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**PROSPECTS FOR GENE THERAPY OF
DIABETES: CARBOHYDRATE REGULATED
EXTRAPANCREATIC INSULIN EXPRESSION
IN TRANSGENIC MICE**

by

MICHAEL E. RIPPS

A dissertation submitted to the Graduate Faculty in
Biomedical Sciences in partial fulfillment of the
requirements of the degree of Doctor of Philosophy,
The City University of New York.

1996

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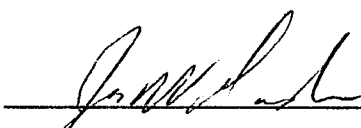
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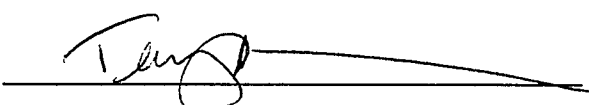
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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

PROSPECTS FOR GENE THERAPY OF
DIABETES MELLITUS: GLUCOSE REGULATED
EXTRAPANCREATIC INSULIN EXPRESSION
IN TRANSGENIC MICE

by

Michael E. Ripps

Advisor: Professor Jon W. Gordon

This thesis research focuses on the development of strategies for genetically engineering physiologically appropriate hepatic insulin production as an approach to the treatment of diabetes mellitus. Diabetes mellitus is a prevalent metabolic disorder resulting from a loss of insulin-producing pancreatic islet cells or a decrease in the peripheral response to insulin. Despite current treatment, many patients develop major complications that represent a great health burden. The benefits of intensive insulin therapy in delaying progression of the disease provide an impetus for developing improved modes of insulin delivery. The liver possesses several attributes that make it an attractive candidate for genetically engineering insulin production with regulatory properties similar to pancreatic beta cells. Hepatocytes express the glucose transporter isoform GLUT-2 and the glucose phosphorylating enzyme glucokinase, which are key components of the glucose-sensing apparatus in pancreatic beta cells. Hepatocytes also express the liver-type pyruvate kinase (L-PK) gene, which is regulated at the transcriptional level by glucose

responsive elements. The L-PK gene thus represents a hepatic analog to the pancreatic-expressed insulin gene, which contains similar regulatory elements. To examine the potential for engineering physiologically appropriate hepatic insulin production, transgenic mice expressing the human insulin gene under control of the L-PK promoter were produced. Results demonstrate that insulin expression in liver, intestine, and kidney is stimulated by carbohydrate feeding. Hepatic insulin mRNA levels were induced up to 100 fold when fasted mice were refed with a high sucrose diet and there were rapid increases in the mRNA level after injection of a glucose load. Mice subjected to beta cell ablation had a partial correction of hyperglycemia, with decreases in both non-fasting and fasting blood glucose levels and without episodes of hypoglycemia. These findings indicate significant potential for L-PK:insulin constructs to provide a homeostatic control mechanism for the endogenous production of insulin that is responsive to changes in blood glucose concentration in the physiologic range. Improvements in construct design may lead to the development of gene therapy vectors suitable for the treatment of diabetes mellitus.

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The past seven years have been challenging and exciting ones for me. I want to extend my sincere appreciation to my advisor Dr. Jon Gordon, for providing an environment that allowed me to learn, discover, and freely explore one's ideas. His willingness to share his knowledge and experience with me and his continuing enthusiasm for science has set the path down which I have traveled. While this thesis focuses on the prospects for gene therapy of diabetes, I also engaged in stimulating and productive research on the superoxide dismutase gene leading to the development of a mouse model for amyotrophic lateral sclerosis. In addition, Appendix 2 contains a brief presentation related to the development of a transcriptional regulatory system with potential for gene therapy applications. The Gordon lab has indeed been a place where ideas and hard work, not just genes, amplify a million fold.

The dedicated interest and professionalism of the faculty have greatly contributed to the completion of this thesis. I thank Dr. Robert McEvoy, Dr. Mary Rifkin, Dr. Heidi Stuhlmann, Dr. Savio L.C. Woo of the Baylor College of Medicine, and Dr. Robert Lazzarini, Chair of the Brookdale Center for Molecular Biology.

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I. Brief Introduction: Control systems for gene therapy

The techniques of gene therapy and cellular engineering are being applied to an increasing number of metabolic diseases (1-4). Although initial efforts have focused on the replacement of a functional gene in the context of single gene defects, applications to the treatment of more complex disorders may soon be possible (5). Somatic gene transfer may be employed to engineer the endogenous production of therapeutic agents, obviating the need for exogenous administration. The difficulties encountered in developing an appropriate delivery vehicle for complex proteins and repeated administration of the therapeutic agent may be avoided by stably transferring a gene encoding the protein. For example, diseases manifested by the lack of an essential hormone, such as insulin-deficient diabetes or growth hormone deficiency, may be treated by delivering a gene encoding the hormone to a tissue which is an appropriate site for synthesis and secretion.

New initiatives in somatic gene therapy will undoubtedly require the development of control systems to achieve the desired level of protein production for varying physiological circumstances. One can envision control systems that operate at the levels of gene transcription, mRNA stability or translational efficiency, and protein stability, activity, or localization. As a general strategy control systems should be specific to the therapeutic gene,

causing little or no interference with normal functions. Appropriate control may be achieved by utilizing endogenous regulatory pathways (Figure 1) or by using small molecule regulators that can be easily administered.

This study focuses on the potential application of gene therapy to the treatment of insulin deficiency. The possibility of achieving appropriately regulated hepatic insulin production is explored by utilizing transcriptional control elements from the glucose regulated liver-type pyruvate kinase gene to drive expression of the human insulin gene in transgenic mice (Figures 1 and 2). In addition, there is a brief presentation of the development of a transcriptional control system based on the mouse metallothionein promoter that incorporates lac operator-repressor regulatory elements. This system is demonstrated by using small molecules to regulate the secretion of human growth hormone from hepatoma cells.

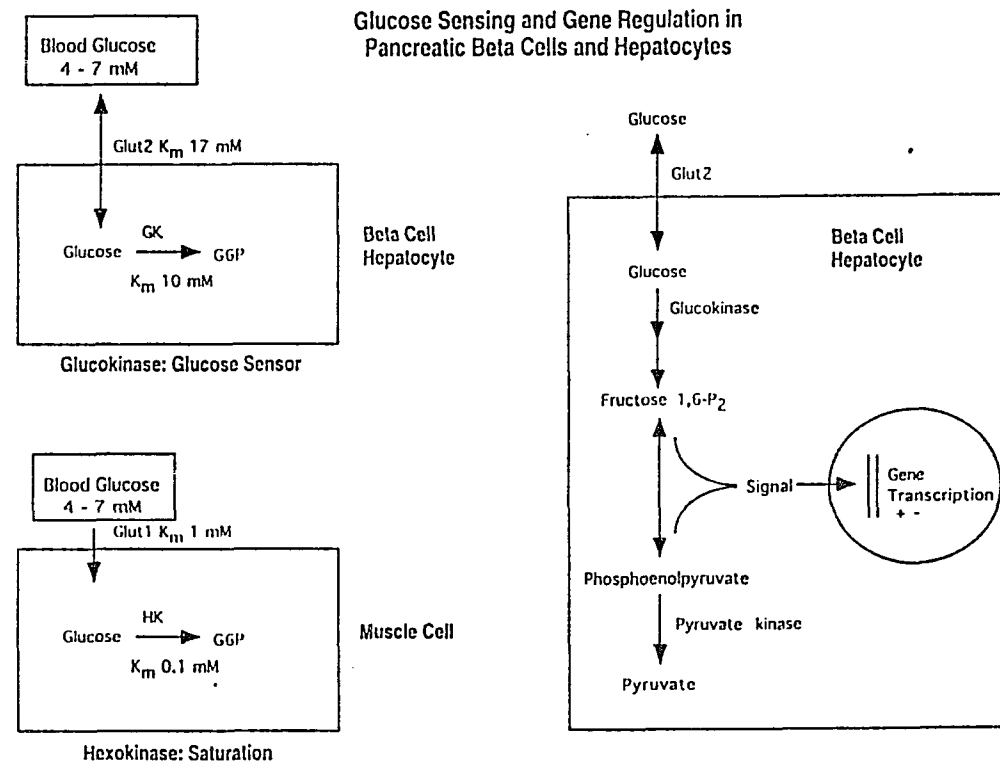


Figure 1. Glucose sensing and gene regulation in pancreatic beta cells and hepatocytes. Hepatocytes express the glucose transporter isoform GLUT2 and the glucose phosphorylating enzyme glucokinase, which combine to make the rate of glycolysis in hepatocytes sensitive to changes in blood glucose concentration in the physiologic range. Hepatocytes express liver-type pyruvate kinase (L-PK), a glycolytic enzyme whose level of expression appears to be regulated by a signalling molecule emanating from a glycolytic intermediate. In this respect, L-PK represents a hepatic analog to the pancreatic expressed insulin gene, which has a similar transcriptional control mechanism. Since the L-PK promoter is activated by carbohydrate feeding and inhibited by fasting, an L-PK:insulin fusion gene may provide a two-way control system for hepatic insulin production that is regulated in a manner similar to a pancreatic beta cell.

II. Introduction

A. Diabetes mellitus - general background and current treatment

Diabetes mellitus is a chronic disorder affecting carbohydrate, fat, and protein metabolism with the main feature being elevated blood glucose levels. Impaired carbohydrate utilization results from a deficiency in pancreatic insulin secretion and/or a decrease in the peripheral response to insulin. Diabetes is characterized by hyperglycemia, excessive urination and thirst, and weight loss. There are two major forms of the disease, insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). IDDM is an autoimmune disorder present in approximately 1:400 persons that results from a severe lack of insulin, caused by a reduction in the mass of insulin-producing pancreatic beta cells (6). IDDM usually develops in childhood and becomes severe at puberty, with untreated patients succumbing to acute metabolic complications, such as ketoacidosis and coma. A genetic susceptibility to altered immune regulation, linked to specific alleles of the class II MHC locus predisposes certain individuals to the development of autoimmunity to beta cells (6). The autoimmunity either develops spontaneously or, more likely, is triggered by an environmental agent such as a virus or toxin. NIDDM is a disorder that affects approximately 1:100 persons, with

onset in the adult years. While a strong genetic component is implied by the 90% concordance rate among identical twins, the pathogenesis of NIDDM is not well understood. NIDDM is characterized by an inability of peripheral tissues to respond to insulin, as well as insulin secretion patterns that are delayed or insufficient relative to the glucose load (6).

Current treatment of diabetes involves the administration of insulin for IDDM and hypoglycemic drugs for NIDDM. Despite treatment, many patients develop major complications including retinopathy, nephropathy, neuropathy, and atherosclerosis. In addition, diabetics have increased rates of infection and skin breakdown. The benefits of intensive insulin therapy in delaying the onset and progression of major complications is underscored by the recent findings of the Diabetes Control and Complications Trial (7). Intensive therapy was administered to IDDM patients either with an external insulin pump, or by three or more daily injections guided by frequent blood glucose monitoring. Patients receiving intensive therapy had markedly reduced incidences of retinopathy, nephropathy, and neuropathy. The major adverse effect of intensive therapy was an increased incidence of severe hypoglycemia. These findings should provide an impetus for developing improved modes of insulin delivery that are appropriately regulated by blood glucose levels.

B. New approaches to the treatment of insulin deficiency

An alternative therapeutic strategy for IDDM is the use of isolated islets or pancreas fragments for transplantation, in the hope of replacing the precise insulin secretion function provided by beta cells (8). Although transplantation of naked islet tissue results in rapid autoimmune destruction or graft rejection, recent work has shown that biocompatible semi-permeable encapsulation devices might be a viable method for the protection of transplanted islets (9,10). A major problem of this approach, however, is the supply of tissue for transplantation. The high cost of islet isolation and the difficulty of assuring functional uniformity make it unlikely that transplantation of primary islet tissue can be employed to treat a significant fraction of individuals with IDDM (11).

Another strategy is to develop insulin-secreting clonal cell lines that can be grown in unlimited quantity and at far less cost compared to isolated islets. The transformation of primary islet cells with viruses or by engineering expression of oncogenes (12,13) has been applied with limited success, since transformed cells display aberrant patterns of insulin secretion. Another approach is to attempt to confer glucose-stimulated insulin release on a non-islet cell line. The engineering of glucose-stimulated insulin secretion in the mouse anterior pituitary cell line

AtT-20ins has been achieved by stable transfection with a cDNA encoding the glucose transporter GLUT-2 (14,15). GLUT-2 and glucokinase are believed to be key components in the glucose-sensing apparatus that regulates insulin secretion from pancreatic beta cells (16-20). However, several problems must be overcome before engineered AtT-20ins cells can be useful, since these cells respond over a low range of glucose concentrations and also secrete ACTH upon stimulation (14).

Gene therapy for diabetes is an intriguing possibility. The introduction and expression of an appropriately regulated insulin gene in non-islet tissues is a potential strategy for the treatment of IDDM. Despite the heterogeneity of factors involved in the pathogenesis of NIDDM, extrapancreatic insulin production may conceivably be useful for the treatment of NIDDM as well. The promise of such an approach was demonstrated by the transplantation of proinsulin-producing fibroblasts into immunosuppressed diabetic mice (21). Ltk- fibroblasts were engineered to express the human insulin gene by stable transfection of a mouse metallothionein I:human insulin fusion construct. Blood glucose dropped to normal levels within two weeks of transplantation. However, due to unrestrained proliferation of transplanted cells and unregulated proinsulin production, the mice developed severe hypoglycemia and died (21).

C. Engineering hepatic insulin expression

Hepatocytes have been shown to be capable of synthesizing and secreting human insulin after delivery of a beta-actin:human insulin construct using liposome-DNA complexes injected into the portal vein of adult rats (22). Serum insulin levels increased between 1-8 days post-injection, then rapidly declined as the transfected plasmid DNA was degraded. In this study, human insulin was measured by a radioimmunoassay employing two monoclonal antibodies with low crossreactivity to rat insulin. However, the relative amounts of proinsulin and insulin produced by transfected hepatocytes was not examined. The processing of proinsulin to mature insulin occurs by removal of the C peptide, which connects the A and B chains. Dibasic residues at the B-C and C-A junctions are recognized by the β -cell specific proteases PC2 and PC3 (23). Recently, the processing of proinsulin was examined in rat hepatoma cells transfected with a human insulin cDNA under control of the Rous Sarcoma virus LTR (24). Insulin-like material released into the medium was analyzed by reversed phase HPLC. Interestingly, although proinsulin and des-31,32 split proinsulin were the dominant products measured, fully processed insulin was also produced. In this study, the amount of fully processed insulin measured underestimates its production, since insulin, but not proinsulin, was rapidly cleared from the medium by the cells. The studies

of insulin production and processing in hepatocyte-like cells thus provide a strong stimulus for further research, since the functional consequences of hepatic insulin production have yet to be explored.

D. Engineering physiologic insulin regulation in extrapancreatic sites

Extrapancreatic insulin production as an approach to the treatment of diabetes demands physiologically appropriate regulation. The pancreatic β -cell increases insulin production in response to increased blood glucose, thus normalizing blood glucose levels. β -cell insulin production is lowered during periods of fasting to prevent hypoglycemia. The β -cell response to a rapid increase in glucose is biphasic; pre-formed insulin is released in the initial phase and continued release of pre-formed and newly synthesized insulin characterize the second phase (25-27). Several approaches can be envisioned for simulating such response properties in extrapancreatic sites. Non-beta cells transfected with the insulin gene may be further modified in an attempt to engineer physiologically appropriate insulin release. Cell types that already possess a regulated secretory pathway are most amenable to this approach. Genetic modifications such as expression of the glucose transporter isoform GLUT-2 and adjustment of the glucokinase/hexokinase ratio in AtT-20ins cells are currently being investigated (11).

Another approach would be to simulate glucose-induced insulin gene transcription (28-30) using control mechanisms that exist in other cell types. Genes with appropriate transcriptional controls in cells possessing constitutive secretory pathways might release newly formed insulin immediately after synthesis, thus providing a direct link between transcriptional control and insulin secretion. Candidate genes possessing appropriate regulatory elements may be found in the pathways of intermediary metabolism.

E. Dietary and hormonal control of gene expression

The transcriptional regulation of several genes encoding proteins that are part of the glycolytic and gluconeogenic pathways are currently being investigated, including glucokinase (31), hexokinase (32), phosphofructokinase-1 (33), phosphofructokinase-2 (34), liver (L) and red blood cell (L') type pyruvate kinase (35), phosphoenolpyruvate carboxykinase (PEPCK) (36), and fructose-1,6-bisphosphatase (37).

The dietary and hormonal regulation of the genes for the gluconeogenic enzyme PEPCK (38,39) and the glycolytic enzyme L-type pyruvate kinase (40) have been analyzed extensively in transgenic mice. The hepatic PEPCK gene is stimulated by a diet high in protein and devoid of carbohydrate, primarily due to an increase in glucagon levels (41,42). Conversely, insulin downregulates PEPCK expression; treatment of diabetic mice with insulin

decreases expression of a PEPCK-growth hormone transgene (43). Insulin may act through an insulin-responsive element located in the PEPCK promoter region (44). The liver-type pyruvate kinase (L-PK) gene has the opposite pattern of dietary and hormonal regulation; dietary glucose and insulin stimulate transcription of the L-PK gene and increase mRNA stability, while glucagon decreases transcriptional activity and mRNA stability (45).

F. Transcriptional regulation of the rat L-type pyruvate kinase gene

Rat liver-type (L) and erythroid-type (L') pyruvate kinase is encoded by a gene which possesses two promoters that are 500 bp apart, with the different forms derived from splicing of either the L or L' first exon to the remaining 10 exons (35,46). An L-type minigene construct containing 2.7 kilobases of 5' flanking sequences and lacking exons 2 to 9 was expressed strongly in liver, and more weakly in intestine and kidney, of transgenic mice (40). Moreover, the minigene was regulated by dietary and hormonal conditions in a manner similar to the endogenous gene; expression was greatly decreased by a 24 hour fast and was strongly stimulated by refeeding with a carbohydrate-rich diet. In another study, transgenic mice were produced using 3.2 kilobases of 5' flanking sequences linked to the chloramphenicol acetyltransferase structural gene (47). Tissue specific and dietary transcriptional control of the

reporter gene were similar to endogenous L-PK, suggesting that all necessary cis-acting elements are contained in the 3.2 kilobase fragment.

Additional studies have further characterized the cis-acting elements responsible for regulation of the L-PK promoter (48-51). The proximal promoter region (-183 to +11, Figure 2) contains binding sites for liver-specific factors HNF1, HNF4, and L5BF, in addition to binding sites for the ubiquitous factors NF1 and MLTF. The proximal promoter is sufficient to confer tissue specificity and dietary/hormonal control, but its activity is modified by distal sequences in a tissue-specific manner. Sequences from -183 to -392 induce expression in liver but repress expression in small intestine. Sequences from -1170 to -2080 have an inhibitory effect that is greater in small intestine than liver. There is an activating sequence between -2080 and -3200, which is required for full expression of the gene in liver (51).

The glucose response element of the L-PK promoter is thought to consist of the MLTF binding site (Figure 2), which cooperates with the HNF4 binding site to give tissue-specific glucose regulation (52,53). Although both insulin and glucose are required for induction, it appears that insulin is not the direct effector, but rather plays a permissive role by stimulating insulin-dependent steps of glycolysis (54-56). The mechanism by which glucose

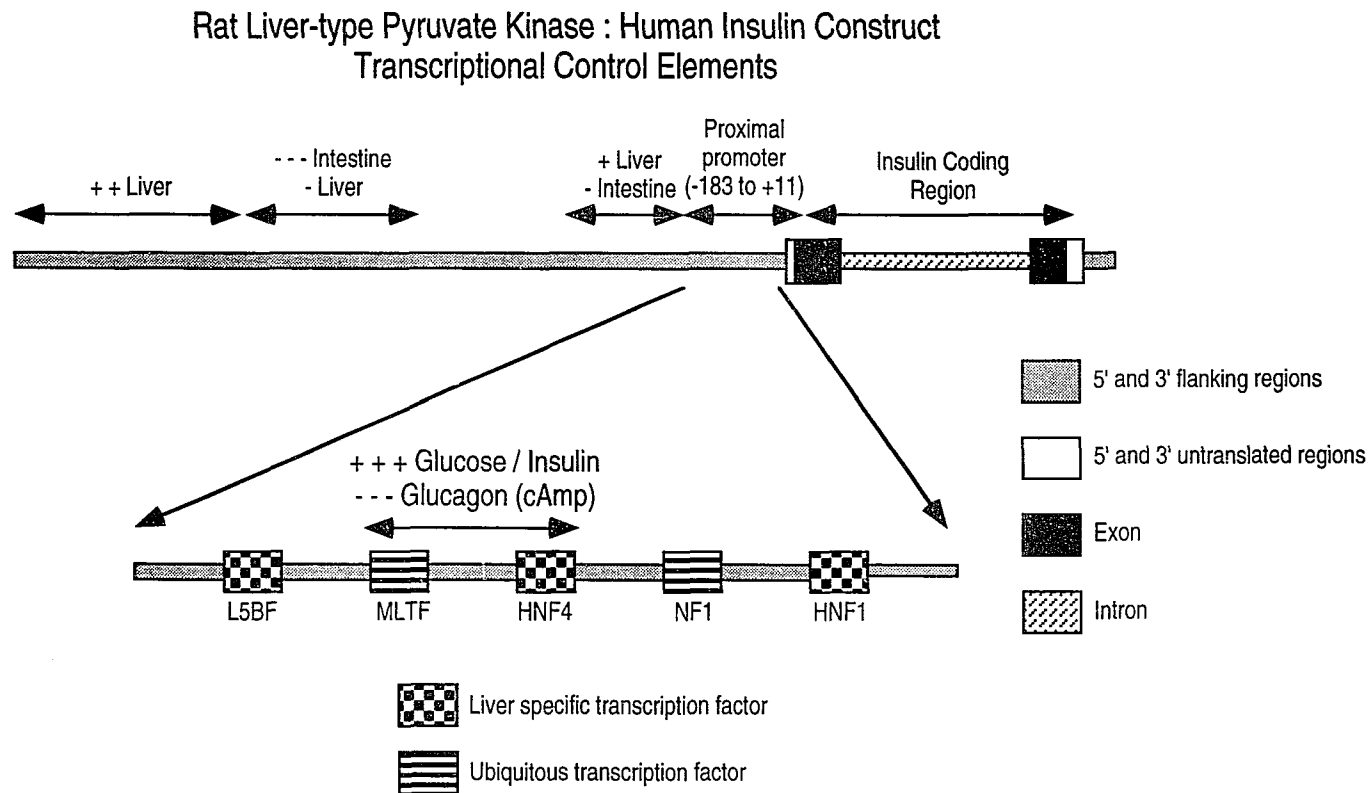


Figure 2. Map of the rat liver-type pyruvate kinase promoter - human insulin fusion gene. The locations of known cis-acting sequences within the 3.2 kb L-PK promoter fragment are indicated. (+) indicates transcriptional activation and (-) transcriptional repression. The transcription factors binding to the proximal promoter region are shown in the expanded view.

stimulates the L-PK promoter is unknown, but is thought to involve the production of a signaling molecule generated by metabolism of glucose (50).

G. Rationale for glucose regulated hepatic insulin production

Here we investigate the potential for regulated hepatic production of insulin using a gene construct that incorporates carbohydrate and hormonal response elements from the liver-type pyruvate kinase (L-PK) gene. There are several reasons why we believe that the liver is an appropriate target for a transferred insulin gene. First, as described above, hepatocytes are capable of synthesizing and secreting both proinsulin and insulin. Second, the liver plays a central role in the regulation of blood glucose, with gene expression patterns that make it an attractive candidate for glucose-stimulated insulin production. Hepatocytes express GLUT-2 and glucokinase, which are involved in the glucose-sensing apparatus in pancreatic beta cells (11,16,17,20). Therefore, hepatocytes may respond to serum glucose concentrations in an appropriate physiologic range (Figure 1). Third, as described above, hepatocytes express the L-PK gene, which contains transcriptional elements mediating stimulation by glucose and inhibition by glucagon. With respect to glucose stimulation, the L-PK gene thus represents a hepatic analog

to the pancreatic-expressed insulin gene, which also contains regulatory elements mediating carbohydrate responsiveness (28-30). Further, the 3.2 kb promoter region of the rat L-PK gene has been shown to be sufficient to confer tissue-specific and glucose/glucagon regulation to a heterologous reporter (47,48,51). Fourth, the blood from the pancreas drains into the portal system and is brought initially to the liver, where much insulin is degraded before delivery to peripheral tissues. The hepatic production of insulin would preserve this physiologic relationship. Fifth, the liver is a promising target organ for somatic gene transfer, with several delivery systems in development (57-59).

H. Study design: transgenic mice and diabetes models

We believe that the regulatory properties of the L-PK gene may lead to physiologically appropriate hepatic insulin production. Since the L-PK promoter is positively regulated by glucose, we expect that insulin production will be directly related to blood glucose levels. Further, since the L-PK promoter is negatively regulated by glucagon, we expect that insulin production will be decreased when glucagon secretion is raised in response to lowered blood glucose. The use of human insulin coding sequences enables us to differentiate products of the transgene from endogenous mouse products. In order to study hepatic

insulin production in vivo, we produced transgenic mice (60,61) carrying the L-PK:insulin gene construct. Transgenic mice offer several advantages as a model system for assessing potential approaches to gene therapy. First, established transgenic lines provide an easily renewable supply of animals which stably express the transferred gene. In contrast, procedures for achieving transgene persistence by somatic gene transfer are in the early stage of development (59). Second, gene expression may be directed to the target of interest by using tissue-specific regulatory elements. The L-PK promoter fragment we have used has been shown to confer predominantly liver-directed expression on a heterologous reporter gene in transgenic mice, with much lower levels of expression in intestine and kidney (47,51). Third, transgenic mice are useful for evaluating the effects of overexpression of the transferred gene, and thus the safety of potential gene therapy approaches (62). The safety of extrapancreatic insulin production is critically important, since unregulated insulin production can lead to severe hypoglycemia (21).

To study the effects of extrapancreatic insulin production in diabetic animals, we ablated pancreatic β -cells using the diabetogenic agent streptozotocin (STZ). A single injection of the STZ produces an extensive necrosis of pancreatic β -cells within 48 - 72 hours, resulting in permanent diabetes in mice (63). An alternative approach would be to breed the insulin transgenic animals to an

existing mouse model for diabetes. The nonobese diabetic (NOD) mouse is a commonly used model for IDDM, but the trait is inherited in a recessive manner. When NOD mice are bred to other strains, there is no development of diabetes (64). However, transgenic mice expressing class II MHC molecules or interferon-gamma under the control of the human insulin promoter develop IDDM at about 8 weeks of age (65). Also, transgenic mice expressing calmodulin under the control of the rat insulin II promoter develop diabetes soon after birth (66). Both of these transgenic models have demonstrated β -cell pathology and would be suitable for cross-breeding experiments.

Thus, in the following study, we examine extrapancreatic insulin expression in transgenic mice using transcriptional regulatory elements of the L-type pyruvate kinase gene. The regulatory and functional properties of extrapancreatic insulin production are explored to assess the feasibility of liver-directed gene transfer as an approach to the treatment of diabetes.

III. Materials and Methods

A. Construction of the rat L-PK:human insulin fusion gene

A 12.5 kb human genomic EcoRI fragment in pBR322 containing the insulin gene was obtained from Graeme Bell (67). The insulin gene has a 3 exon - 2 intron arrangement with the start codon 18 bp from the beginning of exon 2. The insulin gene was isolated from surrounding sequences by subcloning a 2.8 kb BglII-SacI fragment into the BamHI and SacI sites of pGEM3zf+ (Promega), creating the plasmid phI(2.8). The BglII-SacI fragment spans the region from 169 bp upstream of the transcriptional start site to 1214 bp downstream of the end of exon 3. The coding region was further isolated by digesting phI(2.8) with NcoI and SphI, yielding a 1.3 kb fragment spanning the region from the NcoI site 2 bp upstream of the start codon to the SphI site 132 bp downstream of the end of exon 3. The NcoI site was converted to a BamHI site using an adapter made by annealing two 18 mer oligonucleotides, 5'GATCCATCGATAGGCCTC3' and 5'CATGGAGGCCTATCGATG3'. These manipulations change the sequence surrounding the initiator codon from TCTGCCATGG to GCCTCCATGG, moving the GCC motif from the -1:-3 position to the -4:-6 position. GCC motifs at these positions have been shown to increase translational efficiency of the rat preproinsulin mRNA after transfection into COS-1 cells (68). The new BamHI-SphI fragment was cloned into the BamHI and SphI sites of pGEM3zf+, then released as a BamHI-HindIII

fragment and cloned into the BamHI and HindIII sites of pGEM11zf- (Promega), creating the plasmid p11hI(1.3).

The plasmid L-PK(-4300)CAT, which contains the 5'-flanking region of the rat L-type pyruvate kinase gene fused to the chloramphenicol acetyltransferase gene, was obtained from Howard Towle (50). L-PK(-4300)CAT was digested with EcoRI and XhoI, releasing a 3.2 kb fragment spanning the region from -3193 to +12 with respect to the transcription start site of the L-PK gene. Finally, the 3.2 kb L-PK promoter region was cloned into the EcoRI and XhoI sites of p11hI(1.3), creating the plasmid pPI. The plasmid pPI contains 3 kb of vector sequences and a 4.5 kb EcoRI-HindIII fragment encompassing the rat L-PK:human insulin fusion gene (L-PK:hIns). The L-PK:hIns construct contains 2 exons which are referred to as exons 2 and 3, corresponding to their position in the intact human insulin gene (67).

The plasmid pPI was purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient according to established procedures (69). The purified plasmid was digested with EcoRI and HindIII, and electrophoresis was carried out in a 0.6% agarose gel (SeaKem GTG agarose, FMC 50072) at 40 volts in 1 X TBE buffer for 17 hours (MPH electrophoresis unit, IBI). The 4.5 kb fragment was excised and electroeluted from the gel, followed by two phenol extractions and one chloroform extraction of the DNA solution. The DNA was precipitated with ethanol and resuspended in microinjection buffer (10 mM Tris.HCl, 0.18

mM EDTA pH 7.5) at a concentration of 5 ng/ μ l (approximately 1000 molecules/pl). The DNA solution was dialyzed against microinjection buffer at 4 °C for 3 days with one change per day. The dialyzed DNA solution was stored at 4 °C until the time of microinjection.

B. Transient transfection of HepG2 cells

Plasmids used for transfection were: Vector, the cloning vector pGEM3zf+ (Promega); ghIns, a 12.5 kb human genomic EcoRI fragment containing the insulin gene (67) in pGEM3zf+; L-PK:hIns, the 4.5 kb fusion gene in pGEM11zf-. Plasmid DNA was purified by differential precipitation with polyethylene glycol according to established procedures (69).

HepG2 cells, 5×10^4 cells in 1 ml of DMEM/10% FBS, were seeded into individual wells of a 24 well tissue culture plate and placed in a 37°C/5% CO₂ incubator for 20 hours. Plasmid DNA was complexed with cationic liposomes by mixing DNA (1.6 μ g in 0.4 ml DMEM) with Lipofectin (GIBCO BRL 18292-011, 6.4 μ g in 0.4 ml DMEM) and incubating for 10 minutes at room temperature. After washing the cells once with 1 ml of DMEM, 0.2 ml of the DNA-liposome mixture was added to triplicate wells. After incubation at 37°C for 5.5 hours, the mixture was removed and 0.25 ml of MCDB 105 medium (Sigma M6395, D-glucose 72 mg/dl) was added. After a 40 hour culture period, 5 μ l of medium was removed and

glucose concentration was determined with a colorimetric glucose oxidase assay (Trinder reagent, Sigma 315-100).

C. Animals

Mice of the highly inbred strain FVB/N were used to generate transgenic animals. This strain is characterized by vigorous reproductive performance and consistently large litters (70). Transgenic lines were not bred to homozygosity to generate a population of animals for experiments, since this would necessitate the use of separately bred normal mice as controls. All studies were carried out using mice bred from a heterozygous parent (a transgenic male bred to a normal female), allowing the use of nontransgenic littermates as controls. Although this approach required continual screening of mice for experiments, it was felt that every attempt should be made to minimize sources of spurious variation between transgenic mice and normal controls.

