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Insulin is an essential hormone and is produced exclusively in endocrine pancreas β-cells for the control of glucose homeostasis in mammals. The hypothesis tested in this thesis is that increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) contributes to activation of glucose-induced insulin gene transcription. Glucose-induced insulin transcription has been mapped to binding of transcription factors by β-cell sequence motifs from -197 to -247, a glucose-response-enhancer (GRE), in the rat insulin1 gene (rIns1) promoter. Using oligonucleotide probes representing this glucose-response-enhancer (GRE) in electrophoretic mobility shift assays (EMSA), we have examined the Ca^{2+}-sensitivity of transcription factor binding to nuclear extracts from cultured rat insulinoma β-cells (INS-1).

In the presence or absence of kinase inhibitors, Ca^{2+} chelators, and Ca^{2+} channel blockers, binding was assayed for the following cell conditions: 1) *in situ* permeabilized cells exposed to Ca^{2+}; 2) *in vitro* ^{32}P-phosphorylated nuclear extracts; and 3) *in situ* glucose-stimulated and K^+-depolarized intact cells. Binding was Ca^{2+}-sensitive due to activation by K^+ depolarization as well as inhibition by a Ca^{2+}-chelator, a Ca^{2+}-channel blocker, and KN-93, specific for Ca^{2+}/calmodulin kinases, suggesting a phosphorylation-dependent

mechanism. Taken together, these findings identify a role for the Ca^{2+} second messenger in the glucose regulation of the insulin gene which points to novel treatments for type II diabetes.

CALCIUM SENSITIVITY OF β-CELL
TRANCRIPTION FACTOR BINDING TO
TO AN INSULIN ENHANCER

Gary Frank Scott, B.A.

APPROVED:

Major Professor

Committee Member

Committee Member

Chair, Department of Molecular Biology and Immunology

Dean, Graduate School of Biomedical Sciences

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THESIS

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Gary Frank Scott, B.A.

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bHLH	basic helix-loop-helix transcription factor
bZIP	basic leucine zipper transcription factor
BSA	bovine serum albumin
Ca^{2+}	calcium divalent cation
$[Ca^{2+}]i$	intracellular calcium concentration
cAMP	3',5'-cyclic adenosine 5'-monophsphate
CaMK	calcium/calmodulin-dependent protein kinase
CAT	chloramphenicol acetyltransferase
C/EBPβ	CCAAT/Enhancer-binding protein β
CREB	cAMP-response-element protein
Ci	curies
CMV	cytomegalovirus
CPM	counts per minute
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene gycol-bis(β-aminoethyl ether)N,N,N',N'-tetracetic acid
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum

GLUT2	glucose transporter protein
GRE	glucose-response-enhancer (A3E2 motif, rIns1 -197 to -247)
hIns	human insulin gene
IDDM	insulin-dependent diabetes mellitus - type 1
IDX-1	Islet/Duodenum homeoboX-1 transcription factor, also called IUF-1, PDX-1, STF-1
INS-1	rat insulinoma cultured cell line
KRB	Krebs-Ringer Bicarbonate buffer
mRNA	messenger ribonucleic acid
NIDDM	non-insulin dependent diabetes mellitus - type 2
PFK-1	phosphofructokinase-1
PP	pancreatic polypeptide
PREMSA	phosphoprotein reverse electrophoretic mobility shift assay
poly-dIdC	poly-deoxyinosine-deoxycytosin
[γ^{32}P]-ATP	adenosine 5'-[gamma-^{32}P] triphosphate
RER	rough endoplasmic reticulum
rIns	rat insulin gene
S. aureus	*Staphylococcus aureus* gram-positive bacterial strain
VDCC	voltage-gated, or voltage-dependent, calcium channels

INTRODUCTION

The hypothesis tested by experiments in this thesis is that increased intracellular calcium ($[Ca^{2+}]_i$) contributes a fundamentally important mechanism to glucose-induced insulin gene transcription. This will be evaluated by directly examining the Ca^{2+}-sensitivity of β-cell transcription factor binding to insulin enhancer sequences previously shown to respond to glucose metabolism in reporter constructs.

The insulin gene is expressed exclusively in β-cells of the endocrine pancreas to produce preproinsulin, precursor to mature insulin hormone. The insulin hormone is conserved in mammals and most vertebrates, normally functioning to control circulating glucose concentrations within physiological ranges by regular insulin release upon demand. As soon as a raised glucose metabolic signal is detected, β-cells rapidly secrete insulin pre-stored in secretory granules by exocytosis into islet capillaries (1) for distribution to peripheral tissues. While new insulin synthesis is not required for initial secretion, glucose can stimulate *de novo* biosynthesis (2). Although glucose is considered the main regulator of insulin biosynthesis and secretion, the molecular mechanism by which β-cell sensing of elevated blood glucose induces transcription of its insulin gene is unresolved. This study will examine the intracellular

communication of a glucose-derived signal, i.e. raised intracellular calcium (Figure1), by assessing β-cell nuclear transcription factor protein binding to highly conserved DNA base sequence motifs in enhancer regions of the insulin gene promoter .

β-cell Function and Insulin

Discovered by Banting and Best in 1921, insulin plays a vital regulation role in vertebrate energy metabolism (4). In response to elevations in blood glucose concentrations, insulin is released exclusively by the β-cells of the endocrine pancreatic islets of Langerhans into the bloodstream, through which it travels to its sensitive target tissues - primarily fat, muscle, and liver. Interacting with these tissues, insulin stimulates the influx of nutrients, principally glucose, and blocks the release of energy from storage molecules, e.g. glycogen (5). Thus, insulin promotes glycogen storage and glycolysis, and curtails gluconeogenesis and glycogenolysis in the liver. In cardiac and striated muscle, protein and glycogen synthesis are induced by insulin, and in adipose (fat) cells, lipid storage is favored.

Insulin is manufactured and secreted by the β-cells which form the core of the islets of Langerhans constituting the endocrine pancreas. Although insulin mRNA has been detected in rat brain neurons (6), the pancreatic β-cells are the only cells in the body known to express the insulin gene.

Comprising 85 % of islet cells, the highly vascularized β-cell core is surrounded by lower numbers of α, δ, and PP endocrine cells,

which secrete glucagon, somatostatin, and pancreatic polypeptide, respectively, into circulating blood. Glucagon opposes the action of insulin in peripheral tissues by releasing glucose stores and raising blood sugar, while somatostatin may have paracrine effects to inhibit the secretion of both insulin and glucagon within the endocrine pancreas. The function of islet PP is as yet unknown. Together, these islet hormones regulate glucose homeostasis throughout the mammalian body within normal physiological limits, approximately 4-7 mM in humans..

Insulin and Diabetes

When blood glucose surges above the homeostatic range after feeding, the normal β-cell has evolved to exercise exquisitely tight control of an animal's energy metabolism. The normal response to periodic increased fuel ingestion requires insulin's dispersion from pancreatic islets through the bloodstream for use by peripheral cells and tissues to help absorb glucose and other catabolic products. Insufficient insulin, either due to lack of supply or ineffective cellular uptake, allows development of the hyperglycemic condition known as Diabetes Mellitus, or "sweet urine" literally from the Latin root, in which glucose carbohydrates accumulate in the bloodstream with potentially destructive consequences.

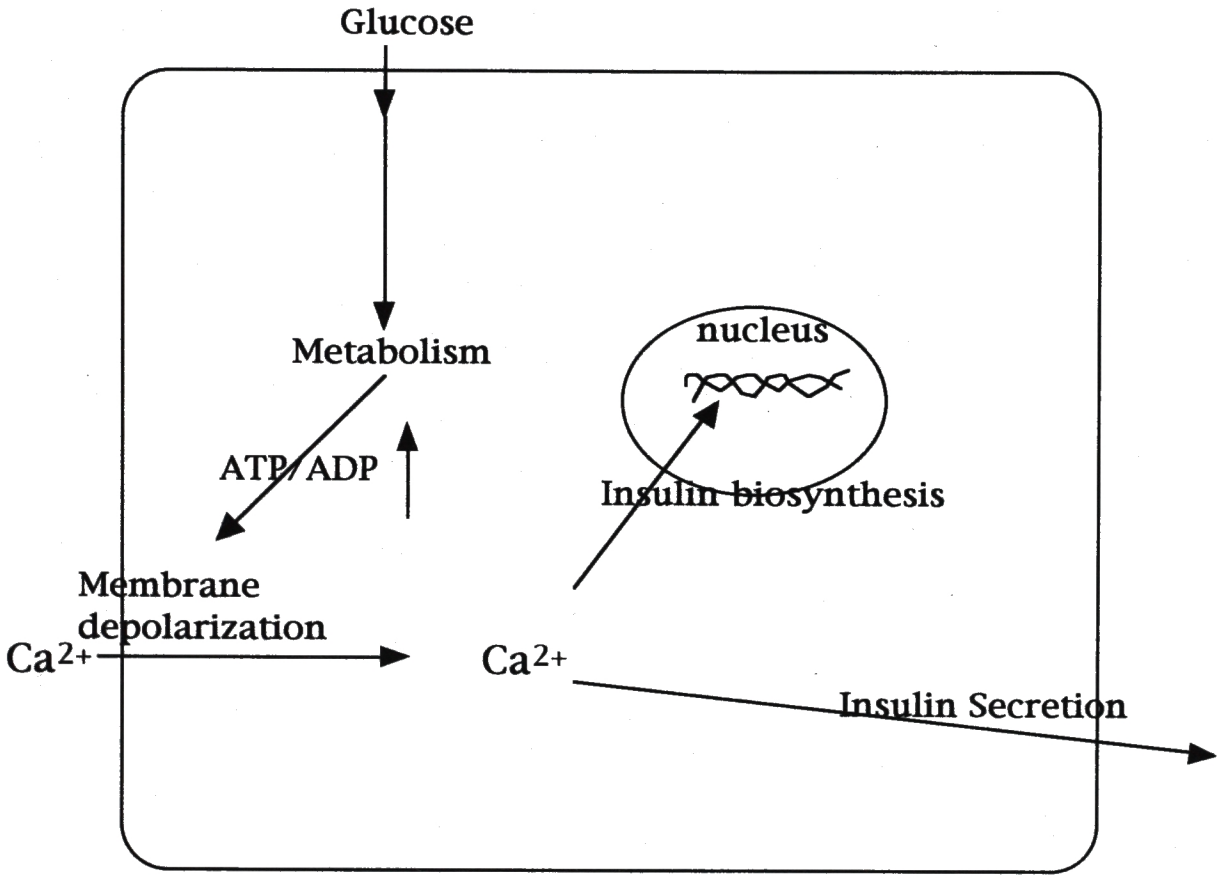
Although diabetes manifests as two distinct syndromes, types I and II, the common feature of both forms of hyperglycemia is impaired β-cell function(3). In contrast to autoimmune β-cell destruction in type I diabetes (IDDM), the pathology of β-cell

dysfunction in type II diabetes (NIDDM), is characterized by insulin resistance and impaired insulin secretion (7). Analysis of NIDDM experimental animals has shown extreme decomposition of insulin secretory granules that occurs concurrently with onset of overt diabetes strongly suggesting the significance of impairment of insulin synthesis as a symptom of disturbed β-cell function (3). Apparently, failure of the β-cell to express the insulin gene upon glucose stimulation will cause diabetes once insulin pre-stored in secretory granules is released. Even with insulin furnished by translation of already transcribed insulin mRNA, fresh preproinsulin mRNA is needed to replenish mature insulin in storage compartments by *de novo* biosynthesis. This is required to ensure adequate supplies to control glucose homeostasis when elevated blood glucose is prolonged. To understand the molecular mechanism for this loss of glucose-induced insulin synthesis, the components of the β-cells' unique signal transduction system that leads from glucose sensing and uptake to activation of insulin gene expression must be explored.

The central role that intracellular calcium cation fluctuations play in the β-cell signal pathway instigated by glucose metabolism (Fig.1) implies an integral function for Ca^{2+}-mediated events in both insulin secretion and biosynthesis. The Ca^{2+}-sensitivity of gene expression regulatory mechanisms will be evaluated and characterized in this study to provide better understanding of β-cell function and insight into its deterioration.

Diabetes mellitus is a heterogeneous endocrine disorder characterized by high levels of circulating glucose (hyperglycemia)

FIG. 1. Simplified Model of Endocrine Pancreas β-cell Signal Transduction Pathway



Proposed Model of Endocrine
Pancreas β-cell Metabolism

resulting from either loss of insulin production {Type I diabetes, or insulin-dependent diabetes mellitus (IDDM)} or insufficient compensation (i.e. increased insulin secretion and biosynthesis) for a reduced sensitivity to insulin's action, {Type II diabetes, or non-insulin-dependent diabetes mellitus (NIDDM)}. Type I diabetes (IDDM) results from autoimmune destruction of β-cells due to an immune system T-cell activation of unknown causes. Type I (IDDM) diabetic β-cell decimation removes adequate insulin biosynthetic capability for secretory homeostasis demands, and requires treatment with insulin supplements. NIDDM is a more prevalent and heterogeneous metabolic disease than IDDM (approximately 5% vs. <1% of the U.S. population) that is caused by defects in insulin action and endocrine β-cell function. Treatments for NIDDM include drugs that stimulate β-cell activity (i.e. sulfonylureas, by raising intracellular calcium ($[Ca^{2+}]_i$) , in use for over 30 years (8)) or enhance insulin action (e.g. thiazolidinediones, by raising insulin receptor sensitivity and thus reciprocally lowering insulin resistance (9), in clinical use since 1997).

In both types of diabetes, there is inadequate insulin secretion to maintain glucose homeostasis, possibly related to the breakdown of normal insulin biosynthesis. In progressive type II (NIDDM) pathology, increases in secretory demand exceed stored secretory granule supply limits, ultimately exhausting the β-cell's synthetic capacity. In animal studies, prediabetic Zucker homozygous *fa/fa* leptin-deficient infant rats exhibited obesity and insulin resistance for 8 to 10 weeks before suffering overt diabtes. During the

progression from insulin resistance to diabetes, insulin secretion increased significantly, islet cell mass was enlarged, and expression of many islet genes was profoundly altered well before hyperglycemia developed (10). These altered patterns of gene expression prior to the appearance of overt diabetes signify a major compensating cellular reaction at the gene transcription level to insulin resistance (i.e. loss of insulin action) at the same time secretion accelerates and β-cell function deteriorates. Aside from important differences between IDDM and NIDDM, the long term prognosis of both diseases of chronic hyperglycemia are connected with many debilitating conditions including neuropathy, retinopathy, nephropathy, and cardiovascular diseases of the nervous system, eyes, kidneys, and heart/circulation, respectively (11,12).

While insulin release responds to a number of metabolic, hormonal and neural influences, the major physiological regulator is D-glucose, although other nutrient secretagogues are leucine, mannose, and ketone bodies (13). Regardless of agonists (or ligands), hyperglycemic loss of homeostatic control of glucose metabolic fluctuations following dietary intake centers on aberrations in β-cell function, including biosynthesis as well as secretion dysfunction. The biosynthesis of insulin that proceeds from preproinsulin mRNA in the nucleus, translation in the rough endoplasmic reticulum (RER), cleavage to proinsulin folded around disulfide links, and proteolytic processing into mature crystals for storage in secretory granules is intricately well-integrated in both molecular and cellular biology (14). Such fine-tuned co-ordination argues for a common signal messenger system such as the one orchestrated by Ca^{2+} and its binding

calmodulin in muliple conformations, capable, in turn, of regulating myriad substrates through numerous kinase isozyme permutations. Pancreatic islets express numerous kinases that require Ca^{2+} and calmodulin and that appear to be activated in response to glucose (22). Since insulin secretion is a well-documented Ca^{2+}-dependent event, it is logically plausible that mediation of insulin gene expression is Ca^{2+}-sensitive as well.

β-cell function and Ca^{2+}

A fundamental response of the β-cell to D-glucose is increased intracellular calcium ($[Ca^{2+}]_i$) (15,16), primarily through plasma membrane channels (17, 18). Increased $[Ca^{2+}]_i$ may also be supplemented from intracellular stores due to parasympathetic nervous system postprandial release of acetylcholine. By activating muscarinic recepters in the β-cell, phospholipase C is stimulated which hydrolyzes phosphatidylinositol 4,5-bisphosphate. This generates $Ins(1,4,5)P_3$, which mobilizes Ca^{2+} from intracellular stores in the ER (endoplasmic reticulium)(30). Although prior investigations have implicated a role for Ca^{2+} in glucose-induced insulin biosynthesis (19,20), the molecular mechanism is unknown. One clue to a Ca^{2+} role in insulin biosynthesis is implied by the ability of sulfonylurea drugs (e.g. tolbutamide, glibenclamide) to stimulate increased insulin secretion by raising $[Ca^{2+}]_i$ in the treatment of type II diabetes (NIDDM) (8). These drugs bind sulfonylurea receptors,

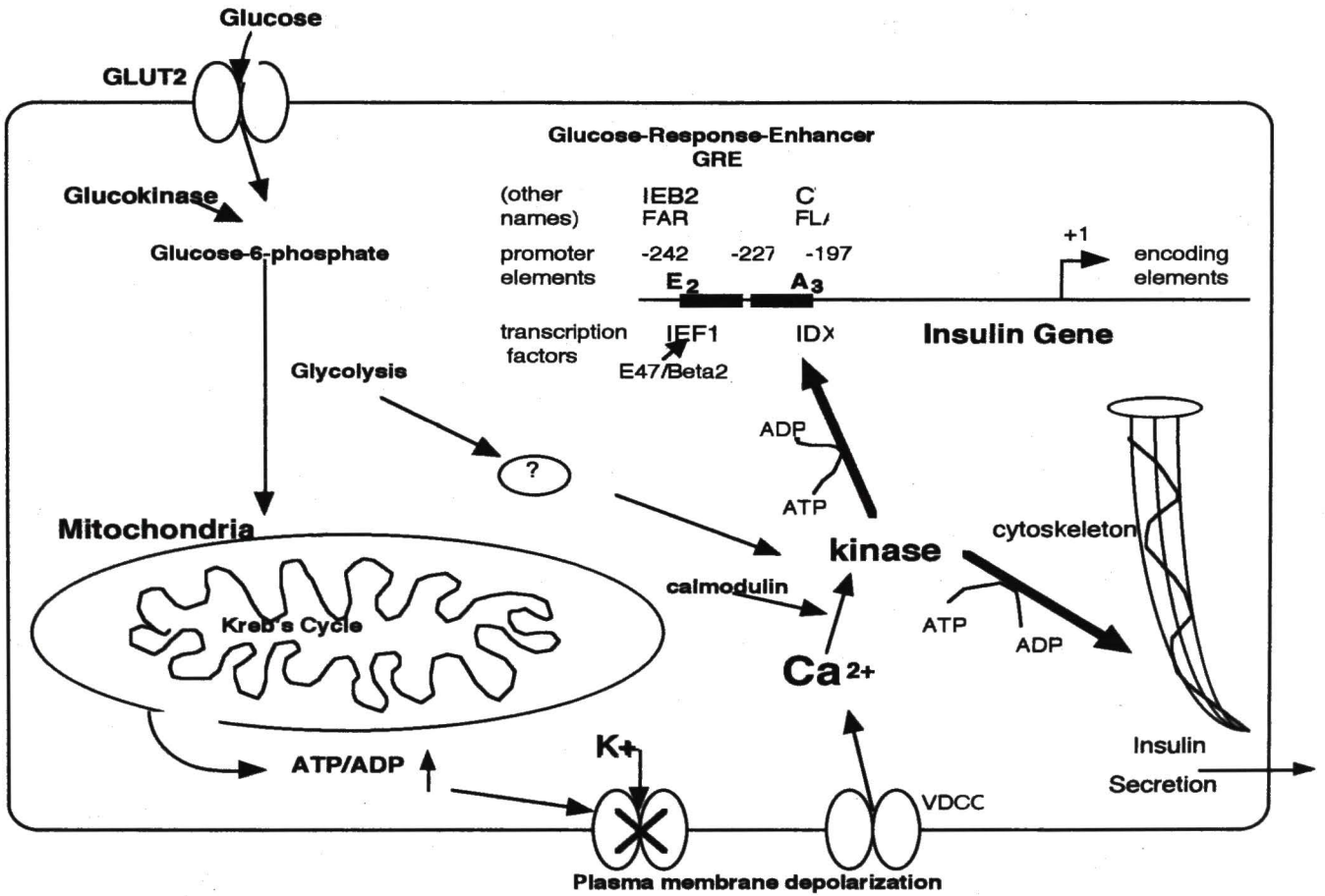
close K^+_{ATP} channels, depolarize the plasma membrane to activate voltage-gated Ca^{2+} channels and rapid Ca^{2+} influx, in a signal transduction pattern that parallels that produced by glucose metabolism in the normal β-cell (Fig. 2). As mentioned above, release of prestored insulin would create an immediate need for additional insulin synthesis to replenish storage compartments before supplies vanish. Since supplies are indeed renewed (insulin shortages eventually can occur after prolonged treatment with secretion-stimulating drugs due to hyperglycemic toxicity and β-cell exhaustion), the raised $[Ca^{2+}]_i$ signal, or some other unknown effect of sulfonylurea drugs, also stimulates insulin biosynthesis by some unidentified mechanism. Given this strong correlation, it became relevant to explore the possible molecular mechanism by which insulin synthesis and transcriptional regulation is sensitive to the $[Ca^{2+}]_i$ signal.

