REGULATION OF PANCREATIC AMYLASE AND LIPASE GENE EXPRESSION BY DIET AND INSULIN

by

An Tsai

A Dissertation Submitted to the Faculty of the

COMMITTEE ON NUTRITIONAL SCIENCES (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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Tsai, An, Ph.D.

The University of Arizona, 1994

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by An Tsai

entitled Regulation of pancreatic amylase and lipase gene expresssion by diet and insulin

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Patsy M. Brannon	July 8, 1994 Date
David K. Y. Lei	July 8, 1994 Date
Bobby L. Reid	July 8, 1994 Date
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Vincent Guerriero, Jr.	July 8, 1994 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Patsy M. Brannon	July 8, 1994 Date

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DEDICATION

This dissertation is dedicated with love to my parents Kuo-Liang and Chou-Lion, and to my brother Ching-Hsien.

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ABSTRACT

Although insulin has been proposed to mediate the dietary regulation of pancreatic amylase, its interaction with diet in the regulation of amylase and lipase is not well understood and was examined in diabetic rats fed diets high in carbohydrate (HC), protein (HP) or fat (HF) and treated with insulin. Diabetes, independent of diet, decreased amylase content (80%; $p < 0.001$), relative synthesis (96%; $p < 0.0001$) and mRNA (92%; $p < 0.0002$), but insulin only restored amylase content, relative synthesis and mRNA to respective dietary control values. Diabetes increased lipase mRNA 60% ($p < 0.004$), but interacted ($p < 0.02$) with diet to affect lipase content, which increased in HC-fed (81%) diabetic rats and decreased (28%) in HF-fed diabetic rats. Insulin partially restored lipase content and mRNA to respective dietary control values. Lipase synthesis tended to parallel the increases in lipase mRNA and content in HC-fed diabetic rats. Diet, independent of diabetes, regulated amylase content ($p < 0.0001$), relative synthesis ($p < 0.0002$) and mRNA ($p < 0.0003$), which were 200-300% greater in HC- than HF-fed rats, and lipase content ($p < 0.001$) and mRNA (rPL-1 - $p < 0.02$; rPL-3 - $p < 0.0001$), which were 80% greater in HF- than HC- fed rats. Lipase relative synthesis was 21% greater in HF-fed than HC-fed control rats. Insulin failed to stimulate maximal amylase gene expression in HP- or HF-fed diabetic rats, suggesting that it is necessary, but not sufficient, for this dietary regulation. The parallel changes in amylase mRNA, relative synthesis and content suggest pre-translational regulation; whereas the differential regulation of lipase activity, relative synthesis and mRNA suggest pre-translational and post-translational regulation.

The interactive effects of diet (HC, HP, and HF), diabetes and insulin on whole body energy metabolism were also examined. Diabetes, independent of diet, decreased body weight (330%), metabolic energy (ME) by 66, 39, and 41% (HC-, HP-, or HF-fed rats, respectively),

and energy retentions (338%). The best diabetes and diet interactions in energy utilization, retention and body weight maintenance were in rats fed the HP diet. Insulin treatment restored carbohydrate utilization with the greatest effect in the HC-fed rats and increased energy retentions (625%). These results suggest that insulin affects the utilization of dietary carbohydrate the most and dietary protein the least.

CHAPTER 1

INTRODUCTION

The pancreas plays a key role in the digestion and utilization of ingested food. The acinar cells of the exocrine pancreas synthesize and secrete digestive enzymes and adapt this production and secretion to accommodate different dietary substrate. Alterations of dietary substrate result in adaption of the mRNA synthesis and content of the pancreatic digestive enzymes, thus optimizing the digestive process. The cellular mediators of this pancreatic adaption to diet are not defined, but insulin and nutrients have been implicated.

Insulin, one of the peptide hormones synthesized by the endocrine pancreas, has important functions not only on the utilization and metabolism of digested foodstuffs, but also has a positive influence on exocrine pancreas secretion. Furthermore, insulin has been proposed to mediate pancreatic adaptation to dietary carbohydrate. Diabetes significantly decreases pancreatic amylase content, and exogenous administration of insulin reverses this effect. Pancreatic amylase mRNA parallels these changes in amylase content in diabetes and insulin-treatment. Transcription of amylase-reporter gene constructs is repressed in diabetic transgenic mice and restored by insulin treatment. A 126-bp fragment of the mouse Amy 2.2 gene (-208 to -82 bp), which includes the insulin-responsive element (IRE), is sufficient for regulation of amylase by dietary carbohydrate; but a smaller 29-bp fragment (-167 to -138) containing only the IRE and part of the pancreatic tissue specific element fails to respond to dietary carbohydrate, suggesting that insulin alone is not solely responsible for the dietary regulation of pancreatic amylase.

Insulin also influences the regulation of pancreatic lipase. Lipase content, synthesis, and mRNA levels increase in streptozotocin (STZ)-induced diabetic rats fed purified or non purified HC diets. Exogenous insulin administration restores lipase mRNA levels as well as lipase activity to control values. Little attention has focused on the mechanism of the interaction of diet and insulin in the regulation of pancreatic amylase and lipase.

Alteration of dietary composition and diabetes also influences energy intake and energetic efficiency. Energetic efficiency is influenced by dietary fat concentration, with more efficient utilization of high fat diets than low fat ones. Insulin may also be an important metabolic satiety signal which may regulate eating behavior and energy balance. Rats with insulin-deficient diabetes, display marked hyperphagia with a preference for carbohydrates. Insulin treatment results in two opposite effects reported: one is normalized food intake; and the other is sustained hyperphagia, leading to excessive weight gain. The interaction of diet and insulin on energy intake and utilization remains unclear and controversial.

The objectives of these studies were to determine the interactive effects of diet, diabetes and insulin on 1) the content, synthesis, and mRNA levels of pancreatic amylase and lipase and 2) whole body energy metabolism. STZ-induced diabetic rats were fed HC, HP or HF diets and treated for 7 days with insulin or saline. Pancreatic enzyme relative synthesis, as well as cellular content and mRNA levels, were determined. Energy balance and utilization was determined by indirect calorimetry, and body composition was estimated from total body electrical conductivity measurements (TOBEC).

CHAPTER 2

LITERATURE REVIEW

PANCREAS

The pancreas is a soft, lobulated, and glandular organ which has both exocrine and endocrine functions. The exocrine portion comprises 84% of its total volume, whereas the endocrine cells, the islets of Langerhans, occupy only 2% of total volume (Gorelick and Jamieson, 1987). The remaining portion is made up of ductular cells, blood vessels, and extracellular matrix. The islets, which synthesize various peptide hormones including insulin, glucagon, somatostatin, and pancreatic polypeptide (Korc, Williams and Goldfine, 1980), are unevenly distributed among the exocrine pancreatic cells, which secrete both bicarbonate-rich fluid (ductal cells) and digestive enzymes (acinar cells; Korc et al., 1980). Together, the exocrine and endocrine enable the digestion and utilization of ingested food.

THE EXOCRINE PANCREAS

Ultrastructure

The basic subunit of the exocrine pancreas is the acinus, which consists of polarized epithelial acinar cells, bound by a connective matrix continuous with that surrounding the ductular epithelium. The main subcellular organelles involved in the formation and storage of proteins for export are arranged in a highly polarized fashion, reflecting their functional and temporal inter-relationships. The plasma membrane of the acinar cell is surrounded by an extracellular matrix containing collagen type IV, laminin, and proteoglycans (Kern, 1986), and the basal portion of the cell contains its nucleus and copious rough endoplasmic reticulum (RER). There are also hormone receptors, such as those for cholecystokinin (CCK),

exclusively localized on the basolateral plasmalemma (Rosenzweig, Miller and Jamieson, 1983). The apical membrane faces the lumen of the duct and contains the Golgi apparatus and the secretory vesicles, zymogen granules (ZG). Connecting acinar cells are gap junctions (Kern, 1986) through which small molecules can pass and may regulate neighboring acinar cells.

Digestive Enzymes

Function. The pancreatic digestive enzymes hydrolyze macromolecules in food into smaller molecules, which can be absorbed. Proteins, starch, and triglycerides make up the majority of the macromolecules in food and are hydrolyzed by the major pancreatic enzymes: the serine proteases, amylase, and lipase, respectively.

The pancreatic serine proteases [trypsin (E.C. 3.4.21.4), chymotrypsin (E.C. 3.4.21.1), and elastase (E.C. 3.4.21.11), are a family of endopeptidases that cleave natural substrates at specific sites along the peptide chain. The members of this group exhibit considerable homology in their amino acid sequences, share a similar three-dimensional structure (Schotton and Hartley, 1970) and contain a reactive serine in the active site. Unlike pancreatic amylase and lipase, these proteases are not secreted by the pancreas in their active form, but are secreted as proenzymes (trypsinogen, chymotrypsinogen, and proelastase), which must be activated by specific proteolytic cleavage through the action of enterokinase or trypsin. Enterokinase, a brush border protease, is released from the membrane by the action of bile acids and activated by trypsinogen itself. It selectively cleaves a hexapeptide from the amino terminus of trypsinogen to produce trypsin (Nordstrom, 1972). Trypsin then activates the other pancreatic proproteases and autocatalytically activates trypsinogen. Trypsin has several isozymes, depending on the species, and cleaves peptide bonds between basic amino acids (arginine and lysine) and the next amino acid (Rinderknecht, 1986). Chymotrypsin has multiple

isoenzymes, depending on the species, and cleaves peptide bonds between the aromatic residues (phenylalanine, tyrosine, and tryptophan) and the next residue (Rinderknecht, 1986). The resulting products of these proteases are oligopeptides, with specific C-terminal amino acids, that are subsequently hydrolyzed by intestinal oligopeptidases.

Amylase (E.C. 3.2.1.1) is secreted in its active form and hydrolyzes α-1,4-glucosidic bonds in oligosaccharides with at least four subunits (Roberts and Whelan, 1960). It binds five α-1,4-linked glucose residues and rapidly cleaves the glucosidic linkage between the second and third residues, forming maltose and small oligosaccharides (Robyt and French, 1970), which are further hydrolyzed by intestinal glucosidases prior to absorption.

Pancreatic lipase (E.C. 3.1.1.3) is secreted in its active form (Rinderknecht, 1986), acts at oil/water interfaces to hydrolyze triglycerides at the sn1 and sn3 positions, and yields fatty acids and 2-monoglycerides (Mattson and Beck, 1956). The emulsification of fats in the intestine is aided by bile salt micelles. However, bile salts have two major actions on pancreatic lipase. First, bile salts occupy the oil-water interface of the micelle and displace lipase from the surface of the emulsions reducing its lipolytic activity (Borgstrom and Erlanson, 1973). Second, bile salts shift the pH optimum to 6-8 for pancreatic lipase. This inhibitory effect of bile salt micelles is, however, reversed by colipase (Morgan and Hoffman, 1971). Colipase (E.C. 3.1.1.1) is secreted as procolipase (102-107 amino acids), which is proteolytically cleaved to its active form (96 amino acids with MW = 10,000; Borgstrom, Wieloch and Erlanson-Albertsson, 1979). Colipase binds to lipase in a 1:1 molar ratio and also with bile salts (Borgstrom and Erlanson, 1973; Borgstrom and Donner, 1975). It acts as an anchor that displaces bile salts from the micellar interface and brings lipase to its site of action, allowing complete hydrolysis of triglycerides (Borgstrom, 1975). The products, monoglycerides

and free fatty acids, partition into mixed micelles, cross the unstirred water layer, and are absorbed by the enterocyte.

Synthesis. In the nucleus, mRNAs for these enzymes are transcribed, processed to their mature polyadenylated forms and translocated to the cytoplasm. Initiation of protein synthesis begins at the AUG initiation codon on free ribosomes. Translation continues on the RER through the interaction of (1) a signal peptide, which is encoded in the N terminus of these mRNAs; (2) a signal peptide recognition particle (SRP), which is found free in the cytoplasm; and (3) the SRP receptor (SRPR), which is an integral endoplasmic reticulum (ER) membrane protein.

First, the signal peptide, after emerging from the channel in the large ribosomal subunit, binds to the SRP. And then, the interaction of the SRP with the SRPR in the RER membrane allows the polypeptide chain translocation across the membrane to proceed. Once the complex binds to the RER, the SRP dissociates from the complex in a GTP-dependent manner (Connolly and Gilmore, 1989). Prior to chain termination, the signal peptide is cleaved from the nascent protein by a protease associated with the cisternal leaflet of the RER membrane. In the absence of a "stop-transport" sequence, chain termination occurs and the completed polypeptide chain is released to the intravesicular space with synthesis and translocation completed, the nascent proteins are further processed and transported to the Golgi complex.

Secretion. In the Golgi complex (cis, mid, and trans element), the secretory proteins are segregated from lysosomal and plasma membrane proteins (Pfeffer and Rothman, 1987). They leave the trans-Golgi complex in condensing vacuoles (Palade, 1975), which mature into zymogen granules (ZG) as their contents are concentrated. The ZG, which serve as storage

vesicles and exocytosis vehicles, move toward the apical membrane, where the ZG membranes fuse by an unknown process with the apical membrane and release their contents into the ductal lumen. This fusion and release represents the classical regulated secretion pathway. Alternatively, Beaudoin and Grondin (1991) have proposed two other pathways from which secretory proteins could potentially originate: (1) the constitutive pathway that would correspond to small vesicles derived from the Golgi-network and (2) the paragranular pathway that would correspond to small vesicles derived from the maturation process of the secretory granule. In summary, the digestive enzymes are synthesized in the nucleus; segregated and transported to the Golgi complex; concentrated and stored in the ZG; and discharged from the ZG into the ductal lumen. This is an orderly process that proceeds from the basal to the apical membrane of the cell.

Acinar cells have been one of the best studies models of the "regulated pathway" of secretion. These cells are programed to direct more than 90% of their biosynthetic effort toward the production and storage of a mixture of approximately 20 different enzyme precursors (Bruzzone, 1990). Upon appropriate stimuli, the latter are secreted into the pancreatic ducts and activated in the intestinal lumen and hydrolyze macromolecules in the diet (Scheele, 1986b). The ability of pancreatic acinar cells to respond to a given stimulus from hormones or neurotransmitters known as secretagogues with the coordinated release of secretory granule content implies the existence of intracellular messengers capable of transducing the external signal into an increased rate of vesicle membrane fusion. The term "stimulus-secretion coupling", coined by Douglas and Rubin (1961), defines this transduction of the interaction of a stimulus with its specific plasma membrane receptor and the accelerated discharge of secretory products. Pancreatic secretagogues have been classically subdivided into two categories: (1)

those that increase cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) without affecting cyclic adenosine monophosphate (cAMP) as exemplified by CCK, acetylcholine and bombesin (Schulz and Stolze, 1980) and (2) those that elevate cellular cAMP but do not raise $[Ca^{2+}]_i$ as exemplified by secretin and vasoactive intestinal peptide (VIP). The key feature of the former transduction pathway is the receptor-mediated hydrolysis of phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2] by a specific phosphodiesterase (termed phospholipase C). This event generates two intracellular messengers: inositol[1,4,5]trisphosphate [Ins(1,4,5)P_3], which releases Ca^{2+} from intracellular stores (Berridge and Irvine, 1984), and diacylglycerol, which activates protein kinase C (Kikkawa and Nishizuka, 1986). In the latter transduction pathway upon secretagogue-receptor interaction, cAMP increases 8- to 30-fold (Gardner et al., 1982), and activates adenylate cyclase in the acinar cell plasma membrane (Long and Gardner, 1977). Cyclic AMP then binds to and activates protein kinase A, which phosphorylates intracellular proteins. However, most of the specific enzymes or regulatory proteins involved in the distal steps of transduction pathway are not understood.

Tissue-Specific Gene Expression. Pancreatic digestive enzymes increase markedly during pancreatic development and differentiation. This differentiation involves the well-coordinated expression of a selective subset of genes that determine the differentiated phenotype of acinar cells. The common underlying mechanism of this differentiation is the selective transcriptional activation of cell-specific genes. Transcriptional regulatory sequences associated with cell-specific genes, the transcription factors that bind those sequences, and the productive consequence of the binding to activate transcription in specific cell types (MacDonald and Swift, 1993) are needed for tissue specific expression. Considerable effort has identified

transcriptional regulatory sequences and putative transcriptional factors and their actions in the exocrine pancreas over the last ten years.

The pancreas-specific expression of rat elastase I (EI) has been demonstrated to be a cell-specific gene and so set the stage for identifying the transcriptional elements responsible for its regulation. The initial transgenic experiments with the rat EI gene, including the entire 11 kilobase pairs (kbp) of the gene plus extensive flanking sequences upstream (7 kb) and downstream (5 kb), showed pancreatic levels of the EI transgene mRNA as being equal to or greater than those of the gene in the normal rat, whereas the levels in the seven nonpancreatic tissues examined were generally more than 1000-fold lower and often below detection (Swift et al., 1984a). Transcriptional runoff experiments with isolated nuclei from the pancreas and liver of transgenic animals demonstrate that this pancreatic specificity is controlled transcriptionally (MacDonald et al., 1986a).

To localize transcriptional control regions, the 4.2 kb of the flanking sequence immediately upstream of the rat EI gene has been fused to a passive reporter gene, in this instance the human growth hormone (hGH) structural gene. This fusion transgene is expressed at high levels in the pancreas, but not detectably in nonpancreatic tissues (Ornitz et al., 1985a,b). Immunofluorescent localization of hGH protein in the pancreas of these and similar transgenic mice bearing an hGH fusion gene with 0.5 kb of elastase I flanking sequences demonstrate that all acinar cells express the fusion gene, consistent with all acinar cells expressing the complete complement of exocrine digestive enzymes (MacDonald et al., 1986b).

Stepwise trimming of the flanking region from -4200 to -71 bp defined the region necessary for pancreatic transcription in transgenic mice (Ornitz et al., 1985a). Deletion from -4200 to -205 bp had no discernible effect on tissue-specific expression of fusion transgenes.

Further deletion to -71 bp removes sequences required for pancreatic expression; thus, EI-hGH fusion transgene containing EI gene sequences from -71 to +8 bp was inactive in all tissues. These experiments define the 134-bp region from -205 to -72 bp as important for pancreatic expression in vivo (Ornitz et al., 1985a).

Compact and potent tissue-specific regulatory sequences near the transcriptional start site may be characteristic of many pancreatic genes. Transcriptional control elements sufficient for pancreatic expression of amylase 2.2 and trypsin I transgenic mice (Osborn et al., 1988; Davis et al., 1992) and chymotrypsin B in transfected cultured cells (Boulet, Erwin and Rutter, 1986) are within 225 bp of the 5' end of each gene as well. Meisler and co-workers (Osborn et al., 1987) first demonstrated that the complete mouse amylase 2.2 gene with 9 kb of 5' flanking and 5 kb of 3'-flanking sequences was correctly expressed in a pancreas-specific manner in transgenic mice. The amylase 2.2 gene region from -208 to +19 is sufficient to direct expression of the bacterial CAT (chloramphenicol acetyl transferase) gene to the pancreas of transgenic mice without detectable expression in 11 other tissues examined (Osborn et al., 1988). Moreover, both of these transgenes are correctly regulated by insulin and diet, demonstrating the presence of the appropriate DNA sequence information for these regulatory processes as well (see below). Similarly, the rat trypsin I gene region from -225 to +4 bp is sufficient for expression of the hGH reporter gene in a transgenic mouse pancreas (Davis et al., 1992).

The common characteristic of the elastase I, amylase, and trypsin I gene control regions is the presence of a recognizably conserved sequence of 21 bp (Swift et al., 1984a; Boulet et al., 1986), termed the pancreas consensus element (PCE), which binds transcription

factors (PTF-1 or XPF-1) present selectively in the pancreas or tumor cell lines derived from pancreatic acinar cells (Cockell et al., 1989; Weinrich, Meister and Rutter, 1991).

The 134-bp region from -205 to -72 of the elastase I transcriptional enhancer has been further analyzed systematically to verify that three individual functional elements exist and to determine their role in acinar cell-specific transcription. By transfection analysis of the effects of 10-bp scanner mutations, Kruse and coworkers (1988) identified three mutation-sensitive elements of 15 to 30 bp each (elements A, B, and C in Figure 2-1) that are necessary for enhancer activity in pancreatic acinar tumor cells in culture. The regulatory information within each of the three elastase enhancer elements also has been defined in transgenic mice by assaying the sites of expression of transgenes driven solely by homomultimeric tandem repeats of each element (Kruse et al., 1988).

From these results and analyses of the transcription factors that bind each element (Kruse et al., 1988; Cockell et al., 1989; Petrucco, Wellauer and Hagenbuchle, 1990; Weinrich et al., 1991), a regulatory strategy (Figure 2-1) for the acinar cell specificity of the elastase I gene has been proposed (Kruse et al., 1988).

1. The A element, which contains the PCE, binds an acinar cell-specific transcription factor (PTF1 or XPF1) and is the sole positively acting determinant of acinar cell specificity.
2. The C element, which is required for activity in cultured acinar tumor cells and important in animals, binds ubiquitous transcription factors and augments the strength of the enhancer without affecting cell specificity.
3. The B element plays an interesting dual role. In acinar cells it binds ubiquitous transcription factors and contributes to enhancer strength without affecting cell specificity. However, when multimerized or in the context of the truncated enhancer/promoter (-205

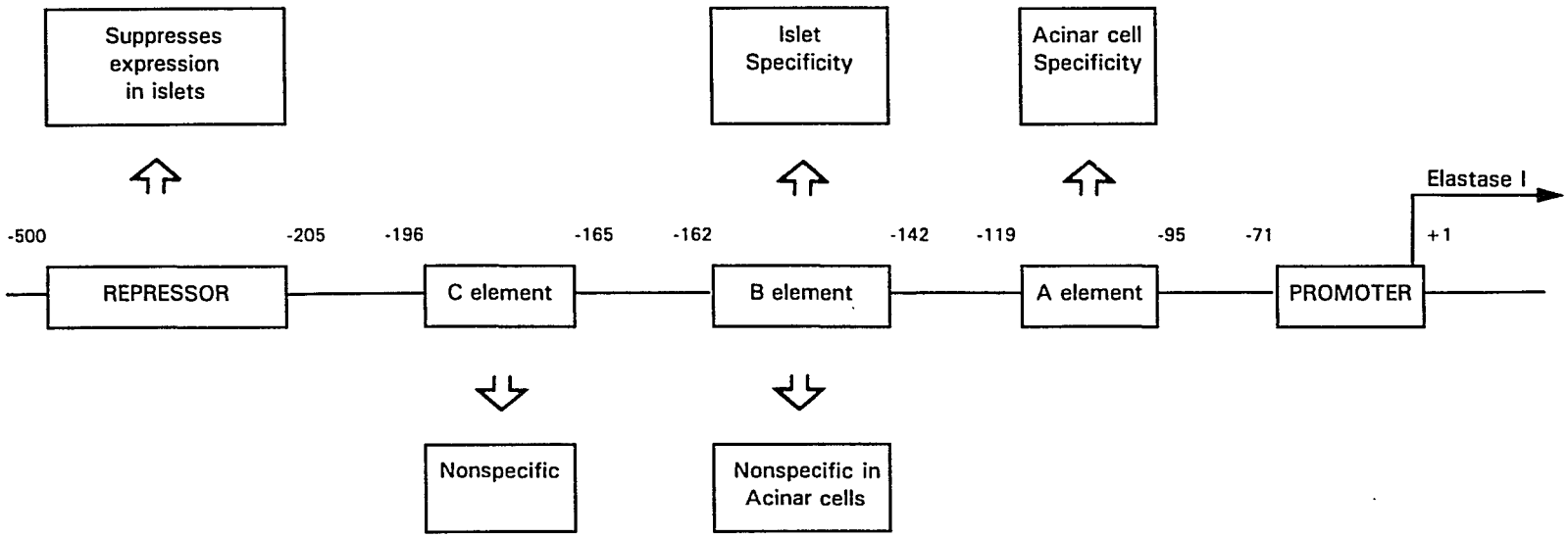

Figure 2-1. The function of individual elements within the elastase I transcriptional enhancer.

to +8); the B element directs expression selectively to β-cells of the islets of Langerhans through the selective action of a β-cell-specific transcription factor (βTF-1).

4. Expression is limited to acinar cells by an upstream region (Repressor, Figure 2-1) that suppresses islet expression, leaving acinar cell expression intact.

THE ENDOCRINE PANCREAS

The mammalian endocrine pancreas consists of groups of hormone-secreting cells that are dispersed throughout the exocrine tissue. These so-called endocrine islets were described by Langerhans in 1869. It is now evident that the islets of Langerhans are complex structures containing different types of hormone-secreting cells, nerve cells, and a rich vascular supply.

Ultrastructure

Pancreatic endocrine cells are highly differentiated and exhibit the phenotypic characteristics that allow these cells to perform their specialized functions. There are four major types of pancreatic endocrine cells. The pancreatic β-cell possesses the typical morphology of an endocrine secretory cell and synthesizes insulin, which is packaged into heterogeneously shaped granules that have electron-dense cores. The α-cells are smaller than the β-cells, and possess uniform electron dense granules that contain glucagon (Falkmer and Ostberg, 1977). The pancreatic γ-cells are small, somatostatin-containing cells, and often have a dendritic shape (Falkmer and Ostberg, 1977). The pancreatic polypeptide- (PP) containing cells have relatively oblong, electron-dense granules (Falkmer and Ostberg, 1977).

Insulin

Synthesis, Secretion, and Its Regulation. The control of glucose and energy metabolism is highly dependent on hormones secreted by the islets of Langerhans, most notably insulin. The human insulin gene is located on chromosome 11 (Owerbach et al., 1980). It contains 3 exons and 2 introns (Steiner et al., 1985). The encoded insulin mRNA is translated into preproinsulin, a 110 amino acid peptide that consists of a 24 amino acid signal sequence, the A and B peptides, and a connecting C peptide (Orci et al., 1987; Davidson, Rhodes and Hutton, 1988). The signal sequence is cleaved into proinsulin in the endoplasmic reticulum by signal peptidases that act in a cotranslational manner (Orci et al., 1987; Davidson et al., 1988) as described above for the synthesis of digestive enzymes. Proinsulin is then packaged in the Golgi and processed into mature insulin by two enzymes in coated secretory vesicles released from the trans-face of the Golgi apparatus (Orci et al., 1987; Davidson et al., 1988). One processing enzyme is a trypsin-like one that removes the intervening C peptide by cutting at two dibasic amino acid sequences, and the other is a carboxypeptidase B-like enzyme that removes the dibasic peptide from the carboxyl terminal end (Smeekens and Steiner, 1990; Seidah et al., 1991). The final product, insulin, is a 51 amino acid peptide that consists of A and B peptides that are linked by two disulfide bonds at positions A7 to B7 and A20 to B19 (Orci et al., 1987).

