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Compositions and methods for detecting intracellular glucose and analogs thereof

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US008008071B2

(12) **United States Patent**
Cooper et al.(10) **Patent No.:** **US 8,008,071 B2**
(45) **Date of Patent:** ***Aug. 30, 2011**(54) **COMPOSITIONS AND METHODS FOR
DETECTING INTRACELLULAR GLUCOSE
AND ANALOGS THEREOF**(75) Inventors: **Denise R. Cooper**, St. Petersburg, FL
(US); **Niketa A. Patel**, Wesley Chapel,
FL (US)(73) Assignee: **University of South Florida**, Tampa, FL
(US)(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 1210 days.This patent is subject to a terminal dis-
claimer.(21) Appl. No.: **11/054,024**(22) Filed: **Feb. 8, 2005**(65) **Prior Publication Data**

US 2005/0197311 A1 Sep. 8, 2005

Related U.S. Application Data(63) Continuation-in-part of application No. 09/435,471,
filed on Nov. 8, 1999, now Pat. No. 6,852,529.(51) **Int. Cl.****C12N 5/00** (2006.01)**C12N 15/00** (2006.01)**C07H 21/04** (2006.01)(52) **U.S. Cl.** **435/325**; 435/320.1; 435/455;
536/24.1; 536/24.31(58) **Field of Classification Search** None
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**5,703,054 A 12/1997 Bennett et al.
5,795,961 A 8/1998 Wallace et al.
6,852,529 B1 * 2/2005 Cooper et al. 435/325**OTHER PUBLICATIONS**GenBank Accession No. AA875851 (Mar. 25, 1998) alignment with
SEQ ID No. 9.*Amara, F.M. et al. "Defining a Novel *cis*-Element in the
3'-Untranslated Region of Mammalian Ribonucleotide Reductase
Component R2 mRNA" *J. Biol. Chem.*, Aug. 1996, 271(33):20126-
20131.Blobe, G.C. et al. "Protein Kinase C β II Specifically Binds to and Is
Activated by F-actin" *J. Biol. Chem.*, Jun. 1996, 271(26):15823-
15830.Cañete-Soler, R. et al. "Characterization of Ribonucleoprotein Com-
plexes and Their Binding Sites on the Neurofilament Light Subunit
mRNA" *J. Biol. Chem.*, May 1998, 273(20):12655-12661.Chalfant, C.E. et al. "Regulation of Alternative Splicing of Protein
Kinase C β by Insulin" *J. Biol. Chem.*, Jun. 1995, 270(22):13326-
13332.Hara, K. et al. "1-Phosphatidylinositol 3-kinase activity is required
for insulin-stimulated glucose transport but not for RAS activation in
CHO cells" *Proc. Natl. Acad. Sci., USA*, Aug. 1994, 91:7415-7419.McClain, D.A. et al. "Glucose and glucosamine regulate growth
factor gene expression in vascular smooth muscle cells" *Proc. Natl.
Acad. Sci., USA*, Sep. 1992, 89:8150-8154.Nakshatri, H. and P. Bhat-Nakshatri "Multiple parameters determine
the specificity of transcriptional response by nuclear receptors HNF-
4, Arp-1, PPAR, RAR and RXR through common response ele-
ments" *Nucleic Acids Res.*, 1998, 26(10):2491-2499.Niino, Y.S. et al. "Positive and Negative Regulation of the Transcrip-
tion of the Human Protein Kinase C β Gene" *J. Biol. Chem.*, Mar.
1992, 267(9):61513-6163.Obeid, L.M. et al. "Cloning and Characterization of the Major Pro-
moter of the Human Protein Kinase C β Gene" *J. Biol. Chem.*, Oct.
1992, 267(29):20804-20810.Palmer, R.H. et al. "Activation of PRK1 by Phosphatidylinositol
4,5-Bisphosphate and Phosphatidylinositol 3,4,5-Trisphosphate" *J.
Biol. Chem.*, Sep. 1995, 270(38):22412-22416.Prokipcak, R.D. et al. "Purification and Properties of a Protein That
Binds to the C-terminal Coding Region of Human *c-myc* mRNA" *J.
Biol. Chem.*, Mar. 1994, 269(12):9261-9269.Quilliam, L.A. et al. "Isolation of a NCK-associated Kinase, PRK2,
an SH3-binding Protein and Potential Effector of Rho Protein Sig-
naling" *J. Biol. Chem.*, Nov. 1996, 271(46):28772-28776.Ron, D. et al. "Cloning of an intracellular receptor for protein kinase
C: A homolog of the β subunit of G proteins" *Proc. Natl. Acad. Sci.
USA*, Feb. 1994, 91:839-843.Ross, J. "mRNA Stability in Mammalian Cells" *Microbiology
Reviews*, Sep. 1995, 59(3):423-450.Sayeski, P.P. and J.E. Kudlow "Glucose Metabolism to Glucosamine
Is Necessary for Glucose Stimulation of Transforming Growth Fac-
tor- α Gene Transcription" *J. Biol. Chem.*, Jun. 1996, 271(25): 15237-
15243.Vincent, S. and Settleman, J. "The PRK2 Kinase Is a Potential Effec-
tor Target of both Rho and Rac GTPases and Regulates Actin
Cytoskeletal Organization" *Mol. Cell. Biol.*, Apr. 1997, 17(4):2247-
2256.

(Continued)

Primary Examiner — Anne-Marie Falk

(74) Attorney, Agent, or Firm — Saliwanchik, Lloyd &
Eisenschenk

(57)

ABSTRACT

The subject invention pertains to nucleic acid constructs for post-transcriptional control of expression of a polynucleotide encoding a protein in a cell, wherein the constructs include a metabolite responsive instability element such as the glucose-regulated mRNA instability element. The subject invention further pertains to host cells and vectors comprising the nucleic acid constructs of the invention, as well as probes, methods, and kits for detecting metabolite responsive instability elements or mutations thereof. The present invention further concerns a reporter vector useful for detecting intra-cellular glucose and glucose-analogs, host cells genetically modified with the reporter vector, and methods for detecting intracellular glucose. The present invention utilizes an element that regulates messenger RNA (mRNA) stability in response to a metabolite such as glucose or a glucose analog. This glucose-regulated mRNA instability element has been mapped to the protein kinase C β II (PKC β II) mRNA that was found to decrease in the presence of elevated glucose levels. When cloned into a reporter vector, the region of PKC β II containing the mRNA instability element imparts glucose-sensitive instability to the mRNA that is transcribed, thereby down-regulating the expression of the reporter gene when glucose is elevated. The reporter vector of the present inven-tion may be introduced into host cells, allowing detection of intracellular glucose and glucose analogs within intact, living cells in real-time and, optionally, in a high-throughput format.

OTHER PUBLICATIONS

- Amano, M. et al. "Identification of a Putative Target for Rho as the Serine-Threonine Kinase Protein Kinase N" *Science*, Feb. 1996, 271(5249):648-650.
- Amara, F.M. et al. "A *cis-trans* Interaction at the 3'-Untranslated Region of Ribonucleotide Reductase mRNA Is Regulated by TGF- β_1 , TGF- β_2 , and TGF- β_3 " *Biochem. and Biophysical Res. Comm.*, 1996, 228:347-351.
- Benbow, U. and C.E. Brinckerhoff "The AP-1 Site and MMP Gene Regulation: What Is All the Fuss About?" *Matrix Biology*, 1997, 15:519-526.
- Bernstein, P.L. et al. "Control of c-myc mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant" *Genes Dev.*, 1992, 6:642-654.
- Chung, J. et al. "PDGF- and insulin-dependent pp70^{S6K} activation mediated by phosphatidylinositol-3-OH kinase" *Nature*, Jul. 1994, 370:71-75.
- Hansen, W.R. et al. "The 3'-nontranslated region of rat renal glutaminase mRNA contains a pH-responsive stability element" *Amer. J. Physiology*, Jul. 1996, 271:F126-F131.
- Jia, Z. "Protein phosphatases: structures and implications" *Biochem. Cell Biol.*, 1997, 75:17-26.
- Kemp, B.E. and R.B. Pearson "Protein kinase recognition sequence motifs" *Trends Biochem. Sci.*, Sep. 1990, 15(9):342-346.
- Mukai, H. and Y. Ono "A Novel Protein Kinase with Leucine Zipper-Like Sequences: Its Catalytic Domain is Highly Homologous to That of Protein Kinase C" *Biochem. Biophys. Res. Comm.*, Mar. 1994, 199(2):897-904.
- Newton, a.C. "Regulation of protein kinase C" *Curr. Opin. Cell Biol.*, 1997, 9:161-167.
- Nishizuka, Y. "Studies and Perspectives of Protein Kinase C" *Science*, Jul. 1986, 233(4761):305-312.
- Nishizuka, Y. "Intracellular Signaling by Hydrolysis of Phospholipids and Activation of Protein Kinase C" *Science*, Oct. 1992, 258(5082):607-614.
- Nishizuka, Y. "Protein kinase C and lipid signaling for sustained cellular responses" *Faseb J.*, 1995, 9:484-496.
- Orehov, A.N. et al. "Cellular Composition of Atherosclerotic and Uninvolved Human Aortic Subendothelial Intima" *Am J. Pathol.*, 1984, 115:17-24.
- Palmer, R.H. and P.J. Parker "Expression, purification and characterization of the ubiquitous protein kinase C-related kinase I" *Biochem. J.*, 1995, 309:315-320.
- Patel, N.A. et al. "Acute hyperglycemia regulates transcription and posttranscriptional stability of PKC β II mRNA in vascular smooth muscle cells" *Faseb J.*, 1999, 13:103-113.
- Perrotti, D. et al. "TLS/FUS, a pro-oncogene involved in multiple chromosomal translocations, is a novel regulator of BCR/ABL-mediated leukemogenesis" *The EMBO J.*, 1998, 17(15):4442-4455.
- Prekeris, R. et al. "Identification and Localization of an Actin-binding Motif That Is Unique to the Epsilon Isoform of Protein Kinase C and Participates in the Regulation of Synaptic Function" *J. Cell Biol.*, Jan. 1996, 132(1-2):77-90.
- Ridley, A.J. et al. "The Small GTP-Binding Protein rac Regulates Growth Factor-Induced Membrane Ruffling" *Cell*, Aug. 1992, 70:401-410.
- Ross, R. "The Pathogenesis of Atherosclerosis—An Update" *N. Eng. J. Med.*, Feb. 1986, 314(8):488-500.
- Santen, R.J. et al. "Atherosclerosis in Diabetes Mellitus. Correlations With Serum Lipid Levels, Adiposity, and Serum Insulin Level" *Arch. Intern. Med.*, Dec. 1972, 130(6):833-843.
- Schwartz, S.M. et al. "Replication of Smooth Muscle Cells in Vascular Disease" *Circ. Res.*, Apr. 1986, 58(4):427-444.
- Shiba, T. et al. "Characterization of the Activation of Protein Kinase C Isoenzymes in the Retina of Diabetic Rats" *Diabetes*, 1990, 39(Supplement 1):31A. Abstract No. 123.
- Takai, Y. et al. "Studies on a Cyclic Nucleotide-independent Protein Kinase and Its Proenzyme in Mammalian Tissues" *J. Biol. Chem.*, Nov. 1977, 252(21):7603-7609.
- Watanabe, G. "Protein Kinase N (PKN) and PKN-Related Protein Rhophilin as Targets of Small GTPase Rho" *Science*, Feb. 1996, 271(5249):645-648.
- Patel, N.A. et al. "The protein kinase C β II exon confers mRNA instability in the presence of high glucose concentrations" *J. Biol. Chem.*, 2003, 278(2):1149-1157.
- Patel, N.A. et al. "Phosphoinositide 3-kinase mediates protein kinase C β II mRNA destabilization in rat A10 smooth muscle cell cultures exposed to high glucose" *Arch. Biochem. Biophys.*, 2002, 403:111-120.
- Yamamoto, M. et al. "A shift from normal to high glucose levels stimulates cell proliferation in drug sensitive MCF-7 human breast cancer cells but not in multidrug resistant MCF-7/ADR cells which overproduce PKC- β II" *Int. J. Cancer*, 1999, 83(1):98-106.
- Benjamin, D. et al. "A GFP-based assay for rapid screening of compounds affecting ARE-dependent mRNA turnover" *Nucleic Acids. Res.*, 2004, 32(11):1-8.
- Chalfant, C. et al. "Insulin regulates protein kinase C β II expression through enhanced exon inclusion in L6 skeletal muscle cells" *J. Biol. Chem.*, 1998, 273(2):910-916.
- Patel, N. et al. "The protein kinase C β II exon confers mRNA instability in the presence of high glucose concentrations" *J. Biol. Chem.*, 2003, 278(2):1149-1157.
- Patel, N. et al. "Insulin regulates alternative splicing of protein kinase C β II through a phosphatidylinositol 3-kinase-dependent pathway involving the nuclear serine/arginine-rich splicing factor, SRp40, in skeletal muscle cells" *J. Biol. Chem.*, 2001, 276(25):22648-22654.
- Yamamoto, M. et al. "Acute glucose-induced downregulation of PKC- β II accelerates cultured VSMC proliferation" *Am. J. Physiol. Cell Physiol.*, 2000, 279:C587-0595.

