormonal regulator of blood glucose homeostasis. The regulation by glucagon occurs via 4 principle mechanisms: potentiation of glycogenolysis/inhibition of glycogenesis, and potentiation of gluconeogenesis/inhibition of glycolysis. These 4 mechanisms are a direct result of glucagon binding to its receptor.

Glucagon Regulation of Blood Glucose Levels

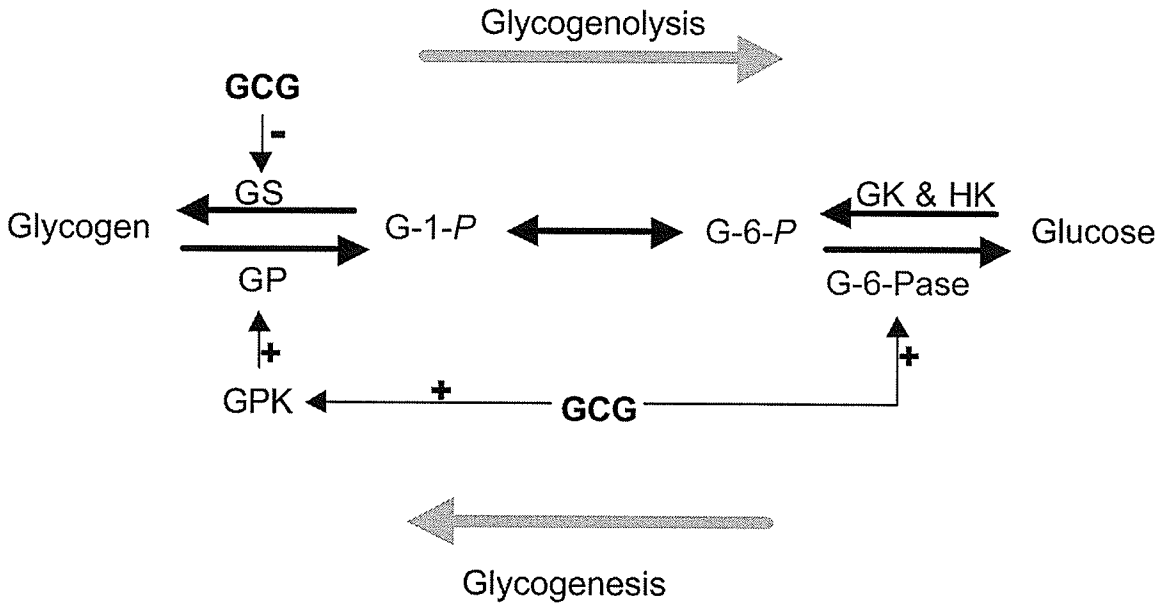

Figure 7: Glucagon regulation of glycogenolysis and glycogenesis. Glucagon promotes glycogen breakdown to glucose. GCG (glucagon); GPK (glycogen phosphorylase kinase); GP (glycogen phosphorylase); GS (glycogen synthase); GK & HK (glucokinase and hexokinase); G-1-P (glucose-1-phosphate); G-6-P (glucose-6-phosphate); G-6-Pase (glucose-6-phosphorylase). Reproduced with permission from Jiang (2003).

Following glucagon (GCG) binding to its receptor in liver, activation of PKA leads to potentiation of glycogenolysis and inhibition of glycogenesis (Figure 7). Activated PKA phosphorylates and activates glycogen phosphorylase kinase (GPK). Activated GPK subsequently phosphorylates glycogen phosphorylase (GP) leading to its activation. Glycogen phosphorylase then phosphorylates glycogen resulting in increased glycogen breakdown and an increase in glucose levels. Glucagon also increases transcription of the glucose-6-phosphatase (G-6-Pase) gene. Glucagon also inhibits glycogen synthesis by

inducing the phosphorylation of the glycogen synthase (GS) enzyme, thereby inactivating it.

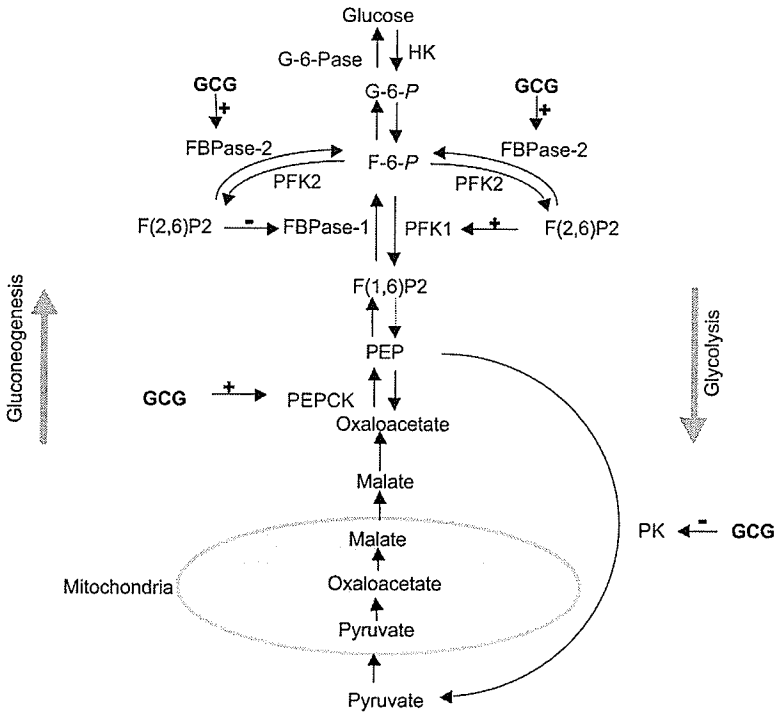

Figure 8: Glucagon regulation of glycolysis and gluconeogenesis. Glucagon promotes conversion of oxaloacetate to phosphoenolpyruvate (PEP) and inhibits glycolysis. Concurrently, glucagon stimulation of activated protein kinase A leads to phosphoryation of the PFK-2/FBPase-2 enzyme which shifts the equilibrium in favor of F-6-P. HK (hexokinase); G-6-Pase (glucose-6-phosphatase); GCG (glucagon); F-6-P (fructose-6-phosphate); FBPase-1 (fructose-1,6-bisphosphatase); FBPase-2 (fructose-2,6-bisphosphatase); PFK (phosphofructokinase); F(1,6)P2 (fructose-1,6-bisphosphate); F(2,6)P2 (fructose-2,6-bisphosphate); PEP (phosphoenolpyruvate); PEPCK (phosphoenolpyruvate carboxykinase). Reproduced with permission from Jiang (2003).

Concurrent with the activation of glycogen breakdown, glucagon inhibits the breakdown of glucose to pyruvate (glycolysis) and therefore promotes gluconeogenesis by activating phosphoenolpyruvate carboxykinase (PEPCK), which is an early and rate-limiting step (Figure 8). Another major regulatory mechanism of glycolysis is the conversion between

fructose-6-phosphate (F-6-P) and fructose-2,6-bisphosphate [F(2,6)P2] which is catalyzed by a single protein that has both phosphofructokinase-2 (PFK2) and fructose-2,6-bisphosphatase (FBPase-2) enzymatic activities. Glucagon stimulation of activated PKA leads to the phosphorylation of PFK-2/FBPase-2 which simultaneously activates the FBPase-2 activity and inhibits the PFK2 activity, shifting the reaction in favor of F-6-P and decreasing the amount of F(2,6)P2. F(2,6)P2 is an allosteric inhibitor of fructose-1,6-bisphosphatase (FBPase-1) and also an allosteric activator of phosphofructokinase-1 (PFK1). A decrease in the amount of F(2,6)P2 promotes the formation of fructose-6-phosphate from fructose-1,6-bisphosphate, which is a rate-limiting step in glycolysis/gluconeogenesis. Thus, glucagon activation of PKA leads to activation of one of the rate-limiting steps of glucose formation. The activity of insulin opposes that of glucagon. Insulin promotes glycogen synthesis, glycolysis and glucose uptake by cells.

Pancreas Development

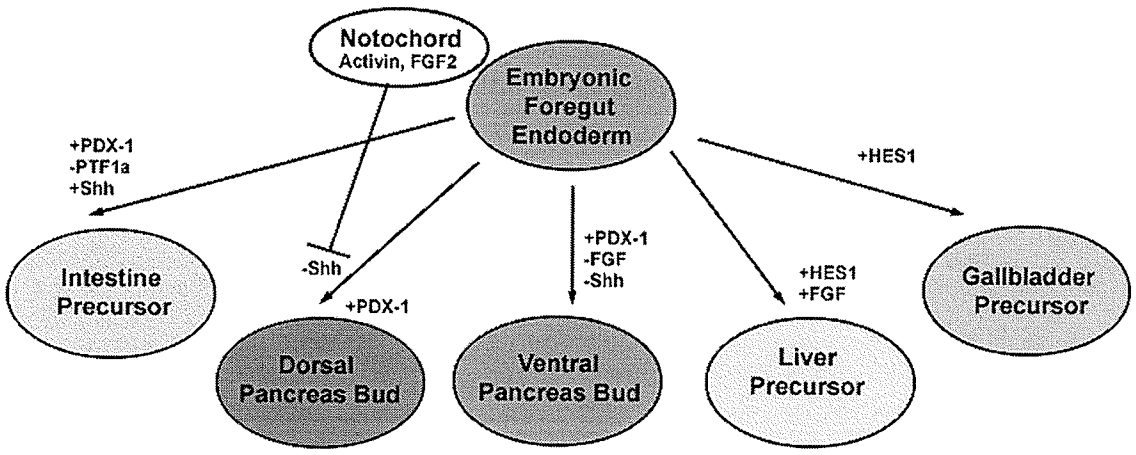

Figure 9: Embryonic foregut endoderm development. The pancreas develops from a region of the foregut in close proximity to cells which will become liver, intestine, stomach or gallbladder. Regulation of development in this region is dependent both on extrinsic signaling such as Activin and FGF2 from the notochord and on intrinsic factors present within the cells such as the pancreas transcription factor Pdx-1. Adapted from Bort, 2002.

During embryogenesis, the endoderm is partitioned along the anterior-posterior axis of the embryo. The pancreas develops from the foregut region of the endoderm. This region also gives rise to liver, gallbladder, stomach and intestine at almost the same time (Figure 9). Specification of these different organ cell fates requires both intrinsic and extrinsic factors. In the developing foregut there exists a population of cells which are competent to become the pancreas but require additional extrinsic signaling from adjacent tissues to differentiate. However, there are also different populations of cells (which will give rise to liver, gallbladder, stomach and intestine) in the same region which cannot form pancreas even in the presence of the same extrinsic signals. Thus, both extrinsic and intrinsic factors are necessary to specify pancreatic cell fates. The intrinsic signals are transcription factors

present within cells which will become pancreas: the combination of transcription factors, and their temporal expression determines cell fate (Bort, 2002). In addition to their role in development, many of these intrinsic factors are present in the mature adult pancreas where they regulate gene expression and/or maintain terminal differentiation. Extrinsic factors are transcription factors or signaling molecules in adjacent tissues outside of the pancreatic epithelium. Currently, the extrinsic signaling is proposed to initially derive from the notochord and the surrounding pancreatic mesenchyme. Together, extrinsic and intrinsic factors regulate the pancreatic developmental program which begins with a specification of a pancreas cell-fate. Once the appropriate foregut region becomes destined to form pancreas, a pool of progenitor cells must give rise to the three classes of pancreatic cell types: exocrine, endocrine and ductal. Endocrine cells then differentiate into the four different islet cell types: α, β, δ and PP. Pancreas development has been studied in great detail in mice, and our current understanding is summarized in numerous papers (Jensen, 2004; Kemp, 2003; Murtaugh, 2003, Wilson 2003; Kim, 2001; Sander 1997).

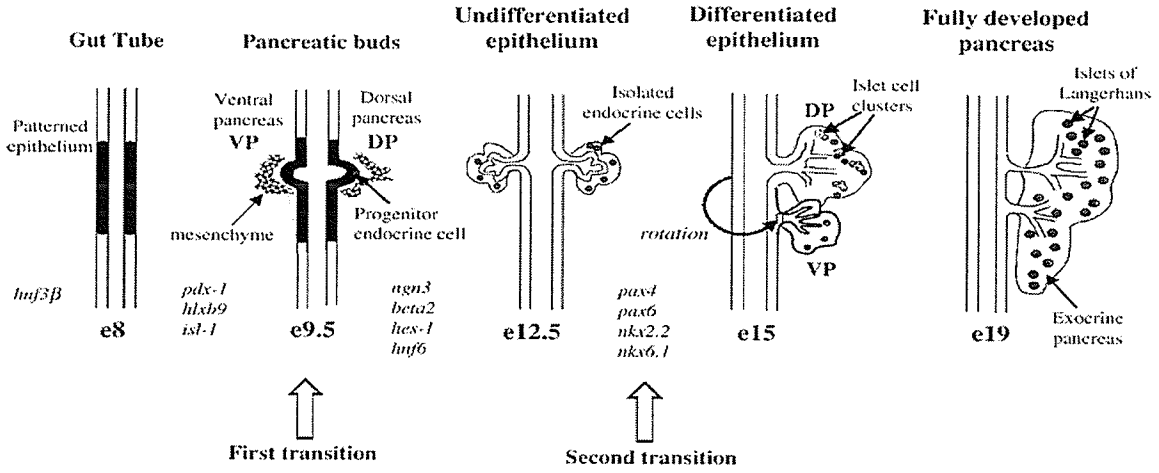

Figure 10: Mouse pancreas development. In mice, gastrulation occurs at E7. Specification of a pancreas cell-fate is dependent on HNF-family transcription factors and occurs at E8. At E9.5, a primary transition occurs with formation of dorsal and ventral pancreatic buds. During a secondary transition, an exponential increase in endocrine cells and the rate of β-cell formation occurs. The dorsal and ventral buds fuse at E16, and proper islets form at E18.5. Reproduced with permission from Kemp (2003).

In mice, which have a gestational period of roughly 19-20 days, the pancreas develops from two distinct regions of foregut endoderm: dorsal pancreatic endoderm which is adjacent to the notochord until E8.5, and ventral pancreatic endoderm which is adjacent to splanchnic mesoderm and aortic endothelial cells. The pancreatic developmental program begins at E8-8.5 with commitment of endodermal cells at the foregut/midgut junction of the endoderm (immediately posterior to the region which will become stomach) to a pancreatic cell fate (Figure 10). A "primary transition" occurs 24 hours later at E9.5 with the formation of a two distinct and unequal anlages or "buds". First, the "dorsal bud" forms from the posterior foregut region which was adjacent to the notochord. The "ventral bud" forms later at E10 and develops in close proximity to the liver and gallbladder. The different temporal and spatial properties of these two buds allow them to receive different

extrinsic signals. Following bud formation, proliferation of the pancreatic epithelium occurs, concurrently with a process of branching morphogenesis (reviewed in Jensen, 2004) from E9.5-E12.5. The proliferating pancreatic epithelium eventually invades the surrounding mesenchyme. During this time, the majority of cells formed are α-cells. A "secondary transition" occurs from E13.5-E15.5 during which there is an exponential (100-1000 fold) increase of endocrine cells and a very large increase in the rate of β-cell formation. Ducts and acini become visible histologically at E14.5. During pancreas development, the ventral bud rotates toward the dorsal side. The mechanism underlying this rotation remains poorly defined. At E16-E17, the two buds fuse together forming the pancreas-proper. Therefore, the dorsal and ventral pancreatic anlages require specification along both the dorsal-ventral and anterior-posterior axes. Late in embryonic development, at E18.5, endocrine cells aggregate, forming the Islets of Langerhans. Postnatally, a massive growth of exocrine tissue occurs, which then condenses to form compact acini.

Extrinsic Pancreas Signaling

Several known signaling pathways are known to regulate pancreas development and function, such as fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), Notch and Hedgehog, which are all expressed in the surrounding pancreatic mesenchyme (reviewed in Kim, 2001).

Prior to formation of the dorsal bud, notochord signaling conditions dorsal pancreatic endoderm by secretion of fibroblast growth factor 2 (FGF2) and the TGF-β family member activin-βB, which repress expression of Sonic Hedgehog (Shh) (Figure 11). Shh is also

repressed in the ventral bud, but the mechanism is unknown and is notochord independent. Interestingly, Shh expression is found in intestinal tissue in a sharp molecular boundary between the pancreas and the intestine/stomach (Hebrok, 2003) suggesting that it might restrict pancreas development and separate pancreas from the intestine.

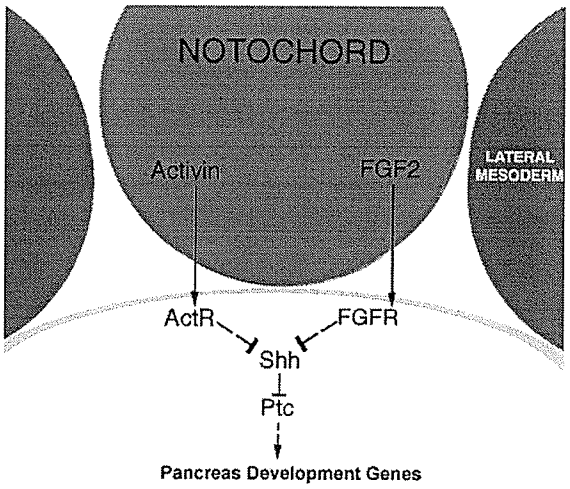

Figure 11: TGF and FGF signaling from notochord. Sonic hedgehog (Shh) expression in the dorsal bud of the pancreas is repressed by FGF-2 and the TGF-β family member Activin-βB which are produced in the notochord. Modified from Kim (2001).

TGF-β signaling is a major regulator of pancreatic endocrine and exocrine cell fates (reviewed in Kim, 2001). Components of this pathway, including the ligands activin and TGF-β1; their receptors; and ligand antagonists such as follistatin, noggin and gremlin, are expressed in embryonic pancreatic epithelium and mesenchyme. Follistatin inhibits differentiation of endocrine cells, whereas activin and TGF-β1 promote development of endocrine cells (Figure 12). The full role of TGF-β in pancreas development is not known, and it is unclear which cell types transmit signals and which cell types receive them.

It is known that fibroblast growth factor signaling (FGF) from cardiac mesoderm specifies liver cell fate. Absence of FGF signaling allows the formation of the ventral pancreatic bud, suggesting that the pancreas is the default program for a cell which has the potential to become either pancreas or liver. Evidence that Pdx-1 fused to a VP16 transactivation domain can convert liver to pancreas *in vivo* supports pancreas as being the default state (Horb, 2003; McLin, 2003). FGF10 has been found to be expressed in pancreatic mesenchyme (Bhusan, 2001). The FGF10 null mouse has dorsal and ventral bud hypoplasia, decreased epithelial proliferation and absence of the pancreatic progenitor transcription factor Pdx-1. This positions Pdx-1 downstream of FGF10 signaling. After organ specification, FGF signaling regulates the proliferation of endocrine and exocrine cell types.