The regulation of insulin biosynthesis is coordinated by several hormones and nutrients, but most notably by glucose. Insulin synthesis increases markedly within minutes of exposure of the β-cell to glucose in vitro, as a result of transcriptional and posttranscriptional events (Welsh et al., 1986; German, Moss and Rutter, 1990). Transcriptional activation of the insulin gene is dependent on the presence of 5'-flanking sequences that contain promoter regions, including a TATA box, and the FAR and NIR boxes (Edlund et al., 1985; Karlsson

et al., 1988; Moss, Moss and Rutter, 1988). These DNA sequences are important regions that regulate both the site of initiation of gene transcription and the rate of transcription. The FAR and NIR boxes dictate the cell-specific expression of the insulin gene and contain the transcription initiation site, GCCATCTG, that interacts with insulin enhancer binding protein 1 (IEF1), a DNA binding protein (Karlsson et al., 1988; Moss et al., 1988; Whelan et al., 1990). Other DNA-binding proteins have also been implicated in the regulation of insulin gene expression. However, the exact mechanisms by which DNA-binding proteins confer β-cell specificity for insulin gene expression in the adult pancreas remain to be determined.

In order for glucose to exert its effects on insulin secretion and synthesis, it must first get into the β-cell. This is accomplished through facilitated diffusion, which is mediated by a glucose transporter. Five distinct isomers of the glucose transporter (GLUT-1 through GLUT-5) have been cloned (Bell et al., 1990). GLUT-2 has a high Km for glucose uptake and allows the β-cell to transport glucose in proportion to the extracellular glucose concentration, even at high physiological levels of glucose.

After uptake, glucose is metabolized by a rate-limiting glucokinase leading to the generation of glucose metabolites and ATP. This results in the closing of potassium channels, which causes the cell membrane to depolarize, thereby activating voltage-dependent calcium channels and raising cytosolic free calcium levels. The rise in calcium induces microtubule contraction and the release of insulin. The β-cell also possesses a variety of cell-surface receptors that bind specific ligands, leading to the activation of adenylate cyclase (AC) and phosphoinositides (PIP_2) hydrolysis through the actions of phospholipase C (PLC). PIP_2 hydrolysis generates diacylglycerol (DG), which activates protein kinase C, and inositol

trisphosphate (IP_3), which mobilizes calcium from intracellular stores and is converted to inositol tetrakisphosphate (IP_4) (Korc, 1993).

Insulin Receptor and Signaling Mechanisms. After its release by the β-cells, insulin binds to the insulin receptor on the surface of cells throughout the body and leads to the actions of insulin. The receptors are heterotetrameric transmembrane glycoproteins composed of two α- and two β-subunits. The α-subunits are completely extracellular and contain the insulin-binding domain, whereas the β-subunits are composed of an extracellular domain, a membrane-spanning domain, and a 402-amino acid intracellular tail (Ullrich et al., 1985). This intracellular domain contains a Tyr-specific protein kinase similar to that of certain cellular proto-oncogene products (e.g., $p60^{c\text{-}src}$, c-ras) and the receptors for certain peptide growth factors [e.g., platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)] (Ullrich et al., 1985; Hanks, Quinn and Hunter, 1990).

Insulin binding immediately activates the Tyr kinase in the β-subunit and results in autophosphorylation of Tyr residues in three regions, including the juxtamembrane region, the regulatory region, and the COOH-terminal region (White and Kahn, 1989; Myers et al., 1991; Feener et al., 1992). The Tyr kinase activity of the receptor is crucial for insulin action; naturally occurring mutations of the insulin receptor that inhibit kinase activity and block autophosphorylation are associated with severe insulin resistance (Odawara et al., 1989; Moller et al., 1990). Moreover, point mutations of the insulin receptor designed to destroy ATP binding (and thus abolish kinase activity) also abrogate insulin signaling in cultured cells (Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987). Thus, insulin-stimulated autophosphorylation is the key initial event for signal transmission.

In order to continue the signal transduction, two possible models (autophosphorylation-based signaling and substrate-based signaling) of insulin downstream signal transmission have been proposed. The autophosphorylation-based signaling model suggests that Tyr autophosphorylation causes a conformational change in the β-subunit of the insulin receptor (Perlman et al., 1989), facilitating the interaction of the insulin receptor with cellular elements responsible for downstream signaling. This model is especially attractive because it is also used by the EGF and PDGF receptors to interact with PLC-γ, p21^{ras}-GAP, and GRB-2/sem-5 (Coughlin, Escobedo and Williams, 1989; Kazlauskas and Cooper, 1989; 1990a; Meisenhelder et al., 1989; Kaplan et al., 1990; Kazlauskas et al., 1990b; Morrison et al., 1990; Reedikj, Liu and Pawson, 1990; Molloy et al., 1991; Lowenstein et al., 1992). However, the direct association of these or other molecules implicated in signaling has not been clearly demonstrated for the insulin receptor. The substrate-based signaling model suggests that signaling by the insulin receptor may alternatively be secondary to the Tyr phosphorylation of intracellular substrate proteins. The discovered insulin receptor substrate, IRS-1, suggests that the substrate-based signaling model is more important in insulin signaling than previously thought, which will be discussed below. However, autophosphorylation is essential to substrate-based signaling, as it activates the insulin receptor Tyr kinase and allows it to phosphorylate IRS-1 more readily.

This recently discovered IRS-1 provides an innovative and simple way to explain the pleiotropic effects of insulin including its regulation of membrane transport, gene expression, protein synthesis and DNA synthesis and its modulation of enzyme activities. IRS-1 is a cytosolic and tyrosyl-phosphorylated protein, which is purified from insulin-stimulated rat liver by affinity chromatography on antiphosphotyrosine antibodies immunoprecipitates or immunoblots; migrates as a broad band of about 185,000 Mr during SDS-PAGE; and has

predicted molecular weight of 131,000 Mr (Rothenberg et al., 1991; Sun et al., 1991). IRS-1 contains 20 possible Tyr phosphorylation sites. These phosphorylated sites have been associated with high affinity to cellular proteins that contain SH2 (src homology-2) domains. This association is specific and depends on the amino acid sequence surrounding the phosphotyrosine residue and the isoform of the SH2 domain.

A revised model of insulin receptor signaling mechanisms has been proposed by Myers and White (1993). Insulin binding to the extracellular α-subunits of the insulin receptor activates the Tyr kinase activity of the β-subunit. The insulin receptor then phosphorylates the endogenous cellular protein, IRS-1, allowing Ptdins 3'-kinase (a complex of p85-α and p110), GRB-2, p85-B, SHPTP2 (a novel SH2 domain-containing Tyr phosphatase), and other unidentified SH2 domain-containing proteins to associate with IRS-1. This association is mediated by the binding of SH2 domains to Tyr-phosphorylated amino acid motifs on IRS-1 and activates Ptdins 3'-kinase. Therefore, this binding may also regulate the activity of other associated enzymes. The interaction between phosphorylated IRS-1 and multiple SH2 domain-containing proteins may ultimately explain the pleiotropic effects of insulin.

Metabolic Action of Insulin. As mentioned above, the metabolic consequences of insulin binding to the cell surface are multiple and depend on the nature of the target tissue. Many different key enzymes are stimulated or inhibited by insulin (Clauser, Leconte and Auzan, 1992). Glucose metabolism is modified by the combined stimulatory effect of insulin on glucose transport and glycolysis in all tissues and glycogen synthesis in the liver, muscle and adipose tissue and its inhibitory effect on gluconeogenesis and glycogenolysis in the liver. These physiological effects are the consequences of the activation of the insulin-dependent glucose transporter (GLUT4) and multiple enzymes such as glucokinase, glycogen synthase

(glycogen synthesis), phosphofructokinase, pyruvate kinase and pyruvate dehydrogenase (glycolysis) (Clauser et al., 1992). Inhibition of hexokinase, glycogen phosphorylase and phosphoenolpyruvate carboxykinase are also observed. In adipose tissue, lipid synthesis is stimulated by insulin via an activation of citrate lyase, acetyl-CoA carboxylase, fatty acid synthase, glycerol-3-phosphate dehydrogenase and lipoprotein lipase (Girard and Ferre, 1990). All these protein activations or inhibitions result from different mechanisms. Some of these enzymes are activated by phosphorylation (acetyl-CoA carboxylase) or dephosphorylation (pyruvate dehydrogenase). In other cases, insulin modifies the transcription of the genes for some enzymes, with increased glucokinase or pyruvate kinase transcription and decreased phosphoenolpyruvate carboxykinase transcription. In these cases, phosphorylation of trans-acting factors regulating the gene transcription is the more likely explanation. In particular, insulin regulates pancreatic amylase gene expression, which will be discussed below.

INSULIN-ACINAR INTERACTIONS

The islets of Langerhans are dispersed throughout the pancreatic tissue. The existence of portal circulation within the pancreas results in insulin-acinar interactions. In a comprehensive study of rabbit pancreas, Lifson and coworkers (1980) found that 11 to 23% of pancreatic blood went directly to the islets and 77 to 89% to acini. Of more interest, after retrograde perfusion of microspheres into pancreatic veins, essentially none of the microspheres lodged in the islets. These results support that essentially all efferent islet blood flow goes into acinar capillaries before leaving the pancreas. In canines, blood emerging from pancreatic veins after glucose challenge contains up to 10 nM insulin, a concentration of about 20 times that measured in the femoral vein (Kanazawa, Kuzuya and Ide, 1968). In addition, insulin acts directly on the exocrine pancreas through saturable insulin receptors found on pancreatic acinar

cells (Korc et al., 1978; Mossner et al., 1984). These receptors have a high affinity for insulin and are abundant (about 10,000 per acinar cell) (Sankaran et al., 1981; Sjodinn, Holmberg and Lyden, 1984).

Insulin positively influences pancreatic exocrine function. In vitro, insulin acts directly to increase glucose transport and protein synthesis in pancreatic acini (Korc et al., 1978). In vivo, insulin acts as a tropic factor to maintain the tissue level of amylase in the acini of diabetic animals (Malaisse-Lagae, 1975) and also potentiates the action of CCK on pancreatic enzyme secretion in perfused rat pancreas (Kanno and Imai, 1976). Saito, Williams and Kanno (1980), using an isolated perfused rat pancreas preparation, showed that both exogenous and endogenous insulin potentiated the action of CCK on pancreatic enzyme secretion. Lee and coworkers (1990) further demonstrated that the immunoneutralization of insulin in normal rats almost abolished the pancreatic secretory response to CCK. Clearly, insulin acts directly on acinar cells to regulate digestive enzymes and acinar cell metabolism.

PANCREATIC AMYLASE

MULTIGENE FAMILY

Mammalian α-amylase (α-1,4-glucan-4-glucanohydrolase, E.C. 3.2.1.1) is encoded by a multigene family whose members have diverged with respect to tissue specificity and dietary and hormonal regulation. In humans, the major tissues that produce amylase are the pancreas and salivary glands (Merritt et al., 1973). Based on differences in electrophoretic mobility and antigenicity, pancreatic and salivary amylases were postulated to be the product of different genes (Merritt and Karn, 1977). During the 1980s, amylase cDNAs were cloned from the pancreas and parotid gland and the differences between their sequences confirmed that they were different gene products.

The human amylase genes have been cloned independently by three groups of investigators. Meisler and colleagues used a mouse amylase cDNA to screen a cosmid library of human genomic DNA (Gumucio et al., 1988). Overlapping cosmids spanning 230 kb were ordered on the basis of their restriction maps. This 230-kb region contains five complete amylase genes and one truncated pseudogene. A similar chromosomal organization has been observed by Groot and coworkers (1989; 1991), who generated overlapping cosmid clones by screening a human genomic library with a human pancreatic amylase cDNA. As analyzed by Nishide and colleagues, the human salivary amylase gene is approximately 10 kb in length with 11 exons and 10 introns (Nishide et al., 1986). Based on the results of both groups, the amylase gene cluster on human chromosome 1 p21, contains two pancreatic amylase genes (Amy2A and Amy2B) and three salivary amylase genes (Amy1A, Amy1B and Amy1C).

The human amylase gene family was generated during primate evolution by the expansion of one ancestral gene copy with a complete γ-actin processed pseudogene (Amy2B) during primate evolution (Samuelson et al., 1990). Later, the γ-actin pseudogene was itself interrupted by an endogenous retroviral-like element which formed the salivary amylase genes (Amy1A, Amy1B and Amy1C) (Emi et al., 1988; Samuelson et al., 1988). In contrast, the pancreatic Amy2A gene contains only a residual LTR left by excision of the retrovirus. Insertion of the retroviral-like element appears to have activated a cryptic promoter within the γ-actin sequences that now serve as the initiation sites for transcription of the salivary amylase gene. Excision of the retrovirus from Amy2A is associated with reversion to pancreas-specific expression (Meisler and Ting, 1993).

Molecular analysis of the mouse amylase genes has also demonstrated the presence of distinct salivary and pancreatic amylase genes. Two types of amylase genes, Amy-1 and

Amy-2, have been isolated from the mouse genome by molecular cloning techniques (Hagenbuchle, Bovey and Young, 1980; Hagenbuchle et al., 1981; Schibler at al., 1982; Mikkelsen et al., 1986). Amy-1 produces an abundant mRNA in parotid gland and a minor mRNA in liver, while Amy-2 produces an abundant pancreatic mRNA (Hagenbuchle et al., 1980; 1981). In the mouse, Amy-2 comprises a subfamily of amylase genes. Amy-2.1 and Amy-2.2 have been isolated from a cosmid library of mouse strain YBR/ki genomic DNA (Gumucio et al., 1985). These two closely linked genes encode two isozymes of pancreatic amylase in mouse strain YBR/ki that are independently regulated. Amy-2.1 encodes the α_1 isozymes of YBR/ki pancreatic amylase, while Amy-2.2 encodes the insulin-dependent β_1 isozyme. An insulin-responsive element (IRE) and a dietary response unit (DRU) have been localized in the Amy-2.2 gene. (see Mechanism of Adaptation).

In the rat, analysis of the α-amylase enzymes and the mRNAs encoding them shows that the rat genome contains multiple related amylase genes which are expressed selectively and to varying extends in the pancreas, parotid, and liver. In the early 80's, the cDNAs derived from both pancreatic and parotid amylase mRNAs were cloned (MacDonald et al., 1980). Amylase mRNA from the liver differs slightly in sequence from the pancreatic RNA (Harding and Rutter, 1978) and is larger than either the pancreatic or parotid mRNAs (Crerar et al., 1983). In contrast, the mouse parotid and liver amylases are encoded by a single gene with overlapping regions (Young, Hagenbuchle and Schibler, 1981).

Although the amylase multigene family is complicated, it shares many similarities. Human pancreatic and salivary amylase genes have a 95% homology (Meisler and Ting, 1993). Even among species, the human, mouse, and rat pancreatic amylase gene (Amy-2) share a high degree of homology. However, amylase content and mRNA accumulate to different levels

between the two tissues, pancreatic and parotid. Understanding the tissue-specific, hormonal and dietary regulation in this conserved gene family is important.

ADAPTATION TO DIETARY CARBOHYDRATE

In the early 1900s, Pavlov (1902) first reported the adaptation of the exocrine pancreatic secretions to changes in nutritional substrates in the diet. Since then, many studies have confirmed and extended these findings (Brannon, 1990). Pancreatic cellular content and secretion of the major digestive enzymes (amylase, lipase, proteases) change with alterations in their respective substrates: starch, triglyceride, protein (Grossman, Greengard and Ivy, 1942; Ben Abdeljlil, Visani and Desnuelle, 1963; Gidez, 1973). These adaptations occur through changes in enzyme synthesis (Reboud et al., 1966a,b; Dagorn and Lahaie, 1981; Wicker and Puigserver, 1987), mRNA levels (Giorgi et al., 1984; 1985; Wicker, Puigserver and Scheele, 1984; Wicker, Scheele, and Puigserver, 1988) and transcription (Wicker and Puigserver, 1990a; Schmid and Meisler, 1992).

In response to a high carbohydrate diet (HC; 65-73% of the total energy as carbohydrate), amylase content increases by 50-500% (Grossman, Greengard and Ivy, 1942; Ben Abdeljlil and Desnuelle, 1964; Reboud et al., 1966a,b; Deschodt-Lanckman et al., 1971; Poort and Poort, 1981). Its synthesis increases by 200-800% (Marchis-Mouren, Paséro and Desnuelle, 1963; Reboud et al., 1966a,b; Johnson, Hurwitz and Kretchmer, 1977; Wicker and Puigserver, 1987), and its mRNA levels increase by 900% (Giorgi et al., 1984). However, to provide sufficient essential amino acids, the diets must contain greater than 10% protein; or amylase content decreases (Schick et al., 1984b). This adaptation occurs whether carbohydrate increased at the expense of protein (Schick et al., 1984b) or fat (Wicker and Puigserver, 1987; Snook, 1971), indicating that the carbohydrate is primarily responsible for the adaptation.

Intravenously administered glucose affects pancreatic amylase similarly to dietary starch, supporting a role of starch digestive end-products in this dietary adaption. However, increasing blood glucose concentrations stimulates insulin release, so it is unclear whether glucose is a sole mediator or acts in conjunction with insulin.

The effect of insulin on amylase synthesis has been studied in experimental diabetes induced by the β-cell toxins, streptozotocin and alloxan, in rodents. Chemically induced diabetes reduces amylase protein and mRNA, and insulin treatment restores amylase protein and mRNA (Ben Abdeljlil, Palla and Desnuelle, 1965; Korc et al., 1981). Insulin clearly regulates transcription of the amylase gene expression. This evidence suggests that insulin may mediate pancreatic amylase adaption to dietary carbohydrate.

However, insulin alone does not appear to be the sole mediator of this adaptation (Dagorn, 1986; Brannon, 1990). Exogenous administration of insulin to non-diabetic rats fed a carbohydrate-free, protein-rich diet fails to change pancreatic levels of amylase (Palla, Ben Abdeljlil and Desnuelle, 1968). Diabetic rats treated with insulin still have decreased amylase activity when fed a high fat (HF) diet, but have restored amylase activity when fed a high carbohydrate (HC) diet (Bazin and Lavau, 1979), suggesting that glucose interacts with insulin in the regulation of amylase.

Increased intracellular glucose, either alone or in conjunction with insulin, may stimulate changes in amylase gene expression (Snook, 1971). An independent role for glucose in regulating amylase is reported in pancreatic AR42J cells (Stratowa and Rutter, 1986; Estival et al., 1991). Removal of glucose from the medium decreases amylase mRNA by five-fold within 30 h (Stratowa and Rutter, 1986); but reduction of the media glucose from 5 to 1 mM decreases amylase synthesis, but not mRNA levels (Estival et al., 1991). Thus, the role of

glucose alone and its interaction with insulin in the dietary regulation of amylase remains controversial. Amylase synthesis and mRNA levels are also increased by glucocorticoids (Logsdon et al., 1987, 1985; Mossner, Bohm, and Fischbach, 1989), but there is little evidence linking glucocorticoid regulation of amylase to its dietary regulation.

MECHANISMS OF REGULATION OF AMYLASE GENE EXPRESSION BY HORMONES AND DIET

Over the last 10 years, transgenic approaches have been used to investigate the molecular basis of the dietary and insulin regulation. Pancreatic expression fusion transgenes were created that contained only upstream transcriptional control regions of the pancreatic amylase gene linked to an unrelated reporter gene such as CAT, which is not present in normal tissue. Such transgenes usually are not regulated post-transcriptionally in a physiologically meaningful manner because their transcripts do not typically contain the relevant regulatory information. Consequently, if the expression of this transgene is correctly regulated by insulin or diet, such regulation must be transcriptional; and the appropriate regulatory elements must necessarily be contained within the upstream gene fragment used in the fusion gene.

The amylase 2.2 gene flanking region from -208 to +19, bp, is sufficient for pancreatic-specific expression in mice and for regulation by insulin (Keller et al., 1990). Diabetes decreases more than 100-fold this transgene's expression (Keller et al., 1990). To localize the insulin-responsive sequences more precisely, Keller and coworkers (1990) investigated the ability of different segments within this flanking region (-205 to +19 bp) to confer insulin regulation on a CAT reporter fusion transgene. The expression of CAT transgenes with as little as 30 bp of amylase gene flanking sequences from -167 to -138 bp decreases up to 500-fold in diabetes and is restored with insulin treatment. This 30-bp insulin-

responsive element (IRE) contains the conserved pancreas consensus element (PCE) that binds the pancreatic nuclear factor PTF1 (Howard et al., 1989). More recent studies in transgenic animals indicate that the insulin response may be mediated by a conventional repression mechanism in which, in the absence of insulin, a factor binds to the PCE region of the amylase gene, thereby preventing the binding of the PTF1 transcription factor essential for amylase gene transcription (MacDonald and Swift, 1993). The role of insulin appears to be the maintenance of the repressor in an inactive state.

Schmid and Meisler (1992) have recently shown that the same region of the amylase 2.2 gene (from -208 to +19 bp) also contains the region for dietary carbohydrate transcriptional regulation. The 0.2Amy.CAT transgene is expressed 14-fold higher in the pancreas of animals fed high (71%) carbohydrate versus low (11%) carbohydrate isoenergetic diets, which is comparable to the nine-fold difference in expression of the endogenous amylase 2.2 gene. A dietary response unit (DRU) containing the essential transcriptional elements is in the 127-bp region between -208 and -82 bp of the amylase 2.2 gene. Although the DRU and the minimal insulin response element are contained within the same 127-bp region of the amylase gene, the two are not congruent. Thus, even though the 30-bp IRE and a 63-bp fragment that include the IRE are necessary for dietary regulation, neither fragment is sufficient alone to mediate the dietary response (Schmid and Meisler, 1992). It remains to be determined whether the dietary response is mediated in part by the conditional binding of the PTF1 transcription factor to the PCE.

PANCREATIC LIPASE

GENE STRUCTURE

Pancreatic lipase gene structure has been studied intensively for the last several years. The sequences of rat pancreatic lipase mRNAs have been determined (Wicker-Planquart and

Puigserver, 1992; Payne et al., 1994). Wicker-Planquart and Puigserver (1992) screened rat pancreatic libraries using a canine pancreatic lipase cDNA and isolated two positive clones. One clone (from the pUC9 cDNA library; designated rPL-2 by Wicker-Planquart and Puigserver, 1992) has 692 nucleotides and encodes only the half the protein (beginning at nucleotide 840, which corresponds to lysine 239). The other clone (from λgt11 library; designated rPL-1 by Wicker-Planquart and Puigserver, 1992) is a full length cDNA (accession number in the EMBL data library, X61925) with 1531 nucleotides and a poly (A) stretch of about 60 nucleotides. The deduced amino acid sequences of both clones contain all the residues, which have been demonstrated to be important in the catalytic activity or in the binding of the substrate, and share almost identical sequences, except for 12 nucleotides, which represent less than 2% base replacement. According to Wicker-Planquart and Puigserver, the existence of two closely related lipase sequences may be explained by two allelic genes. The mature enzyme (rPL-1) shares 65, 66 and 82% identity from porcine, human and canine lipases, respectively.

Another rat pancreatic lipase sequence (designated rPL-3 by Wicker-Planquart and Puigserver, 1992) has been reported in Genbank (M58369) by Lowe and coworkers and published recently (Payne et al., 1994). This cDNA was isolated from a rat pancreatic library with a probe derived from the human pancreatic lipase (hPL) cDNA. The clone is 1,492 nucleotides encoding an open reading frame of 465 amino acids with a predicted 16-amino acid signal peptide. The rPL-3 sequence has 78% identity to the hPL sequence, 66% identity to human pancreatic lipase related protein 1(hPLRP-1), 62% identity to hPLRP-2, and 63% identity to mouse pancreatic lipase related protein 2(mPLRP-2). The comparison of amino acid sequences of rPL-1 and rPL-3 shows only a 65% identity. When rPL-3 is expressed in *Sf*9

cells, lipolytic activity is expressed, which can be inhibited by bile salts and reactivated by (pro)colipase (Payne et al., 1994).

A cDNA encoding human pancreatic lipase has been isolated by Lowe (1992), by screening a λgt11 cDNA library with a rabbit polyclonal anti-human pancreatic lipase antibody. The full length cDNA clone of 1477 base pairs contains an open reading frame encoding a 465-amino acid protein, including a 16-amino acid signal peptide. The nucleotide sequence is 69% identical to the canine pancreatic lipase cDNA. The predicted amino acid sequence is 85 and 70% identical to that of porcine and canine pancreatic lipase, respectively. In vitro translation of the mRNA transcribed from this cDNA results in a protein of the expected molecular size that is processed by microsomal membranes to yield a glycosylated protein with proper signal peptide cleavage. Further, expression of hPL in COS cells yields a pancreatic lipolytic activity that is colipase dependent (Giller et al., 1992) and demonstrates tissue-specific expression when transfected in rat pancreatic acinar cell line, AR42J, but not in cells from other tissues (Giller et al., 1992).