Mice were housed in the Center for Laboratory Animal Sciences and were maintained in accordance with institutional guidelines. Routinely, five mice were kept in a shoebox-type polycarbonate cage with dimensions 26 cm long X 16 cm wide X 12 cm high. For some experiments, one to three mice were kept in a cage. Cage bottoms were covered with bedding (Bed-o-cobs, Andersons 6090-01). The mouse room was kept lighted from 8 AM to 10 PM and was maintained

at a temperature of 72 °C. Except for breeding cages, male and female mice were caged separately beginning at the weaning age of approximately 3 weeks. Mice were given a standard laboratory chow diet (Purina 5001) ad libitum and tap water. Mice were maintained on the chow diet for at least 3 weeks (3 - 9 weeks) prior to dietary changes. Breeding mice were often given a breeding diet (Purina 5015) instead of the chow diet. When mice were fasted, they were placed in a clean cage without bedding and given fresh tap water. If mice were fasted longer than 12 hours, they were changed to a new clean cage without bedding every 12 hours. When mice were refed after a period of fasting, they were placed in a clean cage with bedding, tap water, and the new diet. The chow diet (Purina #5001) contained 5.9% sugars, 32% starch, 4.5% fat, and 23% protein. The breeding diet (Purina #5015) contained 3.7% sugars, 39% starch, 11% fat, and 18% protein. A high sucrose diet (USB #16990), which contained 68% sucrose, 8% fat, and 18% protein, was used in many fast-refeeding experiments.

D. Production of transgenic mice

Transgenic mice were produced by pronuclear microinjection according to procedures established by Gordon et. al. (60,61,71). Collection of embryos, microinjection, and embryo transfer were performed by Jon Gordon. Michael Ripps observed and assisted with these procedures and

performed procedures related to preparation of DNA for microinjection, as well as superovulation and mating of mice to obtain embryos (71). In brief, one-celled embryos were collected from the oviducts of superovulated immature FVB/N females prior to fusion of the male and female pronuclei. A microneedle was used to inject the male pronucleus with about 1 pl of a DNA solution containing approximately 1000 copies/pl. After microinjection, surviving embryos were implanted into the oviducts of pseudopregnant CD-1 females obtained by mating with vasectomized CD-1 males. The embryos were then allowed to develop through birth. At approximately 4 weeks of age, genomic DNA was prepared from tail biopsies and the polymerase chain reaction (PCR) was performed to screen for animals with integrated copies of the introduced gene.

E. Preparation of genomic DNA from tail biopsies

Tail lysates were prepared by cutting a 7 - 10 mm section from the end of the mouse tail and placing it into a 1.5 ml tube containing 0.2 ml of tail lysis buffer (100 mM Tris.HCl pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 µg/ml Proteinase K). The tail samples were lysed by incubation for 5 hours in a 55°C waterbath. The samples were spun for 1 minute in a microcentrifuge to clear the lysate of insoluble material. The lysate was extracted once with 0.1 ml of phenol-chloroform (a 1:1 vol/vol mixture) by gentle

rocking for 10 minutes, a vigorous shake for 2 seconds, and phase separation by centrifugation at 12,000 g for 2 minutes at room temperature. The upper aqueous phase was combined with 1/10 volume 3M sodium acetate pH 5.2, followed by 2 volumes of cold 100% ethanol. Precipitated genomic DNA was pelleted by centrifugation at 12,000 g for 2 minutes at room temperature, the supernatant was removed, and the pellet was allowed to air dry for 5 minutes. The DNA pellet was resuspended in TE/10 (10 mM Tris.HCl, 0.1 mM EDTA pH 8.0) by incubation at 55 °C for 2 hours, followed by repeated pipetting up and down to mix. DNA concentration was determined by A260 reading (1 OD unit = 50 µg/ml) using a Beckman DU-64 spectrophotometer.

F. Identification of transgenic mice by PCR using genomic DNA

Two 19 mer oligonucleotides, hI-E2 and hI-E3C, were synthesized corresponding to sequences within exons 2 and 3, respectively, of the human insulin gene. The primer sequences are within the region that codes for the C peptide, where there is a 23% sequence dishomology between the human insulin gene and the two mouse insulin genes (67,72). In comparison, the dishomology is 14% in the B chain region and only 5% in the A chain region. hI-E2 was designed from the sense strand and has the sequence 5'CGGGAGGCAGAGGACCTGC3'; there are 6 mismatches between this

sequence and the cognate sequence of the mouse insulin genes, including changes at positions 17 and 18 of the oligonucleotide near the 3' end (67,72). hI-E3C was designed from the antisense strand and has the sequence 5'AGGGACCCCTCCAGGGCCA3'; there are 4 mismatches between this sequence and the cognate sequence of the mouse insulin genes, including a change at position 15 of the oligonucleotide near the 3' end (67,72). hI-E2 and hI-E3C are predicted to amplify a 878 bp region from the intron-containing transgene construct, including 92 bp of exonic sequences and the 786 bp of intron 2.

To serve as an internal control for PCR screening, a 21 mer oligonucleotide, mI-E2, and a 22 mer oligonucleotide, mI-E3, were synthesized corresponding to sequences within the mouse insulin I and II genes. The mouse insulin II gene has a 3 exon-2 intron arrangement, similar to the arrangement of the human insulin gene (67,72). The mouse insulin I gene lacks intron 2, and has the sequences which correspond to exons 2 and 3 of the mouse insulin II gene within a single exon (72). mI-E2 and mI-E3 lie within exons 2 and 3 respectively of the mouse insulin II gene, and within exon 2 of the mouse insulin I gene. mI-E2 and mI-E3 are derived from sequences that have 100% homology between the mouse insulin I and II genes. mI-E2 was designed from the sense strand and has the sequence 5'GCCGTGAAGTGGAGGACCCAC3'; there are 6 mismatches between this sequence and the cognate sequence of the human insulin

gene, including changes at positions 17 and 18 of the oligonucleotide near the 3' end (67,72). mI-E3 was designed from the antisense strand and has the sequence 5'GCAGATGCTGGTGCAGCACTGA3'; there are 3 mismatches between this sequence and the cognate sequence of the human insulin gene, including changes at positions 19 and 22 of the oligonucleotide near the 3' end (67,72). mI-E2 and mI-E3 are predicted to amplify a 625 bp region from the mouse insulin II gene, including 137 bp of exonic sequences and the 488 bp of intron 2. The amplified region from the mouse insulin I gene is predicted to be 131 bp, since this gene lacks intron 2 and has a 6 bp deletion in the C peptide region (72). The four oligonucleotides described above (hI-E2 T_m 68.5, hI-E3C T_m 68.5, mI-E2 T_m 69.7, mI-E3 T_m 68.4) have no extensive complementary sequences and calculated T_m s in the range 68.4 - 69.7 °C, thus allowing them to be used simultaneously.

For the PCR, 500 ng of genomic DNA was mixed with 20 pmol each of primers hI-E2 and hI-E3C, and 5 pmol each of primers mI-E2 and mI-E3, to a volume of 50 μ l in water. Primers were diluted to working concentrations of 10 - 20 pmol/ μ l with TE/10 (10 mM Tris.HCl, 0.1 mM EDTA pH 8.0). Mineral oil (Sigma M-5904), 100 μ l, was used to overlay the aqueous phase. For some experiments, positive controls containing cloned human insulin DNA were included. Positive controls were made to simulate transgenes present at 1, 10, and 100 copies per diploid genome by mixing 0.375 pg, 3.75

pg, and 37.5 pg, respectively, of the 4.5 kb L-PK:hIns construct (the same DNA preparation that was used for microinjection to generate transgenic mice) with 500 ng of normal FVB/N genomic DNA. Reactions were performed in 0.5 ml tubes using a DNA Thermal Cycler (Perkin Elmer Cetus). Initially, the samples were heated to 98°C for 10 min, then held at 80°C while a 50 μ l reaction mix was added to the aqueous phase. The final 100 μ l reaction contained 10 mM Tris.HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 2.5 units of Taq polymerase (Boehringer Mannheim). The initial annealing step was carried out at 65°C for 2 minutes, followed by an extension step at 72°C for 2 minutes. The reaction was completed by performing 34 cycles of 94°C for 45 seconds, 65°C for 1 minute, and 72°C for 1 minute. The samples were loaded on a 1.2% agarose (GIBCO BRL 5510UB) gel containing 0.5 μ g/ml ethidium bromide, electrophoresis was carried out at 100 volts in 1 X TBE buffer for 1-2 hours (MPH electrophoresis unit, IBI), and gels were photographed under UV illumination.

G. Screening of mice by PCR using tail lysates

For routine screening of mice, a procedure was optimized for performing PCR directly from lysates of tail biopsies. A 19 mer oligonucleotide, hI-I2, was synthesized corresponding to a sequence within intron 2 of the human insulin gene (67), beginning 146 bp upstream of exon 3. The

corresponding regions of the mouse insulin I (11 of 19 bp mismatch) and II (15 of 19 bp mismatch) genes (72) show no homology to the human intronic sequence. A 20 mer oligonucleotide, hI-E3A, was synthesized corresponding to a sequence within exon 3 of the human insulin gene (67), in a region with 100% homology to the mouse insulin I and II genes (72). hI-I2 was designed from the sense strand and has the sequence 5'CCTGGGTCCAGCCCGGCTG3'; hI-E3A was designed from the antisense strand and has the sequence 5'CTCCAGCTGGTAGAGGGAGC3'. hI-I2 and hI-E3A are predicted to amplify a 277 bp region from the intron-containing transgene construct, including 146 bp from intron 2 and 131 bp from exon 3. To serve as an internal control for PCR screening, primers specific for the mouse insulin genes were also included in PCR reactions. The four oligonucleotides described above (hI-I2 T_m 70.6, hI-E3A T_m 67.1, mI-E2 T_m 69.7, mI-E3 T_m 68.4) have no extensive complementary sequences and calculated T_m s in the range 67.1 - 70.6 °C, thus allowing them to be used simultaneously.

Tail lysates were prepared by cutting a 7 - 10 mm section from the end of the mouse tail and allowing it to fall directly into a 1.5 ml tube containing 0.2 ml of tail lysis buffer (100 mM Tris.HCl pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 μ g/ml Proteinase K). Since the high sensitivity of the PCR can result in false positives by cross-contamination, rigorous care was taken in washing utensils between mice and the manipulation of tail samples

was reduced to a minimum. The tail samples were lysed by incubation for 5 hours in a 55°C waterbath, and then stored at 4°C for at least 1 hour prior to use. Samples stored for up to a month at 4°C also gave adequate results. Prior to setting up reactions, the samples were centrifuged at 12,000 g for 5 minutes at room temperature to clear the lysate of insoluble material.

For the PCR, 1 μ l of tail lysate was mixed with 10 pmol each of primers hI-I2 and hI-E3A, and 0.4 pmol each of primers mI-E2 and mI-E3, to a volume of 10 μ l in water. Primers were diluted to working concentrations of 10 - 20 pmol/ μ l with TE/10 (10 mM Tris.HCl, 0.1 mM EDTA pH 8.0). Mineral oil (Sigma M-5904), 30 μ l, was used to overlay the aqueous phase. The reaction was performed in 0.5 ml tubes using a DNA Thermal Cycler (Perkin Elmer Cetus). Initially, the samples were heated to 98°C for 10 min, then held at 80°C while a 10 μ l reaction mix was added to the aqueous phase. The final 20 μ l reaction contained 10 mM Tris.HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM dNTPs, and 0.5 units of Taq polymerase (Promega M1861). The reaction was completed by performing 30 cycles of 94°C for 45 seconds, 65°C for 1 minute, and 72°C for 1 minute. The samples were loaded on a 2% agarose (Metaphor Agarose, FMC 50182) gel containing 0.5 μ g/ml ethidium bromide, electrophoresis was carried out at 100V in 1X TBE buffer for 1-2 hours (MPH electrophoresis unit, IBI), and the gel was photographed under UV illumination.

H. Isolation of total RNA from mouse tissues

Total cellular RNA was isolated from various mouse tissues using TRI Reagent (Molecular Research Center, TR-118), which is an RNA/DNA/Protein isolation reagent developed from the acid guanidinium thiocyanate-phenol-chloroform method for RNA isolation (73,74). For some isolations, the UltraSpec reagent was used (BIOTECX BL-10050). Mice were sacrificed by cervical dislocation and freshly dissected tissue samples were rinsed under a thin stream of distilled water for 2 seconds. Dissecting equipment was rinsed frequently with distilled water to prevent cross-contamination of tissue samples. Tissue samples (typically 12 per experiment) of 50 - 100 mg were homogenized in 0.5 ml of TRI Reagent in a 50 ml conical polypropylene tube for 45 seconds using a polytron homogenizer (Kinematica CH-6010, setting 5). The tip of the homogenizer was rinsed thoroughly with a thin stream of distilled water after homogenization of each sample. After homogenization samples were kept at room temperature for 5 minutes. Samples were then placed on ice for a maximum of 60 minutes until all dissected tissue samples were homogenized and ready for phase separation. Homogenates were then transferred to a 1.5 ml microfuge tube containing 0.1 ml of chloroform, vortexed for 1 second, and kept at room temperature until all samples were combined with

chloroform. Samples were then vortexed again for 20 seconds (3 tubes at a time) and kept at room temperature for 2 minutes. For phase separation, the samples were centrifuged at 12,000 g for 15 minutes at 4 °C. A 0.2 ml portion of the upper aqueous phase was transferred to a 1.5 ml tube containing 20 µl of isopropanol, the samples were vortexed for 1 second, and then kept at room temperature for 5 minutes to precipitate contaminating genomic DNA. The samples were centrifuged at 12,000 g for 10 minutes at 4 °C, then 180 µl of the supernatant was transferred to a 1.5 ml tube containing 200 µl of isopropanol. The samples were inverted to mix, kept at room temperature for 5 minutes, and centrifuged at 12,000 g for 5 minutes at 4 °C to pellet the RNA precipitate. Most of the supernatant was carefully decanted away, the tubes were spun in a microfuge for 5 seconds, and the remaining supernatant was removed with a pipette tip. After air drying the pellets for about 5 minutes, the RNA pellet was resuspended in 54 µl of RNase free water by pipetting up and down approximately 20 times. A 4 µl aliquot was mixed with 396 µl of water and absorbances at 260 nm, 280 nm, and 320 nm were obtained using a Beckman DU-64 spectrophotometer. RNA concentration was determined using the formula: RNA concentration (ug/ml) = $(-36.0 \times (A_{280} - A_{320})) + (62.9 \times (A_{260} - A_{320}))$ (75). RNA samples were either used immediately or stored at -70 °C until the time of assay.

I. Human insulin RT-PCR with mouse β -actin internal control

Primers hI-E2 and hI-E3C are predicted to amplify a 92 bp region from the properly spliced mRNA product of the transgene. The antisense primer hI-E3A was used to prime cDNA synthesis by reverse transcriptase. To serve as an internal control, two 19 mer oligonucleotides, mBA1 and mBA2, were synthesized corresponding to sequences within exon 2 of the mouse β -actin gene (76). mBA1 was designed from the sense strand and has the sequence 5'GGGTGACGAGGCCAGAGC3'; mBA2 was designed from the antisense strand and has the sequence 5'CTCCTCAGGGGCCACACGC3'. mBA1 and mBA2 are predicted to amplify a 139 bp region from mouse β -actin mRNA. An additional antisense primer, the 20 mer mBA3 (5'GGCTACGTACATGGCTGGGG3'), was synthesized corresponding to sequences within exon 3 of the mouse β -actin gene (76). The primer mBA3 was used to prime cDNA synthesis by reverse transcriptase. The four oligonucleotides used during the amplification step of the assay (hI-E2 T_m 68.5, hI-E3A T_m 67.1, mBA1 T_m 68.5, mBA2 T_m 68.5) have no extensive complementary sequences and calculated T_m s in the range 67.1 -68.5 °C, thus allowing them to be used simultaneously.

For the reverse transcription, 2 μ g of total RNA was mixed with 0.4 pmol of hI-E3A, 0.01 pmol of mBA3, 10 mM Tris.HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.4 mM dNTPs, 0.1 mg/ml BSA, 5 mM DTT, 16 units of RNasin ribonuclease

inhibitor (Promega N2511), and 40 units Superscript reverse transcriptase (GIBCO BRL 18053-017) to a volume of 10 μ l in RNase free water. The reaction was performed in 0.5 ml tubes using a DNA Thermal Cycler (Perkin Elmer Cetus). The aqueous phase was overlaid with 30 μ l of mineral oil (Sigma M-5904). For reverse transcription, the samples were incubated at 20°C for 10 minutes, followed by 42°C for 50 minutes. The samples were heated to 98°C for 5 minutes, then held at 80°C while a 10 μ l PCR reaction mix was added to the aqueous phase. The PCR reaction mix contained 40 pmol each of primers hI-E2 and hI-E3C, 1 pmol each of primers mBA1 and mBA2, 10 mM Tris.HCl pH 8.3, 50 mM KCl, 0.63 mM MgCl₂, and 0.5 units of Taq polymerase (Boehringer Mannheim 1146 165) in water. For some experiments, 2 microcuries of ³³P-alpha-dATP (Dupont NEN, NEG-312H) was included during the amplification step to label the PCR products. The reaction was completed by performing 30 cycles of 94°C for 45 seconds, 65°C for 1 minute, and 72°C for 1 minute. For reactions without ³³P labeling, the samples were incubated with 50 μ g/ml DNase-free RNase A for 30 minutes at 37 °C and loaded on a 3% agarose (Metaphor Agarose, FMC 50182) gel containing 0.5 μ g/ml ethidium bromide. Electrophoresis was carried out at 100 volts in 1 X TBE buffer for 2 hours (MPH electrophoresis unit, IBI) and the gel was photographed under UV illumination. For reactions with ³³P labeling, the samples were loaded on an 8% polyacrylamide gel (20 cm X 20 cm X 0.75 mm) containing 5

M urea and electrophoresis was carried out at 20 milliamperes in 1 X TBE buffer for 2 hours (VCV electrophoresis unit, IBI). The gel was then vacuum dried and exposed to X-ray film (Kodak XAR, 1651454) for 21 hours at -70 °C.

J. Quantitative RT-PCR assays

An in vitro transcribed insulin template was prepared from a cloned section of the transgenic insulin mRNA in the following manner. Transgenic liver RNA was subjected to RT-PCR as described above; however, the antisense primer hI-E3A was used both to prime reverse transcriptase and in the amplification reaction with the sense primer hI-E2. The 153 bp cDNA product was isolated on a 2% agarose gel (Metaphor Agarose, FMC 50182) and ligated to the pGEM-T vector (Promega A3600) according to the manufacturer's instructions. The pGEM-T vector is a linearized plasmid which contains single 3'-terminal thymidine residues. Since Taq polymerase catalyzes the addition of a single 3'-terminal adenosine residue, PCR products can easily be ligated directly to the pGEM-T vector. The cloned 153 bp region was sequenced to confirm its nature and determine its orientation in the plasmid cloning site. To synthesize a sense RNA strand, the plasmid was linearized with Not I prior to in vitro transcription with T7 RNA polymerase. A 50 µl transcription reaction was assembled, which contained

1 μ g of linearized template, 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine-HCl, 10 mM DTT, 100 μ g/ml BSA, NTPs at 0.5 mM each, and 50 units of T7 RNA polymerase (NEB 251S). The reaction was carried out at room temperature for 2 hours, then 2 units of RQ1 Dnase (Promega M6101) was added and the template DNA was eliminated by incubating at 37 °C for 90 minutes. The synthesized RNA was recovered by phenol-chloroform extraction and ethanol precipitation then resuspended in RNase-free water. The RNA was electrophoresed on 2% Metaphor agarose to confirm its size and was quantified by spectrophotometry. Aliquots were stored at -70 °C until the time of assay. The RNA was diluted in total RNA isolated from normal mouse liver to make standards for the quantitative RT-PCR assay.

For the RT-PCR assay, 1 μ g of total RNA (added in a volume of 4 μ l) from various mouse tissues or standards were reverse transcribed with the 0.4 pmol of primer hI-E3A in a reaction containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, 16 units of RNasin ribonuclease inhibitor (Promega N2511), and 120 units of MMLV reverse transcriptase (GIBCO BRL 28025-013) to a volume of 10 μ l in RNase free water. The reaction was performed in 0.5 ml tubes using a DNA Thermal Cycler (Perkin Elmer Cetus). The aqueous phase was overlaid with 30 μ l of mineral oil (Sigma M-5904) and assembled reactions were kept on ice until all tubes were ready for the initiation of the reaction. The samples were incubated at 37 °C for 30

minutes, heated to 98 °C for 5 minutes, then held at 80 °C while a 10 µl PCR reaction mix was added to the aqueous phase. PCR was carried out using the sense primer hI-E2 and the antisense primer hI-E3B (5'GAGGGAGCAGATGCTGGTACA3') which amplify a 139 bp region from spliced insulin mRNA. The PCR reaction mix contained 20 pmol each of primers hI-E2 and hI-E3B, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1 % Triton X-100, 2 units of Taq polymerase (Promega M1861), and 4 microcuries of ³²P-α-dCTP (Dupont NEN, NEG-013H) in water. The reaction was completed by performing 30 cycles of 94 °C for 45 seconds, 65 °C for 1 minute, and 72 °C for 1 minute. A portion of the completed reaction, 12 µl, was removed from under the mineral oil and mixed with 8 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). A 10 µl portion was loaded on an 8% polyacrylamide gel (20 cm X 20 cm X 0.75 mm) containing 5 M urea and electrophoresis was carried out at 20 milliamperes in 1 X TBE buffer for 2 hours (VCV electrophoresis unit, IBI). The gel was wrapped carefully in plastic wrap and exposed to X-ray film (Kodak XAR, 1651454) for 0.5 - 3 hours at -70 °C. Bands were quantified by densitometry using Quantity One software on a Sun computer workstation.

Mouse β-actin mRNA was quantified in separate reactions as described above, except that the antisense primer mBA3 was used for reverse transcription, and primers mBA1 and mBA2 were used for the PCR.

K. Ten minute insulin mRNA experiment

Transgenic mice feeding on the chow diet were fasted for 6h prior to intraperitoneal injection of 4 g/kg glucose or water. Ten minutes after injection the mice were bled for 30s to 60s, then immediately sacrificed for dissection of liver samples. For glucose measurements, blood was collected in heparinized capillary tubes and immediately placed on ice. Plasma was separated by centrifugation and assayed for glucose by the hexokinase method (Sigma #16-50).

L. Antibodies and peptide standards

Antibodies and standards used for the measurement of insulin, human C peptide and mouse C peptide were obtained from Linco Research. All antibodies were raised in guinea pigs. A rat insulin antibody (#1013) with 100% crossreactivity to human and mouse insulins was used to measure total insulin-like immunoreactivity. Human C peptide immunoreactivity was measured using an antibody with less than 4% crossreactivity to human proinsulin and undetectable crossreactivity to mouse C peptide (#1020). A rat C peptide antibody (#1021) with 100% crossreactivity to mouse C peptide, less than 0.1% crossreactivity to human C peptide, and less than 0.01% crossreactivity to human proinsulin was used to measure mouse C-peptide immunoreactivity. Lyophilized antibodies

were dissolved in 20 ml of antibody buffer, dispensed into 1 ml aliquots, and stored at -20 °C. One liter of antibody buffer was prepared by dissolving 4.6 g sodium phosphate dibasic, 0.91 g sodium phosphate monobasic, and 1 g bovine serum albumin (BSA, RIA grade, Sigma A-7888) in water and adjusting the pH to 7.4. Purified human insulin (#8014), human C peptide (#8020), and rat C peptide (#8021), were also obtained for use as standards in competitive ELISAs. Rat and mouse C peptide I differ at amino acid positions 8 and 15 (there are total of 31 residues in rat, mouse, and human C peptides); rat and mouse C peptide II differ only at position 30). The insulin standard was diluted in insulin calibrator, which is human serum that is processed to remove insulin (Diagnostic Products 10INZ). The C peptide standards were diluted in C peptide calibrator, which is human serum that is processed to remove C peptide (Diagnostic Products 25PEZ).

M. Preparation of mouse serum

Mice were fasted for 24 hours and then refed with the high sucrose diet for 26 hours. Mice were placed in aluminum container under a heat lamp for 2 minutes and then bled by cutting the end of the tail with a razor blade. Blood was collected (100 - 200 µl) in 1.5 ml polypropylene tubes and allowed to stand for 15 minutes at room temperature for clot formation. Samples were centrifuged at

12,000 g for 2 minutes at room temperature to separate the serum from the clot. Aprotinin (Boehringer Mannheim 236624) was added to the serum at a concentration of 500 KIU/ml, and the samples were stored at -20 °C until the time of assay.

N. Enzyme linked immunosorbent assay (ELISA)

Competitive ELISAs were used to quantify the amounts of specific proteins in mouse sera. Goat anti-guinea pig IgG (Pierce 31114, 2.6 mg/ml), 0.5 µl diluted in 150 µl of coating buffer, was attached to the well surface of a microtiter plate (Pierce 15040, 15038) by incubating for 2 hours in a 37 °C cabinet incubator. One liter of coating buffer was prepared by dissolving 1.59 g sodium carbonate, 2.93 g sodium bicarbonate, and 0.2 g sodium azide in water and adjusting the pH to 9.6. The plate was washed by dispensing 200 µl of wash buffer into each well with a multichannel pipettor, immediately emptying the plate by inverting it, and blotting 3 times on a paper towel. A total of 3 washes was performed at this step. One liter of wash buffer was prepared by dissolving 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g sodium phosphate dibasic, 0.2 g potassium phosphate monobasic, and 0.5 % Tween 20 (Pierce 28320) in water and adjusting the pH to 7.2. For successive washes, the plate rows were alternately processed from top to bottom followed by from bottom to top. Insulin or C peptide antibodies, 10 µl diluted in 100 µl of antibody

buffer, were then applied to the wells and the plate was again incubated at 37 °C for 2 hours. To check for non-specific binding, some wells did not receive primary antibodies. After washing the plate four times, non-specific binding sites were blocked by dispensing 150 µl of blocking buffer into each well and storing at 4 °C for 18 - 24 hours. Blocking buffer was prepared by dissolving 5.9 g RIA grade BSA, 0.6 g sodium chloride, and 0.05 % Tween 20 in 100 ml of antibody buffer and adjusting the pH to 7.4. Particulates were removed from the blocking buffer by syringe filtration (0.22 µm glass/cellulose acetate, Denville Scientific F5713).

After the blocking step, the plate was washed 4 times and 100 µl of insulin or C peptide calibrator was dispensed into each well. Mouse serum samples and peptide standards, 20 µl, were then added to individual wells and the plate was stored at 4 °C for 24 - 30 hours to allow binding to the fixed primary antibody. After washing the plate 4 times, 100 pg of biotinylated insulin (bovine, Sigma I2258) or 50 pg of biotinylated C peptides (Peninsula) was added to each well in 100 µl of blocking buffer. The plate was stored at 4 °C for 14 - 18 hours to allow binding of biotinylated peptides to unbound antibody sites. The plate was then washed 6 times prior to detection of the bound biotinylated peptides.

To form the detecting enzyme conjugate (for a 42 well experiment), 24 µl of biotinylated horseradish peroxidase

(Pierce 29139, 100 µg/ml in peroxidase stabilizer, Pierce 31503) was added to a 1.5 ml polypropylene tube containing 48 µl of PBS (100 mM phosphate pH 7.2, 150 mM sodium chloride). Then, 48 µl of neutravidin (Pierce 31000, 100 µg/ml in peroxidase stabilizer) was added, the tube was vortexed for 1 second, and the tube was allowed to stand at room temperature for 30 minutes. The conjugate was then diluted in 4.8 ml of blocking buffer and 100 µl of the mixture was dispensed into each well of the washed microtiter plate. The plate was allowed to stand at room temperature for 30 minutes to allow binding of the enzyme conjugate to fixed biotinylated peptides. After washing the plate 6 times, 100 µl of the peroxidase substrate tetramethylbenzidine (TMB, Pierce 34022) was added and the plate was kept at room temperature for 2 - 5 minutes. To stop the peroxidase reaction, 100 µl of 1 M sulfuric acid was added to each well. Absorbance at 540 nm was measured using a microtiter plate reader (Dynatech). % Bound was calculated using the formula $\% \text{ Bound} = ((X - \text{NSB}) / (\text{MAX} - \text{NSB})) * 100$, where X is the A540 reading for the unknown sample or standard, NSB is the A540 reading of the non-specific binding wells, and MAX is the A540 reading from wells for which only biotinylated peptides were added. % Bound of standards was plotted versus log(standard concentration) and standard curves were generated using the four parameter logistic equation with Prism 2.0 software (GraphPad).

O. Immunfluorescence

Mice were fasted for 24 hours and then refed for 26 hours with the high sucrose diet. Mice were perfused through the heart with 4% paraformaldehyde for approximately 5 minutes, then pieces of liver and pancreas were dissected and placed in 4% paraformaldehyde for 4 hours at room temperature. The tissue samples were then rinsed with 20% sucrose and stored in fresh 20% sucrose at 4 °C overnight. Tissue samples were cut into cubes, covered with freezing medium (Triangle Biomedical H-TFM), and frozen in a methylbutane-dry ice bath. After storage overnight at -20 °C, sections were cut with a cryostat and placed on microscope slides coated with poly-L-lysine. Detection was carried out using an insulin immunofluorescence kit (Peninsula IFK-7303G) according to the manufacturer's instructions. Processed slides were observed with a darkfield microscope for incident light fluorescence.

P. Plasma glucose measurement

Blood was drawn by cutting a small section from the tail tip with a razor blade. For each glucose determination, approximately 8 microliters of blood was collected in heparinized capillary tubes (Baxter B4393-1), transferred to a 0.5 ml polypropylene centrifuge tube, and immediately placed on an ice-water bath. Plasma was

separated by centrifugation at 12,000 g for 30 seconds at room temperature and 2 μ l was assayed for glucose by the hexokinase method, in reactions using 750 μ l of reagent (Sigma 16-50). Glucose standards were made by dissolving glucose in phosphate buffered saline pH 7.4 containing 6% bovine serum albumin.

Q. Glucose tolerance tests

Mice were fasted without food or water for 14 hours and then administered a 50% glucose solution equivalent to 1.75 milligrams of glucose per gram of body weight. The glucose solution was given by allowing the mice to drink from a round-ended 18 gauge 1.5 inch needle (Tiemann 160-8904) attached to a 1 ml syringe. Mice consumed the glucose load within 30 to 45 seconds from the initiation of drinking. Mice were placed in a plastic rodent restrainer (Plas-Labs 551BSRR) for the duration of the test, a maximum of 2 hours.

R. Streptozotocin treatment

Mice feeding on the chow diet were fasted for 16 hours prior to the injection of streptozotocin (STZ, Sigma S0130). Mice were weighed after the fasting period to determine the amount of STZ to be administered. STZ, 50 mg, was dissolved in 3.5 ml of cold citrate buffer pH 4.5 by inverting the container 6 times and pipetting up and down 20 times with a P1000 tip. Three non-transgenic and three transgenic mice

were alternately injected with STZ within 3 minutes of dissolution in the citrate buffer. A new 50 mg STZ vial was used for each group of six mice. Injections were given intraperitoneally using a 30 gauge needle attached to a 1 ml syringe. Untreated mice were injected with buffer only. After injection the mice were fasted for an additional 3 hours and then allowed to feed on the chow diet for the duration of the experiment. Three mice were kept in each cage. Measurements of water and food consumption were based on the total consumption by a cage of three mice for the given time period.

IV. Results

A. Construction of the rat L-PK:human insulin fusion gene

To enable us to study hepatic insulin production in transgenic mice, a fusion gene was constructed using the rat liver-type kinase promoter to drive expression of human insulin coding sequences (See Methods IIIA). A 12.5 kb human genomic clone containing the insulin gene (67) was obtained. The coding region was isolated as a 1.3 kb fragment spanning the region from 2 bp upstream of the start codon in exon 2 to 132 bp downstream of the end of exon 3 (Figure 3). This region contains the sequences that code for the 110 amino acids that comprise preproinsulin (67). The sequences that code for the 24 amino acid signal peptide, the 30 amino acid B chain and the first 8 amino acids of the C peptide are contained in exon 2. The sequences that code for the remaining 27 amino acids of the C peptide and the 21 amino acid A chain are contained in exon 3. Exons 2 and 3 are interrupted by the 786 bp intron 2. Since the presence of introns has been shown to enhance the expression of transgenes, intron 2 was included in the construct. Intron 2 of the human insulin gene has not been shown to contain transcriptional regulatory elements. The construct also includes the polyadenylation site located 76 bp downstream of the stop codon in exon 3.