In order to explain the rationale upon which the experiments of this thesis research were designed to test this hypothesis, further details of β-cell glucose metabolism and transcriptional regulation will be reviewed.

Glucose metabolism and $[Ca^{2+}]_i$ signal in β-cell

Figure 2 illustrates the currently accepted β-cell signal transduction pathway in which glucose stimulation of insulin secretion requires glucose metabolism leading to a rise in $[Ca^{2+}]_i$ (21

FIG. 2. Model of Endocrine Pancreas β-cell Signal Transduciton Pathway Linking Ca^{2+} to Insulin Gene Transcription



β-Cell of Endocrine Pancreas

- 24). This diagram is modified to contain a proposed link from $[Ca^{2+}]_i$ to transciptional regulation through the insulin promoter. As has been well established for insulin secretion, glucose also regulates insulin synthesis (20,25,27,28,29). Given that Ca^{2+} signals arising from glucose metabolism mediate insulin secretion, possibly through Ca^{2+}-sensitive kinases (30-32), glucose-induced insulin synthesis likely also exhibits Ca^{2+}-sensitivity.

Glucose initiates insulin secretion by mechanisms that are principally dependent on a rise in intracellular calcium ($[Ca^{2+}]_i$) due to calcium influx across the plasma membrane (Fig.1 & 2), more than from mobilization from intracellular stores (33). This calcium influx follows from a series of steps in glucose metabolism culminating in plasma membrane depolarization which opens voltage-dependent Ca^{2+} ion channels (VDCC). In brief, β-cell glucose uptake and entry is facilitated by GLUT2, a high Km glucose transporter also present in hepatocytes (34,35). Once inside the cell, glucose is irreversibly phosphorylated by β-cell specific glucokinase (βGK - a putative high Km glucose sensor) to glucose-6-phosphate and processed by PFK-1 through glycolysis to pyruvate. Pyruvate converted to acetyl-CoA enters mitochondria and then after passing through the Kreb's, or citric acid, cycle, subsequent O_2 respiration produces substantial ATP. The resultant increased cytoplasmic ATP/ADP ratio triggers closure of ATP-sensitive potassium channels (K^+_{ATP} channels) in the plasma membrane leading to cell depolarization (from resting potential of approximately -70 mV), immediate opening of L-type voltage-

dependent calcium channels (VDCC) and rapid Ca^{2+} influx. (18)

Ca^{2+}-sensitive points of regulation in β-cell function

In evaluating a Ca^{2+} role in the regulation of insulin transcription, several steps in the β-cell signal tranduction pathways which may be influenced by the $[Ca^{2+}]_i$ signal should be considered (Figure 2). Pyruvate dehydrogenases in the mitochondrial matrix, required for conversion of pyruvate to Acetyl-CoA for entry in the Kreb's cycle, are Ca^{2+}-sensitive, as are the mitochondrial dehydrogenases, isocitrate and α-ketoglutarate, that catalyze the cycle itself(36). This possible effect of Ca^{2+} upon the increase of ATP/ADP ratio will, in turn, modulate the ATP sensitivity of K^+-ATP channels, their closure and membrane depolarization, as well as subsequent activation of voltage-gated calcium channels (VDCC). Stimulation of Ca^{2+}-sensitive adenylate cyclases (42) which produce cAMP and facilitate PKA phosphorylation(37) of VDCC proteins can prolong channel activation and increase Ca^{2+} influxes. Upon Ca^{2+} entry into the cytoplasm, $[Ca^{2+}]_i$ is bound by calmodulin in groups of up to four Ca^{2+} cations, inducing a conformational change. The Ca^{2+}/calmodulin complex can then bind to and stimulate Ca^{2+}-dependent kinases, i.e. CaMK's (Ca^{2+}/calmodulin-dependent kinases), MLCK (myosin light chain kinase), etc., as well as phosphatases, e.g. calcineurin, etc. (22,35), to phosphorylate or dephosphorylate, respectively, a large number of substrates. Thus, when bound by

calmodulin, the Ca^{2+} signal may activate kinases and phosphatases which attach and remove high-energy phosphate groups to and from substrates to regulate insulin secretion (via modification of cytoskeletal motor proteins (32)) and insulin synthesis (via modification of transcription factors and/or co-activators (Fig.2) (27,80)).

Ca^{2+} and insulin biosynthesis

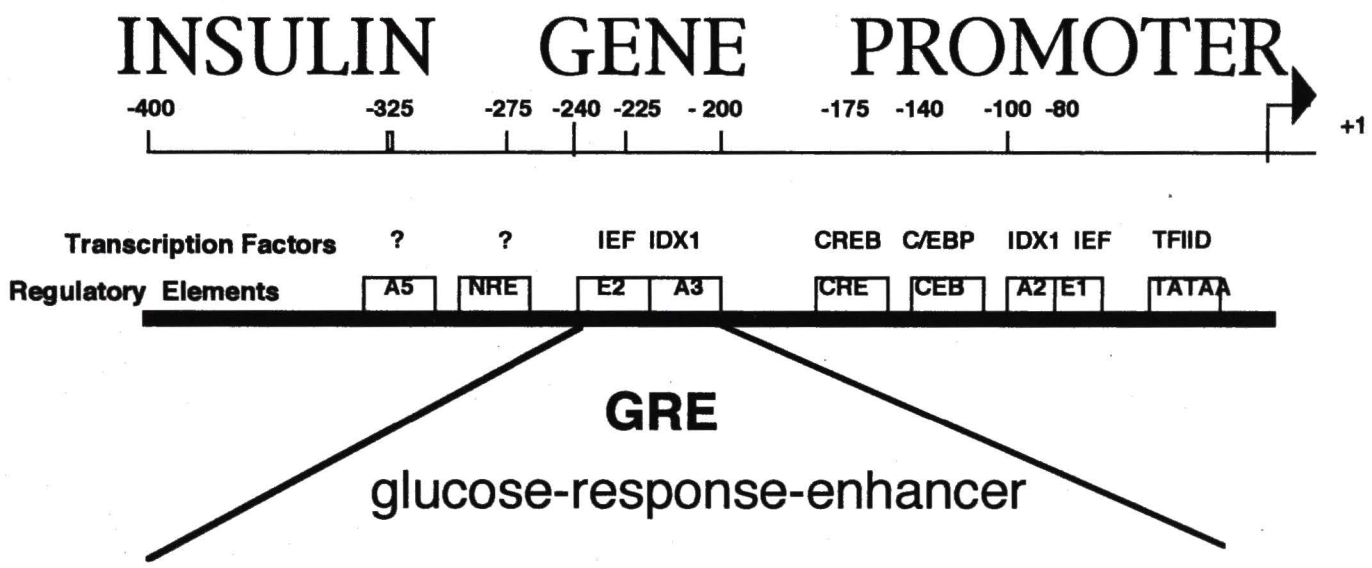
Since both insulin secretion and biosynthesis are sensitive to circulating glucose levels, it is reasonable to assume that Ca^{2+} signals involved in stimulus-secretion coupling may also contribute stimulation to biosynthesis. Tight coupling of integrated molecular and cellular regulation was reported for oscillating glycolysis in β-cells that induced oscillations in $[Ca^{2+}]_i$, ATP/ADP ratios, plasma membrane potentials, and insulin secretion through apparently coupled, synchronized signals (39). While recent studies have detected synchrony of oscillations of $[Ca^{2+}]_i$ and insulin secretion in single islets (95,96), normal oscillatory, or pulsatile, insulin secretion is perturbed in Type II diabetes (97,98). These findings imply that synchronous coupling to $[Ca^{2+}]_i$ oscillations, i.e. cycling concentration levels, may exemplify normal β-cell function. Since β-cells, as others, self-regulate their energy metabolism at constant temperatures for maximum economy (36), tight coupling is the favored strategy. In that several insulin secretagogues also stimulate biosynthesis, synthesis and secretion are potentially coupled by unidentified

common signals, even though the threshold for glucose-stimulated insulin secretion (4.2-5.6 mM) differs from preproinsulin synthesis (2.5-3.9 mM) (43). Activation of L-type calcium ion channels (VDCC) allows Ca^{2+} influx during the sustained glucose-induced increase in insulin secretion (40), while such a response is blocked by dihydropyridine Ca^{2+}-channel blockers (41). Ca^{2+} channel blocker verapamil dramatically reduced CAT reporter mRNA transcription linked to a glucose responsive insulin gene enhancer in chimeric genes transfected into fetal rat islets (20). Stimulation of insulin gene expression as well as secretion was inhibited in βTC3 murine insulinoma cells by D-600, another Ca^{2+}-channel blocker (19). Overall, glucose metabolism is required for insulin release due to increased ATP/ADP ratios that inhibit K_{ATP} channels and activate Ca^{2+} influx. Products of glucose metabolism also stimulate insulin promoter/enhancer activity when a promoter-reporter construct is transfected into cell cultures (76), so insulin transcriptional regulation associated with a Ca^{2+}-sensitive mechanism is strongly suggested.

Promoter control of insulin expression

Prior studies have traced insulin gene regulation in the β-cell through a metabolic signal from glucose fluxes transmitted through the insulin upstream enhancer involving interactions of *cis*-acting elements with *trans*-acting promoter factors (Figure 3). These promoter elements, located from -196 to -247 nucleotide bases 5'

FIG.3. Insulin Gene Promoter and β-cell Transcription Factors with Comparison of *cis*-acting Enhancer Motifs Conserved between Mammalian Species



RAT I 5' CTTCA++TCAGGCCATCTG+++GGCCCCTTGTTAATAATCTAATTACCCTA+GGTC+TA 3'

RAT II 5' GTTCA++TCAGGCCACCCAGGAGCCCCTG+TTAAGACTCTAATTACCCTA+AGGC+TA 3'

HUMAN 5' GGACGGTTCTGGCCACCGG+++GCCCCTGGTTAAGACTCTAATGACCCGCTGGTCCTG3'

MOUSE 5' GTTCA++TCAGGCCATCTG+++GTCCCTTATTAAGACTATAATAACCCTA+AGAC+TA 3'

DOG 5'+++++++++GGCCATCAGG+++CCCCTCGCCAAGACTCTAACGACCCCGAGG+CCCG 3'

CONSERVED MOTIFS **E BOX** **A BOX**

upstream of the transcription start site, function as a β-cell-specific enhancer and impart glucose responsiveness when linked to a thymidine kinase (TK) and a CAT (chloramphenicol acetyltransferase) gene in a reporter plasmid transfected into fetal rat islets. Polymerase chain reaction technique was used to demonstrate that mRNA transcription level increases paralleled CAT reporter enzyme activity to show that glucose responsiveness actually represented stimulation of β-cell transcription of the insulin gene. Moreover, the glucose-response of this plasmid was severely lowered when calcium influx to the transfected cells was blocked by verapamil(20). Therefore, as postulated in the hypothesis of this thesis, any evidence indicating a Ca^{2+}-sensitive mechanism for modulation of transcription factor binding to these glucose responsive enhancer elements would provide valuable and relevant insight into β-cell function and insulin biosynthesis.

β-cell restricted transcription of the insulin gene requires the interaction of *trans*-acting protein factors with *cis*-acting elements in the insulin promoter (79)(Fig. 2 & 3). The promoter is also the region of assembly of the transcriptional initiation complex, comprised by the general transcription factors, which operates in all eukaryotic gene expression in response to external stimuli to recruit RNA polymerase II through TBP recognition of and TFIID binding to the TATAA motif (TATA box) approximately 20 to 30 nucleotides upstream from the transcription start site (45, 46). Unlike prokaryotes which employ repressive negative regulation, eukaryotic DNA-binding gene regulatory proteins positively control expression by activating and turning genes on and are called transcriptional

activators or *trans*-acting factors, although negative regulation has been reported for the insulin promoter (47-50). Negative eukaryotic regulation has been reported for the basic leucine zipper transcription factor, CCAAT/Enhancer-binding protein β (C/EBPβ), in which the transcription factor expression rises in chronically elevated glucose in HIT-T15 and INS-1 β-cells, and paradoxically inhibits insulin gene transcription by competing for dimer partnership with the normal transactivator DNA-binding protein (50).

As well as directing the mRNA encoding start site (51), the insulin promoter restricts expression to the β-cell (52), and regulates the rate of transcription in response to glucose and other physiological regulators (20). Most previous reports on the insulin promoter are concerned with the proximal -350 base pairs of the rat insulin I and II (rat has two non-allelic insulin genes) and the human insulin gene which share sequence homology over 70% for this region (Figure 3) (35). When removed by endonuclease digestion from the whole gene and linked to a heterologous reporter gene in a plasmid, the proximal promoter retains the functional activity potential of the intact promoter when transfected in cultured cells. (47,52-54). Studies of the proximal insulin promoter using this recombinant technique have identified sequence elements whose deletion or mutation alters gene expression under control of that promoter segment (47,55). Since the promoter sequence elements serve as binding sites for transcription factor recognition, their modification interferes with protein factor ability to *trans*-activate these *cis*-acting DNA motifs to influence transcription. The E-box

elements, E1 and E2, with the consensus CANNTG motifs, may participate in tissue-specific expression in insulin-producing cells although combinations of E1 and E2 fail to confer tissue specificity on transgenic mice (57). The E boxes bind transcription factors of the basic helix-loop-helix (bHLH) family, which possess two amphipathic helices separated by a loop structure next to a basic domain (58). The basic domain mediates DNA binding while the HLH structure is required for dimerization by protein partners which may derive from separate genes, such as E12 and E 47, products of the E2A gene in humans (59). The A box elements (A1-A5), named for their AT-rich sequence, contain the conserved TCTAAT motif and serves as binding site for members of the homeodomain class of transcription factors, including isl-1 (60), cdx , lmx1 (61), and IDX-1 (also called IUF-1, IPF-1, STF-1, or PDX-1)(62-66). TAAT motif cognate binding site also occur in β-cell GLUT2 and glucokinase promoters. Conserved sequences elements in the A and E arrays are reported to account for over 90% of the transcriptional activity of the insulin gene promoter (56) .

The principal A-box IDX-1 (Islet/Duodenum homeoboX-1), a 283-amino acid transcription factor expressed only in β and δ pancreatic cells and duodenum, has conserved central DNA-binding homeodomain and N- and C-terminal proline-rich regions (62). The IDX-1 C-terminus may be involved in homodimerization with another IDX-1 molecule (66), while the N-terminus activates the insulin promoter in close synergism with other trans-acting factors, such as E47 which binds E box elements (68,69). Knockout experiments have found IDX-1 to be required for pancreas

development and subsequent β-cell function in differentiated phenotypes (70, 2). IDX-1 is detected during early pancreatic development in pluripotent precursor cells (70) becoming restricted to β-cell nuclei along with insulin gene expression (71). Since loss of IDX-1 expression correlates with non-insulin producing α, δ or PP phenotypes, sustained capacity for IDX-1 expression appears required for islet β-cell differentiation (73).