The role of Notch signaling has been well studied in mammalian and *Drosophila melanogaster* neuronal development and the pathway has been found to be highly evolutionarily conserved. Expression of neurogenin genes leads to production of extracellular ligands such as Jagged, Serrate and Delta, which bind the Notch receptor on adjacent cell surfaces and result in the proteolytic cleavage of an intracellular notch receptor domain. This domain associates with the intracellular DNA-binding protein RBP-J_K in the nucleus and promotes transcription of the hairy/enhancer-of-split (HES) genes. Hes genes encode bHLH transcription factors which regulate neurogenin genes and other target genes. This "lateral inhibition" model provides a mechanism where one cell can express neurogenins and thereby inhibit neurogenin expression in neighboring cells,

forcing them to adopt a different cell fate. Vertebrates have 4 Notch genes, named Notch 1, 2, 3 and 4, which regulate differentiation and proliferation during development. In the pancreas, Notch 1–4 are expressed temporally in the order they are numbered (Lammert, 2000). Notch 1 is expressed in pancreatic epithelium along with Hes-1, its downstream target, beginning at E9.5. Notch 2 is also co-expressed with Hes-1 at E10.5 in the embryonic ducts, while Notch 3 and 4 are expressed in the pancreatic mesenchyme and later in endothelial cells with onset at E11.5. The Hes-1 knockout mouse was originally found to have defects in brain, eye, thymus and pituitary development and a small pancreas (Kageyama, 2000) while mice deficient for Delta1 or RBP-J_K have accelerated differentiation of Neurogenin-3 (Ngn-3) expressing cells. Ngn-3 is a critical transcription factor which promotes endocrine cell formation in pancreas. In agreement with the lateral inhibition found in neuronal development, pancreatic Hes-1 is expressed only in non-endocrine cells. It is thought that Ngn-3 expression in endocrine cells leads to activation of Hes-1 and repression of Ngn-3 in adjacent cells, directing them to adopt a non-endocrine fate (Figure 12). In addition to its importance in pancreas development, Hes-1 has been found to be essential for the specification of the gall bladder and bile ducts from foregut endoderm. Upon further investigation, Hes-1 knockout mice are found to have gallbladder agenesis and ectopic expression of Ngn-3 in liver (biliary) bile duct epithelium (Sumazaki, 2004), demonstrating the close developmental relationship between liver, gallbladder and pancreas development, and the role of Hes-1 in regulating the specification of the foregut endodermal region. A recent study (Fukada, 2006) using Hes-1 knockout mice along with Ptf1a (Ptf1a is downstream of Hes-1, Figure 14) reporter mice found that in the absence of

Hes-1, Ptf1a was ectopically expressed in the intestine and bile duct. These results are in agreement with the general understanding of foregut development (Figure 9).

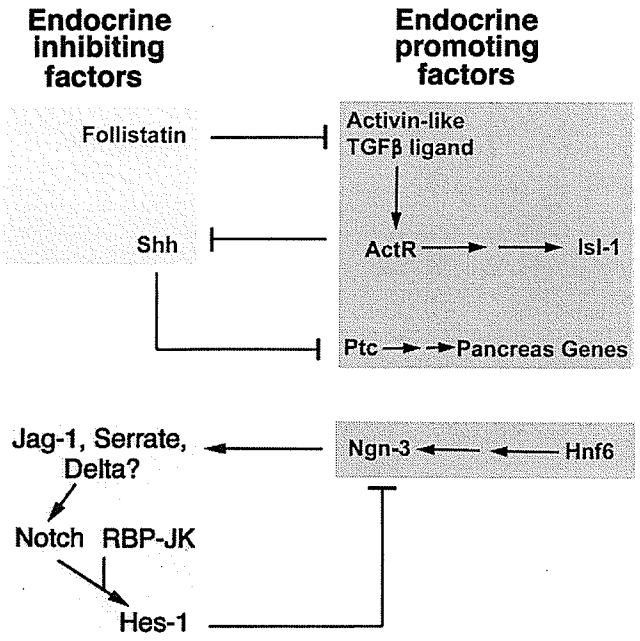

Figure 12: Notch signaling and pancreas development. The pancreas endocrine transcription factor Ngn-3 promotes expression of the extracellular Notch ligands Jagged, Serrate and Delta, which bind to Notch receptors on adjacent cells. Activation of Hes-1 in these adjacent cells leads to inhibition of Ngn-3, forcing them to adopt a non-endocrine cell-fate. Modified from Kim (2001).

Hedgehog proteins are secreted molecules that bind to their cell surface receptors and regulate development. Shh is not detectable in the embryonic or adult pancreas (due to repression as described above). As expected from its absence in the pancreas, experiments in chick, mice and fish show that elevated levels of Shh impair pancreas formation (Hebrok, 2003). However, the related proteins Indian hedgehog (Ihh) and Desert hedgehog (Dhh) as well as the Hedgehog receptor Patched (Ptc) are expressed in the developing pancreas and adult islets (Figure 13). Single knockouts of Ihh, Dhh have increased

proliferation of endocrine cells suggesting that Hedgehog signaling inhibits endocrine cell growth. The Ptc homozygous null dies at E9.5 and fails to express Pdx-1 in the dorsal bud. Thus Hedgehog signaling clearly plays an essential role in pancreas development, but much remains to be discovered.

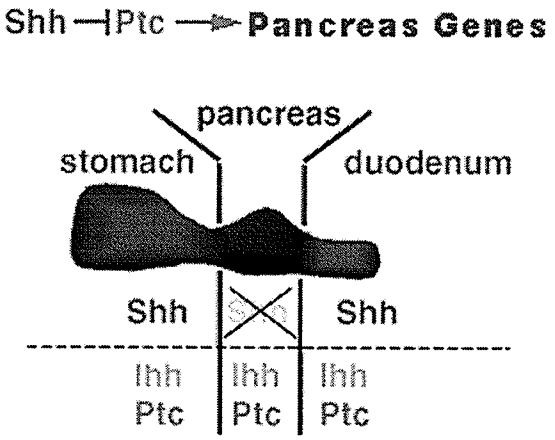

Figure 13: Shh signaling and pancreas development. Shh activates target genes by blocking the function of its receptor, Ptc. Shh blocks pancreas development in stomach and duodenum. Shh is excluded from pancreatic tissue during embryogenesis. However, other hedgehog signaling components such as Ihh and Ptc are expressed in pancreas during development. Modified from Hebrok (2003).

Recent work has shown that there are many other signaling pathways involved in pancreas development including the β-catenin protein in the Wnt pathway (Murtaugh, 2005) and epidermal growth factor receptor (EGFR) signaling (Means, 2005).

Intrinsic Pancreas Signaling (Transcription Factors)

Pancreas development can be divided into several stages which are regulated both intrinsically and extrinsically: specification of foregut cells to a pancreatic cell fate; growth

of pancreatic primordium (beginning with formation of dorsal and ventral buds); and specification, differentiation and maturation of endocrine, exocrine and ductal cells. Current knowledge of this process comes mostly from mouse models by two types of experiments. Firstly, knockout mice models were used to assess the pancreatic phenotype resulting from loss of a specific regulatory factor. This type of experiment is useful to determine where and when a factor functions during development but may not be able to distinguish the altered cell-fate of factor-deficient cells from normal cells which normally adopt that fate. Therefore, lineage analysis experiments have been carried out. Generally, these experiments involve replacing the coding region of a gene with Cre-recombinase and crossing this knockout into a reporter mouse. Any cell which expresses the gene of interest will give rise to descendents which are marked by Cre-mediated recombination at the reporter gene locus. A clear example of the advantage of lineage analysis is the experiments looking at the transcription factor PTF1a (also called PTF1-p48 or p48) which was shown to be expressed in progenitors of exocrine, endocrine and ductal cells (Kawaguchi, 2002) rather than being exocrine-specific as had been previously reported. The result of these two transgenic approaches is that a cascade of transcription factors has been identified which regulate the differentiation of specific pancreatic cell types (Figure 14).

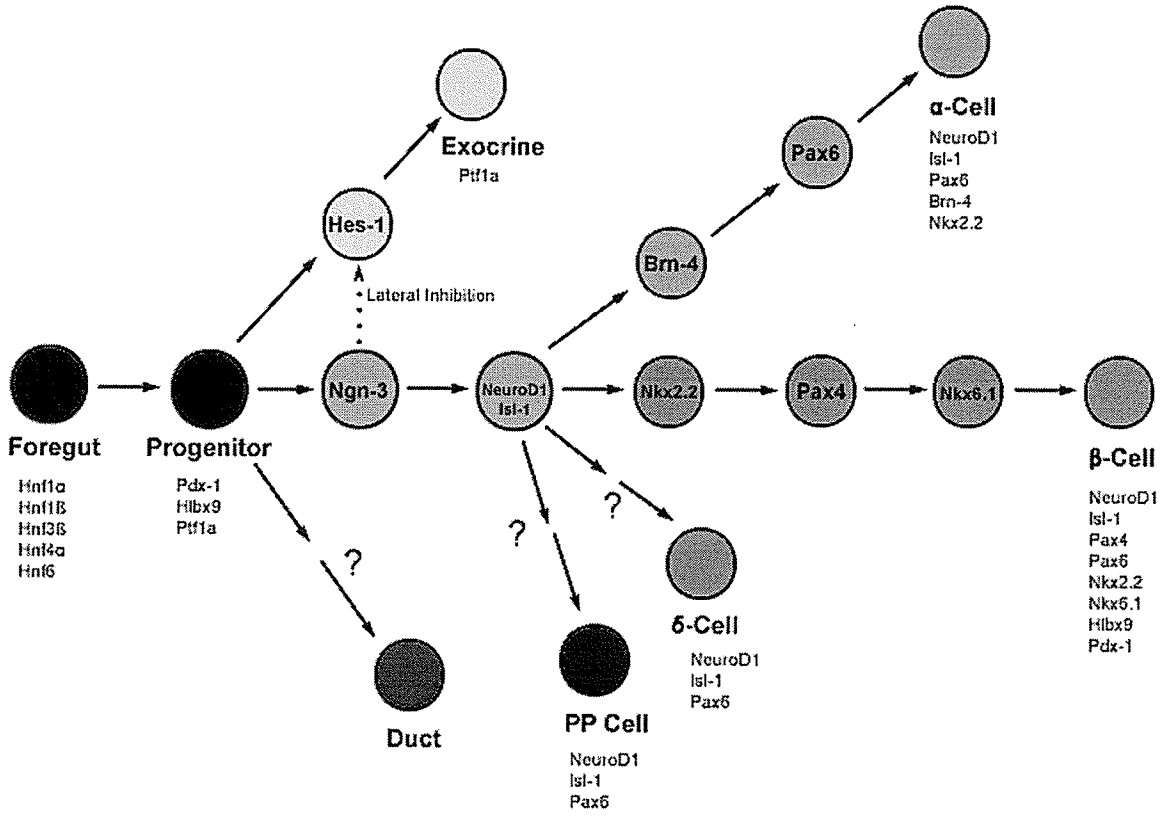

Figure 14: Pancreas development transcription factor cascade. At E8, expression of transcription factors from the HNF-family specify pancreas cell fate. All pancreas cells are derived from a progenitor cell which expresses Pdx-1, Hlbx9 and Ptf1a at E9.5. Different transcription factors specify endocrine, exocrine or ductal cell fates. Adapted from Kemp (2003) and Wilson (2002).

Genes expressed within the endoderm prior to E8.5 help to regulate tissue specific transcription factors. This "core endodermal program" involves several genes from the hepatocyte nuclear factor family (HNF-family) including Hnf1α, Hnf1β, Hnf3β, Hnf4α and Hnf6 (Jensen, 2004). In the pancreas, HNF1α regulates expression of Ngn-3 and Pdx-1, and Hnf1α knockout mice have small islets and reduced insulin secretion. HNF1β and HNF4α both bind the Pdx-1 promoter. HNF6 positively regulates Ngn-3 and the Hnf6 knockout has reduced Ngn-3 expression and a decreased number of endocrine cells. Hnf3β

(also called Foxa2) regulates Pdx-1 and Ngn-3 and the mouse knockout has a very obvious phenotype: the foregut does not form. At least three HNF family members are involved in MODY, a subset of Type II diabetes with a heavy monogenetic basis. HNF genes are not unique to pancreas however, and they are important throughout the developing foregut region. Pancreas specification begins with the expression of HB9, PTF1a and the ParaHox family member Pdx-1.

Pdx-1 (also called Ipf1) is a homeodomain protein which is expressed at E8.5 and found uniformly along the region of the foregut which will give rise to both the dorsal and ventral anlages. The Pdx-1 mouse knockout has pancreatic agenesis, although the dorsal bud does undergo limited proliferation (Jonsson, 1994). In wild-type adults, Pdx-1 is expressed in β–cells and is required for maintenance of insulin expression and proper β-cell differentiation as determined by Cre-lox β-cell specific knockouts. Activity of Pdx-1 is modulated by Pbx-1 which forms Pdx-1/Pbx-1 heterodimers. These heterodimers are important early in development, as the Pbx-1 knockout mouse has pancreatic hypoplasia.

HB9 (encoded by the Hlxb9 gene) is another transcription factor expressed in early pancreatic progenitors. In the HB9 knockout, the dorsal lobe fails to develop (Harrison, 1999). The ventral lobe develops normally and contains both exocrine and endocrine cells, but has reduced number of β-cells. HB9 is expressed at E8 in notochord and gut endoderm and in both dorsal and ventral buds at E10.5. In wild-type adults, HB9 expression is restricted to β-cells.

PTF1a (also called PTF1-p48 or p48), a bHLH protein, is the DNA-binding subunit of the PTF1 tetrameric transcription factor complex composed of p48, p64 and p75. It is expressed at E9.5 and is restricted to the acinar (exocrine) cells in wild-type adults. The PTF1a knockout mouse undergoes a cell-fate switch where progenitor cells form intestine instead of pancreas. Pdx-1 expressed from the PTF1a promoter rescued a Pdx-1 null mouse demonstrating that it is expressed in pancreatic progenitors. (Kawaguchi, 2002). PTF1a is also essential for exocrine formation.

Unlike Pdx-1, HB9 and PTF1a which are expressed in the pancreatic epithelium, the LIM-domain family member Isl-1 is expressed in the dorsal pancreatic mesenchyme (in addition to other important developmental roles). The Isl-1 knockout mouse has a phenotype similar to that of the HB9 null (dorsal bud agenesis) and the mice die at E9.5 due to dorsal aorta agenesis (Ahlgren, 1997). Isl-1 is expressed in all islet cell types in mature wild-type mice and may also play a role in endocrine development.

Endocrine, Exocrine and Ductal Cell Differentiation

Endocrine cell-fate specification begins with the critical neurogenin-family transcription factor Ngn-3, which is a bHLH protein (non-endocrine cells are laterally inhibited by Ngn-3 as mediated by Hes-1 and Notch signaling). Experiments have shown that Ngn-3 is both necessary (Gradwohl, 2000) and sufficient (Grapin-Botton, 2001) to promote endocrine cell development, and mice null for Ngn-3 fail to develop any endocrine cells or putative

endocrine precursors. Wild-type expression of Ngn-3 begins E9-9.5 and peaks at E15.5 with little or no Ngn-3 expressed in adult pancreas.

Downstream of Ngn-3 is the bHLH transcription factor NeuroD1 (also called Beta-2). NeuroD1 is not absolutely required for islet development, but the knockout mouse has reduced islet cells and severe β-cell hypoplasia (Naya, 1997). NeuroD1 is expressed in mature islet cells in the adult mouse. Interestingly, NeuroD1 activates the promoter of Ngn-3, but Ngn-3 expression is independent of NeuroD1, and the Ngn-3 knockout does not express NeuroD1 showing that NeuroD1 is downstream of Ngn-3.

Nkx2.2 is a member of the NK class of homeodomain proteins. Its expression begins at E8.5-E9 in dorsal and ventral pancreatic epithelium and becomes restricted to α, β and PP cells only in mature islets. The knockout mouse has reduced α, β and PP cells, and a complete lack of insulin production (Sussel, 1998). However there is a population of islet cells which express other β-cell markers suggesting that Nkx2.2 is responsible for the terminal differentiation of islets. Nkx6.1 is another NK homeodomain transcription factor and is downstream of the paired homeodomain protein Pax4. Nkx6.1 is expressed in β-cells in the adult mouse pancreas. The Nkx6.1 knockout mouse has inhibited formation of β-cells during the secondary transition and a ~95% reduction of β-cells at birth (Sander, 2000). The Nkx2.2/Nkx6.1 double-knockout has same phenotype as the Nkx2.2 single-knockout showing that Nkx6.1 is downstream of Nkx2.2.

Pax4 contains both a paired domain and a homeodomain. It is expressed at E9.5 in the pancreatic buds. In the adult, its expression is restricted to β-cells. The Pax4 knockout mouse lacks β and δ-cells (Sosa-Pineda, 1997). Pax6, another paired homeodomain gene, is expressed in all mature islet cells. The Pax6 knockout displays a phenotype reciprocal to that of the Pax4 knockout, in that there are no α cells and reduced β, δ and PP cells (St-Onge, 1997). Conditional inactivation of Pax6 in pancreas (Ashery-Padan, 2004) results in mice which die shortly after birth and display diabetic symptoms including hyperglycemia and low insulin levels. This would suggest a role for Pax6 in maintaining β-cell function in adults. It is unclear if the Pax4 and Pax6 genes have overlapping functions which may be masked in these single-knockout experiments.

Brn-4 is a member of the class III family of POU-homeodomain proteins that are highly expressed in neural stem cells and regulate stem cell-specific genes. In pancreas, it is expressed in the dorsal and ventral buds at E10 and becomes specifically restricted to progenitor cells that later differentiate into α-cells. Brn-4 has not been detected in either differentiating or mature β cells. Brn-4 also directly stimulates glucagon gene expression by binding to the G1 promoter element within the glucagon gene (Hussain, 1997) and misexpression of Brn-4 by the Pdx-1 promoter results in the ectopic expression of the proglucagon gene in insulin-expressing pancreatic cells (Hussain, 2002). These results suggest that Brn-4 promotes an α-cell fate.

A recent study has shown that the homeodomain transcription factor Prox1 is expressed in pancreas at E9.5 and is important in regulating pancreatic growth and morphology. A

substantial decrease in endocrine cells is found after E13.5, concurrently with an increase in the number of exocrine cells in the Prox1 null mouse (Wang, 2005), suggesting that Prox1 prevents exocrine differentiation.

Very little is known about exocrine or duct specific transcription factors. The protein PTF1a (also PTF1-p48 or p48) was previously thought to be exocrine specific but is now known to be important for the early differentiation of all pancreatic cell types.