The human insulin coding region was fused to a 3.2 kb fragment from the promoter of rat liver-type pyruvate kinase gene. The 3.2 kb fragment, which spans the region from -

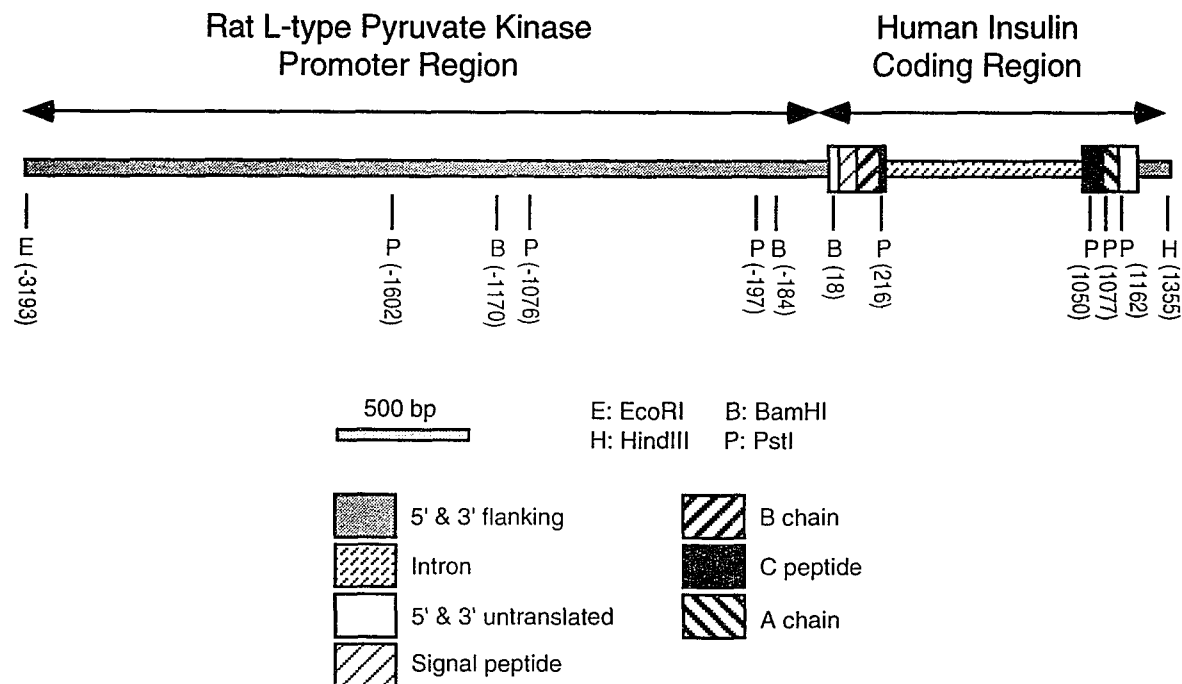


Figure 3. Structure and restriction map of the L-PK:insulin transgene. The fusion construct is a 4.5 kb EcoRI/HindIII fragment. The positions of the 5' and 3' flanking regions, the regions coding for preproinsulin, and the single intron are shown. The location of restriction sites for EcoRI, BamHI, PstI, and HindIII are given relative to the transcription start site.

3193 to +12 with respect to the transcription start site, has been shown to be sufficient to confer tissue-specific and carbohydrate regulation to a heterologous reporter in transgenic mice (40).

The L-PK:insulin fusion gene is a 4.5 kb EcoRI/HindIII fragment which is designated PI. The structure of PI and a predicted restriction map are shown in Figure 3. The structure of the transgene was confirmed by diagnostic BamHI and PstI restriction digests (Figure 4A). The 4.5 kb EcoRI/HindIII fragment (PI) is shown in lane 3. BamHI digestion of PI predicts fragments of 2024 bp, 1338 bp, 987 bp, and 202 bp. The confirmatory BamHI digest of PI is shown in lane 4. PstI digestion of PI predicts fragments of 1592 bp, 880 bp, 835 bp, 527 bp, 413 bp, 197 bp, 86 bp, and 28 bp. The 413 bp PstI fragment spans the junction between the L-PK promoter and the insulin coding region. The confirmatory PstI digest of PI is shown in lane 5. Fragments less than 300 bp were not resolvable in this gel. For fragments greater than 300 bp (3 BamHI fragments and 5 PstI fragments), the pattern of restriction fragments seen matches the predicted structure of PI. Thus, construction of the L-PK:insulin fusion gene was confirmed.

After purification of the plasmid pPI by CsCl-ethidium bromide gradient centrifugation, the 4.5 kb EcoRI/Hind III fragment was isolated. The isolated PI fragment is shown in Figure 4B, lanes 2 and 3. The characteristic BamHI digest is shown in lane 4. The PI fragment was diluted to a

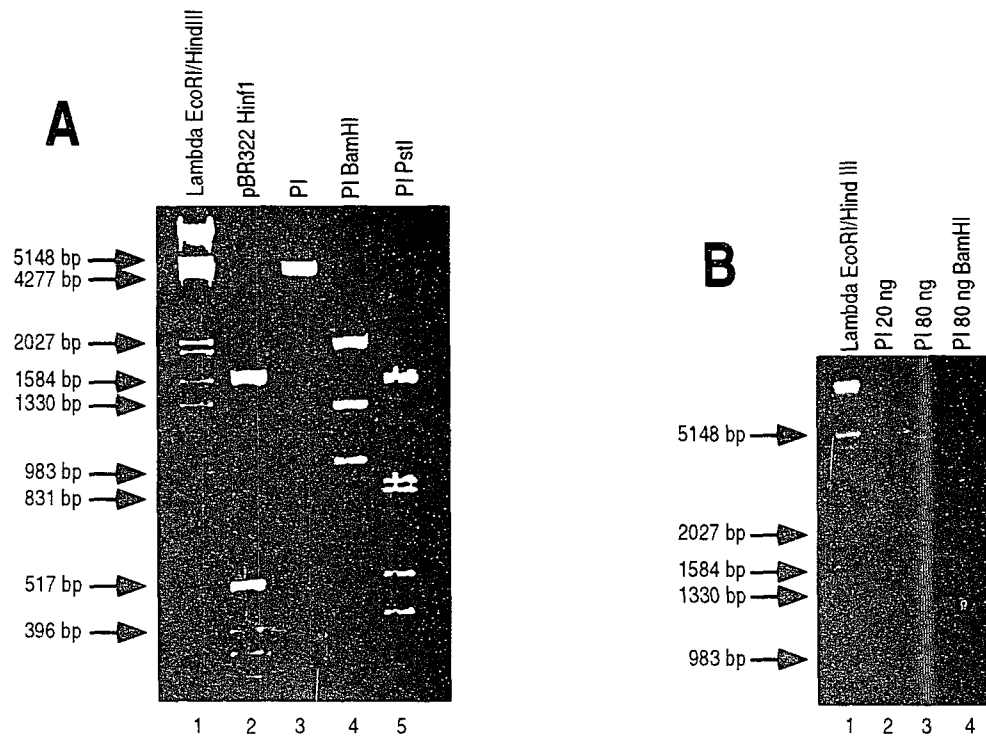


Figure 4. Confirmation of transgene structure.

A, The transgene is a 4.5 kb EcoRI/HindIII fragment (PI, lane 3). BamHI and PstI digests of PI are shown in lanes 4 and 5. The arrows show the positions of marker bands in lanes 1 and 2.

B, Verification of the purified PI fragment prior to microinjection of mouse embryos. Lanes 2 and 3 show the PI fragment (20 ng, 80 ng). Lane 4 shows a BamHI digest of the PI fragment. The arrows show the positions of marker bands in lane 1.

concentration of approximately 1000 molecules/pl prior to microinjection of mouse embryos.

B. Functional expression of human insulin in HepG2 cells

The L-PK:insulin construct was tested by transient transfection of HepG2 cells, a human hepatocellular carcinoma cell line. Hepatoma cell lines, including HepG2, do not show carbohydrate regulation of the L-PK gene, a property that has only been observed in vivo and in primary hepatocyte cultures (49). However, HepG2 cells have previously been shown to express L-PK:CAT constructs at high levels (48,77). Thus, HepG2 cells are partially useful for examining expression of L-PK promoter constructs. We employed HepG2 cells to determine if the transfected L-PK:insulin construct could produce functional insulin/proinsulin.

Rat hepatoma (FAO) cells have been shown to secrete insulin immunoreactive material by the constitutive pathway after transfection with a RSV:human insulin construct (24,78). Since secreted insulin/proinsulin would be expected to rapidly associate with cell surface insulin receptors and activate signal transduction pathways, functional expression may be assessed by measuring an appropriate physiological response. In hepatocytes, a major action of insulin is the modulation of metabolic pathways involved in carbohydrate and fat metabolism. Insulin action leads to activation of glucokinase, glycogen synthase,

phosphofructokinase-1, and the pyruvate dehydrogenase complex, resulting in increased glucose uptake and increased glucose utilization by the pathways of glycolysis and glycogen synthesis (79). Insulin also acts as a growth stimulator, which may result increased mitotic activity of cultured cells (79). Increased cellular growth rate would also be expected to increase glucose utilization. In this experiment, glucose utilization by transfected cells was measured by determining the decrease in extracellular glucose concentration. We expected that secretion of insulin/proinsulin from transfected cells would stimulate glucose utilization.

HepG2 cells were transfected with three separate plasmid constructs: the vector without an insert, the intact human insulin gene (ghIns), and the L-PK:insulin construct (L-PK:hIns). After transfection using DNA-liposome complexes, the cells were cultured in serum-free medium, so that there was no exogenous source of insulin. As shown in Figure 5, there was a significant decrease in medium glucose concentration when cells were transfected with ghIns and L-PK:hIns ($p < 0.05$) as compared to the control vector transfection. The most likely interpretation of this result is that insulin/proinsulin material is being secreted from the cells, binding cell surface receptors, and stimulating glucose utilization and cell growth. Insulin action by an intracellular mechanism is possible, but seems unlikely. The most reasonable conclusion is that L-PK:hIns transfected

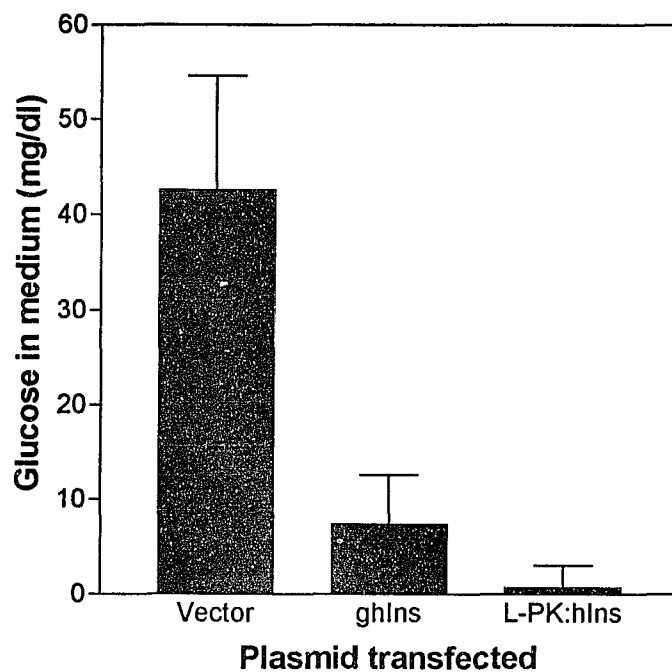


Figure 5. Glucose utilization of insulin transfected HepG2 cells. HepG2 cells were transiently transfected with 3 separate plasmids: Vector, the cloning vector pGEM3zf+; ghIns, the intact human insulin gene in pGEM3zf+; L-PK:hIns, the L-PK:human insulin fusion gene in pGEM11zf-. Transfected cells were cultured in serum-free medium without insulin for 40 hours and the glucose concentration of the medium was determined.

HepG2 cells are capable of producing insulin/proinsulin material that is functional.

C. Establishment of L-PK:hIns transgenic mouse lines

Transgenic mice were produced by pronuclear microinjection of the 4.5 kb L-PK:hIns fragment into mouse embryos (See Methods IIIC & IIID). The highly inbred FVB/N strain (70) was used to generate transgenic mice. A homogeneous genetic background was expected to be advantageous, since genetic variability in response to dietary and pharmacologic manipulations would be minimized. In addition, to minimize environmental variation, transgenic lines were maintained in a heterozygous state, so that normal littermates could be used as controls for all experiments.

For identification of transgenic founders, polymerase chain reaction (PCR) was performed using genomic DNA isolated from tail biopsies (See Methods IIIE & IIIF). The strategy for PCR is shown in Figure 6, with primers hI-E2 and hI-E3C predicted to amplify a 878 bp region from the L-PK:hIns transgene. The screening process employed an internal control amplification of the mouse insulin genes using primers mI-E2 and mI-E3. Primers mI-E2 and mI-E3 are predicted to amplify a 131 bp region from the mouse insulin I gene and a 625 bp region from the mouse insulin II gene. Primer sets were designed from sequences that differed between the human and mouse genes (See Methods IIIF).

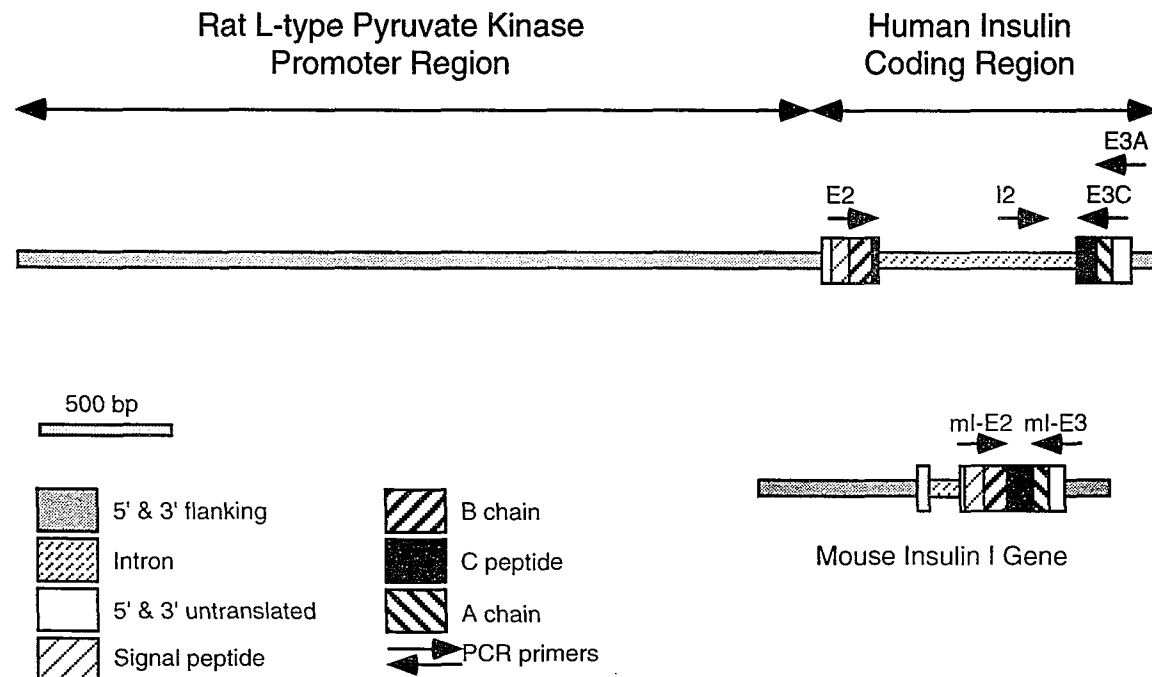


Figure 6. Strategy for identification of transgenic mice. The transgene is shown with arrows indicating the positions of PCR primers hI-E2, hI-I2, hI-E3A, and hI-E3C, which were used to detect human insulin sequences in transgenic mice. The screening process employed an internal control amplification of the mouse insulin I gene using primers mI-E2 and mI-E3.

The specificity of the human insulin primers hI-E2 and hI-E3C was tested by performing PCR using human insulin plasmid DNA diluted in normal FVB/N mouse genomic DNA (Figure 7A). Lanes 2 and 3 show detection of 160 fg and 16 fg, respectively, of the 878 bp target region diluted in 500 ng of normal genomic DNA. The sensitivity for detection of the plasmid DNA template was between 16 fg and 1.6 fg, since 1.6 fg and 0.16 fg of template were not detected (lanes 4 and 5). This sensitivity appears more than adequate for identification of transgenic mice, since 500 ng of genomic DNA containing a single copy transgene would present approximately 73 fg of the target region. However, the sensitivity for plasmid DNA template and genomic DNA template may not be equivalent.

Optimization of primer concentrations was essential to the development of an internally controlled PCR. Optimization of the mouse insulin primer concentrations is shown in Figure 7B. The concentration of the mouse primer set was varied between 4 and 16 pmol per 20 μ l reaction, while the concentration of the human primer set was held constant at 20 pmol per reaction. The 131 bp band corresponding to the mouse insulin I gene was not detected at 4 pmol (lanes 1 and 7), but was detected with increasing intensity from 8 to 16 pmol (lanes 2-4, 8-10). However, detection of the 878 bp human insulin target was influenced by the mouse primer concentration. Higher concentrations of mouse primers were associated with decreasing intensity of

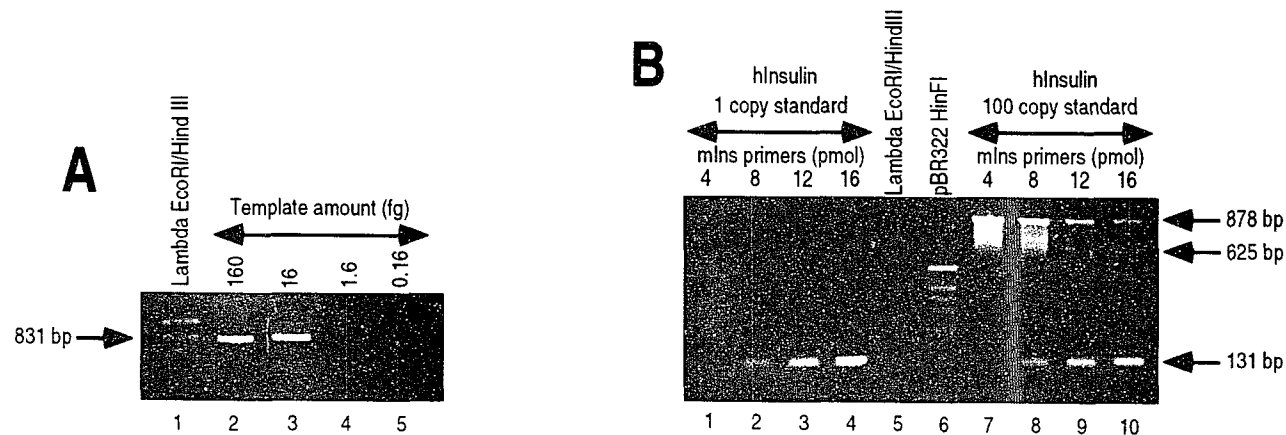


Figure 7. Optimization of PCR screening process for identifying transgenic mice. A, Plasmid DNA containing human insulin sequences was amplified with PCR primers hI-E2 and hI-E3C. Lanes 2-5 show PCR amplification of serial tenfold dilutions of the insulin template in normal mouse genomic DNA. Lane 1 contains DNA markers. B, Optimization of primer concentration for the internal control amplification of mouse insulin gene sequences. Transgene standards (1 copy or 100 copies) were amplified with human insulin primers hI-E2 and hI-E3C, as well as mouse insulin primers mI-E2 and mI-E3. The concentration of the mouse insulin primers was varied as shown in lanes 1-4 and 7-10. Arrows show the predicted sizes of PCR products from human insulin sequences (878 bp), the mouse insulin II gene (625 bp), and the mouse insulin I gene (131 bp). Lanes 5 and 6 contain DNA markers.

the 878 bp band (lanes 7-10). This could result from competition between mouse and human primers for annealing to target sequences; however, there is little homology between the human and mouse primers (See Methods IIIF). Another possibility is that amplification of two targets results in competition for other reaction components, such as Taq polymerase. Smaller amplification products appear to dominate larger products. The mouse insulin I product (131 bp) was strongly detected while the mouse insulin II product (625 bp) was weakly detected (lane 10). Also, when primer and target concentrations were approximately equivalent for mouse and human amplifications (lane 4), the 131 bp mouse band is strongly detected while the 878 bp human band is not detected at all. These results suggest that many factors should be considered in developing an internally controlled assay, including primer concentrations, primer homologies, size of amplification products, and concentration of other reaction components such as Taq polymerase.

Once PCR conditions were optimized, founder litters were screened for mice carrying the L-PK:hIns transgene. Ten founder litters (A-J) with a total of 57 mice were screened and 4 transgenic mice were identified. Litter C had 1 transgenic mouse and 4 normal mice, but all 5 mice died of unknown causes before weaning. The PCR screening of litter H is shown in Figure 8A. Mice H4, a female, and H6, a male, were positive for the transgene (lanes 4 and 6). The screening assay included a negative control

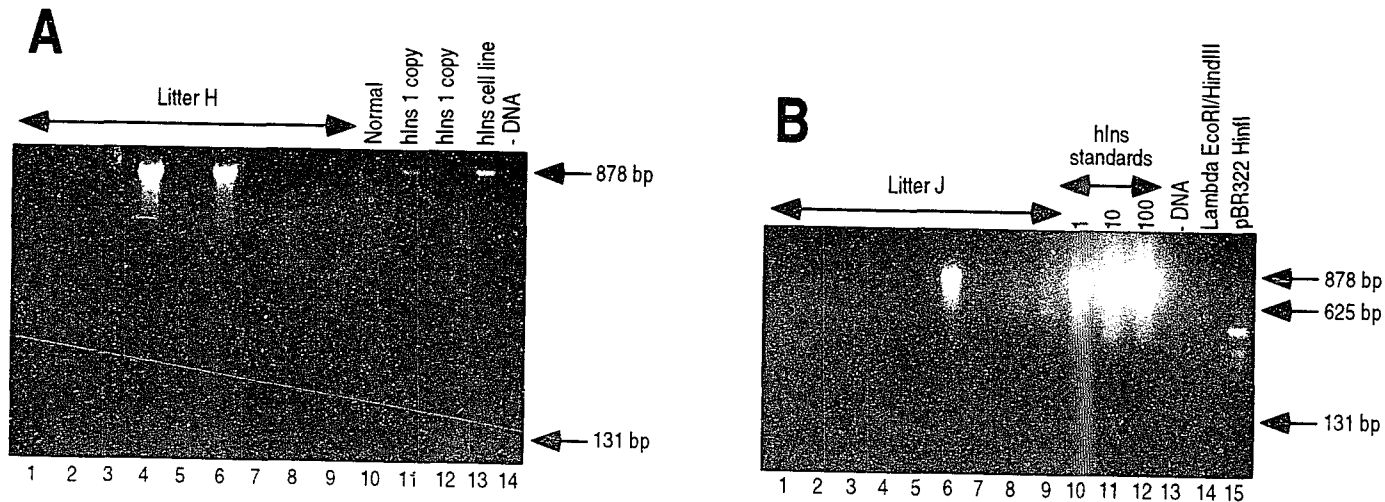


Figure 8. Identification of founder transgenic mice.

A, PCR screening of founder litter H identifying transgenic founders H4 (lane 4) and H6 (lane 6). Negative littermates are shown in lanes 1-3, 5, & 7-9. Amplification of normal mouse genomic DNA is shown in lane 10. Amplification of a 1 copy transgene standard is shown in lanes 11 and 12. Amplification of genomic DNA from a human insulin transfected cell line is shown in lane 13. Lane 14 shows amplification with no added DNA template. Arrows show the predicted sizes of PCR products from human insulin sequences (878 bp) and the mouse insulin I gene (131 bp).

B, PCR screening of founder litter J identifying transgenic founder J6 (lane 6). Negative littermates are shown in lanes 1-5 & 7-9. Amplification of 1, 10, and 100 copy transgene standards is shown in lanes 10-12. Lane 13 shows amplification with no added DNA template. Lanes 14 and 15 contain DNA markers. Arrows show the predicted sizes of PCR products from human insulin sequences (878 bp), the mouse insulin II gene (625 bp), and the mouse insulin I gene (131 bp).

amplification of normal genomic DNA (lane 10), a 1 copy transgene standard (lanes 11 and 12), and a reaction without added genomic DNA to control for possible cross contamination (lane 14). Lane 13 shows amplification of genomic DNA isolated from a mouse hepatocyte cell line, mhAT2 (77), that was stably transfected with the L-PK:hIns construct. The PCR screening of litter J is shown in Figure 8B, showing that mouse J6, a male, was also positive for the transgene.

The three transgenic founders, H4, H6, and J6, appeared healthy and each was bred to normal FVB/N mice to establish transgenic lines. The rates of transmission for the three lines are shown in Table 1. In the F1 generation, transmission rates were 16 % for H4, 33% for H6, and only 7% for J6. F1 transmission rates less than 50% could be due to mosaicism resulting from integration of the transgene after the one cell stage. A transmission rate of 25% would be predicted from an integration event at the two cell stage. Another possibility is that the transgene prevents normal development and birth of transgenic mice. However, there was no evidence that the transgene caused abnormal development. Litter sizes were normal in the J6 line, for which only 2 of 30 F1 mice carried the transgene. In addition, transmission rates for the F2 generation were 48% for H4, 57% for H6, and 50% for J6, which is consistent with a Mendelian pattern of inheritance of the transgene. Transgenic mice from all three lines appeared normal; as

compared to nontransgenic littermates, no differences were observed with regard to food or water intake, size, level of activity, health, or sexual behavior.

Since we intended to carry out studies using heterozygous lines, we wanted to develop an efficient process for the routine screening of transgenic and nontransgenic mice. A procedure was optimized for

Table 1. Establishment of L-PK:hIns transgenic lines

Line	F1 screened	F1 (%) transgenic	F2 screened	F2 (%) transgenic
H4	32	16%	23	48%
H6	36	33%	28	57%
J6	30	7%	24	50%

performing PCR directly from lysates of tail biopsies, thus eliminating the need for isolation of genomic DNA (See Methods IIIG). To avoid inhibition of Taq polymerase by SDS in the lysis buffer, SDS was used at a concentration of 0.2%. Since 1 μ l of tail lysate was used in a 20 μ l reaction, the final SDS concentration was 0.01%. To compensate for chelation of magnesium ions by EDTA in the lysis buffer, magnesium chloride was used at a concentration of 2.5 mM in the PCR, instead of the 1.5 mM concentration used for amplification of purified genomic DNA. The PCR

employed human insulin primers hI-I2 and hI-E3A (Figure 6), which amplify a 277 bp region of the transgene.

Amplification of the shorter 277 bp product was associated with increased band intensity compared with the 878 bp product amplified by primers hI-E2 and hI-E3C. The assay included an internal control amplification of the 131 bp region from the mouse insulin I gene using primers mI-E2 and mI-E3. The concentration of hI-I2 and hI-E3A was 10 pmol each per 20 μ l reaction; the concentration of mI-E2 and mI-E3 was 0.4 pmol each per 20 μ l reaction.

An example of the lysate screening PCR is shown in Figure 9. Amplification of a normal lysate (lanes 2-4) shows detection of the 131 bp band only in the presence of the mouse primer set (lanes 2 and 4). Amplification of a transgenic lysate (lanes 6-8) shows detection of the 277 bp band in the presence of the human primer set (lanes 7 and 8), and detection of the 131 bp band in the presence of the mouse primer set (lanes 6 and 8). Reactions with no added lysate (lanes 5 and 9) were included to control for possible cross contamination. Lysate PCR was a very efficient procedure for screening mice; 16 mice could be screened within 24 hours with less than 2 hours of hands-on work time.

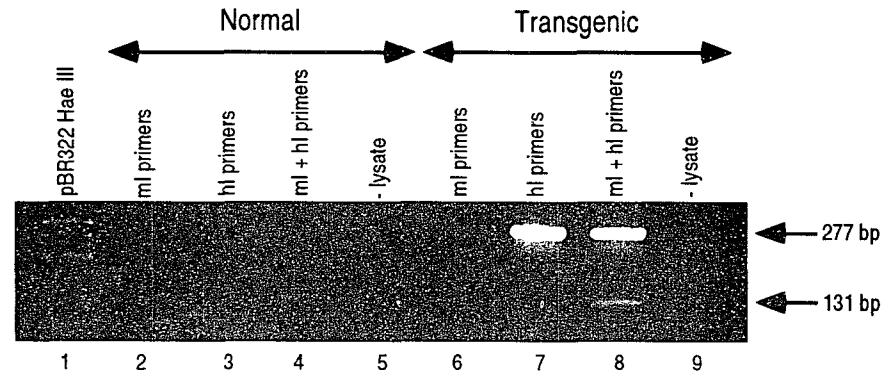


Figure 9. Screening of mice by PCR using tail lysates. An example of lysate screening showing the specificity of the human insulin (hI) and mouse insulin (mI) PCR primers. Amplification of a normal lysate is shown in lanes 2-5, and a transgenic lysate in lanes 6-9. Reactions employing only mouse insulin primers (mI-E2 and mI-E3) are shown in lanes 2 and 6. Reactions employing only human insulin primers (hI-I2 and hI-E3A) are shown in lanes 3 and 7. Reactions employing both primer sets are shown in lanes 4 and 8. Lanes 5 and 9 show amplification with no added lysate. Lane 1 contains DNA markers. Arrows show the predicted sizes of PCR products from human insulin sequences (277 bp) and the mouse insulin I gene (131 bp).

D. Sites of transgene expression

The sites of transgene expression were determined using reverse transcription polymerase chain reaction (RT-PCR). The strategy for RT-PCR is shown in Figure 10. Primers hI-E2 and hI-E3C amplify a 878 bp region from the intron containing transgene (Figure 6) and are predicted to amplify a 92 bp region from spliced mRNA. To serve as an internal control for the RT-PCR, a 139 bp region of the mouse β -actin mRNA was amplified with primers mBA1 and mBA2 (See Methods III I).

Optimization of primer concentrations was essential to the development of an internally controlled RT-PCR. Optimization of the β -actin primer concentration is shown in Figure 11. Total RNA prepared from transgenic liver was reverse transcribed with 20 pmol of the antisense insulin primer hI-E3C and varying amounts (1, 2, 3, or 4 pmol) of the antisense β -actin primer mBA2 (lanes 1-4). For the PCR amplification step, 20 pmol of the sense insulin primer hI-E2 was added, along with varying amounts (1, 2, 3, or 4 pmol) of the sense β -actin primer mBA1. The 92 bp band corresponding to spliced insulin mRNA was detected in transgenic liver at all 4 concentrations of the actin primers. Actin mRNA was strongly detected at 3 and 4 pmol of actin primers (lanes 1 and 2) and weakly detected at 1 and 2 pmol (lanes 3 and 4).