* cited by examiner

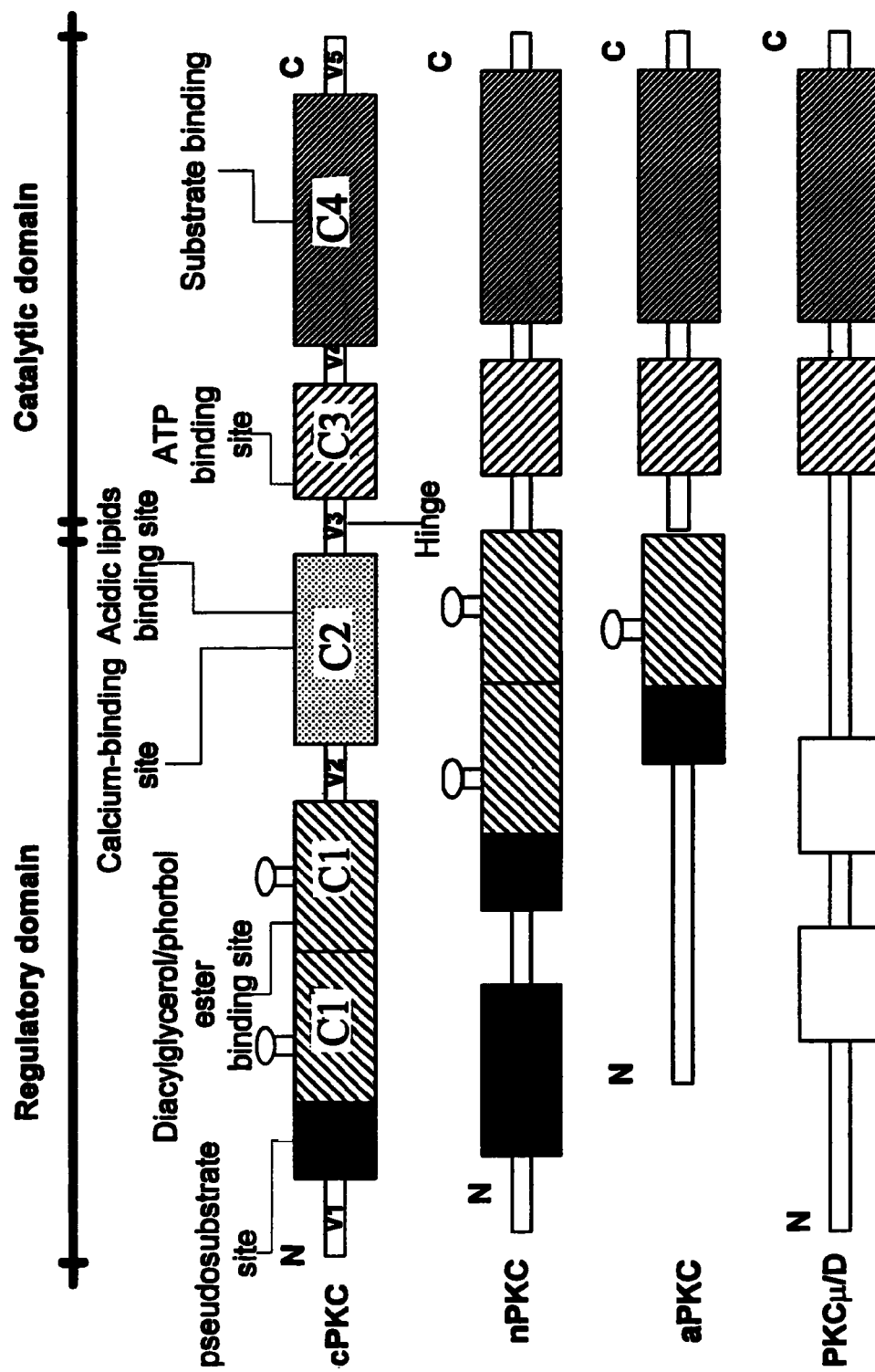
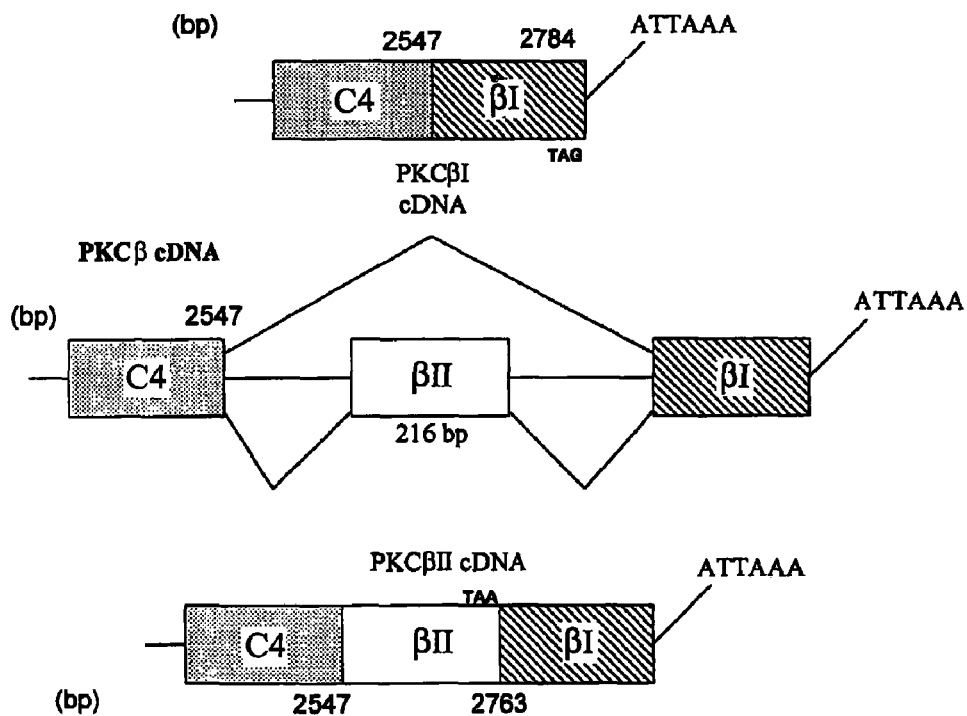
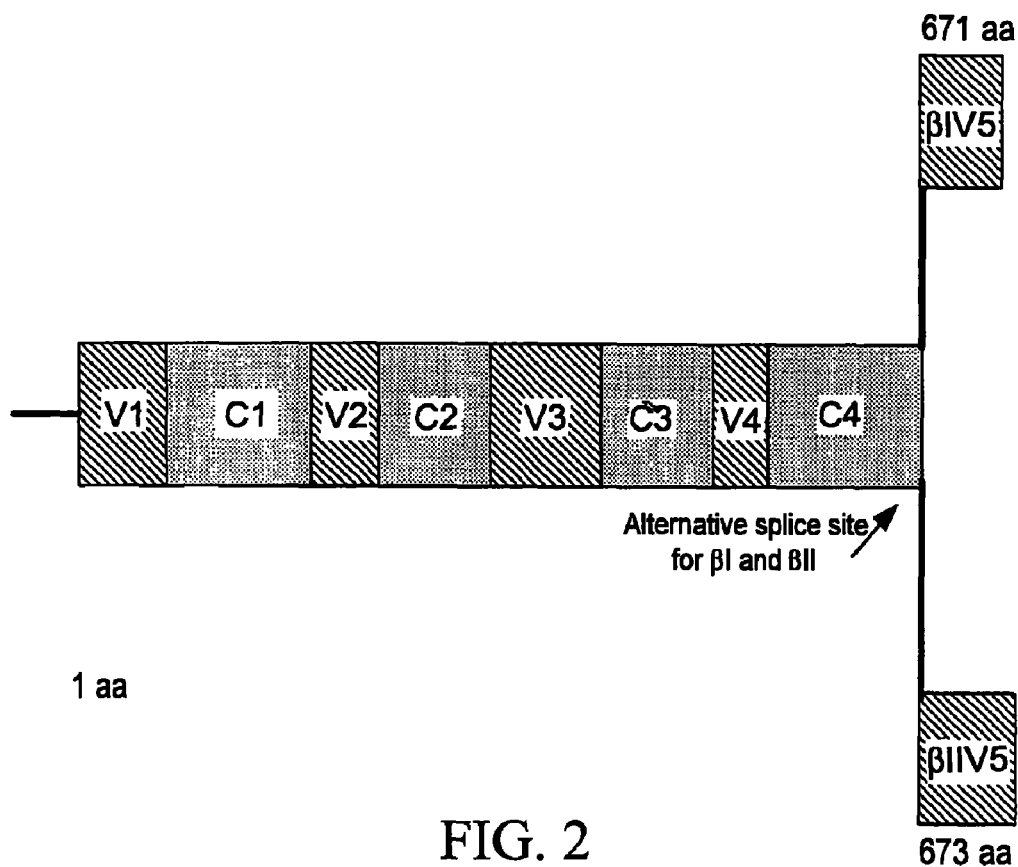