Homeobox Genes

Homeobox genes encode homeodomain (or homeobox) proteins. This class of proteins was first discovered in *Drosophila melanogaster* (Lewis, 1978) and its members all contain a related DNA binding region called the homeodomain (HD) which can bind to consensus DNA sequences often containing one or more TAAT tetranucleotide motifs. Homeodomain proteins are transcription factors which regulate expression of other genes and are important in the adult and also during development (Gehring, 1994). This regulation is often mediated by their binding to DNA via the HD and subsequent regulation of transcription. The importance of HD proteins during development is evident in that there are over 1000 HD encoding genes known including over 100 from humans, many of which are known to result in disease when mutated (D'Elia, 2001). It is known that one HD protein may bind to several TAAT sites on different genes, or, that many different HD proteins may bind to the same TAAT site on one gene (reviewed in Mannervik, 1999). Structural and biochemical studies demonstrate that HD proteins are folded into a highly

conserved structure composed of three alpha-helices. Helix 3 (C-terminal) contacts the major grove of DNA, while helices 1 and 2 pack against helix 3. The N-terminal portion of the HD makes additional contacts with the minor grove of DNA (Simon, 2004). A number of studies have been carried out on the Engrailed HD, which is considered to be the prototypical HD and recognizes the consensus sequence TAATTA. These studies show that base-specific contacts in the major groove are mediated by at least three residues in helix 3. Residue N51 of the HD is highly conserved and the N51A mutation abrogates binding to DNA (Ades, 1994). Two other residues are also highly conserved and are important in DNA binding: Q50 and I47. The Q50A and I47A mutations of engrailed lead to ~20 and ~2 fold reductions in binding affinity respectively (Ades, 1994 and Ades, 1995). Thus the contribution of these I47 and Q50 residues is considered to be relatively small in comparison to N51. However, residue Q50 in particular was subsequently studied in detail after it was found that the Q50K mutation altered DNA-binding specificity from TAATTA to TAATCC, suggesting that this residue might be important for recognition of bases 5 and 6. However, it was later found that Q50A has preference for TAATTA with only slight loss of DNA-binding affinity (Ades, 1995). The Q50A crystal structures show only subtle changes in protein-DNA structure (Grant, 2000) but a recent mutation study in phage has demonstrated a binding advantage for Q50 (the wild-type) over any other residue (Simon, 2004). In *Drosophila melanogaster*, the subtle Q50E mutation was found to abrogate DNA binding (Gehring, 1994).

Specificity of homeodomain binding to a particular target sequence is conferred by the nucleotides surrounding the core TAAT motif. However, it has been suggested that an additional level of regulation of binding to DNA may be provided by additional homeodomain functions such as protein-protein interactions, either with other transcription factors or with transcription machinery. There are two proposed models for regulation of homeodomain protein binding to DNA through protein-protein interactions (Biggin, 1997). The first is the "co-selective binding" model where each homeodomain protein first binds a specific co-factor which confers increased binding affinity to a specific target. The second model is "widespread-binding" which proposes that many homeodomain proteins can bind to the same sequences, but their activity is modulated through binding to specific co-factors. Although the majority of literature thus far has investigated only the DNA-binding function, there have been some studies which demonstrate that homeodomain proteins interact with TATA binding protein in addition to the normal DNA-binding (Um, 1995; Zhang, 1996). Some *in vivo* cross-linking experiments also support the "widespread-binding" model of homeodomain protein-protein interactions (Carr, 1999; Walter 1996).

Dlx Genes

The Dlx family of homeodomain proteins are the vertebrate homologues of *Drosophila* Distal-less. Distal-less is required in the fruit fly for distal limb development. There are six known Dlx genes in mice and humans which are found in three bigenic clusters (convergently transcribed). Each pair is linked to a Hox cluster. Dlx1/Dlx2 are linked to Hoxd; Dlx3/Dlx4 are linked to Hoxb; and Dlx5/Dlx6 are linked to Hoxa (reviewed in

Panganiban, 2002). Pairs of Dlx genes exhibit similar patterns of expression and are known to have partially redundant functions. In many studies of knockout mice, no significant phenotype is present if only one Dlx gene is deleted, but such a phenotype becomes detectable if a pair of Dlx genes is knocked out. Extensive research has been conducted on the role of Dlx genes in development (reviewed in Panganiban, 2002) and they are known to be important for correct development of many parts of the body such as neuronal differentiation and migration, bone and cartilage formation, patterning of the branchial arch, and limb development. Only one post-translational modification of a DLX protein has been currently described: the phosphorylation of DLX3 by protein kinase C (Park, 2001). The homeodomain of Dlx family members is 60 amino acids long and is highly conserved among family members.

Dlx1 and Dlx2

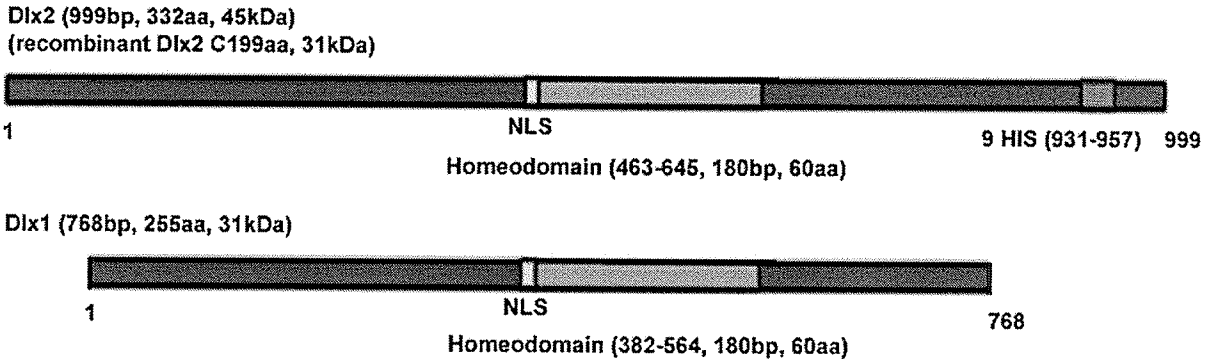

Figure 15: Dlx1 and Dlx2. Dlx1 and Dlx2 share a high degree of homology, especially in the 60aa homeodomain. Dlx2 is slightly larger, and has extra N and C-terminal portions, including a 9-Histidine amino acid sequence near the C-terminus. Recombinant Dlx2 used in our laboratory consists of the C-terminal 199aa of the protein.

Dlx1 and Dlx2 are linked in a bigenic cluster on mouse chromosome 2. They are known to have nearly identical expression patterns (McGuinness, 1996), suggesting that they function and are regulated similarly. There have been some studies on the regulation of Dlx1 and Dlx2. Bone morphogenetic protein 4 can induce Dlx1/Dlx2 expression in murine dental mesenchyme and FGF8 can induce Dlx1/Dlx2 expression in murine dental mesenchyme (Bei, 1998). Dlx1 and Dlx2 are also known to be expressed in the developing central nervous system. Dlx1 and Dlx2 double-knockout mice die at birth and display severe craniofacial (Qiu, 1997) and central nervous system defects (Anderson, 1997a & Anderson, 1997b). In addition to striatum cells which do not fully differentiate, there is marked reduction of tangential migration of GABAergic interneurons to the cortex. In the developing retina, Dlx1 and Dlx2 are both expressed by E12.5 (Eisenstat, 1999). However, there is a difference in the expression of Dlx1/2 as expression of DLX1 is largely restricted to the ganglion cells while DLX2 is restricted to the ganglion cells, amacrine and horizontal cells. Temporally, DLX1 expression is downregulated after birth, while DLX2 expression is maintained throughout adult life. Both Dlx1 and Dlx2 are important for terminal differentiation and survival of ganglion cells in the retina (de Melo, 2005). Thus Dlx1 and Dlx2 genes appear to regulate cell differentiation in neuronal cells. Although they share very similar function and patterns of expression, some differences between DLX1 and DLX2 have been discovered. Both proteins share a high degree of homology especially in their homeodomains, which are 60 aa long. However, DLX2 is slightly longer and has extra N and C-terminal portions compared to DLX1, including a 9-His poly-histidine motif

at its C-terminus. Both DLX1 and DLX2 have a nuclear localization signal (NLS) before the homeodomain.

Dlx1 and Dlx2 in Pancreas

The Dlx1/Dlx2 double knockout mouse dies at birth with cleft palate and abnormalities in the differentiation of several cell types in the CNS and retina as described above. Of interest, experiments from our laboratory have found that many DLX transcriptional targets are also expressed in the pancreatic islets of Langerhans (Ho, Qiu and Eisenstat, unpublished observations). Several important transcription factors in the pancreatic developmental cascade are homeodomain proteins including: Nkx2.2, Nkx6.1, Pax4 and Pax6. Of particular interest, Dlx1 and Dlx2 are highly expressed in developing pancreas with their onset of expression at least as early as E14.5. Preliminary chromatin immunoprecipitation (ChIP) experiments with our well-characterized specific antibodies to DLX1 and DLX2 (Zhou, 2004) and pancreatic islet tissues obtained at birth and in the adult shows that both Dlx homeoproteins bind to the promoter regions of the mouse pre-proinsulin I and glucagon genes *in situ*. Hence, Dlx1 and Dlx2 may play a role in pancreatic islet cell development by direct transcriptional regulation of pancreatic endocrine genes.

Research Aims and Hypothesis

Hypothesis: Dlx1 and Dlx2 homeobox genes play a direct role in regulating glucagon expression in the developing and/or the adult pancreas.

Specific Aim 1. Determination of onset, localization and level of DLX1/DLX2 expression in embryonic and adult pancreas.

The onset of pancreatic DLX1/DLX2 expression as well as their localization at both adult and various embryonic time points will be determined by immunofluorescence microscopy on mouse pancreas tissue sections, using specific antibodies to DLX1 and DLX2. Antibodies to the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and various pancreas transcription factors, such as Ngn-3 and Pdx-1, will also be used to identify specific cell types in which DLX proteins are expressed.

Specific Aim 2. Characterize the interaction of DLX1/DLX2 proteins with the regulatory region(s) of the pre-proinsulin I and proglucagon genes.

Previous chromatin immunoprecipitation (ChIP) studies in our laboratory show that DLX1 and DLX2 bind to the promoter regions of proglucagon and pre-proinsulin I. To confirm this binding, electrophoretic mobility shift assays (EMSA) using recombinant DLX1/DLX2 proteins will be carried out. Binding of DLX1/DLX2 to the insulin/proglucagon promoters will also be examined using EMSA with pancreatic islet nuclear extracts obtained from mice at various developmental time-points.

Specific Aim 3. Effect of DLX1/DLX2 binding to the proglucagon promoter on reporter gene expression.

The functional consequences of binding of DLX1 and DLX2 proteins to the proglucagon gene promoter will be studied by luciferase reporter gene assay in the relevant cell line α-TC1, which is derived from a mouse adenoma that expresses glucagon.

Specific Aim 4. Study the pancreatic phenotype of DLX1/DLX2 double knockout mouse.

If DLX1/DLX2 are shown to bind to the pre-proinsulin I and proglucagon gene promoters and regulate insulin and glucagons expression *in vitro*, then it can be expected that insulin and glucagon expression will be abnormal in the mutant mouse lacking these genes. The levels of insulin and glucagon hormones will be also determined by radioimmunoassay at various time points during embryonic development.

Chapter II: Materials and Methods

Mouse Pancreas Tissue Procurement

Wild-type CD-1 or Dlx1/2 double-knockout mice were sacrificed by cervical dislocation at the appropriate age using protocols approved by Central Animal Care (University of Manitoba). For transcription factor and hormone analysis, cervical dislocation provides the advantage of rapid death with minimal chemical contamination, hypoxia and necrosis of tissue in contrast to other widely used methods such as CO_2-mediated asphyxiation. Timed-pregnant mice were used to obtain embryos at the appropriate developmental stage. Tissues were kept in phosphate-buffered saline (PBS) on ice, and the pancreas was dissected (under stereo-microscope if necessary). The pancreas was then immediately prepared for embedding/sectioning or processed using an islet preparation method as detailed below. Verification of Dlx1/2 genotype in embryos was done by PCR-genotyping with specific primers to Dlx1 and Dlx2 (Qiu, 1997). For PCR genotyping, mice tails were added to 500 µl of tail lysis buffer (100 mM NaCl, 50 mM Tris-HCl, 50 mM EDTA, 0.5% SDS, pH 8.0) with 0.1 mg/ml proteinase K and incubated overnight at 60°C. They were then centrifuged at maximum speed (13,000 rpm) in a tabletop microcentrifuge for 5 minutes. 100 ul of supernatant was collected, boiled for 10 minutes, placed on ice for 10 minutes and then 1 ul of this solution was used for each PCR reaction.

Mouse Islet Preparation

A protocol for mouse islet preparation incorporating collagenase digestion and gradient

separation procedures widely described in literature was developed (a typical protocol can be found in Simeonovic, 1988). This protocol is summarized below:

Pancreas from two CD1 mice (25-30 mg) were cut into small pieces in a sawed-off conical tube containing ice cold Hank's Balanced Salt Solution (HBSS) with 20 mM HEPES pH=7.4, then suspended in 3 mL of HBSS with 2mg/ml Collagenase (Type V, Sigma, make a 20 mg/mL stock in HBSS) in a 15 mL conical tube. After shaking at 185 rpm for 20 minutes in a 37°C water bath, the digest was diluted with 10 ml ice cold HBSS and mixed thoroughly (centrifuge at 250 x g, and ensure supernatant is clear after each spin). Finally, the digest was washed 2 additional times with ice cold HBSS, and 25 ul of DNase solution (10 ug/ml) was added. Islets were enriched using a Percoll (Amersham) step gradient. The above sample was resuspended in 6 mL of 1.089 g/mL Percoll and mixed, with 5 mL of 1.060 g/mL Percoll layered on top. The tube was centrifuged at 800 x g for 10 minutes at 10°C. The islets were harvested from the gradient by removing the interface between the low and high density solutions using a Pasteur pipette (aspirate off top layer using pipette then remove interface). The islets were washed twice with ice cold HBSS and were then ready for use.

Islet purity was assessed by endocrine tissue staining with Dithiazone (DTZ, Sigma). Islets were collected in 1.5 mL tubes, washed once with 1X phosphate-buffered saline (PBS) and then collected in a 35 mm Petri dish. 10ul of a 39 mM DTZ in DMSO solution was added to islets suspended in 1 mL Krebs-Ringer Bicarbonate buffer (pH 7.4) with 10 mM HEPES

and incubated at 37°C for 10-15 minutes. Red/orange staining cells were counted under an inverted microscope.

Cell viability was assessed by 0.2% Trypan Blue exclusion (more appropriate here than staining with acridine orange or propidium iodide). A suitable number of cells was placed in 0.5 ml of 1X PBS (dilute cells to approximate concentration of 1×10^5 to 2×10^5 cells/ml) in a screw cap test tube. 0.1 ml of 0.4% of Trypan Blue stain was added and mixed thoroughly. The mixture was allowed to stand for 5 min at room temperature. A hemocytometer was used for cell counting with blue stained cells scored as non-viable and unstained cells scored as viable.

Tissue Embedding and Sectioning

Whole pancreas and/or embryos were washed once with 1X PBS, then incubated in 4% paraformaldehyde at 4°C overnight on a rotating shaker. The tissue was then transferred to a 10% sucrose solution and incubated rotating at 4°C until it sedimented at the bottom of the tube. This step was repeated using 15% sucrose and then 20% sucrose. Tissue was then incubated in a 1:1 mixture of 20% sucrose and OCT Compound (#4583, Sakura USA) and quickly frozen in a dry-ice/ethanol bath in embedding molds ("blocks") and stored at -80°C until use. Frozen "blocks" were sectioned at 12 μM thickness on a cryostat and transferred to frosted microscope slides (Fischer).

Immunofluorescence (IF)

Frozen sections on microscope slides were blocked for 1 hour at room temperature with 10% horse or goat serum as appropriate. Primary antibodies to DLX1, DLX2, insulin, glucagon, somatostatin, pancreatic polypeptide and PDX-1 were incubated overnight on frozen sections on microscope slides at 4°C. Each slide was washed three times with PBS containing 0.05% Triton X-100 (PBS-T), then incubated with the appropriate secondary antibody for 2 hours, then washed 3X times with PBS-T. For double-labeling, the second primary antibody was incubated overnight and the process repeated. Slides were then mounted using Permount with DAPI and imaged by confocal microscopy (Olympus).

Antibody	**Catalogue#, Company**	**Secondary**
Mouse anti-insulin 1:3000	Ab8305, Cedarlane	Goat anti-mouse Texas Red
Mouse anti-glucagon 1:400	Mab1249, R&D	Goat anti-mouse Texas Red
Goat anti-somatostatin 1:100	Sc7819, Santa Cruz	Donkey anti-goat Alexa 594
Guinea Pig anti-pancreatic polypeptide 1:75	4041-01, Linco	Goat anti-GP Texas Red
Goat anti-PDX1 1:100	14662, Santa Cruz	Donkey anti-goat Alexa 594
Rabbit anti-Dlx1 1:100 Rabbit anti-Dlx2 1:300	Eisenstat Laboratory Eisenstat Laboratory	Biotinylated goat/mouse - anti rabbit followed by tertiary strepavidin Alexa

Table 2: Antibodies used for immunofluorescence

For immunofluorescence of cultured αTC1-9 cells, cells were grown on microscope coverslips and transfected using Lipofectamine 2000 (Invitrogen). 48 hours post-transfection, cells were fixed for 10 minutes in 4% paraformaldehyde and then incubated overnight with primary antibody. Immunofluorescence was then carried out as described

above.

Chromatin Immunoprecipitation (ChIP)

The ChIP protocol consists of cross-linking, immunoprecipitation with specific antibodies and then reverse cross-linking. Embryonic or adult pancreas tissue was obtained as described above. Hindbrain (no Dlx1/Dlx2 expression) was used as a negative control and forebrain was used as a positive control. The pancreas was then cut into small pieces manually, dispersed by pipetting, and incubated in 1% paraformaldehyde with protease inhibitors (Complete, Roche) for 2 hours. The cells were then washed twice with PBS and collected at 5000 rpm (JA-20 rotor) for 5 minutes. Cells were resuspended in 400 µl of SDS Lysis-Buffer (1% SDS, 10 mM Tris-HCl pH 8.1, 10 mM EDTA) in a 1.5 ml centrifuge tube and incubated on ice for 10 minutes. The cells were then sonicated five times for 10 seconds each time (30% pulse output), incubating on ice between each pulse. The resulting lysate was run on an agarose gel and the major DNA band was verified to be ~300-500bp. Lysate was then centrifuged at 13000 rpm for 10 minutes at 4°C on a table-top centrifuge. 5µl of lysate was resuspended in 995µl of 2M NaCl/5M urea and the OD_{260} was measured. Four A_{260} units of lysate were diluted in 1 ml of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 150 mM NaCl) in a fresh 1.5 ml tube. The lysate was then pre-cleared with 60µl of Protein A-sepharose (50% slurry) for 30 minutes at 4°C with agitation. The Protein A-sepharose beads were pelleted at 4000 rpm for 1 minute and the supernatant was transferred to a fresh 1.5 ml tube. 30ul of DLX1 or DLX2 polyclonal antibody or no antibody (control) was added to the chromatin

solution (supernatant) and incubated overnight at 4°C with agitation. 60 µl of Protein A+G-sepharose (50% slurry) was added to the tube and incubated for 3 hours. The beads were pelleted at 8000 rpm for 1 minute. Supernatant was removed, and the beads were washed for 5 minutes with 1ml of each of the following buffers in order: Low Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl); High Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl); LiCl Buffer (0.25M LiCl 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1, 1% NP-40), TE buffer pH 8.0; and TE buffer pH 8.0. Following the washes, elution buffer was added (1% SDS, 0.1M $NaHCO_3$) and incubated at room temperature for 15 minutes with agitation. Beads were pelleted at 13000 rpm for 1 minute, and the supernatant was removed and transferred to a fresh 1.5 mL tube. The elution was repeated and pooled into the same 1.5 mL tube. Cross-linking was then reversed by adding 25 µl of 5 M NaCl to the combined eluates and incubating for 4 hours at 68°C. 20 µl of 0.25 M EDTA, 20 µl of 1 M Tris-HCl pH 6.5, and 2 µl of proteinase K were added to the eluates and incubated at 45°C for 1 hour. DNA was then extracted using the phenol/chloroform method. Subsequently, 10 µg of carrier (glycogen) was added. Then the DNA was precipitated with ethanol. DNA was then washed with 70% ethanol and resuspended in TE buffer. DNA samples were analyzed with specific PCR primers to putative Dlx1/2 promoter binding site regions (pre-proinsulin I and proglucagon).