To confirm the nature of the regions amplified by the insulin and actin primers, the 92 bp band and the 139 bp

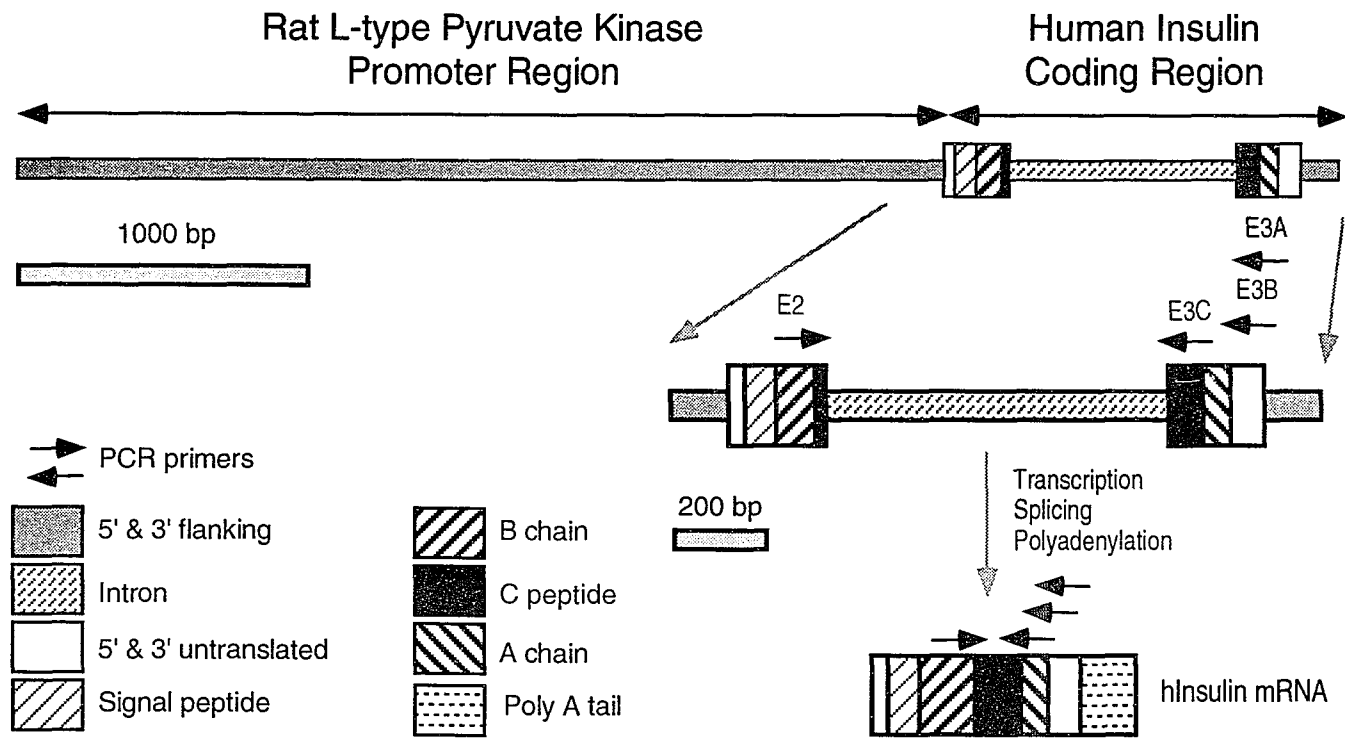


Figure 10. Strategy for RT-PCR analysis of transgene expression. Antisense primers hI-E3A, hI-E3B, and hI-E3C are separated from the sense primer hI-E2 by an intron in the transgene. The detection of the smaller PCR products thus identifies spliced mRNA. Primers hI-E2 and hI-E3C are predicted to amplify a 92 bp region from spliced mRNA; primers hI-E2 and hI-E3B amplify a 139 bp region. Primer hI-E3A was used for specific priming in the reverse transcriptase reaction.

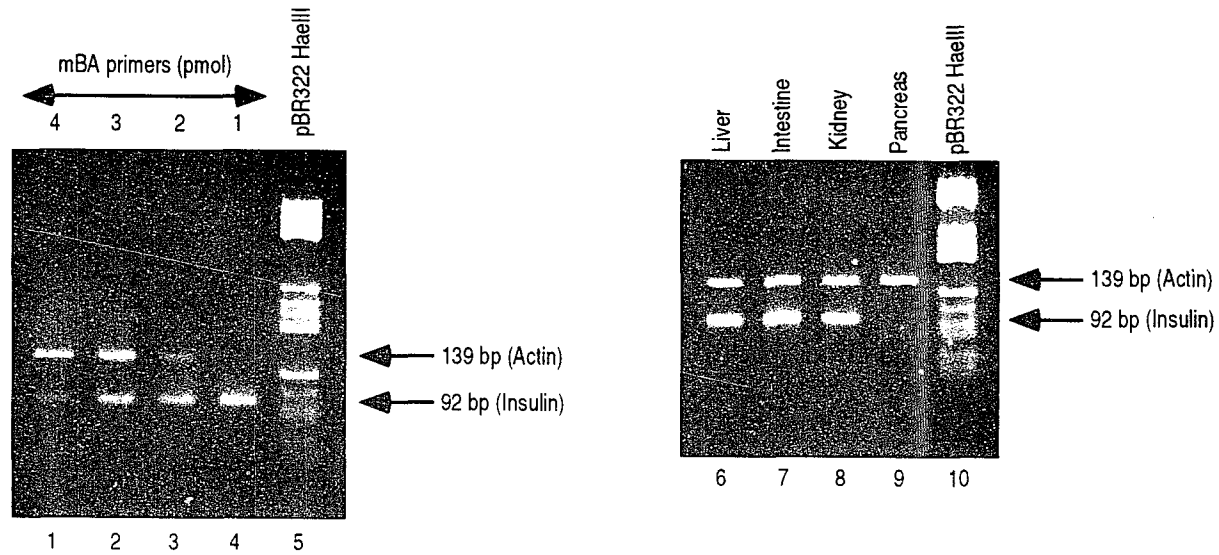


Figure 11. Optimization of internally controlled RT-PCR. Total RNA from transgenic mouse tissues was amplified with human insulin primers (hI-E2 and hI-E3C) and mouse beta actin primers (mBA1 and mBA2). Amplification of liver RNA is shown in lanes 1-4, while the concentration of the actin primers was varied as shown. Amplification of RNA from liver, intestine, kidney, and pancreas using a heminested strategy is shown in lanes 6-9. Arrows show the predicted sizes of PCR products from human insulin mRNA (92 bp) and mouse beta actin mRNA (139 bp). Lanes 5 and 10 contain DNA markers.

band were cloned into a plasmid vector and sequenced (See Methods IIII). DNA sequencing of the 92 bp band revealed 54 bp of human insulin sequence flanked by the sequences of primers hI-E2 and hI-E3C. The sequence was in accordance with the predicted structure of insulin mRNA after splicing of the intron (Figure 6). Sequencing of the 139 bp band verified amplification of the mouse β -actin mRNA with primers mBA1 and mBA2. Thus, human insulin mRNA was detected in transgenic liver, with an internal control amplification of β -actin mRNA.

The insulin/actin RT-PCR was modified to increase specificity by using a heminested priming approach. The antisense insulin primer hI-E3A and the antisense actin primer mBA3 were used for the reverse transcription reaction. After addition of the sense primers hI-E2 and mBA1, ten cycles of PCR were carried out. An aliquot of this reaction was used to initiate 30 cycles of PCR using insulin primers hI-E2 and hI-E3C, in addition to actin primers mBA1 and mBA2. Thus, the antisense primer used for the 30 cycle PCR, hI-E3C, is located internal to the antisense primer used for reverse transcription and the initial 10 cycle PCR, hI-E3A (Figure 10). Similarly, the antisense actin primer, mBA2, lies internal to the antisense primer mBA3. The heminested insulin/actin RT-PCR was employed to analyze RNA isolated from transgenic liver, intestine, kidney, and pancreas (Figure 11, lanes 6-9). Human insulin mRNA was strongly detected in liver,

intestine, and kidney, which are the primary sites of endogenous L-PK expression (40). Human insulin mRNA was weakly detected in pancreas, which has been shown to express the L-PK gene minimally (80). β -actin mRNA was strongly detected in all four tissue samples. The heminested strategy (lanes 6-9) yielded fewer non-specific bands and stronger detection of insulin and actin mRNAs when compared with the non-nested RT-PCR (lanes 1-4). The number of cycles in the second amplification step was varied in the experiment shown in Figure 12. Actin mRNA was detected in all four tissues after 20 cycles, although detection was stronger at 25 and 30 cycles. Detection of insulin mRNA required at least 25 cycles and was strongest after 30 cycles. Actin detection with fewer cycles may be due to higher initial concentrations of mRNA, but could also be explained by greater amplification efficiency of the actin primer set. Since the 30 cycle condition yielded strong detection of both insulin and actin mRNAs, this condition was used for subsequent experiments.

A survey of ten transgenic tissues was conducted using the insulin/actin RT-PCR (Figure 13). Insulin mRNA was detected in liver, intestine, kidney and testis, but not in brain, heart, lung, spleen, skeletal muscle, or pancreas. To check for amplification of DNA which may be present in the RNA preparations, RT-PCR was carried with and without reverse transcriptase. The insulin primers are located within different exons; they amplify a 878 bp region from

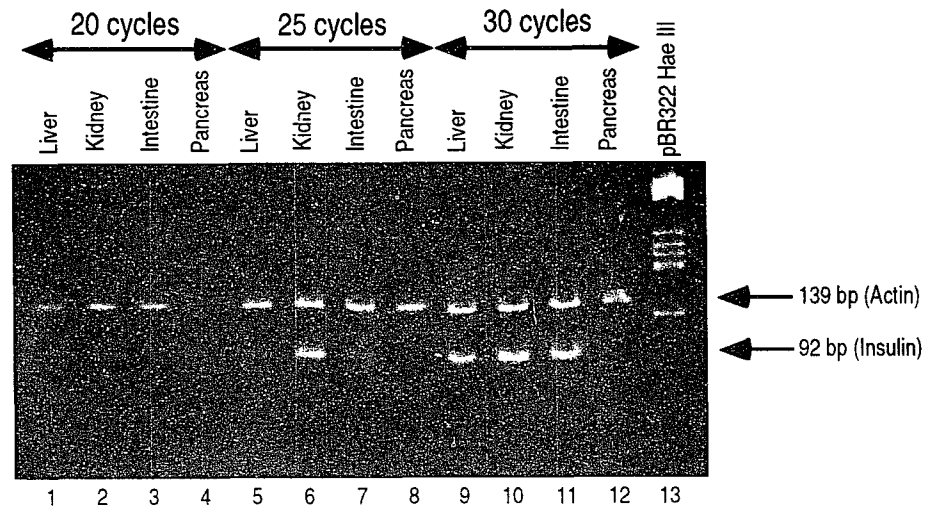


Figure 12. Optimization of PCR cycle number for internally controlled RT-PCR. Total RNA from transgenic mouse tissues was amplified with human insulin primers and mouse beta actin primers. The PCR cycle number was varied as follows: 20 cycles (lanes 1-4), 25 cycles (lanes 5-8), and 30 cycles (lanes 9-12). Lane 13 contains DNA markers.

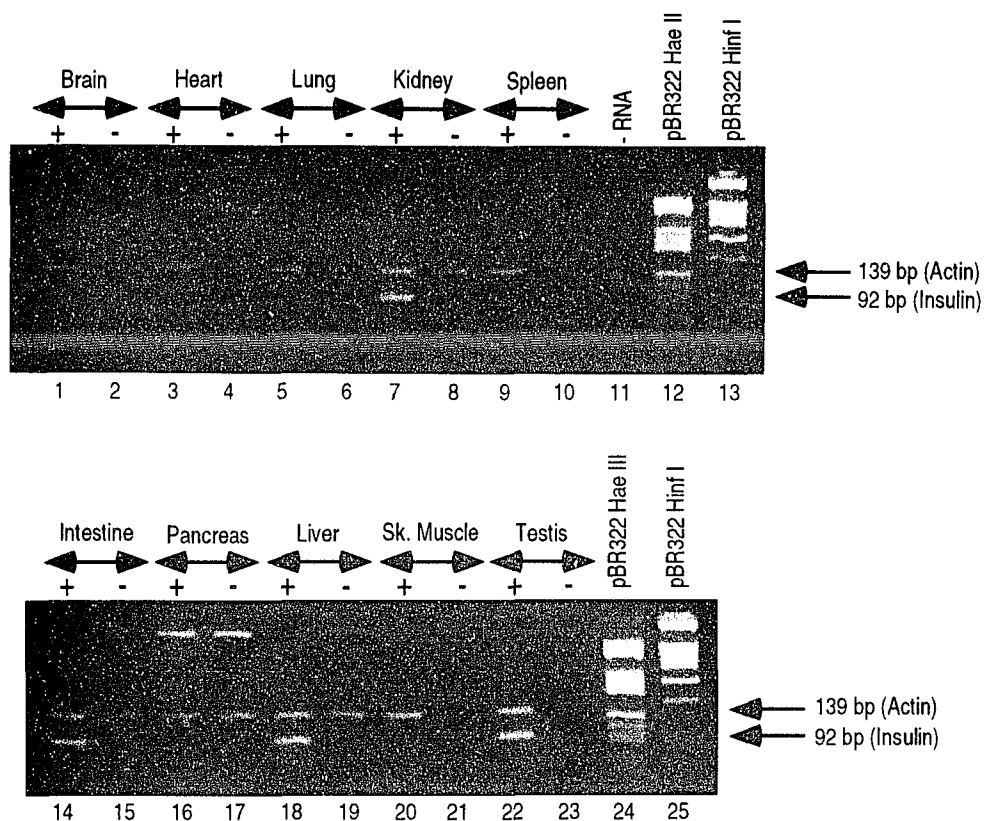


Figure 13. Survey of transgenic tissues by RT-PCR. Total RNA from 10 different transgenic mouse tissues was amplified with human insulin primers and mouse beta actin primers. Reactions were performed with (+) and without (-) reverse transcriptase as shown in lanes 1-10 and 14-23. Lane 11 shows RT-PCR without added RNA. Lanes 12, 13, 24, and 25 contain DNA markers.

the transgene (Figure 6) and a 92 bp region from spliced mRNA (Figure 10). Since the 878 bp region may not be as efficiently amplified as the shorter 92 bp target, small amounts of contaminating genomic DNA could easily be missed. However, the actin primers mBA1 and mBA2 are located within the same exon of the mouse β -actin gene; they are predicted to amplify a 139 bp region from either genomic DNA or mRNA. Detection of the 139 bp band in the absence of reverse transcriptase would indicate that significant amounts of contaminating genomic DNA are present.

In the experiment shown in Figure 14, total RNA was prepared using the UltraSpec reagent (See Methods IIIH). RNA preparations from brain, heart, lung, skeletal muscle, and testis did not contain significant DNA contamination, since the 139 bp band was not detected in the absence of reverse transcriptase. However, RNA preparations from kidney, spleen, intestine, pancreas, and liver did show the 139 bp band in the absence of reverse transcriptase. The presence of contaminating DNA was correlated with the total yield of each RNA preparation. Contaminating DNA was detected in the five RNA preparations with a total yield greater than 100 μ g. Contaminating DNA was not detected in the five RNA preparations with a total yield less than 100 μ g. This indicates that the processing of greater amounts of material resulted in significant DNA contamination. After DNase treatment of the preparations from kidney, spleen, intestine, pancreas, and liver, contaminating DNA was no

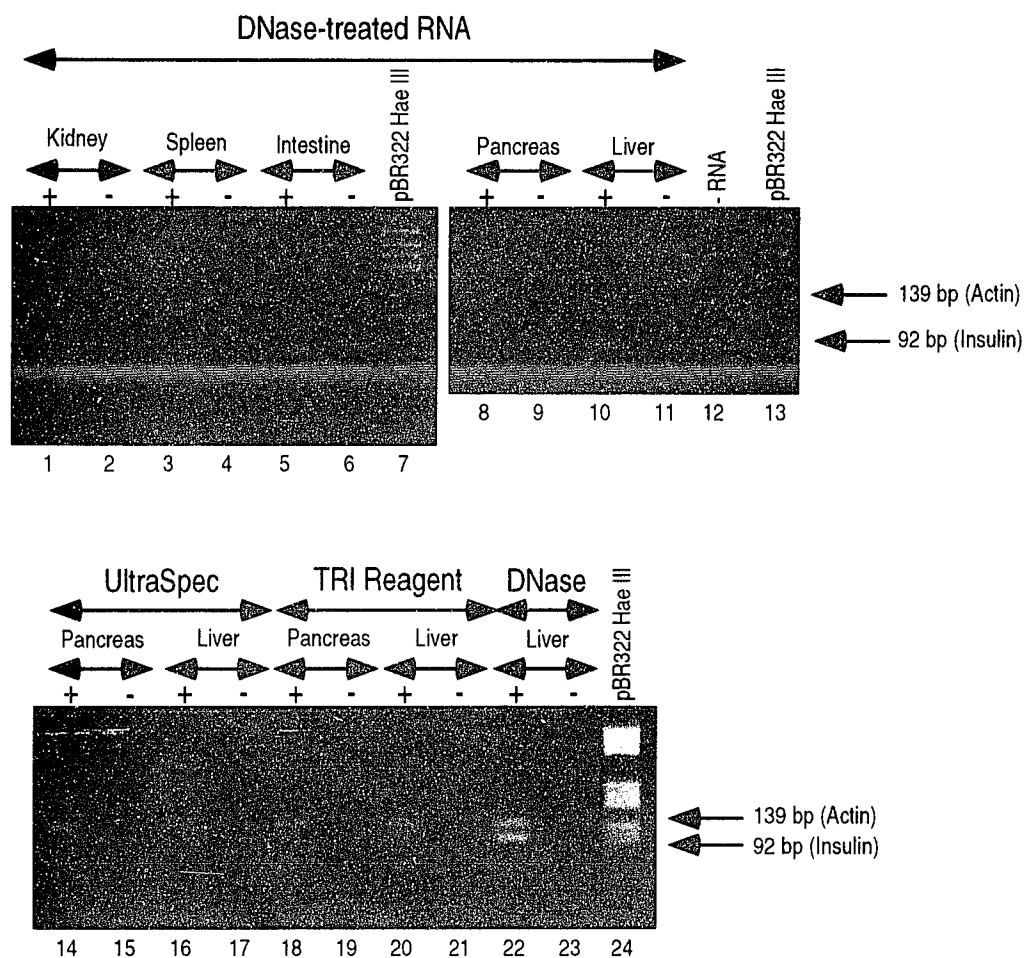


Figure 14. Optimization of RNA preparation methodology for RT-PCR tissue survey. Total RNA from transgenic kidney, spleen, intestine, pancreas, and liver was amplified with human insulin primers and mouse beta actin primers. Reactions were performed with (+) and without (-) reverse transcriptase. The RNA samples for reactions shown in lanes 1-11 and 22-23 were prepared with the UltraSpec reagent and subsequently treated with DNase. The RNA samples for reactions shown in lanes 14-17 (UltraSpec reagent) and 18-21 (TRI reagent) were prepared using an additional precipitation step to eliminate contaminating DNA. Lane 12 shows RT-PCR without added RNA. Lanes 13 and 24 contain DNA markers.

longer detected (Figure 14, lanes 1-11). However, the 139 bp band was not detected in pancreas in the presence of reverse transcriptase (lane 8). This suggests possible degradation of the RNA prepared from pancreas, which is a tissue that contains large amounts of RNase. The RNA preparation method was then modified in two ways: dissected mouse tissues were immediately homogenized after removal from the animal, and an additional isopropanol precipitation step was performed to eliminate contaminating DNA (See Methods IIH). In addition, two different RNA isolation reagents were tested, the UltraSpec reagent and the TRI reagent (Figure 14, lanes 14-23). The modified method yielded RNA from pancreas and liver that was free of contaminating DNA, with detection of actin mRNA in both tissues (lanes 14, 16, 18, and 20). Insulin mRNA was again weakly detected in pancreas (lanes 14 and 18), as seen previously (Figure 12). In subsequent experiments, RNA was prepared by immediate homogenization of mouse tissues with TRI reagent, and the additional isopropanol precipitation step was included.

A survey of 11 tissues from transgenic and normal mice was then conducted (Figure 15). Insulin mRNA (92 bp band) was again detected in transgenic liver, intestine, kidney, and pancreas, but not in brain, heart, lung, spleen, skeletal muscle, ovary, or blood. The 92 bp band was not detected in any of the normal tissues. Actin mRNA was detected in all tissue samples. The insulin/actin RT-PCR

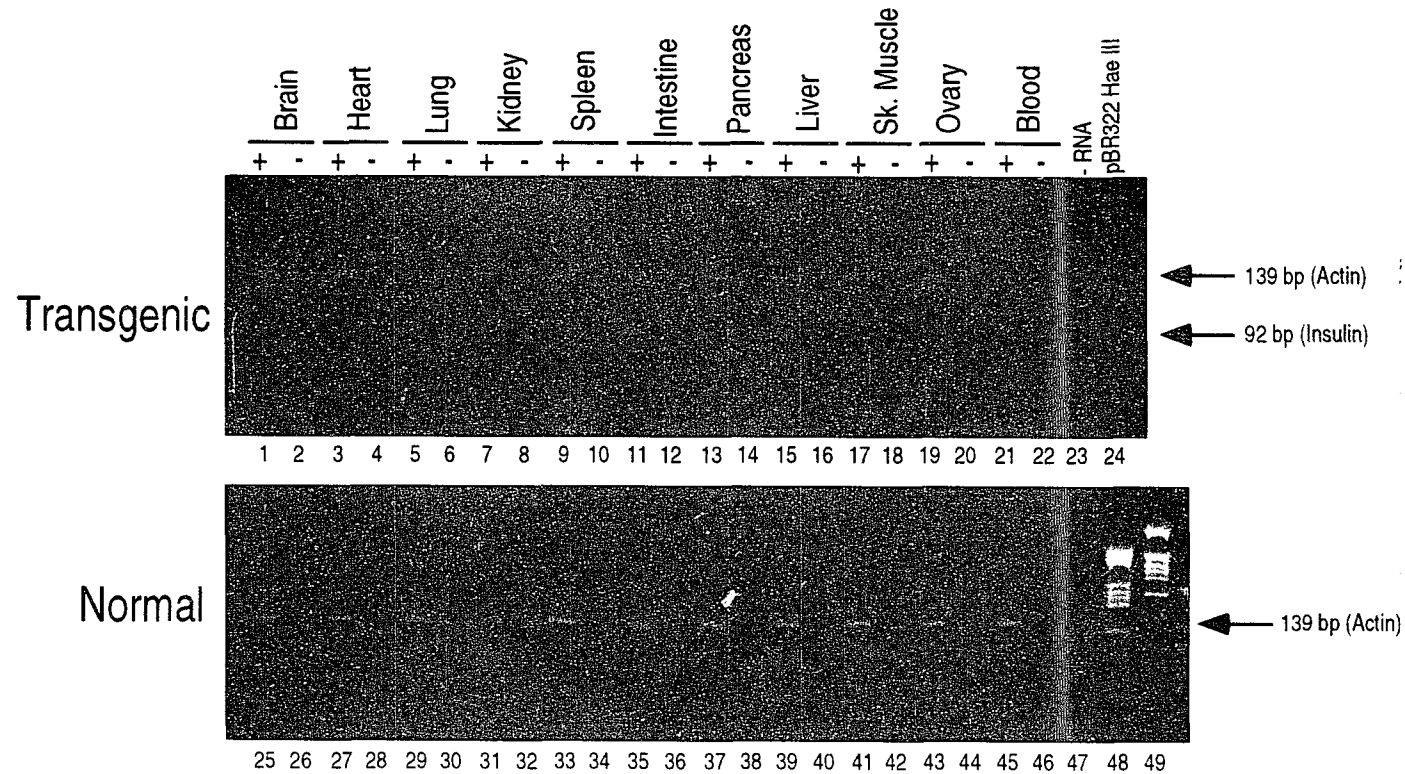


Figure 15. Survey of transgenic and normal tissues by RT-PCR. Total RNA from 11 different tissues was amplified with human insulin primers (92 bp) and mouse beta actin primers (139 bp). Reactions were performed with (+) and without (-) reverse transcriptase. Transgenic tissues are shown in lanes 1-22 and the corresponding normal tissues are shown below in lanes 25-46. Lanes 23 and 47 show RT-PCR without added RNA. Lanes 24 and 48 contain pBR322 Hae III markers, and lane 49 contains pBR322 Hinf I markers.

was modified to increase the efficiency of screening many tissue samples. A single tube assay was performed by priming reverse transcriptase with low concentrations of hI-E3A and mBA3, followed by a 30 cycle PCR with primers hI-E2/hI-E3C and mBA1/mBA2. The 10 cycle initial amplification was eliminated and all of the reverse transcription reaction was used to initiate the 30 cycle PCR. (See Methods III I). The single tube heminested insulin/actin RT-PCR is demonstrated in Figure 16; amplification of transgenic hepatic and pancreatic RNA with and without reverse transcriptase is shown. Although the single tube assay resulted in increased background ethidium bromide staining, this could be greatly reduced by incubating completed PCRs briefly with RNase prior to gel electrophoresis (Figure 16, lanes 6 and 9). Alternatively, ^{33}P - α -dATP incorporation during the amplification step followed by autoradiography was used to detect PCR products (Figure 18). Additional RT-PCRs confirming the sites of transgene expression are shown in Figure 17. The 92 bp band was again detected in transgenic testis (lane 7), but not normal testis (lane 23). In addition, expression of the transgene was detected in bone marrow (transgenic: lanes 6, 16, 18, and 20; normal: lanes 22 and 25).

A summary of the insulin/actin RT-PCR tissue survey is shown in Figure 18. Amplification of transgenic hepatic RNA was performed with either insulin (lane 17) or actin (lane 18) primers alone, demonstrating once again that the 92 bp

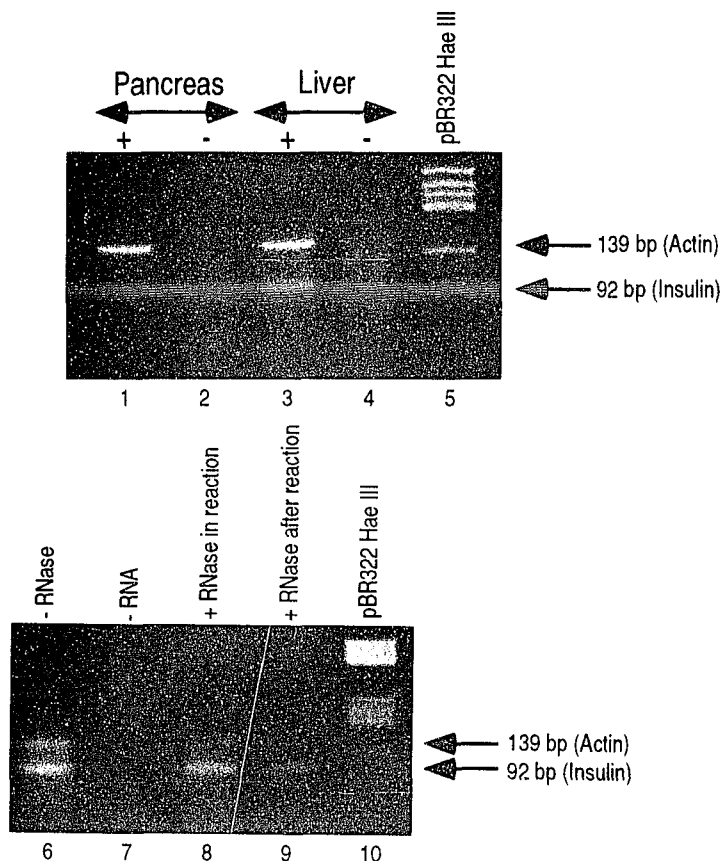


Figure 16. Optimization of a single-tube RT-PCR assay. Amplification of total RNA from transgenic pancreas and liver is shown in lanes 1-4. Reactions were performed with (+) and without (-) reverse transcriptase. Background ethidium bromide staining from added RNA could be reduced by treatment of PCR products with RNase (lanes 6-9, transgenic liver). Lane 6 shows an untreated reaction; RNase was added either during PCR amplification (lane 8) or after PCR amplification (lane 9). Lane 7 shows RT-PCR without added RNA. Lanes 5 and 10 contain DNA markers.

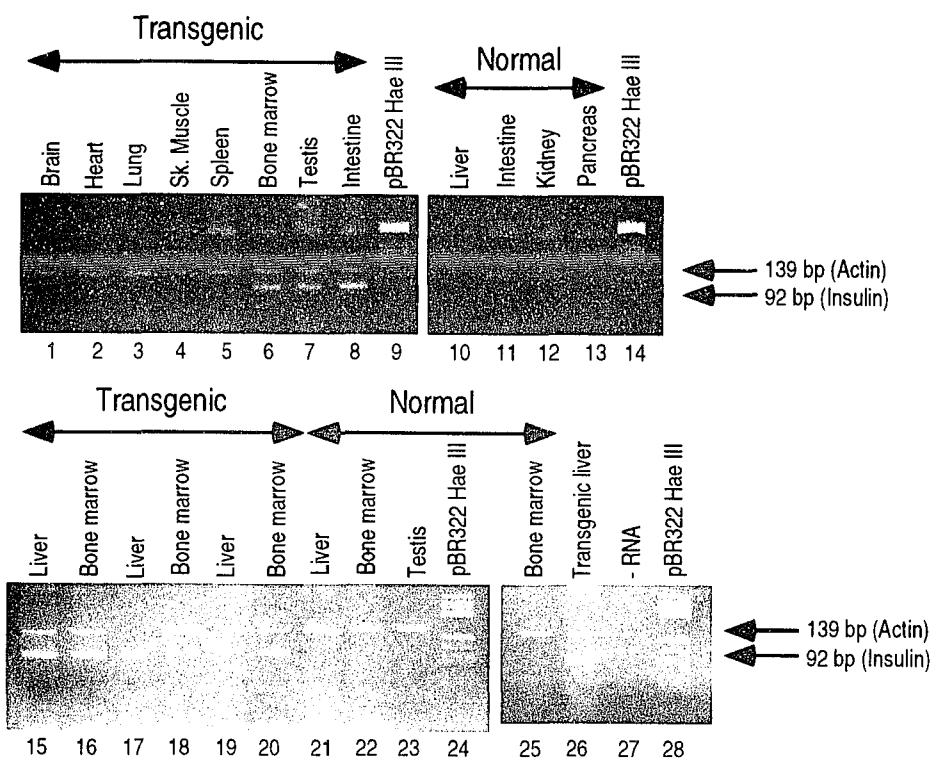


Figure 17. Confirmation of sites of transgene expression by RT-PCR. Total RNA from transgenic and normal tissues was amplified using the single tube RT-PCR assay. Transgenic tissues are shown in lanes 1-8, 15-20, and 26. Transgene expression was not detected in brain, heart, lung, skeletal muscle, or spleen (lanes 1-5). Expression of the transgene was confirmed in bone marrow (lanes 6, 16, 18, & 20) and testis (lane 7). Normal liver, intestine, kidney, pancreas, bone marrow, and testis do not show the 92 bp band corresponding to insulin mRNA (lanes 10-13, 21-23, & 25). Lane 27 shows RT-PCR without added RNA. Lanes 14 and 28 contain DNA markers.

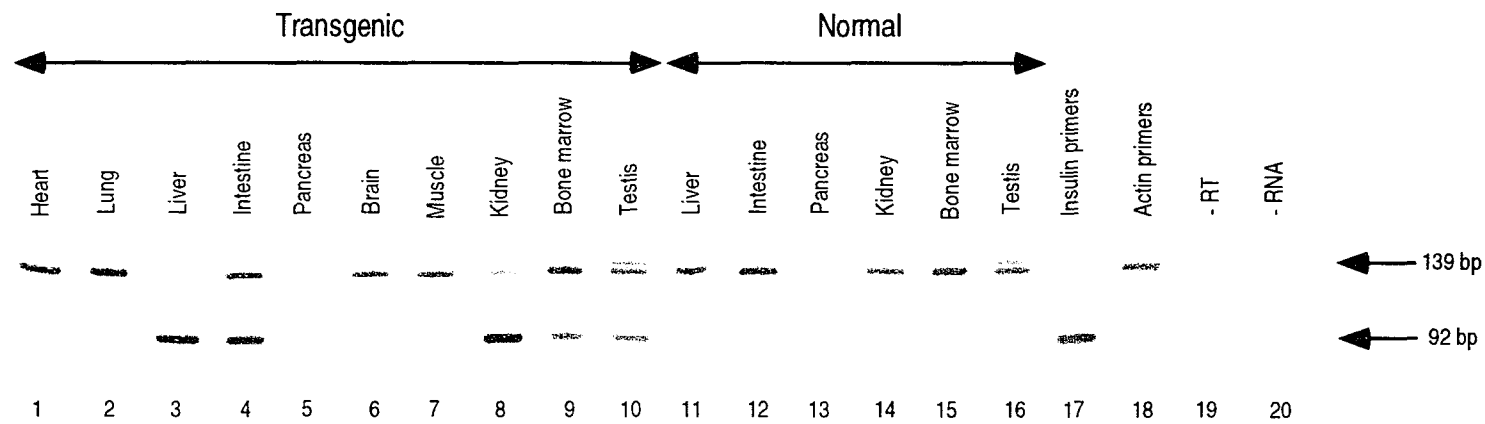


Figure 18. Summary of the RT-PCR tissue survey for transgene expression. Total RNA from transgenic (lanes 1-10) and normal (lanes 11-16) tissues was amplified using human insulin primers (92 bp) and mouse beta actin primers (139 bp). ^{33}P -alpha-dATP was included in the reaction to label the products in a single-tube RT-PCR assay. Transgenic liver RNA was amplified with insulin primers only (lane 17), actin primers only (lane 18), and without reverse transcriptase (lane 19). Lane 20 shows RT-PCR without added RNA.

band detects insulin expression and the 139 bp band detects actin expression. Transgene expression was strongly detected in liver, intestine, and kidney (lanes 3, 4, and 8), which are the primary sites of endogenous L-PK expression. Expression was weakly detected in pancreas, bone marrow, and testis (lanes 5, 9, and 10). Pancreas has been shown to express L-PK minimally (80), bone marrow expression may be due to an upstream erythroid promoter (40), and testis is a common site of ectopic expression in transgenic mice (81,82). The 92 bp band did not appear in reactions analyzing these six tissues from normal mice (lanes 11-16). Expression was not detected in transgenic heart, lung, brain and skeletal muscle (lanes 1, 2, 6, and 7); these tissues do not express the endogenous L-PK gene (40). Thus, the pattern of expression seen was consistent with previous studies of endogenous L-PK expression (35) and the expression of L-PK reporter genes in transgenic mice (40,47).