FIG. 1



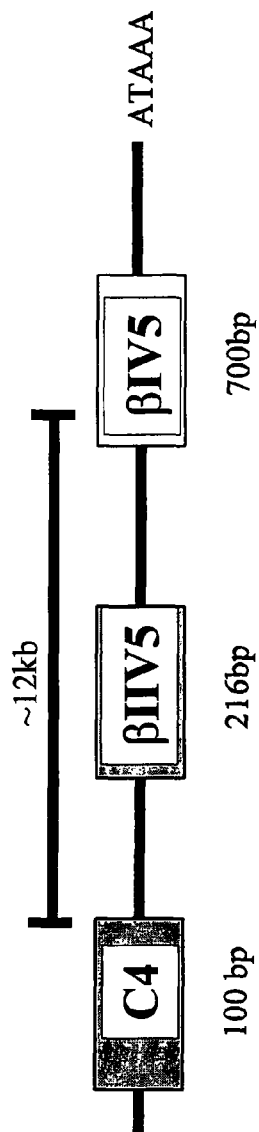


FIG. 3A

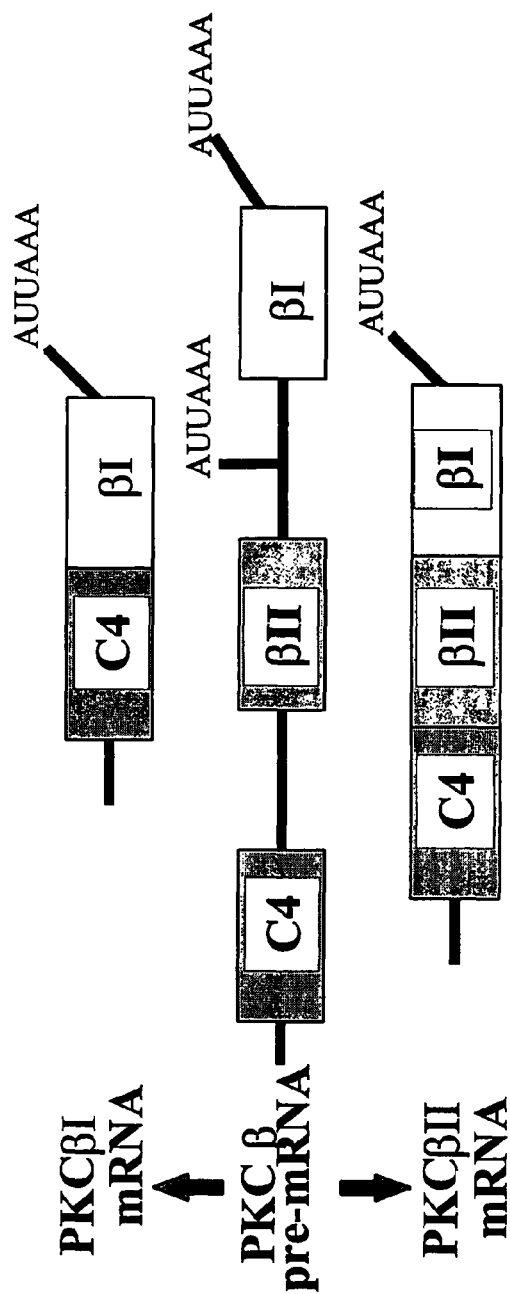


FIG. 3B

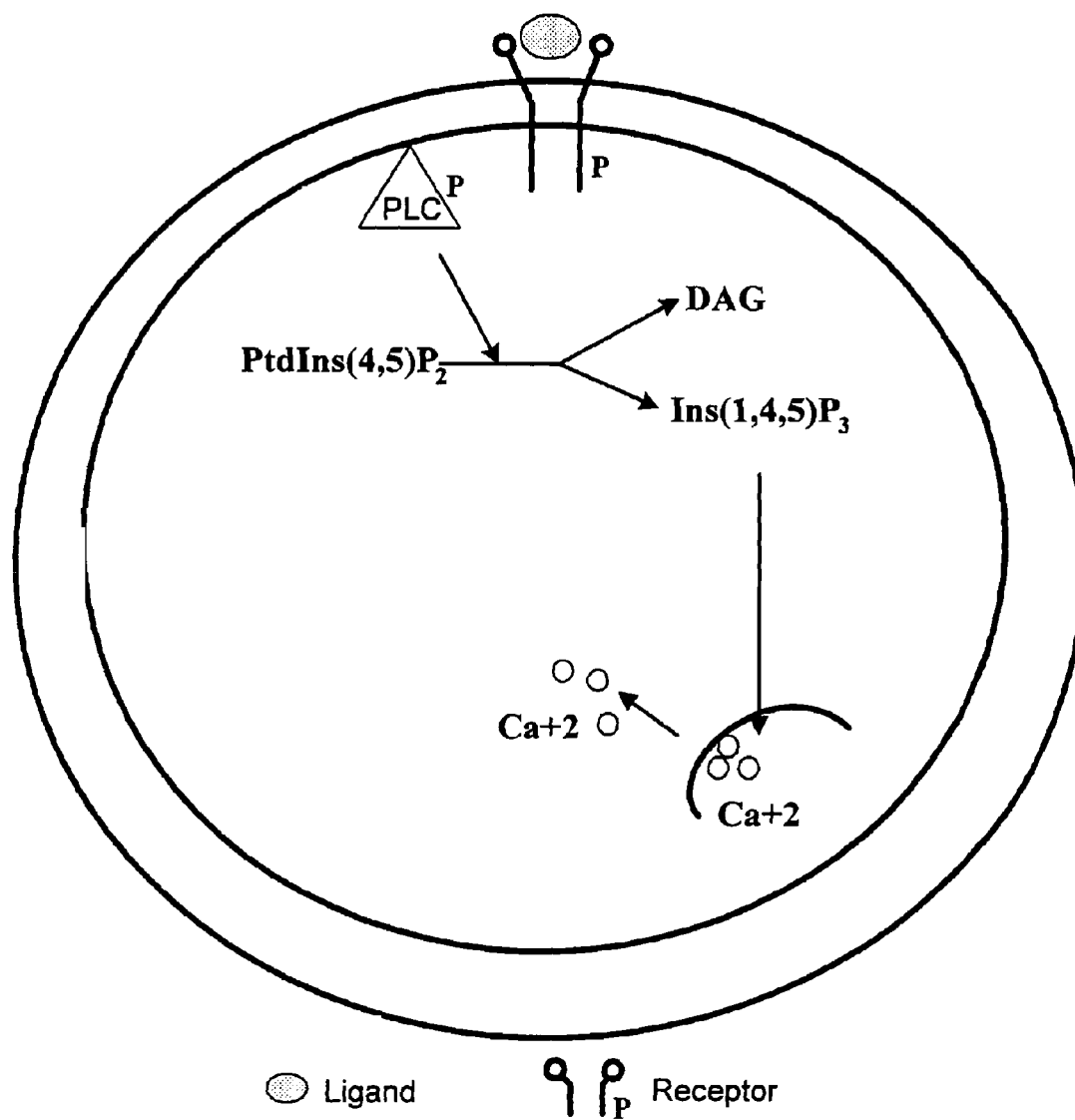


FIG. 5

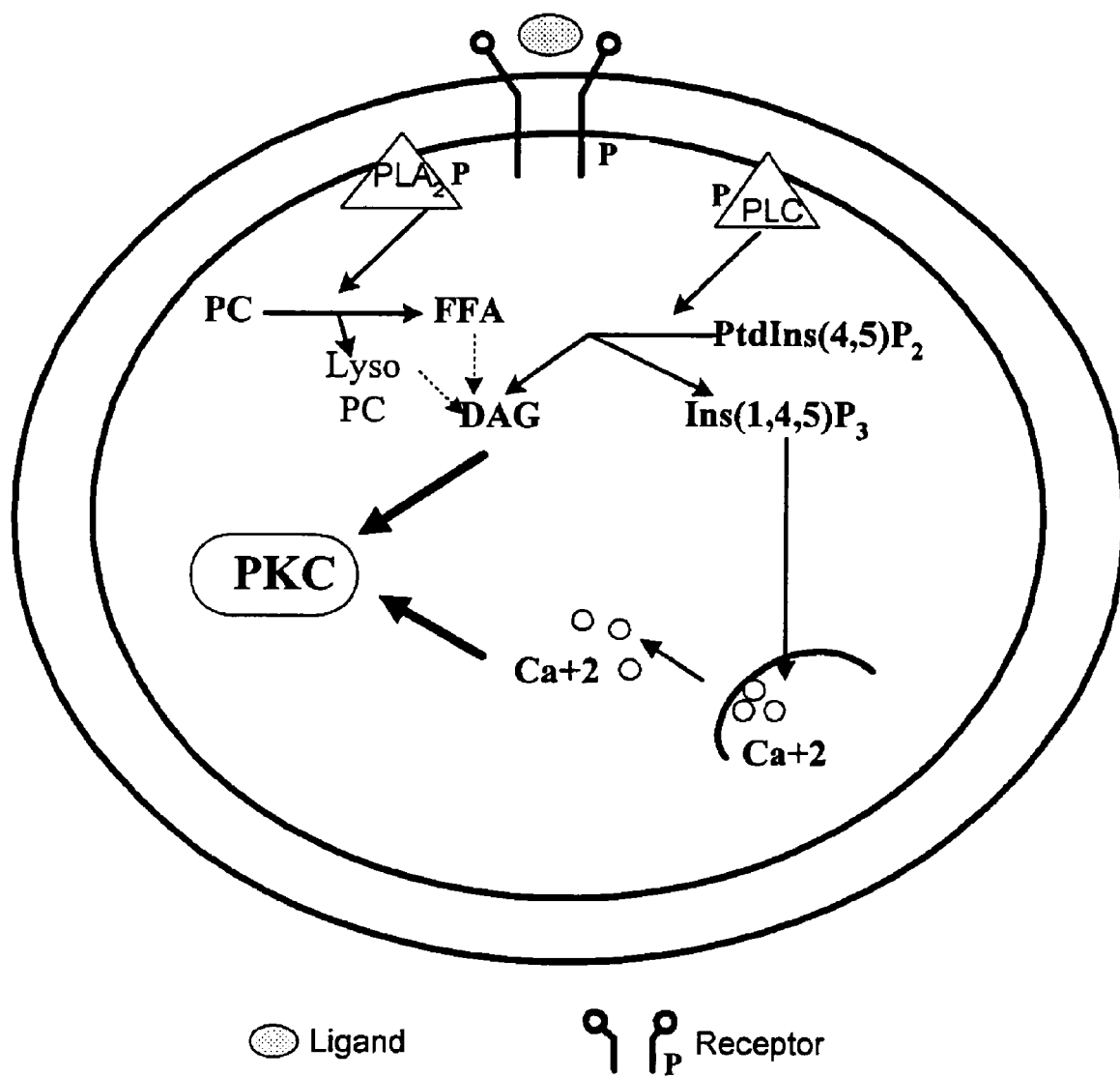
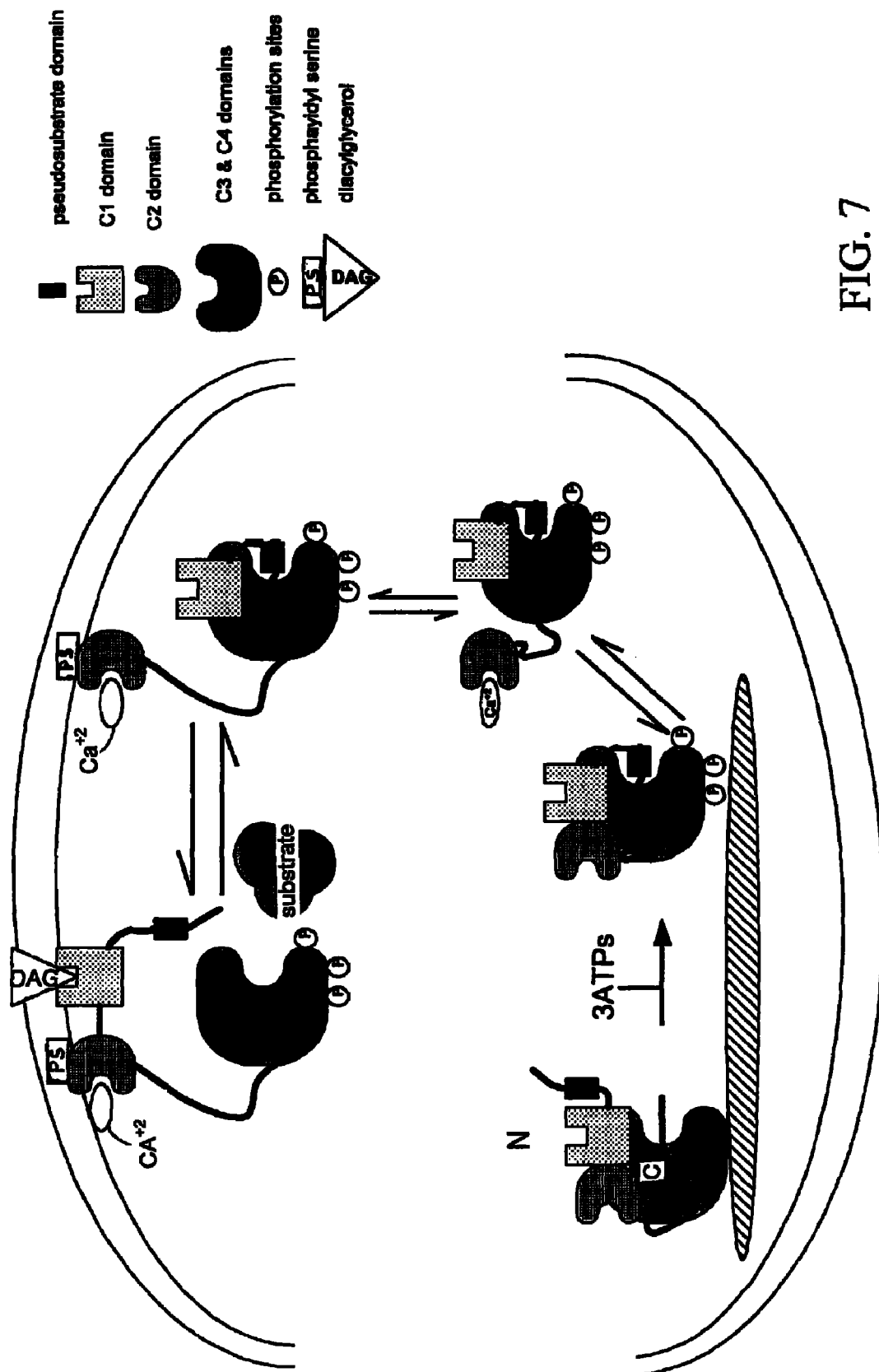