Glucose response enhancer (GRE) and synergism

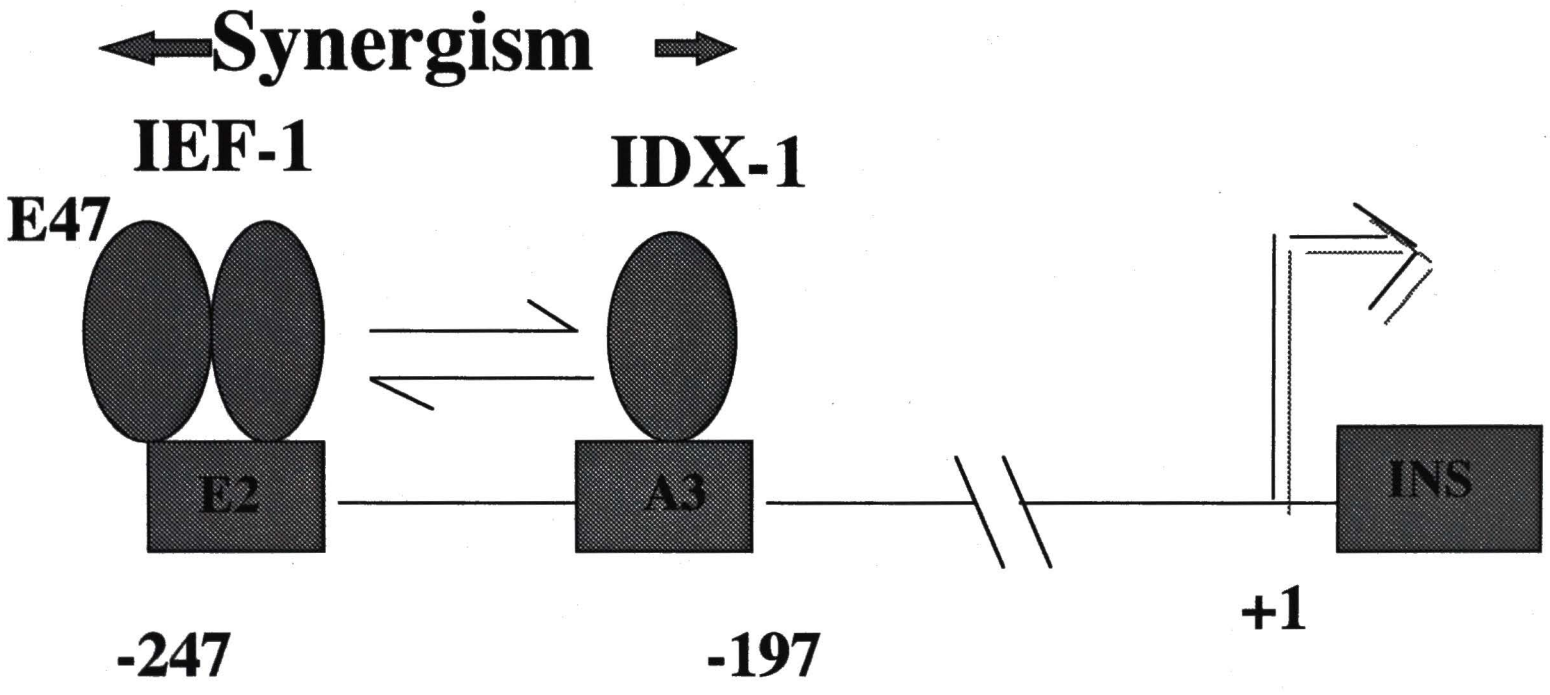
Recombinant mutational and deletion strategies have mapped insulin promoter elements that are sensitive to stimulation by glucose metabolism and its products to sequences the -197 to -227 A3 motif (also called CT2) (47,73,74,81). In the rat insulin 1 (rInsI) gene, a glucose response enhancer (GRE) has been mapped to a 50 nucleotide sequence located between position -197 to -247 (75,20,61,68,78), just upstream from the cAMP response element (CRE)(77). Within the rInsI GRE (Fig. 3), there are three adjacent elements, designated E2 for its conserved E box, and A3, both possessing the TAAT motif. These distal E box (CANNTG) and A box (TCTAAT) box core sequences are highly conserved showing approximately 70% homology between human (hIns) and rat I (rInsI) genes. Separately, the E2 and A3 sequences in the rInsI GRE show little activity, yet,when combined, they function in a synergistic fashion in promoter assays using chimeric reporter constructs (78,79). Response to glucose stimulation requires recognition of

regulatory A box sequences containing the TAAT motif by β-cell specific transcription factors, specifically IDX-1 (20,73,74,80). Such IDX-1 binding to both rat I and human A3 sites is modulated by extracellular glucose dose-dependently (73,74). Glucose-stimulated transcription in the rat insulin I gene was shown to be mediated by the A3E2 elements, a glucose-response-enhancer (GRE), in fetal islets (20).

When insulin promoter/CAT reporter and CMV/IDX-1 expression plasmids were co-transfected into non-insulin producing α-cells, IDX-1 protein binded to DNA in insulin promoter sites and activated insulin gene reporter constructs (64,68,73) This induced insulin gene expression relies on cooperation between IDX-1 and IEF-1 (Insulin Enhancer Factor-1; (81,68)), a heterodimer made up of Beta 2, which is expressed in both α and β-cells, in partnership with products of the E2A gene (E12 or E47) and ubiquitous class A HLH factors like Beta 1/rat E-box binding protein (REB; (59)). Additional pancreas-specific factors were shown to be necessary for this gene regulation since rat embryo fibroblasts failed to be induced in its absence (82).

Taken together, maximal insulin gene expression in response to glucose requires synergistic interaction by A box-binding proteins, e.g. IDX-1 (β-cell specific), with co-activator transcription factors of the bHLH family (pancreas specific) that bind as heterodimers to adjacent E elements in the insulin promoter (Figure 4). Therefore, study of the glucose response of the insulin promoter when separated from the intact wildtype form will be more physiologically relevant if both A and E elements are observed in tandem activity

FIG. 4. Synergism interaction model of insulin gene transcriptional activation.In the normal β-cell, interaction between two adjacent enhansons comprised of contiguous enhancer A and E elements through which *trans*-activating factors synergistically energize the maximal induction of the insulin gene. Binding to the E box elements by E47-containing heterodimers interacts with IDX-1which is bound by adjacent A box elements.



Proposed Synergism Combinatorial Control Of Insulin Gene Transcription

because A and E elements never perform alone *in vivo*. In the attempt to furnish physiologically valid findings, albeit with cultured transformed cells, this research of Ca^{2+} sensitivity of transcriptional activation will assess β-cell transcription factor binding to the entire A3E2 glucose-response-enhancer (GRE).

Of greatest biomedical consideration in the study of β-cell insulin promoter activity is understanding the molecular mechanism of insulin gene transcriptional activation which deteriorates in patients with type II diabetes. The chronic hyperglycemia of type II diabetes (NIDDM) may cause permanent β-cell dysfunction by effects of glucose toxicity (10). Studies using *in vivo* animal models and *in vitro* cultured β-cells have reported a correlation between reduced insulin gene transcription and loss of IDX-1 (IPF-1/STF-1) transcription factor expression (83,84). In 90% pancreatectomized rats, lower levels of IDX-1 protein coincide with loss of insulin mRNA and protein (84). If the derangement of β-cell function in diabetes is due to impaired activation of insulin gene transcription, the defective molecular mechanism may interfere with normal transcription factor, i.e. IDX-1, binding to the insulin promoter control elements, i.e. GRE. Given that β-cell transcription factor binding to GRE sequences may be essential for initiation of insulin gene transcription in response to glucose metabolism, defective communication by Ca^{2+} intracellular signals may disrupt β-cell function and and insulin gene expression.. Although previous findings have suggested that the signal provided by calcium influx contributes to the activation of

insulin gene transcription by glucose (20), the mechanism for this induction remains unclear.

Binding of the β-cell transcription factor, IDX-1, to a GRE (glucose-response-enhancer) (55) in the insulin promoter 5' upstream region has been shown to depend on phosphorylation(80). In the widespread communication of signals and information throughout biological systems using Ca^{2+} as a second messenger, these cations' effects are orchestrated through its interaction and binding by calmodulin, as well as other calcium-binding proteins, e.g. troponin in heart. Most of the downstream effects through which these Ca^{2+}-transduced signals are enacted involve activation of a Ca^{2+}/Calmodulin-dependent kinase which in turn phosphorylates, i.e. covalently modifies by transfer of a phosphate group, one of a voluminous numbers of substrates, including transcription factors like CREB, C/EBPβ (85), and IDX-1.

Combinatorial control of transcription appears to be mediated by multiprotein complexes through protein-protein interactions of factors bound to adjacent elements, as it is in most eukaryotes (86). Together, these A and E elements function as a 'minienhancer' or glucose-response-enhancer (GRE) which is required for the full β-cell reponse to glucose stimulation of insulin gene expression (20). Intracellular signals, or conditions which modulate this binding, must therefore be essential for proper insulin hormone and β-cell function in reponse to D-glucose.

Of possible candidates for regulatory roles, this thesis focuses on the Ca^{2+} sensitivity of binding to both A and E

elements in the composite A3E2 (-197 to -247) GRE by β-cell-specific multiprotein complexes of transcription factors containing GSF (glucose-sensitive factor, also called IDX-1/PDX-1/STF-1) due to differential activation by Ca^{2+}/calmodulin-dependent kinases through phosphorylation of a serine, threonine, or tyrosine amino-acid residues possessing a viable hydroxyl (-OH) side group. Post-translational modification of regulatory transcription factor proteins by phosphorylation and dephosphorylation is recognized as a universal mechanism for modulating potential fates of transcription factor regulation (45). These fates include cytoplasmic or nuclear localization, gain or loss of DNA binding, stability, intramolecular oligomerization or intramolecular folding. Since many different types of stimuli that affect gene expression also lead to the activation of protein kinases, it is likely that transcription factor function is partly regulated by phosphorylation by protein kinases.

Three main levels of regulation modulate transcription factor activity by phosphorylation. First, transcription factors can be sequestered in the cytoplasm and rendered inactive though lack of access to their target promoter sequences. Phosphorylation of the factor itself, or of a cytoplasmic anchor protein, may allow translocation of the transcription factor into the nucleus, where it acts. Second, the DNA binding activity of nuclear transcription factors can be modulated by phosphorylation, either positively or negatively. Third, phosphorylation can affect the interaction of transcription factor transactivation domains with the transcriptional machinery. These possibilities are by no means mutually exclusive,

and in principle phosphorylation at multiple sites by different protein kinases can allow regulation at several distinct levels. Three dimensional structural analysis has shown that phosphorylation can affect protein activity by inducing allosteric conformational changes which alter DNA binding capacities and thus regulation (87).

DNA-protein binding affinity and avidity can be detected by radiolabeling the nucleotide binding site sequences in an *in vitro* reaction with nuclear proteins and visualizing bands on electrophoresis gels using densitometric quantification of radiographs. This assay is termed an EMSA (Electrophoresis Mobilty Shift Assay) since the mobility of free DNA probe is retarded, or shifted, by protein complexed to its sequence binding sites causing bands to be detectable (92). Although the potentiating effects of external stimuli upon binding and gene induction can be assessed, the mediating role of transcription factor phosphorylation on binding and transcription initiation cannot be distinguished. In view of the primary role of transcription factor phosphorylation in regulation of gene expression (45,86), the development of a methodology capable of distinguishing such phosphorylation-dependent DNA-protein interactions would be of great importance for molecular genetics research with broad applications for all cell types.

To enable identification of DNA-protein electrophoresis gel bands which require factor phosphorylation, we reversed labelling of protein and oligonucleotides, measuring the binding of labelled nuclear phospho-proteins to unlabelled DNA probes. This innovation allows only bands incorporating a phosphorylated nuclear protein bound to a target DNA promoter binding site to be visible on

radiographs. In this way, modulation of binding, increase or decrease, can be attributed to factor phosphorylation.

Experimental Design Rationale

Chronic hyperglycemia can produce toxic effects which impair insulin biosynthesis related to a reduction in β-cell transcription factor binding to insulin promoter elements (50, 83, 88). 'Glucose toxicity' from chronic high glucose exposure of insulin-secreting clonal cells impairs binding of a protein which had otherwise been found glucose-sensitive (74) to a promoter glucose-response-element (GRE) binding site (20), which was disrupted by diabetic conditions(88).

Therefore, to demonstrate a Ca^{2+} role in insulin gene regulation, experiments were designed and performed to measure Ca^{2+}-sensitivity of binding of β-cell nuclear proteins to probes representing GRE (A3E2-Fig. 2) sequences. Clonal cultured INS-1 β-cell lines which produce insulin in response to glucose ranges of concentration roughly equivalent to normal islets were used in the experiments. Although removed from the normal physiological situation within the islets of Langerhans in the rat pancreas, these insulinoma cells have been shown to retain normal transcriptional inducibility of their β-cell-specific promoter elements within the approximate range of normal human blood glucose concentrations (4-7 mM). As the experimental findings progressively emerged, more concerted measures were devised to minimize extraneous

transcripitional activation by supraphysiological glucose concentrations and other stimulating agents incorporated in the culture medium.

Three experimental models were employed:

I. Intact INS-1 β-cells were permeabilized with *Staphylcoccus aureus* α-toxin, following incubations in low (3mM) glucose and in high glucose (11 mM), and directly stimulated by Ca^{2+} concentrations which closely approximated the *in vivo* range of $[Ca^{2+}]_i$ - 0.05 μM to 10 μM. Binding of nuclear extracts to both A3 (IDX-1 binding site) and A3E2 (glucose-response-enhancer (GRE) which includes the A3 IDX-1 recognition motif) was measured by EMSA to assess Ca^{2+}-sensitivity.

II. Since binding Ca^{2+}-sensitivity differed very slightly between partial (A3) and composite (A3E2) sequences (while rat recognition of nuclear factors was more sensitive than human (Fig. 5)), only composite rIns1 GRE sequences were used in EMSA binding assays to recognize nuclear extract proteins from INS-1 cells incubated (4 hr) in low glucose (2 mM) and high glucose (20mM) following *in vitro* phosphorylation reaction with Ca/CaM-dependent kinase II or EGTA (Ca^{2+}-chelator). Similar to an immunoprecipitation strategy for identifying a radiolabelled phosphoprotein by means of antibody recognition of its antigenic epitope (which distinctively alters electrophoretic mobility), phosphorylated transcription factor proteins incorporated in nuclear extracts are likewise distinguished by their recognition by the consensus binding sites within an unlabelled GRE oligonucleotide probe to create radiographically

identifiable bands.

III. Next, after *in situ* glucose stimulation (at 0.5 mM and 20 mM) of INS-1 cells following passage at 11.1 mM glucose and18-24 hr equilibration at physiologic 5.5 mM, nuclear extracts' binding to GRE minienhancer probes was measured by EMSA. To confirm that effects of glucose mediation of binding was actually due to Ca^{2+} sensitivity, culture medium of cells incubated in low glucose (0.5 mM) was replaced for the final 15 min before nuclear extraction by K^+ (40mM)/KRB to induce plasma membrane depolarization and Ca^{2+} influx. To determine the Ca^{2+} sensitivity and kinase specificity of the modulation of binding activity by high (20 mM) glucose metabolism, the 4 hour incubations prior to nuclear extractions and EMSA assays were supplemented by Ca^{2+}-channel blocker Verapamil and KN-93, the CaMK II-specific inhibitor.

Experimental findings from these procedures indicate that for *in situ* and *in vitro* conditions, β-cell nuclear protein transcription factor binding by insulin gene enhancer is Ca^{2+}-sensitive and is specifically reduced when CaMK II modification of transcription factors is disrupted. The finding of Ca^{2+}-sensitivity and an implied CaMK II role in activating insulin gene expression supports the thesis hypothesis illustrated by Figure 2 representing my proposed β-cell signal transduction model.

EXPERIMENTAL PROCEDURES

Materials - INS-1 cells, a clonal rat insulinoma cell line, were a generous gift of Dr. Christopher Rhodes (UT-Southwestern Medical School, Dallas, Texas). CMRL-1066, RPMI-1640, L-glutamine, amphotericin, and gentamycin were purchased from Life Technologies (Gaithersburg, MD) and Hepes was from Fisher Scientific (Nepean, CA). Ficoll, ATP (disodium salt), leupeptin, verapamil, *S. aureus* α-hemolysin, pyruvic acid, ditheothreitol, β-mercaptoethanol, EGTA were purchased from Sigma Chemical Co. (St. Louis, MO). [γ^{32}P]-ATP was purchased from NEN Research-DuPont Chemicals, (Boston, MA). Ca^{2+}/calmodulin-dependent protein kinase II (CaMK IIα) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). KN-93 was obtained from Calbiochem (La Jolla, CA). [γ^{32}P]-ATP radiolabelling of reactants was measured using a Beckman Model LS3801 (Beckman Instruments, Fullerton, CA) scintillation counter. Densitometry by Cerenkov counting of labelled binding complex components was quantitated by the InstantImager Electronic Autoradiography System (Packard Instrument Co., Meriden, CT). All other chemicals were of the finest reagent grade available.

Cell culture - Cells were cultured in RPMI 1640 medium (11.1 mM

glucose or other concentrations as indicated) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml amphotericin, 50 units/ml gentamycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM β-mercaptoethanol at 37°C under an atmosphere of 5% CO_2. After weekly passage at 11.1 mM glucose, cultures grown to 50-70% confluency were detached from monolayer by treatment with trypsin/EDTA and transferred to 60 mm dishes (Nalge Nunc Intl., Copenhagen, Denmark). for 18-24 hrs reattachment of monolayer culture growth. Cells were allowed to equilibrate in a physiological concentration of 5.5 mM glucose for an additional 18-24 hrs to return gene transcription to basal levels of activation before attempting to assay protein-DNA binding events. Cells were then exposed for a minimum 4 hrs to low (0.5, 2, or 3 mM) or high (11.1, or 20 mM) glucose conditions, as stated, to stimulate or suppress gene transcription activation levels prior to nuclear extractions for gel shift assay determinations. K^+ (40 mM) stimulation to low glucose (0.5 mM) conditions replaced RPMI 1640 media with Krebs-Ringer Bicarbonate buffer (KRB) without glucose (75 mM NaCl, 45 mM KCl, 24 mM $NaCO_3$, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM Hepes, 0.1% BSA) in which KCl was raised (5 to 45 mM) in place of equal proportion NaCl (lowered 115 mM to 75 mM) to maintain osmolarity. Verapamil (100 μM), a VDCC blocker, EGTA (20 mM), a Ca^{2+} chelator, or KN-93 (10-50 μM), a specific CaMK II inhibitor, was added to *in situ* or in *vitro* conditions, as indicated. When KN-93 (30 μM) or K-252a (200 μM) inhibitors were added from DMSO suspensions, equivalent DMSO was include in reactions to

normalize conditions

Permeabilization of INS-1 Cells - Cells were permeabilized following a method described by Wollheim *et al.* (86). INS-1 cells (approximately 6 x 10^6/flask) were detached from monolayer tissue culture-treated flasks by trypsinizing (37°C for 5 minutes) and resuspended in RPMI 1640 media (supplemented with 11.1 mM glucose, 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml amphotericin, 50 units/ml gentomycin, and 50 μM β-mercaptoethanol) for two hours re-equilibration in 60 mm non-tissue culture-treated petri dishes at 37 °C in 5% CO_2. Before permeabilization, cells were washed twice in Ca^{2+}-free KRB containing 6 mM glucose, 1 mM EGTA, and 0.1% fetal calf serum and cell number counted by hemocytometry. After counting, cells were washed once in Ca^{2+}-free permeabization buffer (20 mM Hepes, pH 7.0, 140 mM K-glutamate, 5 mM NaCl, 4 mM $MgSO_4$, 1 mM EGTA, and 0.3 mM Na_2ATP). Cells in suspension were made permeable by adding *Stapholococcus aureus* α-hemolysin, at a concentration of 225 U/10^6 in 100 ml/10^6 (counted cells) in Ca^{2+}-free permeabilization buffer. Permeabiliztion proceeded in a 37 °C water bath for 15 min and the efficiency was monitored by visualizing trypan blue penetration to >50%. To terminate the permeabilization reaction, cells were then washed in equal volumes of permeabilization buffer, pelleted and resuspended in 50 nM Ca^{2+}

(determined by a Ca^{2+}sensitive electrode (ORION, Inc.), using a Ca^{2+}/EGTA standard curve as described by Bers *et al* (87)).

Protein Phosphorylation in Permeabilized Cells - Cells were pelleted and resuspended in a reaction volume of 200 μl containing either low (0.05 μM Ca^{2+}), or high (10 μM Ca^{2+}). To radiolabel protein phosphorylation, 5 μCi of [γ-^{32}P]ATP was added to the prewarmed reaction mixture and incubated at 37°C for 100-110 s. Reactions were stopped by rapid cooling on ice.