Recently, controversy arose over whether the rPL-1 reported by Kern and coworkers (Steinhilber et al., 1988) and sequenced by Wicker-Planquart and Puigserver (1992) is a rat pancreatic lipase isozyme or lipase related protein. Earlier, Giller and coworkers (1992) reported a cDNA clone for hPLRP1, which does not yield lipolytic activity in transfected COS cells, but has the closest homology to rPL-1. This report leads to the possibility that the rat may have PLRPs just like the human does. This has just been confirmed by Payne and coworkers (1994). The rPL-1 isolated by Wicker-Planquart and Puigserver (1992) is a rat pancreatic lipase related protein (designated rPLRP-1 by Payne et al., 1994). When expressed in *Sf*9 cells, rPL-1 (or rPLRP-1) shows barely detectable lipolytic activity against triolein

emulsified solely with bile salts with (pro)colipase present. Of interest is the developmental expression of rPL-3 (designated rPL by Payne et al., 1994), which begins at weaning and predominates in the adult, and of rPL-1 (rPLRP-1) which is high in the postnatal period and decreases during weaning. Lowe and coworkers (Payne et al., 1994) demonstrated that another rat pancreatic lipase related protein (rPLRP-2) has full colipase dependent lipolytic activity in *Sf*9 cells. This is puzzling because the highly homologous hPLRP2 does not have colipase dependent activity when expressed in COS cells (Giller et al., 1992), and assayed with a phosphatidylcholine emulsified triolein. Possibly, differences in processing of the proteins in *Sf*9 and COS cells, as well as substrate differences or species differences contribute to these conflicting results.

STRUCTURE-FUNCTIONAL RELATIONSHIP

Pancreatic lipase plays a key role in fat digestion in the intestine by converting insoluble triacylglycerols into more polar products, fatty acids and 2-monoacylglycerides, which able to cross the brush border membrane of enterocytes. Because of the insolubility of its substrate, lipase must absorb at the water/lipid interface. However, this step is impaired by bile salts, but the pancreas also synthesizes and secretes a small protein, colipase, to counteract the inhibitory effect of bile salts. Colipase anchors lipase on the bile salts coated interface. Thus, fat digestion proceeds through protein/protein interaction mediated by an organized lipid phase.

The predicted protein sequence of rat and human pancreatic lipase reveals significant homology with hepatic lipase and lipoprotein lipase (Hide, Chan and Li, 1992). These three lipases have been included in the lipase superfamily. They share structural similarities and are derived from a common ancestral gene. The most highly conserved feature in all lipases is a 9-amino acid segment containing some hydrophobic side-chains ranging in hydropathy indices

from 0.7 to 14.3 (Mickel et al., 1989), with a consensus sequence Gly-Xaa-Ser-Xaa-Gly. In human and porcine pancreatic lipase, the corresponding sequence is VHVIGHSLG (residues 146-154; one letter amino-acid code), which contains the active site Ser 152 (Winkler, D'Arcy and Hunziker, 1990). This 9-residue sequence has been described by some investigators as part of a lipid-binding segment (Hide et al., 1992).

X-ray crystallography of human pancreatic lipase has shown that Ser 152 is the nucleophilic residue essential for catalysis (Winkler et al., 1990). It is located in the larger N-terminal domain at the C-terminal edge of a doubly-wound parallel β-sheet and is part of an Asp-His-Ser triad. The crystalline structure of human pancreatic lipase suggests that a substantial conformational change occurs before it can bind substrate in this postulated active site. There is a surface loop between disulfide-bridged residues (237 to 261) that covers the active site with a short one-turn α-helix. Interfacial activation, a characteristic property of lipolytic-enzymes acting on water-insoluble substrates at water-lipid interfaces, probably involves a reorientation of this flap, not only in pancreatic lipases but also in the homologous hepatic and lipoprotein lipase (Winkler et al., 1990).

Furthermore, Lowe (1992) demonstrated that Ser 153 in human pancreatic lipase (which corresponds to Ser 152 in previous human studies and porcine and rat pancreatic lipase) is involved in the catalytic site of pancreatic lipase, but is not a critical part of the interfacial binding site. By site-specific mutagenesis of the cDNA for human pancreatic lipase, amino acid substitutions were made at Ser 153; and the mutant cDNAs were expressed in transfected COS-1 cells. The mutant lipases lost their lipase activity, which was measured with triolein as substrate in the presence of deoxycholate and porcine colipase. The interfacial binding is measured by the capacity to bind to octyl-Sepharose. All of the mutant and wild-type lipases

show a similar level of binding to octyl-Sepharose. Other amino acid residues (His 264 and Asp 177) are also involved in catalysis. A Ser-His-Asp catalytic triad similar to that present in serine proteases, appears to be present in human pancreatic lipase.

The catalytic activity of pancreatic lipase, like that of a number of other lipases, is greatly increased when the enzyme comes into contact with a lipid/water interface, a phenomenon known as interfacial activation (Sarda and Desnuelle, 1958). The most peculiar property of pancreatic lipase is the inhibition of this interfacial activation by bile salts and phospholipids (Borgstrom and Erlanson, 1973). To overcome this inhibition, pancreatic lipase must bind another protein, colipase, which, by adsorbing to the bile-salt covered interfaces, allows lipase access to the substrate (Maylié et al., 1971).

Recently, van Tilbeurgh and coworkers (1992; 1993) have crystallized and determined the structure of a human pancreatic lipase-colipase complex in the presence of mixed phospholipid/bile salt micelles. A series of conformational rearrangements in the protein complex create a binding site for the lipid substrate. The most dramatic change is seen in the α-helical "lid" (residues 273-261 of lipase), which covers the active site of enzyme and blocks access to the substrate in the "closed", inactive, complex. In the presence of micelles, the lid opens. This movement exposes the active site, consisting of the catalytic triad (discussed above), which lies at the bottom of a hydrophobic canyon in the catalytic amino-terminal domain of the protein.

A further consequence of the opening of the lid is the restructuring of the β5-loop (residues 76-85) (van Tilbeurgh et al., 1992; 1993). In the closed complex, this loop makes van der Waals contacts exclusively with the lid. When the lid opens, these contacts are lost and the loop folds back onto the core of the protein. This creates an electrophilic region, the

"oxyanion hole," around the active site serine; the oxyanion hole helps stabilize the transition-state intermediate formed during catalysis. The movements of the lid and the β5-loop expose hydrophobic residues and bury hydrophilic ones on the active-site face of the complex, further increasing the affinity of the complex for the lipid substrate.

Colipase, which binds to the non-catalytic carboxyl-terminal domain of pancreatic lipase, has three hydrophobic "fingers" pointing out from the protein complex on the same face as the active site of the lipase. These fingers are thought to enhance binding of the complex to the surface of the micelles, overcoming the inhibitory effect of bile salts. This new structure reveals a second important role for colipase. Once bound, interfacial activation brings the colipase molecule close to the lid and three hydrogen bonds between the fingers and the lid stabilize the conformation of the open complex. This interaction seems crucial for the activity of the enzyme.

The resulting conformational change from the interactions of lipase, colipase and the interface of mixed micelles serves many purposes. First is the creation of the interfacial lipid-binding site; second is the resulting shape and access to the active-site canyon; third is the stabilization of the oxyanion hole loop; and last is the strengthening of the interaction with colipase. The α-helical lid may regulate the different substrate specificity, interfacial behavior, and cofactor dependency of mammalian lipases.

ADAPTATION TO DIETARY FAT

High fat (HF) diets (41-75% of total energy as triglyceride) result in increased the lipase content by 170-800% (Deschodt-Lanckman et al., 1971; Gidez, 1973; Vandermeers-Piret et al., 1977; Bazin and Lavau, 1978; Muorot and Corring, 1979; Ouagued et al., 1980; Saraux et al., 1982; Sabb, Godfrey and Brannon, 1986; Wicker and Puigserver, 1987) and increased

synthesis by 200% (Wicker and Puigserver, 1987). Lipase mRNA levels also increase (200-380%) with increasing dietary fat content (Giorgi et al., 1985; Wicker et al., 1988; Wicker and Puigserver, 1990a), and lipase gene transcription increases during the adaptation to the lipid-rich diet (Wicker and Puigserver, 1990a). Thus, the stimulation of lipase transcription may explain messenger accumulation during dietary regulation. This adaptive response of lipase is the same whether dietary fat is increased at the expense of protein (Deschodt-Lanckman et al., 1971; Robberecht et al., 1971) or carbohydrate (Sabb et al., 1986; Wicker and Puigserver, 1987), suggesting that dietary fat is responsible for the adaptation.

The type of fat, chain length or degree of saturation, also regulates the lipase, but the effects of type of fat are controversial. Deschodt-Lanckman and coworkers (1971) and Bazin, Lavau and Herzog (1978) reported that unsaturated fats stimulate lipase to a greater extent than saturated fats do. In contrast, Saraux and coworkers (1982) reported that the degree of saturation of fat does not influence lipase, but that long-chain triglycerides stimulate lipase more than medium-chain triglycerides do. Sabb and coworkers (1986) reported that the differences in saturation (polyunsaturated/saturated (P/S) ratio of 0.1 to 7.9) and chain length (C12 to C18) affect lipase activity similarly after consumption of a HF diet (67% of energy as fat), but only highly unsaturated fat (safflower oil; P/S ratio = 7.9) leads to increased lipase content after consumption of a moderate fat diet (MF; 40% of energy as fat). A recent study by Ricketts and Brannon (1994) reports that lipase activity increases 300% when MF-safflower diet is fed, but is unchanged when MF-lard diet is fed. However, the LF-lard diet fed groups have higher lipase activity (190%) compared to the LF-safflower diet fed groups. Both rPL-1 and rPL-3 mRNA levels increase 200-300% in MF-fed groups, independent of the type of fat (Ricketts and Brannon, 1994). Further, the safflower oil fed groups, independent of the amount

of dietary fat, have a slight increase (50%) in mRNA levels when compared to the lard-fed groups. Thus, type of fat appears to regulate pancreatic lipase through a poorly understood interaction with amount of fat.

Colipase also responds to dietary fat (Girard-Globa and Simond-Cote, 1977; Muorot and Corring, 1979; Saraux et al., 1982; Wicker and Puigserver, 1990b). Since colipase is needed in a 1:1 molar ratio to lipase for optimal activation, the content of these two proteins often changes in the same direction but differs in kinetics. For example, the change in lipase activity in general occurs before that of colipase (Wicker and Puigserver, 1987; Duan and Erlanson-Albertsson, 1989b) and also to a larger extent (Muorot and Corring, 1979; Duan and Erlanson-Albertsson, 1990b). When rats are fed a high fat diet, lipase synthesis already increases after 1 day, whereas colipase synthesis increases only after 3 days (Wicker and Puigserver, 1990b). A similar delay in colipase response compared to that of lipase occurs after induction of diabetes (Duan and Erlanson-Albertsson, 1992a).

Unlike lipase, increasing the amount of protein (Ougued et al., 1980) or carbohydrate (Wicker and Puigserver, 1987) in the diet has a specific simulating effect on colipase synthesis. Therefore, the adaptation of colipase to dietary fat is still controversial because dietary fat can only be increased by concomitant changes in protein or carbohydrate.

Insulin influences the regulation of pancreatic lipase and colipase. A significant increase in lipase and colipase contents, synthesis, and mRNA levels has been demonstrated in streptozotocin (STZ)-induced diabetes in rats fed commercial (non-purified) or semi-purified HC diets (Bazin and Lavau, 1979; Bendayan and Levy, 1988; Duan et al., 1989a; Duan and Erlanson-Albertsson, 1989b; 1990a; 1992a). The mRNA levels, synthesis and activity for both

lipase and colipase increase one week after STZ-induction of diabetes, and return to control values with insulin treatment (2 U/100 g/d) (Duan and Erlanson-Albertsson, 1992a).

Whether insulin plays an inhibitory role in regulating levels of pancreatic lipase and colipase has been questioned. Possible inhibitory effect of insulin administration on synthesis of pancreatic lipase and colipase has been reported. Administration of insulin (0.5/100 g/d) decreases lipase and colipase contents, synthesis and mRNA levels within 4 h (Duan, 1991). Starvation of normal rats for 72 h increases the synthesis of lipase and colipase and decreases insulin secretion. However, this latter observation is difficult to interpret because long-term starvation increases circulating fatty acids and triglycerides, which also induce pancreatic lipase and colipase. The nature of the regulation of lipase and colipase is not yet clear.

Secretin, gastric inhibitory polypeptide (GIP), and ketones are proposed to regulate the adaptation of pancreatic lipase to dietary fat. In vivo infusion of secretin increases the relative synthesis of lipase (Rausch et al., 1985a,b; 1986), but does not alter mRNA levels (Brannon, 1990), which suggests a translational control mechanism. GIP, a gut hormone that belongs to the secretin family, has a specific stimulatory effect on the synthesis of pancreatic lipase and colipase at both pretranslational and translational levels (Duan and Erlanson-Albertsson, 1992b). Short-term, 24 h, GIP significantly increases pancreatic lipase content, but has no effect on colipase content. No change in mRNAs encoding for these proteins has been found in this short-term infusion. Long-term, 5 days, GIP increases the content and mRNA levels of both lipase and colipase. However, the dosage varied from a 3 μg/kg dose once in 24 h to doses of 5-60 μg/kg three times a day for 5 days, making it difficult to distinguish short and long term effects from dosage effects or their interaction.

Ketones, end products of lipid metabolism, are also involved in the regulation of pancreatic lipase. Increasing blood ketone levels parallel the increasing lipase content in HF-fed, fasting, or diabetic animals. Both lipase activity and ketones are restored in diabetic rats treated with insulin and fed a HC diet, but are increased further in diabetic rats treated with insulin and fed a HF diet (Bazin and Lavau, 1979). In vitro, β-(OH)-butyrate increased lipase activity in cells isolated from rats fed low fat diet but did not affect lipase activity in cells isolated from rats fed HF diet (Hirschi, Sabb and Brannon, 1991). It is still unclear whether ketones regulate pancreatic lipase response to dietary fat alone or with other factors. Possibly, a complex and interactive mechanism(s), which involve(s) secretin, ketones, GIP, or other factors regulate pancreatic lipase adaptation to dietary fat.

MECHANISMS OF ADAPTATION

The mechanism of regulation of lipase and colipase genes by hormones and diet is still unknown, and the isolation and characterization of these genes is a first step in understanding these mechanisms. In humans, a 28-bp region (from -122 to -150 bp) in the 5'-flanking region of the pancreatic colipase gene is homologous to the rat pancreatic-specific enhancer (Sims and Lowe, 1992), and directs the tissue-specific expression of a reporter gene construct in the rat pancreatic acinar cell line, AR-42J (Sims and Lowe, 1992), but not in the non-pancreas cells as such MEPG2, C2C12, and COS-1 cells (Sims and Lowe, 1992). This region is also a binding site for the putative trans-acting factor (PTF-1; Sims and Lowe, 1992). Two approaches have been used to determine the tissue-specific regulatory sequences in the canine pancreatic lipase gene in transfected AR42J cells: one using site-directed mutagenesis to the 5'-flanking sequence of the gene, and the other using various restriction fragments from the 5'-flanking or 3'-flanking sequences placed upstream of the hGH gene with the tk promoter

(Mickel et al., 1989). Unfortunately, neither homologous nor heterologous promoter studies have identified the tissue-specific enhancer. Possibly, the cell line (AR42J cell) lacks the necessary transcription factors required for expression of the heterologous lipase gene constructs, but this cell line has been successfully used to identify tissue specific elements in pancreatic colipase. Alternatively, the regulatory elements for tissue-specific expression of this gene are positioned outside of the sequences studied.

Neither a dietary regulatory element nor any hormonal regulatory elements for insulin, GIP or secretin have been identified in either gene. In contrast to mammalian pancreatic lipase, two chromosomal lipase genes from *Geotrichum candidum* have the putative promoter sequences which might participate in the lipase induction by long chain fatty acids (Nagao et al., 1993). More studies are required to elucidate the mechanism of regulation of pancreatic lipase and colipase at the molecular level.

DIABETES

Diabetes mellitus is a chronic disease due to insulin deficiency or ineffectiveness of insulin present in the system, which affects primarily carbohydrate metabolism, but also alters fat and protein metabolism (Ganong, 1975). Two types of diabetes occur in humans, juvenile-onset (Type I) and mature-onset (Type II). In Type I, destruction of pancreatic β-cells by some means results in absolute deficiency of insulin, whereas in Type II insulin resistance results in ineffectiveness of available insulin even in the presence of hyperinsulinemia (Eli Lilly, 1980).

In general, diabetes results primarily in a malfunction in carbohydrate metabolism. Lack of insulin from the β-cells (Type I) or insulin resistance (Type II) decreases glucose uptake by peripheral tissues leading to hyperglycemia, the major symptom of uncontrolled diabetes. Protein catabolism increases to provide glucogenic amino acids for gluconeogenesis in the liver

and kidneys, and increasing urinary nitrogen loss. This consequent increase in glucose production exacerbates the hyperglycemia because of the inability of target cells to transport glucose. When the renal glucose threshold is exceeded, glycosuria results. The hyperglycemia and glycosuria cause osmotic diuresis (polyuria) and dehydration, which in turn causes polydipsia. Insulin deficiency or its resistance also causes malfunctions in lipid metabolism with increased lipolysis, plasma free fatty acids, ketones, and ketosis. Ketosis can exacerbate any dehydration already present and may lead to acidosis.

CHEMICALLY-INDUCED DIABETIC ANIMAL MODEL

To elucidate the mechanism of dietary and insulin regulation of exocrine pancreatic enzymes, a chemically-induced diabetic animal model has been used. Chemicals like streptozotocin (STZ) or alloxan, which selectively destroy the β-cells by oxidant production, deplete insulin synthesis and secretion and cause severe diabetes (Dulin and Soret, 1977; Cooperstein and Watkins, 1981; Mossman et al., 1985).

STZ is a naturally occurring antibiotic produced by *Streptomyces achromogenes* (Wiggans et al., 1958). It is a nitrosamide methylnitrosourea (MNU) linked to the C-2 position of D-glucose (Wilson and Leiter, 1990). The glucose moiety appears essential to direct STZ specifically to the β-cells. MNU, alone, is much less toxic to β-cells (LeDoux et al., 1986). Further, β-cells that have lost their responsiveness to glucose also lose sensitivity to STZ toxicity (LeDoux et al., 1984). Like most of the nitrosamides, STZ is able to decompose spontaneously inside the cell forming an isocyanate compound and a methyldiazohydroxide (Tjalve, 1983). The isocyanate component can carbamoylate various cellular components or itself. The methyldiazohydroxide decomposes further to form a highly reactive carbonium ion, capable of alkylating DNA or protein or reacting with H_2O to form methanol. These carbonium

ions react with nucleophilic centers, nitrogens and oxygens, in DNA by a unimolecular (Sn1) reaction. Lesions in DNA of this type are removed by excision repair, through the action of poly (ADP-ribose) synthetase to form poly (ADP-ribose) using NAD as a substrate (LeDoux et al., 1986; Wilson et al., 1988). One hypothesis is that in the β-cell NAD is critically depleted, resulting in cell death (Yamamoto, Uchigata and Okamoto, 1981).

Although this hypothesis has been widely accepted, other studies have demonstrated that the toxic action of STZ is more complex than the overactivation of a single enzyme. A new hypothesis explaining the lethal effects of a single high dose of STZ speculates that STZ alkylates DNA and other key components in the generation of ATP (e.g., glycolytic or mitochondrial enzymes). This hypothesis suggests that the fall of NAD levels is not by itself lethal, but that the concomitant drop in ATP formation would impair the resynthesis of NAD, causing the levels of this key cellular component to drop below critical levels (Wilson et al., 1988). The combination of these two critical processes occurring simultaneously would allows a single administration of STZ to destroy β-cells selectively and rapidly.

Other hypotheses also have been proposed. Kawada (1992) proposes that STZ is transported into pancreatic β-cells through the glucose transporter, attacks mitochondria, and inhibits mitochondrial ATP generation, resulting in high concentrations of intracellular ADP and its degradation product hypoxanthine. Xanthine oxidase (XOD) activity is high in β-cells, and increased O_2^- radicals would be produced. However, β-cells scavenge these radicals inefficiently because of their low activity of superoxide dismutase. Alternatively, STZ can directly activate XOD and enhance O_2^- generation. Consequently, pancreatic β-cells suffer two-fold from O_2^- or hydroxyl radicals following the exposed to STZ.

Wolff (1993) proposes that STZ produces an inappropriate nitric oxide response. Nitric oxide, a free radical, targets intracellular iron-containing enzymes, resulting in the loss of their activity. Desferrioxamine binds iron, prevents the formation of oxygen free radicals, and reduces the incidence of diabetes associated with multiple low-dose STZ injections (Mendola, Wright and Lacy, 1989). Recently, nitric oxide generation by streptozotocin has been confirmed (Kwon et al., 1994).

In summary, several mechanisms may trigger diabetes by STZ. First, its decomposition products can alter cellular membrane proteins by both alkylation and carbamoylation reactions so that they are no longer recognized as self. Second, STZ can alter DNA such that a previously silent gene may be expressed or a normal protein may be altered by point mutation. This could happen as a result of alkylation at the O^6 position of guanine, allowing mispairing with thymine, or by alkylation of the phosphate backbone, conformationally altering the DNA. Third, the production of free radicals and peroxides are also implicated in the cytotoxic action of STZ.

Alloxan, the ureide of mesoxalic acid, has been used to induce diabetes mellitus in experimental animals since the 1940's (Dunn, Seehan and McLetchie, 1943). Jacobs (1937) observed the following triphasic action by alloxan: (1) an immediate hyperglycemia, peaking within 2-3 hours; (2) a severe and often fatal hypoglycemia; and (3) chronic, final hyperglycemia (if the animal survives the second phase). Goldner and Gomoria (1944) reported that the first phase hyperglycemia can be prevented by administering insulin simultaneously with alloxan, but the ensuing two phases still occurred. The first hyperglycemic phase results from increased glycogenolysis. The second phase (hypoglycemia) may occur after β-cell

degeneration due to increased insulin levels as insulin leaches from dying or dead β-cells (Ridout, Ham and Wrenshall, 1944).

Alloxan acts within the β-cell by (1) initially stimulating the pentose-monophosphate shunt with the formation of H_2O_2; and (2) subsequently inhibiting both the pentose-monophosphate shunt and the tricarboxylic acid cycle pathways through the formation of OH^- from O_2^- and H_2O_2 (Ishibashi and Howard, 1981; Malaisse, 1982; Sener, Malaisse-Lanae and Malaisse, 1982). The β-cells accumulate alloxan very rapidly (Malaisse, 1982), within 2-5 minutes after treatment and are the most sensitive to peroxide compounds. These two factors, rapid accumulation and sensitivity, may explain the diabetogenic action of alloxan.

ENERGY BALANCE AND UTILIZATION

Bioenergetics is the conversion of various forms of energy into a form that is biologically useful to the organism. The basis of bioenergetics, as defined by the laws of thermodynamics, can be stated by the following formula [National Research Council (NRC), 1981]:

IE (Energy Intake) = Fecal E + Gaseous E + Urinary E + Surface E + Heat E + Net E

Energetic efficiency, a basic concept in bioenergetics, is the efficiency with which energy is utilized to deposit or gain new tissue or recovered as a product, and can be defined as a ratio of the energy deposited to the metabolizable energy (ME) intake corrected for maintenance costs.

Energy utilization can be determined by direct or indirect measurements of heat production or by evaluation of carcass deposition of protein and fat. Heat production measurements involve direct or indirect calorimetry, where heat produced by the animal is

measured directly in a calorimeter (thermal exchange) by the first method, or indirectly by O_2 consumed and CO_2 produced (respiratory exchange) in the latter method. Measurements of carcass composition involve determination of the heats of combustion of carcass components, feed and feces (Blaxter, 1971; NRC, 1981).

The alterations in metabolism associated with diabetes mellitus in experimental animals provides an opportunity for study of energy utilization and energetic efficiency in relation to insulin. Most early work regarding the effect of insulin on energy balance and utilization was done in the alloxan-induced diabetic animal models. Kumaresan and Turner (1965) reported that after an initial 67% decrease in food intake (compared to control period) following a single alloxan injection, intake increased up to 56% above the control period. This hyperphagia may be caused by decreased glucose utilization in the food intake regulating area of the brain (Mayer and Bates, 1952). Exogenous insulin ameliorated the high rate of food intake, but excessive overeating did not occur in diabetic animals fed high-fat diets (Friedman, 1972). Diabetic rats adjusted food intake only when the caloric density of the diet was changed by altering dietary fat levels, whereas normal rats adjusted intake when either dietary fat or carbohydrate content was altered (Friedman, 1978). Further, diabetic rats utilize fat more readily than carbohydrates for energy needs after body-fat stores are depleted. Alloxan-diabetic rats use fat as the major fuel, and intake increased as dietary fat was diluted with filler,

A study by Dudas (1984) compared the energy utilization in normal and alloxan-induced diabetic rats fed Ralston-Purina rat chow. Energy efficiency decreased in diabetic rats. Maintenance dietary digestible energy (DE) requirements were considerably higher in diabetic rats. Net efficiency values of converting DE to net energy (NE) were decreased in diabetes.

Efficiencies of lean tissue deposition were reduced in diabetic rats as well as fat deposition efficiencies were.

In contrast to alloxan-induced diabetic animals, STZ-induced diabetic animals have higher energy and protein intakes per unit of metabolic body weight than the control animals (Krishnamachar and Canolty, 1986). The energy efficiencies were not affected when protein was held constant and fat as a proportion of nonprotein energy increased. When fat was held constant and protein, as a proportion of nonfat energy increased, energetic efficiency for diabetic rats increased significantly when 22.25% of Fat given, whereas that for control rats increased at all levels except 44.5% of Fat given. However, less investigation on how energy is utilized has been done in STZ-induced diabetic animal models. More research is needed to compare the different effects on energy balance and utilization of macronutrients by these two diabetogenic agents (STZ and alloxan) and to elucidate the mechanism of energy utilization and energetic efficiency in relation to insulin.

CHAPTER 3

REGULATION OF PANCREATIC AMYLASE AND LIPASE GENE EXPRESSION BY DIET AND INSULIN IN DIABETIC RATS

INTRODUCTION

Alterations of dietary substrates result in adaptation of the mRNA, synthesis and content of the pancreatic digestive enzymes, thus optimizing the digestive processes. Insulin has been proposed to mediate this adaptation to dietary carbohydrate due to the anatomical relationship between the endocrine and exocrine pancreas and the evidence from experimentally-induced diabetes using alloxan or streptozotocin (STZ) (Brannon, 1990). Diabetes markedly decreases pancreatic amylase nine to ten fold, and exogenous administration of insulin reverses this effect (Ben Abdeljlil et al., 1965; Palla et al., 1968; Snook, 1968; Soling and Unger, 1972). Pancreatic amylase mRNA parallels these changes in amylase content in diabetes and insulin-treatment (Korc et al., 1981). Transcription of amylase-reporter gene constructs is repressed in diabetic transgenic mice (Keller et al., 1990), demonstrating an insulin-responsive element (IRE) in the 5' flanking region of this gene.