FIG. 6



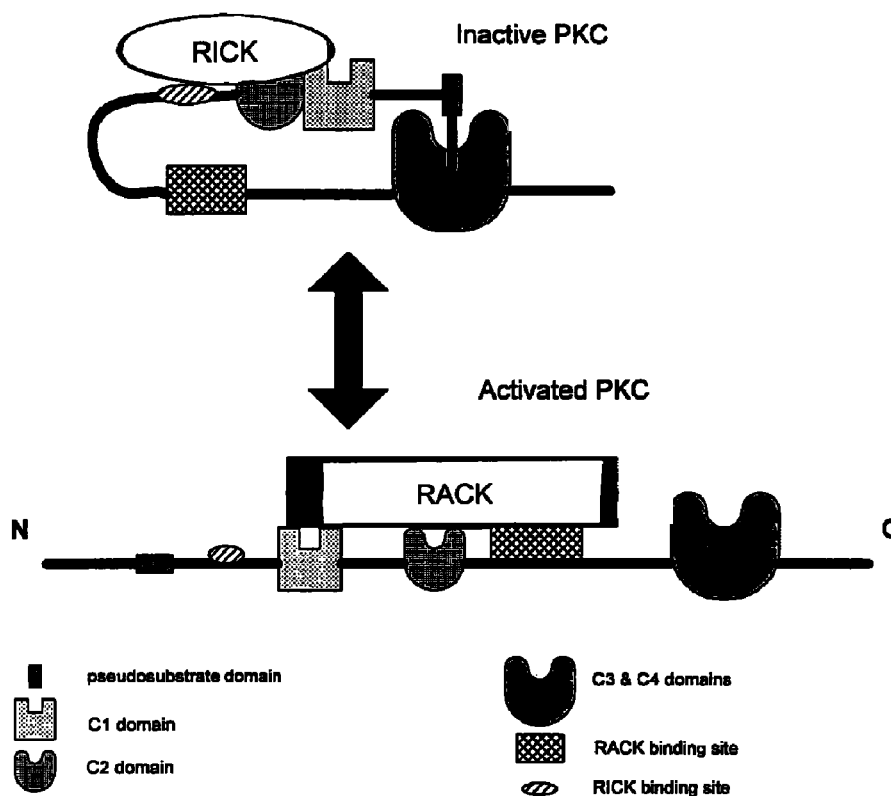


FIG. 8

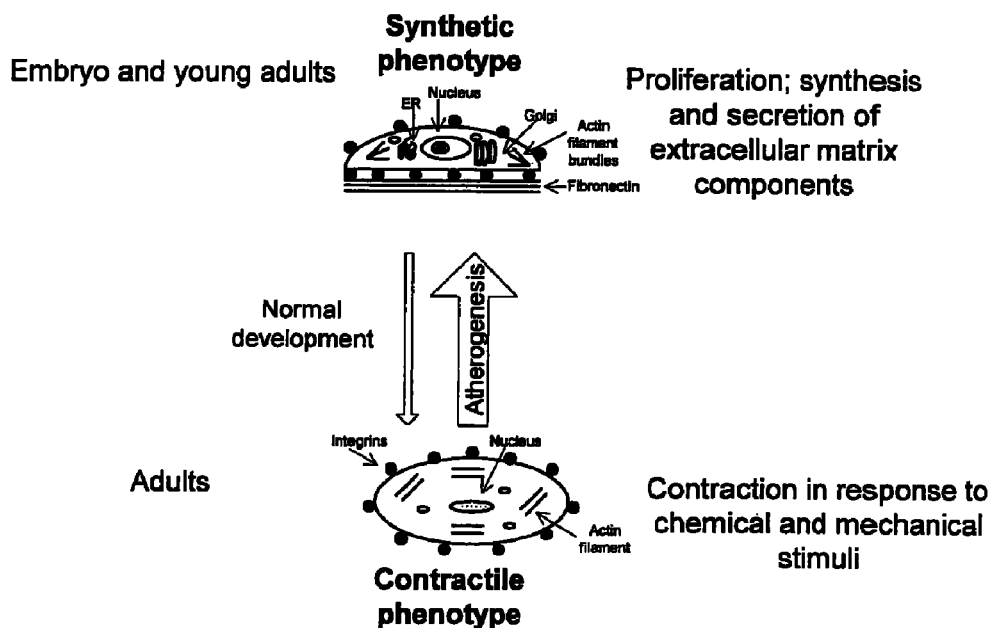


FIG. 9

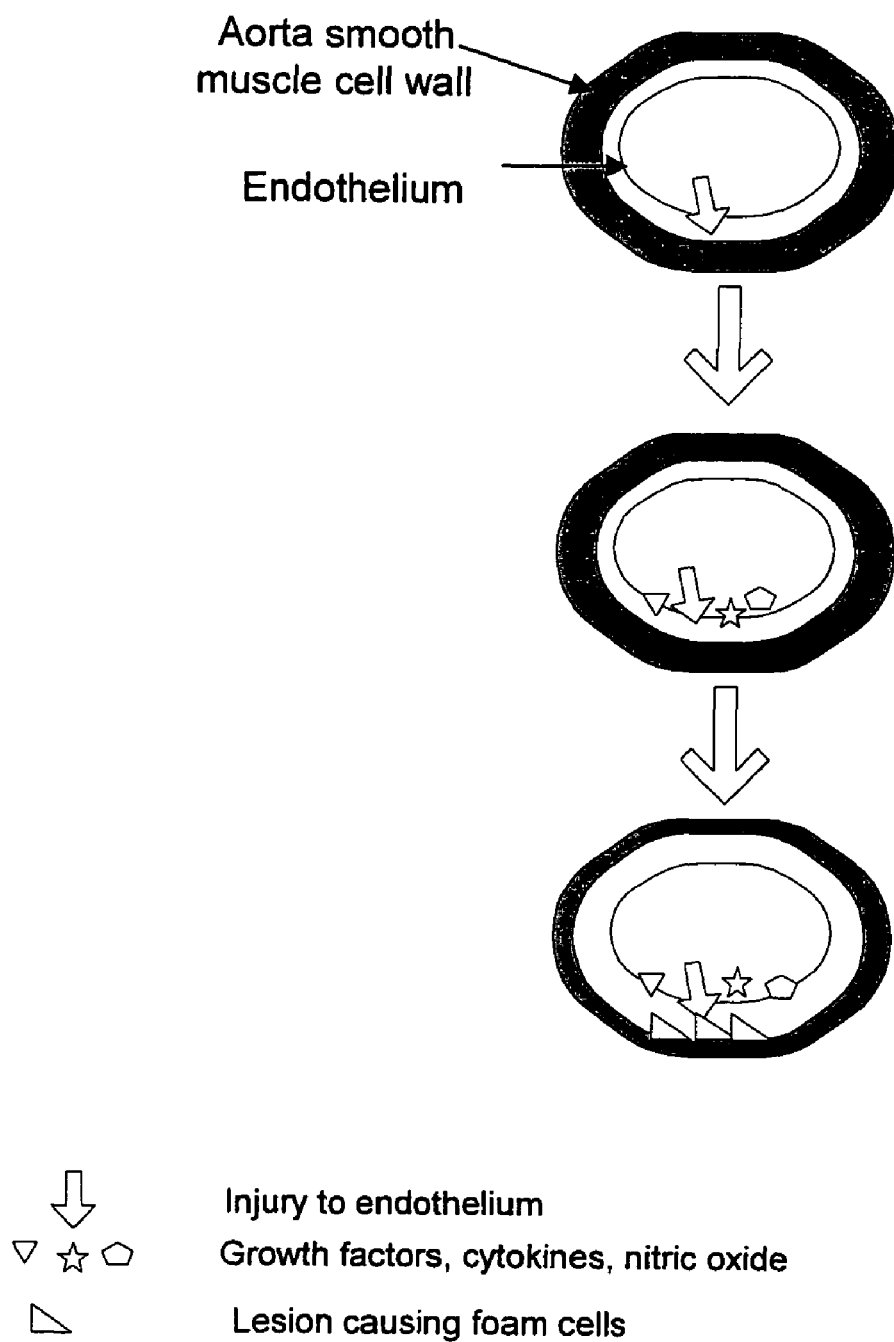