E. Dietary regulation of transgene expression

To assess the relative level of transgene mRNA in the expressing tissues and the pattern of dietary regulation, a quantitative RT-PCR assay was developed. The number of PCR cycles for which the amplification reaction distinguished between differences in initial template concentration was determined (Figure 19). An in vitro transcribed insulin template diluted in normal liver RNA gave rise to signals of

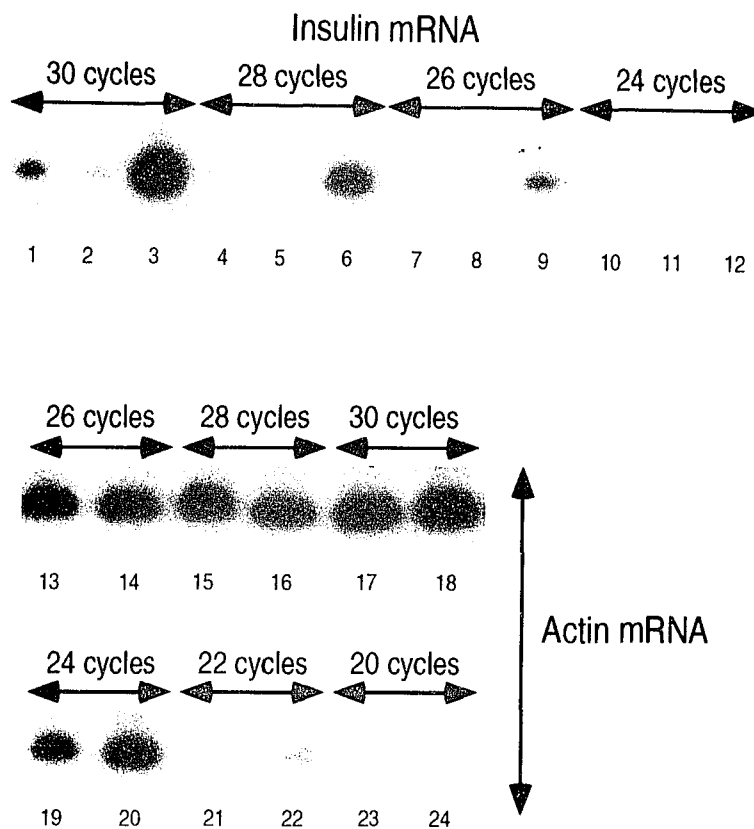


Figure 19. Optimization of PCR cycle number for quantitative RT-PCR assays. Lanes 1-12 show cycle optimization for the insulin assay. Lanes 1, 4, 7, & 10 were amplified from 1 ug of transgenic liver RNA; lanes 2, 5, 8, & 11 were amplified from 0.25 ug of transgenic liver RNA + 0.75 ug of normal liver RNA. Lanes 3, 6, 9, & 12 were amplified from an in vitro transcribed insulin template diluted in 1 ug of normal liver RNA. Lanes 13-24 show cycle optimization for the actin assay. Lanes 14, 16, 18, 20, 22, & 24 were amplified from 1 ug of transgenic liver RNA; lanes 13, 15, 17, 19, 21 & 23 were amplified from 0.25 ug of transgenic liver RNA + 0.75 ug of yeast RNA.

increasing intensity between 24 and 30 cycles of PCR (lanes 12, 9, 6, & 3). In the same experiment, 1 μ g of transgenic liver RNA from a mouse feeding ad libitum on the chow diet was also amplified (lanes 10, 7, 4, & 1). Insulin mRNA was detectable at 28 cycles (lane 4), and with greater intensity at 30 cycles (lane 1). To determine if the reaction was sensitive to differences in the amount of initial mRNA template, 0.25 μ g of the same transgenic liver sample was mixed with 0.75 μ g of normal liver RNA and amplified (lanes 11, 8, 5, & 2). Insulin mRNA was only detectable after 30 cycles (lane 2), and with less intensity than for the fourfold greater amount of transgenic RNA at 30 cycles (lane 1). Thus, the 30 cycle condition appeared to be appropriate for further testing of the assay: it was sensitive to the initial amounts of transgenic RNA under dietary conditions that were expected to yield a relatively low level of expression, and there remained a considerable range of band intensity (lane 3 versus lane 1) for expected increases in expression under stimulating conditions.

A similar experiment was carried out to determine appropriate RT-PCR conditions for assessing the level of β -actin mRNA in mouse tissues (Figure 19, lanes 13-24). Amplification from 1 μ g of transgenic liver RNA for 20 to 30 cycles is shown in lanes 24, 22, 20, 14, 16, & 18. Amplification from 0.25 μ g of the same transgenic sample mixed with 0.75 μ g of yeast RNA is shown in lanes 23, 21, 19, 13, 15, & 17. Cycle numbers of 22 (lanes 21 and 22) and

24 (lanes 19 and 20) seemed the most appropriate for quantifying β -actin mRNA; between 26 and 30 cycles the reaction appeared to reach a maximum intensity level (lanes 13-18), with no sensitivity to differing initial amounts of mouse RNA.

The reproducibility of the quantitative insulin RT-PCR was assessed prior to further refinements of the assay (Figure 20). The reagents used for assembling the reactions (except for the enzymes) were prepared in large batches which would be enough for hundreds of assays. A series of aliquots were made for each reagent, using the amount required for a typical experiment size of 20 assays. In vitro transcribed RNA standards were stored at -70°C and other reagents were stored at -20°C . Thus, repeated freeze-thaw cycles and opening-closing of reagent tubes was avoided. The assay was designed so that all components were added in a volume that could be pipetted with good accuracy (See Methods III J). RNA samples were prepared from the livers of 12 different transgenic mice and $1\text{ }\mu\text{g}$ ($4\text{ }\mu\text{l}$) of each sample was subjected to RT-PCR (lanes 1-12). The RNA samples were stored at -70°C for 1 week and then assayed again in the same sequence (lanes 13-24). There was a close correspondence between the relative band intensities for the same sample in the two experiments. Densitometry readings of band intensity are shown in Table 2. The correlation (r^2) between the two experiments was 0.97. Thus, the

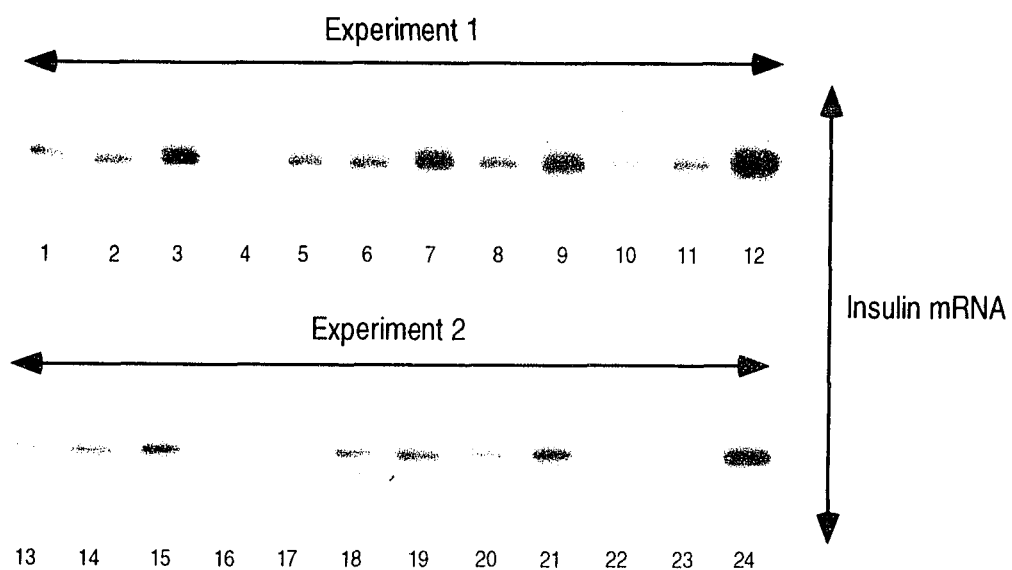


Figure 20. Reproducibility of the quantitative insulin RT-PCR assay. In experiment 1, 12 samples of transgenic liver RNA were assayed (lanes 1-12). In experiment 2, the same 12 samples were assayed again (lanes 13-24).

measured level of insulin mRNA by this assay is highly reproducible.

The level of human insulin mRNA and the degree of dietary regulation was assessed for the six expressing tissues in line H6 (liver, intestine, kidney, pancreas, bone marrow, and testis, Figures 21 and 22). Mice were either fasted for 36 hours or fasted for 24 hours and refed for 12 hours with a high sucrose diet. The standard curve was adjusted to cover the range of expression by varying the amount of in vitro transcribed insulin template that was diluted in normal liver RNA (Figure 21, lanes 13-16 and Figure 22, lanes 13-18). The two separate experiments, each using 6 tissue samples from a transgenic and a non-transgenic mouse, yielded almost identical results (lanes 1-12). Hepatic insulin mRNA levels were 50 to 100 fold higher in mice fed a high sucrose diet compared with fasted mice (lanes 1 and 2). Intestinal levels increased about 20 fold under these conditions (lanes 3 and 4), and kidney showed a relatively high fasting level with a moderate increase in fed mice (lanes 5 and 6). Insulin mRNA levels in bone marrow, pancreas, and testis were at the lower limit of detection and showed no differences between fasted and fed mice (lanes 7-12).

The levels of expression found in line J6 upon sucrose refeeding were approximately 10 fold lower than line H6 in liver and intestine (Figure 23, lanes 2 and 4) and barely detectable in kidney (lane 6). Mice from line H4 showed no

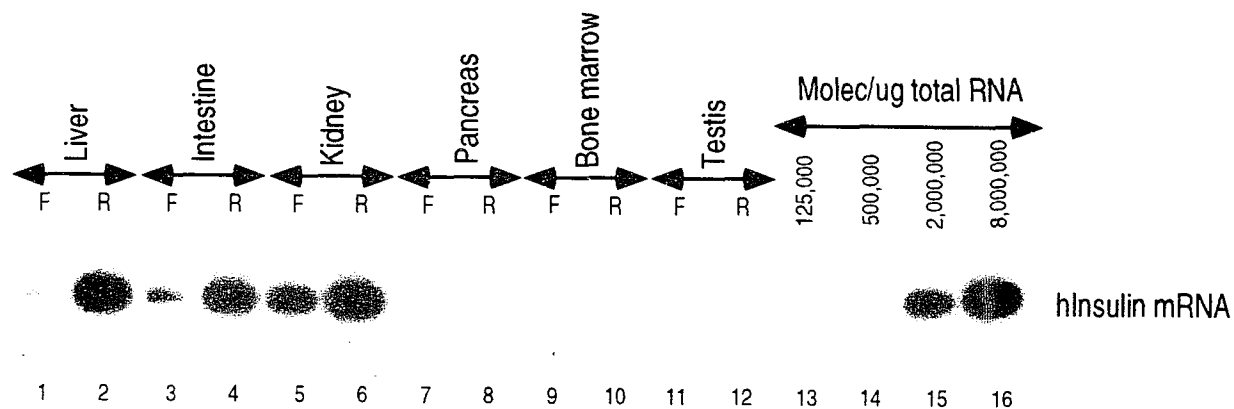


Figure 21. Fast-refeed regulation of insulin mRNA in expressing tissues. Human insulin mRNA was quantified by RT-PCR using total RNA isolated from the six expressing tissues (lanes 1-12). Mice were either fasted for 36 hours (F) or fasted for 24 hours and refed for 12 hours with a high sucrose diet (R). An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 13-16).

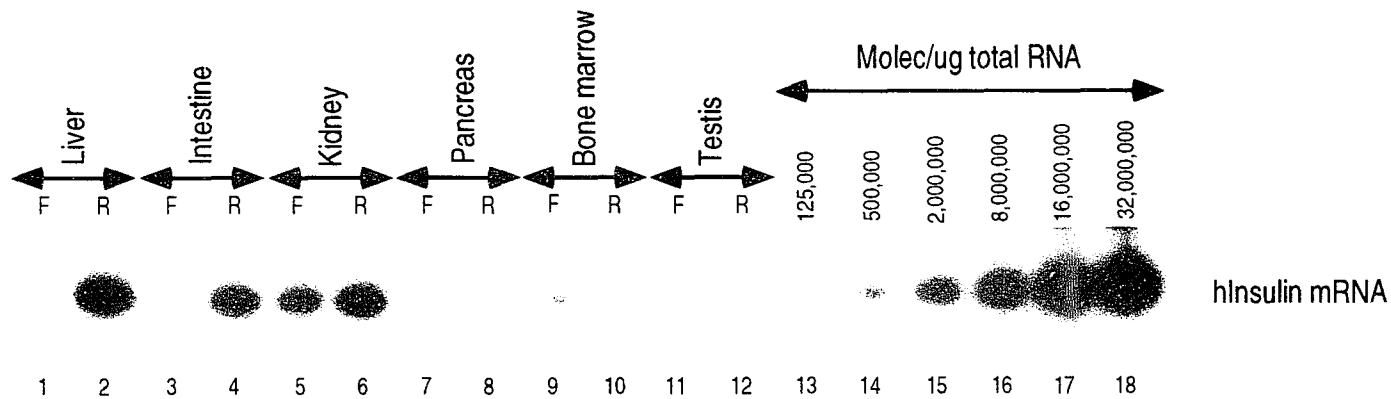


Figure 22. Fast-refeed regulation of insulin mRNA in expressing tissues. Human insulin mRNA was quantified by RT-PCR using total RNA isolated from the six expressing tissues (lanes 1-12). Mice were either fasted for 36 hours (F) or fasted for 24 hours and refed for 12 hours with a high sucrose diet (R). An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 13-18).

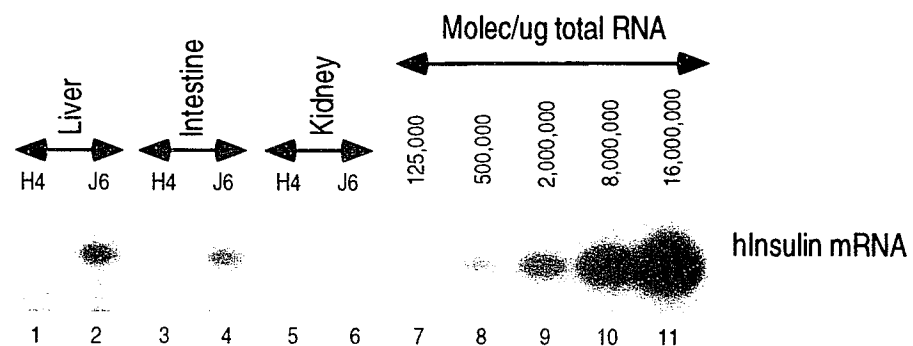


Figure 23. Expression level in transgenic lines H4 and J6. Human insulin mRNA was quantified by RT-PCR using total RNA isolated from liver, intestine, and kidney of H4 and J6 mice (lanes 1-6). Mice were fasted for 24 hours and then refed with a high sucrose diet for 12 hours. Each lane shows the RT-PCR product generated from the pooled RNA of three mice. An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 7-11).

detectable expression in liver, intestine, or kidney (Figure 23, lanes 1, 3, and 5). Since mice from lines J6 and H4 showed low or absent expression in the major sites of L-PK expression, and mice from line H6 showed higher expression with appropriate dietary regulation, line H6 was used for all subsequent experiments.

Since liver and intestine showed a marked increase in insulin mRNA over the course of a 12 hour refeeding period (Figures 21 and 22, lanes 1-4), these tissues were examined at various time points to ascertain the rapidity of induction and the maximum levels of expression (Figure 24). Mice that were fasted for 36 hours with no refeeding showed expression at the lower limit of detection (lanes 1 and 7). For both liver and intestine, there was no detectable expression within 2 hours (lanes 2 and 8) or 4 hours (lanes 3 and 9) of sucrose refeeding after a 24 hour fast. Liver showed detectable expression at 6 hours of refeeding (lane 4), with a marked increase between 6 and 12 hours (lanes 4-6). Intestine showed a high level of expression beginning at 6 hours (lanes 10-12). For both liver and intestine, there were no further increases after 12 hours for the time points examined (16, 20, and 24 hours, lanes 18-29). In addition, male (lanes 18, 20, 22, 24, 26, & 28) and female (lanes 19, 21, 23, 25, 27, & 29) mice showed no marked differences in expression level at the 16, 20, or 24 hour time points.

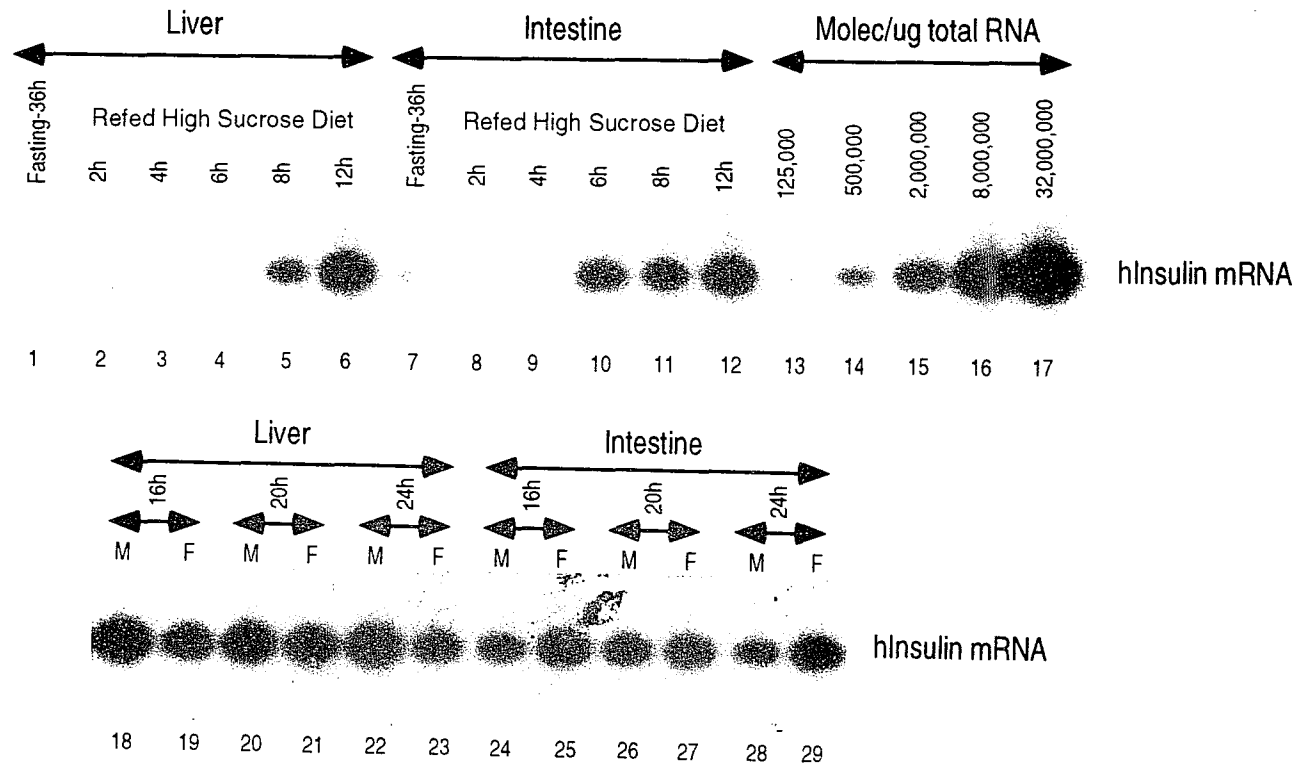


Figure 24. Time course of insulin mRNA induction in liver and intestine. Human insulin mRNA was quantified by RT-PCR using total RNA isolated from liver and intestine (lanes 1-12 & 18-29). Mice were either fasted for 36 hours (lanes 1 and 7) or fasted for 24 hours and refed for varying times with a high sucrose diet (lanes 2-6, 8-12, & 18-29). For the 16, 20, and 24 hour time points, a male (M) and a female (F) mouse were used (lanes 18-29). An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 13-17).

To further assess the potential of the L-PK promoter system to simulate beta cell insulin production, a more detailed examination of temporal response patterns was conducted (Figures 25 - 28). Groups of three transgenic mice were fasted for 24 hours and refed with the high sucrose diet for various times (2, 4, 6 hours: Figure 25; 8, 12, 18, 26 hours: Figure 26; 40 hours: Figure 27). In addition, groups of mice that had been refed for 40 hours were fasted again for 12, 24, and 36 hours (Figure 27). The results of these experiments are summarized in Figure 28, for which pooled RNAs from the three mice for each time point were assayed, and in Tables 3 and 4, which show densitometric readings of band intensities. The mean expression level of the groups was in close correspondence to the level obtained by analysis of pooled samples (Table 4, $r^2=0.99$). The results show that insulin mRNA was first detectable 6 hours after refeeding (Figure 28, lane 4) and that there was a marked increase (5.5 fold) between 8 and 12 hours after refeeding (lanes 5 and 6). Peak levels were seen at 18 and 26 hours (lanes 7 and 8) and there was a 3.6 fold decrease between 26 and 40 hours (lanes 8 and 9). However, there was considerable variability within the groups (Figures 25 - 27 and Table 3), such that the increase between 8 and 12 hours and the decrease between 26 and 40 hours were not statistically significant. The groups of mice that were refasted for 12, 24, and 36 hours after the 40 hour refeeding period showed very low or undetectable

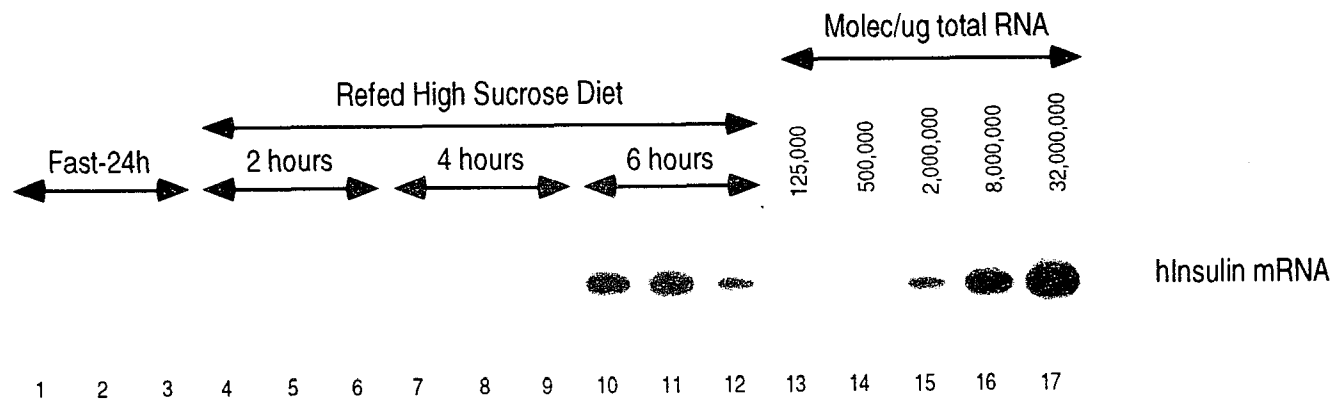


Figure 25. Time course of insulin mRNA induction in liver.

Human insulin mRNA was quantified by RT-PCR using total RNA isolated from liver (lanes 1-12). Mice were either fasted for 24 hours (lanes 1-3) or fasted for 24 hours and refed with a high sucrose diet for 2 hours (lanes 4-6), 4 hours (lanes 7-9), or 6 hours (lanes 10-12). An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 13-17).

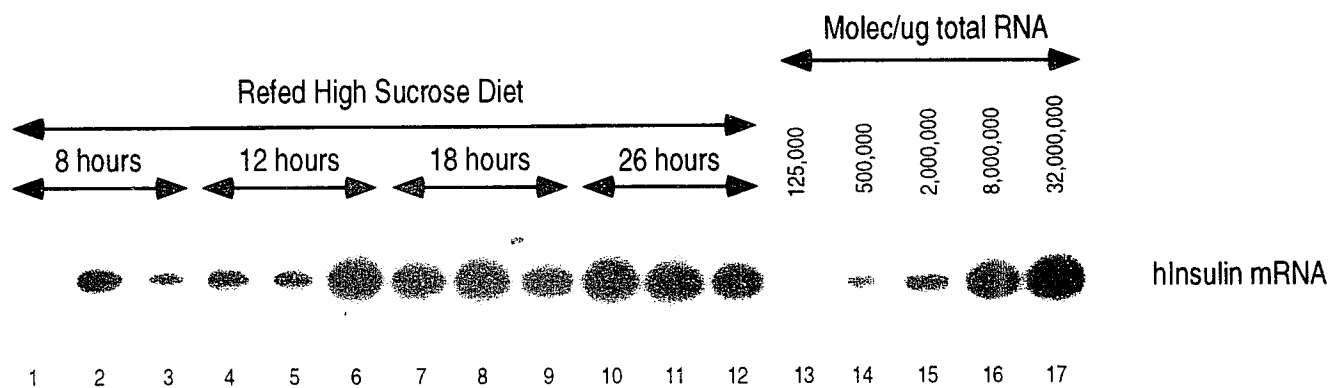


Figure 26. Time course of insulin mRNA induction in liver.

Human insulin mRNA was quantified by RT-PCR using total RNA isolated from liver (lanes 1-12). Mice were fasted for 24 hours and refed with a high sucrose diet for 8 hours (lanes 1-3), 12 hours (lanes 4-6), 18 hours (lanes 7-9), or 26 hours (lanes 10-12). An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 13-17).

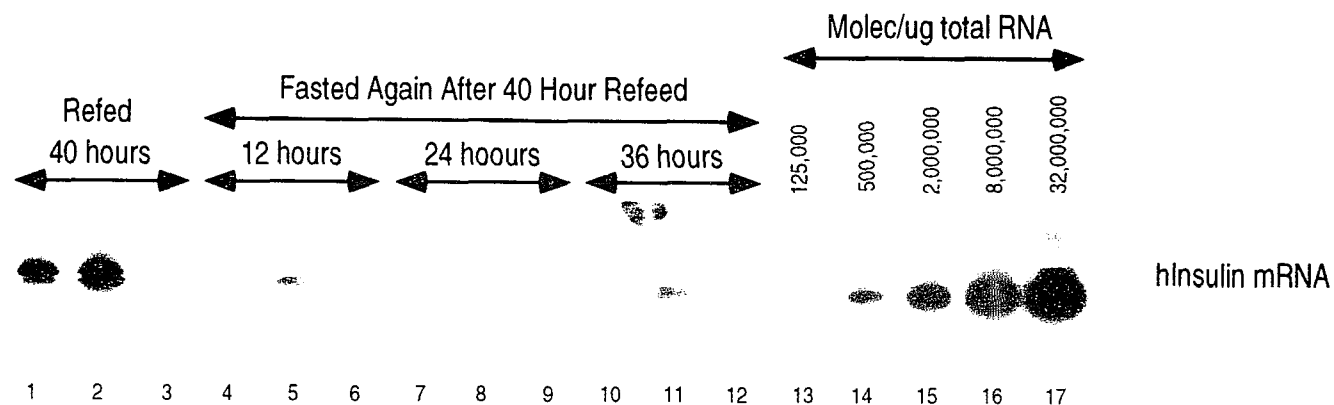


Figure 27. Time course of insulin mRNA induction in liver.

Human insulin mRNA was quantified by RT-PCR using total RNA isolated from liver (lanes 1-12). Mice were fasted for 24 hours and refed with a high sucrose diet for 40 hours (lanes 1-3). After the 40 hour refeeding period, mice were fasted again for 12 hours (lanes 4-6), 24 hours (lanes 7-9), or 36 hours (lanes 10-12). An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 13-17).

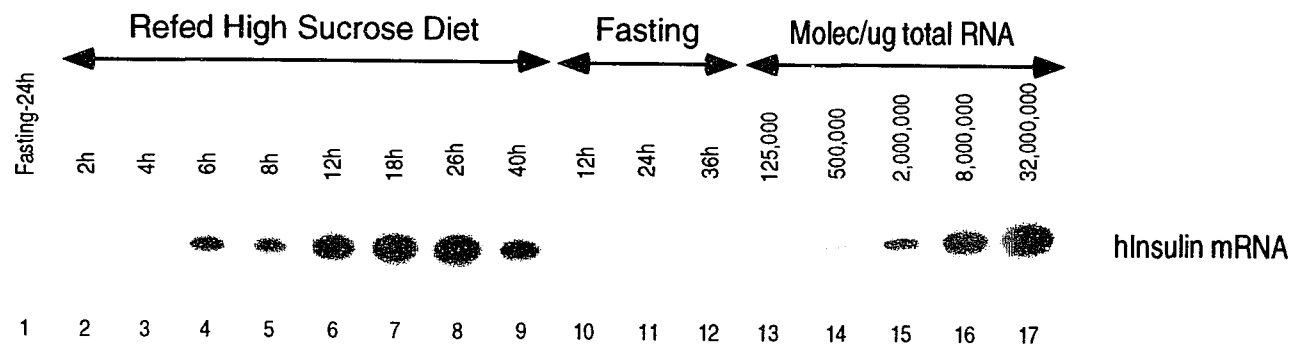


Figure 28. Time course of insulin mRNA induction in liver. Human insulin mRNA was quantified by RT-PCR using total RNA isolated from liver (lanes 1-12). Mice were fasted for 24 hours (lane 1) and refed with a high sucrose diet for varying times up to 40 hours (lanes 2-9). After the 40 hour refeeding period, mice were fasted again for 12 hours (lane 10), 24 hours (lane 11), or 36 hours (lane 12). Each lane shows the RT-PCR product generated from the pooled RNA of three mice. An in vitro transcribed insulin template was progressively diluted in normal liver RNA and subjected to RT-PCR to generate a standard curve (lanes 13-17).

levels of mRNA (Figure 27, lanes 4-12, and Figure 28, lanes 10-12). The changes in expression level associated with fasting and refeeding with the high sucrose diet did not appear to result from a generalized effect on total hepatic mRNA, since levels of β -actin mRNA were not influenced by dietary conditions (Figure 31, lanes 1 and 3).

To examine the possibility that changes in transgene expression during the refeeding period might be associated with variations in the rate of food intake, the amount of food consumed by each mouse was measured during the 40 hour refeeding period (Figure 29 and Table 5). There was little variation in food intake between mice or throughout the refeeding period, with a constant rate of 0.20 g of the sucrose diet consumed per hour.