Insulin alone, however, does not appear to be the sole mediator of this adaptation (Dagorn, 1986). Exogenous administration of insulin to non-diabetic rats fed a carbohydrate-free, protein-rich diet fails to change pancreatic levels of amylase (Palla et al., 1968). In diabetes, pancreatic acinar cells are deprived not only of insulin but also of glucose. Treatment of diabetic animals with insulin replenishes acinar cells with both insulin and glucose as long as the diet provides carbohydrate. Diabetic rats treated with insulin still have decreased amylase activity when fed a high fat (HF) diet, but have restored amylase activity when fed a high carbohydrate (HC) diet (Bazin and Lavau, 1979), suggesting that glucose interacts with insulin

in the regulation of amylase. Further, the IRE in the 5'-flanking region of the amylase gene is insufficient by itself for dietary regulation (Schmid and Meisler, 1992), and a putative dietary response element has been localized in the same region (-208 to -82 bp) as the IRE (-167 to -138 bp).

Increased intracellular glucose, either alone or in conjunction with insulin, may stimulate changes in amylase gene expression (Snook, 1968). An independent role for glucose in regulating amylase is reported in pancreatic AR42J cells (Stratowa and Rutter, 1986; Estival et al., 1991). Removal of glucose decreases amylase mRNA by 5 fold within 30 h (Stratowa and Rutter, 1986), while reduction of the media glucose from 5 to 1 mM decreases amylase synthesis, but not mRNA levels (Estival et al., 1991). Thus, the role of glucose alone and its interaction with insulin in the dietary regulation of amylase remain controversial.

Evidence gathered from source of energy fed or diabetic conditions, suggests an anti-coordinate regulation of pancreatic amylase and lipase. Rats fed a HC diet have increased amylase and decreased lipase, whereas the reverse is true when fed a HF diet (Christophe et al., 1971; Deschodt-Lanckman et al., 1971; Snook, 1971; Sabb et al., 1986; Wicker et al., 1988). In diabetic rats, amylase content and synthesis decrease whereas those of lipase increase (Deschodt-Lanckman et al., 1971; Bazin and Lavau, 1979; Duan et al., 1989a,b; Duan and Erlanson-Albertsson, 1990a). Little attention has focused on the mechanism of this anti-coordination, but glucose may be involved (Bazin and Lavau, 1979).

In order to investigate the role of dietary carbohydrate and its interaction with insulin in regulation of amylase and lipase, diabetic rats were fed HC, HF or HP diets and were treated for seven days with insulin or saline. Pancreatic enzyme content and mRNA levels were determined. Both diet and insulin independently affected pancreatic amylase and lipase gene

expression, but interactive effects of diet and insulin were only observed for lipase content and amylase mRNA.

METHODS

EXPERIMENTAL PROTOCOL

Male Sprague-Dawley rats (230 g; Harlan, Indianapolis, IN) were housed individually in metabolic chambers in a temperature- and humidity-controlled environment with a 12-h light-dark cycle. Rats were weight matched into three groups and fed ad libitum purified HC, HP or HF diet for 21 days (control group). The composition of the diets (Werner, Korc and Brannon, 1987) is shown in Table 3-1. These diets were isoenergetic but varied in content of cellulose, which has been shown not to affect the exocrine pancreas (Schneeman and Gallaher, 1980). On day 7, diabetes was induced by a single intraperitoneal injection of STZ (50 mg/kg) after an overnight fast. Diabetes (plasma glucose > 300 mg/dl) was confirmed after 6 days following a 6-h fast. Diabetic animals were randomly subdivided into two groups and injected subcutaneously daily with saline (Db-group) or NPH insulin (Eli Lilly, Indianapolis, IN; Db+I-group). The dosage of insulin (U/100 g of body weight) varied to normalize plasma glucose: 3.9 for HC, 3.0 for HP, and 1.75 for HF. The insulin dose response of plasma glucose and pancreatic amylase and lipase in HC-fed diabetic rats is shown in Figure 3-1. Animals fed HF diet were highly sensitive to insulin dosage, and overdose caused hypoglycemic shock. Food consumption was measured daily, and body weights were measured periodically. Rats were fasted 6 h and killed (12:00-1:00 pm) by exsanguination from the abdominal aorta while anesthetized with ether. Pancreata were removed, and a portion frozen immediately on dry ice and stored at -80°C for subsequent enzyme analyses. The remainder of the pancreas was used immediately for RNA isolation as described below.

Table 3-1

Dietary composition

Component	HC		HP		HF	
	Weight	kcal	Weight	kcal	Weight	kcal
	%					
Casein	20.0	20.7	65.0	67.0	20.0	20.7
Corn oil	5.0	10.4	5.0	10.4	28.9	67.0
Corn starch	65.0	67.0	20.0	20.7	11.3	10.4
DL-Methionine	0.3	--	0.3	--	0.3	--
Salts[1]	3.5	--	3.5	--	3.5	--
Vitamins[2]	1.0	--	1.0	--	1.0	--
Choline bitartrate	0.2	--	0.2	--	0.2	--
Cellulose	5.0	--	5.0	--	34.2	--

[1]AIN Mineral mixture 76

[2]AIN Vitamin mixture 76

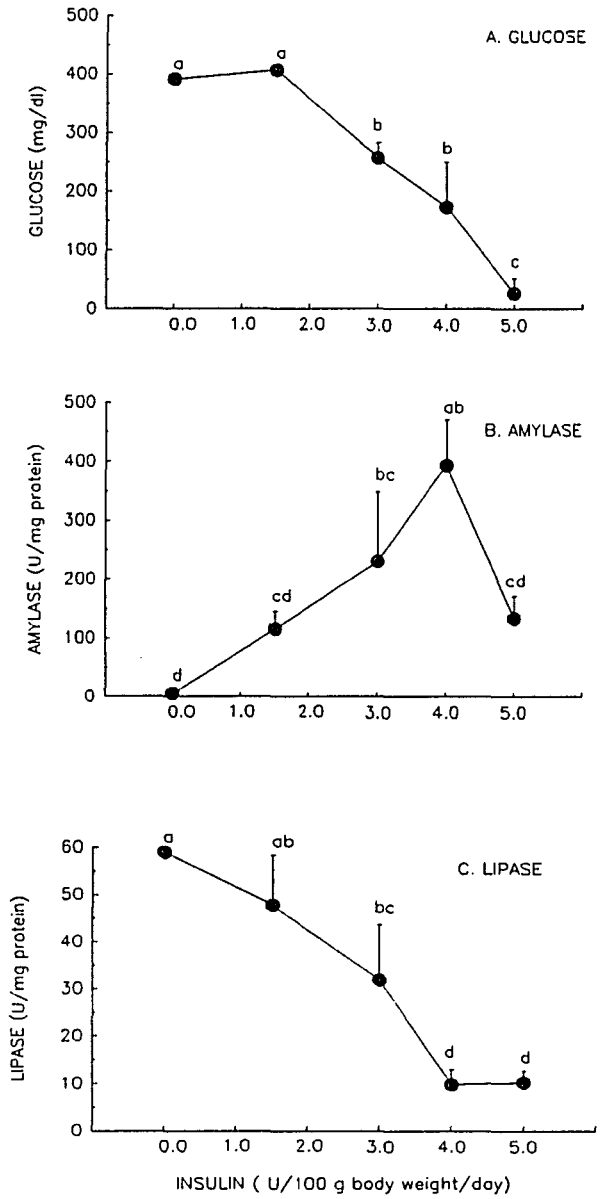

Figure 3-1. The effects of insulin on plasma glucose (A), pancreatic amylase (B) and lipase (C) in HC-fed diabetic rats. Values represent mean ± SEM from 4-7 rats; non-diabetic control values were 175.2 ± 17.42 for plasma glucose (A), 443.74 ± 50.85 for amylase activity (B), and 29.27 ± 1.81 for lipase activity (C). $^{a\text{-}d}$Values for a given parameter (A, B, or C) not sharing a superscript differ significantly ($p < 0.05$; one-way ANOVA).

PLASMA INSULIN AND GLUCOSE ANALYSIS

Blood was collected in heparinized tubes. Plasma was removed after centrifugation for 10 min at 1000×g. Aliquots were frozen for the determination of plasma insulin by radioimmunoassay using rat insulin as the standard for controls and diabetics and porcine insulin as the standard for insulin-treated diabetics (Zaharko and Beck, 1968). All blood samples were taken during a 1-h period from 12:00-1:00 pm after a 6-h fast. Plasma glucose was determined by using the coupled glucose oxidase method (Raabo and Terkildsen, 1960).

PANCREATIC ENZYME ANALYSES

Pancreatic fragments were homogenized in nine volumes of 0.15 M NaCl-5 mM PO_4 (PBS, pH 7.4) with a Polytron homogenizer. Homogenates were centrifuged at 14,000×g at 4°C for 30 min. An aliquot of supernatant was removed for proteolytic analysis, and soybean trypsin inhibitor was added to the remainder (final concentration = 0.01%). The supernatant was used for determination of enzyme activity and protein content. Amylase activity was assayed by the Phadebas blue starch method (Ceska, Birath and Brown, 1969) using Sigma Enzyme 2E standard. Lipase activity was determined by a titrimetric method (Sabb et al., 1986) with 20 mM NaOH using a gum arabic-stabilized emulsion of neutralized triolein with excess crude colipase. Protein was determined by the method of Lowry et al. (1951), using bovine albumin as a standard. Proteolytic enzymes were activated with equal volumes of enterokinase (30% in PBS) for 1 hour at room temperature (Brannon, Collins and Kore, 1987). Protease activity was determined for trypsin by the procedure of Erlanger, Kokowsky and Cohen (1961), chymotrypsin by that of Erlanger, Edel and Cooper (1966) and elastase by that of Bieth, Spiess and Wermuth (1974). Enzyme activities were expressed as units (μmol released/min) per mg protein.

RNA EXTRACTION AND HYBRIDIZATION

RNA was isolated as described by Steinhilber et al. (1988). This method is a cold guanidine thiocyanate and guanidine hydrochloride procedure. A freshly-isolated pancreatic fragment was immediately homogenized with an Polytron for 2×20s at 70% power in ice-cold 4 M guanidine thiocyanate-0.1 M Tris-Cl, pH 7.5, 0.14 M β-mercaptoethanol. RNA was differentially precipitated with ethanol. The pellet was resuspended in 6 M guanidine hydrochloride-25 mM EDTA-10 mM DTT, pH 7.0, reprecipitated with ethanol and dissolved in 10 mM Tris, pH 7.5-25 mM EDTA. The RNA was extracted with chloroform:butanol (3:1) and then phenol:chloroform:isoamyl alcohol (25:24:1). The integrity of RNA was checked by 1% agarose gel electrophoresis for the presence of intact 18S and 28S bands and by Northern hybridization using formaldehyde-MOPS gel electrophoresis (Steinhilber et al., 1988), transfer to nitrocellulose and hybridization with cDNA probes.

Recombinant plasmids used in these studies were the generous gifts of Dr. H. F. Kern, University of Marburg, Germany [rat pancreatic amylase cDNA (1.5 kb insert in PstI site of pGEM3) and rat pancreatic lipase 1 (rPL-1) cDNA (0.82 kb insert in PstI site of pUC9)]; Dr. John Williams, University of Michigan [rat pancreatic lipase 3 (rPL-3) cDNA (1.5 kb insert in EcoR I of pUC 18] and Dr. G. T. Bowden, University of Arizona [β-actin (0.5 kb insert in Hind/EcoRI sites of pGEM1)]. Each probe resulted in a single, intact band by Northern hybridization (Figure 3-2). Two different rat pancreatic lipase (rPL) cDNAs were used in this study. These two rPL cDNAs are designated rPL-1 [isolated by Steinhilber and coworkers (1988) and sequenced by Wicker-Planquart and Puigserver (1992)] and rPL-3 [isolated and sequenced by Lowe and co-workers in an unpublished report to GenBank M58369 and confirmed

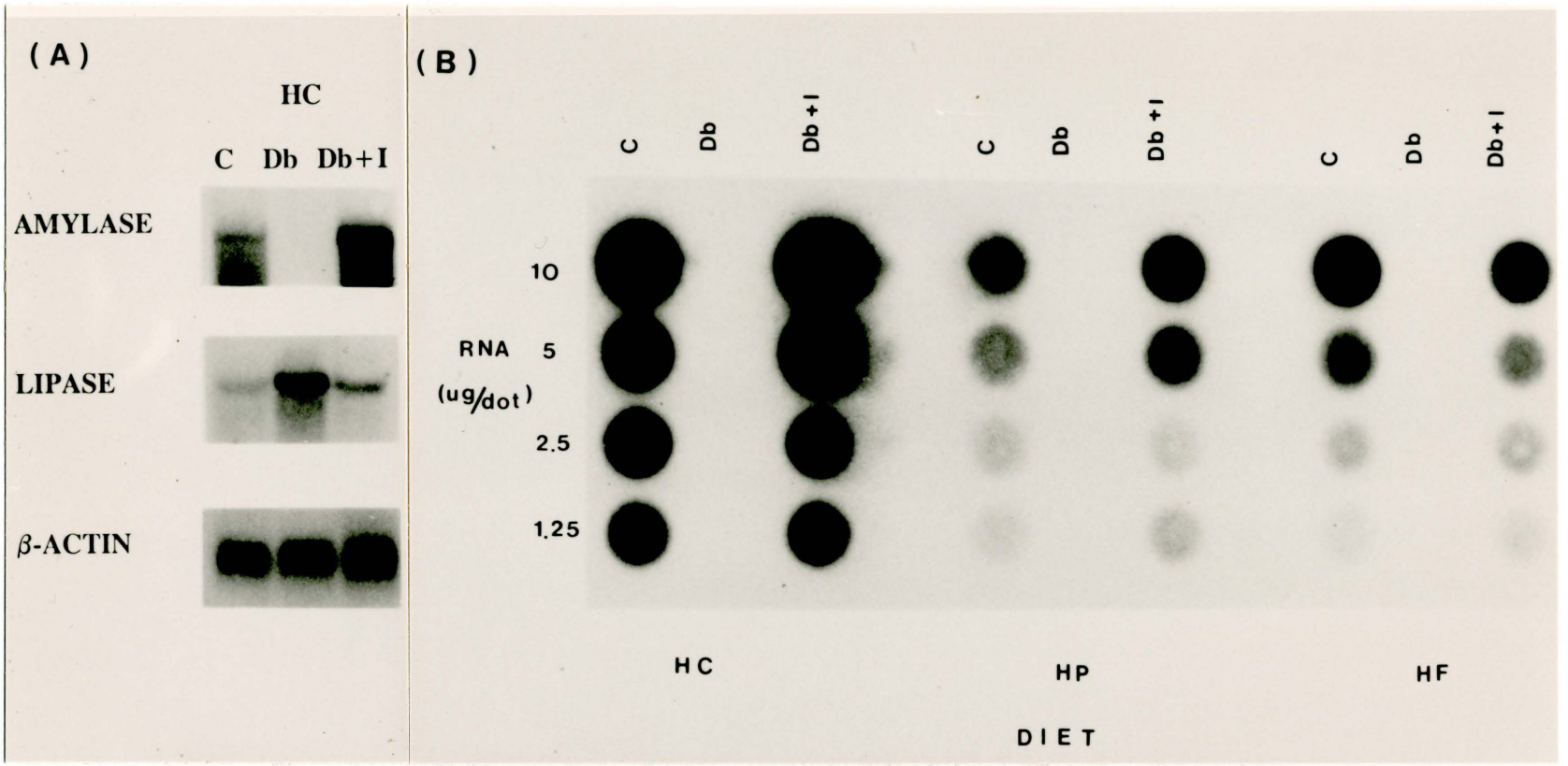

Figure 3-2. Northern (A) and Dot (B) blot analysis of total pancreatic RNA from control (C), diabetic (Db), and insulin-treated diabetic (Db+I). For Northern hybridization, total RNA from HC-fed control was loaded into each lane and hybridized for amylase (5 μg/lane), lipase (rPL-1; 30 μg/lane) and β-actin (10 μg/lane). For dot blots, a total RNA sample from each treatment group was blotted at 1.25-10 μg/dot and hybridized for amylase cDNA.

by Wicker-Planquart and Puigserver (1992) and Wishart et al. (1993)], based on the nomenclaturesuggested by Wicker-Planquart and Puigserver (1992). To date, studies of dietary and hormonal regulation of pancreatic lipase have only used rPL-1, but the true nature of this probe has been questioned following reports of a highly homologous human pancreatic lipase related protein that fails to express pancreatic lipolytic activity in COS-cells (Giller et al., 1992). This controversy and its ramifications are discussed in the RESULTS and DISCUSSION sections.

Specific mRNAs were quantitated by dot-blot hybridization of at least two concentrations of total RNA within the range of linear hybridization. Total RNA was diluted in diethyl pyrocarbonate (DepC)-treated water to the appropriate concentration, verified by Absorbance at 260 nm. For denaturation, the samples were mixed with 6.15 M formaldehyde in 0.75 M NaCl-0.075 M trisodium citrate (5×SSC) and heated for 15 min at 65°C. The denatured samples were spotted onto a nitrocellulose filter using a Millipore dot-blot apparatus. The filters were crosslinked by UV with optimal dosage (120 mJ/cm^2) and prehybridized at 42°C for 2 h in a solution containing 50% formamide, 5×SSC, 5×Denhard's solution, 0.1% sodium dodecyl sulfate (SDS) and 100 μg tRNA. Hybridization was performed at 42°C for 16-18 h after adding ^{32}P-labelled cDNA probe containing 5 × 10^5 cpm/ml of amylase cDNA, 1 × 10^6 cpm/ml of rPL-1 and rPL-3 cDNA, or 1 × 10^7 cpm/ml of β-actin cDNA. Plasmids containing cDNA inserts were labeled by nick translation (Steinhilber et al., 1988) for amylase and rPL-1 or by random-primer (*Promega* Prime-a-gene labeling system) for rPL-1, rPL-3 and β-actin. Labeled control plasmids without inserts yielded no hybridization to total RNA (data not shown). After hybridization, filters were washed under increasingly stringent conditions (2×SSC with 1% SDS to 0.2×SSC with 1% SDS) and air-dried prior to autoradiography

overnight at -80°C. Autoradiographic films were quantitated by an area laser densitometer (Molecular Dynamics) and volume integration. Each filter contained a sample of total RNA obtained from the pancreas of one untreated rat to control the variability among hybridization assays (Steinhilber et al, 1988). The data were expressed as relative units of each sample to within filter control per μg RNA. To check the linear relationship between concentration of RNA and specific hybridization, 0.5-10 μg of total RNA from HC-control fed rats were spotted and quantitated directly by liquid scintillation counting of each dot or indirectly by laser densitometric analysis of the autoradiogram.

Amylase mRNA levels were linear in the range of 0.5 to 10 μg RNA ($r = 0.98$, Figure 3-3). Quantitation of mRNA by either area-scanning laser densitometer of the autoradiography or liquid scintillation counting (LSC) of the filter yielded a linear relationship ($r = 0.87$-0.98; Figure 3-3), and all samples were subsequently quantitated by laser densitometry. Both rPL-1 and rPL-3 mRNA levels were linear in the range of 0.1 to 2.0 μg RNA ($r = 0.96$, data not shown). There was also a highly significant ($p<0.005$) linear correlation of rPL-1 mRNA versus rPL-3 mRNA ($r = 0.99$), data not shown). Further after identical hybridization and washing conditions, the levels of rPL-3 mRNA were 2-3 fold higher than rPL-1 mRNA levels. We examined the specificity of the hybridization of rPL-1 and rPL-3 and their cross-hybridization by homologous and heterologous hybridizations of random prime-labeled rPL-1 and rPL-3 cDNA inserts with 0.01 to 0.2 μg of linearized plasmid DNA containing each insert. Hybridization and washing conditions were identical as described above. The rPL-1 cDNA hybridized linearly to the rPL-1 plasmid in this range ($r = 0.89$), but the cross-hybridization of rPL-1 to the heterologous rPL-3 plasmid averaged 0.2% (range 0-0.7%

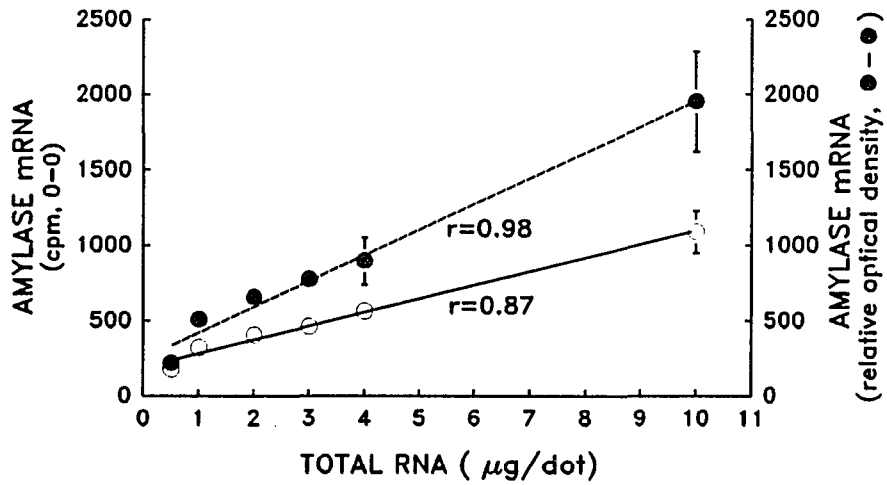

Figure 3-3. Linearity curve of amylase cDNA hybridization. Six different concentrations of total RNA from HC-fed control rats were spotted on the same filter and probed with labeled amylase cDNA. The linear regression coefficient (r) was 0.98 for laser densitometer (●) quantitation and 0.87 for liquid scintillation counting (○).

of its hybridization to the homologous rPL-1 plasmid) (Table 3-2). The rPL-3 cDNA hybridized linearly to the rPL-3 plasmid in this range (r = 0.99), but the cross-hybridization of rPL-3 to the heterologous rPL-1 plasmid averaged 3.4% (range = 0-4.0%) of its hybridization to the homologous rPL-3 plasmid (Table 3-2). These results demonstrate the specificity of the hybridization of each probe under the highly stringent conditions used in this study, which is to be expected given the low homology (65%) between rPL-1 and rPL-3.

DATA ANALYSIS

All data, expressed as mean ± SEM, were analyzed by one- or two-way analysis of variance (ANOVA) and least significant differences (LSD; Steel and Torrie, 1960). Results were considered to be significantly different if $p < 0.05$.

Two way ANOVA was the primary analysis because of the 3×3 factorial design of experiments with main, independent, treatments of 1) *Diet* (HC, HP or HF) and 2) *Insulin* status (Control, Db or Db+I). Most importantly, this analysis is the only statistical analysis that enables the assessment of the interactive effects of *Diet* × *Insulin* as well as the overall independent effects of *Diet* and *Insulin*. When ANOVA was significant, post-hoc tests were done by LSD to determine differences among the nine individual treatment groups. Results of the two way ANOVA for amylase and lipase gene expression are presented in Table 3-3.

RESULTS

FOOD CONSUMPTION AND BODY WEIGHT

Food consumption was similar among the HC-, HP- and HF-fed controls, but was increased in the HC- and HP-Db and Db+I and HF-Db+I groups (64-85%; Table 3-4). Only the HF-Db rats had decreased consumption (18%; Table 3-4). Final body weights (Table 3-4)

Table 3-2

Cross-hybridization of rPL-1 cDNA and rPL-3 cDNA

Probe	rPL-1	rPL-3
Plasmid DNA (μg)	rPL-3	rPL-1
	(% rPL-1-rPL-1 hybridization)	(% rPL-3-rPL-3 hybridization)
0.01	0	2.0
0.05	0	3.5
0.1	0.1	3.9
0.2	0.7	2.8
Average	0.2	3.4

Table 3-3

Two-way ANOVA for independent and interactive effects of diet and insulin on pancreatic amylase and lipase gene expression[1]

	Amylase		Lipase		
	Activity (U/mg protein)	mRNA (Relative unit)	Activity (U/mg protein)	rPL-1 mRNA (Relative unit)	rPL-3 mRNA (Relative unit)
I. Insulin effect					
Control	238 ± 48 (14)	230 ± 44 (9)	51 ± 9 (14)	878 ± 135 (9)	832 ± 132 (9)
Db	8 ± 3 (22)	23 ± 9 (8)	40 ± 4 (22)	1740 ± 254 (11)	1305 ± 169 (9)
Db+I	212 ± 35 (28)	251 ± 77 (8)	28 ± 4 (28)	901 ± 164 (12)	830 ± 140 (9)
	$p<0.0001$	$p<0.0001$	$p<0.001$	$p<0.004$	$p<0.0003$
II. Diet effect					
HC	254 ± 47 (25)	291 ± 79 (9)	30 ± 4 (25)	954 ± 179 (10)	803 ± 116 (9)
HP	90 ± 20 (20)	112 ± 26 (9)	31 ± 3 (20)	989 ± 121 (11)	648 ± 79 (9)
HF	67 ± 14 (19)	92 ± 22 (7)	53 ± 7 (19)	1516 ± 302 (11)	1516 ± 105 (9)
	$p<0.0001$	$p<0.0003$	$p<0.001$	$p<0.04$	$p<0.0001$
III. Interactive insulin × diet effect (see Figures 3-6 and 3-7)					
	n.s.	$p<0.004$	$p<0.0003$	n.s.	n.s.

[1]Values represent mean ± S.E. with sample size (n).