Preparation of Nuclear Extracts - Nuclear extracts were prepared using a modification of the method of Dignam *et al.* (89). After passsage in 11.1 mM RPMI 1640, monolayer cell cultures were detached by trypsinization, aliquoted in 1.5 ml tubes and pelleted. Supernatant fluids were discarded and cells were resuspended in low (0.5, 2, or 3 mM) or high (11.1 or 20 mM) glucose for 4 hr incubations. Cells were microcentrifuged for 1 min and resuspended in 250 μl of Buffer A [10 mM Hepes, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 10 mM NaF, 10 mm ammonium molybdate, 10 mM sodium pyrophosphate, and 10 mM orthovanadate]. Cells were allowed to swell on ice for 15 min before adding 25 μl of 10% (v/v) Nonidet P-40. Cells were then vortexed for 15 sec and centrifuged for 4 min at 4°C. The pellet, now enriched

with nuclei, was resuspended in 50 μl of Buffer B [20 mM Hepes, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/μl leupeptin, 10 mM NaF, 10 mM ammonium molybdate, 10 mM sodium pyrophosphate, and 10 mM orthovanadate]. Nuclear extracts were then centrifuged for 10 min at 4°C. The supernatant was collected, aliquoted into small volumes, and stored at -70°C.

In Vitro Phosphorylation of Nuclear Proteins - INS-1 β-cell nuclear extracts were phosphorylated by purified rat forebrain calcium/calmodulin-dependent protein kinase II (CaMKII), the 50 kDa α isoform. In a total volume of 30 μl, 1 μl [γ-^{32}P]-ATP (1 μCi/μl in dH_2O) was added to a reaction mixture of 5 μl INS-1 nuclear extract (10-15 μg), 22 μl binding buffer (10 mM Tris (pH7.9), 50 mM KCl, 1 mM EDTA, 5 mM DTT), 10 mM MgAc, 1 μM calmodulin, and either 0.55 mM $CaCl_2$ plus CaMKII (1.3 μg/μl), with or without KN-93 (10,30 or 50 μM, as indicated), or 1 mM EGTA. After 8-10 min at 30°C, aliquots were combined directly with oligonucleotide probes in a binding reaction (PREMSA, see below), or loaded on SDS-PAGE for molecular weight size determination.

Oligonucleotide Probes - Oligonucleotide sequences corresponding to portions of the glucose-reponse-enhancer (GRE) in the rat I (rInsI) insulin promoter were purchased from Genosys

Biotechnologies, Inc. (The Woodlands, Texas) - conserved core recognition motifs of the A and E elements (see explanation in INTRODUCTION) are underlined:

(1) human A3E2 (also termed GRE, CT2-IEB2 or FAR-FLAT)(5' upstream bp - hIns -197 to -247)

5"GGACGGTTCTGGCCACCGGGCCCCTGGTTAAGACTCTAATGACCCGCTGGTCCTG3'

(2) human A3 (also termed CT2 or FLAT)(5' upstream bp hIns -197 to -230)

5'CTGGTTAAGACTGTAATGACCCGCTGGTCCTG3'

(3) rat A3E2 (also termed GRE, CT2-IEB2 or FAR-FLAT)(5' upstream bp - rIns -197 to -247)

5'CTTCATCAGGCCATCTGGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTA 3',

(4) rat A2 (also termed CT2 or FLAT)(5' upstream bp rIns -197 to -230)

5' CTTGTTAATAATCTAATTACCCTAGGTCTA3'.

Annealing and 5' End-Labelling of Oligonucleotide Probes- Single-stranded complementary oligonucleotides were annealed by slow cooling (RT for 8 hrs) from boiling (5' at 97^{O} C). Double-stranded oligonucleotide probes (of indicated base sequences) were 5'end-labeled with 1 μL [γ-^{32}P] ATP (3000Ci/mmol, 10 μCi/μl) using Ready-To-Go™ T4 Polynucleotide Kinase kit (Pharmacia) in a final reconstituted volume of 50 μl (containing 8-10 units of T4 Polynucleotide Kinase, 50 mM Tris-HCl, (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA (pH8.0), 0.2 mM ATP and

stabilizers) incubated at 37°C for 30 minutes and terminated with 5 μL of 250 mM EDTA. In the presence of 4 μG poly dIdC (poly-deoxyInosine-deoxyCytosine-(Pharmacia)-non-specific competitor - to prevent non-specific nuclear protein binding to the probe), the reaction mixture was ethanol precipitated, dried and suspended in TE buffer (1mg/ml) at 4 °C.

Electrophoretic Mobility Shift Assay (EMSA) - The conventional EMSA measurement of DNA-protein binding detects complexes formed between unlabeled nuclear proteins and [γ-^{32}P]-ATP radiolabeled oligonucleotide DNA sequences (90). Binding reactions were carried out using radiolabeled oligonucleotide probes with unlabeled INS-1 nuclear extracts (EMSA). For both reactions, nuclear extracts (6-14 μg of protein as normalized by Bradford assay) were pre-incubated in a total volume of 22 μl of reaction buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA), 1 mM dithiothreitol, and 5 mM glycerol for 10 min at 25°C prior to combining with the oligonucleotide probes. Unless otherwise stated, all binding reaction incubations contained 1 μg of poly(dIdC) (Pharmacia) as a nonspecific competitor. Aproximately 1-2 ng of double-stranded probe (labeled (40-60K cpm/ml- measured by Beckman scintillation counter) was added to the reaction and incubated for 30 min at 25°C before immediate loading to gel. Total volume of 20 μl was loaded on a 4% TBE (Tris-borate-EDTA) non-denaturing polyacrylamide gel in a 0.5 M TBE buffer. Affinity-

purified anti-STF-1 antibody (2-4 μl) (provided by Dr. Mark Montminy, Joslin Diabetes Center, Harvard Medical School, Boston, Mass.) was added to certain binding reactions for 15 min preincubation prior to adding probe. Verapamil (100 μM), EGTA (20 mM) or 10-50 μM of KN-93, a specific CaMK II inhibitor, was added to *in situ.* Dried gels were visualized and quantitated on an Instant-Imager (Packard) and then radiographed on XOMAT film (Kodak).

Phosphoprotein Reverse EMSA (PREMSA) -By reversing the labeled components of the EMSA binding reaction, binding of unlabelled rIns1 GRE probes to [γ-^{32}P]-labelled phosphoproteins (see *In Vitro Phosphorylation of Nuclear Proteins*, in above METHODS) was induced so that only phosphorylated transcription factors or other nuclear proteins forming complements to the DNA sequences of the probes are detected. Binding reactions were carried out using unlabeled rIns1 GRE oligonucleotide probes with radiolabeled phosphorylated INS-1 nuclear extracts (termed PREMSA). EGTA (20 mM), a Ca^{2+} chelator, or 10-50 μM of KN-93, a specific CaMK II inhibitor, was added to the *in vitro* phosphorylation reaction, as indicated. An amount of the *in vitro* phosphorylation reaction mixture estimated to contain 5-10 μg of nuclear protein extracts (approximately 7-14 μl of the 25 μl total, as previouly determined by Bradford protein concentration assay (88)), equivalent to a conventional EMSA binding reaction mixture, was added directly to a total volume of 25 μl of reaction buffer (

10mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA), 1 mM DTT, 1 μg of poly dIdC, and 5 mM glycerol for 10 min incubation at room temperature (25°C) prior to combining with oligonucleotides. Approximately 1-2 ng of double stranded rIns1 GRE probe was added to the reaction mixture for a 30 min incubation at 25°C before loading a total volume of 25 μl to a 4% non-denaturing TBE (Tris-Borate-EDTA) polyacrylamide gel. Dried gels were visualized and quantitated on an Instant-Imager (Packard) and then radiographed on XOMAT film (Kodak).

Quantification of protein-DNA binding activity - Quantitative analysis of [γ-^{32}P]-ATP labelled oligonuleotides, or phosphoproteins, in EMSA, or PREMSA, binding complexes, respectively, was performed using direct nuclear counting function of the InstantImager Electronic Autoradiography System (Packard).

Protein Determination - Protein concentrations were estimated using BSA as standard and by a method described by Bradford (88).

Statisical Treatment of Data - Data here contained is presented as the mean $\pm$ the standard error of the mean of at least 3 independent observations or data points, unless otherwise noted. Statistical significance was assessed from the Student's *t*- test.

RESULTS

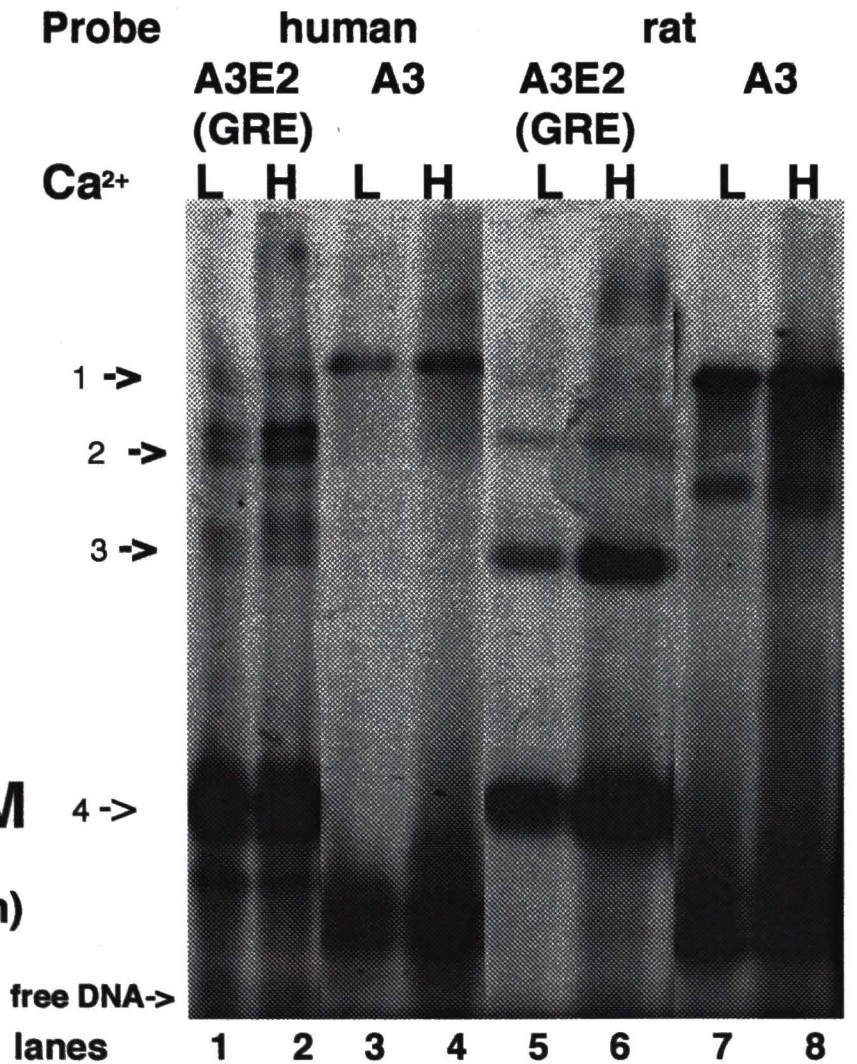
Stimulation of INS-1 β-cell Transcription Factor Binding to Glucose Response Enhancer (GRE) in Insulin Promoter by Calcium in α-toxin Permeabilized Cells - To determine if β-cell transcription factors recognizable by specific Glucose-Response-Enhancer (GRE) DNA sequences were subject to Ca^{2+}-induced activation of their promoter binding, INS-1 clonal insulinoma β-cells were permeabilized and directly stimulated with low and high concentrations of calcium which approximate the extremes of the physiological range of intracellular Ca^{2+}. Thus, the exposure of intracellular constituents would simulate the variation between baseline, Low (0.05 μM), and the raised $[Ca^{2+}]_i$, High (10 μM), levels produced by glucose-induced Ca^{2+}-influx following plasma membrane depolarization (18). In order to more clearly distinguish increased protein-DNA binding, the cells were incubated for 4 hr in sub-physiological 3 mM glucose media to reduce the level of transcription factor binding to the intact insulin promoter. For these evaluations, nuclear proteins were extracted and mixed with ^{32}P-radiolabelled oligonucleotide probes representing the glucose-response-enhancer sequences of the human and rat insulin promoters, as discussed in the INTRODUCTION and PROCEDURES. The protein-DNA binding complexes formed in this reaction were

determined in the EMSA gel shift assay and visualized by radiography (Fig. 5) and quantified by digital CPM-counting densitometry (InstantImager) (Fig. 6).

INS-1 insulinoma β-cells were incubated in low (3 mM) glucose concentrations for 4 hr prior to protocols of α-toxin permeabilization (86), followed quickly by 100 -110 sec of Low (50 nM) Ca^{2+} or High (10μm) Ca^{2+} stimulation at 30°C, and immediate extraction of nuclear proteins as described in METHODS. In the β-cell which had been incubated in 3 mM glucose, Ca^{2+} stimulated nuclear extract binding to both an A3 (-197 to -230, a known IDX-1 binding site) and the full GRE minienhancer A3E2 (-197 to -247 - including the binding site for the IEF-1 heterodimer) to a similar degree (Fig. 5). Although Ca^{2+}-sensitivity of binding stimulation appears comparable for both partial (A3) and full mini-enhancer probes, the number and relative mobility of binding complexes varies greatly. The position, but not the number of complexes is similar, yet unique, between human and rat probes and the rat nuclear proteins, perhaps revealing the high homology of the consensus binding sites in the probes. In comparison to basal binding to insulin enhancer probes as control (50 nM Ca^{2+} (Low)- 100% control) in Figure 6 , 10 μM Ca^{2+} (High) increased binding as follows:

in Band 1 to 157 ±7.2% with human, and 208.5±4.5% with rat;
in Band 2 to 196.7±3.7%, with human, and 290.7±36% with rat;
in Band 3 to 134.5±8.8% with human, and 260.6±58.4% with rat;
in Band 4 to174.1±48% with human, and 263.3±77.8% with rat probes, respectively. These findings suggest that factor binding to

FIG. 5. Effects of Ca^{2+} on INS-1 β-cell transcription factor binding to human (hIns1) and rat (rIns1) promoters. After 4 hr incubation in 3 mM glucose media, *S. aureus* α-toxin permeabilized INS-1 rat β-cells were stimulated by Low (0.05 μM) Ca^{2+}, and High (10.0 μM) Ca^{2+} buffers. Proteins in the nuclei were extracted, and nuclear transcription factor binding to human (hIns1) and rat (rIns1) -197 to -227 (A3) and -197 to -247 (A3E2 (GRE)) 5' upstream oligonucleotide probes assayed by EMSA on a 4% non-denaturing electrophoresis gel run at 36 mA constant current for 80-90 minutes. Aliquots of 6-10 μg of nuclear extracts (normalized by Bradford assay) were added to approx 1 ng of oligonucleotide in the binding reaction



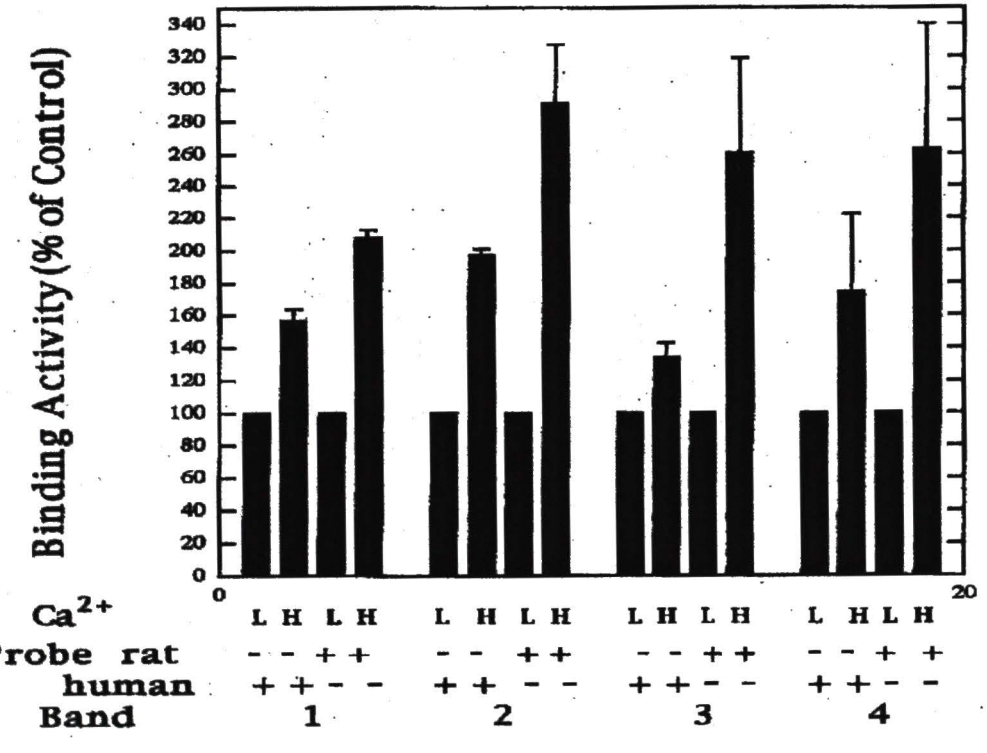
Glucose 3 mM

(4 hr incubation)

Ca^{2+} Sensitivity of Rat β-cell Factor Binding to Rat/Human Enhancer Probes

FIG.6. Ca^{2+} stimulates β-cell transcription factor binding to insulin glucose-response-enhancer (GRE). 10 μM Ca^{2+} stimulation of α-toxin permeabilized β-cells incubated in 3 mM glucose increased binding activity of rat nuclear protein extracts (6-10 μg) to rat and human insulin gene enhancers (1-2 ng per binding reaction). Compared to 0.05 μM Ca^{2+}-sensitivity (as 100% control), measured binding activity increased in Band 1 to 157 ±7.2% with human, and 208.5±4.5% with rat; in Band 2 to 196.7±3.7%, with human, and 290.7±36.4% with rat; in Band 3 to 134.5±8.8% with human, and 260.6±58.4% with rat; and in Band 4 to174.1±48% with human, and 263.3±77.8% with rat probes, respectively. Probes representing rat insulin 1 (rIns1) sequences (A3E2 (-197 to -247, also called GRE) and A3 (-197 to -230)) bind to INS-1 rat factors with nearly twice the Ca^{2+}-sensitivity than do human insulin (hIns) DNA sequences.

3 mM Glucose
(4 hr incubation)



Binding Activity
(% of Control)

Band	Human	Rat
1	157.1%	208.5%
2	196.7%	290.7%
3	134.5%	260.6%
4	174.1%	263.3%

the A3 portion of the GRE sequence of the insulin promoter is sensitive to $[Ca^{2+}]_i$ elevations. Ca^{2+} sensitivity of β-cell factor binding to the glucose-response-enhancer of the insulin promoter implies a role for Ca^{2+} in signal transduction between glucose metabolism and transcription initiation mechanisms.