While the previous experiments show that the L-PK:hIns gene can respond to dietary changes over the course of many hours, it was also of interest to know if the system is capable of responding rapidly to changes in plasma glucose. In an initial experiment, groups of four mice were fasted for six hours and injected intraperitoneally with a glucose load (Figure 30 and Table 6). Mice were sacrificed for preparation of liver RNA at 0 (uninjected), 10, and 20 minutes after injection. The glucose injections resulted in a rapid increase in plasma glucose concentration, with an increase of 214 mg/dl at 10 minutes (Figure 30). The insulin mRNA level was 47% higher in the 10 minute group, and 34% higher in the 20 minute group, compared to the

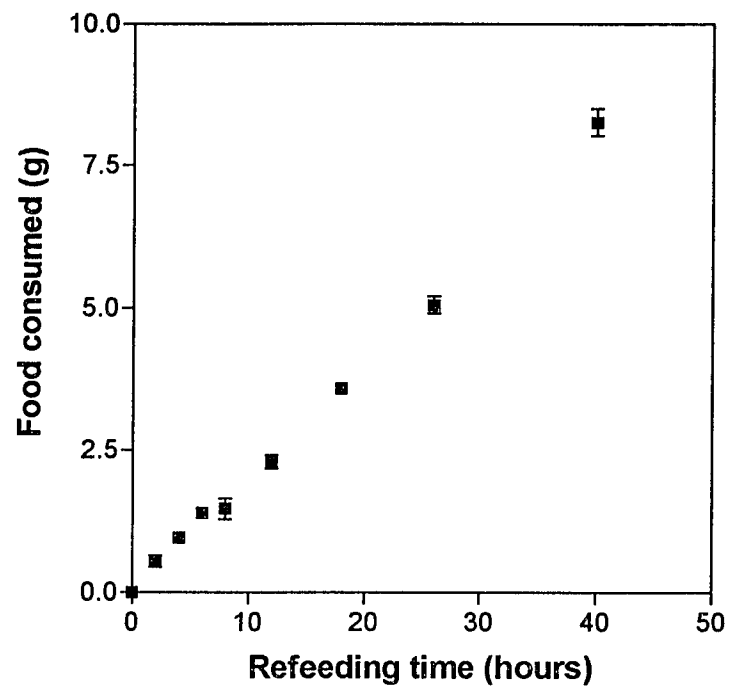


Figure 29. Rate of food consumption during the 40 hour refeeding experiment. Mice were fasted for 24 hours and then refed for 2, 4, 6, 8, 12, 18, 26, or 40 hours. Consumption of the high sucrose diet was measured for three individual mice at each time point.

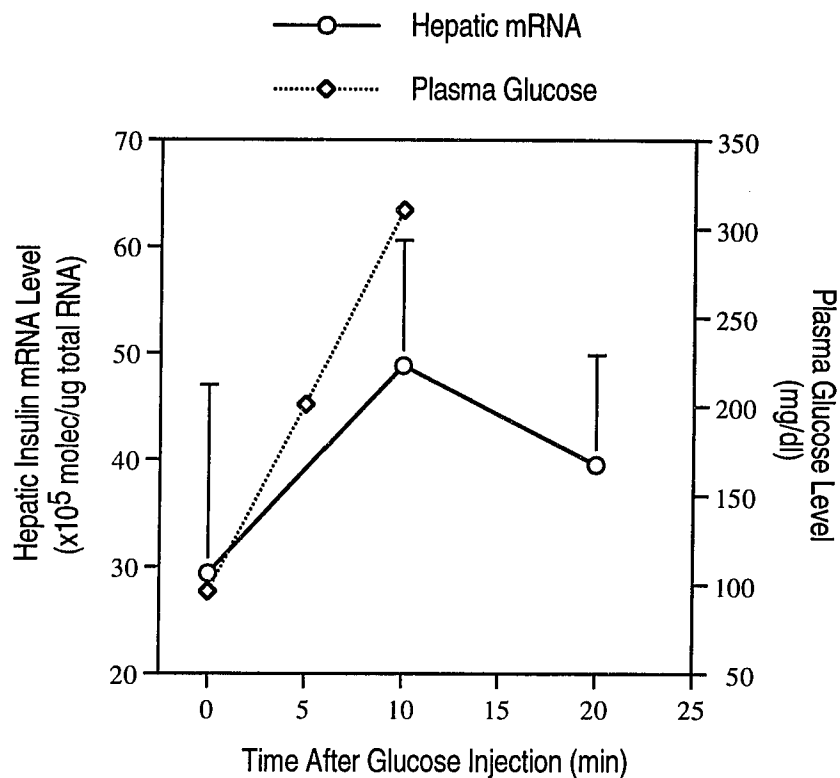


Figure 30. Hepatic insulin mRNA levels after injection of a glucose load. Groups of four mice were fasted for six hours and then injected intraperitoneally with a glucose load. For both non-injected mice (0 minutes) and glucose-injected mice (10 and 20 minutes), insulin mRNA and plasma glucose were determined.

uninjected group. However, there was large variability within each group and these differences were not statistically significant (Table 6). Since a small, but potentially physiologically important effect, might remain undetected in the presence of large intra-group variability and small sample sizes, another series of experiments was conducted. Eighteen transgenic mice were fasted for 6 hours, 9 were injected with a glucose load, and 9 were injected with water for controls. At 10 minutes post-injection, each mouse was bled to determine the plasma glucose level and immediately sacrificed for the preparation of liver RNA. The levels of insulin mRNA and plasma glucose for each mouse are shown in Table 7. There was an increase in mean glucose level from 114 mg/dl in water-injected mice to 452 mg/dl in glucose-injected mice, with a range of 217 - 510 mg/dl for individual mice. There was a 2.4 fold

Table 7: Hepatic insulin mRNA 10 minutes after glucose injection

Mouse	Plasma Glucose (mg/dl)		Insulin mRNA ($\times 10^5$ molec/ug RNA)	
	Water injected	Glucose injected	Water injected	Glucose injected
1	121	470	<20*	<20*
2	114	539	<20*	20
3	94	311	<20*	352
4	100	443	356	374
5	106	444	372	653
6	130	352	503	1071
7	143	443	526	1152
8	112	445	701	1394
9	110	620	822	3082
Mean	114	452	547	1288
SEM	5.0	30.3	75.0	388.8

* mRNA level was below the lowest assay standard

increase in mean insulin mRNA levels in the glucose injected group compared to controls. In the glucose injected group, 4 of 9 mice had insulin mRNA levels greater than 1000 (1071, 1152, 1394, 3082), while the highest value in the control group was 822. Also, for the six pairs of mice whose mRNA levels could be accurately measured (pairs 4-9), there was a statistically significant ($p = 0.045$, one-tailed) 2.4 fold increase in mean insulin mRNA levels in the glucose-injected group. Thus, these data suggest that the L-PK:hIns transgene may be capable of responding rapidly to increases in plasma glucose.

Dietary regulation was further examined by varying the content of the refeeding diet and comparing changes in insulin mRNA levels with actin mRNA levels (Figure 31). For liver and intestine, a low sugar chow diet resulted in small increases over fasting levels when compared with the sucrose diet (lanes 1-3 & 5-7). A breeding diet with a low sugar content but approximately 3 times greater fat content resulted in a marked stimulation in liver (lane 4) but not intestine (lane 8). As seen previously, the level of expression in kidney was relatively insensitive to dietary changes (lanes 9-12). Although there were significant differences in β -actin mRNA levels between liver, intestine, and kidney (lower panel, lanes 1-12), the levels were not influenced by dietary manipulations, indicating that the regulation of insulin mRNA by diet is not the result of a generalized effect on total mRNA.

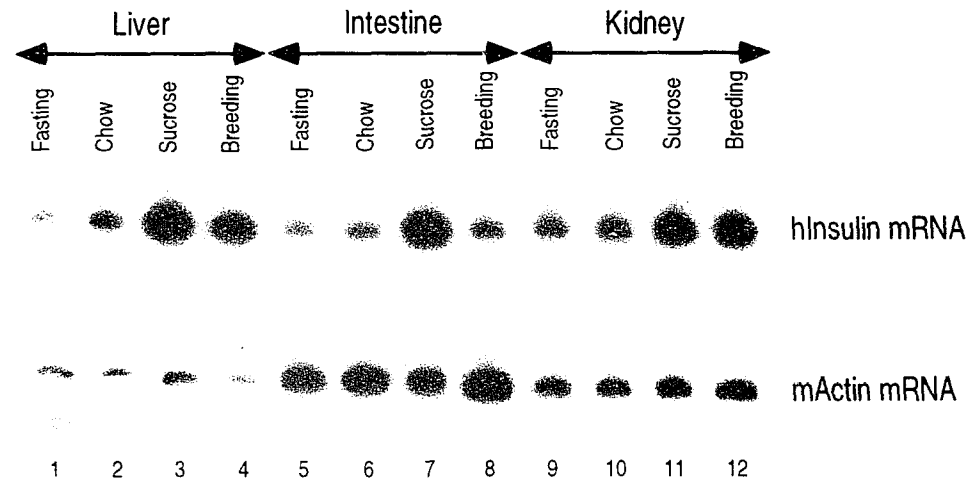


Figure 31. Dietary regulation of insulin expression. Human insulin and mouse beta actin mRNAs were quantified by RT-PCR using total RNA isolated from liver (lanes 1-4), intestine (lanes 5-8), and kidney (lanes 9-12). Mice were fasted for 36 hours (lanes 1, 5, & 9) or fasted for 24 hours and refed for 12 hours with three different diets (Chow:lanes 2, 6, & 10; Sucrose:lanes 3, 7, & 11; Breeding:lanes 4, 8, & 12). Each lane shows the RT-PCR product generated from the pooled RNA of three mice.

F. Measurement of protein products in serum

Competitive ELISAs were developed based on a procedure for measuring rat insulin (83). This assay is characterized by indirect binding of the anti-insulin antibody by an antiglobulin antibody and uses an insulin-peroxidase conjugate to compete for binding to the immobilized anti-insulin antibody. The assay could detect approximately 4.9 pg of insulin in a single ELISA plate well. The assay developed here differs principally in these respects: 1) biotinylated insulin acted as the competitor for binding of the anti-insulin antibody; 2) the detecting conjugate was formed by complex formation between avidin and biotinylated-peroxidase, which likely results in several detecting enzyme molecules being bound by a single target molecule; 3) the peroxidase substrate used for detection was tetramethylbenzidine (TMB) instead of o-phenylenediamine dihydrochloride (OPD). The insulin assay developed here has more than 10 times greater sensitivity than the previous assay, with a detection limit of approximately 0.36 pg of insulin in a single ELISA plate well. In addition, the assay is approximately 40 times more sensitive than a typical clinical diagnostic radioimmunoassay (Coat-a-Count Insulin, Diagnostic Products TKIN1). A low fasting insulin level of 2 μ U/ml can be measured from a serum sample of 5 μ l. This assay may be of considerable value in future studies using small animals such as mice, from which limited

amounts of blood are available, and perhaps as a clinical tool.

A similar approach was used to measure human and mouse C-peptide immunoreactivities in mouse serum (See Methods IIIN). Standard curves for the insulin, human C-peptide, and mouse C-peptide ELISAs are shown in Figures 32-34. Serum immunoreactivities for sucrose-fed normal and transgenic mice are shown in Tables 8 & 9, and summarized in Figure 35. Total insulin immunoreactivity, measured using a rat insulin antibody with 100% crossreactivity to mouse and human insulins, was 22% higher in transgenic mice, but this difference was not statistically significant.

Human insulin/proinsulin protein was detected in transgenic mouse serum using a human C-peptide antibody with undetectable crossreactivity to mouse C-peptide. Human C-peptide immunoreactivity was significantly increased in transgenic mice relative to controls ($p < 0.05$, Figure 35). However, the antibody used has only 4% crossreactivity to human proinsulin, which may be the major product secreted from extrapancreatic sites of expression (84). An antibody specific for human proinsulin was not available in these studies. Thus, the relative amounts of processed human insulin and C-peptide versus proinsulin could not be determined.

Possible alterations of pancreatic β cell function in transgenic mice were assessed by measuring mouse C-peptide using a rat C-peptide antibody with 100% crossreactivity to

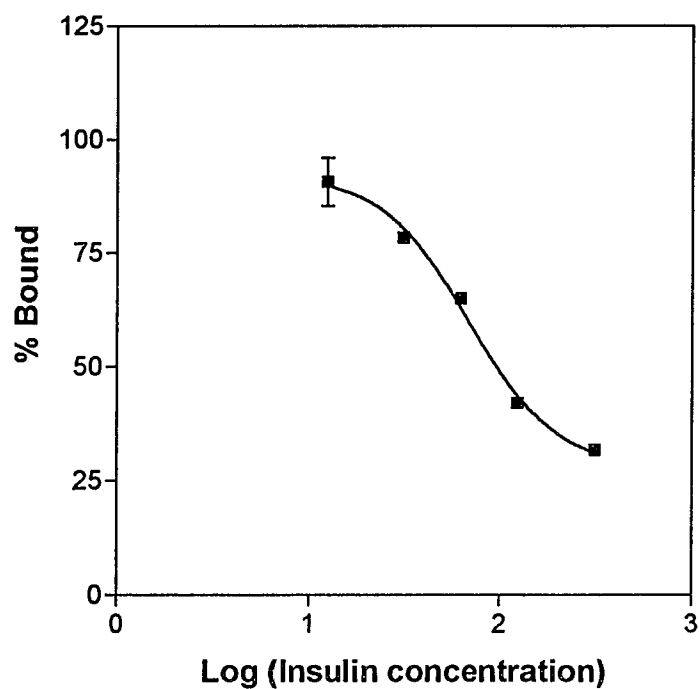


Figure 32. Insulin ELISA standard curve. ELISA was performed using insulin standards of 0, 2, 5, 10, 20, and 50 uU/ml in duplicate. The percent of labeled insulin bound is plotted versus the log(insulin concentration) and the standard curve was generated by fitting to the four parameter logistic equation.

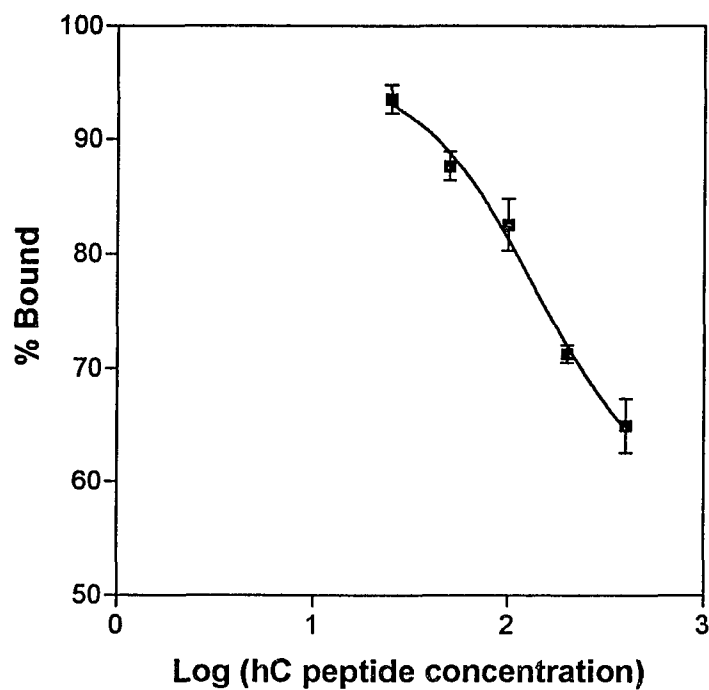


Figure 33. Human C peptide ELISA standard curve. ELISA was performed using human C peptide standards of 0, 25, 50, 100, 200, and 400 pM in duplicate. The percent of labeled C peptide bound is plotted versus the log(hC peptide concentration) and the standard curve was generated by fitting to the four parameter logistic equation.

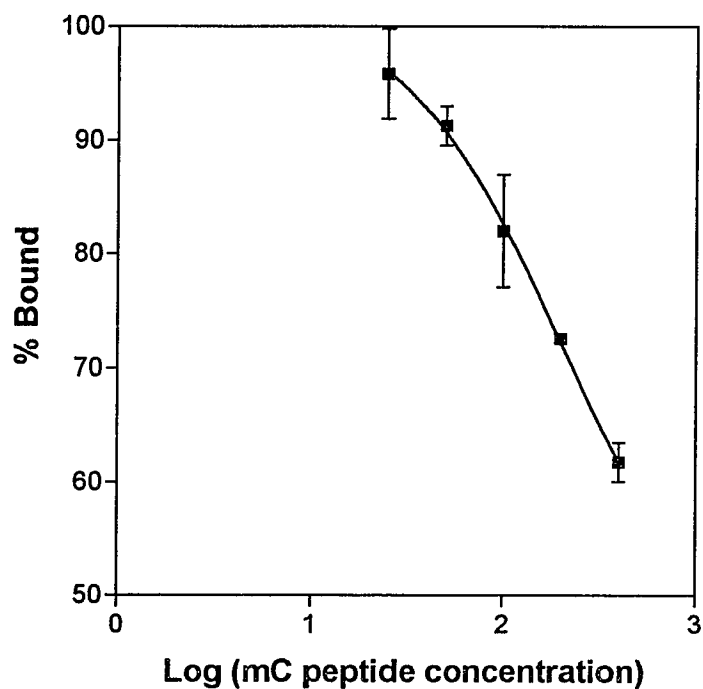


Figure 34. Mouse C peptide ELISA standard curve. ELISA was performed using rat C peptide standards of 0, 25, 50, 100, 200, and 400 pM in duplicate. The percent of labeled C peptide bound is plotted versus the log(mC peptide concentration) and the standard curve was generated by fitting to the four parameter logistic equation.

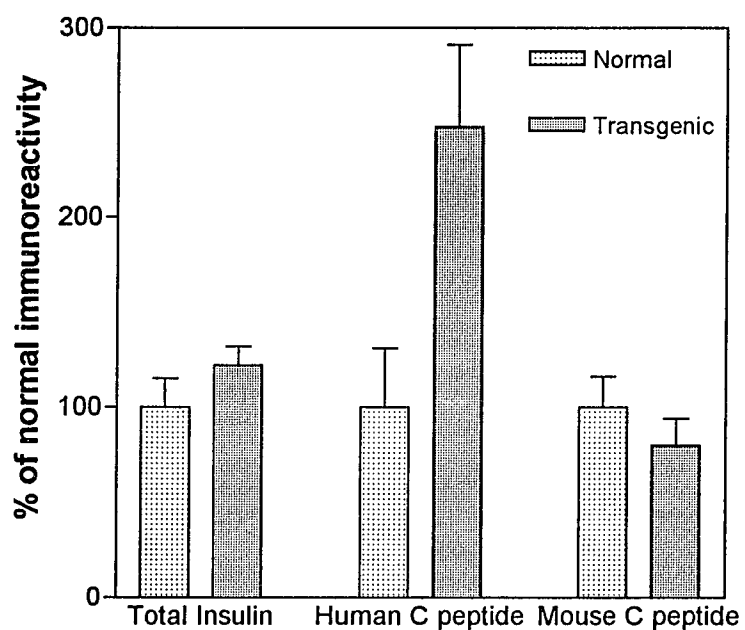


Figure 35. Serum immunoreactivities for insulin and C peptides. Serum immunoreactivity for total insulin, human C peptide, and mouse C peptide was determined for normal and transgenic mice. Values are expressed as a percentage of the immunoreactivity of normal serum.

mouse C-peptide and less than 0.1% crossreactivity to human C-peptide. Mouse C-peptide levels were 20% lower in transgenic mice (Figure 35), but this difference was not statistically significant. However, the absence of a marked decline in mouse C-peptide levels in association with a small increase in total insulin immunoreactivity in transgenic mice suggests that most of the insulin-like protein present in serum is of pancreatic origin.

G. Insulin immunofluorescence of liver tissue

Frozen liver sections were prepared from 3 transgenic and 3 normal mice that had been fasted for 24 hours then refed for 26 hours with the high sucrose diet. As a positive control for immunofluorescent detection of insulin, sections of pancreatic tissue from normal mice were also prepared. No increase in incident light fluorescence was observed in transgenic liver compared to control, while fluorescent islets were clearly observed in pancreatic sections. The amount of insulin present may be below the limit of detection with this assay. However, these results are consistent with the interpretation that newly synthesized insulin does not accumulate intracellularly, rather that it is secreted along the constitutive pathway in non-beta cells (24,85).

H. Growth and general health

Growth curves for male and female (both transgenic and

normal) from 3 to 6 weeks of age are shown in Figure 36 and Tables 10 & 11. There was a statistically significant ($p=0.04$) increase in body weight of 3 week old transgenic females compared to normal females. However, the increase was only 5% above that for normal mice and the difference between transgenic and normal mice did not persist after 3 weeks. Otherwise, the growth curves for transgenic and normal mice were almost identical, with both male and female mice reaching the same adult body weights. Transgenic mice of all ages appear normal; as compared to non-transgenic littermates, no differences have been observed with regard to health, level of activity, or sexual behavior.

I. Oral glucose tolerance tests

To determine if transgenic and normal mice differed with respect to plasma glucose levels and metabolism of a glucose load, glucose tolerance tests were performed. Mice were fasted for 14 hours and then given an oral glucose load of 1.75 mg/g body weight. Plasma glucose levels were determined over the course of 2 hours after the glucose load (Figure 37 and Table 12). Transgenic and normal mice showed no differences in fasting plasma glucose (time= 0 minutes) or at the 10 and 20 minutes time points. However, beginning at the 30 minute time point, transgenic mice showed statistically significant decreases in plasma glucose levels compared to controls. The rise in plasma glucose leveled

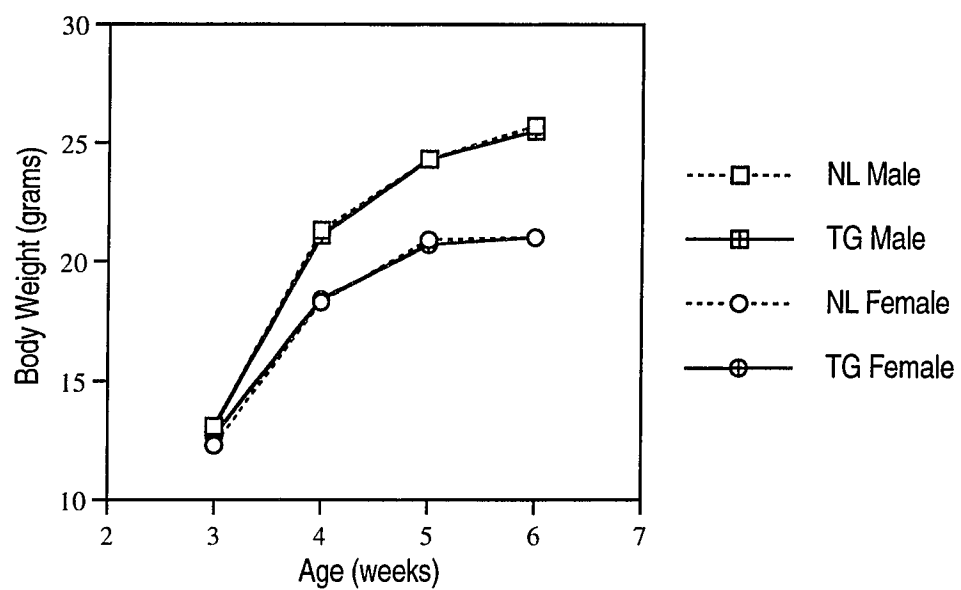


Figure 36. Growth curves for transgenic and non-transgenic mice. Body weight was measured at 3, 4, 5, and 6 weeks of age for groups of 12 male and female mice.

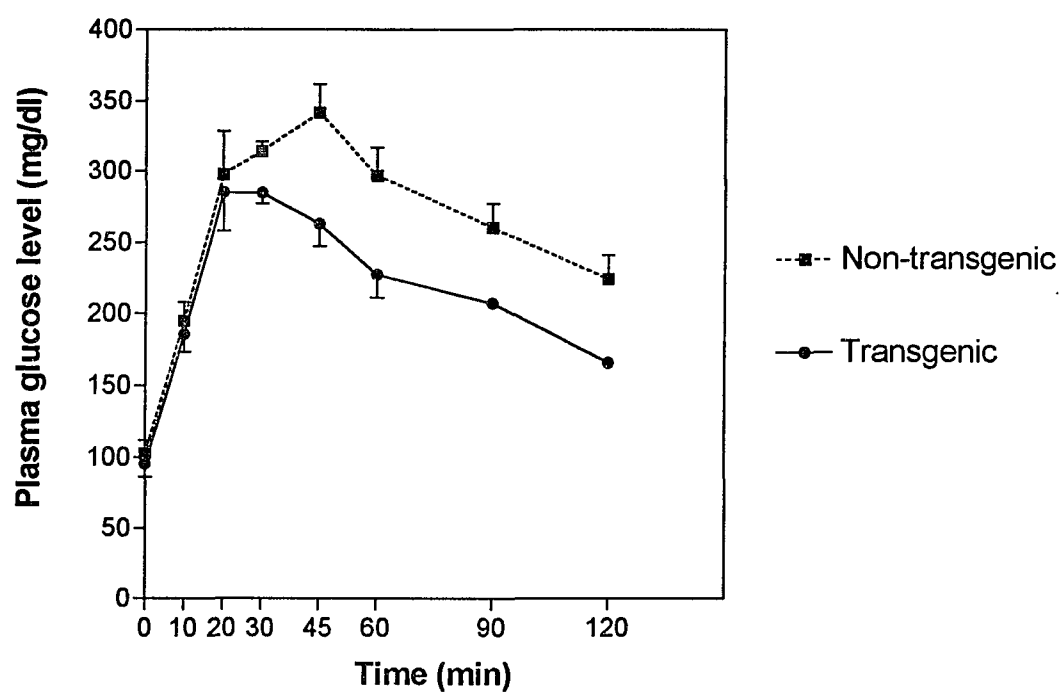


Figure 37. Oral glucose tolerance test. Mice were given an oral glucose load (1.75 mg/kg), and blood samples were drawn at various time points to determine plasma glucose concentration.

off at 20 to 30 minutes for transgenic mice, but continued to rise at 45 minutes for normal mice. The largest difference between the groups was at 45 minutes, when normal mice had a mean glucose level of 342 mg/dl and transgenic mice had a mean of 263, a 23% decrease ($p=0.02$). In addition transgenic mice had statistically significant 9% ($p=0.02$) and 23% ($p=0.03$) declines at 30 and 60 minutes. Thus, while not differing from normal mice in fasting glucose levels, transgenic mice showed significant differences in disposal of an oral glucose load. Differences in the rates of absorption and/or clearance of plasma glucose in transgenic mice could account for these effects. However, glucose levels during the rapid absorptive phase from 0 - 20 minutes were not significantly different from controls. Thus, increased plasma clearance of glucose would seem to be a more likely mechanism.

J. Treatment with diabetogenic agent streptozotocin

A single injection of the drug streptozotocin (STZ) produces an extensive necrosis of pancreatic β -cells within 48 - 72 hours, resulting in permanent diabetes in mice (63). To arrive at an effective STZ dose for these experiments, a dose-response curve was generated (Figure 38 and Table 13). The dose of streptozotocin was correlated with 4 hour fasting plasma glucose levels measured 6 days after injection. STZ was administered to fasted mice by intraperitoneal injection. Doses of 70 and 100 mg/kg

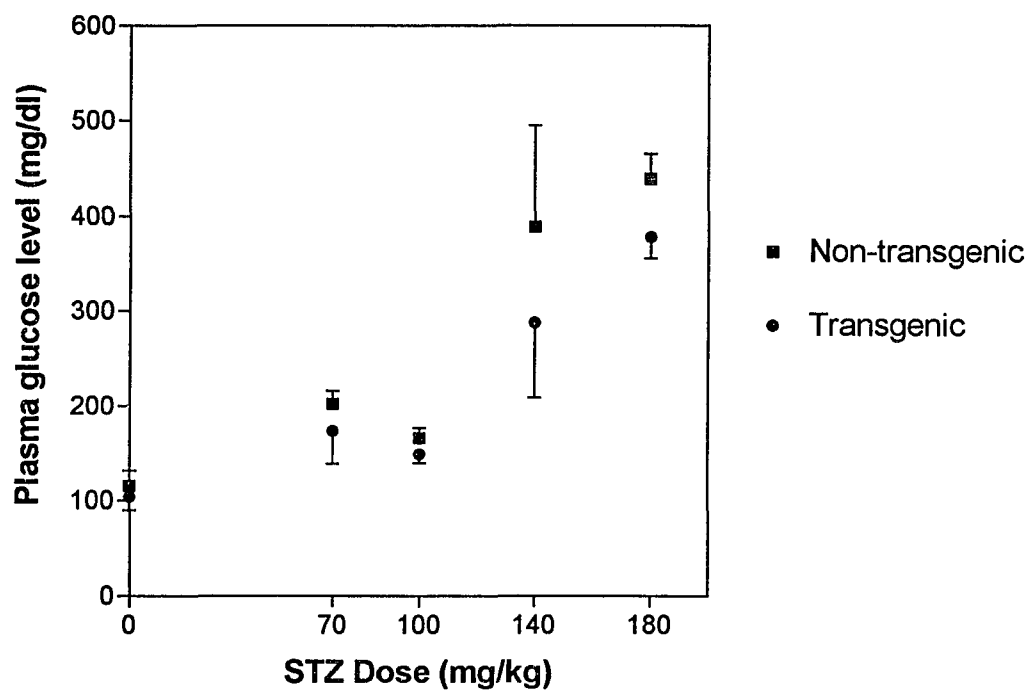


Figure 38. STZ Dose-response curve. Mice were given streptozotocin (STZ) at doses of 0, 70, 100, 140, and 180 mg/kg, and plasma glucose concentration was determined after a 4 hour fast on day 6 post-injection.

resulted in small elevations of blood glucose for both non-transgenic and transgenic mice, while doses of 140 and 180 mg/kg resulted in elevations of blood glucose typical of mice treated with STZ doses in this range (63,86). There was substantial variability in plasma glucose levels in response to the drug, as has been seen previously (86) (Figure 38). The mice treated with 180 mg/kg STZ were severely debilitated within 3 days of treatment. One transgenic mouse and one non-transgenic mouse died on day 4 before blood glucose could be determined; the remaining mice were inactive and the collection of blood samples was problematic due to poor flow. The mice treated with 140 mg/kg STZ were active and there was no difficulty with collection of blood samples; thus, this dose was used for subsequent experiments.

K. Plasma glucose levels in STZ treated mice

Transgenic and non-transgenic mice were either treated with 140 mg/kg STZ (+STZ) or injected with buffer alone (-STZ), and allowed to feed on the standard chow diet for 6 days. Random (non-fasting) plasma glucose levels for the 4 groups at 6 days post-injection are shown in Figure 39 and Table 14. There was no significant difference between untreated transgenic (mean of 147 mg/dl) and non-transgenic mice (mean of 150 mg/dl). STZ treated non-transgenic mice had a mean plasma glucose level of 524 mg/dl, while STZ

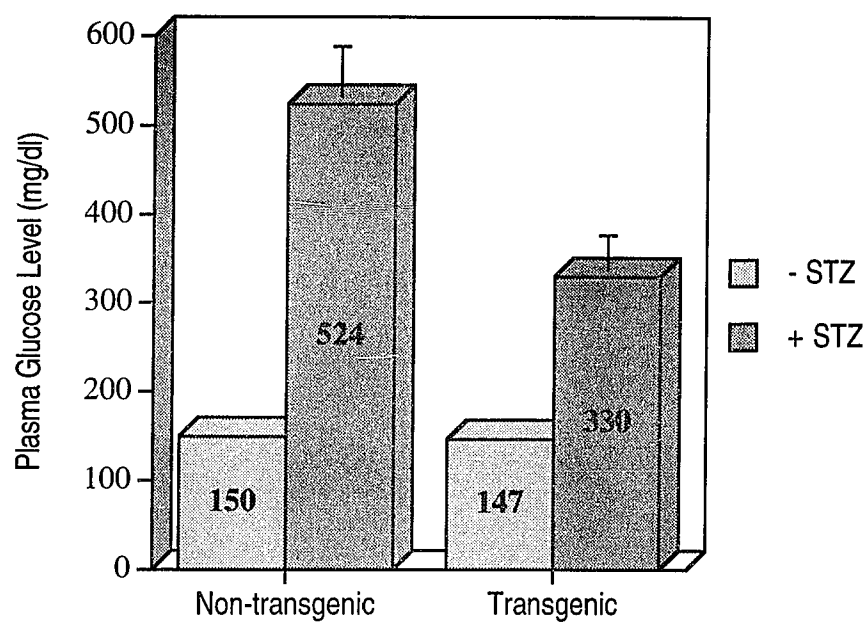


Figure 39. Random plasma glucose after STZ treatment. Non-fasting plasma glucose levels of non-transgenic and transgenic mice were determined six days after STZ treatment (+STZ, n=12). Glucose levels of untreated mice are also shown (-STZ, n=6).

treated transgenic mice had a significantly lower ($p<0.05$) mean of 330 mg/dl.