Table 3-4

Effects of diet, diabetes and insulin on average food consumption, final body weight and pancreatic weight[1]

Diet	Treatment	n	Avg. Food Consumption (g/d)[2]	Final Body Weight (g)[3]	Pancreatic Weight (mg)[4]
HC	Control	4	15.5 ± 0.4^{c}	275 ± 23^{a}	853 ± 107^{cd}
	Db	6	27.9 ± 3.2^{a}	189 ± 5^{d}	729 ± 107^{de}
	Db+I	15	25.6 ± 0.5^{a}	266 ± 6^{ab}	915 ± 55^{bc}
HP	Control	5	13.8 ± 0.8^{cd}	254 ± 18^{ab}	899 ± 94^{bcd}
	Db	8	22.6 ± 1.1^{b}	221 ± 6^{c}	1042 ± 53^{ab}
	Db+I	7	22.2 ± 0.2^{b}	257 ± 7^{ab}	1098 ± 74^{a}
HF	Control	5	13.7 ± 0.8^{cd}	273 ± 18^{a}	786 ± 22^{cde}
	Db	8	11.3 ± 0.9^{d}	190 ± 8^{d}	661 ± 47^{e}
	Db+I	6	25.3 ± 1.0^{a}	244 ± 8^{bc}	847 ± 58^{cd}

[1]Rats were fed diets for total 21 d; after the first week; made diabetic with 50 mg/kg STZ (second week); and the third week for daily insulin treatment (Db+I) or saline treatment (Db). Values represent mean ± SEM from 4-15 rats. [a-e]Values not sharing a superscript differ significantly ($p<0.05$; ANOVA).

[2]The third week daily food consumption was measured. Diet ($p<0.0001$; HC > HP > HF) and Insulin ($p<0.0001$; Db+I > Db > Control) had independent and interactive ($p<0.01$) effects on average food consumption.

[3]Final body weight was taken on the day before killing. Insulin, independent of diet, decreased final body weight ($p<0.0001$; Control = Db+I > Db).

[4]Both Diet ($p<0.03$; HP > HC > HF) and Insulin ($p<0.0001$; Db+I > Db) independently affected pancreatic weight. There was no interactive effect of diet and insulin.

were not different among the HC, HP, and HF Control and Db+I groups, but were significantly decreased in all three Db groups (13-30%). Pancreatic weights differed significantly with diet or insulin treatment (Table 3-4). HP-fed animals had the highest pancreatic weights comparedto HC- or HF-fed groups. Insulin treatment significantly increased pancreatic weight 12% compared to diabetic rats.

PLASMA GLUCOSE AND PLASMA INSULIN

Due to the different glycemic response among rats fed these semi-purified diets, a preliminary dose response was performed to determine the optimal dosage of insulin to normalize the plasma glucose; these were (per 100 g of body weight): 3.9 U for HC, 3.0 U for HP and 1.75 U for HF (data not shown). A representative insulin dose response curve for plasma glucose, amylase activity, and lipase activity of HC-diabetic animals is shown in Figure 3-1. Doses of insulin below 3.0 U and above 4.0 U/100 g body weight resulted in plasma glucose values significantly different from non-diabetic control values of 175.2 $\pm$ 17.4 mg/dl. The selected insulin dose of 3.9 U (per 100 g of body weight) for HC-diabetic rats reversed plasma glucose levels to normal and maximally stimulated amylase activity. Plasma glucose (Figure 3-4A) was significantly increased in Db rats, independent of diet, and returned to respective dietary control values after insulin treatment, except in the HF-Db+I group in which plasma glucose was within the normal range even though significantly greater than the HF-control values. Fasting (6 h) plasma insulin (Figure 3-4B) tended to decrease with diabetes, but was not significantly different between the control group and Db groups. After administration of insulin, plasma insulin, independent of diet, increased significantly in Db+I groups compared to control and Db groups. For specific diets, plasma insulin increased significantly

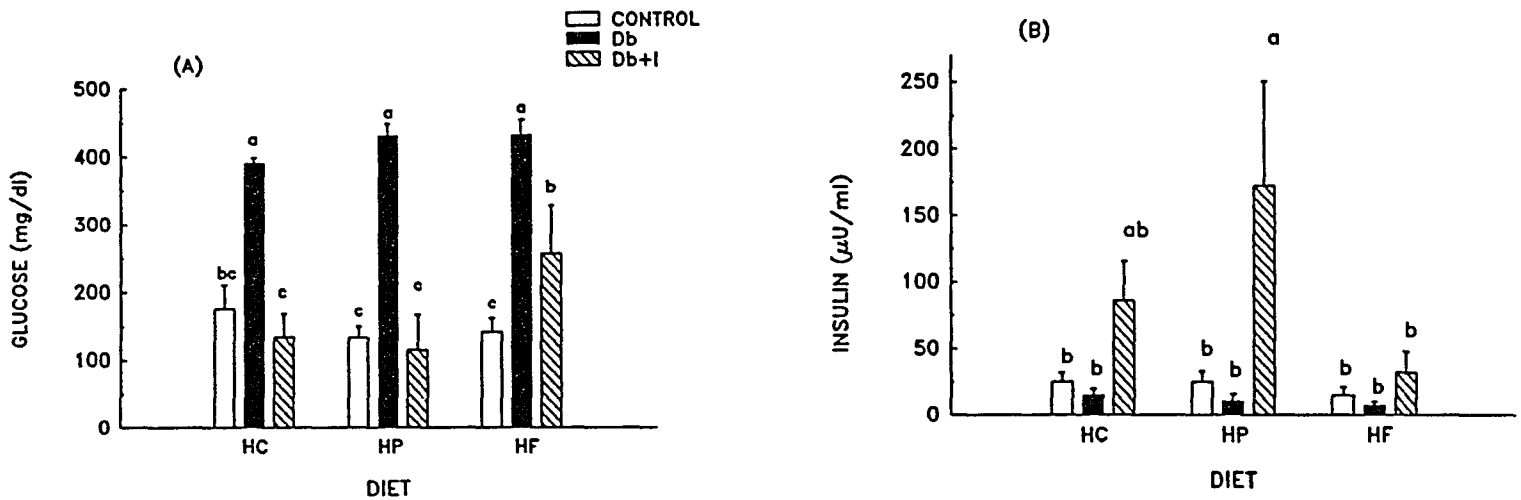

Figure 3-4. Effects of diet, diabetes and insulin on plasma glucose (A) and plasma insulin (B). Rats were fed diets 6 d; made diabetic with 50 mg/kg STZ; and treated daily d 15-21 with 1.75-3.9 U insulin. Values represent mean ± SEM from 4-15 rats. ^{a,b,c}Values for a given parameter not sharing a superscript differ significantly ($p<0.05$; ANOVA). For plasma glucose, there were significant independent effects of Insulin ($p<0.0001$, Db > Control = Db+I) and of Diet ($p<0.01$, HF > HC). There was no interactive effect of Insulin × Diet. For plasma insulin, there was a significant independent effect of Insulin ($p<0.004$, Db+I > Control = Db), no significant independent effect of Diet or interactive effect of Insulin × Diet.

in the HP-Db+I and tended to increase in the HC- and HF-Db+I groups compared to respective dietary control and Db groups.

PANCREATIC AMYLASE AND LIPASE ACTIVITIES AND mRNA LEVELS

To investigate the effects of diet and insulin on pancreatic amylase and lipase gene expression, amylase, lipase 1 and 3 and β-actin mRNAs were examined qualitatively by Northern blot analysis and quantitatively by dot blot analysis.

Both diet and insulin independently regulated amylase gene expression. In Figure 3-2A is a representative Northern blot analysis and in Figure 3-2B is a representative dot blot analysis of total RNA from HC fed control, Db and Db+I rats. Amylase mRNA is markedly reduced in Db and restored to control levels in Db+I. In contrast to the marked effects of diet and insulin on amylase mRNA, β-actin mRNA did not change significantly after either diet or insulin treatment (Figure 3-5), demonstrating the specificity of the changes in amylase and lipase mRNAs.

Diabetes, independent of diet, decreased amylase activity over 97% (Table 3-3 and Figure 3-6A) and mRNA levels over 90% (Table 3-3 and Figure 3-6B). Amylase activity and mRNA values were restored to respective dietary control values by insulin treatment. Diet independently affected amylase activity and mRNA, which were greatest in HC-fed rats. Despite elevation of plasma insulin to or above HC control values, insulin treatment of HP- and HF-fed diabetic rats did not maximally increase amylase activity and mRNA levels to HC control values. Diet and insulin did not interact in the regulation of pancreatic amylase activity, but interacted on amylase mRNA levels, which significantly decreased in diabetes and were restored by insulin treatment only in the HC and HP fed rats. The HF fed rats tended to exhibit

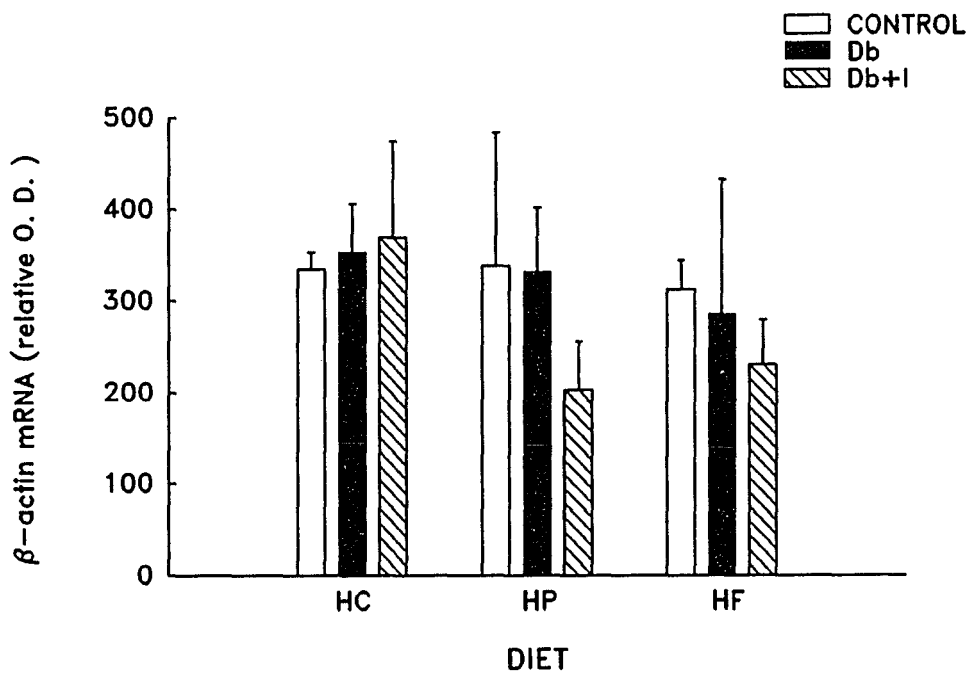

Figure 3-5. Effects of diet, diabetes and insulin on pancreatic β-actin mRNA. Values represent mean ± SEM of triplicate samples from three or four rats. There was no independent effect of Diet or Insulin and no interactive effect of Diet × Insulin on pancreatic β-actin mRNA.

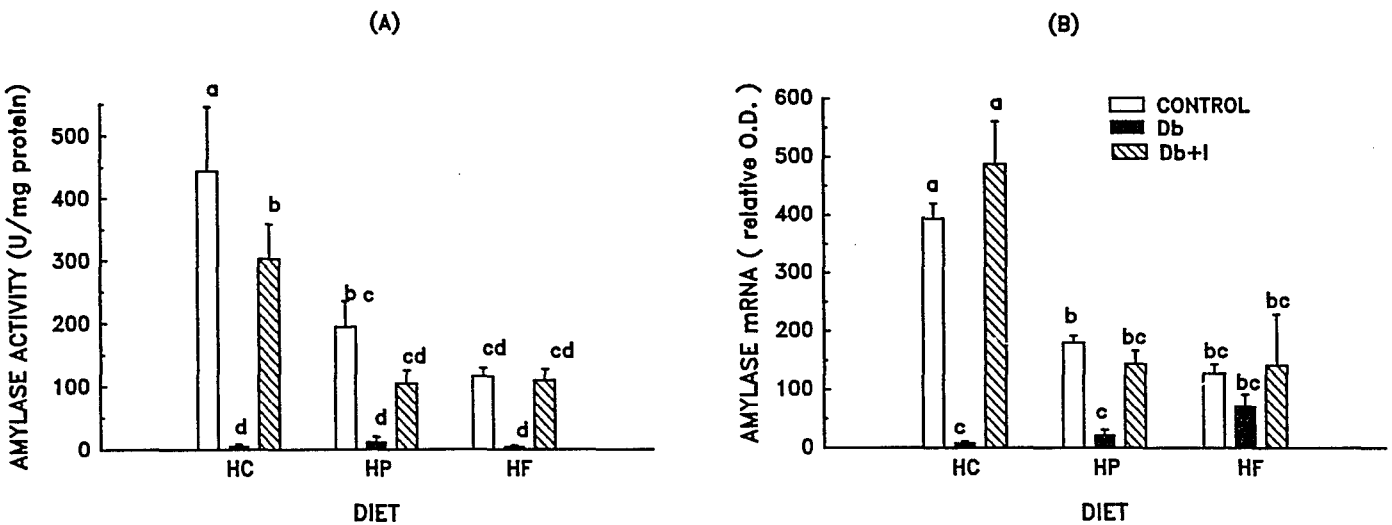

Figure 3-6. Interactive effects of diet, diabetes and insulin on amylase activity (A) and mRNA (B). Values represent mean ± SEM from 4-15 rats (activity) or 3-4 rats (mRNA). [a,b,c,d]Values for a given parameter not sharing a superscript differ significantly ($p<0.05$; two-way ANOVA). There were significant independent effects of Diet (HC > HP = HF) and Insulin (Control = Db+I >> Db) for both amylase activity ($p<0.0001$ for diet; $p<0.0001$ for insulin) and mRNA ($p<0.0003$ for diet; $p<0.0001$ for insulin). Only for amylase mRNA, was there an interactive effect of Diet × Insulin ($p<0.004$) (see Table 3-3).

decreased mRNA levels in diabetes, but the initial levels in control were already significantly lower than HC control.

Presently, a major controversy exists over whether the rat pancreatic lipase cDNA (rPL-1) reported by Kern and co-workers (Steinhilber et al., 1988) and sequenced by Wicker-Planquart and Puigserver (1992) is a rat pancreatic lipase isozyme or lipase related protein 1. The most recent evidence suggests rPL-1 is actually a rat pancreatic lipase related protein (Payne et al., 1994). In light of this controversy and the exclusive use of rPL-1 to date in studies of the dietary and hormonal regulation of lipase, we quantitated lipase mRNA with two cDNAs: the controversial rPL-1 (Steinhilber, 1988) and rPL-3 (Wishart et al., 1993) that is unquestionably a pancreatic lipase (Wishart et al., 1993). The hybridization of these probes was highly specific as demonstrated by the extremely low (0.2-3.4%) cross hybridization (see METHODS). After identical hybridization and washing conditions, the mRNA quantitated with rPL-3 showed 2-3 fold higher levels than that quantitated with rPL-1. These results suggest that rPL-3 is the predominant pancreatic lipase mRNA expressed in the rat.

Diabetes, independent of diet, significantly increased both rPL-1 and rPL-3 mRNA (Table 3-3 and Figure 3-7B and 3-7C) and lipase activity (Table 3-3 and Figure 3-7A). Insulin-treatment of diabetic rats restored lipase mRNA levels to dietary control values, but only restored lipase activity in HC-fed rats (Figure 3-7A). Diet, independent of diabetes, also increased lipase content and mRNA, which was greatest in the HF-fed rats (Table 3-3 and Figure 3-7) for both rPL-1 and rPL-3. Diet and insulin (diabetes) significantly interacted in the regulation of lipase activity; which was increased (202%) in HC-fed diabetic rats, unchanged in HP-fed diabetic rats, and decreased (50%) in HF-fed diabetic rats.

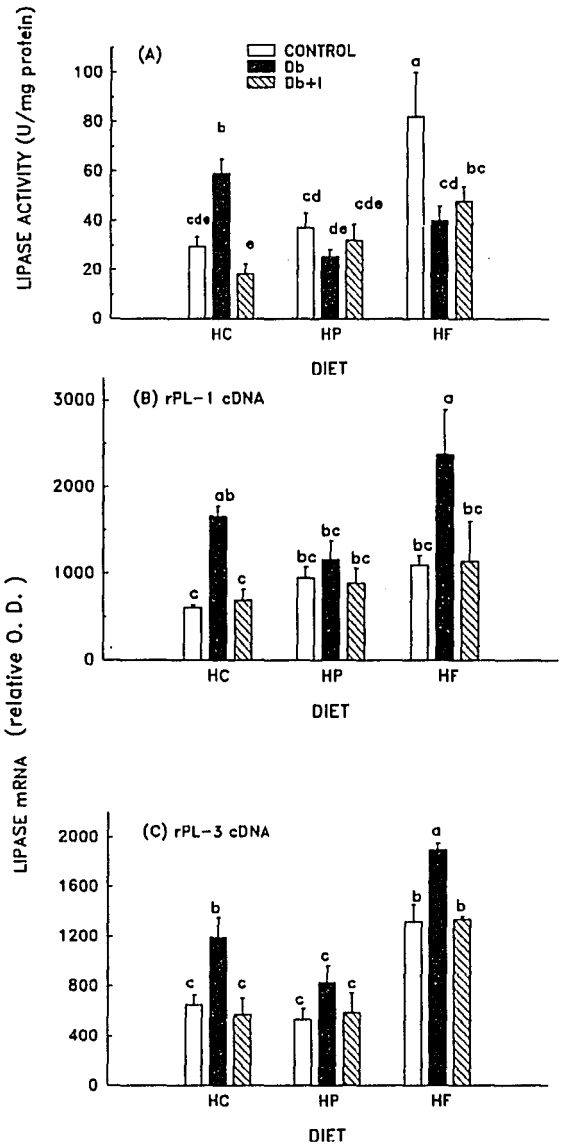

Figure 3-7. Interactive effects of diet, diabetes and insulin on lipase activity (U/mg protein) (A) and mRNA probed with rPL-1 cDNA (B) and rPL-3 cDNA (C). Values represent mean ± SEM from 4-15 rats (activity) or 3-4 rats (mRNA). [a-e]Values for a given parameter not sharing a superscript differ significantly ($p<0.05$; two-way ANOVA). For lipase activity, there was a significant independent effect of Diet ($p<0.001$, HF > HC = HP) and Insulin ($p<0.001$, Control > Db > Db+I) and a significant interactive effect of Diet × Insulin ($p<0.0003$). The lipase mRNA quantitation with rPL-1 cDNA or rPL-3 cDNA had similar results: a significant independent effect of Diet ($p<0.04$ for rPL-1, HF > HP = HC; $p<0.0001$ for rPL-3, HF > HP = HC) and Insulin ($p<0.004$ for rPL-1 and $p<0.0003$ for rPL-3, Db > Control = Db+I). There was no interactive effect of Diet × Insulin for lipase mRNA. (see Table 3-3)

PANCREATIC CHYMOTRYPSIN, TRYPSIN AND ELASTASE ACTIVITIES

There was a significant independent effect of Diet on trypsin activity, which was greater in HP fed groups than in HC fed or HF fed groups (Table 3-5). Diet and insulin (diabetes) also had a significant interactive effect on trypsin activity, which increased in HC-Db but decreased in HF-Db. However, neither diet nor insulin affected chymotrypsin or elastase activities. These results demonstrate the specificity of the effects of diet and insulin on exocrine pancreatic gene expression as neither chymotrypsin nor elastase were affected by either diet or insulin status.

DISCUSSION

Numerous studies in the past have investigated the effects of either insulin or diet on pancreatic amylase and lipase gene expression, but few have examined their interactive effects on the regulation of these enzymes.

Concerning the effects of diet, our results confirmed those of previous studies (Wicker and Puigserver, 1987; Wicker et al., 1988; Brannon, 1990; Wicker and Puigserver, 1990a) that different dietary substrates had different effects on enzyme activity and mRNA level. High carbohydrate diet maximally increased amylase mRNA and activity compared to high protein or high fat diet. The parallel changes in amylase mRNA and its activity, despite different diets fed and insulin status, strongly support a pretranslational mechanism of dietary regulation of pancreatic amylase gene expression.

Concerning the effects of insulin and diabetes, our results agree with those of Bazin and Lavau (1979), Duan et al. (1989b), and Korc et al. (1981). Amylase content and mRNA decreased in diabetes, independent of diet, and were restored after insulin treatment. The reversal of diabetic changes in amylase activity and mRNA levels by insulin treatment indicates

Table 3-5

Effects of diet, diabetes and insulin on pancreatic chymotrypsin, trypsin and elastase activities (U/mg protein)[1,2]

Diet	Treatment	n	Chymotrypsin[4]	Trypsin[3]	Elastase[4]
HC	Control	4	3.5 ± 0.8^{de}	874 ± 91^{d}	2.2 ± 0.5^{a}
	Db	6	4.6 ± 0.5^{a}	1431 ± 161^{a}	2.4 ± 0.5^{a}
	Db+I	15	3.3 ± 0.4^{e}	885 ± 79^{d}	1.3 ± 0.2^{bc}
HP	Control	5	3.7 ± 0.3^{cde}	1182 ± 131^{bc}	1.1 ± 0.1^{c}
	Db	8	4.3 ± 0.7^{ab}	1443 ± 155^{a}	1.2 ± 0.5^{bc}
	Db+I	7	4.0 ± 0.4^{bcd}	1382 ± 206^{a}	1.5 ± 0.4^{bc}
HF	Control	5	4.2 ± 0.5^{abc}	1104 ± 167^{c}	1.6 ± 0.6^{b}
	Db	8	4.2 ± 0.3^{abc}	938 ± 159^{d}	1.5 ± 0.6^{bc}
	Db+I	6	4.2 ± 0.5^{abc}	1322 ± 186^{ab}	2.2 ± 0.7^{a}

[1]Values represent mean ± SEM of 4-15 rats. [a-e]Values not sharing a superscript differ significantly ($p < 0.05$; ANOVA).

[2]Rats were fed diets for total 21 d; after the first week, made diabetic with 50 mg/kg STZ (second week); and the third week for daily insulin treatment (Db+I) or saline treatment (Db).

[3]There was a significant ($p < 0.02$) independent effect of diet on trypsin activity: HP > HC = HF. There was also a significant ($p < 0.04$) interactive effect of diet and treatment on trypsin activity.

[4]There was no significant effect of diet or insulin on chymotrypsin and elastase activities.

that insulin regulates this gene. Meisler and coworkers (Keller et al., 1990) have identified an insulin-responsive element (IRE) in the mouse Amy 2.2 gene that is located in the 5'-flanking region at -167 to -138 bp and is responsible for the transcriptional regulation of this gene by insulin.

It appears unlikely, however, that insulin is the sole mediator of the dietary regulation of amylase. In our study, insulin treatment of HP- or HF-fed diabetic rats did not maximally increase amylase activity and mRNA levels to HC-control values, despite the elevation of plasma insulin above HC control values. Insulin appears to play a permissive role in the dietary regulation of amylase gene expression, such that its presence is necessary for the actions of some other factor(s) as yet unidentified to effect dietary regulation. A recent study from Schmid and Meisler (1992) reports that a 126-bp fragment of the mouse Amy 2.2 gene (-208 to -82 bp), which included the IRE, was sufficient for regulation of amylase by dietary carbohydrate, but a smaller 29-bp fragment (-167 to -138 bp) containing only the IRE and part of the pancreatic tissue specific element failed to respond to diet. Taken together these results suggest that insulin is necessary, but not sufficient, for the regulation of amylase by dietary carbohydrate.

One possibility that needs to be examined is that glucose derived from dietary carbohydrate interacts with insulin in the regulation of the pancreatic amylase gene. In vitro in pancreatic AR42J tumor cells, the removal of medium glucose decreases amylase mRNA by 5 fold within 30 h (Stratowa and Rutter, 1986), while reduction of the medium glucose from 5 to 1 mM decreases amylase synthesis, but not mRNA levels (Estival et al., 1991). This independent role of glucose, our results in vivo and those of Meisler and coworkers with gene constructs in transgenic mice strongly suggest that glucose and insulin may interact in the

transcriptional regulation of amylase. Future studies need to determine the independent and interactive roles of glucose and insulin in the various regulatory mechanisms of amylase gene expression.

A recent paper by Giller and coworkers (1992) raises concerns that the rPL-1 cDNA (Steinhilber et al., 1988) identified with the canine pancreatic lipase (cPL) isolated by Scheele and used in these studies was in fact pancreatic lipase related protein (PLRP-1), not pancreatic lipase. The hPL cDNA, which yields a pancreatic lipolytic activity in transfected COS cells, has a lower amino acid sequence homology (81%) to human PLRP-1, which does not yield lipolytic activity in transfected COS cells (Steinhilber et al., 1988), and a higher homology (87%) with rPL-3 cloned and sequenced by Lowe and co-workers (Sims, Strauss and Lowe, 1990). The rPL-1 and rPL-3 have an even lower (65%) amino acid sequence homology (Wicker-Planquart and Puigserver, 1992). This latter rPL-1 cDNA is in fact the only probe that has been used in all studies to date of dietary and hormonal regulation of pancreatic lipase (Wicker and Puigserver, 1987; 1990a; Wicker et al., 1988; Duan et al., 1989b; Brannon, 1990; Duan and Erlanson-Albertsson, 1990a). If rPL-1 is shown not to be a true pancreatic lipase isozyme, the effects of diet and hormones reported to date will require re-examination with rPL-3. We report here for the first time that effects of diet and diabetes on the rPL-3 mRNA are similar to those on rPL-1 mRNA, suggesting that reported effects to date of diet and hormonal regulation using rPL-1 will be confirmed using rPL-3. The 2-3 fold greater mRNA levels of rPL-3 cDNA compared to those of rPL-1 are similar to the greater abundance of hPL (>4 fold) than hPLRP-1 in human pancreatic total RNA (Giller et al., 1992). The extremely low cross hybridization of rPL-1 and rPL-3 (0.2 to 3.4%) (Table 3-2) demonstrates that the specificity of each probe. The effects of diet and insulin independently and interactively were

similar on rPL-1 and rPL-3 mRNA; suggesting that whatever the true nature of the rPL-1 species, it is similarly regulated as the validated rPL-3 mRNA.