FIG. 10

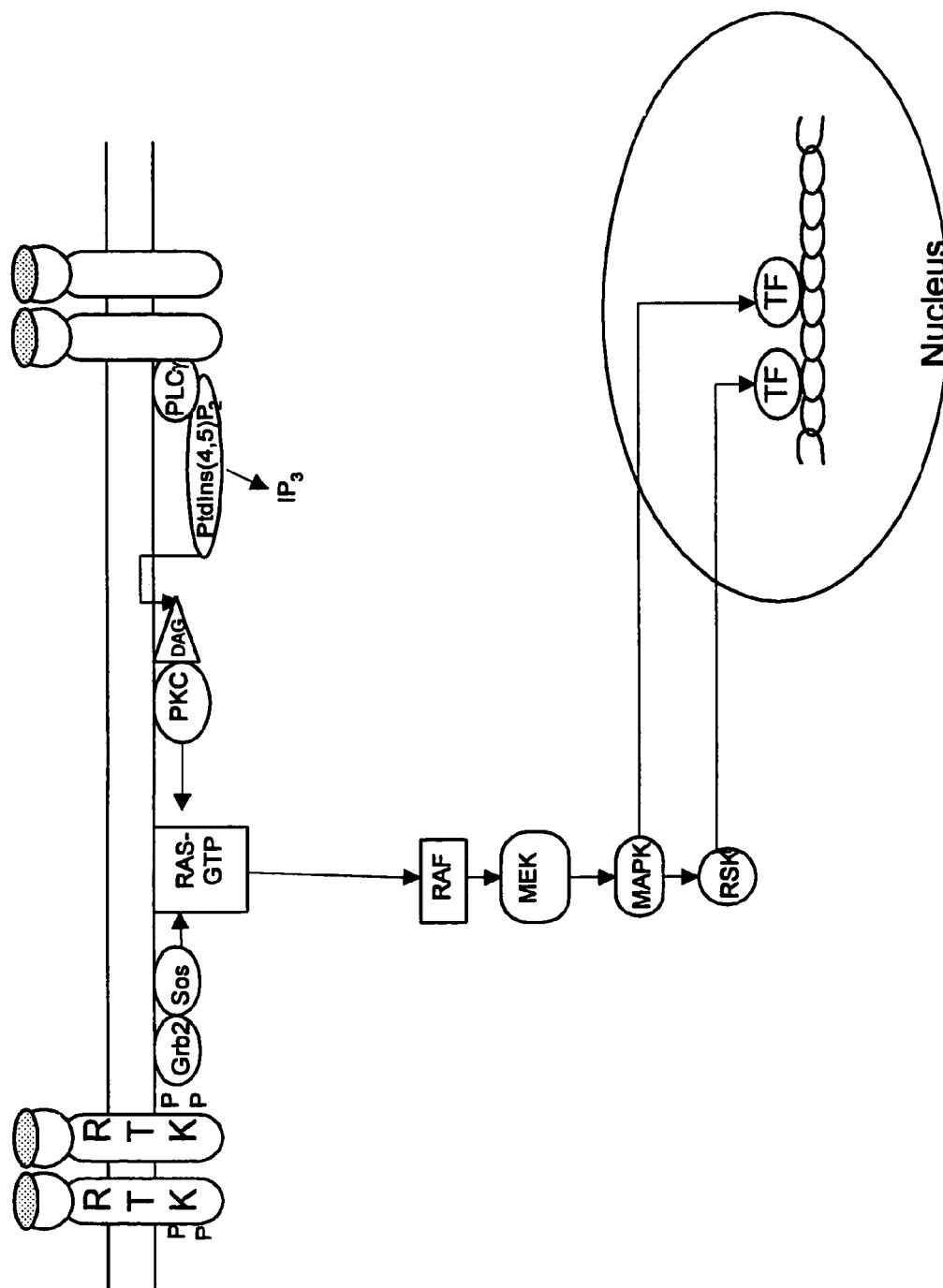
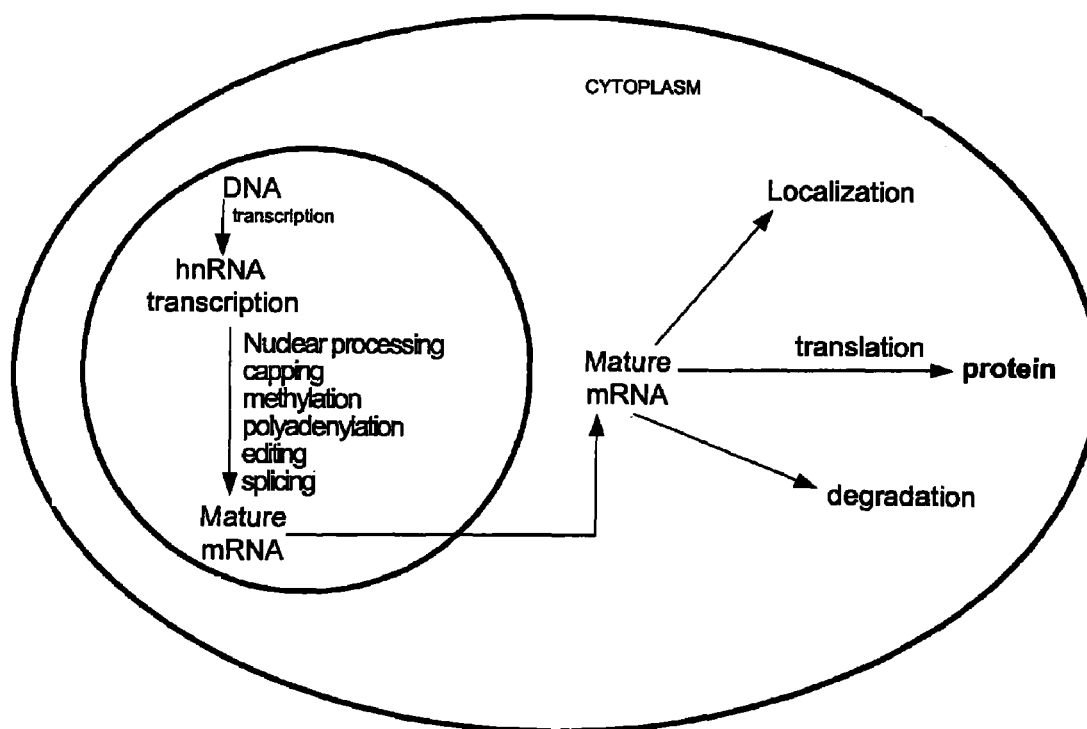
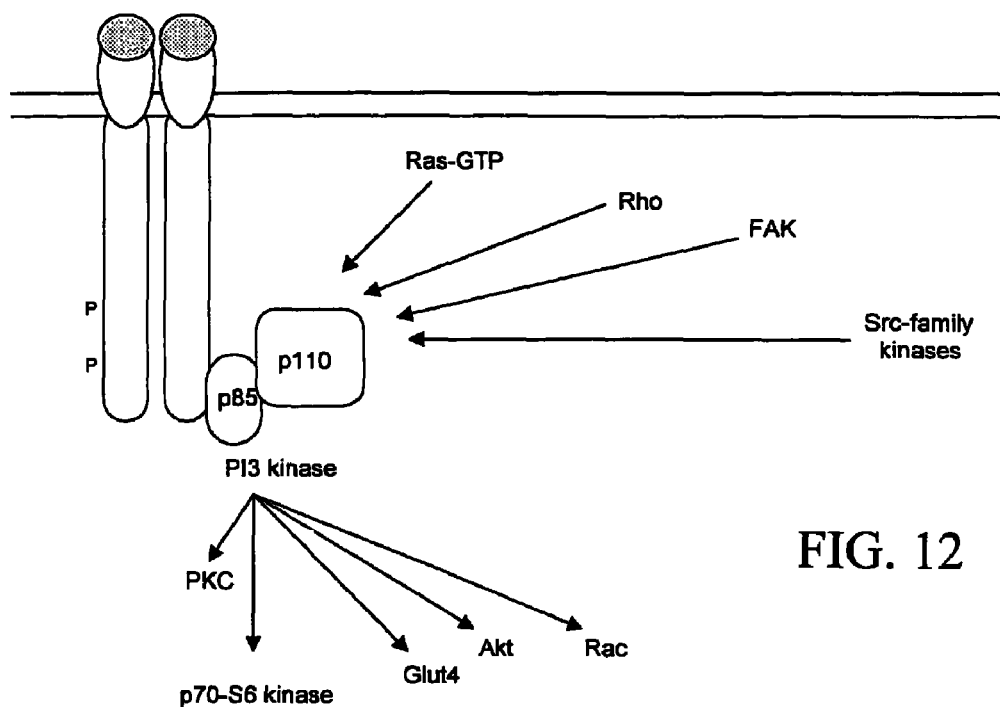