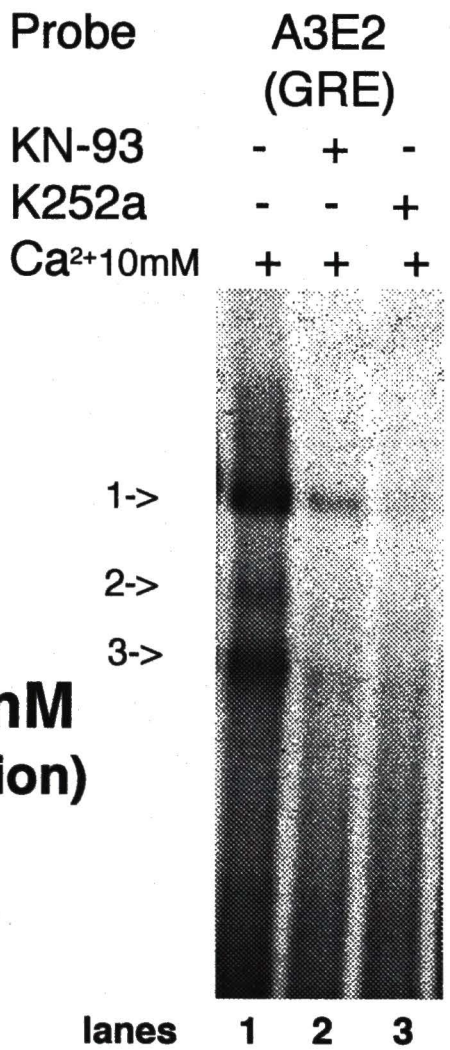
Comparison of binding activity (Fig. 6) shows that 1) probes of rat enhancer sequences consistently bind more avidly to the rat INS-1 transcription factors than do human binding sites and that 2) binding activity is consistently Ca^{2+}-sensitive although increases due to elevated $[Ca^{2+}]i$ vary between bands (i.e. binding complexes containing differing transcription factor components). Therefore, use of rat probes was determinedto be a more sensitive test of Ca^{2+} mediated binding affinity and used in the remaining experiments of this thesis.

Ca^{2+}/Calmodulin-dependent Kinase Inhibitors- KN-93 and K-252a - Reduces α-toxin Permeabilized, Ca^{2+}-stimulated INS-1 β-cell Transcription Factor Binding to Insulin Gene Enhancer - After initially observing the Ca^{2+}-sensitive increases in β-cell factor binding to the GRE probe that consistently occurred in the preceding experiment, it was of interest to determine whether this augmentation was due to activation of Ca^{2+}/calmodulin kinases which regulate many cellular functions by post-translational phophorylation. The *S. aureus* α-toxin permeabilization and 10 μM

Ca^{2+} stimulation of intact INS-1 cultured β-cells was performed as above, with additional 10 μM Ca2+ conditions supplemented by either 30 μM KN-93, a CaMK II-specific inhibitor, or 200 μM K-252a, a non-specific serine/threonine kinase inhibitor. Following an electrophoretic mobility shift assay (EMSA) of β-cell factor binding to a rat (rIns1 -197 to -247) GRE probe, **radiography (Fig. 7) and quantification by digital net CPM counting (Fig.8)** was performed. A substantial reduction in binding complexes was measured (Fig. 7 & 8) by adding either inhibitor in the 10 μM Ca^{2+} stimulating buffer. In comparison to binding activity in extracts of permeabilized cells stimulated by 10 μM Ca^{2+} buffer (100% control), KN-93 decreased measured binding (Fig. 8) in Band 1, lane 2 to 26.9%, in Band 2, lane 5 to 36% and in Band 3, lane 8 to 35.1%. Similarly, K-252a reduced binding activity (Fig. 8) in Band 1, lane3, to 21.3%, in Band 2, lane 6, to 35.3%, and in Band 3, lane 9 to 32% of 10 μM Ca^{2+}-stimulated levels (100% control). These findings are consistent with phosphorylation of transcription factor, or co-activator, substrates by Ca^{2+}/calmodulin-dependent kinases, particularly CaMK II, to effect potentiation of binding to insulin gene enhancer elements.

High Glucose Pre-exposure Reduces Calcium Sensitivity of Transcription Factor Binding to Insulin Gene Enhancer in α-toxin Permeabilized INS-1 β-cells - After observing stimulation of DNA-protein binding by 10 μM Ca^{2+} in intact cells

FIG.7. Inhibition by KN-93 and K252a of Ca^{2+}-sensitive transcription factor binding in permeabilized INS-1 cells. Following permeabilization and Ca^{2+} stimulation (immersion with 200 μl of high (10 μM) Ca^{2+} buffer for 2 min with/without Ca^{2+}-specific kinase inhibitors KN-93 (30μM) and K-252a (200 μM)), nuclear proteins were extracted and protein concentrations determined by Bradford assay. Normalized protein aliquots (6-10 μg) were then added to 1-2 ng of enhancer probe in the EMSA binding reaction as explained in METHODS. Total volume of 20 μl was loaded on 4% non-denaturing polyacrylamide gels and run at constant 36 mA.



Glucose 3 mM
(4 hr incubation)

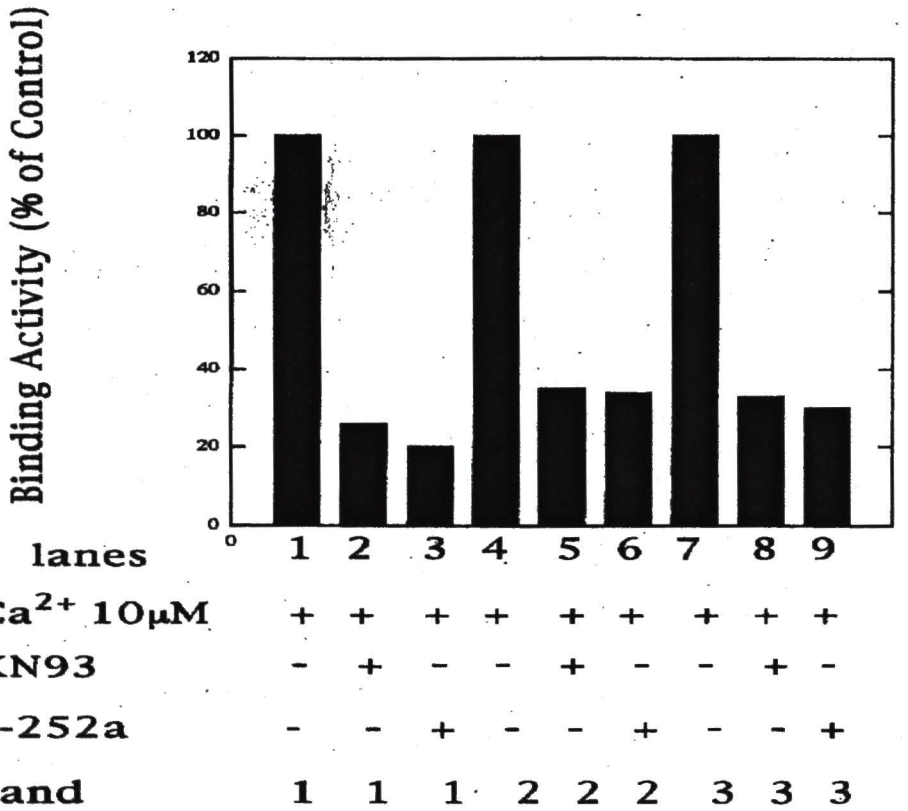
In permeabilized β-cell binding is reduced by inhibition of kinases, specifically Ca^{2+}-sensitive CaMK's

FIG. 8. KN-93 and K252a inhibit binding to insulin enhancer.

Immersion of α-toxin permeabilized INS-1 cells in high (10 μM) Ca^{2+}-supplemented permeabilization buffer stimulated binding activity (lane 1) of 6-10 μg β-cell nuclear extracts (containing transcription factors) to a rat (rIns1) GRE probe (1-2 ng). Addition of 10 μM KN-93 to the 10 μM Ca^{2+} buffer reduced binding in an identical reaction over 60% (lane 2: band 1-26.8%, band 2-35.2%); whereas 10 μM K-252a reduced activity by over 2/3 (lane 3: band 1-21.3%, band 2-31.0%). KN-93 is an inhibitor specifically for Ca^{2+}/calmodulin-dependent kinase II. K-252a is a non-specific protein kinase inhibitor.

Permeabilized INS-1 Cells

3 mM Glucose 4 hr incubation



Binding Activity (% of Control)

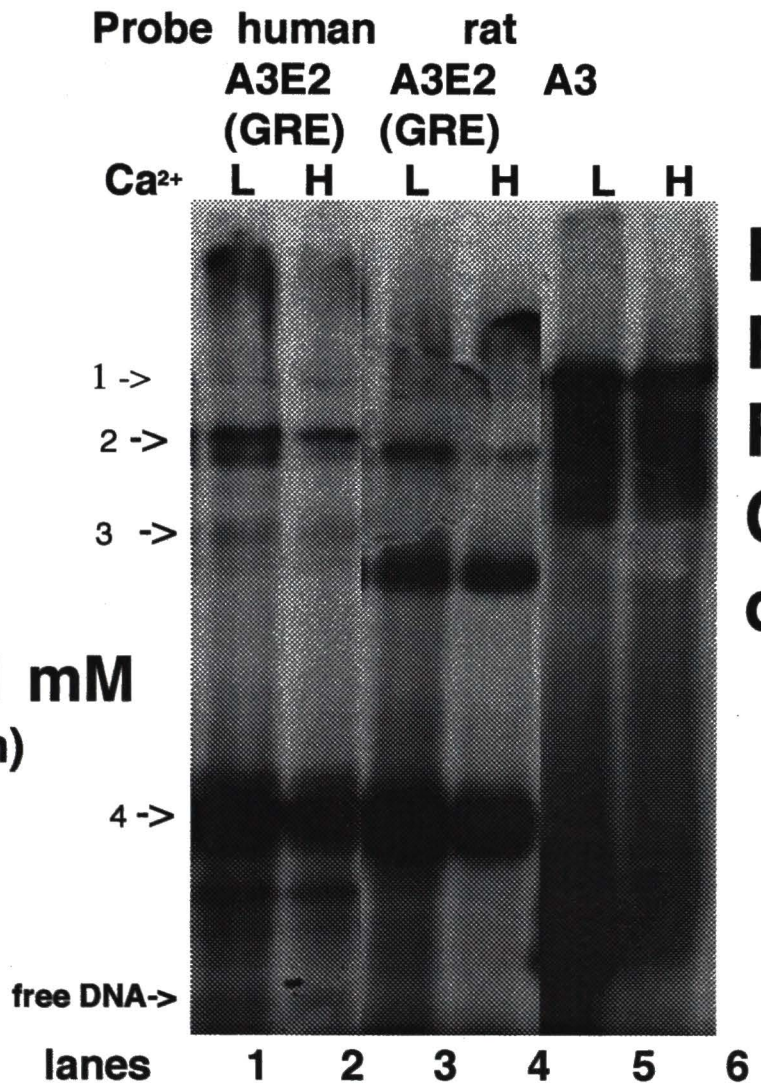
Band	Kn-93	K252a
1	26.9%	21.3%
2	36%	35.3%
3	35.1%	32%

incubated for 4 hrs in 3 mM glucose prior to permeabilization, Ca^{2+} stimulation, and nuclear protein extraction, it was questioned whether high, supraphysiological glucose preincubation might alter this effect. In INS-1 cells incubated at 11.1 mM glucose for 4 hr prior to *S. aureus* α-toxin permeabilization protocols as before (86), 10 μM Ca^{2+} consistently failed to stimulate extract binding to insulin gene promoter probes, either rat or human, A3E2 (GRE) or A3 DNA sequences in EMSA gel shift assays. In contrast to pre-exposure, the capacity for Ca^{2+}-sensitive activation of factor binding to the insulin promoter was reversed and lowered by high 10 μM Ca^{2+} concentrations. Permeabilzed cells were immersed in 10 mM Ca2+ buffer for 2 min and nuclear protein extracts wer prepared and combined with rat and human A3E2/GRE (glucose-response-enhancer), or A3, radiolabelled probes, and run on 4% non-denaturing electrophoresis gels. Gels were visualized by radiography (Fig.9) and quantified by densitometry (Fig. 10) Relative to control (100%=0.05 μM Ca^{2+},Low), binding activity of nuclear extracts (Fig. 10) from cells immersed in 10 μM Ca^{2+}, High, buffer decreased as follows:

Band 1 to 95.2% for human, and 60.6 ±3.5% for rat,
Band 2 to 65.0% for human, and 58.5 ±20.5% for rat,
Band 3 to 79.6% for human, and 98.1 ±3.2% for rat, and
Band 4 to 61.8% for human, and 52.5±3.8% for rat probes, respectively.

At the supraphysiological 11.1 mM glucose concentration, Ca^{2+}-sensitive phosphorylation sites on β-cell factors may be occupied or

FIG.9. High glucose reduces Ca^{2+}-sensitivity of β-cell transcription factor-insulin GRE binding. β-cell transcription factor binding to human and rat insulin enhancer (GRE (A3E2 -197 to -247) and A3 (-197 to -230)) after Ca^{2+} stimulation is *reduced* for INS-1 clonal cells cultured in 11.1 mM glucose. Factors were contained in 6-10 μg nuclear extracts from INS-1 cells following α-toxin permeabilization and treatments with either Low (50nM) or High (10 μM) Ca^{2+}-supplemented buffer and combined in a binding reaction with 1-2 ng of ^{32}P-labelled enhancer probes. This finding may reflect both glucose and Ca^{2+} activation of a common signal pathway.



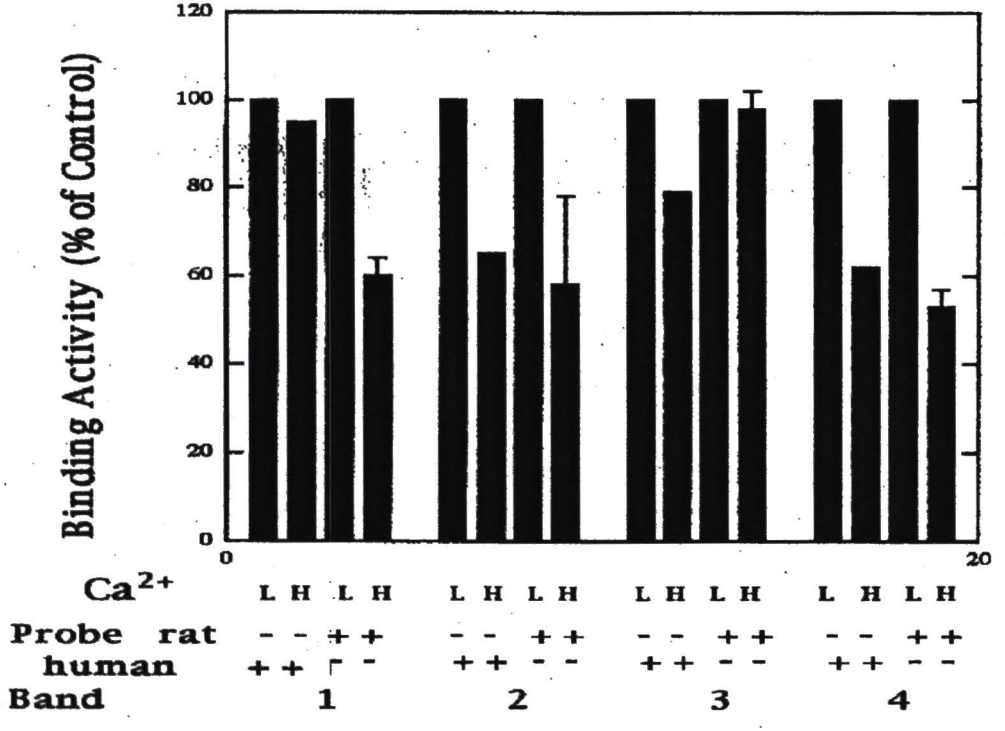
Glucose 11.1 mM
(4 hr incubation)

High Glucose Pre-exposure Reduces Ca^{2+}-Sensitivity of Binding

FIG.10. Effect of 11.1 mM glucose on Ca^{2+}-sensitive transcription factor binding to insulin enhancer . Relative to control (100%=0.05 μM Ca^{2+},Low), EMSA binding activity of nuclear extracts (6-10 μg) from cells immersed in 10 μM Ca^{2+} (High) buffer to 1 ng oligonucleotide probes decreased as follows:
Band 1 was reduced to 95.2% for human, and 60.6 ±3.5% for rat,
Band 2 was reduced to 65.0% for human, and 58.5 ±20.5% for rat,
Band 3 was reduced to 79.6% for human, and 98.1 ±3.2% for rat, and
Band 4 was reduced to 61.8% for human, and 52.5±3.8% for rat probes, respectively.

11.1 mM Glucose

(4 hr incubation)



Binding Activity (% of Control)

Band	Human	Rat
1	95.2%	60.6%
2	65.0%	58.5%
3	79.6%	98.1%
4	61.8%	52.5%

unavailable in cells prestimulated by metabolism of glucose (Fig.9). Findings that Ca^{2+} sensitivity of β-cell transcription factor binding to the GRE of the insulin promoter is reduced by pre-exposure to glucose metabolism suggests that activation of protein-DNA binding, and transcriptional initiation, may involve Ca^{2+}-dependent phosphorylation of sites susceptible to glucose-induced saturation, implying competition for a common signal pathway.

In Vitro Phosphorylation of INS-1 β-cell Transcription Factor Mediates Binding to Insulin Gene Enhancer in a Calcium-Sensitive Manner - In the effort to determine whether the possible phosphorylation of transcription factors was contributing to the glucose-induced or Ca^{2+}-sensitive binding to glucose-response-enhancer probes, a modification of the electrophoretic mobility shft assay (EMSA) was devised. By addition of Ca^{2+}/calmodulin-dependent kinase II (CaMK II), the kinase suggested to be involved from previous experiments using kinase inhibitors, directly in an *in vitro* phosphorylation reaction with INS-1 nuclear proteins extracts, phosphoproteins were radiolabelled with [γ^{32}P]-ATP. By reversing the labeled components of an EMSA, in a so-called Phosphoprotein Reverse Electrophoretic Mobility Shift Assay, or PREMSA, combining radiolabelled phosphoproteins with unlabelled DNA probes in the binding reaction, only binding complexes containing phosphorylated factors will be visible with radiography (Fig.11-lanes 2 through 6). To further characterize the

Ca^{2+}-sensitivity of INS-1 β-cell transcription factor binding to GRE probes, KN-93, the CaMK II-specific inhibitor, and EGTA, a Ca^{2+}-chelator, were added to separate *in vitro* phosphorylation reactions. Ca^{2+} sensitive β-cell factor binding to a GRE probe, normalized to 100% of CaMK II-stimulated levels (100% control, n=7), was inhibited approximately 50% by combination with KN-93 (50.7 ±6.6%, n=4), or EGTA (49.7±3.6%, n=7) (Fig. 12). INS-1 β-cell nuclear extract protein were phosphorylated with [γ^{32}P]-ATP in a reaction containing CaMK IIα (1.3 μg/μl), calmodulin (1 μM), and $CaCl_2$ (0.55 mM) with or without KN-93 (30 μM), a CaMK II inhibitor, or EGTA (1mM), a Ca^{2+}-chelator, instead of Ca^{2+}. The effect of this post-translational modification to transcription factors was measured by net CPM quantification (InstantImager) of PREMSA binding to an unlabelled probe representing the A3E2 (-197 to -247 5' upstream) insulin promoter. Alignment of bands representing binding PREMSA complexes (Fig.11-lanes 2 through 6) showed minimal correspondance to conventional EMSA bands (Fig. 11 - lanes 7 & 8) where probes, not phosphoproteins, are radiolabelled. Such alignments were intended to distinguish phosphorylation-dependent protein-DNA binding. However, failure of alignment of these bands in this study may not exclude the possibility of detecting proteins seen in conventional EMSA by modifying specific variables in this protocol, e.g. excess quantities of kinase and radiolabels may have overdriven the reaction and hyperphosphorylated individual or extraneous substrates; therefore, by reducing reactants, binding complexes may more closely simulate those of conventional EMSAs.