In contrast to amylase, lipase activity was not only affected by diet and insulin, but was also affected by the interaction of diet and insulin. Further, lipase activity did not always parallel mRNA levels (Figure 3-7), suggesting more complicated regulatory mechanisms for lipase gene expression than that of amylase. HF diet resulted in maximal lipase activity and mRNA levels, whereas HC diet resulted in the lowest lipase gene expression. In contrast to amylase, the effect of diabetes depended on the dietary composition. Lipase content and mRNA increased in parallel in HC-fed diabetic rats and were restored to normal values after insulin treatment. These increased lipase activity and mRNA levels in HC-fed diabetic rats are consistent with the results of other studies (Bazin and Lavau, 1979; Duan et al., 1989b) in which non-purified rat chow diets high in carbohydrate were fed. However, our study is the first study to use different macronutrients to examine the dietary effect on lipase gene expression of diabetic rats. In HP-fed rats, neither lipase activity nor mRNA levels were affected by either diabetes or insulin treatment. In HF-fed rats, diabetes decreased lipase activity by 49%, but increased mRNA by over 2 fold. Insulin treatment of HF-fed diabetic rats restored activity and mRNA to control lipase values. The correlation between amylase activity and mRNA levels indicates a pre-translational level of regulation through transcription or stability of mRNA. The preponderance of evidence to date supports a transcriptional regulation of amylase by both diet and insulin. In contrast, there discordance of lipase activity and rPL-3 mRNA in some treatments suggests that complex mechanisms with both pre-translational and translational regulation may be involved in the hormonal and dietary regulation of pancreatic lipase gene expression. Dietary fat has been shown to regulate pancreatic lipase at the

transcriptional level (Wicker and Puigserver, 1990a), but future studies need to examine the independent and interactive effects of insulin and diet on the transcription, mRNA stability and translational efficiency of lipase mRNA. Both transcriptional (Wicker and Puigserver, 1990a) and translational regulation (Wicker and Puigserver, 1987; Steinhilber et al., 1988) have been demonstrated for pancreatic enzyme gene expression.

It is unknown if an IRE exists in the pancreatic lipase gene. Our results suggest, however, that the regulation of pancreatic lipase gene by insulin is dependent on the dietary and metabolic status of the animal and that this regulation is secondary, possibly through alterations of metabolism, rather than primary. Work by Bazin and Lavau (1979) and ourselves (Hirschi et al., 1991) suggest that ketones, a metabolite of fatty acids may play a role in the regulation of pancreatic lipase. Ketones are one possible metabolite through which dietary fat or insulin could act or interact to regulate this gene. Future studies need to determine whether ketones mediate the interactive regulation of pancreatic lipase gene by diet and insulin.

In conclusion, the inability of insulin to stimulate maximal amylase in HP or HF diabetes suggests that insulin is permissive, but not responsible, for the dietary regulation of amylase, and that insulin and dietary carbohydrate interact in regulation of amylase. The parallel changes of pancreatic amylase mRNA and amylase activity support a pretranslational regulation of amylase gene expression that is most probably transcriptional. The differential regulation of lipase activity and mRNA levels with diet and diabetes raises the possibility that lipase gene expression is regulated by an interaction of diet, metabolic state and insulin and through pre-translational and translational multiple regulatory mechanisms.

CHAPTER 4

EFFECTS OF DIET AND INSULIN ON PANCREATIC AMYLASE AND LIPASE ENZYME SYNTHESIS

INTRODUCTION

Insulin, through its IRE in the 5' flanking region, regulate amylase transcription (Schmid and Meisler, 1992) but is permissive for the dietary carbohydrate regulation of amylase (Tsai et al., 1994; Schmid and Meisler, 1992). We have previously shown that exogenous insulin administration in STZ-diabetic rats fails to stimulate maximal amylase gene expression in high protein- (HP) or high fat- (HF) fed diabetic rats (Tsai et al., 1994; Chapter 3). We also demonstrated parallel changes of pancreatic amylase mRNA and activity with diet, diabetes and insulin treatment supporting a pretranslational regulation of amylase gene expression (Tsai et al., 1994; Chapter 3).

Insulin and diabetes has been shown previously to regulate lipase activity, synthesis and mRNA (Duan and Erlanson-Albertsson, 1989; 1990a; 1992a; Duan et al., 1989), and we find that diet and insulin interact in the regulation of lipase. Diabetes increases lipase activity and mRNA levels in rats fed HC diet, and insulin restored these values to normal (Tsai et al., 1994; Chapter 3). However, diabetes did not affect lipase activity or its mRNA levels in rats fed HP diets. In contrast to either its effects on rats fed HC or HP diets, diabetes decreased lipase activity, but increased its mRNA levels in rats fed HF diets. Insulin treatment restored these values in diabetic rats fed HF diets. The differential regulation of lipase activity and mRNA levels with diet, diabetes, and insulin raises the possibility that lipase gene expression is regulated by more complex mechanism (Chapter 3), including translational regulation.

To determine the mechanism of nutrients and the interaction with insulin in regulation of amylase and lipase gene expression, particularly the possible translational regulation of lipase, diabetic rats were fed high carbohydrate (HC) or HF diets and treated for 7 days with insulin or saline. Pancreatic enzyme relative synthesis, as well as cellular content and mRNA levels were determined.

METHODS

EXPERIMENTAL PROTOCOL

Male Sprague-Dawley rats (230 g; Harlan, Indianapolis, IN) were housed as described in Chapter 3 (Tsai et al., 1994). Rats were fed ad libitum purified HC or HF diet for 21 days (control group). The composition of the diets (Werner et al., 1987) is shown in Table 3-1. On day 7, diabetes was induced by a single intraperitoneal injection of STZ (50 mg/kg) after an overnight fast. Diabetes (plasma glucose > 300 mg/dl) was confirmed after 6 days following a 6-h fast. Diabetic animals were randomly subdivided into two groups and injected subcutaneously daily with saline (Db-group) or NPH insulin [dosage (U/100 g) = 3.9 for HC and 1.75 for HF (Tsai et al., 1994; Chapter 3); Eli Lilly, Indianapolis, IN; Db+I-group]. Food consumption was measured daily, and body weights were measured periodically. Rats were fasted 6 h and killed (12:00-1:00 pm) by exsanguination from the abdominal aorta while anesthetized with ether. Pancreata were removed, and a small portion frozen immediately on dry ice and stored at -80° for subsequent enzyme analyses. A second portion of pancreas was used immediately for pancreatic acini isolation and protein synthesis. The remainder of the pancreas was used immediately for RNA isolation as described below and measurement of mRNA levels.

PANCREATIC ENZYME ANALYSES

Pancreatic fragments were homogenized and centrifuged as described previously (Tsai et al., 1994; Chapter 3). An aliquot of supernatant was removed for proteolytic analysis, and soybean trypsin inhibitor was added to the remainder (final concentration = 0.01%). The supernatant was used for determination of enzyme activities and protein content. Amylase activity was assayed by the Phadebas blue starch method (Ceska et al., 1969) using Sigma Enzyme 2E standard. Lipase activity was determined by a titrimetric method as described previously (Tsai et al., 1994; Chapter 3). Protein was determined by the method of Lowry et al. (1951), using bovine albumin as a standard.

PANCREATIC ACINI ISOLATION

Pancreatic acini were isolated according the method of Bruzzone et al. (1985) and modified by Hazlett and Brannon (1988). Pancreas was quickly minced with scissors into small pieces (approximately 2 mm) and then transferred to a sterile 25 ml Erlenmeyer flask to which a volume of KRB-HEPES buffer (12.5 mM-HEPES, 135 mM-NaCl, 4.8 mM-KCl, 1.0 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 5.0 mM-$NaHCO_3$, 5 mM-dextrose, and 0.14 mg of soybean trypsin inhibitor/ml, adjusted to pH 7.4) equal to that of the tissue (usually 1.5-2 ml) was added. For digestion, the pancreatic pieces were vigorously shaken in the presence of 2.0 mg of collagenase/ml (163 U. Worthington CLS 2) at approximately 200 cycles/min at 37°C. The length of the digestion period was variable, being 8-10 min with Db pancreas and 15-20 min with control and Db + I pancreas, by which time the tissue suspension appeared homogeneous to the eye. Db pancreas digested 15-20 min was overdigested with a higher proportion of individual acinar cells and small acini. Further, overdigested Db preparations had poor viability and low incorporation of ^3H phe into total protein (data not shown). Then, 5 ml

of fresh KRB-HEPES with 0.1% bovine serum albumin (BSA) were added and the tissue was centrifuged at 500×g for 2 min. The digested tissue was washed twice, resuspended in 7-8 ml of fresh KRB-HEPES-BSA, and filtered on nylon mesh (54-GG-315 'Nybolt'- 200 μm and PES 74/42 'Polymon'- 75 μm). Pancreatic acini which passed through the fine filter were then centrifuged at 500×g for 2 min and resuspended in fresh KRB-HEPES-BSA. The suspension was gently placed over 40 ml of 3% Ficoll in a sterile 50 ml conical tube for 20 min to separate dense acini from individual acinar cells and cellular debris. The sediment was washed twice with fresh KRB-HEPES-BSA and resuspended in fresh KRB-HEPES-BSA. This was followed by a 30 min preincubation period, during which acini were gassed with 100% O_2 and shaken at approximately 80 cycles/min at 37°C.

PROTEIN SYNTHESIS

Acini were incubated with 10 μCi per ml of ^{3}H-phenylalanine (phe) for 0 or 15 min (a period of linear incorporation of labeled amino acid into total protein) in KRB-HEPES supplemented with amino acids, excluding any unlabeled phe (Korc, 1982; Hirschi, Vasiloudes and Brannon, 1994). Following the addition of cold phe (final concentration = 40 mM) to end the incorporation, cells and media were microfuged; and cellular pellets were washed twice with ice-cold PBS-STI and stored at -80°C until analyzed. Acini were homogenized in deionized water with a probe sonicator for 10 sec. Aliquots of the homogenates were analyzed for protein content and the incorporation of ^{3}H-phe. An aliquot of the homogenate was precipitated with ice-cold 10% trichloroacetic acid (TCA) to measure the incorporation of ^{3}H-phe into total protein. After incubation for 20 min on ice, samples were centrifuged at 4°C for 5 min at 2000×g. After discarding the supernatants, the pellets were washed with 10% TCA and dissolved overnight in 600 μl of 0.1 N NaOH at 4°C. Aliquots of the dissolved pellets were

neutralized with 0.1 N HCl and counted in ACS liquid scintillation cocktail. Incorporation into cellular protein was expressed as net disintegrations per minute (dpm)/mg protein.

Rates of synthesis of individual exocrine proteins were measured by measuring the incorporation of radiolabeled amino acids into individual proteins separated by two-dimensional electrophoresis (Scheele, 1975; Hirschi, 1990). Proteins were first separated by isoelectric focusing (IEF) in 4% polyacrylamide in the presence of 1.0% pH 4-6, 1.0% pH 5-8, 1.0% pH 9-11, 2.5% pH 3.5-10 Ampholines (LKB Instruments, Inc., Gaithersburg, MD), 9.0 M urea, 0.28 M sucrose and 0.2 mM PMSF. The resulting IEF tube gels were soaked for 5 min in 62.5 mM Tris buffer, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 0.01% bromophenol blue dye. Proteins were separated by molecular weight in a 10-20% polyacrylamide gradient gel containing 0.1% SDS in the second dimension. Resulting protein spots were stained with 0.06% Coomassie Blue dye (Figure 4-1), excised from gels with a scalpel and digested overnight in 1 ml 30% hydrogen peroxide at 60°C. The identity of individual proteins was determined by enzymatic activity, PI and MW (Hirschi, 1990). Radioactivity incorporated into individual protein spots was determined by counting digested second dimension gel spots in a scintillation counter (Tri-Carb 460 CD, Packard, Downers Grove, IL).

Relative synthesis of individual exocrine proteins was calculated as the ratio of the amount of radioactivity incorporated into one protein spot to the total amount of radioactivity incorporated into all protein spots. This analysis eliminated the complicating variable of differing intracellular or tRNA specific activities (SA) among the treatments, which we have shown previously varies with hormonal (Brannon, Hirschi and Korc, 1988) and antecedent

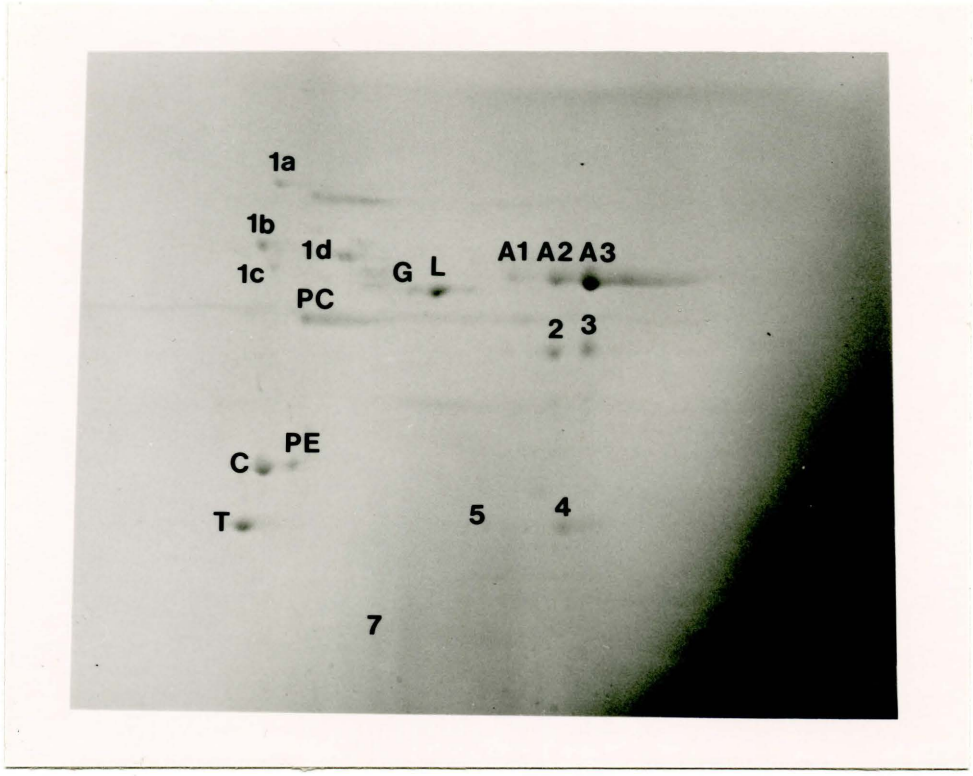

Figure 4-1. Separation of pancreatic acinar cell proteins by 2D gel electrophoresis. Acinar cells were isolated from HC-control rats and sonicated in deionized water. Soluble proteins were separated in the first dimension by IEF and the second dimension by SDS gel electrophoresis using a 10-20% acrylamide gradient. Protein spots were stained with Coomassie Blue dye and are identified by Hirschi (1990). The abbreviations are described as following: A1 - Amylase 1; A2 - Amylase 2; A3 - Amylase 3; C - Chymotrypsinogen; L - Lipase; PC - Procarboxypeptidase; PE - Proelastase; T - Trypsinogen; 1a-d, 2, 3, 4, 5, 6, 7 - Unidentified proteins.

dietary treatment (Brannon et al., 1986). Therefore, data were not analyzed as total incorporation, because intracellular SA were not determined.

RNA EXTRACTION AND HYBRIDIZATION

RNA was isolated as described previously (Tsai et al., 1994; Chapter 3). The integrity of RNA was checked by 1% agarose gel electrophoresis for the presence of intact 18S and 28S and by Northern hybridization. In addition to the described in Chapter 3 (rPL1, rPL3, pancreatic amylase and β-actin), a probe for 28S RNA, the generous gift of Dr. Diane Soprano, Temple University, was used as a control cDNA. Each probe resulted in a single, intact band by Northern hybridization (Tsai et al., 1994; Chapter 3). Two different rat pancreatic lipase (rPL) cDNAs were used in this study. These two rPL cDNAs are designated rPL-1 [isolated by Kern (Steinhilber et al., 1988) and sequenced by Wicker-Planquart and Puigserver (1992)] and rPL-3 [isolated and sequenced by Lowe and co-workers in an unpublished report to GenBank M58369 and confirmed by Wicker-Planquart and Puigserver (1992) and Wishart et al. (1993)], based on the nomenclature suggested by Wicker-Planquart and Puigserver (1992).

Specific mRNAs were quantitated by dot-blot hybridization as described previously (Tsai et al., 1994; Chapter 3). Amylase mRNA levels were linear in the range of 0.5 to 10 μg RNA ($r = 0.98$, data not shown). Both rPL-1 and rPL-3 mRNA levels were linear in the range of 0.1 to 2.0 μg RNA ($r = 0.96$, data not shown). As described in Chapter 3, rPL-1 and rPL-3 are highly specific and little cross hybridization of each probe under the highly stringent conditions used in this study is seen, which is to be expected given the low homology (65%) between rPL-1 and rPL-3.

DATA ANALYSIS

All data, expressed as mean ± SEM, were analyzed by one- or two-way analysis of variance (ANOVA), least significant differences (LSD, Steel and Torrie, 1960), normality Probability plot and Kruskal-Wallis test (StatGraphics Plus, version 6, Manugistics Inc., Rockville, MD). Pancreatic lipase relative synthesis data was not normally distributed, therefore, the non-parametric Kruskal-Wallis test was used to test for the independent and log-transformed data were also analyzed by one- or two-way ANOVA. Log-transformation resulted in a normal distribution of lipase relative synthesis, meeting the assumptions of the parametric ANOVA. Results were considered to be significantly different if $p < 0.05$.

Two way ANOVA was the primary analysis because of the 2×3 factorial design of experiments with main, independent, treatments of 1) *Diet* (HC or HF) and 2) *Insulin* status (Control, Db or Db+I). When ANOVA was significant, post-hoc tests were done by LSD to determine differences among the nine individual treatment groups. Results of the two way ANOVA for amylase and lipase gene expression are presented in Table 4-1 and 4-2.

RESULTS

PANCREATIC AMYLASE mRNA LEVEL, RELATIVE SYNTHESIS, AND ACTIVITY

Both diet and insulin independently regulated amylase gene expression. Diabetes, independent of diet, decreased amylase activity 80% (Table 4-1 and Figure 4-2A), relative synthesis 96% (Table 4-1 and Figure 4-2B) and mRNA levels 93% (Table 4-1 and Figure 4-2C). Amylase activity, relative synthesis and mRNA values were restored to respective dietary control values by insulin treatment. Diet independently affected amylase activity, relative synthesis and mRNA, which were greatest in HC-fed rats (Table 4-1 and Figure 4-2). Diet and insulin interacted ($p < 0.02$) in the regulation of pancreatic amylase activity, relative

Table 4-1

Two-way ANOVA for independent and interactive effects of diet and insulin on pancreatic amylase gene expression[1]

	Amylase		
	mRNA (Relative Units/μg RNA)	Relative Synthesis (%)	Activity (U/mg protein)
I. Insulin effect			
Control	651 ± 198 (6)	23 ± 4 (10)	125 ± 32 (10)
Db	49 ± 15 (6)	1 ± 1 (8)	26 ± 6 (12)
Db+I	1554 ± 470 (9)	21 ± 5 (9)	115 ± 31 (14)
	$p<0.0002$	$p<0.0001$	$p<0.001$
II. Diet effect			
HC	1479 ± 391 (11)	23 ± 6 (14)	144 ± 31 (16)
HF	202 ± 42 (10)	8 ± 2 (13)	44 ± 7 (20)
	$p<0.0001$	$p<0.0002$	$p<0.0001$
III. Interactive insulin × diet effect (see Figures 4-2)			
	$p<0.003$	$p<0.02$	$p<0.002$

[1]Values represent mean ± S.E. with sample size (n).

Table 4-2

Two-way ANOVA for independent and interactive effects of diet and insulin on pancreatic lipase gene expression[1]

	Lipase			
	rPL-1 mRNA (Relative Units/μg RNA)	rPL-3 mRNA (Relative Units/μg RNA)	Relative Synthesis (%)	Activity (U/mg protein)
I. Insulin effect				
Control	534 ± 63 (6)	3320 ± 534 (6)	14 ± 4 (10)	39 ± 5 (10)
Db	1137 ± 172 (6)	4607 ± 794 (6)	26 ± 10 (8)	43 ± 4 (11)
Db+I	665 ± 89 (9)	3457 ± 578 (9)	9 ± 2 (9)	27 ± 5 (14)
	$p<0.004$	$p<0.05$	n.s.	$p<0.04$
II. Diet effect				
HC	616 ± 105 (11)	2451 ± 240 (11)	15 ± 6 (14)	32 ± 5 (16)
HF	923 ± 109 (10)	5171 ± 372 (10)	17 ± 4 (13)	38 ± 4 (19)
	$p<0.02$	$p<0.0001$	n.s.	n.s.
III. Interactive insulin × diet effect (see Figures 4-4 and 4-5)				
	n.s.	n.s.	n.s.	$p<0.02$

[1]Values represent mean ± S.E. with sample size (n).

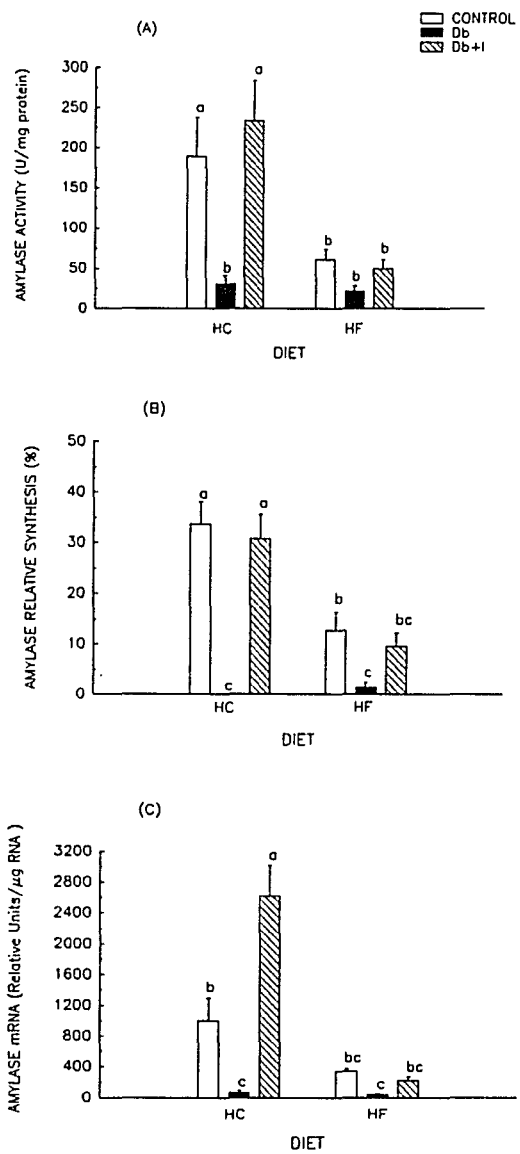

Figure 4-2. Interactive effects of diet, diabetes and insulin on amylase activity (A), relative synthesis (B), and mRNA (C). Values represent mean ± SEM from 5-9 rats (activity), 4-5 rats (relative synthesis) or 3-5 rats (mRNA). ^{a,b,c}Values for a given parameter not sharing a superscript differ significantly ($p<0.05$; two-way ANOVA). There were significant independent effects of Diet (HC > HF) and Insulin (Control = Db+I >> Db) for amylase activity ($p<0.0001$ for diet; $p<0.001$ for insulin), relative synthesis ($p<0.0002$ for diet; $p<0.0001$ for insulin), and mRNA ($p<0.0001$ for diet; $p<0.0002$ for insulin). They all have significantly interactive effect of Diet × Insulin ($p<0.002$ for activity; $p<0.02$ for relative synthesis; $p<0.003$ for mRNA) (see Table 4-1).

synthesis and mRNA levels. In contrast to the marked effects of diet and insulin on amylase mRNA, 28S RNA did not change significantly after either diet or insulin treatment (Figure 4-3A), but β-actin mRNA was affected by diet with increased levels (122%) in HF-fed rats compared with HC-fed rats (Figure 4-3B).

PANCREATIC LIPASE mRNA LEVEL, RELATIVE SYNTHESIS AND ACTIVITY

Diabetes, independent of diet, significantly increased both rPL-1 (200%) and rPL-3 (39%) mRNA (Table 4-2 and Figure 4-4A and B) and lipase activity (10%) (Table 4-2 and Figure 4-5A). There was a trend for increased in relative synthesis (86%) in diabetic rats, but it was not significant ($p=0.15$). Insulin-treatment of diabetic rats restored lipase mRNA levels and lipase activity to dietary control values (Figure 4-4 and 4-5).

Due to the small sample sizes relative to the large variance in relative synthesis data, the independent changes in lipase relative synthesis by diet or insulin or the interactive effect of diet and insulin on relative synthesis were not significant. Unexpectedly, the distribution of lipase relative synthesis was not normal, in contrast to all other data in this study. We, therefore analyzed lipase relative synthesis by non-parametric Kruskal-Wallis analysis (a non-parametric two-way ANOVA equivalent) and by log-transformed ANOVA (best for the Poisson distributed data). Differences were not significant by either alternate analysis. Future studies will need a large sample size to determine if the trends observed in this study are significant. Additional studies of relative synthesis are also needed that measure the specific activities of the immediate precursor pool of $tRNA_{phe}$ so that absolute amounts of lipase synthesized can be determined. Although relative synthesis obviates the effects of SA differences in comparing treatment. It is possible that the amount of lipase synthesized differs if total protein synthesized

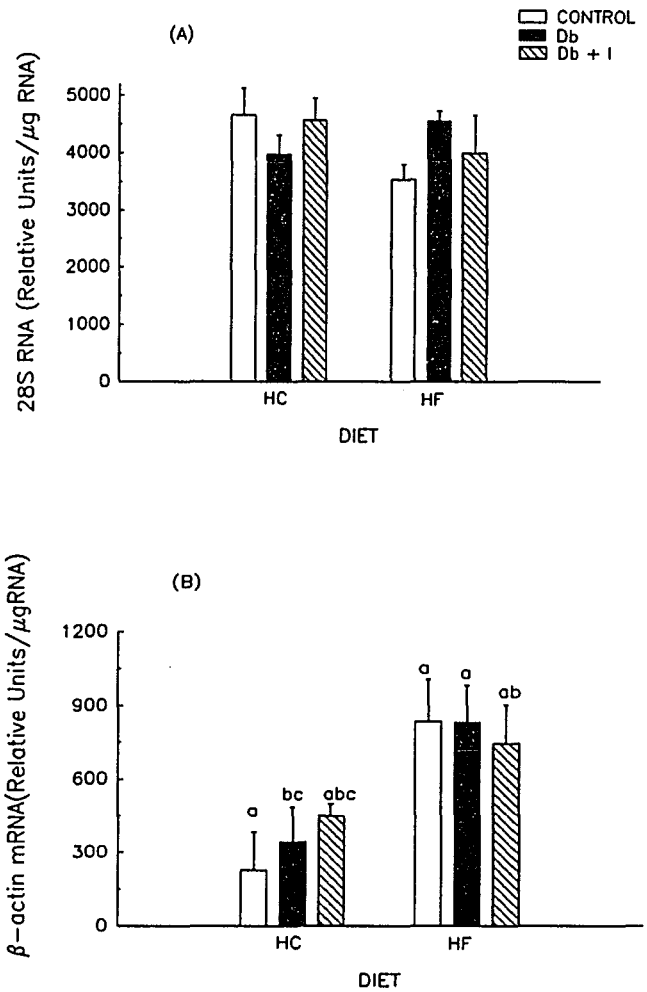

Figure 4-3. Effects of diet, diabetes and insulin on pancreatic 28S RNA (A) and β-actin (B) mRNA. Values represent mean ± SEM of triplicate samples from 3-5 rats. There was no independent effect of Diet or Insulin and no interactive effect of Diet × Insulin on pancreatic 28S mRNA. There was a significant effect of diet on β-actin mRNA level (HF > HC; $p < 0.002$).