Electrophoretic Mobility Shift Assay (EMSA)

Proglucagon and pre-proinsulin I promoters [promoter fragments were created using the

following primers: glucagon, forward 5'-CCTTTCCATTCCCAAACAGA-3' (-219 to -200 nt), reverse 5'-ATAAAAGCACAGCACCCTGG-3' (-17 to +3 nt); insulin forward 5'-TAAGGGCCCAGCTATCAATG-3' (-398 to -379 nt), reverse 5'-ATAAAGCTGGTGGGCATCC-3' (-23 to -5)] were radiolabeled with α-^{32}P-dATP (Perkin Elmer) using Large Fragment of DNA Pol I (#18012-021, Invitrogen). Radiolabeled promoters were then incubated with recombinant DLX1 and DLX2 protein for 30 minutes at RT and then run on an "EMSA acrylamide gel" (Protean-II apparatus, Biorad) as described (Promega technical bulletin #110 "Gel Shift Assay System"). The gel was then dried for 1 hour and exposed overnight with high-performance autoradiography film (RPM-1678 Amersham).

Cell lines, Transfection

α-TC1-9, a mouse adenoma, (#CRL-2350 from ATCC) and Human Embryonic Kidney-293 (HEK-293) cell lines were used for transfection. Cells were grown in T-175 flasks (Falcon). α-TC1-9 was grown in DMEM (#12100-046 Gibco, with 1.5 g $NaHCO_3$/L and 10% fetal bovine serum). HEK-293 was grown in DMEM (#12800-017 Gibco with 3.7 g $NaHCO_3$/L and 10% fetal bovine serum). 24 hours prior to transfection, cells were seeded uniformly into either 6-well plates (for luciferase assays) or 10 cm dishes (for SDS-PAGE) and at a density to be roughly 80% confluent at the time of transfection. The following day, cells were transfected with 0.1-1.5 μg of plasmid DNA (pCDNA3 and pGL3 constructs) or 0.1 μg plasmid DNA (PRSV-β-gal) using Lipofectamine 2000 reagent (Invitrogen). The transfection protocol used is described on the Lipofectamine product sheet. 24-48 hours

post-transfection, cells were harvested using reporter lysis buffer (Promega) and processed for luciferase/β-gal activity as described below or assayed for protein concentration (BCA protein assay kit, Pierce) and separated by SDS-PAGE.

Generation of Plasmid Constructs, PCR

Plasmid constructs used for transfection were obtained from the Eisenstat laboratory or generated using PCR from existing full-length Dlx1, Dlx2 or proglucagon promoter constructs with the primers shown in Table 3.

Site-directed mutagenesis, DNA sequencing

Proglucagon promoter TAAT mutant constructs were generated by site-directed mutagenesis (QuikChange, Stratagene).

Construct	Forward Primer (5'-3')	Reverse Primer (5'-3')
Dlx1-WRPW / pcDNA3	CGCGGATCCATGACCA TGACCACC	AATGAATTCACCAAGGACG CCACATCAGTTGAGGCTG
Dlx2-CTerΔ / pcDNA3	CGCGGTACCATGACTG GAGTCTTTGACAGT	CGGCTCGAGTTAGGTGGGT ATCTCGCC
Dlx2-HISΔ / pcDNA3	CGCGGTACCATGACTG GAGTCTTTGACAGT	CGGCTCGAGTTACGCCTGC GGAGTCTG
Glu_1TAATmutant / pGL3	TCATTTGAACAAAACC CCAGTATTTACAGATG AGAAATTTATATG	CATATAAATTTCTCATCTGT AAATACTGGGGTTTTGTTC AAATGA
Glu_2TAATmutant / pGL3	AGAAATTTATATTGTCA GCGTGATATCTGCAAG GCTAAACAGCC	GGCTGTTTAGCCTTGCAGA TATCACGCTGACAATATAA ATTTCT
Glu_1+2TAATmutant / pGL3	Created by using Glu_2TAATmutant primers on the Glu_1TAAT mutant construct	

Table 3: Primers used for generation of plasmid constructs.

All constructs were cloned into the appropriate vectors (pcDNA3, pGL3) and sequenced. Proper expression of recombinant proteins was verified by transfecting expression plasmids into HEK-293 cells and then Western blotting using specific antibodies.

Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western Blotting

Samples were run on an appropriate (generally 10 or 12%) acrylamide gel (Mini-Protean, Biorad) at 166V. Proteins were then transferred to a nitrocellulose membrane (Biorad) by transfer at 100V for 1.5-2 hours. Following transfer, the membrane was blocked for 1 hour with 6% milk in PBS and then incubated with the appropriate primary antibody overnight, washed 3X with TBS-T, incubated with a horseradish-peroxidase-conjugated secondary antibody for 1 hour, washed three times with TBS-T, incubated for 1 minute with ECL Plus (Amersham) and then exposed to Western Blot film (Kodak).

Luciferase Reporter Gene Assay (Luciferase)

Luciferase samples were analyzed for luciferase activity and β-galactosidase (β-gal) activity as described in technical bulletins 281 ("Luciferase Assay System") and 097 ("β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer), both from Promega.

Radioimmunoassay

Blood and whole pancreas tissue were obtained from adult and embryonic mice as described above and stored at -80°C until use. These samples were assayed for insulin content using a radioimmunoassay kit (Linco).

Chapter III: Results

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed (Figure 16, courtesy of Trung Le) on CD-1 mouse pancreas tissue obtained from P0 or adult using specific antibodies to DLX1 or DLX2 and primers to the pre-proinsulin I and pro-proglucagon promoters. Both DLX1 and DLX2 were found to associate with and possibly bind specific regions within these promoters containing the TAAT homeodomain-binding motif.

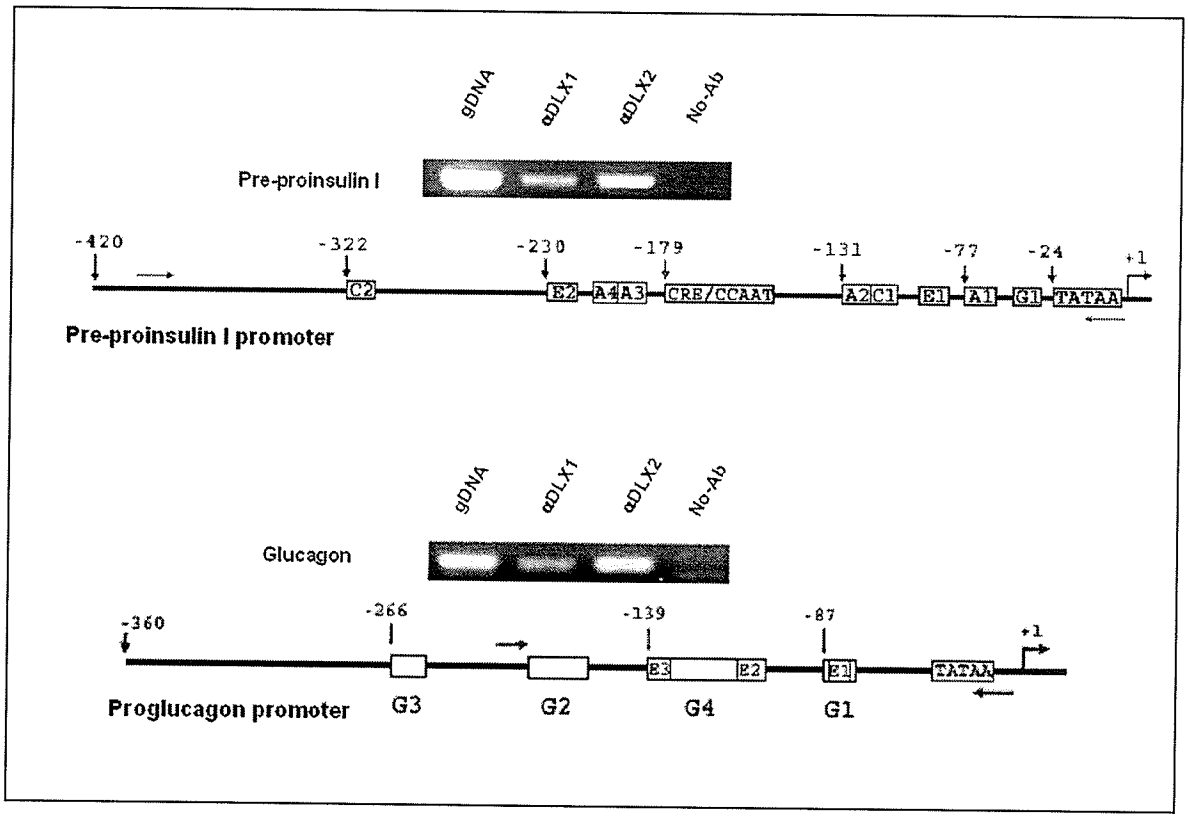

Figure 16: ChIP - DLX1 & DLX2 bind to the pre-proinsulin I and proglucagon promoters in neonatal and adult pancreas (not shown) in situ within the region of DNA flanked by the red arrows. Known DNA binding motifs and sequences are shown as boxes. Genomic DNA (gDNA) was used as a positive control. ChIP without antibody (No-Ab) was used as negative control.

The regions of DNA are shown in Figure 16 (red arrows) and are superimposed onto the mouse proglucagon and pre-proinsulin I promoters with other known regulatory elements shown in boxes.

Electrophoretic Mobility Shift Assay (EMSA)

Binding of DLX1 and DLX2 to proglucagon and pre-proinsulin promoter regions (fragments) as determined by ChIP was confirmed by electrophoretic mobility shift assay using bacterially-derived recombinant DLX1/DLX2 proteins (Figures 17,18). The same binding was also found using total protein extracts from HEK-293 cells transfected with either Dlx1 or Dlx2 expression constructs. EMSA was also attempted using mouse pancreas islet preparations, but no binding was detected. Examination of these islet extracts by Western blot with specific antibodies to DLX1/DLX2 showed low levels of Dlx proteins, possibly due to the abundant proteolytic enzymes present in pancreas with resulting protein degradation. A control sample with only radio-labeled promoter (probe) was run on each gel. In the presence of DLX recombinant protein, a Dlx1/promoter or Dlx2/promoter protein-DNA complex was formed which migrated more slowly and is seen as a higher band. Addition of unlabeled ("cold") promoter was able to specifically compete out this band. Finally, the addition of specific Dlx1/Dlx2 or non-relevant (I) antibodies demonstrated that the protein-DNA complexes do in fact contain DLX1 and DLX2.

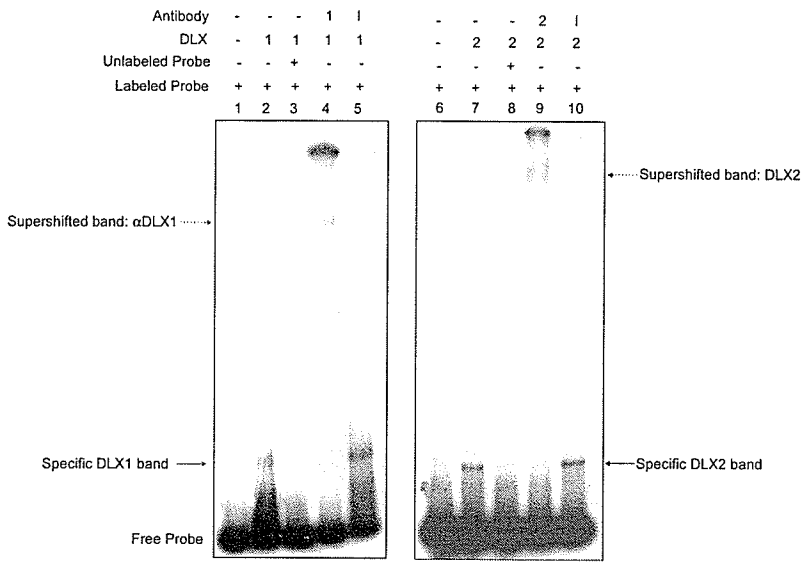

Figure 17: EMSA using recombinant DLX1/2 protein and the pre-proinsulin I promoter region obtained from ChIP assay. Labelled promoter fragment migrates through the gel as a "free probe" band. Incubation with a protein that binds to the promoter results in formation of a DNA-protein complex and shows up as a higher band. Addition of specific antibody to the protein of interest results in an antibody-protein-DNA complex or "supershift".

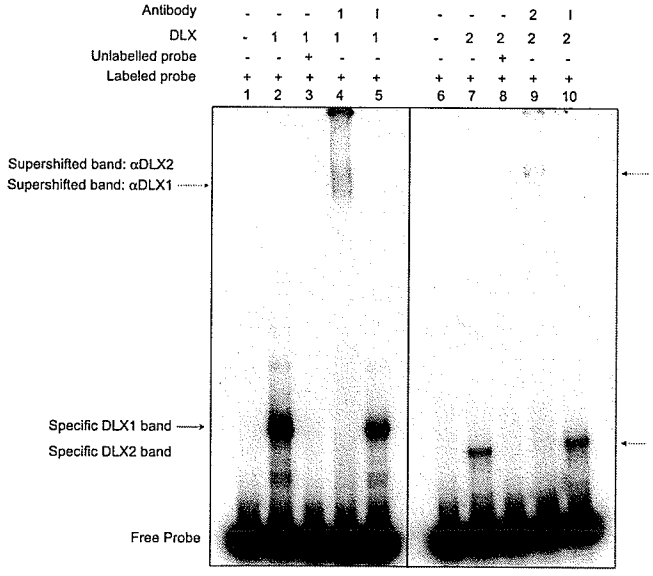

Figure 18: EMSA using recombinant DLX1/2 protein and the proglucagon promoter region obtained from ChIP assay. Labelled promoter fragment migrates through the gel as a "free probe" band. When incubated with a protein that binds to the promoter, a DNA-protein complex is formed, which shows up as a higher band. Addition of specific antibody to the protein of interest results in an antibody-protein-DNA complex or "supershift".

Immunofluorescence

Examination of DLX1 and DLX2 expression in mouse tissues at various developmental stages showed onset of expression to occur as early as E14.5 (Figure 19, courtesy of Niki Boyko). Qualitatively, it was observed that the level of DLX1/DLX2 increased by E18.5 and P0, and then decreased in adult mice (6 weeks old). In newly-formed islets at E18.5, DLX1 and DLX2 were found to be co-expressed with all four islet hormones (insulin, glucagon, somatostatin, pancreatic polypeptide, Figure 20) and also with the important transcription factor PDX-1 (Figure 22). Similar co-expression of Dlx1/2 and insulin/glucagon was found for cultured human islets (Figure 21, human islets courtesy of Dr. Rosenberg, McGill University). Interestingly, DLX1/2 are expressed in the primitive stomach and gut at E14.5 (Figure 19) and are also found in exocrine and ductal cells at E18.5 (Figures 20, 22).

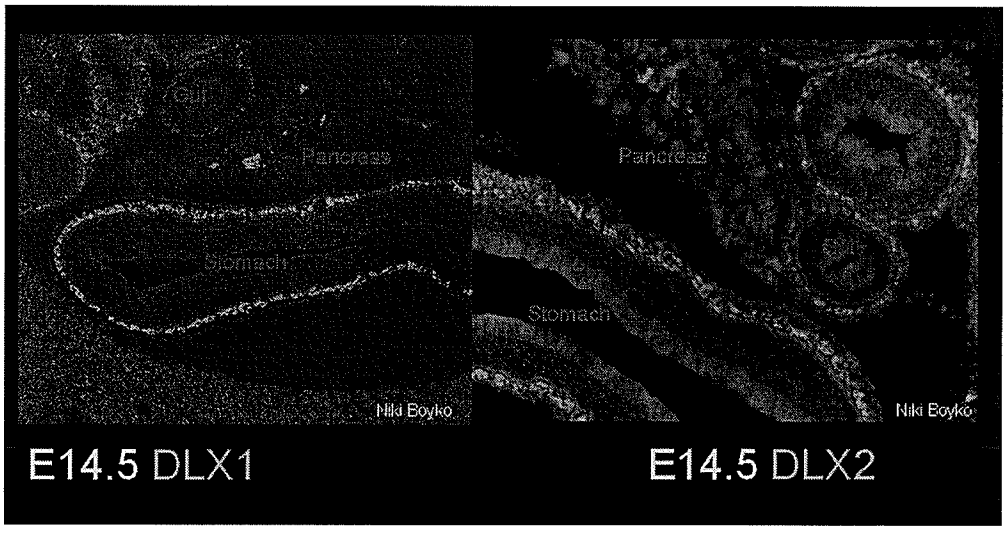

Figure 19: Onset of expression of DLX1 and DLX2 in mouse pancreas was determined to be as early as E14.5 by immunofluorescence (green) of mouse pancreas sections. Interestingly, DLX1 and DLX2 are also expressed within the gut and stomach. (N. Boyko, D. Eisenstat, unpublished observations).