Plasma glucose levels were also examined after a 4 hour fast on days 3 and 6 after STZ treatment (Figures 40 & 41, Table 15). During this period, 22 of 24 non-transgenic mice had an increase in plasma glucose, while 16 of 24 transgenic mice had a decrease in plasma glucose. The change in glucose level from days 3 to 6 differed significantly between the groups (non-transgenic: +27 mg/dl, transgenic: -9 mg/dl, $p<0.001$). For non-transgenic mice, there was no apparent relationship between the 3 day glucose value and the amount of change from days 3 to 6 (Figure 39). Interestingly, however, the transgenic mice with higher glucose levels on day 3 had the larger declines from days 3 to 6 (Figure 41). Also, the 8 transgenic mice that had increases in glucose from days 3 to 6 were among the group with lower glucose values on day 3. This data is summarized in Figure 42, in which mice are grouped according to whether their 3 day glucose level is below or above 120 mg/dl. Non-transgenic mice (bars 1 and 3) and transgenic mice with 3 day values less than 120 mg/dl (bar 2) had a similar amount of increase in blood glucose at 6 days (+26.9 to +27.7 mg/dl), while transgenic mice with 3 day values greater than 120 mg/dl (bar 4) had a decrease in blood glucose (-34.4 mg/dl, $p<0.0001$) at 6 days. Although the physiological basis for these effects is not yet known, these data suggest that a homeostatic mechanism which tends to normalize high glucose

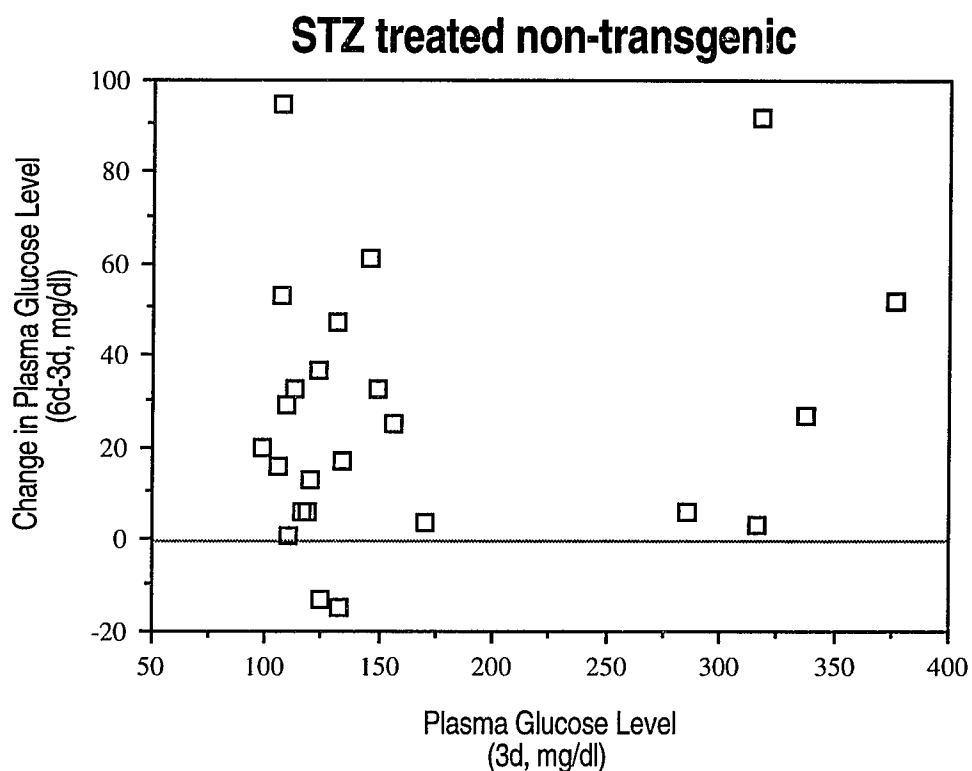


Figure 40. Fasting plasma glucose from 3 days to 6 days after STZ treatment - non-transgenic. Plasma glucose levels of STZ treated non-transgenic mice (n=24) were determined after a 4 hour fast on days 3 and 6. For each mouse, the change in glucose level from 3 days to 6 days (6d-3d) is plotted versus the 3 day glucose level.

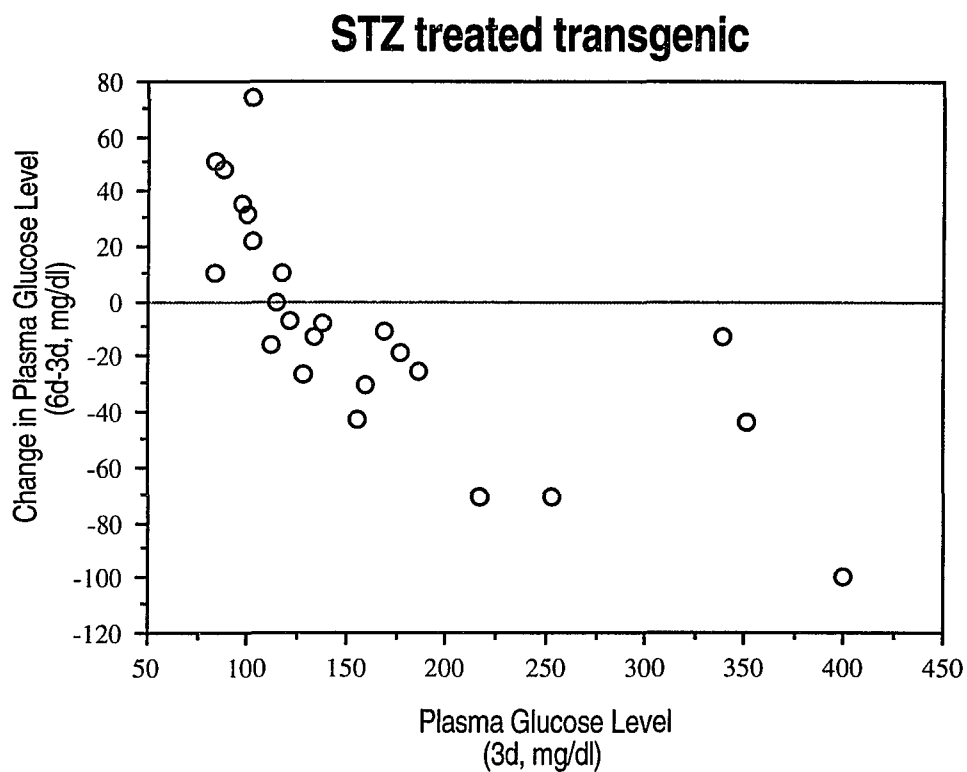


Figure 41. Fasting plasma glucose from 3 days to 6 days after STZ treatment - transgenic. Plasma glucose levels of STZ treated transgenic mice (n=24) were determined after a 4 hour fast on days 3 and 6. For each mouse, the change in glucose level from 3 days to 6 days (6d-3d) is plotted versus the 3 day glucose level.

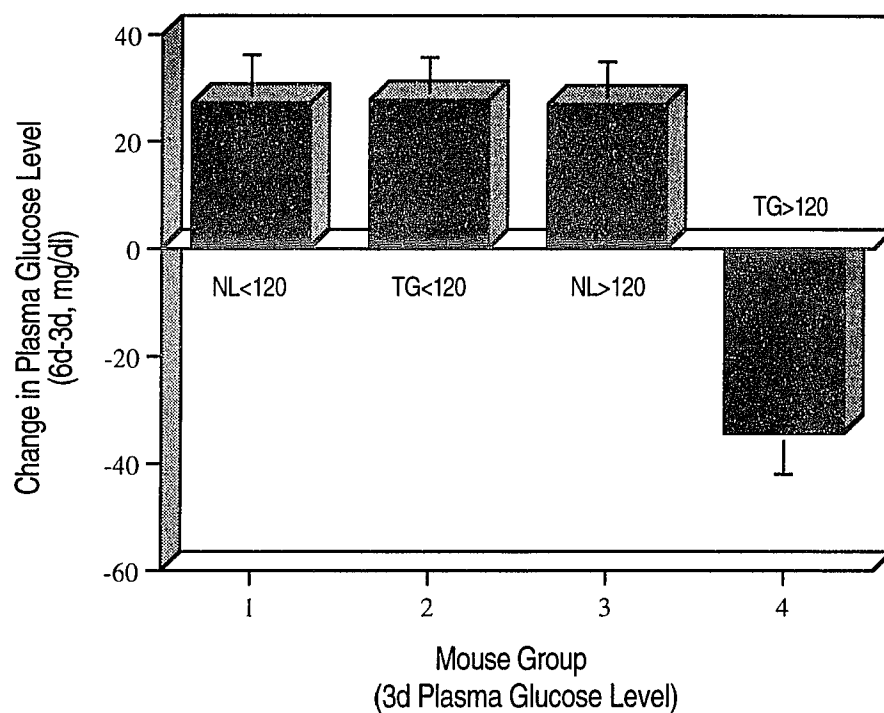


Figure 42. Change in plasma glucose from 3 days to 6 days after STZ treatment. Mice are grouped according to whether their 4 hour fasting plasma glucose value at 3 days was less than or greater than 120 mg/dl. The change in plasma glucose at six days (6d-3d) was determined for each group.

levels may exist in transgenic mice.

L. Metabolic parameters and transgene expression in STZ treated mice

Plasma glucose levels, water and food consumption, body weight changes, and transgene expression were examined at a series of time points up to 21 days after STZ treatment (Figures 43 - 46 and Tables 17 - 21). Transgenic mice consumed 16% less water at 6 days, 16% less at 9 days, 9.5% less at 15 days, and 7% less at 21 days (Figure 43); however, these differences were not statistically significant. Water consumption could not be correlated with plasma glucose levels for individual mice, because it was not practical to cage the 48 mice in the study individually. Similarly, transgenic mice consumed 8.9% less food at 6 days, 5% less at 9 days, 4.7% less at 15 days, and 4.3% less at 21 days (Figure 44); again, these differences were not statistically significant. Body weight increased at approximately the same rate for both groups (Figure 45). Mice lost approximately 3 grams of body weight during the 18 hour fast prior to injection with STZ (Table 22), and weight gains during this period represented a slow return to their pre-fast body weights. Measurement of transgene expression in liver, intestine, and kidney (Figure 46 and Table 21) showed that combined mRNA levels were 38×10^5 molec/ug total RNA 3 days after STZ treatment, compared with 64×10^5 molec/ug total RNA in untreated mice; however, this

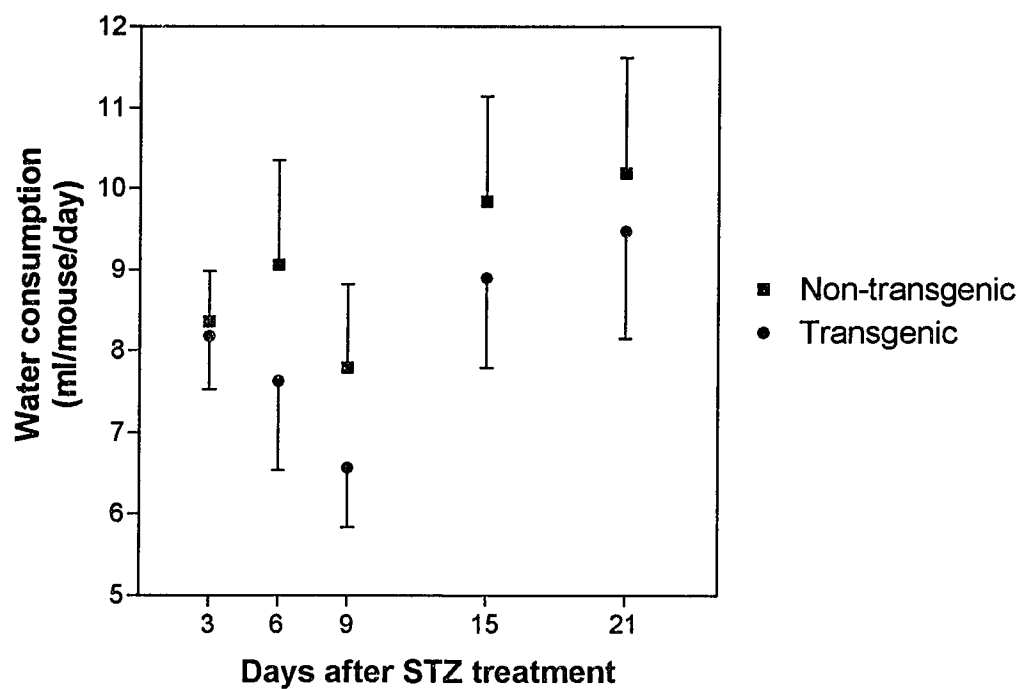


Figure 43. Water consumption of STZ treated mice. Mice were injected with 140 mg/kg of streptozotocin and water consumption was recorded on days 3, 6, 9, 15, and 21 post-injection. Total water consumption for a cage of three mice over 3 days (3,6,9) or 6 days (15,21) was converted to units of ml/mouse/day by dividing by 9 (3,6,9) or 18 (15,21).

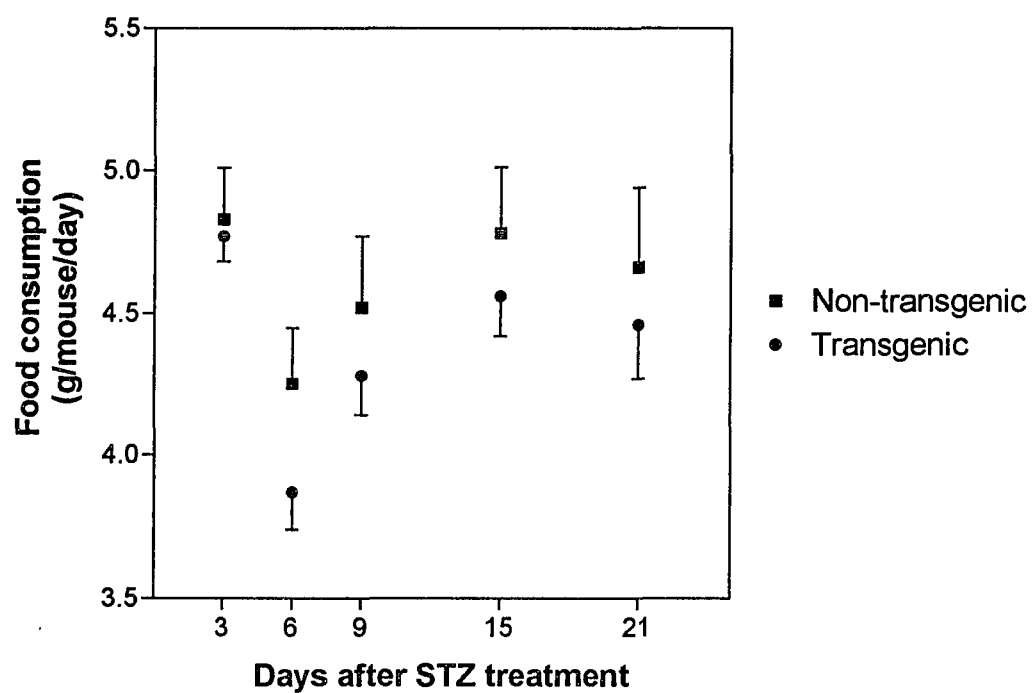


Figure 44. Food consumption of STZ treated mice. Mice were injected with 140 mg/kg of streptozotocin and food consumption was recorded on days 3, 6, 9, 15, and 21 post-injection. Total food consumption for a cage of three mice over 3 days (3,6,9) or 6 days (15,21) was converted to units of ml/mouse/day by dividing by 9 (3,6,9) or 18 (15,21).

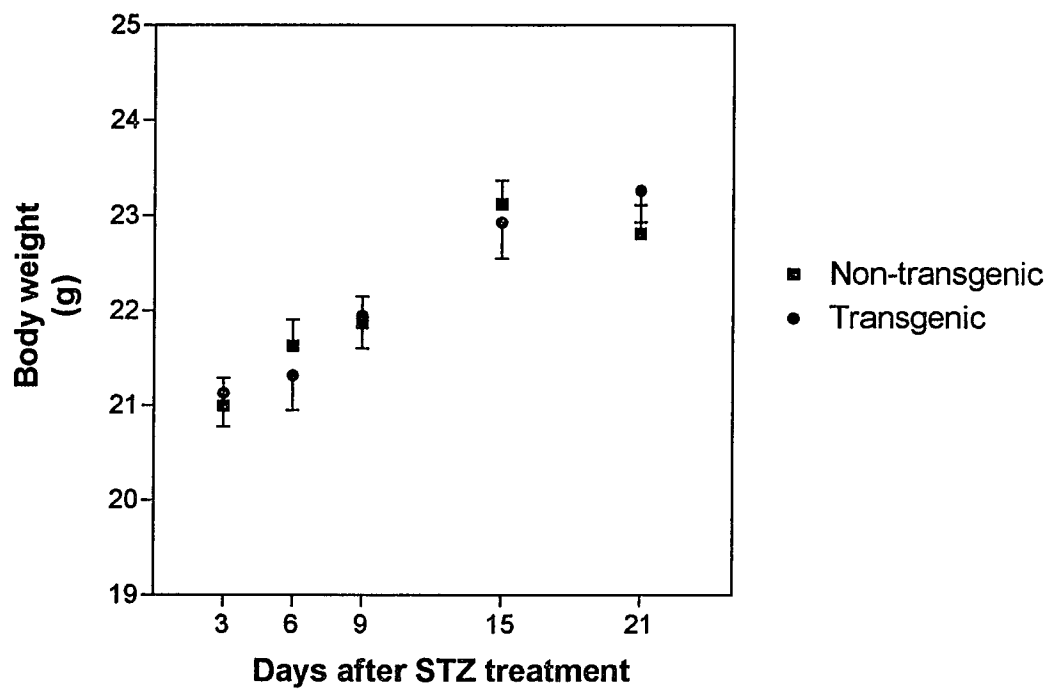


Figure 45. Body weight of STZ treated mice. Mice were treated with 140 mg/kg of streptozotocin and body weight was recorded on days 3, 6, 9, 15, and 21 post-injection.

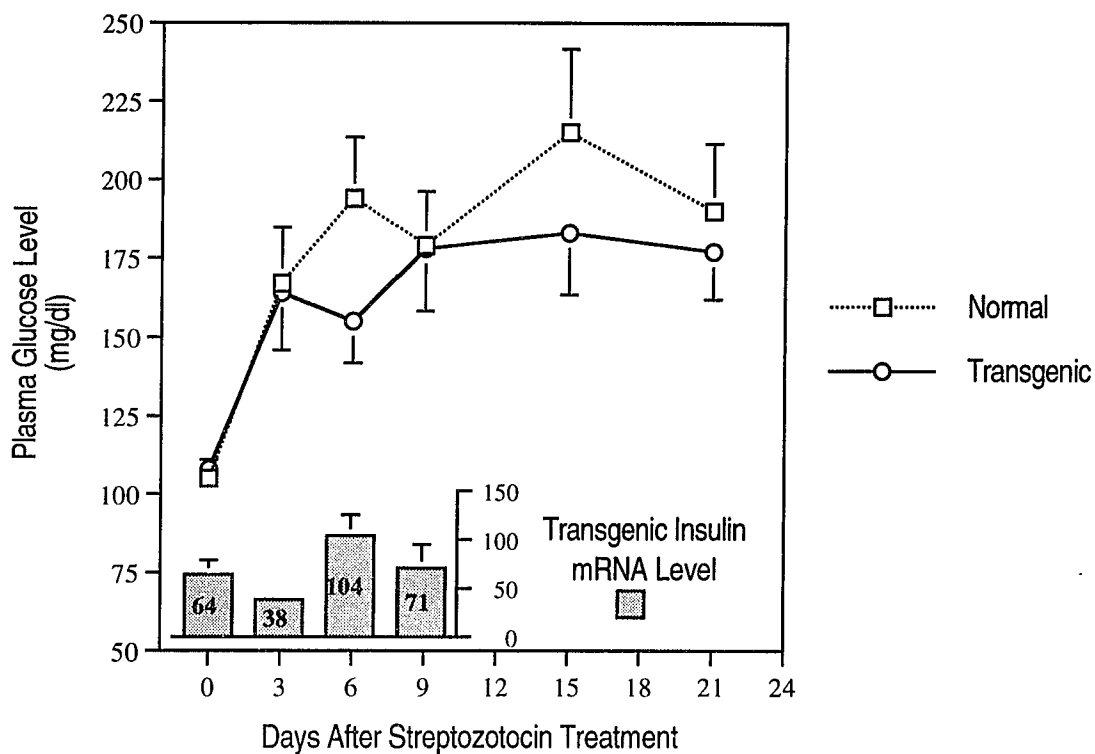


Figure 46. Plasma glucose and insulin mRNA levels in STZ treated mice. Four hour fasting plasma glucose levels were determined for STZ-treated transgenic and non-transgenic mice over the course of 21 days (n=24). In the lower inset, combined liver, intestine, and kidney insulin mRNA levels ($\times 10^5$ molecules/ug total RNA) are shown for untreated (day 0) and STZ treated (days 3, 6, & 9) transgenic mice.

difference was not statistically significant. There was a statistically significant rise in combined mRNA levels between 3 and 6 days, 38×10^5 molec/ug total RNA to 104×10^5 molec/ug total RNA. Interestingly, this was also the time period for which the most marked differences in plasma glucose levels occurred between transgenic and non-transgenic mice.

V. Discussion

In this study, we have explored a possible gene therapy approach for diabetes mellitus by engineering glucose regulated extrapancreatic insulin production in transgenic mice. To enable us to study extrapancreatic insulin production in transgenic mice, a fusion gene was constructed using the rat liver-type pyruvate kinase (L-PK) promoter to drive expression of human insulin coding sequences. Results demonstrate that insulin expression in liver and intestine is tightly regulated by dietary conditions. Fasting mice had low or undetectable levels of insulin mRNA in liver and intestine. Hepatic insulin mRNA levels were induced up to 100 fold, and intestinal levels approximately 20 fold, within 12 hours of refeeding with a high sucrose diet. Furthermore, hepatic insulin mRNA levels were stimulated 2.4 within 10 minutes after injection of a glucose load. Extrapancreatic insulin expression did not result in hypoglycemia in these mice; fasting plasma glucose levels were not significantly different from controls. Transgenic mice treated with the diabetogenic agent streptozotocin had a partial correction of hyperglycemia, with significant declines in both random and fasting glucose levels compared to controls. These findings indicate significant potential for L-PK:insulin constructs in the design of gene therapy vectors for insulin deficiency.

A. Sites of transgene expression

Three transgenic lines, H6, H4, and J6 were produced using the L-PK:hIns transgene construct. The level of expression and degree of dietary regulation was assessed by comparing insulin mRNA levels in fasted and sucrose refeed mice. Lines H6 and J6 both expressed the transgene and showed tissue-specific and dietary regulations patterns typical of those observed in other studies using the L-PK promoter in transgenic mice (40,45,47). Thus, it is prohibitively unlikely that the regulation pattern observed is due to transgene integration sites. Line J6 showed approximately ten fold lower expression than line H6 and line H4 showed not detectable expression in liver, intestine, and kidney. Transgenic mice from all three lines appeared normal; as compared to non-transgenic littermates, no differences were observed with regard to food or water intake, size, level of activity, or general health.

For line H6, transgene expression was strongly detected in liver, intestine, kidney, which are the primary sites of L-PK expression (40,45,47). However, the relative level of expression in these tissues was considerably different from that found previously for L-PK mRNA (40,45). In these studies, the level of L-PK mRNA was several fold lower in intestine than liver and barely detectable in kidney. Since the relative mRNA levels in these tissues reflects not only transcriptional rate, but also mRNA stability, it is useful

to compare these findings with studies using the chloramphenicol acetyltransferase (CAT) gene as a reporter for the 3.2 kb L-PK promoter (47,51). One study showed that CAT activity was 6, 31, and 346 fold higher in liver than intestine for three separate transgenic lines, with barely detectable CAT activity in kidney (51). Another study showed comparable levels of CAT activity for one transgenic line (47). The differing findings of these studies may be attributable to variable transgene expression between independent lines, differences in protein extraction methodology, or quantification of CAT activity. The relative levels of insulin mRNA in the liver, intestine, and kidney of line H6 are similar to the latter study; line J6 showed comparable levels in liver and intestine, with a lower level in kidney.

Transgene expression was also detected in bone marrow, pancreas, and testis for line H6. Bone marrow expression may be derived from transcriptional activity of the upstream erythroid promoter (40), however, additional mRNA analysis would be required to determine the exact site of transcriptional initiation. Pancreas has been shown to express the L-PK:hIns gene minimally (80), and testis is a common site of ectopic expression in transgenic mice (81,82). Even though the L-PK:hIns transgene was expressed in multiple sites in these mice, this does not restrict the usefulness of the L-PK promoter in developing constructs for somatic gene therapy of diabetes. If insulin production is

desirable in a specific cell type, such as hepatocytes, then specific targeting systems may be employed for gene delivery (87).

B. Dietary and temporal response patterns

To evaluate the potential to simulate beta cell insulin production, the dietary and temporal response patterns of the transgene were examined. Hepatic insulin mRNA levels were 50 to 100 fold higher in mice fed the sucrose diet for 12 hours compared with mice fasted for 24 hours. Intestinal levels increased about 20 fold under these conditions, and kidney showed a relatively high fasting level with a moderate increase in fed mice. A detailed examination of the time course of stimulation in liver revealed that, after a 24 hour fast, insulin mRNA was first detectable 6 hours after refeeding, peaked at 18 to 26 hours, and declined markedly within 12 hours when fasting was reinstituted after a 40 hour refeeding period. These experiments employed extreme conditions such as 24 hour fasting periods and a 68% sucrose diet for refeeding. Nevertheless, the findings indicate that insulin levels may be regulated over the course of hours by dietary changes. Such temporal response patterns may be highly desirable for avoiding fasting hypoglycemia in diabetics, which is a common problem with insulin treatment regimens. In addition, increased insulin production during the hours of food consumption may help to achieve better blood glucose control.

The possibility that the L-PK:insulin gene may be capable of responding rapidly to increases in plasma glucose was also examined. A shorter initial fasting period of 6 hours was used so that insulin mRNA levels could be measurable at baseline. There was a 2.4 fold increase in hepatic insulin mRNA at 10 minutes after injection of a glucose load. These data, if proven valid by further, more extensive testing, are consistent with a rapid transcriptional response to glucose, although glucose induced stabilization of insulin mRNA is another possibility. The use of transcriptional inhibitors and nuclear run-on assays could be useful in elucidating the nature of this effect, as would experiments utilizing varying glucose loads and more time points. The significance of the observed 10 minute effect with respect to the treatment of diabetes is not yet clear. The 2.4 fold increase in insulin mRNA was seen after administration of a relatively large glucose load, 3.5 g/kg body weight, twice that which is administered for clinical glucose tolerance test (88). Nevertheless, more extensive investigations of the short time course effects are clearly warranted, since rapid adjustment of insulin production based on changes in ambient glucose concentration may yield substantial therapeutic benefits.

Dietary regulation was further examined by varying the content of the refeeding diet. A standard chow diet resulted in small increases over fasting levels when

compared with the sucrose diet, indicating the importance of dietary sugars in the response. Interestingly, however, a breeding diet that is low in sugar content resulted in marked stimulation in liver and kidney, but not intestine. This effect could be related to the relatively high fat content of the breeding diet. Metabolism of triglycerides may yield significant amounts of glycerol, which is known to stimulate L-PK expression in a similar pattern (55).

C. Physiological effects of extrapancreatic insulin production

Oral glucose tolerance testing revealed that transgenic mice had lower plasma glucose levels from 30 minutes to 2 hours after administration of a glucose load. Differences in the rates of absorption and/or plasma clearance of glucose in transgenic mice may exist as a result of extrapancreatic insulin production. However, since glucose levels were not significantly different during the rapid absorptive phase, increased plasma clearance of glucose would seem to be a more likely mechanism. Since a large percentage of an oral glucose load is cleared by the liver (89), stimulation of glucose-metabolizing pathways resulting from increased intra-hepatic insulin concentrations could have substantial effects on the hepatic extraction of glucose. In addition to insulin secretion by the liver itself, contributions from the intestine through portal

blood flow may lead to increased intra-hepatic insulin concentrations in transgenic mice. A possible mechanism for increases in intra-hepatic insulin within 30 minutes of glucose administration is suggested by the increase in insulin mRNA levels that was observed 10 minutes after injection of a glucose load. However, baseline increases in intra-hepatic insulin may also account for these observations. Further studies of the rapid response patterns of the L-PK promoter, including comparison to non-glucose regulated promoters, may help clarify this issue.

Mice were treated with the diabetogenic agent streptozotocin (STZ) to assess the physiological significance of extrapancreatic insulin production after ablation of pancreatic beta cells. Six days after STZ treatment, non-transgenic mice had a mean random plasma glucose level of 524 mg/dl, while transgenic mice had a significantly lower mean of 330 mg/dl. A recent study of transgenic mice expressing human insulin coding sequences under the control of the PEPCK promoter reported similar results, with STZ treated non-transgenic mice having a mean random plasma glucose level >600 mg/dl and STZ treated transgenic mice having a mean level of 396 mg/dl (90). Fasting plasma glucose levels were not reported for this study. In another recent study, sustained low level expression of the rat insulin 1 gene was achieved by the in vivo administration of a recombinant retroviral vector (9). STZ treated rats exhibited normoglycemia during a 24 hour

fast, but there was no difference in non-fasting levels between insulin-transduced and control rats.

Plasma glucose levels were also examined after a 4 hour fast from 3 to 21 days after STZ treatment. During the period from 3 to 6 days, 67% of transgenic mice had a decrease in plasma glucose, while only 8% of non-transgenic mice had a decrease. Further, the size of the decrease for transgenic mice appeared to be directly related to the 3 day glucose value, suggesting that a homeostatic mechanism which tends to normalize high glucose levels may exist. Interestingly, there was a significant rise in total insulin mRNA levels during this time period. However, insulin mRNA levels and blood glucose levels were not correlated for individual animals, and the mechanism underlying these responses requires further investigation.

The marked differences between transgenic and non-transgenic mice in response to STZ treatment from 3 to 6 days did not persist. During the period from 6 to 21 days after STZ treatment, there was a gradual increase in plasma glucose levels for both transgenic and non-transgenic mice. Water and food consumption also increased for both groups during this time period, indicating a worsening of the diabetic state. Transgenic mice exhibited a tendency toward decreased water and food consumption throughout the 21 day period after STZ treatment, although statistical significance was not reached for individual time points.

Many metabolic factors must be considered in attempting to draw conclusions from these results. At the initiation of the experiment, mice experience a 3 gram weight loss (approximately 13% of total body weight) due to the fasting period required prior to STZ treatment. This fasting and weight loss could affect expression of the transgene. STZ has significant toxicity, especially towards two of the major sites of transgene expression, liver and kidney (63). A rise in transgene expression from 3 to 6 days after STZ treatment might be partially attributable to a recovery from these metabolic stresses. However, since glucose is a known stimulator of L-PK expression, a rise in transgene expression may result from marked increases in plasma glucose associated with the loss of blood glucose control caused by diminished function of pancreatic beta cells.

Since insulin production in transgenic mice is dependent on the activity of the L-PK promoter, metabolic derangements that are known to affect L-PK expression may also account for diminished insulin expression. In severely diabetic rats, the glucagon/insulin ratio is very high and the L-PK gene is not expressed; when insulin treatment is instituted the level of L-PK expression returns to normal (91). Insulin probably acts by stimulating the synthesis of glucokinase, normalizing the glucagon/insulin ratio, and increasing the rate of glycolysis, thus generating the signaling molecule required for activation of the L-PK gene. The transgenic mice in this study may only achieve

significant benefits while the metabolic derangements associated with loss of pancreatic insulin are minor. Hepatic insulin production that is sufficient to bring about significant diminution in plasma glucose in the early phase may be insufficient to maintain normal glucokinase activity and glucagon/insulin ratio. Thus, a gradual decrease in the rate of hepatic glycolysis would occur, and L-PK promoter activity would decrease. A negative cycle would then be established as transgenic insulin production declines further.