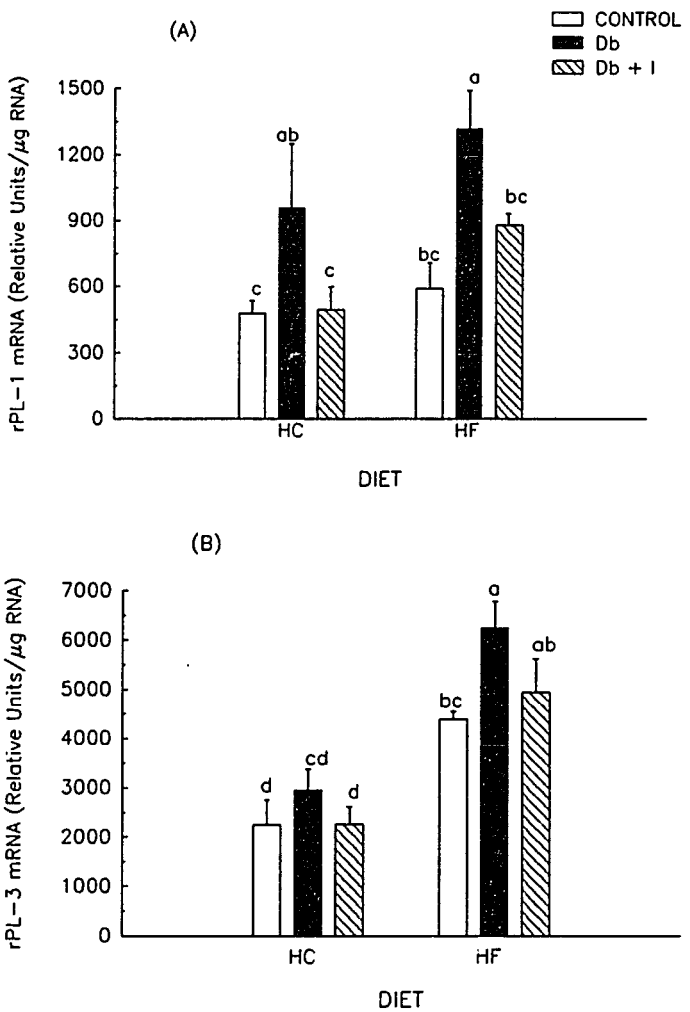

Figure 4-4. Interactive effects of diet, diabetes and insulin on lipase mRNA probed with rPL-1 cDNA (A) and rPL-3 cDNA (B). Values represent mean ± SEM from 3-5 rats (mRNA). [a-d]Values for a given parameter not sharing a superscript differ significantly ($p < 0.05$; two-way ANOVA). The lipase mRNA quantitation with rPL-1 cDNA or rPL-3 cDNA had similar results: a significant independent effect of Diet ($p < 0.02$ for rPL-1, HF > HC; $p < 0.0001$ for rPL-3, HF > HC) and Insulin ($p < 0.004$ for rPL-1 and $p < 0.05$ for rPL-3, Db > Control = Db+I). There was no interactive effect of Diet × Insulin for lipase mRNA. (see Table 4-2)

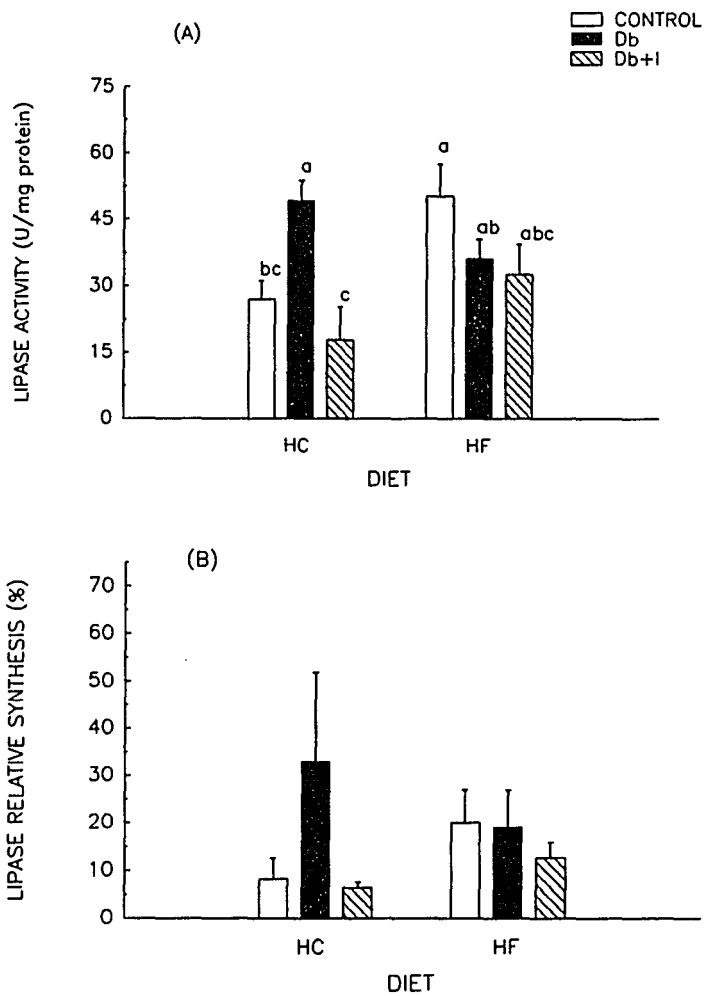

Figure 4-5. Interactive effects of diet, diabetes and insulin on lipase activity (U/mg protein) (A), and relative synthesis (%) (B). Values represent mean ± SEM from 5-9 rats (activity) or 4-5 rats (relative synthesis). [a,b,c]Values for a given parameter not sharing a superscript differ significantly ($p < 0.05$; two-way ANOVA). For lipase activity, there was no significant independent effect of Diet. However, insulin ($p < 0.04$, Db > Control = Db+I) had a significant independent effect on lipase activity and a significant interactive effect of Diet × Insulin ($p < 0.02$). The lipase relative synthesis did not change significantly after diet and insulin treatment. (see Table 4-2)

is affected. The study was not designed to determine absolute synthesis, but future studies should address this.

Diet, independent of diabetes, increased lipase mRNA levels, which were greatest in the HF-fed rats (Table 4-2 and Figure 4-4) for both rPL-1 (49%) and 3 (211%). Although diet was not significant as an independent factor on relative synthesis, control rats fed HF diet had a 21% significantly greater relative synthesis of lipase than did HC-control rats. Diet and insulin (diabetes) significantly interacted in the regulation of lipase activity; which was increased (85%) in HC-fed diabetic rats, and decreased (28%) in HF-fed diabetic rats. Although not significant, there tended to be an increase (86%) in the relative synthesis of pancreatic lipase in diabetic rats fed HC diet, but no change in its relative synthesis of diabetic rats fed HF diets.

DISCUSSION

Concerning the effects of diet and insulin, our results confirmed those of ours (Tsai et al., 1994; Chapter 3) and others previous studies (Wicker and Puigserver, 1987; 1990a,b; Wicker et al., 1988; Brannon, 1990) that 1) high carbohydrate diet maximally increases amylase mRNA, relative synthesis, and activity compared to a high fat diet; and 2) diabetes decreases amylase activity, mRNA and relative synthesis. The parallel changes in amylase mRNA, relative synthesis, and its activity, despite different diets fed and insulin status, strongly support a pretranslational mechanism of dietary regulation of pancreatic amylase gene expression, which is most likely transcriptional based on the transgenic studies by Meisler and co-workers (Schmid and Meisler, 1992).

Recently, Lowe and coworkers (Payne et al., 1994) resolved the controversy over the lipolytic activity of rPL-1 [reported by Kern and coworkers (Steinhilber, 1988) and sequenced by Wicker-Planquart and Puigserver (1992)]. The full-length rPL-1 is, indeed, a rat

pancreatic lipase related protein 1 (designated rPLRP-1 by Payne et al., 1994), which does not express colipase dependent lipolytic activity in the baculovirus system in *Sf*9 cells. This rPL-1 cDNA (or rPLRP-1 as designated by Lowe and coworkers) is the only probe that has been used in all studies of dietary and hormonal regulation of pancreatic lipase (Wicker and Puigserver, 1987; 1990a,b; Wicker et al., 1988; Duan et al., 1989a,b; Duan and Erlanson-Albertsson, 1990a,b) until our present studies. The effects of diet and hormones reported to date now require re-examination with rPL-3. We find that insulin and diet affect both rPL-3 and rPL-1 similarly, but the magnitude of response of rPL1 and rPL3 to diabetes and diet differed. The effects of diabetes were 5-fold greater for rPL-1 than rPL-3 and the effects of diet were 4-fold greater for rPL-3 than rPL-1. It remains, however, to be determined if ketones, secretin and GIP will regulate rPL-3 similarly to their effects on rPL-1.

In contrast to amylase, lipase activity was only affected by the independent effect insulin and the interaction of diet and insulin. There was no significantly independent effect of diet on lipase activity. However, there was a significantly increased lipase activity in HF-fed control rats compared with HC-fed control rats, which is consistent with our previous results (Tsai et al., 1994; Chapter 3) and other studies (Bazin and Lavau, 1979; Duan et al., 1989b). Further, neither lipase activity nor its relative synthesis parallelled either rPL-1 or rPL-3 mRNA levels. Lipase content and mRNA increased in parallel and lipase relative synthesis tended to parallel these increases in HC-fed diabetic rats. Both activity and mRNA levels were restored to normal values after insulin treatment of HC-fed diabetic rats, consistent with the results of our previously study (Tsai et al., 1994; Chapter 3) and other studies (Bazin and Lavau, 1979; Duan et al., 1989b). However, in HF-fed rats, diabetes decreased lipase activity by 28% and

did not affect its relative synthesis, but increased rPL-3 (42%) and rPL-1 (123%) mRNA levels. Insulin treatment of HF-fed diabetic rats restored activity and mRNA to lipase values.

Lipase mRNA levels, relative synthesis, and activity do not change in parallel with the interaction of diet and diabetes suggesting that complex mechanisms with both pre-translational and post-translational regulation may be involved in the regulation of pancreatic lipase gene expression by diet and diabetes interactions. In a pervious study dietary fat regulated pancreatic lipase at the transcriptional level (Wicker and Puigserver, 1987). Our results with control rats fed HC and HF diets support a pre-translational regulation.

In conclusion, the parallel changes of pancreatic amylase mRNA, relative synthesis, and amylase activity support a pretranslational regulation of amylase gene expression that is most probably transcriptional. The differential regulation of lipase activity, relative synthesis and mRNA levels with diet and diabetes raises the possibility that lipase gene expression is regulated by an interaction of diet, metabolic state and insulin through pre-translational and post-translational multiple regulatory mechanisms.

CHAPTER 5

EFFECTS OF DIET AND INSULIN ON ENERGY UTILIZATION

INTRODUCTION

It is well known that diet composition influences energy intake and energetic efficiency. Energetic efficiency is influenced by dietary fat concentration, with high fat diets being utilized more efficiently than low fat diets (Forbes et al., 1946; Carew and Hill, 1964; Schemmel, Mickelsen and Tolgay, 1969; Velu and Baker, 1974; Wood and Reid, 1975). Using dietary protein concentrations ranging from 8 to 60%, Hartsook and Hershberger (1963) demonstrated a curvilinear relationship between dietary protein concentration and energetic efficiency.

Maintaining an acceptable body weight may pose considerable difficulty in the management of patients with insulin dependent diabetes. Optimal glycemic control with insulin and restricted energy intake may achieve this primary goal. Leslie et al. (1986) found that precise glycemic control using continuous subcutaneous insulin infusion does not correct all the metabolic abnormalities of diabetes mellitus, and can produce weight gain if energy intake is unaltered. Diabetic patients do not compensate for decreased metabolic rate by increased noradrenergic and dietary thermic response (Leslie et al., 1986). In a study by Krishnamachar and Canolty (1986), diabetic rats had higher energy and protein intakes per unit of metabolic body weight than the control animals. Little work, however, has been done specifically concerning the relationships among dietary nutrient composition, energy utilization and efficiency, and glycemic control in diabetic rats.

The purpose of the present study was to determine the influence of varying carbohydrate, protein or fat in the diet on energy utilization and energetic efficiency of rats in the control rats (Control), diabetic rats (Db) or diabetic rats treated with insulin state (Db+I).

METHODS

EXPERIMENTAL PROTOCOL

Male Sprague-Dawley rats (230 g; Harlan, Indianapolis, IN) were housed individually in indirect calorimeter metabolic chambers in a temperature- and humidity-controlled environment with a 12-h light-dark cycle as described in Chapter 3. Rats were weight matched into three groups and fed ad libitum purified HC, HP or HF diet for 21 days (Control group). The composition of the diets (Werner et al., 1987) is shown in Table 3-1 (Chapter 3). The metabolizable energy (ME; ME = E intake - E fecal + E urinary) content was determined by bomb calorimetry and chromium oxide (Cr_2O_7) assay for control, Db, and Db + I (Atwater and Benedict, 1903) and is shown in Table 5-1. On day 7, diabetes was induced as described (Chapter 3; Tsai et al., 1994) and confirmed by plasma glucose > 300 mg/dl 6 days later following a 6-h fast. Diabetic animals were randomly subdivided into two groups and treated with saline (Db-group) or NPH insulin (Db+I-group) as described (Chapter 3; Tsai et al., 1994).

ENERGY METABOLISM

Twelve plexiglass chambers, each constructed to house an individual metabolism cage, were incorporated into a multi-chamber indirect calorimeter for the determination of energy utilization. Air flow (2.42 standard liters per minute) was regulated with air mass flow

Table 5-1

Effects of diet, diabetes and insulin on dietary metabolizable energy[1,2]

	HC	HP	HF
	kJ/g		
Control	16.3 ± 0.4[b]	18.2 ± 0.0[a]	14.9 ± 0.0[c]
Db	6.8 ± 0.9[f]	11.2 ± 0.0[d]	8.8 ± 0.0[e]
Db+I	15.6 ± 0.0[bc]	18.2 ± 0.0[a]	14.9 ± 0.0[c]

[1]Determined.

[2]Values represent mean ± SEM from 6-21 rats.

[a-f]Values not sharing a superscript differ significantly ($p < 0.05$; ANOVA).

transducers (Model FMA-507V, Omega Engineering Inc., Stamford, CT) in each chamber. Except during chamber air sampling, the system was open to room air and initial O_2 and CO_2 reading were recorded before each 30-min sampling cycle. Air from each chamber was sampled for 2 min during each sampling cycle and there were 2 sampling cycles per hour for 23 h each day. All rats were monitored daily, and data for energy utilization calculations were collected for at least 6 d at normal food intake and for 2 d at a modest food restriction (30%) at each treatment period (control, Db, or Db+I). Oxygen and CO_2 concentrations were measured at the exhaust of each chamber by an electrochemical O_2 sensor (Model N-22, Ametek-Thermox Instruments, Div., Pittsburgh, PA) and an infrared CO_2 sensor (Model 1-61, Ametek-Thermox Instruments, Div., Pittsburgh, PA), respectively, with sample flow (180 ml/min) controlled by a flow controller (Model R-1, Ametek-Thermox Instruments Div., Pittsburgh, PA). Respective signals from the sensors were processed by O_2 and CO_2 analyzers (Model S-3A1 and Model CD-3A, respectively, Ametek-Thermox Instruments Div., Pittsburgh, PA). Oxygen and CO_2 analyzers were calibrated with an O_2:CO_2 mixture of known concentration. Analog outputs from both analyzers and the flow meters were converted to digital by an ADALAB analog/digital board (Interactive Microware, Inc., State College, PA), and all data were processed by an IBM-compatible computer. Volume corrections for room humidity and barometric pressure to standard conditions were applied to intake flow rates (McArdle, Katch and Katch, 1986), and an air volume correction factor applied to all exhaust O_2 and CO_2 volumes.

Heat production was calculated from O_2 and CO_2 volumes according to the equation derived by Brouwer (1965), and carbohydrate and fat oxidation levels were calculated as described by Abbott et al. (1988). Calorimetry data for each rat, expressed as kJ per kg

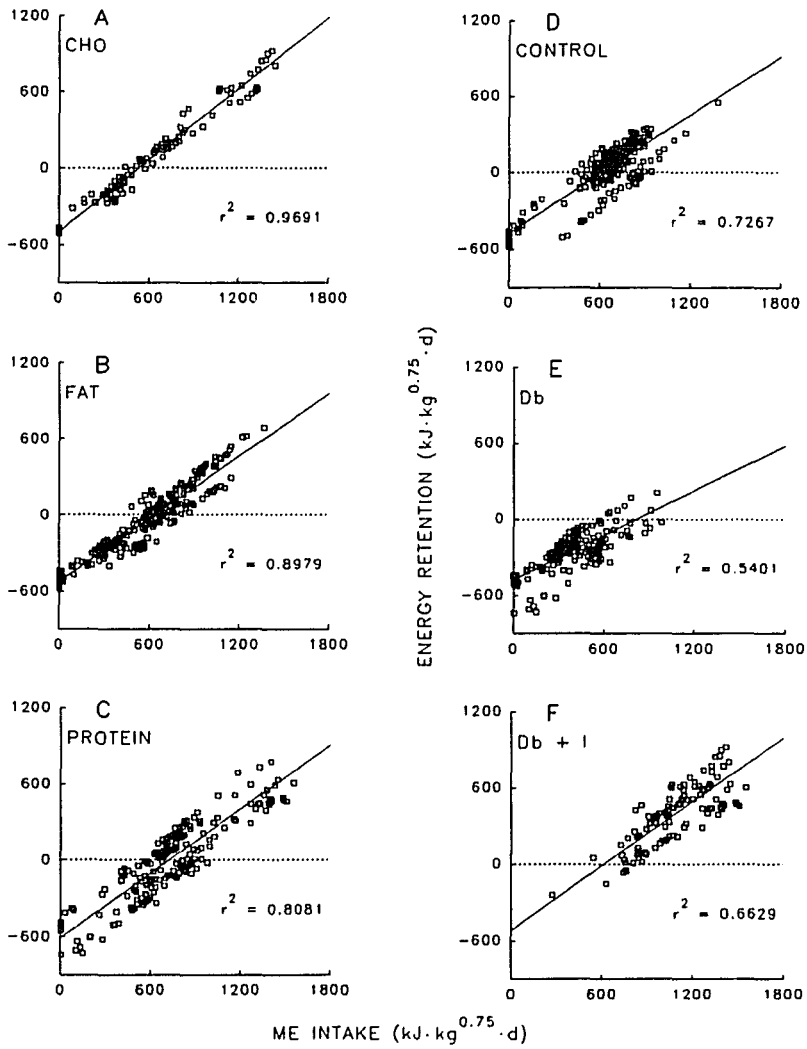

Figure 5-1. Energy retention (energy intake - heat production; kJ/kg$^{0.75}$/d) vs. metabolizable energy intake (ME; kJ/kg$^{0.75}$/d). These regression plots were used to calculate fasting heat production (FHP; Y-intercept), maintenance ME (X-intercept) and net energetic efficiency (NEE; slope). Each symbol in each panel represents data for an individual rat. The calculated regression line and equation shown in each panel represents the mean intercepts and mean slopes for each treatment [n = 8 (HC-control), 8 (HC-Db), 6 (HC-Db+I), 18 (HP-control), 15 (HP-Db), 7 (HP-Db+I), 21 (HF-control), 18 (HF-Db), and 9 (HF-DB+I)].

physiological body weight ($kg^{0.75}$), were averaged for each 23-h day and the daily averages subjected to linear regression analyses using energy retention (ER = energy intake - heat production) as the dependent variable and ME intake as the independent variable (Figure 5-1). The Y-intercept estimates fasting heat production (FHP) and the X-intercept estimates maintenance ME. The slope for the fitted regression above maintenance represents the net energetic efficiency. Slope and intercept comparisons were conducted at $p < 0.05$ as described by Zar (1974).

BODY COMPOSITION ANALYSIS

A total body electrical conductivity (TOBEC) instrument (Model 2A, EM-SCAN, Springfield, IL), interfaced with a computer, was used to determine the percentage of lean and fat (Bracco et al., 1983; Walsberg, 1988) at the end of each experimental period (control-, Db-, or Db+I-period). Total body electrical conductivity is dependent on the differences in conductivity between the fat and lean compartments of the body. The difference between conductivity measurements with the chamber empty and with an animal present is proportional to the lean body mass and body fat, depending on the prediction equations used.

In previous studies, the TOBEC prediction equations used have been validated from TOBEC readings and actual chemical assessments of body fat, water, and lean body mass from 92 carcass analyses (Galaviz-Moreno et al., 1992). Multiple regression analyses were used to derive the prediction equation for body water in $g = ax^2 + bx + cd + e$ (x = TOBEC readout; a = coefficient for x^2; b = coefficient for x; c = coefficient for body weight; d = body weight in g; e = intercept on y axis) which provided an $r = 0.993$. The lean body mass in g was calculated from total body water (g/0.732), and the difference between body weight

and lean body mass provided the fat mass. TOBEC measurements were obtained in triplicate on lightly ether-anesthetized rats, and body weights were recorded prior to killing.

DATA ANALYSIS

All data, expressed as mean ± SEM, were analyzed by one- or two-way analysis of variance (ANOVA) and by the post-hoc least significant difference (LSD, Steel and Torrie, 1960). Results were considered significantly different if $p < 0.05$. Two way ANOVA was the primary analysis because of the 3×3 factorial experimental design with main, independent, treatments of 1) *Diet* (HC, HP or HF) and 2) *Insulin* status (Control, Db or Db+I). This analysis enabled assessment of overall independent effects of diet or insulin and the interactive effects of diet × insulin, as well as differences among the nine individual treatment groups (Tables 5-2, 5-3 and 5-4).

RESULTS

Plasma glucose (Figure 3-3A) was significantly increased in Db rats, independent of diet, and returned to respective dietary control values after insulin treatment, except in the HF-Db+I group in which plasma glucose was within the normal range even through significantly greater than the HF-control values. Fasting (6 h) plasma insulin (Figure 3-3B) tended to decrease with diabetes. After administration of insulin, plasma insulin, independent of diet, increased significantly in Db+I groups compared to control and Db groups.

The daily food intake was similar among the HC, HP, and HF fed controls, but was increased (64-85%) in the HC- and HP-Db and Db+I and HF-Db+I groups (Figure 5-2A). Only the HF-Db rats had decreased consumption (18%; Figure 5-2A). Diet and insulin significantly interacted to affect food intake ($p < 0.00001$). Daily metabolizable energy (ME)

Table 5-2

Two-way ANOVA for independent and interactive effects of diet and insulin on food intake, ME intake, fasting heat production (FHP), maintenance energy, energy retention (ER), and net energetic efficiency (NEE)[1]

	Food Intake ($g/kg^{0.75}/d$)	ME Intake ($kJ/kg^{0.75}/d$)	FHP ($kJ/kg^{0.75}/d$)	Maintenance Energy ($kJ/kg^{0.75}/d$)	ER ($kJ/kg^{0.75}/d$)	NEE (%)
I. Insulin (Diabetes) effect						
Control	43 ± 2	695 ± 21	510 ± 21	594 ± 25	79 ± 25	86 ± 2
Db	56 ± 2	448 ± 21	519 ± 21	703 ± 25	-188 ± 25	77 ± 2
Db+I	72 ± 2	1167 ± 25	393 ± 25	519 ± 33	494 ± 29	76 ± 3
	$p<0.00001$	$p<0.00001$	$p<0.0003$	$p<0.005$	$p<0.00001$	$p<0.02$
II. Diet effect						
HC	66 ± 1	770 ± 29	418 ± 29	519 ± 38	213 ± 33	81 ± 3
HP	55 ± 2	874 ± 21	506 ± 21	661 ± 25	130 ± 25	77 ± 2
HF	50 ± 2	661 ± 21	502 ± 18	636 ± 25	42 ± 21	80 ± 2
	$p<0.00001$	$p<0.00001$	$p<0.03$	$p<0.0001$	$p<0.0001$	n.s.
III. Interactive insulin × diet effect (See Figures 5-2, 5-4, 5-5)						
	$p<0.00001$	$p<0.02$	$p<0.0007$	n.s.	$p<0.05$	n.s.

[1]Values represent mean ± SEM from 6-21 rats.