FIG. 11. *In vitro* phosphorylated β-cell transcription factors Ca^{2+}-sensitive binding to rat GRE. Nuclear proteins were phosphorylated with [γ ^{32}P]-ATP in presence of i) Ca^{2+} and Ca^{2+}/Calmodulin-dependent protein kinase II with or without KN-93 (10 or 30 μg), CaMK II-specific inhibitor, or ii) EGTA (1 mM), a Ca^{2+} chelator. Phosphoproteins are distinguished by recognition of binding site sequences in **unlabelled** GRE probes in a binding reaction (PREMSA) and separated on 4% native gel electrophoresis assays. Conventional EMSA (cold protein with hot probe) is included at right for comparison of band mobilities.

In Vitro Phosphorylated Nuclear Proteins Binding Activity

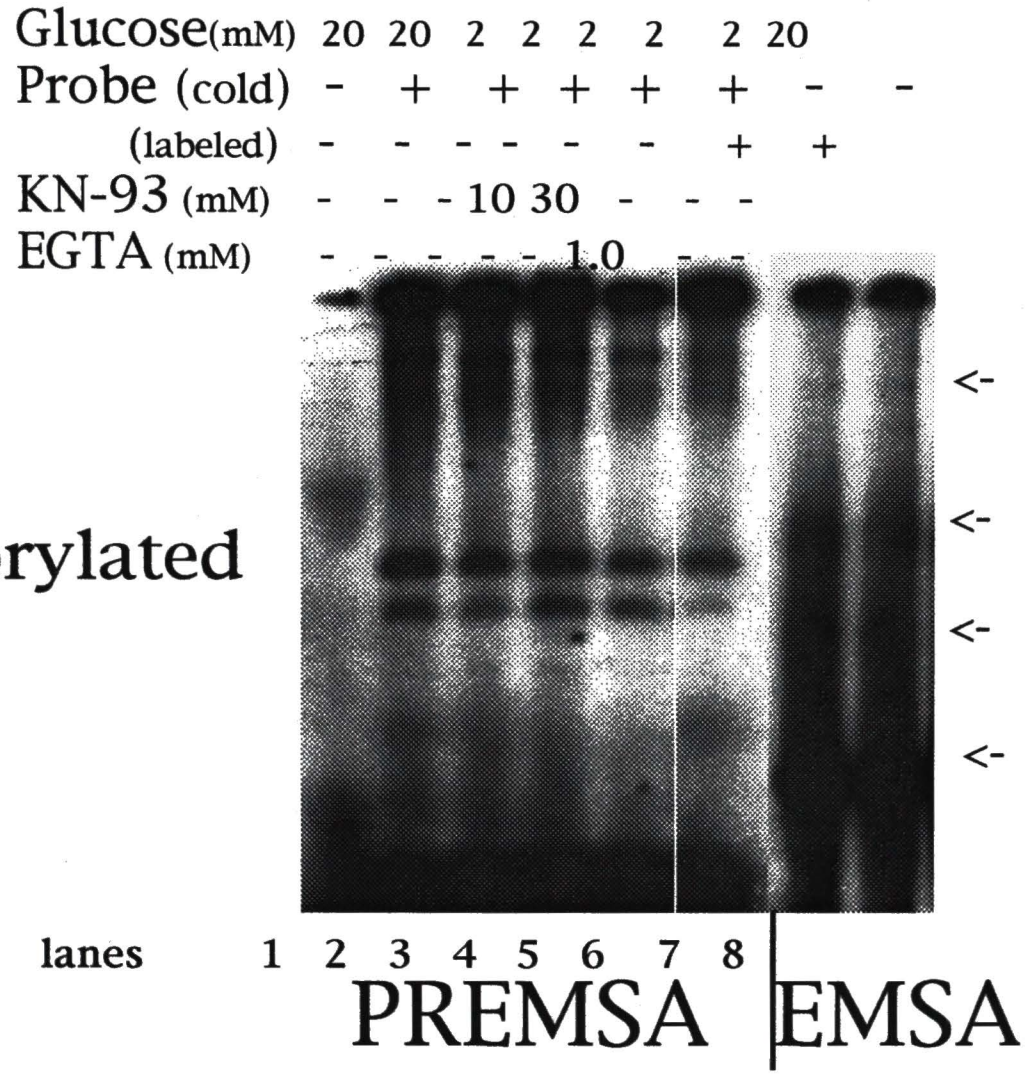
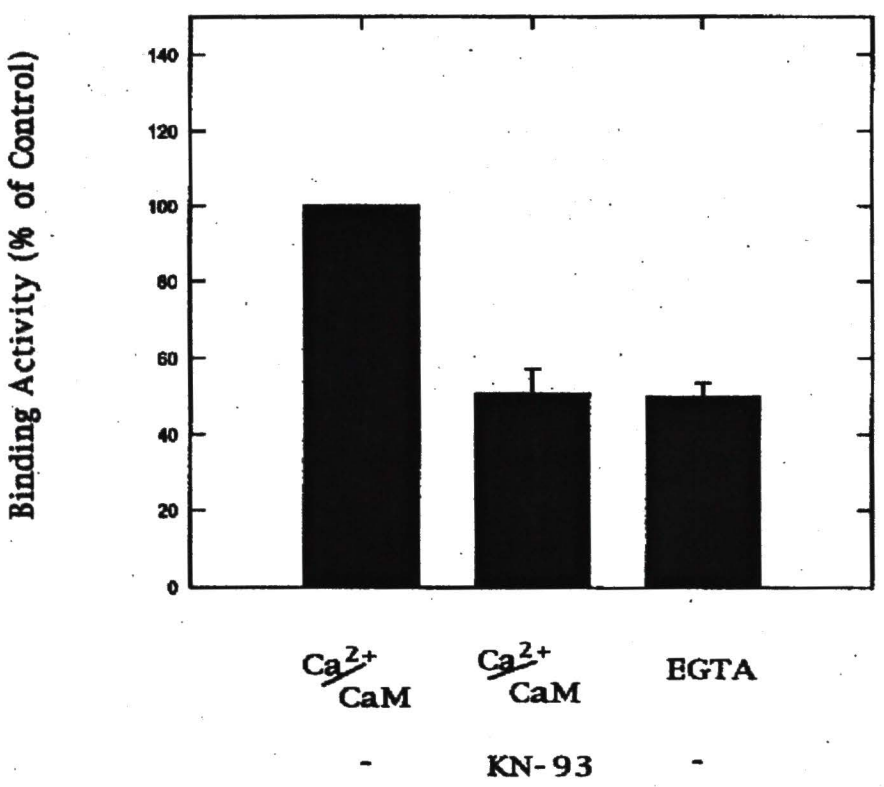


FIG.12. Inhibition by KN-93 and EGTA of *in vitro* phosphorylated factor binding activity in INS-1 cells. Nuclear proteins (10-14 μg/reaction) were phosphorylated with [γ^{32}P]-ATP in presence of i) 0.55 μM Ca^{2+} and 0.13 μg/μl Ca^{2+}/Calmodulin-dependent protein kinase II with or without 30 μM KN-93, CaMK II-specific inhibitor, or ii) 1 mM EGTA, a Ca^{2+} chelator. After two minutes of phosphorylation reaction, 4-8 μg of protein ((1/3 of reaction volume) was combined directly in a binding reaction with 1-2 ng of the unlabelled GRE probe. Binding activity was quantitated for net CPM count by InstantImager and is represented relative to 100% control for factor binding without inhibitor or chelator. Inclusion of 30 μM KN-93 to disrupt Ca^{2+}/calmodulin-dependent kinase II catalysis in the phosphorylation reaction reduced binding activity to 50.7±6.7%, n=4, of control. Removal of Ca^{2+} ions by chelation with 1 mM EGTA similarly lowered binding activity of nuclear proteins to the GRE probe to 49.7+3.6%, n=7, of 100% control, i.e. that amount of binding activity measured for nuclear extracts of cells incubated in 2 mM glucose media. The binding complex exibiting the most pronounced change in activity was quantitated.

In Vitro Phosphorylated
INS-1 Nuclear Extracts'
PREMSA Binding to Cold
Glucose-Response-Enhancer



Binding Activity
(% of Control)

KN-93 50.7%
(30 μM)

EGTA 49.7%
(1 mM)

gels of *in vitro* phosphorylated extracts have revealed multiple β-cell nuclear CaMK II substrate phosphoproteins including prominent bands with molecular weights comparable to histone nuclear proteins, suggesting a possible integral nuclear role for this Ca^{2+}-sensitive kinase.

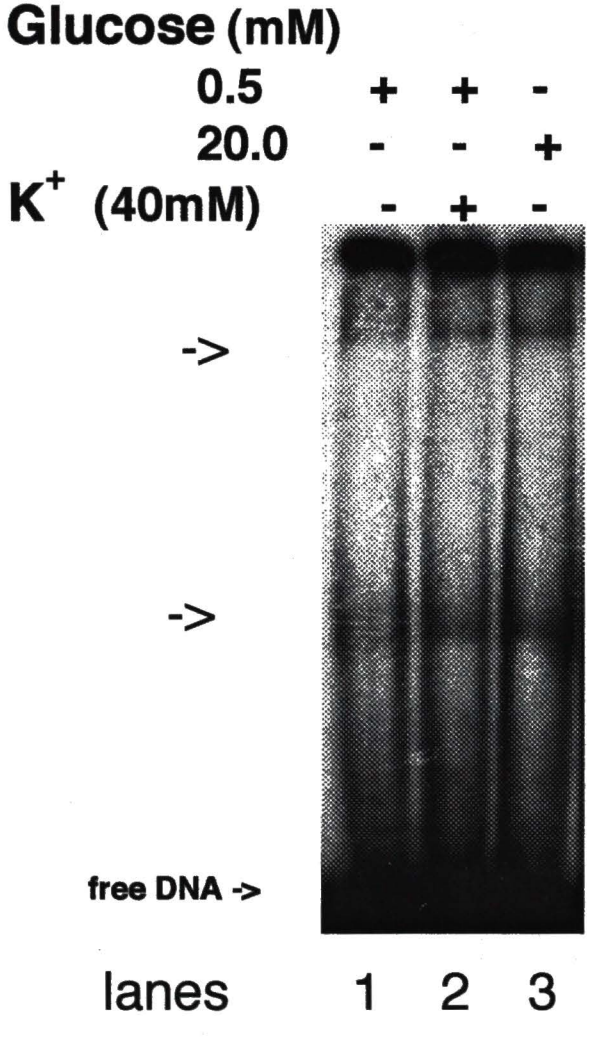
Findings of β-cell nuclear protein phosphorylation by CaMK II *in vitro* demonstrates, for the first time, CaMK II action upon substrates in the β-cell nucleus. This suggests the presence of Ca^{2+}/calmodulin-dependent kinases in the β–cell nucleus localized to perform transcription factor modifications to activate transcription initiation by stimulating promoter binding.

Attempts to distinguish the action of endogenous Ca^{2+}-sensitive *in vitro* phosphorylations were inconclusive although labelled phosphoproteins did indeed form detectable complexes with unlabelled probes in absence of external CaMK II.

In Situ Glucose Stimulation of INS-1 β–cell Transcription Factor Binding to Insulin Gene Enhancer is Mimicked by K^+ - Glucose metabolism is widely thought to induce Ca^{2+} influx into the β–cell by mechanisms which depolarize the plasma membrane (from an approximately -70 mV resting electrical potential) to activate voltage-dependent calcium channels (VDCCs). Depolarization and Ca^{2+} influx is due to closing of K^+_{ATP} ion channels that normally allow K^+ transport out of the cell. Reports have shown that insulin mRNA (in

nuclear run-on assays) and insulin promoter activity (expressed through a CAT reporter) were dramatically reduced by calcium channel blockers, D-600 (19) and verapamil (20), respectively. To test that this Ca^{2+} requirement for transcriptional activation is dependent on extracellular Ca^{2+} in our experimental model, K^+ (40mM) in (glucose-free) KRB replaced the 0.5 mM glucose (low) RPMI medium after a 4 hr incubation for the last 15 minutes prior to extraction of INS-1 nuclei for EMSA assays of binding to a rat GRE (A3E2 -197 to -247) probe. In addition to 0.5 mM (low) glucose incubation and low glucose (4 hr) supplemented by 15 min of 40 mM K^+ (in KRB), a 4 hr incubation in 20 mM glucose tested stimulation of binding activity of nuclear factors to rat GRE probes due solely to effects of glucose metabolism. Relative to 100% normalized control (0.5 mM glucose- low), exposure of INS-1 cells *in situ* for 15 min to K^+ (40mM) stimulated β-cell factor binding by approximately one-fifth (117.7 $\pm$1.7%, n=6) to 44.3% of the increase in binding activity stimulated by 20 mM glucose incubation (4 hr) alone (138.8 $\pm$2.6%, n=10) (Fig. 13 & 14). Not only is binding activity increased by elevated glucose metabolism, the mimickry of almost half of this stimulation by K^+-induced cell depolarization provides very convincing and direct evidence that the rat β-cell transcription factor binding to a rat insulin glucose-response-enhancer (GRE) is in part due to mechanisms which are sensitive to Ca^{2+} influx through VDCCs from external sources. This observation supports the correlation between insulin gene activation mechanisms and insulin secretion being motivated by a common Ca^{2+}

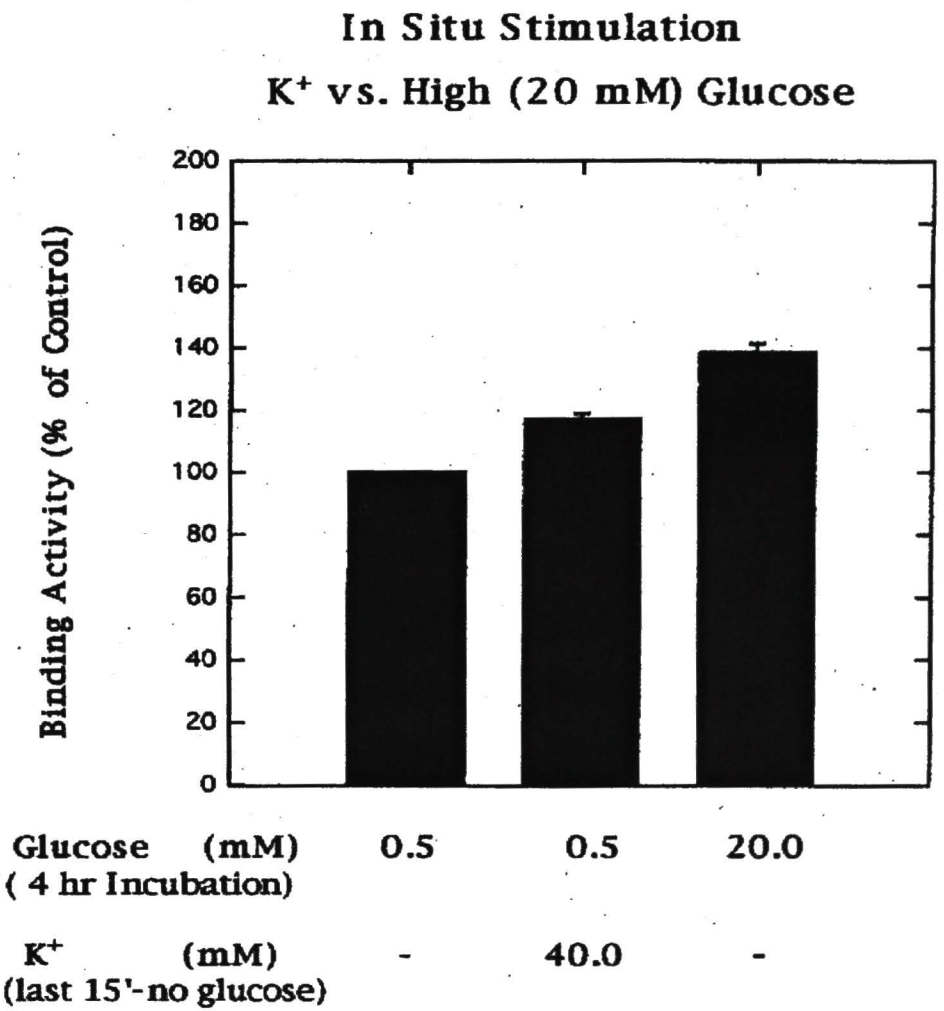
FIG. 13. K^+ depolarization stimulates GRE binding of β-cell transcription factors less than 20 mM glucose stimulation. Cells cultured at 11.1 mM glucose medium were subjected to 5.5 mM glucose equilibration (18-24 hr) in order to simulate physiological conditions of insulin gene expression, INS-1 cells were then incubated for 4 hr in either 0.5 mM (lanes 1 and 2) or 20.0 mM glucose (lane 3). To test whether glucose-induced effects on β-cell binding activity to GRE probes was indeed due to membrane depolarization (and Ca^{2+} influx), the low glucose media (0.5 mM) was replaced by K^+(40mM)/KRB (lane 2) for the final 15 min of incubation prior to nuclear extractions.



K+ Depolarization Mimics Binding Stimulation of 20 mM Glucose

FIG.14. K^+ depolarization (via Ca^{2+} influx) and 20 mM glucose metabolism stimulate binding to GRE. Plasma membrane depolarization induced by 40 mM K^+ increased 0.05 mM glucose-incubated β-cell transcription factor binding to the glucose-response-enhancer (117.2±1.78%,n=6) vs, 100% control (0.5 mM glucose) confirming Ca^{2+} role in high glucose (20.0 mM) stimulated binding (138.8+2.61%,n=10)(Fig. 11-lane 3). Binding reactions contained 6-10 μg of normalized nuclear protein and 1 ng of GRE probes incubated at room temperature for 30 min. 22 μl of reaction mixture was then run for 1 hr at 200 V constant voltage on a 4% non-denaturing polyacrylamide electrophoresis gel to separate mobility retarded binding complexes from free DNA probes.