FIG. 11



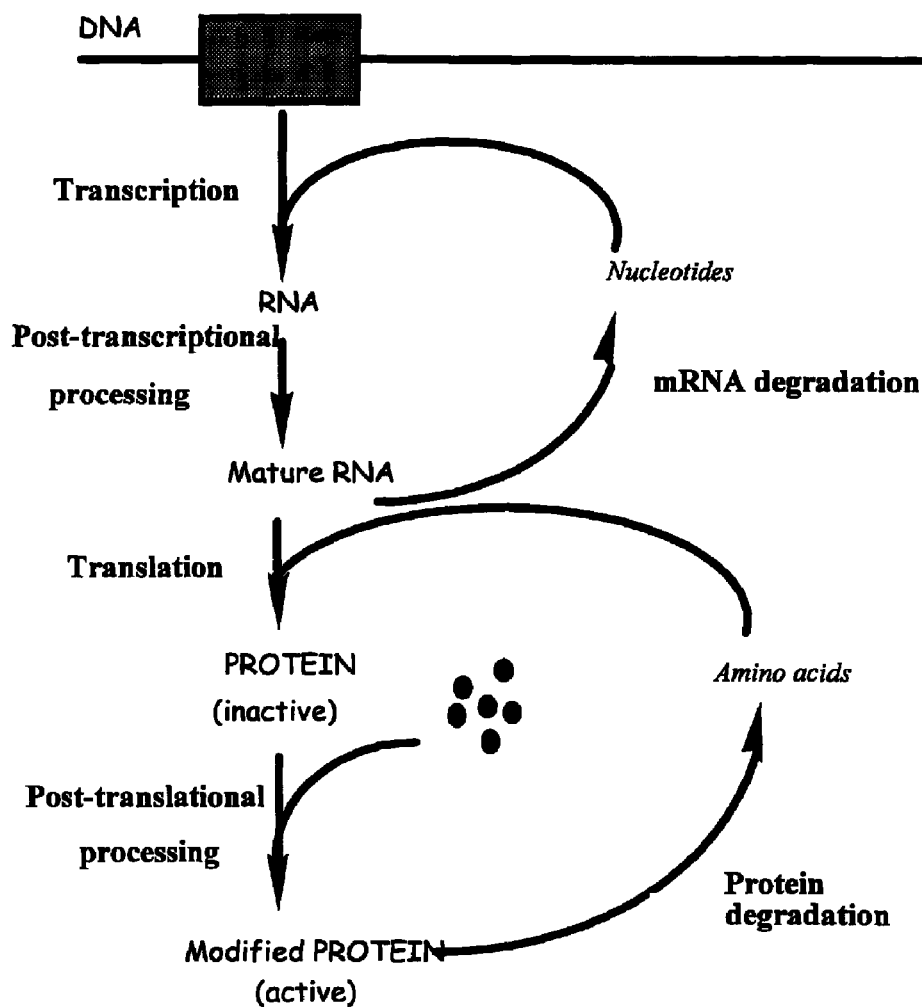
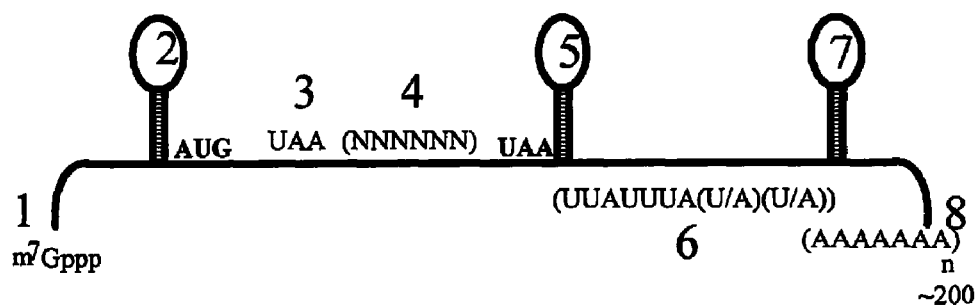


FIG. 13



- | | |
|--------------------------------|-------------------------|
| 1 5' cap | 5 3' UTR sequences |
| 2 5' UTR | 6 ARE sequences |
| 3 premature stop codons | 7 3' terminal stem loop |
| 4 open reading frame sequences | 8 poly(A) tail |