Table 5-3

Two-way ANOVA for independent and interactive effects of diet and insulin on respiratory quotient (RQ) and fat and carbohydrate (CHO) oxidation[1]

	RQ	Fat Oxidized (g/d)	CHO Oxidized (g/d)
I. Insulin (diabetes) effect			
Control	0.8 ± 0.0	4.0 ± 0.3	3.1 ± 0.8
Db	0.7 ± 0.0	5.0 ± 0.3	0.7 ± 0.9
Db+I	1.0 ± 0.0	-0.8 ± 0.4	15.3 ± 1.0
	$p<0.00001$	$p<0.00001$	$p<0.00001$
II. Diet effect			
HC	0.9 ± 0.0	1.5 ± 0.4	7.3 ± 1.1
HP	0.9 ± 0.0	2.0 ± 0.3	9.8 ± 0.8
HF	0.7 ± 0.0	4.6 ± 0.3	2.0 ± 0.7
	$p<0.00001$	$p<0.00001$	$p<0.00001$
III. Interactive insulin × diet effect (see Figure 5-3)			
	$p<0.00001$	$p<0.00001$	$p<0.006$

[1]Values represent mean ± SEM from 6-21 rats.

Table 5-4

Two-way ANOVA for independent and interactive effects of diet and insulin on body weight, fat mass and lean body mass changes[1]

	Body Weight (g)	Fat Mass (g)	Lean Body Mass (g)
I. Insulin (diabetes) effect			
Control	19 ± 3	9 ± 3	10 ± 5
Db	-44 ± 4	7 ± 4	-51 ± 5
Db+I	44 ± 4	-7 ± 4	50 ± 6
	$p < 0.00001$	$p < 0.02$	$p < 0.00001$
II. Diet effect			
HC	8 ± 3	2 ± 3	6 ± 5
HP	11 ± 4	0 ± 4	11 ± 8
HF	1 ± 4	7 ± 4	-7 ± 6
	n.s.	n.s.	n.s.
III. Interactive insulin × diet effect (see Figure 5-6)			
	$p < 0.003$	n.s.	$p < 0.01$

[1]Values represent mean ± SEM from 6-21 rats.

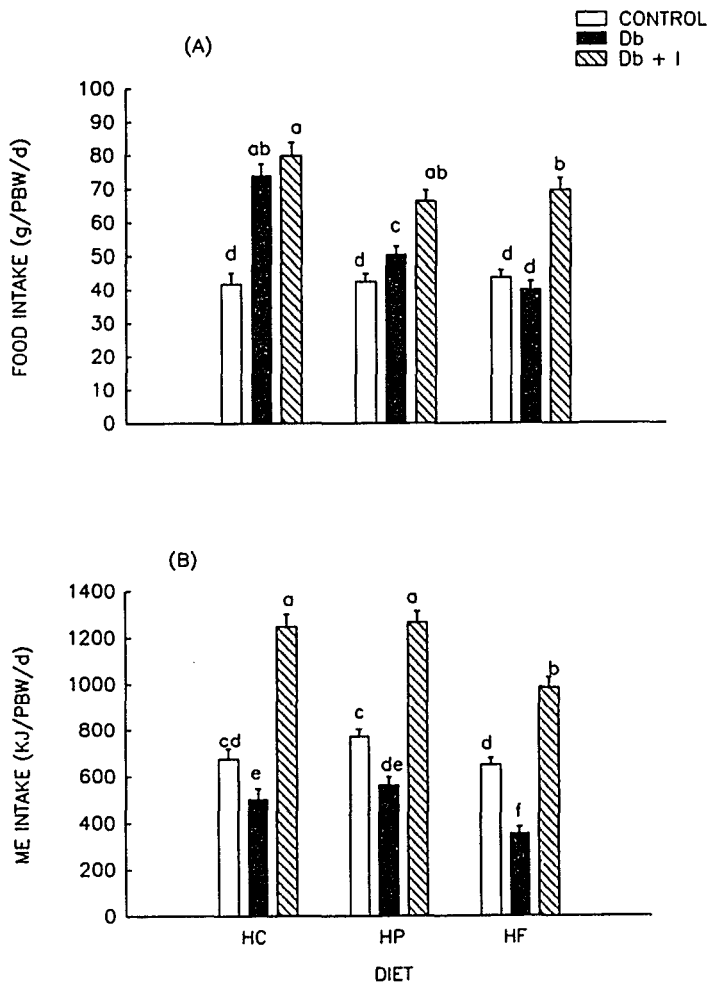

Figure 5-2. Effects of diet and insulin on food intake (A) and energy consumption (B). Values represent mean ± SEM from 6-21 rats. [a-f]Values for a given parameter not sharing a superscript differ significantly ($p < 0.05$; ANOVA). (See Table 5-2 for two-way ANOVA)

intake (Table 5-2) was calculated by the daily food intake multiplied by the determined dietary metabolizable energy (Table 5-1). Diet did not alter the daily ME consumption of control animals (Figure 5-2B). Diabetic rats had significantly lower ME intakes than control rats and insulin treatment increased ME intakes which were significantly greater than the control values (168%; Table 5-2). The HF-Db rats had the lowest ME intake compared to other treatment groups (only 55% of HF-control group; Figure 5-2B). There was a significant interactive effect of diet and insulin on ME intake ($p < 0.02$).

RQ values determined by indirect calorimetry reflected diet composition for control groups. HC-fed control animals had the highest RQ values (0.91), followed by animals fed the HP (0.88) and HF (0.75) diets (Figure 5-3A). Diabetes decreased the RQ in HC and HF fed groups, but not HP-fed groups (Figure 5-3A). Diabetes essentially stopped carbohydrate oxidation in HC- and HF- fed groups and caused net synthesis (indicated by negative values; Figure 5-3B). Fat utilization supplied the majority of the energy during this phase (Figure 5-3C). Insulin caused large increases in carbohydrate utilization in HC and HP fed groups (Figure 5-3B). In HP fed groups, neither carbohydrate nor fat oxidations was affected by diabetes. Diet, insulin and the interaction of diet and insulin significantly altered RQ, fat and carbohydrate oxidation (Table 5-3).

Diet and diabetes also had significant interactive effects on fasting heat production (FHP) ($p < 0.0007$; Table 5-2). HC-fed rats, independent of diabetes, had the lowest FHP compared to HP or HF-fed rats (Figure 5-4A). Independent of diet, control and diabetic rats had similar FHP, but insulin decreased the FHP to about 75% of control values (Table 5-2; Figure 5-4A). Diet, independent of diabetes, affected maintenance energy (HC < HP = HF;

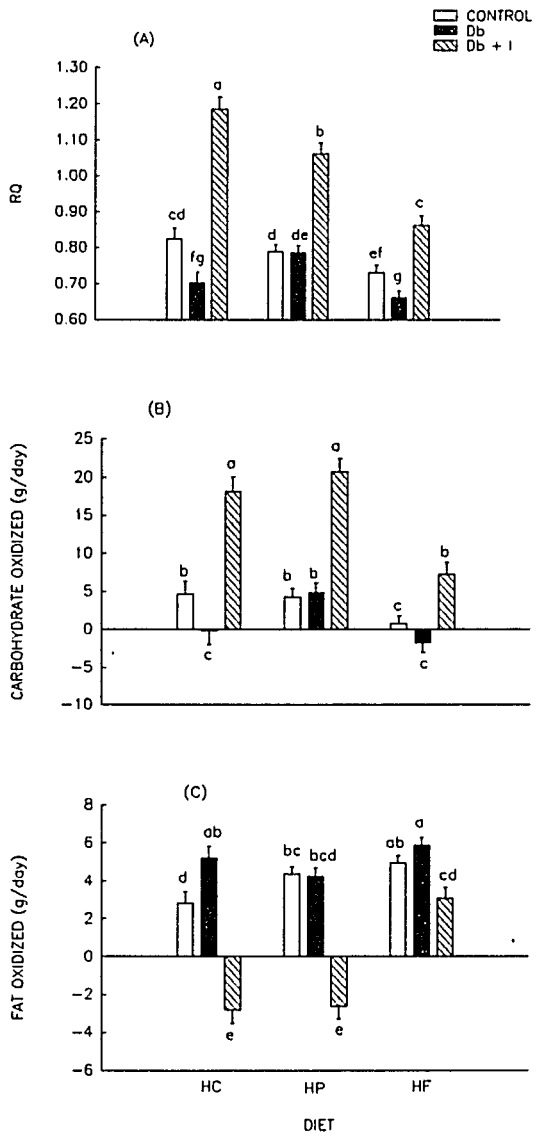

Figure 5-3. Effects of diet, diabetes and insulin on respiratory quotient (RQ) values (A) and carbohydrate (B) and fat (C) oxidation. Values represent mean ± SEM from 6-21 rats. [a-g]Values for a given parameter not sharing a superscript differ significantly ($p<0.05$; ANOVA). (See Table 5-3 for two-way ANOVA)

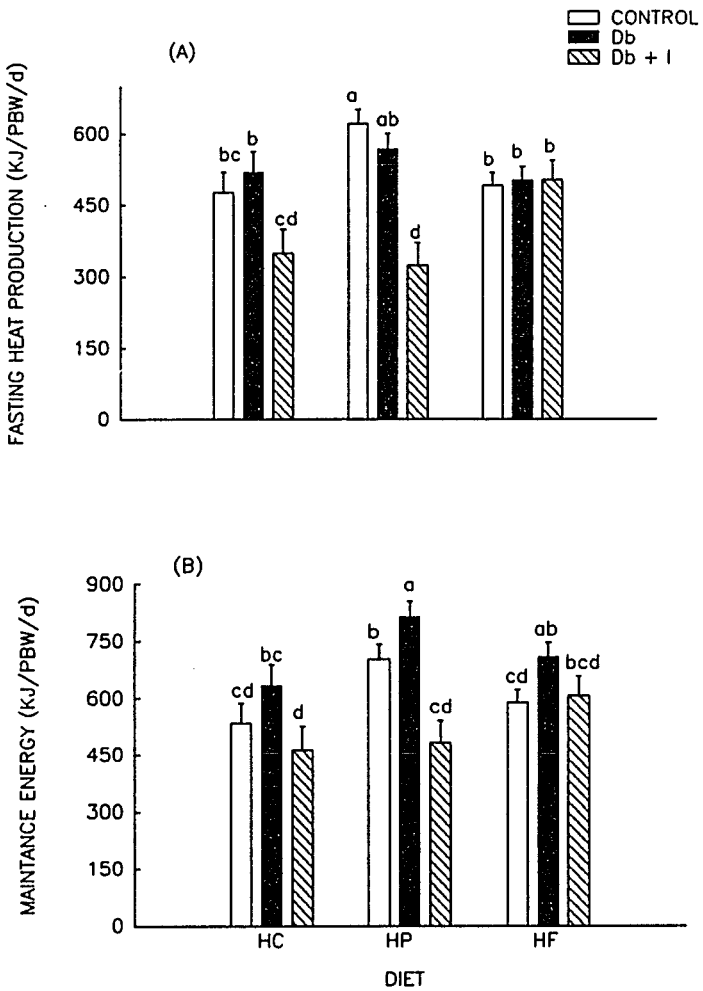

Figure 5-4. Effects of diet, diabetes and insulin on fasting heat production (A) and maintenance energy (B). Values represent mean ± SEM from 6-21 rats. [a-d]Values for a given parameter not sharing a superscript differ significantly ($p < 0.05$; ANOVA). (See Table 5-2 for two-way ANOVA)

$p<0.0001$; Table 5-2). Diabetes, independent of diet, also affected maintenance energy (control = Db+I < Db; $p<0.005$). There was no significant interactive effects of diet and insulin on maintenance energy.

Energy retention, calculated from indirect calorimetry data as the difference between energy intake and heat production, was similar for HC, HP, and HF control groups and significantly decreased and negative in diabetic groups. Insulin treatment significantly increased (6-fold as compared to control groups) the energy retention (Table 5-2; Figure 5-5A). Diet, independent of diabetes, also significantly affected energy retention (HC > HP > HF; $p<0.00001$; Figure 5-5A; Table 5-2). There was a significant interactive effect on energy retention ($p<0.05$; Table 5-2; Figure 5-5A). HF-diabetic rats had the lowest energy retention compared to other groups and insulin treatment of HF fed-diabetic rats did not maximize their energy retention (only 50% of HC-Db+I group; Figure 5-5A). Net energetic efficiencies for conversion of dietary ME to net energy (NE) were only significantly affected by diabetes (control > Db = Db+I; $p<0.02$; Figure 5-5B; Table 5-2). There were no significant effects of diet or interactive effect on net energetic efficiency (Table 5-2).

Diet, independent of diabetes, did not affect body weight change (Table 5-4; Figure 5-6A). Diabetes significantly decreased body weight, and insulin treatment restored the body weight to the control values (Figure 5-6A; Table 5-4). The majority of the body weight loss in diabetic rats was lean mass with little or no fat loss. Diabetes, but not diet, affected the lean body mass change (Db+I > control > Db; $p<0.0001$; Figure 5-6C; Table 5-4). There was a significant interactive effect on lean mass change by diet and diabetes ($p<0.01$) with the largest diabetic effect and the smallest insulin effect in HF-fed rats. Diabetes, independent of diet, affected fat mass (control > Db > Db+I; $p<0.02$; Figure 5-6B; Table 5-4). There was

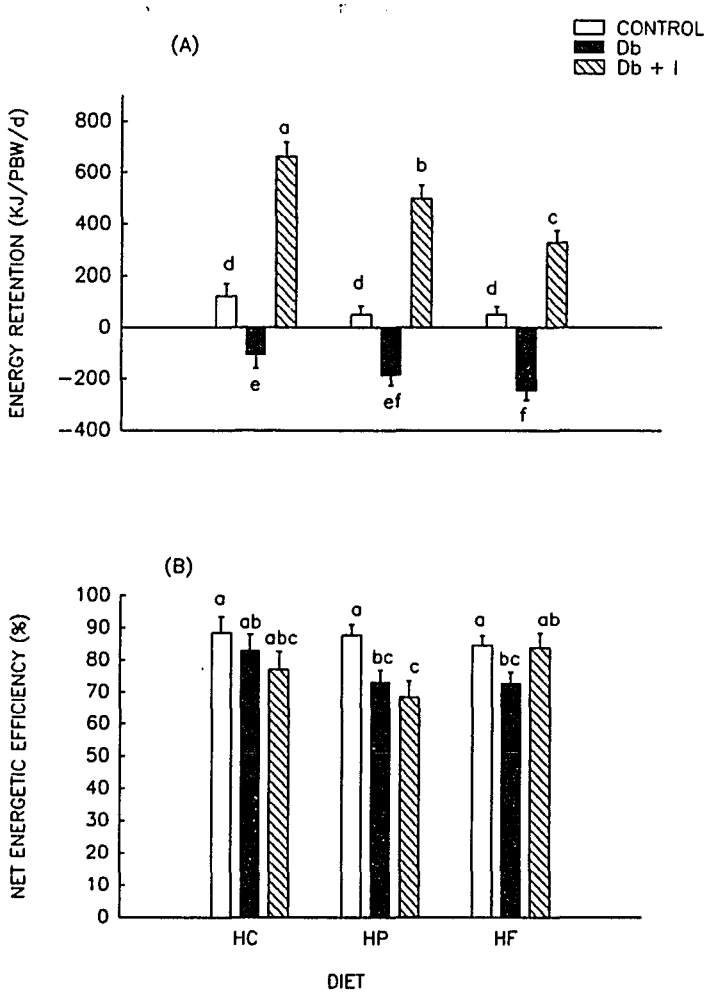

Figure 5-5. Effects of diet, diabetes and insulin on energy retention (A) and net energetic efficiency (B). Values represent mean ± SEM from 6-21 rats. [a-d]Values for a given parameter not sharing a superscript differ significantly ($p < 0.05$; ANOVA). (See Table 5-2 for two-way ANOVA)

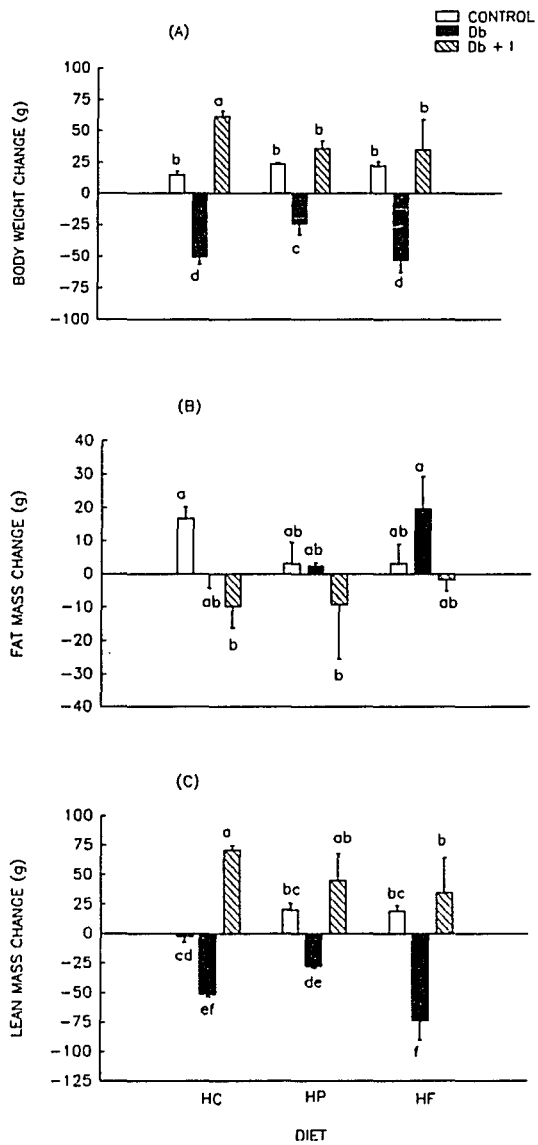

Figure 5-6. Effects of diet, diabetes and insulin on body weight (A), fat mass (B), and lean mass (C) changes. Values represent mean ± SEM from 6-21 rats. [a-f]Values for a given parameter not sharing a superscript differ significantly ($p < 0.05$; ANOVA). (See Table 5-3 for two-way ANOVA)

no significant effect of diet or interactive effect of diet and insulin on fat mass change (Table 5-4).

DISCUSSION

To determine the influence of diet and insulin on energy utilization and energy efficiency of rats, the in vivo STZ-induced diabetic rat model was used in this study, which is the first to examine the independent and interactive effects of diet and insulin administration in diabetic rats on energy utilization. Plasma glucose was elevated in diabetes and restored by insulin treatment; and plasma insulin levels were elevated in insulin-treated groups, which has not been carefully examine in some of earlier studies (Dudas, 1984; Krishnamachar and Canolty, 1986).

The increased food intake in diabetic rats was consistent with previous studies (Duffy, 1945; Kumaresan and Turner, 1965; Thomas, Scharrer and Mayer, 1976; Friedman, 1978). In the present study, diabetic rats ate approximately 31% more than control rats. A growing body of evidence suggests that insulin may be an important metabolic satiety signal, which may regulate eating behavior and energy balance. Rats with insulin-deficient diabetes, either induced by the β-cell toxin streptozotocin (STZ) or occurring spontaneously in the BB Wistar strain, display marked hyperphagia with a preference for carbohydrates (Koopmans and Pi-Sunyer, 1986; Williams et al., 1989a,b; McKibbin et al., 1992). Neuropeptide Y (NYP), a neurotransmitter related to pancreatic polypeptide, is of particular interest in this context because accumulating evidence suggests that it is involved in driving the hyperphagia of insulin-deficient diabetes and that NYPergic neurons in the hypothalamus are regulated by insulin (Schwartz et al., 1992a,b). Insulin-deficient diabetic rats show increased NPY and NPY mRNA levels, together with downregulation of NPY receptor numbers in the hypothalamus and

enhanced NPY release within the paraventricular nucleus (PVN) (Williams et al., 1988; 1989a,b; Sahu et al., 1990; 1992; White et al., 1990; McKibbin et al., 1992; Frankish et al., 1993). Our results support a possible role of insulin in regulating food intake, particularly intake of protein and carbohydrate diets.

Several studies showed that insulin replacement, which corrected hypoinsulinemia and hyperglycemia, could abolish hyperphagia and normalize NPY concentrations in all hypothalamic regions (McKibbin et al., 1992). However, this was not the case in our study. In the present study, the Db+I rats had increased food intake (about 68% more than normal rats) which was also greater than the Db group. The study by Corrin and coworkers (1991) measured NPY concentrations in individual hypothalamic regions in rats with hyperphagia caused by insulin-induced hypoglycemia. Wistar rats were injected with ultralente insulin (20-60 U/kg) to induce either acute or chronic hypoglycemia (Corrin et al., 1991). The plasma insulin and food intake were significantly increased with acute and chronic hypoglycemia, but NPY concentrations were not increased in any region in either acute or chronic hypoglycemia (Corrin et al., 1991). Compared to our results, the dosage of insulin (17.5-39 U/kg) was similar to Corrin's study. Our results showed a decreased plasma glucose in Db+I rats when compared with Db rats with a significantly increased plasma insulin level, support the hypothesis that acute and chronic insulin-treatment stimulates eating. NPY concentrations were not measured in the present study; therefore, it is not known whether NPY is the sole mediator of hyperphagia in the insulin-treated animals.

The theoretical role of that insulin stimulated NPY release in hyperphagia may not apply in diabetic rats fed the HF diet. In the present study, diabetic rats which were glycosuric, polydipsic, and underweight ate similar amounts of a HF diet as the control rats, which is

consistent with previous studies (Friedman and Ramirez, 1985; Koopmans and Pi-Sunyer, 1986). Low insulin levels may enhance the satiating effect of dietary fat, by increasing the capacity for fatty acid oxidation (Friedman, 1990). Insulin treatment of diabetic rats increased the intake of HF diet in this study as has been seen in a previous study (Koopmans and Pi-Sunyer, 1986). These results suggest that any role of insulin in the control of food intake may depend on the type of fuel ingested. Insulin may affect food intake (with regards to different components of the diet) via its metabolic actions rather than acting as a signal itself. The inhibition of fatty acid oxidation and the promotion of fat storage by insulin may reduce the satiating effect of dietary fat.

In the present study, the diabetic rats showed a distinct shift in energy substrate utilization from carbohydrate to fat, resulting in a decreased RQ value except for those fed HP diet. The diabetic rats not only utilized an increased daily amount of fat as substrate for energy metabolism, but their increased fat utilization coincided with a decrease in daily carbohydrate utilization. Insulin inhibits the oxidation of fatty acids, and promotes their esterification, uptake and storage in adipose tissue, as seen in the shifted energy substrate utilization from fat to carbohydrate in Db+I rats, which results in an increased RQ value. In diabetes, insulin-deficiency and excess glucagon and adrenal glucocorticoids accelerate gluconeogenesis, which increase protein catabolism to meet energy needs. The HP-diabetic rats, in the present study, showed an unchanged RQ value and smaller losses of lean body mass than those fed HC or HF diets. HP-fed diabetic rats may use dietary amino acids instead of muscle amino acids as the substrates of gluconeogenesis, thus sparing to a greater extent lean body mass.

We found a significant reduction in daily energy retention expressed percent of physiological body weight in diabetic rats. This finding together with the reduction in net

energetic efficiency may explain in part the depressed body weight gain observed in the diabetic rats. In addition, the mean value for energy retention was negative in the diabetic rats, suggesting that these animals were not able to sustain an adequate energy balance.

In conclusion, dietary composition interacted with insulin to affect energy utilization. In diabetes, energy utilization, retention and weight maintenance were best when rats were fed the HP diet. Insulin treatment restored carbohydrate utilization with the greatest effect in the HC-fed rats. These results suggest that the utilization of dietary energy is most affected by insulin for dietary carbohydrate and least affected for dietary protein.

CHAPTER 6

SUMMARY AND CONCLUSIONS

High carbohydrate diet maximally increased amylase mRNA, relative synthesis, and activity compared with a high fat or high protein diet. Diabetes decreased amylase activity, mRNA and relative synthesis; and insulin restored amylase gene expression, but only to respective dietary control values. The parallel changes in amylase mRNA, relative synthesis, and its activity, despite different diets (HC, HP, or HF) fed and insulin status (control, Db, or Db+I), strongly support a pre-translational mechanism of dietary regulation of pancreatic amylase gene expression that is most probably transcriptional. Insulin has been proposed to mediate this pancreatic adaptation to dietary carbohydrate, but the inability of insulin to stimulate maximal amylase in HP or HF- fed diabetes suggests that insulin is necessary, but not sufficient, for this dietary regulation. Insulin in conjunction with dietary glucose may mediate the adaptation of pancreatic amylase. Future studies are needed to determine the molecular mechanisms involving insulin and diet interactions through the IRE and DRU in the 5' region of this gene to regulate its transcription.

High fat diet significantly increased lipase mRNA and activity. Diabetes increased lipase activity and mRNA levels in HC-fed diabetic rats and, insulin treatment restored these to control values. In HP-fed rats, neither lipase activity nor mRNA levels were affected by either diabetes or insulin treatment. In HF-fed rats, diabetes decreased lipase activity by 49%, but increased mRNA by over 100%. Insulin treatment of HF-fed diabetic rats restored lipase activity and mRNA to control values. Although there were no significant effects of diet, insulin and their interactions on lipase relative synthesis there was a trend of increased lipase relative

synthesis in HC-diabetic rats compared with HC-control rats and in HF-fed rats compared with HC control rats.

The differential regulation of lipase activity and mRNA levels with diet and diabetes, coupled with the lack of change in lipase relative synthesis in HF-fed diabetic rats raises the possibility that lipase gene expression is regulated by an interaction of diet, metabolic state and insulin through pre-translational and post-translational regulatory mechanisms. Additional studies of relative synthesis are needed with a larger sample size in light of the large variability and non-normal distribution of lipase relative synthesis. Future studies also need to measure the specific activities of the immediate precursor pool of $tRNA_{phe}$, so that absolute amounts of lipase synthesized can be determined.

Diabetes decreased body weight. The majority of the weight loss was lean mass. Insulin treatment restored weight gain and lean mass. Diabetic animals were less able to obtain energy from diets, and the ME values were reduced. Diabetes decreased energy retention, and insulin treatment increased energy retention above control values. In diabetes, energy utilization, retention and weight maintenance were best when rats were fed HP diet. Insulin treatment restored carbohydrate utilization, with the greatest effect in the HC-fed rats.

In summary, diet and insulin regulate pancreatic amylase gene expression through a transcriptional regulatory mechanism. Diet and insulin interact to regulate pancreatic lipase through pre-translational and post-translational regulatory mechanisms. The utilization of dietary energy is most affected by insulin for dietary carbohydrate and least affected for dietary protein.

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