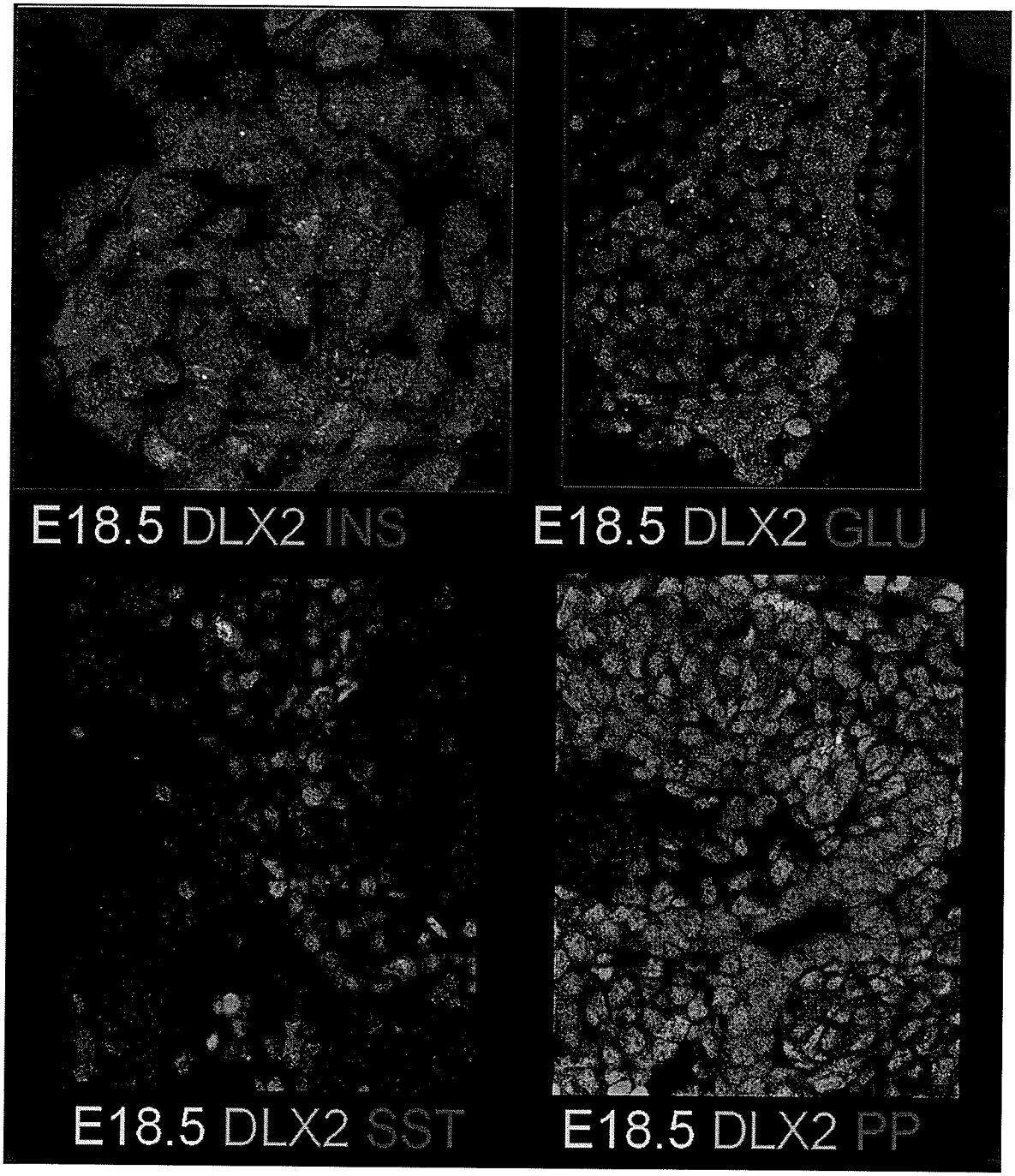

Figure 20: Co-expression of DLX2 (green) with the pancreas hormones (in red): insulin (INS), glucagon (GLU), somatostatin (SST) and pancreatic polypeptide (PP) in mouse pancreas sections at E18.5. DLX2 was found to be co-expressed with all four pancreatic hormones by immunofluorescence double-labeling and confocal microscopy. Similar results were found for DLX1 (data not shown).

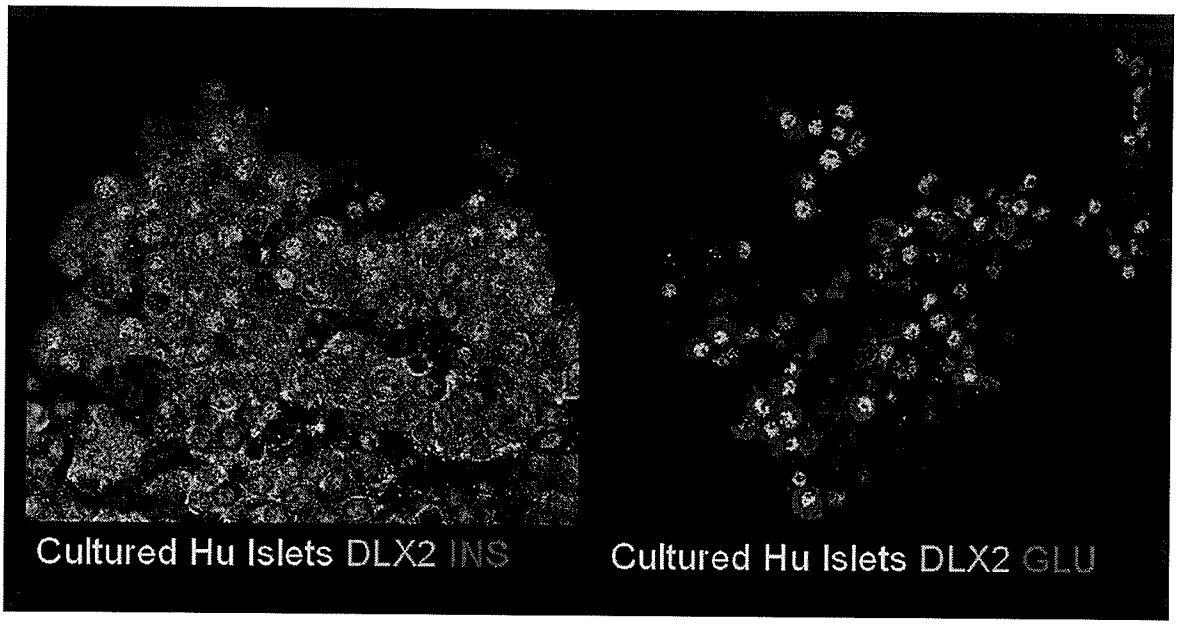

Figure 21: DLX2 (green) co-expression with insulin (red) and glucagon (red) in cultured human (Hu) islets.

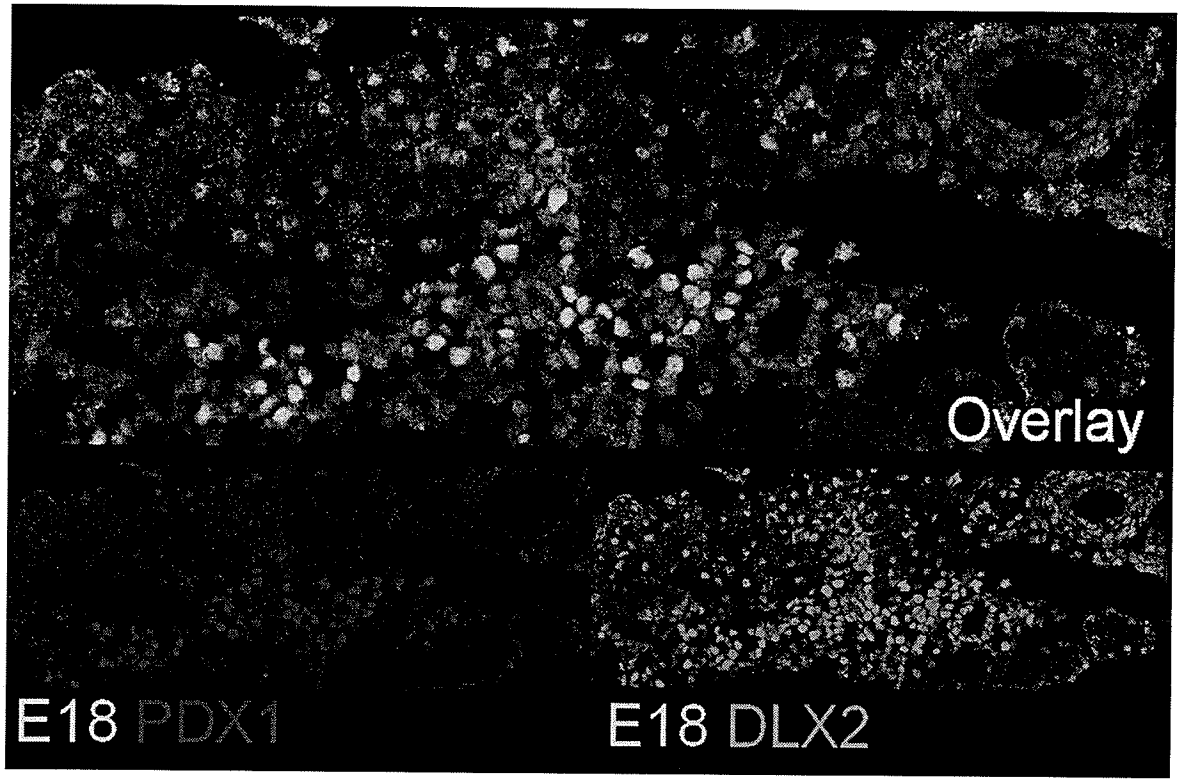

Figure 22: PDX-1 co-expression in E18 mouse pancreas. DLX2 is in green and PDX1 in red. Many cells co-express both transcription factors.

Luciferase Reporter Gene Assay

The consequence of Dlx1/Dlx2 binding to the proglucagon promoter was analyzed by luciferase assay using the αTC1-9 cell line, which is derived from mouse pancreas adenonoma and has a high level of glucagon expression. Endogenous DLX1 and DLX2 protein expression in αTC1-9 cells was determined by Western blot. The cells were found to express low levels of both DLX and DLX2, with high expression upon transfection of Dlx1/2 expressing plasmids (Figure 23).

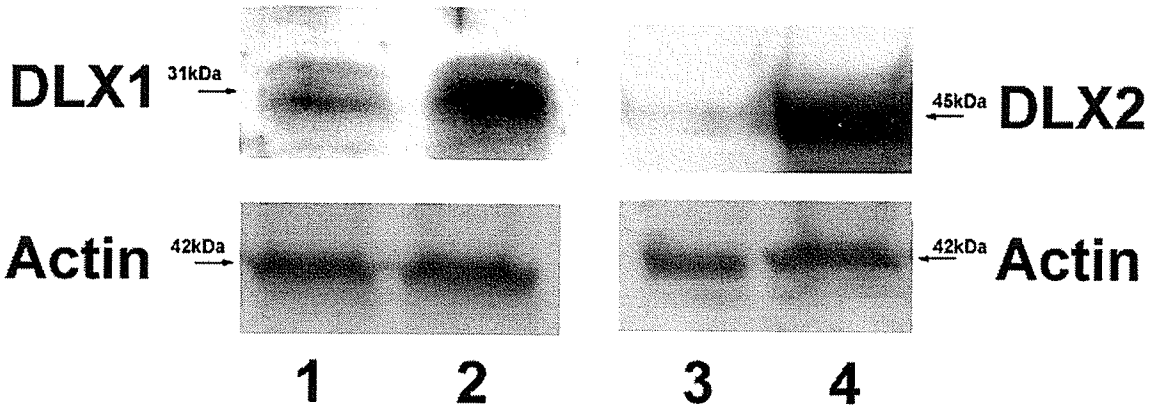

Figure 23: Basal expression of DLX1 and DLX2 (lanes 1, 3) expression in αTC1-9 cells. Expression is greatly increased following transfection with Dlx1 or Dlx2 expressing plasmids (lanes 2, 4).

Luciferase assays were carried out using wild-type Dlx1 and Dlx2 constructs and full-length proglucagon promoter. Expression of either of these DLX proteins were found to down-regulate expression of the proglucagon promoter. To support this Dlx-mediated repression, a number of mutated Dlx1 (Figure 27) and Dlx2 (Figure 31) constructs were created and assayed. These mutant constructs were fully sequenced, and expression of

protein following transfection was verified by Western blot for every mutant (Figure 24) except those where the specific antibody recognition region of the protein was deleted.

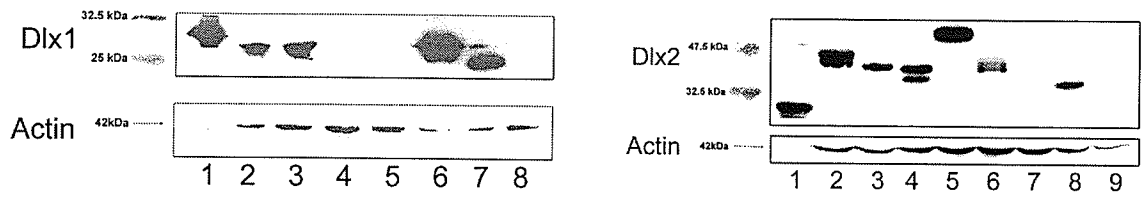

Figure 24: Expression of transfected DLX1 and DLX2 mutant plasmid constructs in αTC1-9 as verified by Western blot. For DLX1 lanes 1-8: (recombinant DLX1; wild-type WT; DLX1-Q50E; VP16-DLX1; ENG-DLX1; DLX1-WRPW; DLX1-HD-Del; untransfected). Lanes 4 and 5 do not have a band due to the DLX1 antibody recognizing the N-terminus (N114 aa) of the DLX1 protein, which was replaced by VP16 or ENG. For DLX2 lanes 1-9: (recombinant DLX2; WT; DLX2-Q50E; VP16-DLX2; ENG-DLX2; DLX2-His-Del; DLX2-CTer-Del; DLX2-HD-Del; untransfected). Lane 7 does not show a band due to the DLX2 antibody recognizing the C-terminus (C199 aa) of the DLX2 protein which was deleted.

Additionally, the cellular localization of the homeodomain deletion constructs was examined by immunofluorescence of αTC1-9 cells transfected with wild-type Dlx2 and homeodomain-deleted Dlx2 (Figure 25). Although the homeodomain deletion construct retains the nuclear localization signal (NLS), the cellular localization of the expressed homeodomain deletion protein was found to be both cytosolic and nuclear, in contrast to that of wild-type which was completely nuclear. It is possible that other NLS sequences are found within the homeodomain or that the deleted homeodomain protein binds to other factors that sequester it in the cytosol (unlike wild-type DLX1 and DLX2 which are expressed in the nucleus). It is known that DLX5 is expressed in both the nucleus and

cytoplasm (Eisenstat, 1999). Isoforms of DLX5 are expressed in the nucleus (DLX5α) and/or in the cytoplasm (DLX5β,γ) (Liu, 1997).

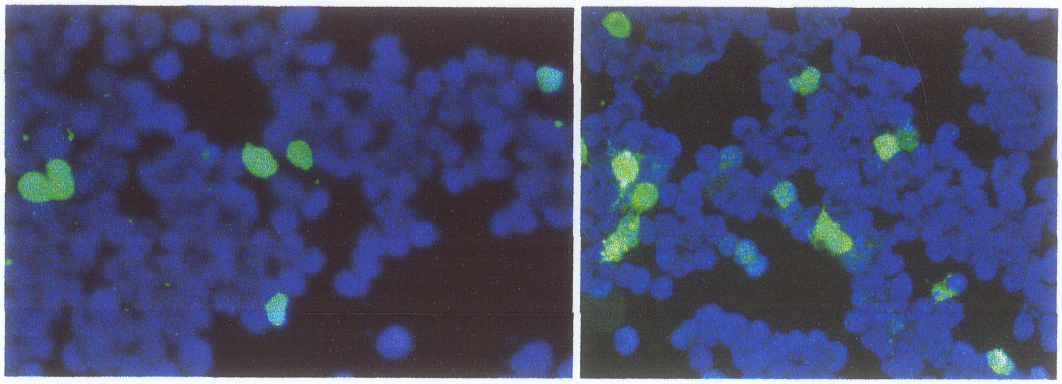

Figure 25: αTC1-9 cells transfected with WT (left) or homeodomain-deleted (Dlx2-HD-Del, right) DLX2. WT DLX2 is localized in the nucleus whereas homeodomain-deleted DLX2 is localized in both nucleus and cytosol.

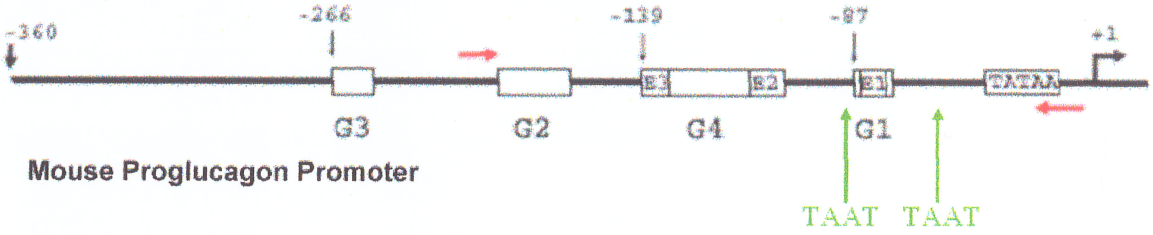

Figure 26: Mouse proglucagon promoter. Binding region of Dlx1/2 obtained by ChIP is within the red arrows. The region contains two TAAT binding sites (in green). Other known binding elements and motifs are shown as black boxes.

From the ChIP assay, the region of Dlx1/2 DNA binding within the proglucagon promoter contained two different TAAT sites which are 30 bp apart. To determine the importance of these sites, mutated proglucagon promoters were created with either or both of these TAAT sites mutated (Figure 26, Table 4) which were then assayed using the luciferase gene reporter system.

WT	TAAT TAAT
Both sites mutated (1+2)	T**G**AT T**G**AT
1st site mutated (1)	T**G**AT TAAT
2nd site mutated (2)	TAAT T**G**AT

Table 4: Mutated proglucagon promoter constructs were generated by mutating either the first or second TAAT site or both sites together. Mutations are shown in bold.