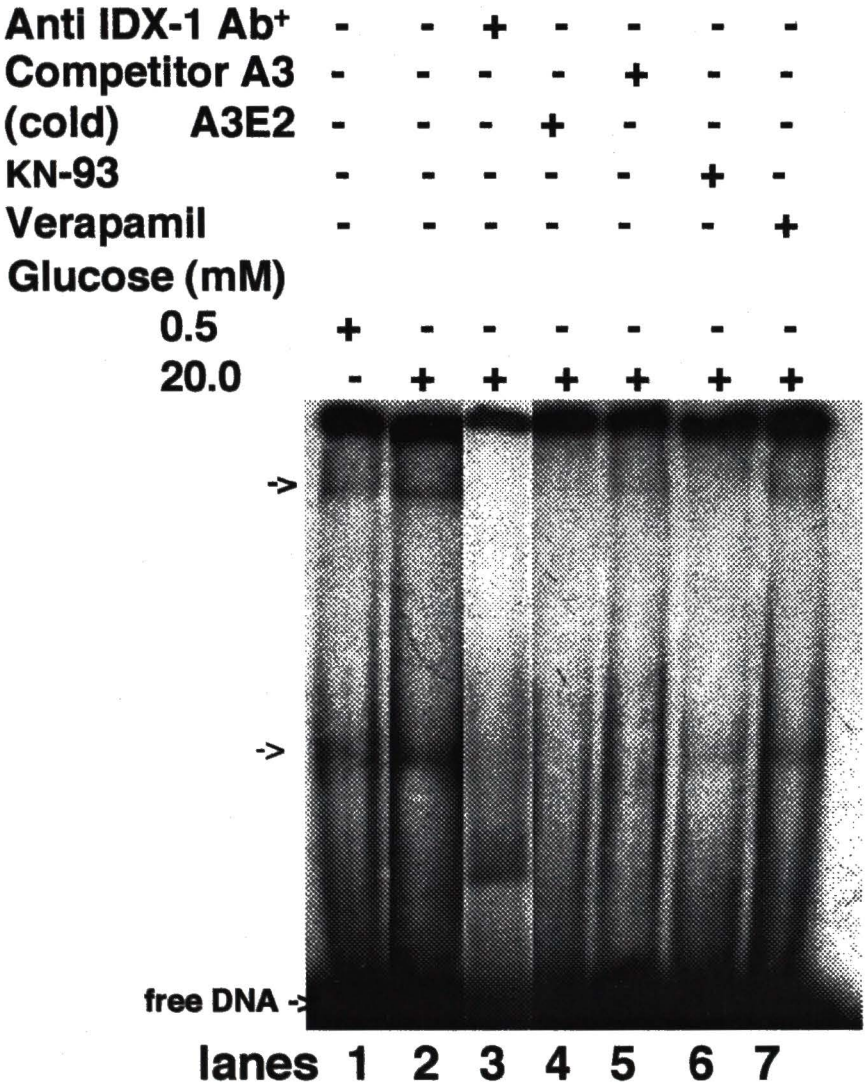
K^+ (40mM) Depolarization Mimics in Part Stimulation of Binding by 20 mM Glucose



signal, as may occur in type II diabetes treatment by sulfonylurea drugs like glibenclamide and others.

In Situ Glucose Stimulation of β-cell Transcription Factor Binding to Insulin Gene Enhancer is Sensitive to Ca^{2+} and Ca^{2+}/Calmodulin-dependent Phosphorylation - As findings in the preceding experiments indicate, glucose-induced β-cell factor binding to glucose response elements in the insulin promoter utilize in part a Ca^{2+} signal which may activate transcription through factor phosphorylation. To distinguish whether binding was mediated by the Ca^{2+} influx in general or by the specific activation by Ca^{2+} of CaMK II, INS-1 cells exposed to 20 mM (high) glucose were co-incubated with either verapamil (100 μM - VDCC blocker) or KN-93 (30 μM - a CaMK II-specific inhibitor). Interestingly, both interference of VDCC opening by verapamil and impairment of kinase activity by KN-93 reduced β-cell factor binding to the GRE promoter by similar degrees to below baseline activity of unstimulated (0.5 mM glucose) INS-1 cells (control=100%, n=10) (Fig. 16). Since verapamil (94.8±3.6%, n=8, Fig.15 - lane 7 & Fig. 14) and KN-93 (88.4±2.4%, n=10, Fig.15-lane 6 & Fig. 16) both reduced binding by virtually equivalent percentages (Fig.15 - lane 6 & Fig. 16), these findings suggest that a large proportion of the Ca^{2+} sensitivity of insulin promoter binding, and probable transcriptional activation, is mediated through factor phosphorylation, likely by CaMK II. In the EMSA binding assay (Fig. 15), the lower band represents the complex

FIG. 15. *In situ* inhibition by KN-93 and verapamil of β-cell transcription factor EMSA binding to GRE. 72In situ stimulation of binding activity of β-cell transcription factors in 6-10 mg of nuclear extracts from INS-1 insulinoma cells with 1-2 ng GRE probes by high (20 mM) glucose is dramatically reduced by 200 μM verapamil (lane 7), a voltage-dependent calcium channel (VDCC) blocker, and by 30 μM KN-93 (lane 6), a CaMK II-specific inhibitor. Inclusion of anti-IDX-1 polyclonal antiserum (4μl) in binding reaction abolished binding complexes (lane 3) suggesting participation of the IDX-1 transcription factor in those complexes. Competition by either A3 (lane 5) or A3E2/GRE (lane 4) cold probes (50-fold excess) blocked binding indicating involvment of these DNA sequences in binding complexes.



Stimulation of Binding by High (20 mM) Glucose Is Reduced by Ca^{2+} blocker (verapamil) and KN-93, CaMK Inhibitor

FIG. 16. *In situ* inhibition by KN-93 and verapamil of INS-1 β-cell transcription factor to GRE. *In situ* stimulation by high (20 mM) glucose (lane 2) of β-cell transcription factor binding to insulin enhancer is dramatically reduced by inclusion in theincubation media of KN-93 (lane 4), a CaMK II-specific inhibitor, and verapamil (lane 3), a voltage-gated calcium channel blocker. This 4 hr incubation immediately preceded nuclear extraction and EMSA binding reactions.. Compared to baseline low glucose binding activity (100% control, n=10), KN-93 (30μM) *in situ* co-incubation decreased binding activity to 94.8±3.56%, n=10, of control (lane 1). Similarly, *in situ* co-incubation with verapamil (100μM) lowered binding complex formation to 88.4±2.35%, n=8, of baseline activity.

containing the IDX-1 transcription factor since its formation was disrupted by an anti-IDX-1 N-terminal antibody (Fig. 15-lane 3) which recognizes epitopes on the factor thereby interfering with and abolishing complex formation with the GRE probe. Repeated attempts to adjust experimental variables failed to produce a clearly distinguishable supershift by the anti-IDX-1 specific antibody, although simple interference of binding is strong evidence of an IDX-1 component. Abolishment of upper and lower binding complexes by competition with both A3 an A3E2(GRE) cold probes for binding complexes (Fig. 15 - lanes 4 & 5) indicate that both lower and upper bands are complexes containing the A3 sequence. Since A3 and A3E2(GRE) cold probes could compete for both bands, and the A3E2/GRE must also contain E element binding complexes, A box complexes bound to a full A3E2/GRE may be inextricably engaged in a combinatorial control synergistic enhanson which includes an E box-binding heterodimer, as recently proposed (50,76).

DISCUSSION

The data obtained from the experiments reported here support the hypothesis that the initial β-cell transcription factor-insulin promoter binding events that lead to insulin gene transcription in response to elevated glucose metabolism are sensitive to increases in intracellular calcium ($[Ca^{2+}]_i$). As illustrated in Figure 1 and Figure 2 models, the intracellular β-cell signals stemming from glucose metabolism intersect at increased influx of calcium, which acts as a second messenger molecule essential for downstream signal cascades that stimulate insulin secretion. For over 30 years, hyperglycemia in Type II diabetes mellitus has been treated by sulfonylurea drugs which stimulate insulin secretion by a mechanism that raises $[Ca^{2+}]_i$ (8). In studies of transcriptionally competent insulin promoter-reporter plasmids transfected into cultured cells, blockage of Ca^{2+} influx by a channel blocker, verapamil, demonstrated a crucial role in glucose-induced insulin gene transcription (20). Given that both

insulin synthesis and secretion are regulated primarily by glucose, this study provides valuable information linking insulin gene transcription to β-cell response to glucose metabolism through a Ca^{2+}-sensitive molecular mechanism.

Since the initial binding of β-cell transcription factors to promoter DNA sequences is the decisive event which targets insulin gene expression exclusively to the β-cell, prior to other supply-mediating influences, i.e. splicing, translation, pre-pro-hormone to prohormone to hormone conversion, transport, storage, release, etc., the relative amount of radiolabelled component, either probe or phosphoprotein, that was incorporated into the binding complex was assumed to indicate the degree of transcriptional induction represented by that specific protein-DNA interaction.

In summary, these findings support the β-cell glucose-induced signal transduction pathway linking $[Ca^{2+}]_i$ to insulin biosynthesis proposed in Figure 2, which is a modification of the consensus models previously presented by German, Rutter et al. (73,74).

I. Pre-exposure to subphysiological glucose (3 mM) of INS-1 cells

before *S. aureus* α-toxin permeabilization and 10 μM Ca^{2+} stimulation was able to potentiate binding activity of transcription factors (contained in nuclear extracts) with DNA sequences representing the glucose-response-enhancer (GRE) IDX-1 recognition motif in the insulin promoter (Fig. 5). A considerable reduction in this 10 μM Ca^{2+} stimulation of binding was observed when non-specific (K252a) kinase inhibitors and calcium/calmodulin dependent kinase II-specific (KN-93) inhibitors were added to the Ca^{2+} buffer (Fig. 7). These inhibitors were tested to assess whether Ca^{2+}-sensitive protein-DNA binding mechanisms may involve Ca^{2+} activated phosphorylation (as has been found for exocytotic proteins in β-cells (31)). Phosphorylation of transcription factors is a universal gene regulation mechanism (45). The Ca^{2+}-sensitivity of binding activity appeared to be reversed by high glucose pre-exposure (Fig. 9) suggesting that binding stimulation by glucose metabolism and Ca^{2+} may compete for a common signal pathway as diagrammed in Fig. 2. The Ca^{2+}-sensitive modulation of binding was more pronounced between INS-1 rat nuclear extracts and rat probes than for probes representing human insulin promoter sequences, likely reflecting the source of nuclear proteins from rodent cell lines. To

attain a more physiologically relevant measure of molecular events in this artificial cell free system and more distinguishable binding activity, the choice was made to use the full rat glucose-response-enhancer (rIns1 GRE/A3E2 - 5' upstream from -197 to -247 bp) in the remainder of these experiments.

II. *In vitro* phophorylation of INS-1 nuclear proteins were found to stimulate binding to the GRE probe in a Ca^{2+}-sensitive manner (Fig. 11). This increase in binding activity was reduced significantly by Ca^{2+} chelation with EGTA as well as by KN-93 inhibition of phosphorylation by Ca^{2+}/calmodulin-dependent kinase II, or possibly other members of the CaM kinase family, such as CaMK IV. This finding substatiates inhibition effects measured in the permeabilized cell model in the previous experiment (Fig. 8). Direct *in vitro* phosphorylation of numerous INS-1 nuclear proteins was performed by adding nuclear protein extracts to a reaction mixture which included Ca^{2+} and [γ^{32}P]-ATP radioisotope in the presence and absence of either exogenous CaMKIIα or calmodulin. Combining of radiolabelled nuclear phosphoproteins with unlabelled oligonucleotide probes in a PREMSA binding reaction permitted the distinguishing of phosphorylated transcription factors, a very

plausible factor modification as was discussed in the INTRODUCTION (87). These phosphoproteins which could be recognized by the A3 motif, and other binding sites within the GRE probe, may represent binding complexes undergoing activation and organization of other 'downstream' components of the 'general transcriptional machinery', such as TFIIB, TFIID, TBP, RNA polymerase II, etc, that require phosphorylation to initiate gene expression. The use of a DNA probe to recognize and 'pull down' proteins resembles the strategy of an immunoprecitation by replacing an antibody fixed to a bead. Indeed, the PREMSA may prove to be an important assay in the study of nuclear phosphoproteins, their binding to promoter and enhancer motifs, and activation mechanisms for transcription in a variety of cell types.

SDS-PAGE determination showed phosphoproteins of molecular weights comparable to phosphorylated IDX-1 (β-cell specific factor - 39-43 kDa), as well as histones (42-50 kDa) and CaMKII (57-60 kDa). However inconclusive the identity of specific substrates, this is the first reported action of CaMK phosphorylation or Ca^{2+}-sensitive kinases in the β-cell nucleus.

Clearly, the identification of specific phosphorylated transcription factor binding to these insulin enhancer core base

sequences is important for the understanding of the relevance of the observed binding complexes, i.e. bands. Attempts were made using factor-specific antibodies in the binding reaction that have been unsuccessful,, although altered bands have appeared. These studies have proven difficult in part due to the unavailability of purified antibodeis. Nevertheless, preliminary evidence not shown here has indicated the involvement of IDX-1 in complexes bound to the GRE, but this observation was not always reproducible. Super-shifting by retarding mobility of a distinct band has failed thus far with the PREMSA assay but some complex disruption due to antibody interaction with factor epitopes has occurred. Competition by identical oligonucleotides, as done with EMSA, cannot be performed since cold probes comprise PREMSA binding complexes.

III. ***In situ* stimulation of INS-1 β-cells by high (20 mM) glucose increased binding** of transcription factors to the GRE probe (which includes the cognate IDX-1 binding motif) (Fig.13 and 15). This important finding recapitulates a previous report of fresh extracts from rat pancreatic islets of Langerhans whose binding to the A3 portion of the GRE sequence of the human required incubation in 20 mM glucose medium (80). Since INS-1 extract

binding to the rat GRE base sequence responds to 20 mM glucose stimulation in an equivalent manner, the relevance of this experimental model of Ca^{2+}-sensitivity to human β-cell mechanisms, and biomedical significance is bolstered.

More remarkably, this binding activity increase was replicated partially in cells incubated in subphysiological glucose by addition of K^+ (Fig. 13) to the culture medium immediately prior to nuclear extraction confirming that plasma membrane depolarization and thus Ca^{2+} influx contribute to initiation of binding activity, in strong support of the hypothesis of this study. This depolarization-dependent Ca^{2+}-sensitivity was substantiated by reduction in binding activity when voltage-gated calcium channels (VDCC) were blocked by co-incubation with verapamil (Fig.15 - lane 7). Activation of 20 mM high glucose-incubated β-cell transcription factor binding to the GRE probe (Fig. 15 - lane 2) was also lowered to below low glucose (0.5 mM) baseline levels by co-incubation with KN-93 (Fig. 15 - lane 6), an inhibitor of the Ca^{2+}/Calmodulin-dependent family of kinases, including CaMK II and CaMK IV. No single band appears visibly

more or less sensitive to Ca^{2+} mediation when examining band inhibition by either verapamil (blocking of extracellular Ca^{2+} entry) or prevention of CaMKII, or possibly CaMK IV, activation by KN-93. Nonetheless, a pivotal mechanism of activation of transcription factors by phosphorylation necessary for increased binding activity is implied for at least some factor component of each complex. The transcription factor IDX-1 which is recognized by the A3 portion of the GRE probe used in these EMSA gel shift assays may be involved in both bands since interaction of an anti-IDX-1 antibody abolished both bands. This interpretation is supported by the competition with unlabelled probes of base sequences identical to either A3 and A3E2/GRE which blocked binding in both complexes. The model in Fig. 4 describes the postulated synergistic interaction between transcription factors binding to adjacent A-box and E-box elements as also illustrated in Fig. 2. Full response to glucose in the β-cell requires the combined A-box and E-box elements in the contiguous GRE (glucose-response-enhancer). Previous research has shown that failure to activate binding to either element, as well as disruption of their synergistic interaction, will result in dysfunction of insulin biosynthesis (50).

The fact that K^{+}-induced depolarization, and thus Ca^{2+} influx,

are only partially required for increased binding of β-cell transcription factors to their cognate sites is supported by the recent report of partial mediation of β-cell transcription factor IDX-1 (also called IUF-1) binding to the A3 portion of the human insulin promoter by stress-activated protein kinase 2 (SAPK2) (27). In that study, SAPK2 (also called p38 MAP kinase) was found to activate an endogenous IDX-1 activating enzyme which only indirectly increases IDX-1 binding in MIN6 cell extracts, leaving open the role of a possible Ca^{2+}-sensitive kinase activation as found in the present study. Significantly, the endogenous nuclear proteins in MIN6 extracts, which may contain CaM kinases, were required for this indirect activation by this MAP kinase of the trancription factor and insulin gene transcription.

Given that the initiation of mRNA encoding by DNA transcription may be mediated by a Ca^{2+} signal, as the findings of this report suggest, the failure in β-cell insulin biosynthesis associated with diabetes may result from disruption Ca^{2+}-dependent kinase or phosphatase modifications of transcription factors upon which tranactivation of the insulin enhancer depends.

FUTURE DIRECTIONS

Further research to clarify the determination of the activating mechanisms of insulin gene transcription can build on the findings of Ca^{2+}-sensitivity in the present report by applying the assessment of β-cell transcription factor binding to extracts from fresh rat islets or human islets. These sources of β-cell nuclear proteins will reflect more accurately the actual physiological responses of transcription factor-insulin enhancer binding activity to glucose and Ca^{2+} conditions. As Fig. 5 shows, the recognition of rat INS-1 insulinoma nuclear proteins by probes containing both human and rat GRE binding site sequences are very similar, presumably due to their high homology. However, the binding activity of human β-cell nuclear extracts to human insulin promoter sequence probes will provide more directly relevant data for β-cell function in human physiology, and application to understanding of diabetes in humans.

The following experiments are envisioned to better

characterize the finer details of a Ca^{2+} role in glucose-induced insulin biosynthesis in β-cells:

A. Identification of specific component transcription factors in discreet binding complexes

1. Differential disruption, or super shift in electrophoretic mobility, of individual complexes by transcription factor-specific antibodies, e.g. IDX-1, E47, Beta2, C/EBPβ etc. for factors in INS-1 nuclear extracts.

B. Differentiation of participant enhancer DNA sequences in discreet binding complexes

1. Differential competition by cold probes representing A box and/or E box consensus motifs in conventional EMSA binding using labelled probes

C. Determination of membrane depolarization parameters of binding stimulation

1. Comparison of dose-dependent binding activity mediation by higher K^+ concentrations

2. Comparison of binding activity mediation ionomycin and by other sulfonylurea drugs of differing modes of action.

To avoid preactivation of gene expression mechanisms, cells must be equilibrated in 5 mM glucose that approximate physiological concentrations (as in the present experiments) after culture and passage at 11.1 mM glucose. Glucose and Ca^{2+} conditions will be provided by a minimum of 4 hr incubation in low glucose (0.5 mM), low glucose replaced for the last 15 min by K^{+} (40 mM or experimentally varied), or high glucose (20 mM) with and without a VDCC blocker such as verapamil. Various kinase inhibitors, particularly KN-93 and other CaMK-specific inhibitors, as well as those specific for MAP kinases, may be used for co-incubation to analyze activation due to phosphorylation. Inhibitors of phosphatases, e.g. okadaic acid and calyculin A, may be included to distinguish mediation by dephosphorylation. Difficulty in distinguishing phosphatase from kinase requirements for mediation is anticipated for an excitable cell such as the β-cell which exhibits oscillations in intracellular calcium concentrations which can synchronize among populations of multiple cells. These fluctuations between high and low levels of Ca^{2+} cation concentrations may alternately activate phosphatases at low concentrations and kinases at higher levels, in effect priming amino acid phosphorylation sites by dephosphorylation just before kinase transfer of a phosphate

group which would require an opening.