FIG. 15

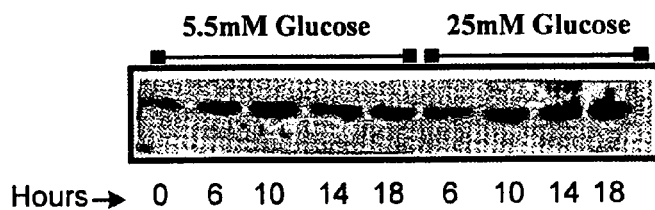


FIG. 16A

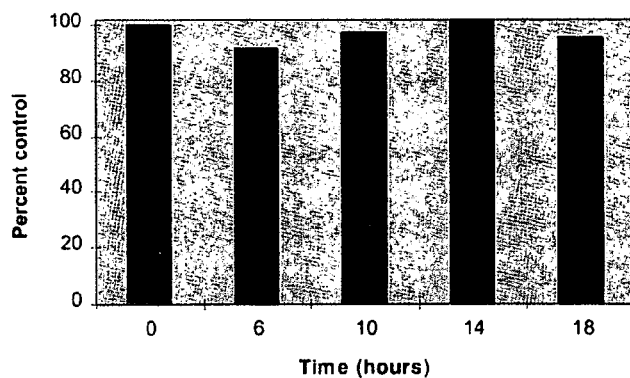


FIG. 16B

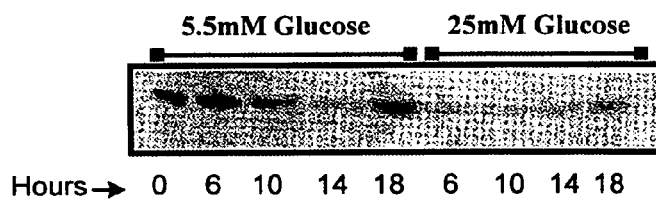


FIG. 17A

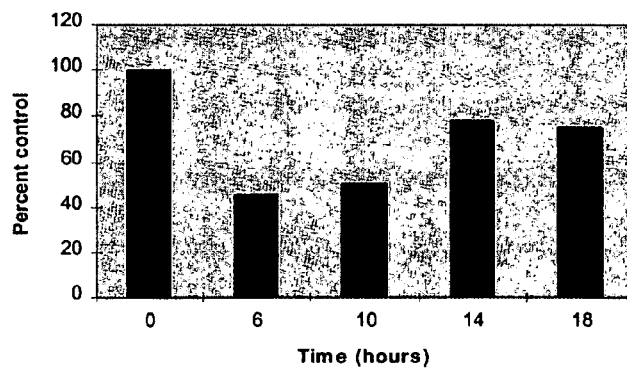


FIG. 17B

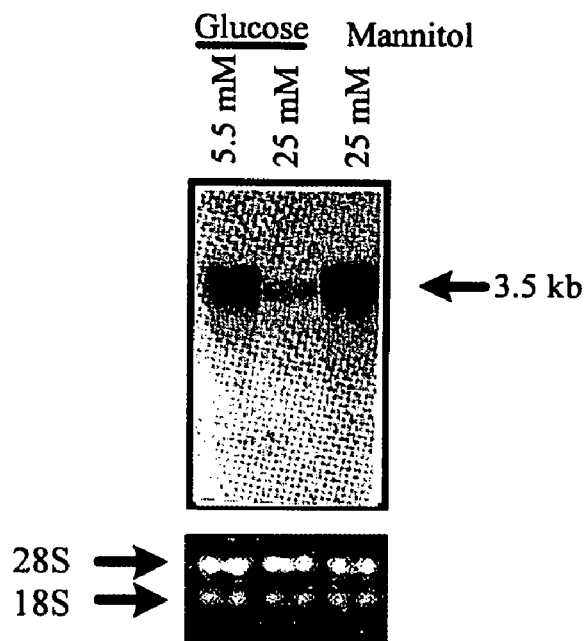


FIG. 18A

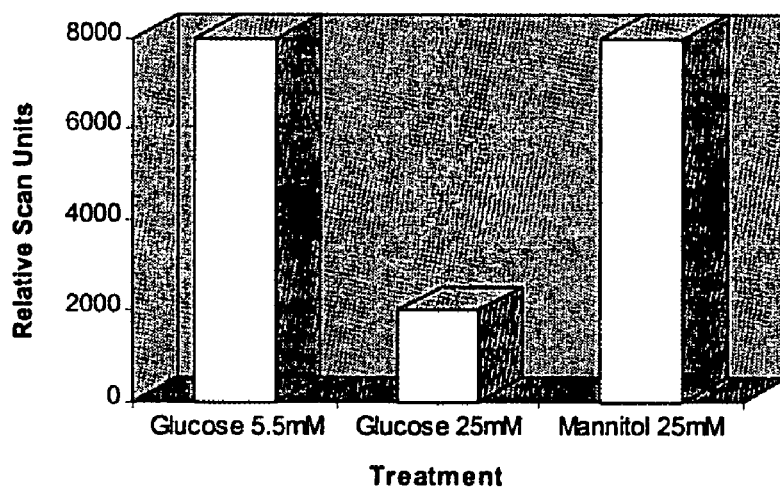


FIG. 18B

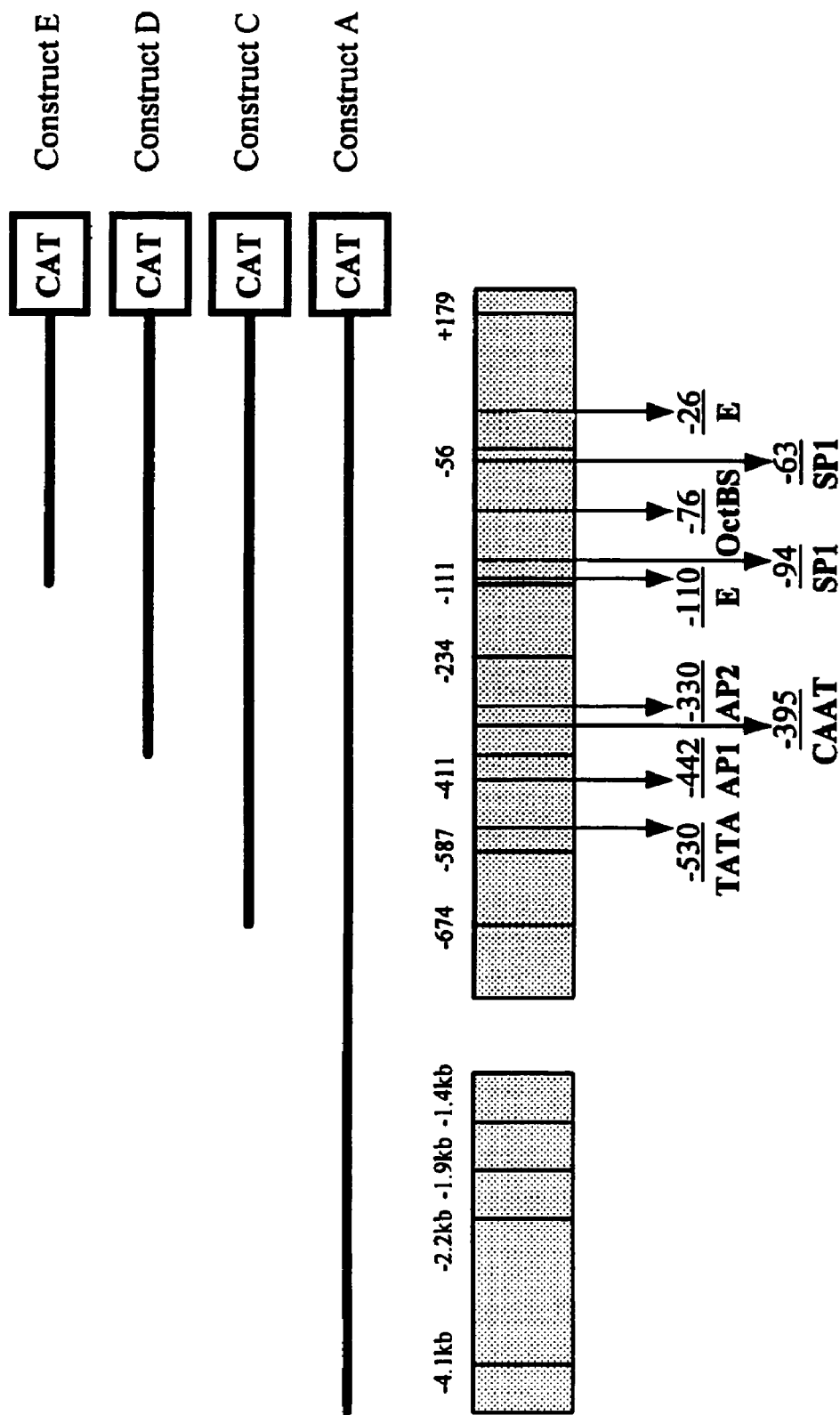


FIG. 19

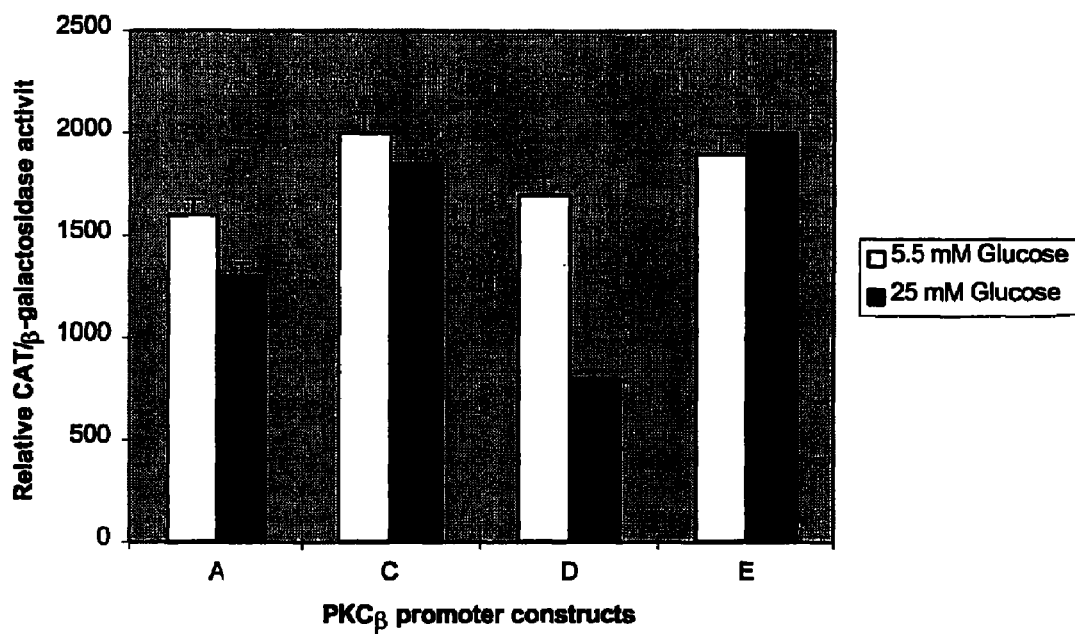
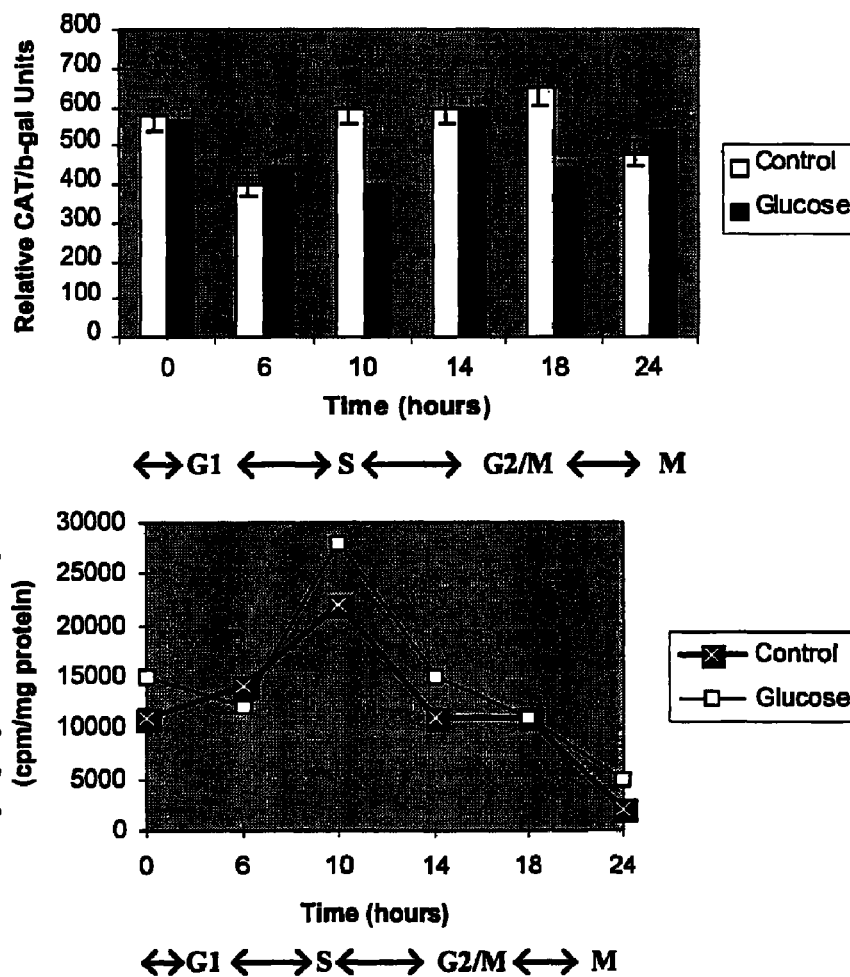