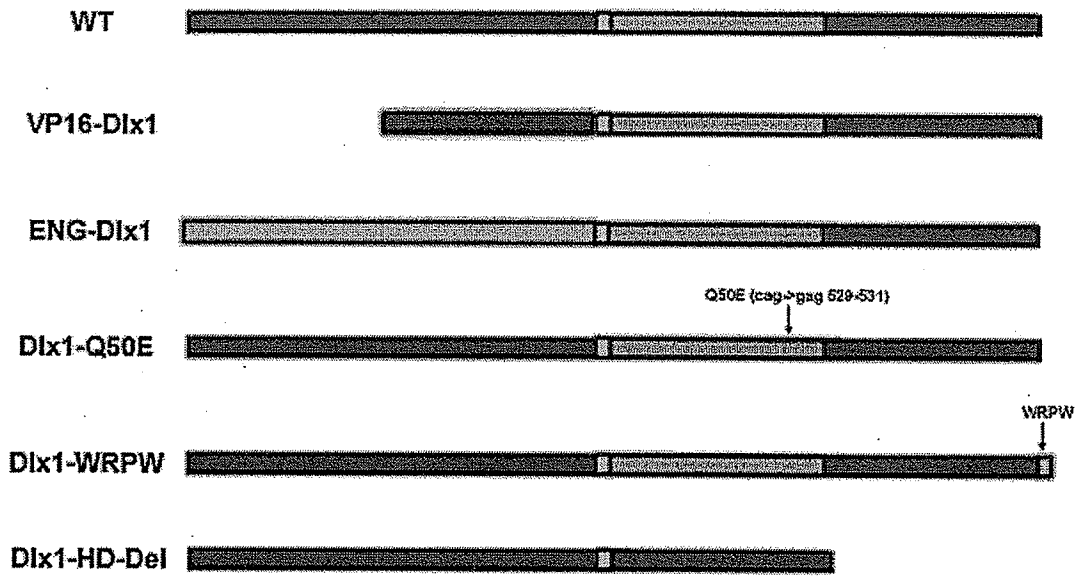

Figure 27: Dlx1 mutant constructs used for luciferase assays. The N-terminus of DLX1 was replaced with either VP16 activation or ENG repression domains (VP16-Dlx1, ENG-Dlx1). A point mutation Q50E at amino acid position 50 of the homeodomain was also generated (Dlx1-Q50E which has been shown to abrogate DNA binding). Also, the tetra-amino acid repression motif WRPW was added to the C-terminus (Dlx1-WRPW). Finally, the entire homeodomain was deleted (Dlx1-HD-Del).

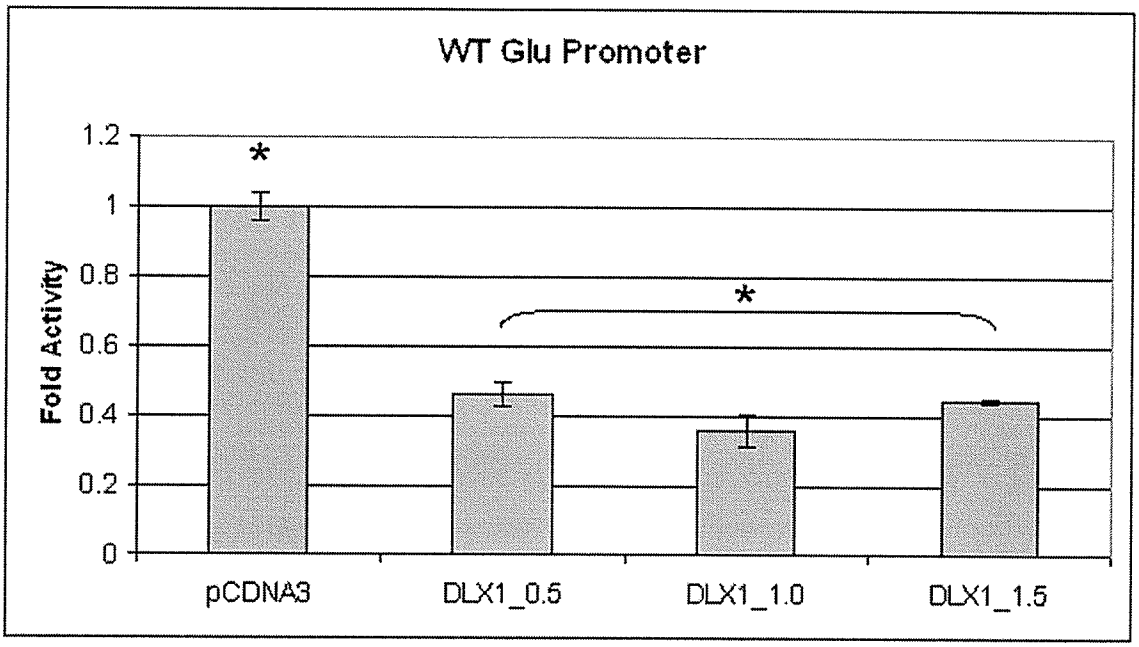

Figure 28: Luciferase assay of Dlx1 and wild-type (WT) proglucagon promoter. αTC1-9 cells were transfected with WT proglucagon promoter expression vector and varying amounts of Dlx1 expression vector (0.5ug, 1.0ug and 1.5ug). DLX1 was found to repress expression from the proglucagon promoter. N=12, (* denotes $p < 0.001$).

Luciferase assays of WT proglucagon promoter with WT Dlx1 showed a 2-fold decrease in activity, suggesting that recombinant DLX1 acts as a repressor (Figure 28). A range of DNA amounts was used from 0.1 ug (not shown) up to 1.5 ug. No dose-dependent response was observed. Thus it appears that a threshold amount of DLX1 can achieve its maximum repression activity *in vitro*.

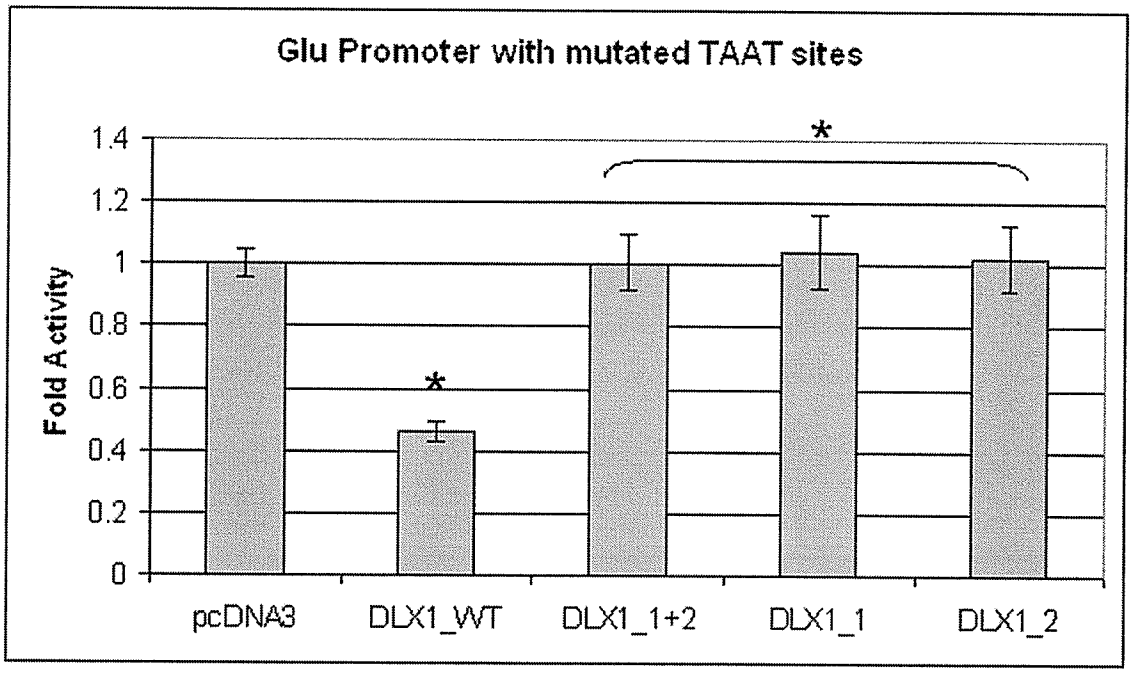

Figure 29: Luciferase assay of Dlx1 and mutated proglucagon promoters. αTC1-9 cells were transfected with WT Dlx1 expression vector and WT or mutated proglucagon promoters (WT, Dlx1_WT; both TAAT sites mutated, Dlx1_1+2; first TAAT site mutated, Dlx1_1; second TAAT site mutated Dlx1_2). N=8, (* denotes $p < 0.004$).

Mutation of either or both of the TAAT sites within the proglucagon promoter region identified by ChIP prevented Dlx1 from repressing luciferase activity compared with wild-type Dlx constructs (Figure 29). This could suggest that DLX1 binding as a multimer to both sites is required. Alternatively, mutation of DNA within this region alters the structure and/or conformation of the DNA and disrupts some other binding mechanism.

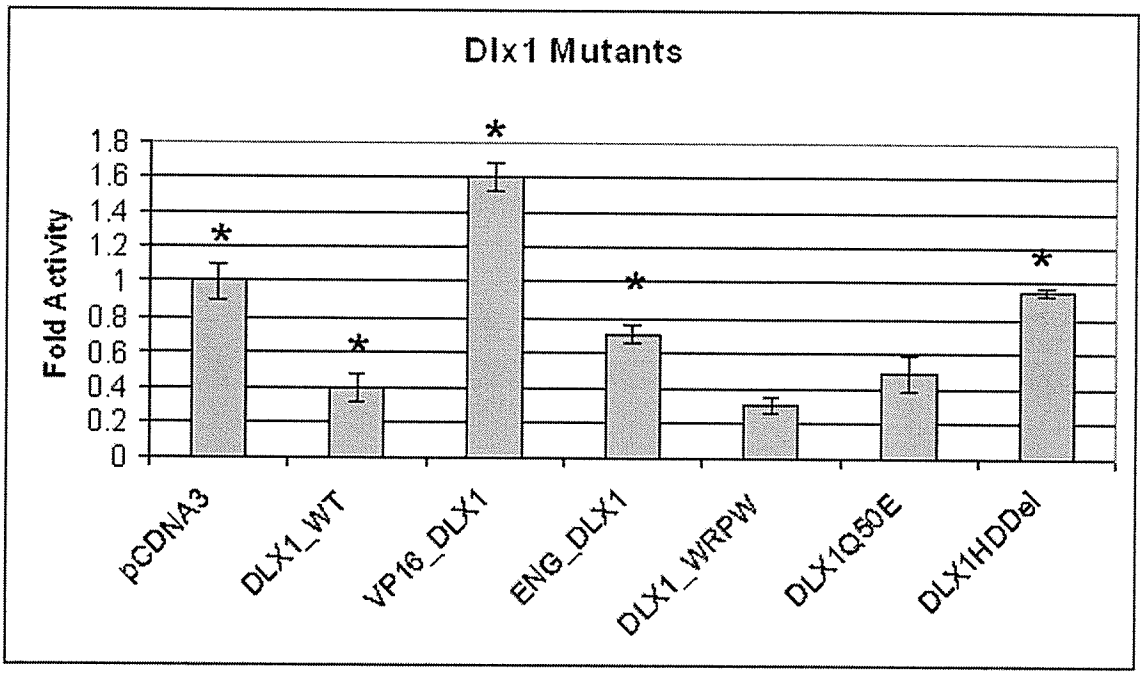

Figure 30: Luciferase assay of mutant Dlx1 constructs and WT proglucagon promoter. αTC1-9 cells were transfected with WT proglucagon promoter expression vector and WT Dlx1 or the different Dlx1 mutant constructs (Figure 27). N=5 for Dlx1_WRPW and Dlx1HDDel. N=9 for all other constructs used. (* denotes $p < 0.009$ compared with DLX1_WT).

To further investigate Dlx1-mediated repression of glucagon expression, a series of Dlx1 mutant constructs were created (Figure 30). VP16-Dlx1 and ENG-Dlx1 replaced the N-terminus of the protein with the VP16 strong activation and Engrailed strong repression domains. The Dlx1-WRPW construct had the four additional amino acids WRPW added to the C-terminus, a motif which has been shown to act as a strong repressor. The function of VP16 is mediated by direct interaction with transcription machinery: TATA-binding protein, TFIIB and the SAGA histone acetylase (Hall, 2002). Engrailed domain represses through interaction with its co-repressor Groucho (Tolkunova, 1998). The tetra-peptide motif WRPW can also recruit Groucho. Groucho represses through a chromatin-remodelling activity that is not currently well understood (Dawson, 1995). The VP16-Dlx1

mutant activated the proglucagon promoter as expected, however the Engrailed mutant showed only a slight repression which was less than for WT Dlx1. This could suggest either that DLX1 is a stronger repressor than Engrailed or that the bulkiness of the Engrailed domain disrupts the structure/activity of DLX1. To determine which of these options was correct, the WRPW construct was created. This construct is expected to repress by a similar Groucho-mediated mechanism. When this mutant was assayed, it was found to repress strongly. Thus the failure of the Engrailed mutant to repress is likely due to the bulky presence of the Engrailed-domain interfering with ENG-Dlx1 protein structure. Finally, two mutant constructs were designed to eliminate homeodomain binding to TAAT. The first, Dlx1-Q50E, had the residue at position 50 of the homeodomain mutated from glutamine (Q) to glutamic acid (E) (polar to negatively charged). Q at position 50 was one of 3 residues (along with N51 and I47 as described in the introduction) identified to be important for homeodomain structure and binding to DNA. The second mutant, Dlx1-HD-Del, is a complete deletion of the homeodomain and is expected to not bind DNA. As predicted, the Dlx1-HD-Del mutant shows no repression and has luciferase activity at the level of the empty plasmid control. Interestingly, Dlx1-Q50E still represses at a level similar to WT Dlx1. It is possible that the glutamine residue at position 50 is not important for homeodomain binding of the proglucagon promoter. Alternatively, DLX1 may be repressing proglucagon expression in a protein complex with one or more other proteins. Based on the mutants created (the portions of Dlx1 deleted), the location of such an interaction would likely be within the homeodomain or the C-terminus.

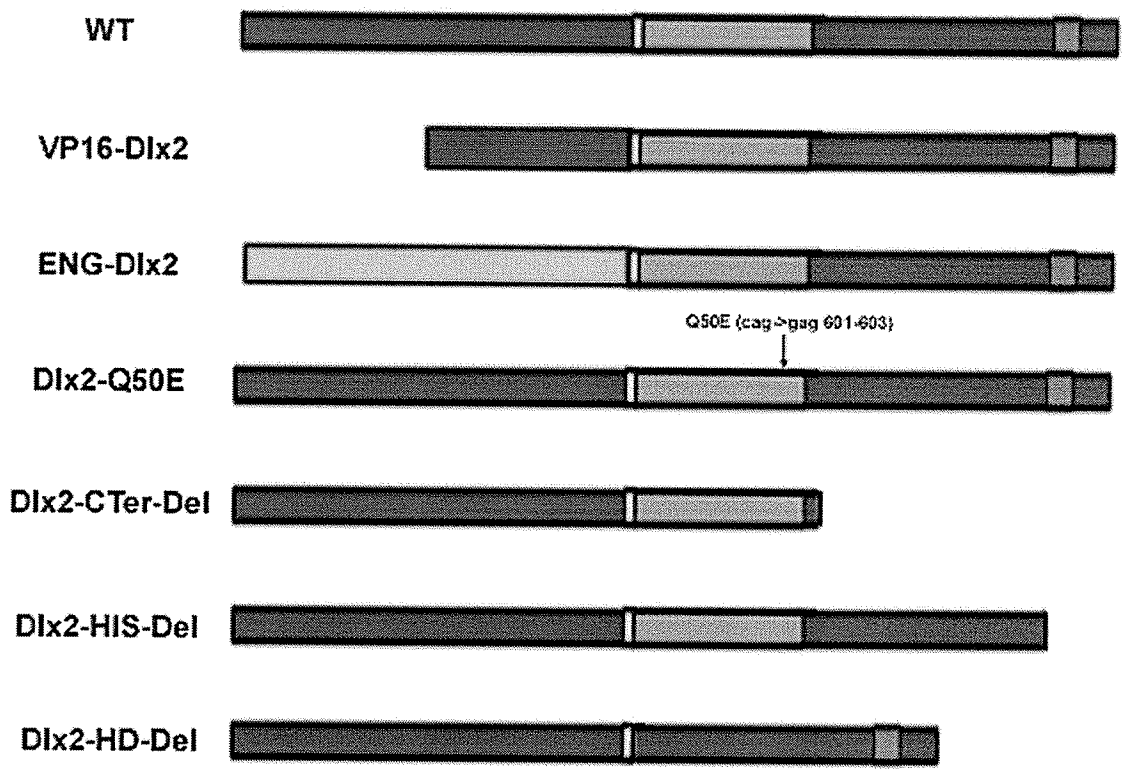

Figure 31: Mutant Dlx2 Constructs used for luciferase assays. The N-terminus of DLX2 was replaced with either VP16 or ENG domains (VP16-Dlx2, ENG-Dlx2). A point mutation Q50E at amino acid position 50 of the homeodomain was also generated (Dlx2-Q50E). Two C-terminal deletion constructs were generated: a deletion of the entire C-terminus after the homodomain (Dlx2-CTer-Del) and a deletion of the 9-HIS C-terminal portion (Dlx2-HIS-Del). Finally, the entire homeodomain was deleted (Dlx2-HD-Del).

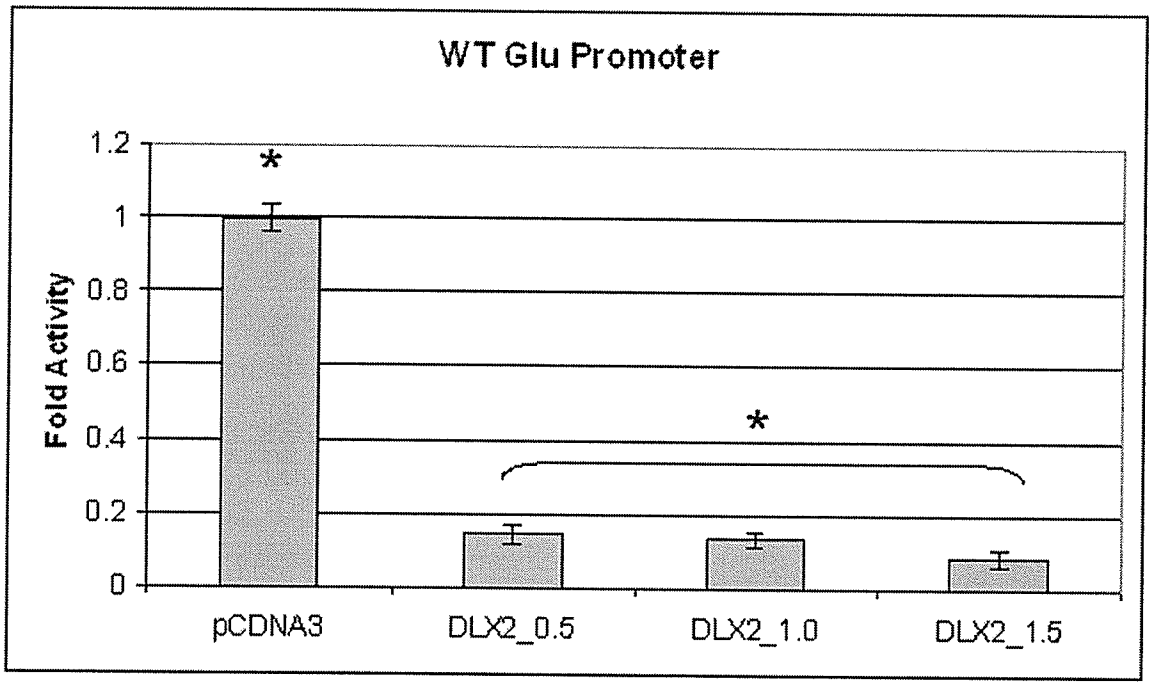

Figure 32: Luciferase assay of Dlx2 and WT Glu promoter. αTC1-9 cells were transfected with WT proglucagon promoter expression vector and varying amounts of Dlx2 expression vector (0.5ug, 1.0ug and 1.5ug). Dlx2 was found to repress expression from the proglucagon promoter. N=12, (* denotes $p < 0.001$).