D. Prospects for gene therapy of diabetes

In this study, the carbohydrate responsive L-PK promoter was used to regulate expression of the human insulin gene in transgenic mice. Previous attempts to engineer extrapancreatic insulin production have been partially successful (14,21,22,84,90), but have not employed promoters with responsiveness similar to that of endogenous insulin (28,30). Although AtT-20 pituitary cells have been engineered for glucose stimulated insulin release, these cells respond to glucose levels below the physiologic range (15,22). Results presented here demonstrate that an L-PK:insulin fusion gene leads to dietary and temporal regulation patterns with significant potential to simulate beta cell insulin production in extrapancreatic sites.

In this study STZ treated transgenic mice had only partial control of hyperglycemia. This may be due to

relatively low serum insulin levels and predominance of the less active proinsulin form (84). Several approaches may be taken to increase the expression level of the construct and the activity of the synthesized protein. Additional transcriptional enhancers could be incorporated, possibly including liver-specific enhancers, general enhancers, or additional regions surrounding the L-PK gene. Constructs could incorporate enhanced ribosome binding sites to increase translational efficiency (68). The use of mutant insulins allowing complete proinsulin processing and greater intracellular stability in extrapancreatic sites (85), may increase the physiologic activity of the synthesized protein and improve glycemic control.

Since L-PK expression is tightly regulated in liver, with animals fasting for 24 hours having undetectable insulin mRNA, it may be necessary to provide a low baseline insulin level as an added component in future studies attempting somatic gene therapy for severe insulin deficiency. This may be necessary to allow sufficient activity of the glycolytic pathway so that glucose signals can act to upregulate L-PK expression. The findings of this study suggest two possible approaches to this problem. A baseline insulin level may be provided either by targeting additional tissues, such as kidney, which maintains high levels of expression with fasting, or by using a second insulin construct driven by a promoter uninfluenced by dietary conditions, such as beta actin. However, such

approaches may not be necessary if the expression level and activity of L-PK:insulin constructs are improved to the point at which fasting insulin levels are sufficient to maintain normal activity of the L-PK signaling pathway in liver. While many issues remain to be explored, improvements in construct design may eventually lead to the development of gene therapy vectors suitable for the treatment of insulin deficiency.

Appendix 1 - Data tables

Table 2. Reproducibility of RT-PCR assay

Sample	Expt #1	Expt #2
1.0	0.3100	0.350
2.0	0.4800	0.420
3.0	0.7200	0.630
4.0	0.2400	0.230
5.0	0.3300	0.360
6.0	0.6000	0.520
7.0	0.7400	0.700
8.0	0.4800	0.420
9.0	0.8200	0.740
10.0	0.2700	0.290
11.0	0.3400	0.350
12.0	1.0000	1.000

Table 3. Insulin mRNA levels during 40 hour refeed experiment (n=3)
($\times 10^5$ molec/ug total RNA)

Refeed time	Insulin mRNA level		
	Y1	Y2	Y3
Fast 24h	0.00	0.00	0.00
2h	0.00	0.00	0.00
4h	0.00	0.00	0.00
6h	5.50	7.00	2.20
8h	0.40	5.30	1.80
12h	4.00	3.80	32.00
18h	14.50	32.00	6.90
26h	32.00	7.50	7.10
40h	2.20	6.90	0.20
Fast 12h	0.00	0.29	0.24
Fast 24h	0.00	0.00	0.00
Fast 36h	0.00	0.32	0.00

(0.00 = undetectable)

Table 4. Insulin mRNA levels during 40 hour refeed experiment
(means of 3 ind. versus pools)
($\times 10^5$ molec/ug total RNA)

Refeed time	Mean (3 ind.)	Pool
Fast 24h	0.00	0.00
2h	0.00	0.00
4h	0.00	0.00
6h	4.90	4.40
8h	2.50	2.70
12h	13.30	14.80
18h	17.80	19.60
26h	15.50	17.20
40h	3.10	4.80
Fast 12h	0.18	0.26
Fast 24h	0.00	0.00
Fast 36h	0.11	0.10

(0.00 = undetectable)

Table 5. Food consumption during 40 hour refeeding experiment (n=3)

Time (hours)	Food consumed (g)		
	Y1	Y2	Y3
0.0	0.00	0.00	0.00
2.0	0.45	0.46	0.75
4.0	1.07	0.88	0.94
6.0	1.26	1.40	1.54
8.0	1.60	1.10	1.70
12.0	2.50	2.10	2.30
18.0	3.70	3.50	3.60
26.0	5.10	5.30	4.80
40.0	8.60	8.40	7.80

Table 6. Human insulin mRNA level after glucose load
 (RNA:mRNA level; PG:Plasma glucose, n=4)
 (RNA:molecx10⁵/ug; PG:mg/dl)

RNA 0 min	RNA 10 min	RNA 20 min	PG 0 min	PG 10 min
43.0	2.50	56.0	66.0	228.0
73.0	62.00	11.0	76.0	376.0
1.5	75.00	38.0	131.0	352.0
0.0	33.00	53.0	112.0	286.0

(0.0= undetectable)

Table 8: Serum immunoreactivities for insulin and C peptides

Immunoreactive	Normal		Transgenic	
	Y	SEM	Y	SEM
Total Insulin	517.0	78.0	633.0	50.0
Human C peptide	130.4	39.8	323.1	55.6
Mouse C peptide	1857.0	291.0	1492.0	252.0

Y = mean value

Table 9. Serum protein concentrations

(n=6)

Total insulin (tIns)

Human C peptide (hCpep)

Mouse C peptide (mCpep)

(pM)

NTG tIns	TG tIns	NTG hCpep	TG hCpep	NTG mCpep	TG mCpep
246.0	595.0	86.00	203.0	1206.0	784.0
605.0	696.0	301.00	449.0	1482.0	826.0
367.0	710.0	19.00	429.0	2684.0	1288.0
632.0	512.0	172.00	240.0	1252.0	2144.0
772.0	486.0	119.00	161.0	1712.0	1766.0
478.0	796.0	86.00	457.0	2804.0	2144.0

Table 10. Male growth curve, 3-6 weeks
(n=12)

Body weight (g)
(NTG:Non-transgenic; TG:Transgenic)

NTG 3w	TG 3w	NTG 4w	TG 4w	NTG 5w	TG 5w	NTG 6w	TG 6w
13.50	12.60	22.30	19.50	25.40	22.90	27.70	24.50
13.30	12.30	21.10	19.70	23.80	23.30	25.00	24.40
13.50	13.50	20.80	21.20	23.80	24.60	24.60	25.60
13.60	13.20	22.40	20.50	25.30	24.00	26.70	24.70
13.90	15.30	22.10	23.10	24.40	25.90	25.30	27.00
13.80	12.90	21.00	20.50	23.70	22.50	24.80	23.00
13.80	14.10	22.50	22.30	25.20	24.50	27.00	25.60
13.90	12.70	21.00	21.30	24.20	23.60	25.40	23.90
13.80	13.50	22.10	22.20	24.70	24.80	26.50	26.30
12.20	13.10	19.80	20.80	23.10	25.30	25.60	27.00
11.00	12.30	19.80	20.60	22.90	25.60	25.50	28.00
11.20	11.40	20.80	21.20	24.80	24.70	24.60	26.40

Table 11. Female growth curve, 3-6 weeks
(n=12)

Body weight (g)
(NTG:Non-transgenic; TG:Transgenic)

NTG 3w	TG 3w	NTG 4w	TG 4w	NTG 5w	TG 5w	NTG 6w	TG 6w
13.50	12.60	22.30	19.50	25.40	22.90	27.70	24.50
13.30	12.30	21.10	19.70	23.80	23.30	25.00	24.40
13.50	13.50	20.80	21.20	23.80	24.60	24.60	25.60
13.60	13.20	22.40	20.50	25.30	24.00	26.70	24.70
13.90	15.30	22.10	23.10	24.40	25.90	25.30	27.00
13.80	12.90	21.00	20.50	23.70	22.50	24.80	23.00
13.80	14.10	22.50	22.30	25.20	24.50	27.00	25.60
13.90	12.70	21.00	21.30	24.20	23.60	25.40	23.90
13.80	13.50	22.10	22.20	24.70	24.80	26.50	26.30
12.20	13.10	19.80	20.80	23.10	25.30	25.60	27.00
11.00	12.30	19.80	20.60	22.90	25.60	25.50	28.00
11.20	11.40	20.80	21.20	24.80	24.70	24.60	26.40

Table 12. Oral glucose tolerance test
(n=4)
Plasma glucose level (mg/dl)
Time (minutes)

Time point	Non-transgenic				Transgenic			
	Y1	Y2	Y3	Y4	Y1	Y2	Y3	Y4
0	94.0	94.0	131.0	93.0	107.0	68.0	100.0	108.0
10	189.0	202.0	226.0	163.0	166.0	162.0	207.0	208.0
20	242.0	343.0	358.0	248.0	223.0	343.0	316.0	259.0
30	326.0	322.0	311.0	297.0	266.0	287.0	283.0	302.0
45	367.0	297.0	319.0	384.0	230.0	244.0	293.0	284.0
60	322.0	248.0	282.0	335.0	206.0	196.0	264.0	244.0
90	277.0	243.0			207.0	208.0		
120	241.0	207.0			162.0	169.0		

Table 13. STZ Dose-response curve
Plasma glucose (mg/dl)
(n=3)

Dose (mg/kg)	Non-transgenic			Transgenic		
	Y1	Y2	Y3	Y1	Y2	Y3
0	100.0	131.0		90.0	118.0	
70	190.0	230.0	187.0	157.0	123.0	239.0
100	184.0	148.0	167.0	141.0	167.0	138.0
140	213.0	581.0	371.0	445.0	218.0	200.0
180	465.0	413.0		401.0	355.0	

Table 14. $\pm$ STZ treatment 6 days
(-STZ, n=6; +STZ, n=12)
Plasma glucose level, random (mg/dl)
(NTG: Non-transgenic; TG: Transgenic)

NTG -STZ	TG -STZ	NTG +STZ	TG +STZ
141.0	144.0	534.0	236.0
132.0	128.0	544.0	337.0
158.0	149.0	203.0	546.0
146.0	138.0	217.0	625.0
149.0	156.0	410.0	198.0
173.0	165.0	196.0	357.0
		857.0	198.0
		550.0	571.0
		694.0	205.0
		650.0	292.0
		691.0	223.0
		743.0	169.0

Table 15. STZ treated 3-6 days (n=24)
Plasma glucose level, 4 hour fast (mg/dl)

Non-transgenic		Transgenic	
3 day	6 day - 3 day	3 day	6 day - 3 day
337.0	27.0	254.0	-71.0
110.0	1.0	112.0	-16.0
156.0	25.0	128.0	-26.0
317.0	92.0	115.0	0.0
134.0	17.0	134.0	-13.0
124.0	-13.0	102.0	22.0
133.0	-15.0	186.0	-25.0
316.0	3.0	155.0	-43.0
109.0	29.0	177.0	-19.0
119.0	13.0	97.0	36.0
149.0	33.0	400.0	-100.0
118.0	6.0	169.0	-11.0
116.0	6.0	100.0	32.0
131.0	47.0	159.0	-30.0
105.0	16.0	137.0	-8.0
107.0	95.0	103.0	74.0
123.0	37.0	88.0	48.0
146.0	61.0	83.0	51.0
170.0	4.0	118.0	10.0
286.0	6.0	217.0	-71.0
112.0	33.0	83.0	10.0
107.0	53.0	122.0	-7.0
377.0	52.0	352.0	-44.0
98.0	20.0	340.0	-13.0

Table 16. Change in plasma glucose from 3d-6d
(mean and SEM, n=12)
(mg/dl)

Group	Glucose (mg/dl)	
	Y	SEM
NL<120	27.20	9.00
TG<120	27.70	8.00
NL>120	26.90	8.00
TG>120	-34.40	7.60

Table 17. Water consumption of STZ treated mice

STZ treated 3-21 days (n=8)
 Water consumption (ml/cage of 3 mice)
 (Values shown are measured since previous time point)
 (NTG: Non-transgenic; TG: Transgenic)

NTG 3d	TG 3d	NTG 6d	TG 6d	NTG 9d	TG 9d	NTG 15d	TG 15d	NTG 21d	TG 21d
92.0	75.0	99.00	75.00	105.0	70.0	230.00	167.00	252.00	180.00
80.0	59.0	82.00	47.00	75.0	50.0	190.00	107.00	239.00	118.00
90.0	70.0	145.00	54.00	113.0	51.0	268.00	148.00	277.00	175.00
65.0	106.0	54.00	116.00	52.0	102.0	108.00	231.00	112.00	260.00
63.0	58.0	54.00	50.00	63.0	50.0	109.00	120.00	106.00	111.00
55.0	70.0	50.00	52.00	45.0	50.0	102.00	118.00	93.00	105.00
62.0	62.0	67.00	49.00	63.0	50.0	167.00	131.00	174.00	136.00
95.0	89.0	101.00	106.00	45.0	50.0	243.00	260.00	215.00	278.00

Table 18. Food consumption of STZ treated mice

STZ treated 3-21 days (n=8)
 Food consumption (g/cage of 3 mice)
 (Values shown are measured since previous time point)
 (NTG: Non-transgenic; TG: Transgenic)

NTG 3d	TG 3d	NTG 6d	TG 6d	NTG 9d	TG 9d	NTG 15d	TG 15d	NTG 21d	TG 21d
45.3	45.0	42.60	35.40	44.0	38.3	93.20	77.70	99.00	84.70
44.8	42.5	37.90	29.00	38.0	33.9	86.10	72.00	95.90	77.10
44.9	43.3	39.20	33.50	50.1	37.6	98.90	79.00	100.40	86.50
47.0	47.3	30.80	39.30	38.4	44.6	73.90	86.90	80.60	95.10
39.6	41.7	35.10	35.20	36.7	36.9	75.00	87.00	65.60	69.20
39.1	42.1	35.60	33.30	32.6	35.2	75.00	78.10	66.20	66.60
36.7	41.4	37.30	34.40	36.5	39.4	81.80	81.40	74.90	77.00
50.3	39.9	47.70	38.70	49.2	42.5	104.30	94.20	87.80	85.30

Table 19. Body weight of STZ treated mice

Pre-fast and post-fast body weight (g) ,(n=24)
(NTG:Non-transgenic; TG:Transgenic)

NTG Pre-fast	NTG Post-fast	TG Pre-fast	TG Post-fast
22.0	18.90	23.50	20.30
22.7	19.00	22.80	19.40
23.2	19.50	23.70	21.10
24.4	20.80	26.80	23.50
22.3	19.00	24.20	20.60
26.3	22.90	24.00	20.20
23.6	19.70	24.40	21.40
24.3	21.20	23.30	20.30
21.2	18.20	22.20	19.10
23.2	20.50	23.40	19.90
23.6	20.20	18.90	16.20
19.2	16.10	22.30	19.30
23.7	21.10	22.80	20.00
21.3	19.10	23.00	19.30
22.3	19.90	22.00	19.60
22.8	20.00	23.20	20.00
20.0	17.50	22.50	19.60
22.2	19.70	19.40	17.20
21.7	18.40	22.20	19.50
21.9	18.90	21.60	18.50
23.3	20.80	21.60	18.30
22.3	19.20	21.30	18.20
21.9	18.60	20.50	17.30
22.5	18.20	22.70	19.50

Table 20. Plasma glucose levels of STZ treated mice

STZ treated 3-21 days (n=24)
 Plasma glucose level, 4 hour fast (mg/dl)
 (NTG:Non-transgenic; TG:Transgenic)

NTG 3d	TG 3d	NTG 6d	TG 6d	NTG 9d	TG 9d	NTG 15d	TG 15d	NTG 21d	TG 21d
337.0	254.0	364.00	183.00	261.0	206.0	446.00	194.00	454.00	352.00
110.0	112.0	111.00	96.00	112.0	96.0	105.00	88.00	111.00	123.00
156.0	128.0	181.00	102.00	181.0	128.0	193.00	99.00	219.00	125.00
317.0	115.0	409.00	115.00	223.0	98.0	446.00	115.00	368.00	123.00
134.0	134.0	151.00	121.00	112.0	148.0	154.00	151.00	126.00	150.00
124.0	102.0	111.00	124.00	104.0	121.0	105.00	130.00	122.00	173.00
133.0	186.0	118.00	161.00	143.0	170.0	169.00	202.00	132.00	188.00
316.0	155.0	319.00	112.00	312.0	198.0	437.00	153.00	312.00	188.00
109.0	177.0	138.00	158.00	138.0	204.0	129.00	182.00	129.00	161.00
119.0	97.0	140.00	133.00	131.0	120.0	114.00	102.00	146.00	128.00
149.0	400.0	162.00	300.00	138.0	406.0	113.00	374.00	157.00	308.00
118.0	169.0	151.00	158.00	139.0	223.0	115.00	135.00	154.00	176.00
116.0	100.0	122.00	132.00	148.0	128.0	171.00	159.00	125.00	134.00
131.0	159.0	178.00	129.00	120.0	144.0	152.00	304.00	117.00	206.00
105.0	137.0	121.00	129.00	139.0	171.0	167.00	211.00	117.00	182.00
107.0	103.0	202.00	177.00	190.0	117.0	167.00	146.00	161.00	125.00
123.0	88.0	160.00	136.00	179.0	115.0	158.00	128.00	122.00	134.00
146.0	83.0	207.00	134.00	203.0	121.0	257.00	129.00	167.00	108.00
170.0	118.0	174.00	128.00	201.0	110.0	228.00	123.00	175.00	127.00
286.0	217.0	292.00	146.00	333.0	244.0	468.00	205.00	388.00	172.00
112.0	83.0	145.00	93.00	144.0	105.0	149.00	97.00	145.00	92.00
107.0	122.0	160.00	115.00	118.0	114.0	146.00	177.00	131.00	137.00
377.0	352.0	429.00	308.00	443.0	359.0	476.00	314.00	386.00	303.00
98.0	340.0	118.00	327.00	85.0	441.0	101.00	479.00	105.00	335.00

No STZ treatment 3-6 days (n=9)
 Plasma glucose level, 4 hour fast (mg/dl)
 (NTG:Non-transgenic; TG:Transgenic)

NTG 3d	TG 3d	NTG 6d	TG 6d
67.0	88.0	88.0	123.0
76.0	74.0	98.0	116.0
61.0	76.0	110.0	133.0
130.0	88.0	88.0	101.0
141.0	94.0	127.0	111.0
114.0	132.0	138.0	112.0
143.0	137.0	93.0	100.0
123.0	149.0	110.0	89.0
157.0	130.0	91.0	88.0

Table 21. Insulin mRNA levels of STZ treated mice

STZ treated 0-9 days (n=4)
 Human insulin mRNA level
 (molecx10⁵/ug total RNA)

Liver 0d	Intestine 0d	Kidney 0d	Liver 3d	Intestine 3d	Kidney 3d
55.0	0.0	56.0	69.0	47.0	29.0
126.0	136.0	133.0	56.0	52.0	39.0
0.0	88.0	66.0	5.0	41.0	3.0
46.0	0.0	0.0	15.0	58.0	43.0
166.0	83.0	0.0			

Liver 6d	Intestine 6d	Kidney 6d	Liver 9d	Intestine 9d	Kidney 9d
69.0	121.0	211.0	122.0	70.0	28.0
176.0	102.0	228.0	49.0	276.0	0.0
92.0	81.0	118.0	172.0	70.0	56.0
0.0	51.0	0.0	9.0	0.0	0.0

(0.0=undetectable)

Table 22. Body weights after 18 hour fast

Pre-fast and post-fast body weight (g) ,(n=24)
 (NTG:Non-transgenic; TG:Transgenic)

NTG Pre-fast	NTG Post-fast	TG Pre-fast	TG Post-fast
22.0	18.90	23.50	20.30
22.7	19.00	22.80	19.40
23.2	19.50	23.70	21.10
24.4	20.80	26.80	23.50
22.3	19.00	24.20	20.60
26.3	22.90	24.00	20.20
23.6	19.70	24.40	21.40
24.3	21.20	23.30	20.30
21.2	18.20	22.20	19.10
23.2	20.50	23.40	19.90
23.6	20.20	18.90	16.20
19.2	16.10	22.30	19.30
23.7	21.10	22.80	20.00
21.3	19.10	23.00	19.30
22.3	19.90	22.00	19.60
22.8	20.00	23.20	20.00
20.0	17.50	22.50	19.60
22.2	19.70	19.40	17.20
21.7	18.40	22.20	19.50
21.9	18.90	21.60	18.50
23.3	20.80	21.60	18.30
22.3	19.20	21.30	18.20
21.9	18.60	20.50	17.30
22.5	18.20	22.70	19.50

Appendix 2: Development of a transcriptional regulatory system using the mouse metallothionein promoter and lac operon elements

The intent of these studies is to develop a transcriptional regulatory system that has a wide range of expression and which is regulatable by small molecule inducers. The regulatory elements of the mouse metallothionein (mMT) promoter have been mapped using deletion mutants and a 12 base consensus metal response element (MRE) has been characterized (92). In addition, deletion of the binding site for the major late transcription factor (MLTF) produced a fourfold reduction in transcriptional efficiency (93).

To engineer a mMT promoter with low basal activity and retained metal responsiveness, a 36 bp region from -70 to -106 with respect to the transcription start site was replaced with 36 bp of new sequence containing two MRE consensus sites (Figure 47) by site directed mutagenesis. This region contains the MLTF binding site (93) and was associated with high basal activity of the promoter in previous studies (92). In initial experiments, transfection of HepG2 cells with the mutated promoter fused to the human growth hormone (hGH) gene resulted in a ten fold reduction in basal activity (Figure 4, compare bars 11 & 13, and 15 & 17). The ratio of metal-induced/basal activity was increased for the mutant promoter (bars 13-14 and 17-18 versus bars 11-12).

Mouse metallothionein - Lac operator - Growth hormone Constructs

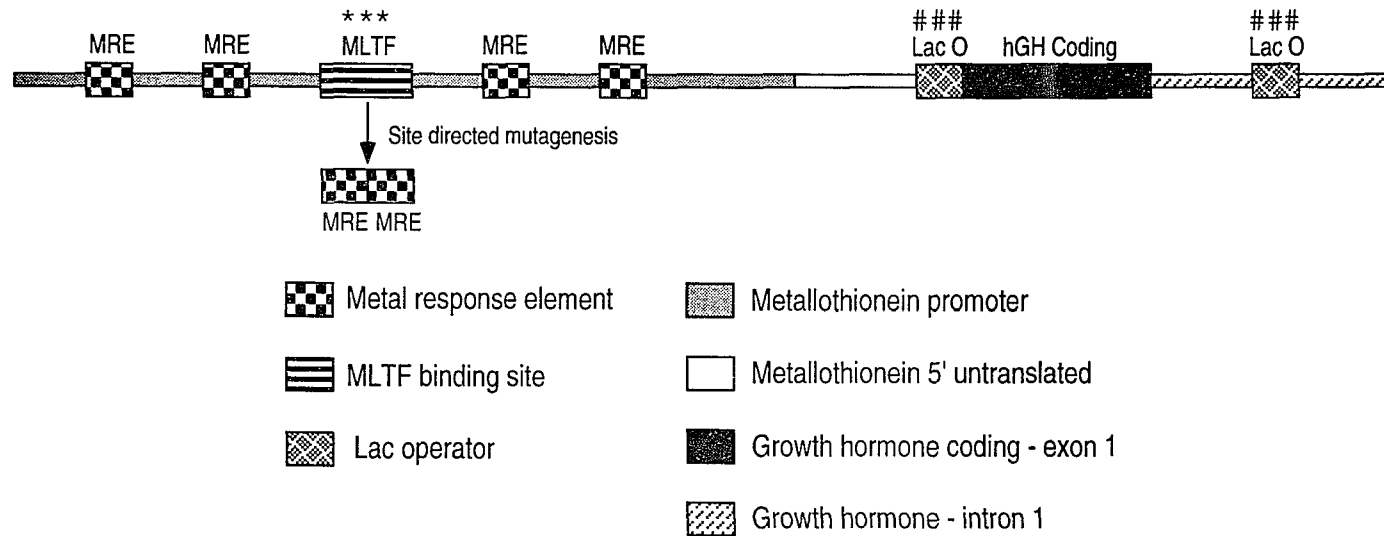


Figure 47. Development of a transcriptional regulatory system using metal response elements and the lac operator-repressor. The range of induction of the mouse metallothionein promoter was increased by replacing the MLTF binding site, which is associated with a high basal level of expression, with additional metal response elements (***). Lac operator sequences (###) were placed within the 5' untranslated region and the first intron of the human growth hormone gene to allow binding of the lac repressor. Binding of the lac repressor creates a block to transcription which can be relieved by applying lac inducers such as IPTG.

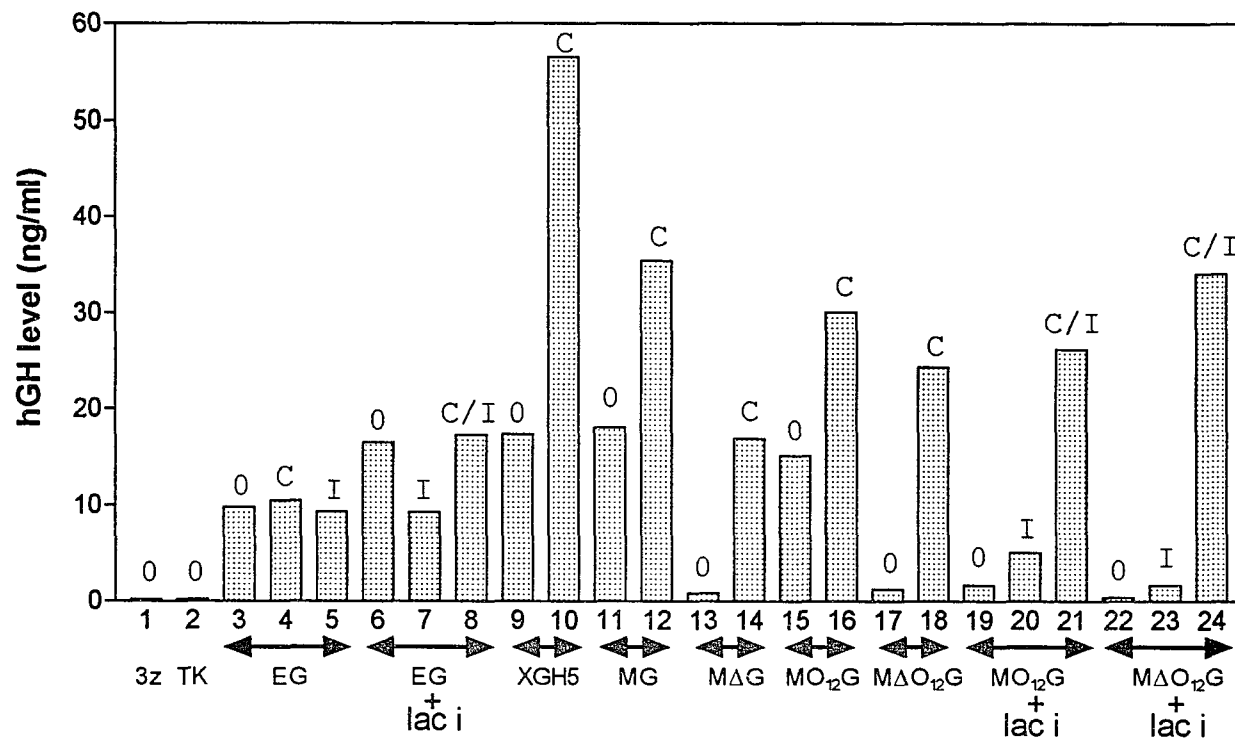


Figure 48. Metallothionein-lac operator-growth hormone constructs. HepG2 cells were transiently transfected with constructs based on the mouse metallothionein (MT) promoter which incorporate lac operon regulatory elements. Secretion of human growth hormone was measured after a 48 culture period. Various promoters were fused to the coding sequences for human growth hormone. The transfected plasmid DNA is indicated below the graph as follows: 3z, the vector pGEM3zf+; TK, herpes thymidine kinase minimal promoter; EG, human elongation factor 1A promoter; XGH5, 1.8 kb MT promoter; MG, 0.5 kb MT promoter; MΔG, MLTF mutant MT promoter; MO₁₂G, MT promoter with 5' UTR and intronic lac operators; MΔO₁₂G, MLTF mutant with lac operators; lac i, CMV-lac repressor construct. The inducers present are indicated above the bars: 0, no inducers; C, 5 uM cadmium; I, 20 mM IPTG.

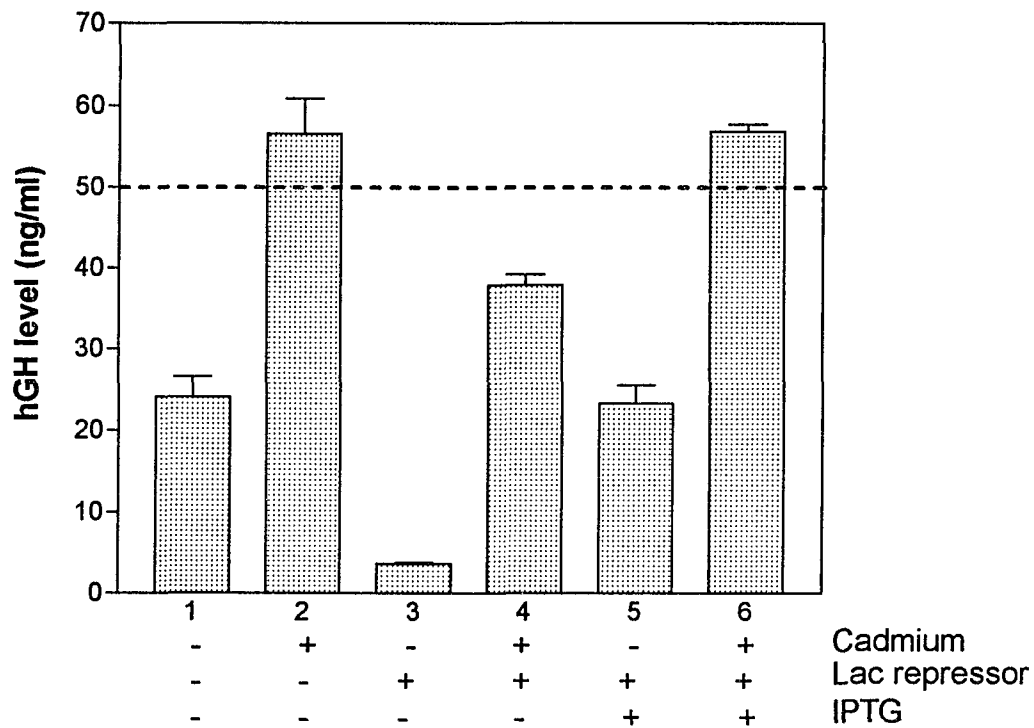


Figure 49. Regulation of growth hormone secretion by small molecule regulators. HepG2 cells were transiently transfected with the MO₁₂G construct, which contains the unmutated 0.5 kb metallothionein promoter with both the 5'UTR and intronic lac operators. For bars 3-6 the CMV-lac repressor construct was cotransfected. For bars 5 and 6, 20 mM IPTG was added, and for bars 2, 4, & 6, 5 uM cadmium was added. For bars 2 and 6 expression exceeded the highest standard in the growth hormone assay.

Transcription from promoters containing lac operator sequences (lac O) can be regulated by IPTG in mammalian cells expressing the lac repressor (94-96). However, these studies also revealed that the insertion of lac O sequences can profoundly affect intrinsic promoter properties. Other studies have shown that the lac operator-repressor complex leads to transcriptional termination by interfering with elongation (97). To determine the effect of lac O positioning, a series of mMT-hGH constructs were made with lac O sequences at various sites (Figure 47). These constructs showed good repression (Figure 48, compare bars 11 & 19, and Figure 49, bars 1 & 3), without a large decrease in intrinsic promoter activity (Figure 48, bars 11 & 15). Moreover, when lac repression was combined with the MLTF mMT mutant, a great range of inducible expression was obtained (Figure 48, bars 22-24). These initial experiments demonstrate significant potential for the development of a tightly controlled transcriptional regulatory system that functions in mammalian cells using small molecule inducers.

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