Further delineation of Ca^{2+}-sensitivity of transcription activating mechanisms in β-cells can provide insights to improve treatment strategies for medical intervention in the pathology of β-cell dysfunction and diabetes. If a specific Ca^{2+}-dependent kinase is determined to be both necessary and sufficient for the activation of insulin biosynthesis, it may be possible to design a delivery system for expression ofthat protein, such as the currently developing adenovirus vectors, to increase quantities of the activator enzyme in the pancreatic islet. If the kinase is found to be necessary but not sufficient, possibly co-transfection with a vector expressing the glucose-sensitive transcription factor IDX-1, or a co-factor, which provides a substrate for activation by phosphorylation, could induce the insulin gene *in vivo* if successfully delivered to target islets and stimulate *de novo* biosynthesis. While efficient delivery of insulin-inducing agonists pose enormous obstacles, the detection of operators in the complex β-cell signal transduction cascades such as transducers of the Ca^{2+} signal will continue to be important goals for diabetic endocrine research.

BIBLIOGRAPHY

1. Myers MG Jr, White MF 1996 Insulin signal transduction and IRS proteins *Annu. Rev. Pharmacol. Toxicol.* 36:615-658

2. Gold G 1989 In*Insulin Secretion* , ed. Draznin B, Melmed S, LeRoith D, pp 25-35, Alan R Liss, New York

3. Eizirik DL, Korbutt GS, Hellerstrom C 1992 Prolonged exposure of human pancreatic islets to high glucose concentrations in vitro impairs the β-cell function. *J Clin Invest* 90:1263-1268

4. Bliss M 1982 *The Discovery of Insulin* The University of Chicago Press, Chicago

5. Birnbaum MJ. 1993 The insulin-sensitive glucose transporter *Intl Rev Cytol* 137:239-97

6. Devasker SU, Giddings SJ, Rajakumar PA, Carnaghi LR, Menon RK Zahm DS 1994 Insulin gene expression and insulin synthesis in mammalian neuronal cells *J Biol Chem* 269:8445-8454

7. Warram JH, Rich SS, Krowlewski AS 1995 Epidemiology and genetics of diabetes mellitus. In *Joslin's Diabetes Mellitus,* ed. CR

Kahn, GC Weir, pp.24-56, Williams and Wilkins, Baltimore

8. Garcia-Barrado MJ, Jonas JC, Henquin JC 1996 Sulfonylureas do not increase insulin secretion by a mechanism other than a rise in cytoplasmic Ca^{2+} in pancreatic β-cells *Eur J Pharmacol* 298:279-286

9. Saltiel AR, Olefsky JM 1996 Thiazolidinediones in the treatment of insulin resistance and type II diabetes *Diabetes* 45:1661-1669

10. Polonsky KS, Sturis J, Bell GI 1996 Non-insulin-dependent diabetes mellitus - a genetically programmed failure of the beta cell to compensate for insulin resistance *N Engl J of Medicine* 334:777783

11. Lernmark A 1995 Insulin dependent (type I) diabetes: etiology, pathogenesis, and natural history *Endocrinology* , ed. DeGroot LJ , WB Saunders, Philadelphia

12. Olefsky JM. 1995 Diabetes Mellitus (type II): etiology and pathogenesis *Endocrinology* , ed. Degroot LJ, WB Saunders, Philadelphia

13.Malaisse WJ, Senner A, Herchuelz A, Hutton JC 1979 Insulin release: the fuel hypothesis *Metabolism* 28:373-386

14. Halban PA 1991 Structural domains and molecular lifestyles of insulin and its precursors in the pancreatic beta cell *Diabetologia* 34:767-778

15. Rorsman P, Abrahamsson H, Gylfe E, Hellman 1984 Dual effects of glucose on the cytosolic Ca^{2+} activity of mouse pancreatic β-cells 1984 *FEBS Lett* 170:196-200

16. Grapengiesser E, Gylfe E, Hellman B 1992 Glucose sensing of individual pancreatic beta cells involves transitions between steady-state and oscillatory cytoplasmic Ca^{2+} *Cell Calcium* 13:219-226

17. Ashcroft FM, Rorsman P 1990 Electrophysiology of the pancreatic β-cell *Prog Biophys Mol Biol* 54:87-143

18. Cook DL, Hales CN 1984 Intracellular ATP directly blocks K^+ channels in pancreatic β-cells *Nature* 311:269-271

19. Efrat S, Surana M, Fleischer N 1991 Glucose induces insulin gene transcription in a murine pancreatic β-cell line *J Biol Chem* 66:11141-11143

20. German MS, Moss LG, Rutter WJ 1990 Regulation of insulin gene expression by glucose and calcium in transfected primary islet cultures *J Biol Chem* 265:22063-22066

21. Meglasson MD, Matschinsky FM 1986 Pancreatic islet glucose metabolism and regulation of insulin secretion *Diabetes Metab Rev* 2:163-214

22. Prentki M, Matschinsky FM 1987 Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion *Physiol Rev* 67:1185-1248

23. MacDonald MJ 1990 Elusive proximal signals of β-cell signal transduction *Diabetes* 39:1461-1466

24. Newgard CB, McGarry JD 1995 Metabolic coupling factors in pancreatic β-cell signal transduction *Annu Rev Biochem* 64:689-719

25. Howell SL, Taylor KW 1966 Effects of glucose concentration on incorporation of [3H]leucine into insulin using isolated human islets of Langerhans*Biochim Biophys Acta* 130:519-521

26. Steiner DF, Chan SJ, Welsh JM, Kwok SCK 1985 Structure and evolution of the insulin gene *Annu Rev Genet* 19:463-484

27. MacFarlane WM, Smith SB, James RFL, Clifton AD, Doza YN, Cohen P, Docherty K 1997 The p38/reactivating kinase mitogen-activated protein kinase cascade mediates the activation of the transcription factor insulin upstream factor 1 and insulin gene transcription by high glucose in pancreatic β-cells *J Biol Chem* 272:20936-20944

28. Goodison S, Kenna S, Ashcroft JH 1992 Control of insulin gene expression by glucose *Biochem J* 285:563-568

29.Philippe J, Pacheco I, Meda P 1994 Insulin gene transcription is decreased rapidly by lowering glucose concentrations in rat islet cells *Diabetes* 43:523-528

30. Easom RA, Filler NR, Tarpley J, Landt M 1997 Correlation of the activation of Ca^{2+}/calmodulin protein kinase II with the initiation of insulin secretion from perifused pancreatic islets *Endocrinology* 138:2359-2364

31. Krueger KA, Bhatt H, Landt M, Easom RA 1997 Calcium-stimulated phosphorylation of MAP-2 in pancreatic βTC3-cells is mediated by Ca^{2+}/calmodulin-dependent kinase II *J Biol Chem* 272:27464-2746

32. Babb EL, Tarpley J, Landt M, Easom RA 1996 Muscarinic activation of Ca^{2+}/calmodulin-dependent protein kinase II in pancreatic islets *Biochem J* 317:167-172

33. Roe MW, Lancaster ME, Mertz J, Worley III JF, Dukes ID 1993 Voltage-dependent intracellular calcium release from mouse islets stimulated by glucose *J Biol Chem* 68:9953-9956

34. Thorens B 1992 Molecular and cellular physiology of GLUT2, a high Km facilitated diffusion glucose transporter *Int Rev Cytol* 137A:209-237

35. Johnson JH, Newgard CB, Milburn JL, Lodish HF, Thoren B 1990 The high Km glucose transporter of islets of Langerhans is functionally similar to the low affinity transporter of liver and has an identical primary sequence *J Biol Chem* 265:6548-51

36. Lehninger AL, Nelson DL, Cox MM 1993 *Principles of Biochemistry* Worth Publishers, New York p468

37. Ammala C ,Ashcroft FM, Rorsman P 1993 Calcium-independent potentiation of insulin release by cAMP in single beta cells *Nature* 363: 356-358

38. Easom RA, Landt M, Colca JR, Hughes JH, Turk J, McDaniel ML 1990 Effects of insulin secretagogues on protein kinases in isolated islets *J Biol Chem* 265:14938-14946

39. Civelek VN, Deeney JT, Fusonie GE, Corkey BE ,Tornheim K 1997 Oscillation in oxygen consumption by permeabilized clonal pancreatic β-cells (HIT) incubated in an oscillating glycolyzing muscle extract *Diabetes* 46:51-56

40. Henquin JC 1992 Adenosine triphosphate-sensitive K^+ channels may not be the sole regulators of glucose-induced electrical activity in pancreatic B-cells *Endocrinology* 131:127-31

41. Ashcroft FM, Kelly RP, Smith PA 1990 Two types of Ca^{2+} channels in rat pancreatic β-cells *Pflugers Arch* 415:504-6

42. Krausz Y, Eylon L, Cerasi E 1987 Calcium-binding proteins and insulin release: different effects of phenothiazines on first and second phase secretion and on islet cAMP response to glucose *Acta Endocrinol* (Copenh) 116:241-6

43. Docherty K, Clark AR 1994 Nutrient regulation of insulin gene expression *FASEB J* 8:20-27

44. Efrat S, Leiser M, Wu Y-J, Fusco-DeMane P, Emron OA, Wang X 1995 Conditional transformation of a pancreatic β-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene *Proc Natl Acad Sci USA* 91:2051-2055

45 Johnson PF, McKnight SL 1989 Eukaryotic transcriptional regulatory proteins *Annu Rev of Biochem* 58:799-839

46. Edlund T, Walker MD, Barr PJ, Rutter WJ 1985 Cell-specific expression of the rat insulin gene: evidence for the role of two distinct 5' flanking sequences *Science* 230:912-916

47. Boam DS, Clark AR, Docherty K 1990 Positive and negative regulation of the insulin gene by multiple trans-acting factors *J Biol Chem* 265:8285-8296

48. Nir U, Walker MD, Rutter WJ 1986 Regulation of the rat insulin 1 gene expression: evidence for negative regulation in non-pancreatic

cells *Proc Natl Acad Sci USA* 83:3180-3184

49. Laimins L, Holmgren-Konig M, Khoury G 1986 Transcriptional 'silencer' element in rat repetitive sequences associated with the rat insulin 1 gene locus *Proc Nat Acad Sci USA* 83:3151-3155

50. Lu M, Seufert J, Habener JF 1997 Pancreatic β-Cell-specific Repression of Insulin Gene Transcription by CCAAT/Enhancer-binding Protein β *J Biol Chem* 272:28349-28359

51. Hanahan D 1985 Heritable formation of pancreatic β-cell tumors in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes *Nature* 315:115-122

52. Walker MD, Edlund T, Boulet AM Rutter WJ 1983 Cell-specific expression controlled by the 5' flanking regions of the insulin and chymotrypsin genes *Nature* 306:557-581

53. Whelan J, Poon D, Weil PA, Stein R 1989 Pancreatic β-cell-type-specific expression of the rat insulin II gene is controlled by positive and negative transcriptional control elements *Mol Cell Biol* 9:3253-3259

54. Crowe DT, Tsai M-J 1989 Mutagenesis of the rat insulin II 5' flanking region defines sequences important for expression in HIT-cells *Mol Cell Biol* 9:1784-1789

55. Ohlsson H, Edlund T 1986 Sequence-specific interaction of nuclear factors with the insulin gene enhancer *Cell* 45:35-44

56. Ohlsson H, Karlsson O, Edlund T 1988 A beta-cell-specific protein binds to the two major regulatory sequences of the insulin gene enhancer *Proc Natl Acad Sci USA* 85:4228-4231

57. Dandoy-Dron F, Monthioux E, Jami J, Bucchini D 1991 Regulatory regions of the rat insulin 1 gene necessary for expression in transgenic mice *Nucleic Acids Res* 18:4925-4930

58. Murre C, McCaw PS, Baltimore 1989 A new DNA binding and dimerization motif in immunoglobin enhancer binding, daughterless, MyoD, and myc proteins *Cell* 56:777-783

59. Peyton M, Moss LG, Tsai M-J 1994 Two distinct class A helix-loop-helix transcription factors, E2A and BETA1, form separate DNA binding complexes on the insulin gene E box *J Biol Chem* 269:25936-25941

60. Karlsson O, Thor S, Norberg T, Ohlsson H, Edlund T 1990 Insulin gene enhancer binding protein isl-1 is a member of a novel class of proteins containing a Homeo- and Cys-His domain *Nature* 344:879-882

61. German M, Chang J, Chadwick RB, Rutter WJ 1992 Synergistic activation of the insulin gene by a LIM-Homeo-domain protein and a

bHLH protein; building a functional minienhancer complex *Genes Dev* 6:2165-2176

62. German M, Ashcroft S, Docherty K, Edlund H, Edlund T, Goodison S, Imura H, Kennedy G, Madsen O, Melloul D, Moss L, Olson LK, Permutt MA, Phillipe J, Robertson RP, Rutter WJ, Serup P, Stein R, Steiner D, Tsai M-J, Walker MD 1995 The insulin promoter: a simplified nomenclature *Diabetes* 44:1002-1004

63. Boam DS, Docherty K 1989 A tissue specific nuclear factor binds to multiple sites in the human insulin gene enhancer *Biochem J* 264:233-239

64. Ohlsson H, Karlsson K, Edlund T 1993 IPF-1, a homeodomain containing transactivator of the insulin gene *EMBO J* 12:4251-4259

65. Leonard J, Peer B, Johnson T, Ferreri K, Lee S, Montminy M 1993 Characterization of somatostatin transactivating factor-STF-1: a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells *Mol Endocrinol* 7:1275-1283

66. Miller CP, McGehee Jr RE, Habener JF 1994 IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene *EMBO J* 13:1145-1156

67. Lu M, Miller CP, Habener JF 1996 Functional regions of the

homeodomain protein IDX-1 required for transactivation of the rat somatostatin gene *Endocrinology* 137:2959-2967

68. Peers B, Leonard J, Sharma S, Teitelman G, Montminy MR 1994 Insulin expression in pancreatic islets cells reliews on cooperative interactions between the helix lop helix factor E47 and the homeobox factor STF-1 *Mol Endocrinol* 8:1798-1806

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

70. Jonsson J, Carlsson L, Edlund H, Edlund T 1994 Insulin-promoter-factor 1 is required for pancreas development in mice *Nature* 371:606-609

71. Guz Y, Montminy MR, Stein R, Leonard J, Garner LW, Wright CVE, Teitelman G 1995 Expression of murine Pdx1, a putative insulin gene transcription factor, in β-cells of pancreas, duodenal epithelium, and pancreatic exocrine and endocrine progenitors during ontogeny *Development* 121:11-18

72. Offeld MF, Jetton TL, Labosky PA, Ray M, Stein R, Magnuson MA, Hogan BLM, Wright CVE 1996 PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum *Development* 122:983-995

73. Petersen HV, Serup P, Leonard J, Michelsen BK, Madsen OD 1994 Transcriptional regulation of the human insulin gene is dependent on the homeodomain protein STF1/IPF1 acting through CT boxes *Proc Natl Acad Sci USA* 91: 10465-10469

74. Melloul D, Ben-Neriah Y, Cerasi E 1993 Glucose modulates the binding of an islet-specific factor to a conserved sequence within the rat I and the human insulin promoters *Proc Natl Acad Sci USA* 90: 3865-3869

75. German MS 1993 Glucose sensing in pancreatic islet beta cells: the key role of glucokinase and glycolytic intermediates *Proc Natl Acad Sci* 90:1781-1785

76. German MS, Wang J 1994 The insulin gene contains multiple transcriptional elements that respond to glucose *Mol Cell Biol* 14:4067-4074

77. Philippe J, Missotten M 1990 Functionial characterization of a cAMP-responsive element of the rat insulin 1 gene *J Biol Chem* 265:1465-1469

78. Karlsson O, Walker MD, Rutter WJ, Edlund T 1989 Individual protein-binding domains of the insulin gene enhancer positively activate β-cell specific transcription *Mol Cell Biol* 9:823-827

79. German MS, Moss LG, Wang J, Rutter WJ 1992 The insulin and amyloid polypeptide genes contain similar cell-specific promoter elements that bind identical β-cell nuclear complexes *Mol Cell Biol* 12:17778-17788

80. MacFarlane WM, Read ML, Gilligan M. Bujalska I, Docherty K 1994 Glucose modulates the binding activity of the β-cell transcription factor IUF1 in a phosphorylation-dependent manner *Biochem J* 303:625-631

81. Serup P, Petersen HV, Petersen EE, Edlund H, Leonard J, Petersen JS, Larsson L-I, Madsen OD 1995 The homeodomain protein IPF1/STF1 is expressed in a subset of islet cells and promotes rat insulin 1 gene expression dependent on an intact E1 helix-loop-helix factor binding site *Biochem J* 310:997-1003

82. Serup P, Jansen J, Andersen F, Jorgensen MC, Blume N, Holst JJ, Madsen OD 1996 Induction of insulin and islet amyloid polypeptide production in pancreatic islet glucagonoma cells by insulin promoter factor 1 *Proc Natl Acad Sci USA* 93:9015-9020

83. Olson LK, Sharma S, Peshavaria M, Wright CVE, Towle HC, Robertson RP, Stein R 1995 Reduction of insulin gene transcription in HIT-T15 β–cells chronically exposed to a supraphysiologic glucose concentration is associated with loss of STF-1 transcription factor expression *Proc Natl Acad Sci USA* 92:9127-9131

84. Zangen DH, Bonner-Weir S, Lee CH, Latimer JB, Miller CP, Habener JF, Weir GC 1997 Reduced insulin, GLUT2 and IDX-1 in β-cells after partial pancreatectomy *Diabetes* 46:258-264

85. Schulman H, Hanson P 1993 Multifunctional Ca^{2+}/calmodulin-dependent protein kinase *Neurochem Res* 18:65-77

86. Batchelor AH, Piper DE, Brousse FC, McKnight SL, Wolberger C 1998 The structure of GABPα/β: An ETS domain-ankyrin repeat heterodimer bound to DNA *Science* 279:1037-1041

87. Hunter T, Karin M 1992 The regulation of transcription by phosphorylation *Cell* 70:375-387

88. Olson LK, Redmon JB, Towle HC, Robertson RP 1993 Chronic exposure of HIT cells to high glucose concentrations paradoxically decreases insulin gene transcription and alters binding of insulin gene regulatory protein *J Clin Invest* 92:514-519

89. Jonas JC, Li G, Palmer M, Weller U, Wollheim CB 1994 Dynamics of Ca^{2+} and guanosine 5'-[gamma-thio] triophosphate action on insulin secretion from alpha-toxin-permeabilized HIT-T15 cells *Biochem J* 301:523-529

90. Bers, DM 1982 A simple method for the accurate determination of free[Ca] in Ca-EGTA solutions *Amer J Physiol* 242:C404-C408

91. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal Biochem* 72:248-254

92. Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei *Nucleic Acids Res* 11:1475-1489

93. Garner MM, Revzin A 1981 A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system *Nucleic Acids Res* 9:3047-3060

94. Olson LK, Qian J, Poitout V 1998 Glucose rapidly and reversibly decreases INS-1 cell insulin gene transcription via decrements in STF-1 and C1 activator transcription factor activity *Mol Endocrinology* 12:207-219