Similar to DLX1, DLX2 repressed proglucagon promoter expression (Figure 32). Again, it was found that a threshold level of DNA was able to achieve maximum repression rather than a dose dependent response. DNA amounts varying from 0.1ug (data not shown) up to 1.5ug were used. DLX2 repressed more strongly than DLX1 (~8-fold vs 2-fold).

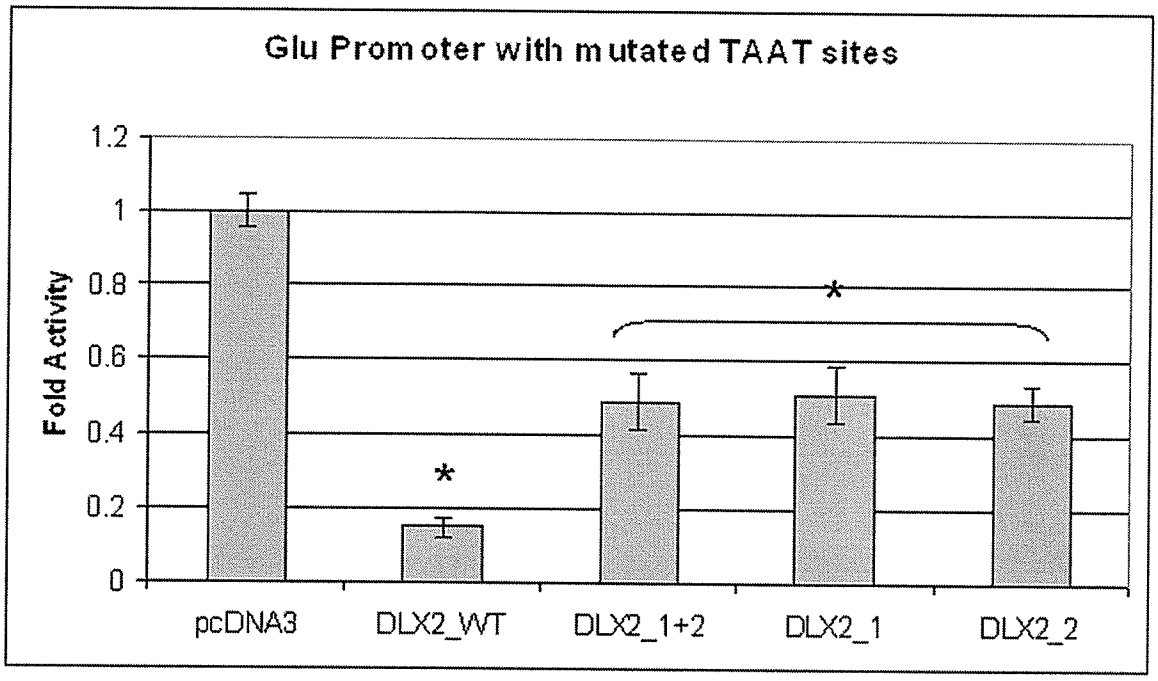

Figure 33: Luciferase assay of Dlx2 and mutated proglucagon promoters. αTC1-9 cells were transfected with WT Dlx2 expression vector and WT or mutated proglucagon promoters (WT, Dlx2_WT; both TAAT sites mutated, Dlx2_1+2; first TAAT site mutated, Dlx2_1; second TAAT site mutated Dlx2_2). N=8, (* denotes $p < 0.03$).

For the TAAT homeodomain binding motif glucagon mutants (generated by site-directed mutagenesis), it was found that both sites are necessary for repression, as mutation of either or both TAAT motifs reduces but does not eliminate repression (Figure 33). In contrast to DLX1, this suggests that there is an additional mechanism of repression for DLX2 that is independent of TAAT binding. This might involve a protein-protein interaction, or binding of some other region of DLX2 to another sequence within the proglucagon promoter other than the canonical TAAT motifs.

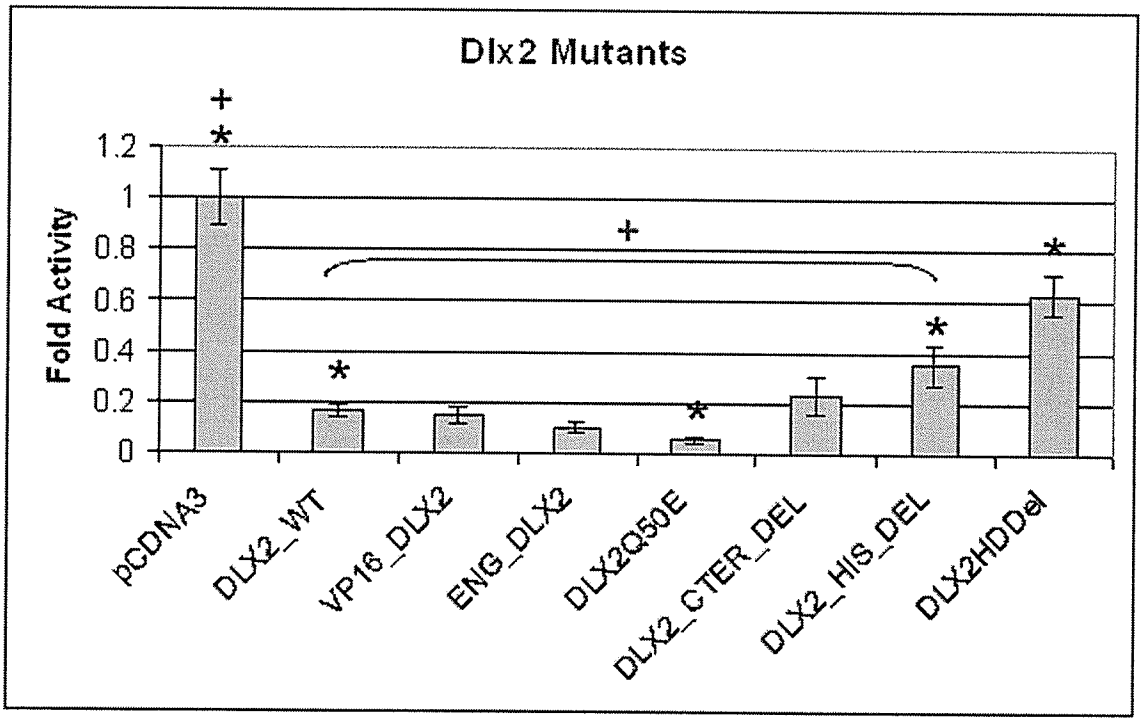

Figure 34: Luciferase assay of mutant Dlx2 constructs and WT proglucagon promoter. αTC1-9 cells were transfected with WT proglucagon promoter expression vector and WT DLX2 or the different DLX2 mutant constructs (Figure 31). N=5 for Dlx2HDDel. N=9 for all other constructs. (* denotes $p < 0.007$ compared with pCDNA3, + denotes $p < 0.03$ compared with DLX2_WT).

To further investigate DLX2 mediated repression of proglucagon promoter expression, a series of mutant Dlx2 constructs were examined using luciferase assays (Figure 34). ENG-Dlx2 was found to repress strongly. Unexpectedly, VP16-Dlx2 repressed similar to WT Dlx2 suggesting that repression may be mediated by a domain within the homeodomain or C-terminus that cannot be overcome by VP16 activation. To address this issue, two C-terminal deletion constructs were created. A Dlx2-His-Del where the 9 Histidine residues near the C-terminus were deleted, and Dlx2-CTer-Del, where the entire C-terminus after the homeodomain was deleted. However, both these mutants repressed proglucagon promoter reporter gene expression strongly, at a level similar to WT DLX2, although the

Dlx2-His-Del construct had significantly less repression. Additionally, two homeodomain deletion constructs were created: Dlx2Q50E with the amino acid at position 50 mutated, and Dlx2-HD-Del with the entire homeodomain deleted. Dlx2-Q50E had repression levels similar to WT suggesting either that Q50 does not affect DLX2 binding to DNA, or that there is another binding mechanism independent of the homeodomain. For DLX1, the TAAT proglucagon promoter mutants and the homeodomain deletion mutant did not repress. By comparison, for Dlx2, the TAAT glucagon mutants and the homeodomain mutants had reduced repression. Since these mutants mutate the putative DNA binding site and the protein-DNA binding domain respectively and had similar reduced but significant levels of repression, it suggests that there is another mechanism of DLX2 repression that does not involve homeodomain binding to TAAT motifs. This homeodomain mutation data, taken together with the data for the N and C-terminal deletions indicates that there might be multiple repression domains within the DLX2 protein or DLX2-protein interactions with other transcription factors that bind the proglucagon promoter.

In light of the unexpected results from the Q50E and HD deletion mutants, EMSA was performed on cell extracts obtained from HEK-293 cells transfected with either WT or mutant Dlx1/2 (Figure 35). Both Dlx1-Q50E and Dlx2-Q50E were found to bind the proglucagon promoter *in vitro* by EMSA, in agreement with the luciferase assay repression results and suggesting that for this DNA sequence, DNA binding may be mediated by N51 rather than Q50. The Dlx1-HD-Del did not bind the proglucagon promoter also in agreement with eliminated repression (Figure 30). Finally, Dlx2-HD-Del was able to bind

the proglucagon promoter, also in agreement with reduced but not eliminated luciferase reporter gene repression (Figure 34). However, binding by EMSA does not always indicate direct protein-DNA interactions. It is possible that these mutant constructs interact with DNA through some other protein present in the transfected cells.

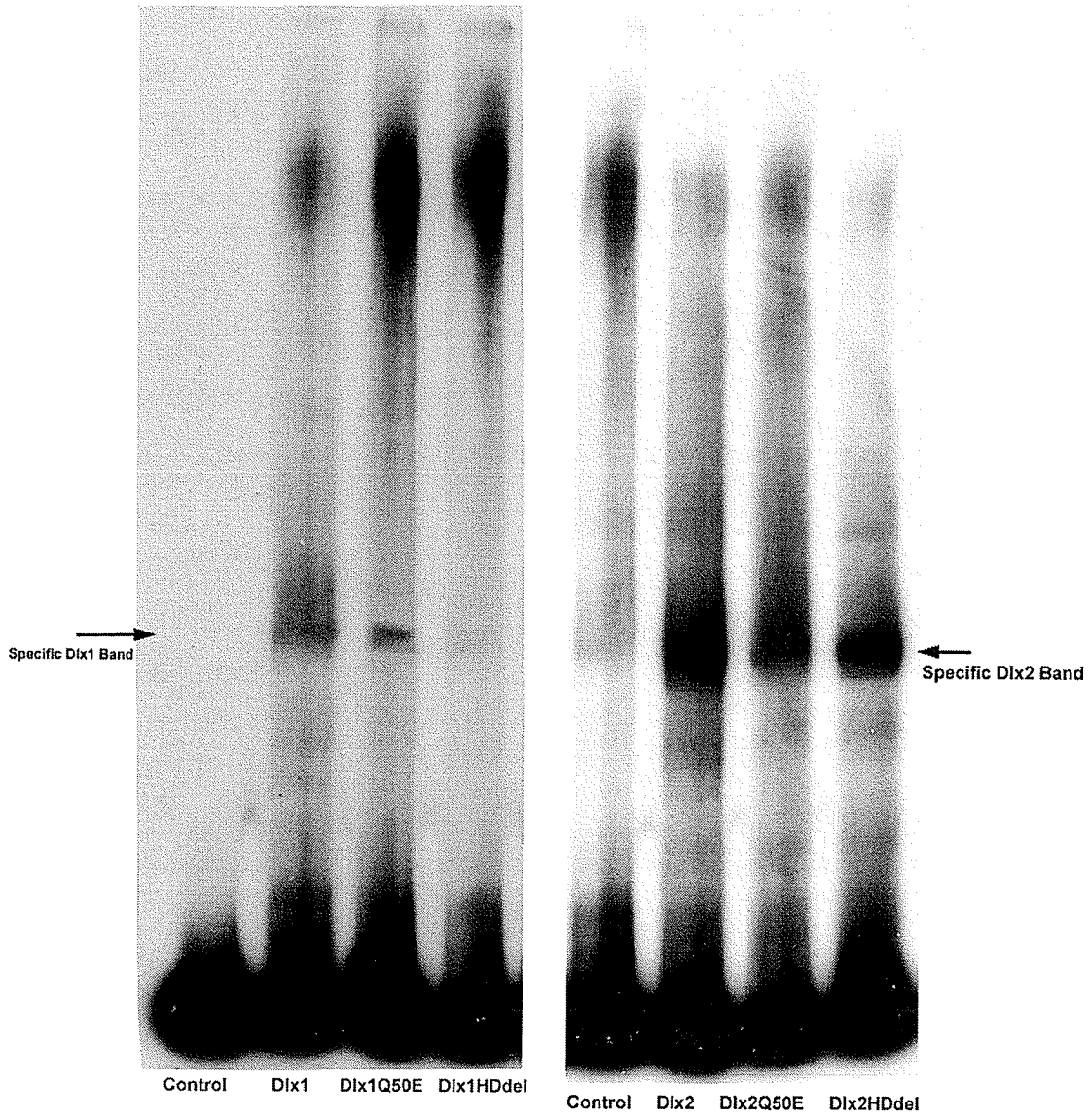

Figure 35: EMSA of WT and mutant Dlx1 and Dlx2 obtained from protein extracts of HEK-293 cells transfected with the appropriate plasmid construct.

Radioimmunoassay and Cell Counting

Insulin levels in the blood of adult and P0 mice were quantified using a radioimmunoassay procedure in collaboration with Drs. J. Silha and L. Murphy (Figure 36). Current commercial radioimmunoassay kits available for glucagon are not considered reliable and therefore glucagon levels were not measured. Additionally, P0 and adult mice tissue sections were labeled with either DLX1 or DLX2 antibody and the number of cells expressing each protein was counted using immunofluorescence microscopy (Figure 37, courtesy of Xiangguo Qiu and Haolin Shi). Statistically significant reductions were found in the number of both insulin and glucagon immunopositive cells in DLX1/2 double-knockout mutant mice. By radioimmunoassay, a 40% reduction in circulating insulin ($p < 0.03$) was observed. By cell counting (N=50,000 cells from WT and mutant pancreas for each of insulin and glucagon labeling), insulin positive cells were reduced by 24.3%, whereas glucagon positive cells were reduced by 11.3% in the DLX1/2 double-knockout mice as compared to wild-type littermate controls.

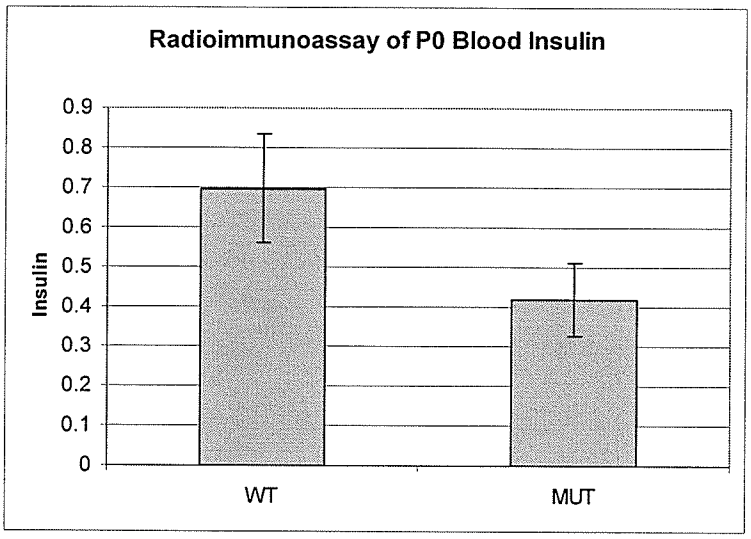

Figure 36: Radioimmunoassay of blood insulin levels taken from P0 mice at birth shows a 40% reduction [$p < 0.03$, 11 wild-type (WT) and 11 mutant (MUT) mice were assayed].

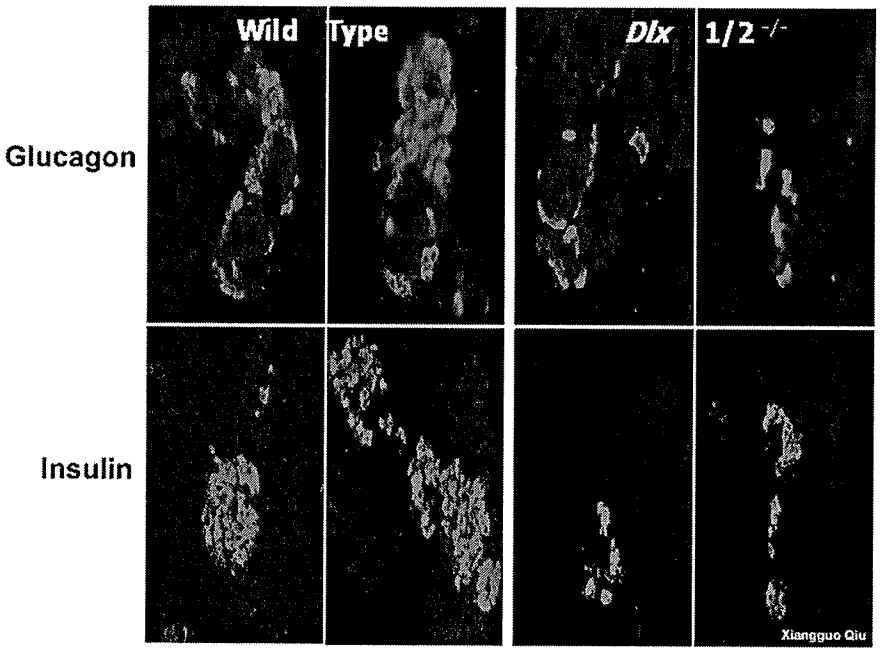

Figure 37: Immunofluorescence (green) counting of glucagon and insulin expression. Wild-type or Dlx1/2 double-knockout mouse pancreas sections were labeled by immunofluorescence with insulin or glucagon antibodies. Double-knockout mice were found to have a 24.3% reduction ($p < 0.05$) in the number of insulin positive cells and a 11.3% ($p < 0.05$) reduction in the number of glucagon positive cells when compared to wild-type littermate controls. (5×10^4 cells counted for each of insulin labeled WT, insulin labeled double-knockout, glucagon labeled WT, and glucagon labeled double-knockout).

Chapter IV: Discussion

The series of studies in this Thesis began with a chromatin immunoprecipitation (ChIP) experiment. This initial experiment found that DLX1 and DLX2 both bound to regions of the pre-proinsulin I and glucagon (proglucagon) promoters. The nature of the paraformaldehyde cross-linking step used in ChIP is such that at least 3 types of complexes will be formed: DNA-DNA, protein-DNA and protein-protein. Subsequently, PCR primers were used to identify a region of Dlx1/2 binding within the promoters. The overall protocol of ChIP and in particular, the two processes described above result in some limitations which are widely known. For example, the ChIP assay fails to document exactly where a protein binds to DNA *in vivo*. Thus for proglucagon, a 360 bp region of the promoter was identified as being bound by Dlx1/2 but the exact region or DNA sequence is still in question. Additionally, the immunoprecipitation of a specific segment of DNA with antibodies directed against DNA-binding proteins does not necessarily indicate that the protein directly interacts with a DNA sequence in the precipitate but could instead reflect protein–protein interactions. As well, the functional effect of protein binding to DNA cannot be determined. Moreover, protein binding to DNA does not necessarily have an effect on transcription. These limitations require that ChIP be used in conjunction with additional studies such as DNase I footprinting (Kang, 2002). In this Thesis, EMSA, immunofluorescence and luciferase assays were performed to verify results obtained by the ChIP assay.

EMSA was performed on the pre-proinsulin I and proglucagon promoters using recombinant and

transfected cell-extract derived DLX1 and DLX2. These experiments are an *in vitro* confirmation of the ChIP results. Confirmation of binding with endogenous DLX1 or DLX2 was also attempted by using mouse pancreas islet extracts, but failed due to technical difficulties when obtaining sufficient protein from pancreas. Such technical difficulties are not uncommon when dealing with pancreas, since this organ is the major site of digestive enzyme production and has numerous enzymes which break down carbohydrates, proteins and lipids. Western blot analysis of prepared islet extracts showed low protein levels of Dlx1/2, probably due to protease degradation.

Immunofluorescence data at E14.5 in mouse pancreas shows DLX1/2 expression in the pancreas (Figure 19). Interestingly, the embryonic mouse sections also show strong expression levels in the developing stomach and intestine. This is not surprising given the close proximity in which these organs develop and the many regulatory factors which they have in common. At E18.5 Dlx1/2 are expressed in the endocrine pancreas, but also in the exocrine and ductal cells to a lesser extent. The presence of Dlx1/2 throughout the developing foregut during development might suggest some role in organ specification, but this remains to be determined. The role of Dlx1/2 in non-endocrine cells was not addressed in these studies but could be followed up in the future. Based on previous studies, Dlx1/2 are generally responsible for terminal differentiation late-embryonic or post-natal in development. In the pancreas, given that these transcription factors bind both the pre-proinsulin I and proglucagon promoters, this would place them around or downstream of Pax6 and Nkx6.1 factors (Figure 14) in determining α and β cell maturation.

Another *in vitro* assay, the luciferase reporter gene assay, was used to obtain more data on the effect of Dlx1/2 binding to the proglucagon promoter *in vitro*. These experiments were carried out in the cell line α-TC1-9 which is derived from a mouse pancreas adenoma and was selected primarily for its high glucagon expression level. This cell line is probably the most relevant for studies involving glucagon and has been widely used (Efrat, 1988 and Hamaguchi, 1990). Luciferase assays of DLX1 on proglucagon promoter reporter gene expression showed that Dlx1 repressed expression. Since DLX1 is a homeodomain protein, the binding site was considered to be one of the two TAAT motifs identified using the ChIP assay (Figure 38, 1a). This was later confirmed by site-directed mutagenesis. For DLX1, the luciferase assays of the VP16 activation and ENG repression mutants as well as the HD-Del mutant yielded the expected results. It is interesting that Dlx1-Q50E continues to bind DNA in EMSA. This is in contrast to previous studies which show that Q50E abolishes DNA binding (Gehring, 1994 and Le, 2006 submitted). Binding in EMSA does not always indicate a direct protein-DNA interaction. Since the Dlx1-Q50E used for EMSA was derived from transfected cell extracts, it is possible that it is interacting with the proglucagon promoter through another protein. In order to determine a direct *in vitro* EMSA interaction, the Dlx1-Q50E would need to be purified by antibody beads, or produced as a purified recombinant protein.

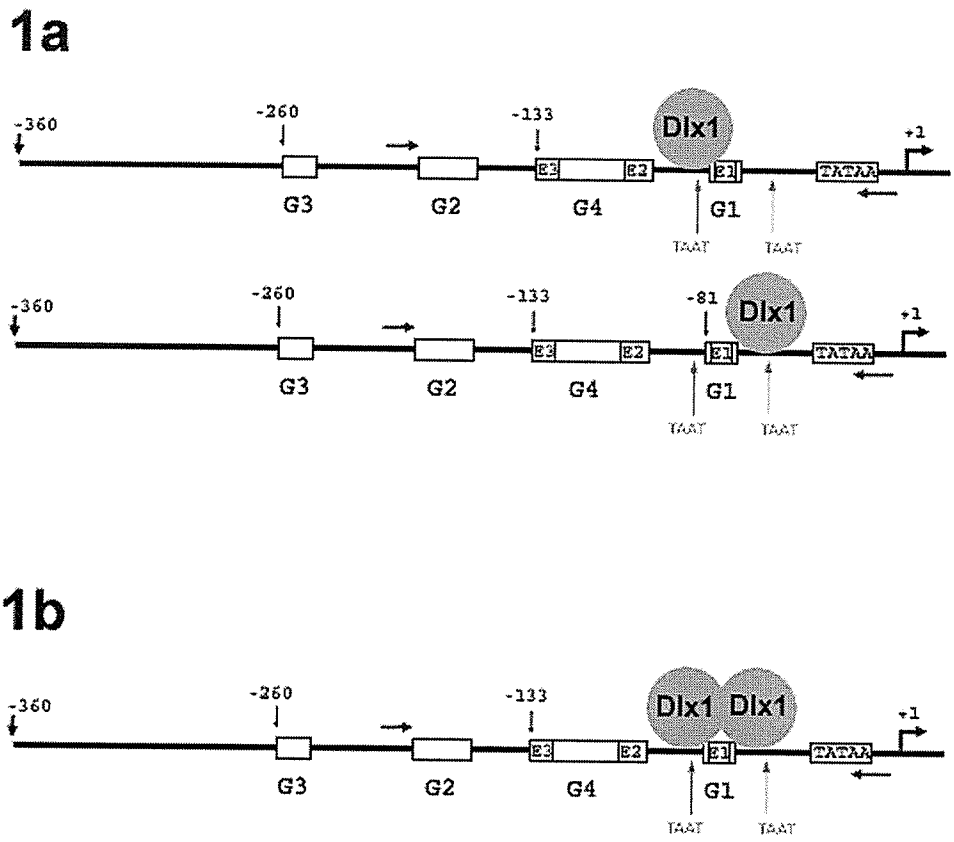

Figure 38: Models for binding of DLX1 to proglucagon promoter: 1a. DLX1 may bind as a monomer to individual TAAT motifs; 1b. Dlx1 may bind as a homodimer or multimer to both TAAT motifs.

Since, previous studies indicate that of the 3 important residues identified (N51, I47 and Q50) in homeodomain binding, Q50 may be the least important. DLX1-N51A and DLX1-I47A mutants could be generated to determine whether these residues are critical for DLX1 binding to the proglucagon promoter DNA sequence *in vitro*. We propose that DLX1 binds to TAAT motifs within the proglucagon promoter via its homeodomain, but the mechanism may be independent of the glutamine at residue 50. The requirement for both TAAT sites within the proglucagon promoter to be intact for repression suggests that DLX1 binds as a homodimer. In support of dimerization, the TAAT sites are 30 bp apart, and this would allow a protein-protein dimer to

bind to 2 major grooves of B-DNA (B-form DNA, the most common conformation, 10 bp per turn). Thus it seems more likely that DLX1 binds to both TAAT sites in the proglucagon promoter simultaneously as a homodimer (Figure 38, 1b) or multimer rather than binding the TAAT sites individually.

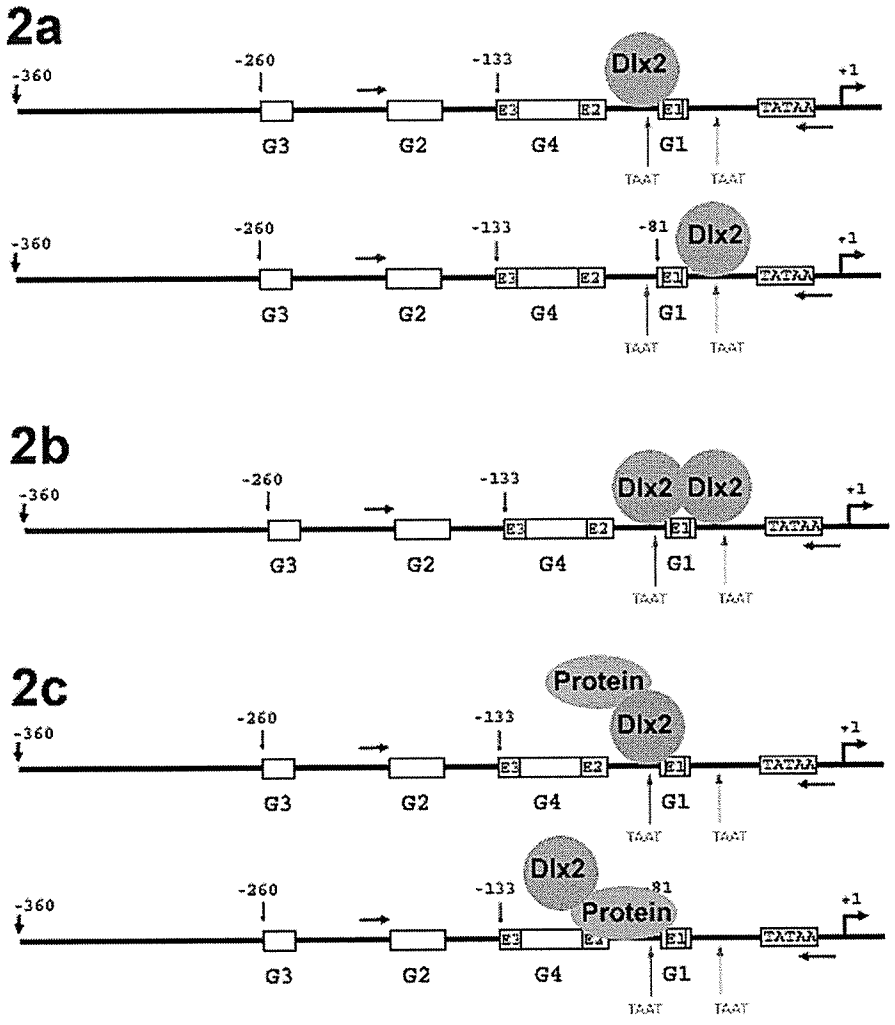

Figure 39: Models for binding of DLX2 to proglucagon promoter: 2a. DLX2 may bind as a monomer to individual TAAT motifs; 2b. DLX2 may bind as a homodimer or multimer to both TAAT motifs; 2c. DLX2 may form protein-DLX2 complexes which then bind via DLX2 to TAAT motifs, or through the other protein elsewhere on the proglucagon promoter.

For DLX2, luciferase assays again showed repression of the proglucagon promoter gene,

although much stronger (2.5 fold greater) than for DLX1. This is consistent with other studies for which DLX2 acts as a stronger transcriptional activator (Zhou, 2004) or repressor (Le, 2006 submitted) than DLX1. Results from selected mutations of either or both of the TAAT sites suggests binding as a homodimer in similar manner as for DLX1 (Figure 39, 2a/2b). However, unlike for DLX1, mutation of both TAAT sites reduced but did not eliminate repression. Unexpected results of persistant and strong repression obtained with N-terminus, C-terminus and homeodomain deletion constructs strongly suggests the presence of one or more additional DNA-binding or protein-interacting domains. It is possible that DLX2 binds to the proglucagon promoter as a DLX2-DLX2 homodimer in addition to binding as a DLX2-protein heterodimer. There are other transcription factors known to bind in close proximity to the TAAT motifs in the G1 region of the proglucagon promoter. This would implicate BRN4, CDX2/3 and PAX6 as possible binding partners of DLX2 (Figure 40).

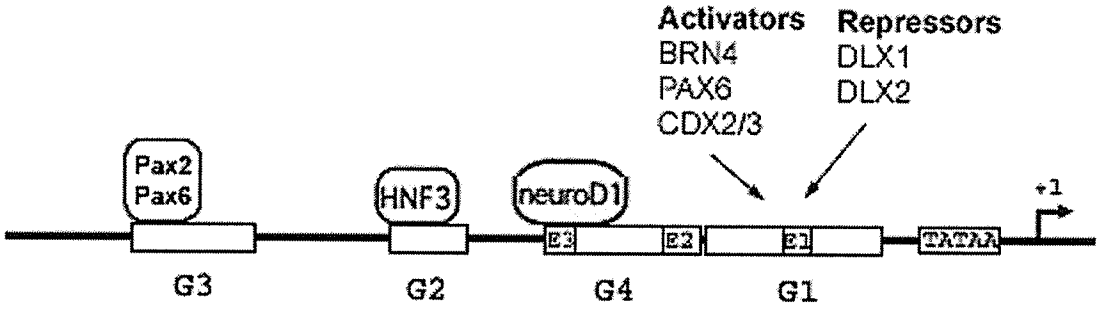

Figure 40: Activators and repressors of glucagon. Three known activators (BRN4, PAX6 and CDX2/3) all bind in close proximity to DLX1 and DLX2.

Since these three transcription factors are known activators of glucagon expression, it is possible that they would interact with DLX2 to result in less activation of proglucagon repression. Alternatively, DLX2 may be binding to some protein which is not yet known to bind the

proglucagon promoter. For the known transcription factors, the possibility of DLX2 binding could be addressed by a co-immunoprecipitation experiments using specific antibodies and would certainly be very interesting in light of the luciferase assay results obtained for DLX2.

By radioimmunoassay and cell-counting of immunofluorescence labeled cells, a decrease in insulin and glucagon levels was found in Dlx1/2 double-knockout mice, which was statistically significant. However, statistical significance does not always correlate with a biologically relevant phenotype. In particular, the 11.3% reduction in the level of glucagon may not have a significant biological effect. Analysis of this question is hampered by the fact that the Dlx1/2 double-null mouse dies at birth before an adult phenotype can manifest. The question arises as to what the relative contributions of DLX1 and DLX2 transcription factors are to the overall regulation of insulin and glucagon. The pancreatic hormones insulin and glucagon have opposing actions and are inversely regulated, with stimulation of insulin accompanied by repression of glucagon and vice versa. It is surprising then that the double-knockout displays a decrease in both hormones rather than an increase in one and decrease in the other. One possible explanation for this observation is that Dlx1/2 play a very small role in the overall regulation of insulin/glucagon in the adult. With numerous other factors tightly regulating insulin and glucagon expression, it is possible that Dlx1/2 play some role in tweaking or refining expression of these hormones in specific cells or under certain conditions. Thus, they might negatively regulate both hormones without significantly affecting overall expression levels. Another possibility is that Dlx1/2 are involved in pancreas development and promote either endocrine cell specification at the secondary transition (Figure 10), or α and β cell maturation during

terminal differentiation. A Dlx1/2 double-knockout would have less α and β cells and would therefore have lower insulin and glucagon expression. These possibilities are not mutually exclusive, and it is possible that the knockout could have decreased numbers of endocrine cells with each cell producing slightly more insulin/glucagon (due to absence of Dlx1/2 repression). Besides direct regulation of promotion of α and β cell fate, there might be an indirect regulation of pancreas development through insulin and glucagon. Previous studies have found that insulin levels are important during pancreas development, and in mice lacking the insulin gene, there is islet hyperplasia (Duvillie, 2002) and other abnormalities, suggesting that insulin itself acts as a negative regulator of islet development. Dlx1/2 regulation of glucagon during development might have a similar effect. Thus the overall contribution of Dlx1/2 to insulin and glucagon regulation in the adult is likely to be small due to there being many other regulatory factors for these hormones. Based on the data from this study, and on previous studies, the function of Dlx1/2 during development is likely specification or maturation of α and β cells late in development.

To further investigate the possible role of Dlx1/2 early in pancreas development, additional immunofluorescence experiments were performed (N. Boyko, D. Eisenstat, unpublished observations). No change in expression of the pancreas transcription factors Ngn-3 and NeuroD1 was found between wild-type or Dlx1/2 double-knockout mice at E14.5. As well, examination of the exocrine factor PTF1a and the exocrine marker pancreatic amylase showed no difference between wild-type and mutant at E16.5 and E18.5. Creation of a pancreas-specific conditional Dlx1/2 double-knockout mouse would be interesting, in order to see which cell types express

Dlx during pancreas development as well as to determine whether these mutant mice may be prone to diabetes (MODY in particular).

Certainly, any research involving hormones and transcription factors will have some relevance to disease. Knowledge of pancreas development will be essential to successfully culturing islet cells from stem cells or pancreas progenitors, which is a major goal of islet transplantation research. Once the role of Dlx1/2 in pancreas development is better understood, it is possible that these transcription factors or their upstream regulators and downstream targets could be useful as clinical markers for diabetes or pancreatic cancers.

Chapter V: Conclusion and Future Directions

The results of these studies provide evidence for Dlx1/Dlx2 mediated regulation of the mouse proglucagon promoter and glucagon hormone expression in the pancreas. DLX1/2 were found to be co-expressed not just with glucagon, but with all pancreas hormones within the islets and also to be co-expressed with the pancreas progenitor transcription factor PDX-1. Although no luciferase assays were carried out for the pre-proinsulin I promoter, preliminary studies by ChIP and EMSA suggest that Dlx1/Dlx2 may directly bind to and regulate this promoter as well. Therefore it remains to be determined what role Dlx1/2 play in regulation of pancreas hormone expression and whether or not it has a place in the pancreas development transcription factor cascade. These questions could be addressed by analysis of cell division and differentiation in pancreas during development in the Dlx1/2 double-knockout mouse. Additionally, a pancreas conditional double-knockout could be made (the Dlx1/2 double-knockout mouse dies at birth) to assess the adult phenotype and look for abnormalities in differentiation and function of the pancreas.

The mechanism of Dlx1/2 repression of the proglucagon promoter is unknown. The unusual behavior of the Dlx2 mutants in luciferase assays suggests a probable protein-protein interaction, possibly with Cdx2/3 or Pax6 (the two proteins known to bind the same region of DNA) or with other proteins. Co-immunoprecipitation studies using either mouse pancreas extracts or transfected cell lines could be used to identify these protein complexes. Studies have shown that insulin levels are important during pancreas development, and in mice lacking the insulin gene,

there is islet hyperplasia (Duvillie, 2002) and other abnormalities, suggesting that insulin acts as a negative regulator of islet development. It is possible that glucagon levels are also important for the correct development of the pancreas. Thus, the contribution of Dlx1/2 to mouse pancreas development and maintenance of adult pancreas phenotype may involve the regulation of different transcription factors or hormones but the exact mechanisms involved remain to be elucidated.

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