FIG. 20

FIG. 21



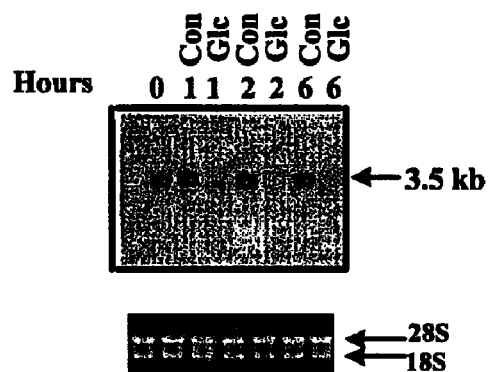


FIG. 22A

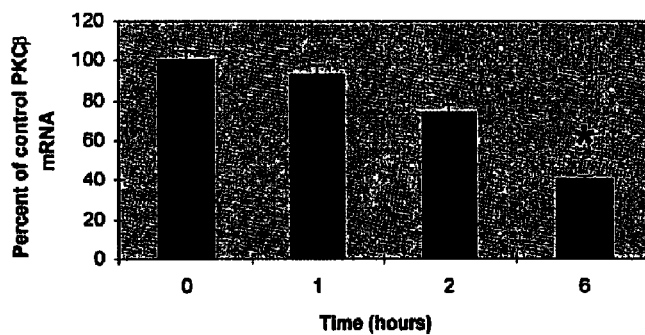


FIG. 22B

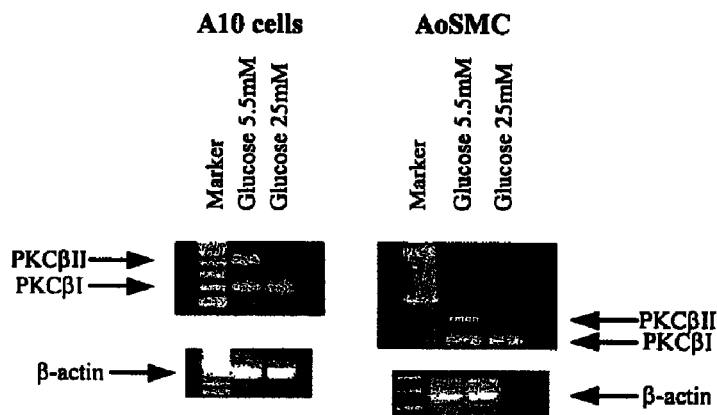


FIG. 26A

FIG. 26B

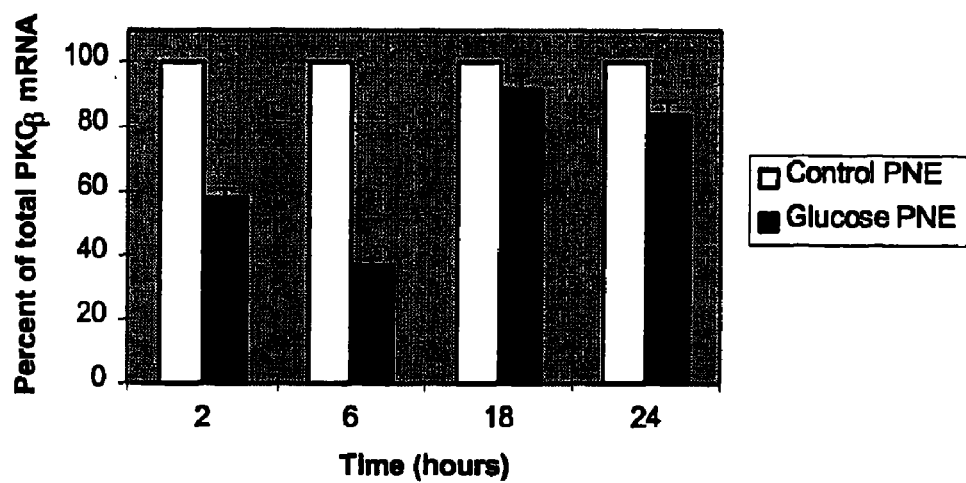


FIG. 23

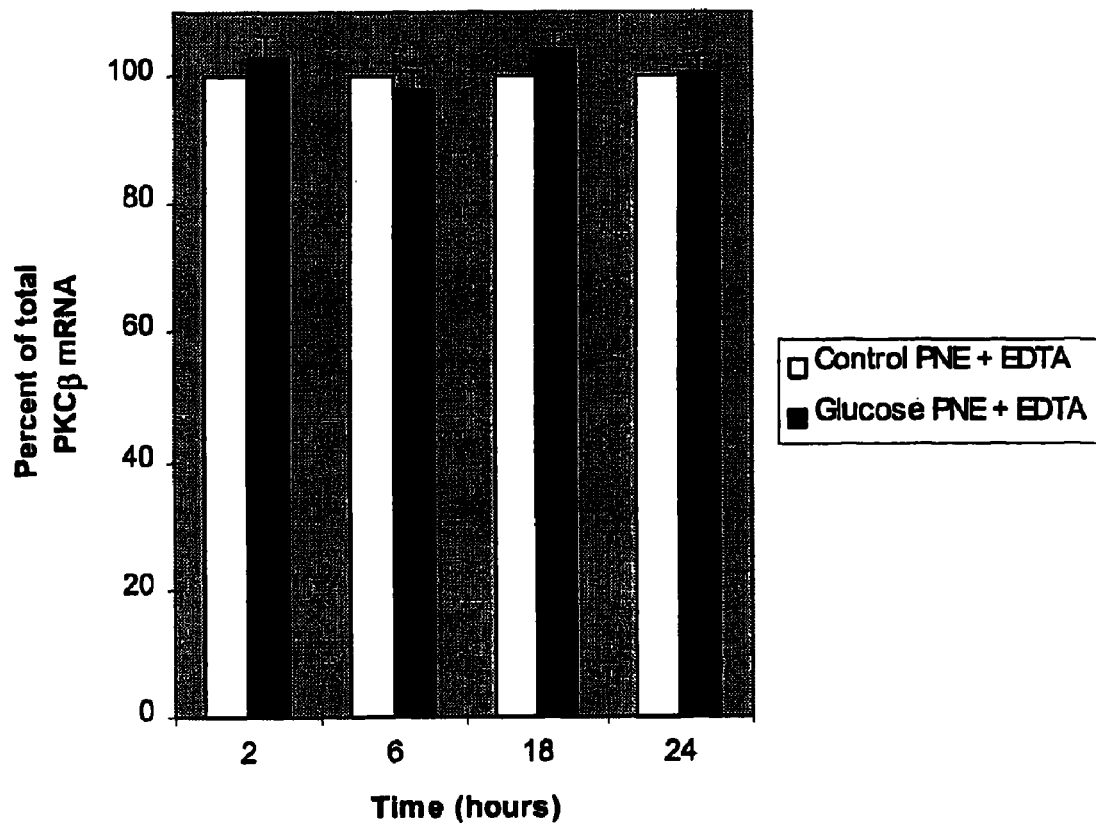


FIG. 24

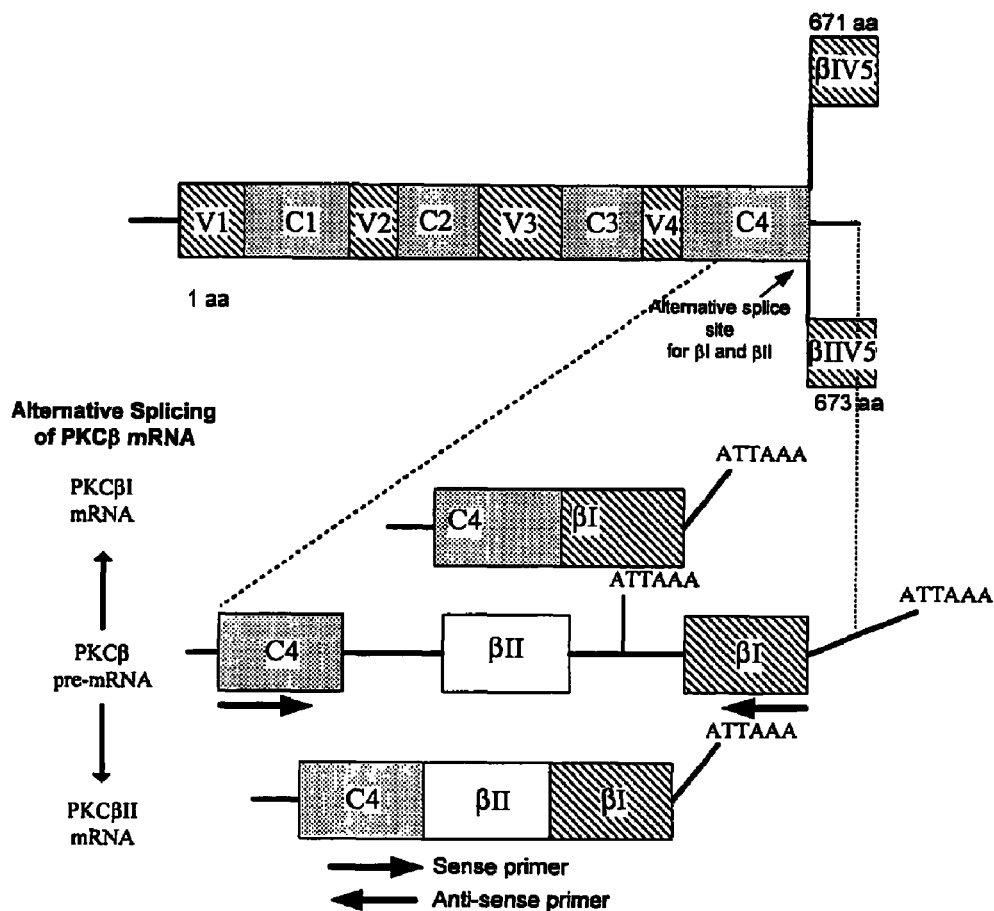


FIG. 25

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5'TTTTAAACCAAAGCTTTTGGGCGAAACGCTGAAACTTC
GACCGGTTTTTACCCGCCATCCACCAGTCCTAACACCTCC
GACCAGGAAGTCATCAGGAATATTGACCAATCAGAATTCGA
AGGATTTCTTTGTAACTCTGAATTTTAAAACCCGAAGTC
AAGAGCTAGTAGATCTGTAGACCTCCGTCCTTCATTTCTGTC
ATTCAAGCTCACAGCTATCATGAGAGACAAGCGAGACACCT
CCAAGTTGACAAAAGTTCACCAGGCAGCCTGTGGAAGTGA
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TGCAGATATCCATCACACTGCG 3'

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FIG. 27

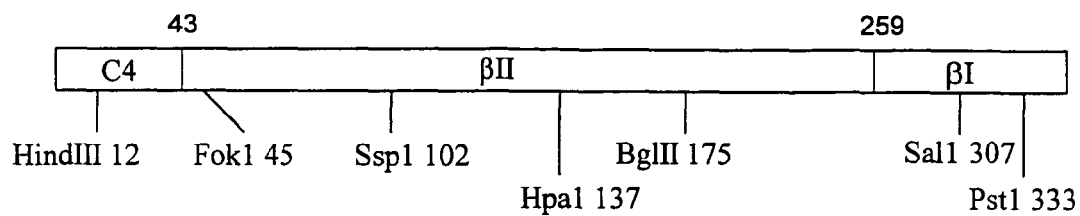


FIG. 28

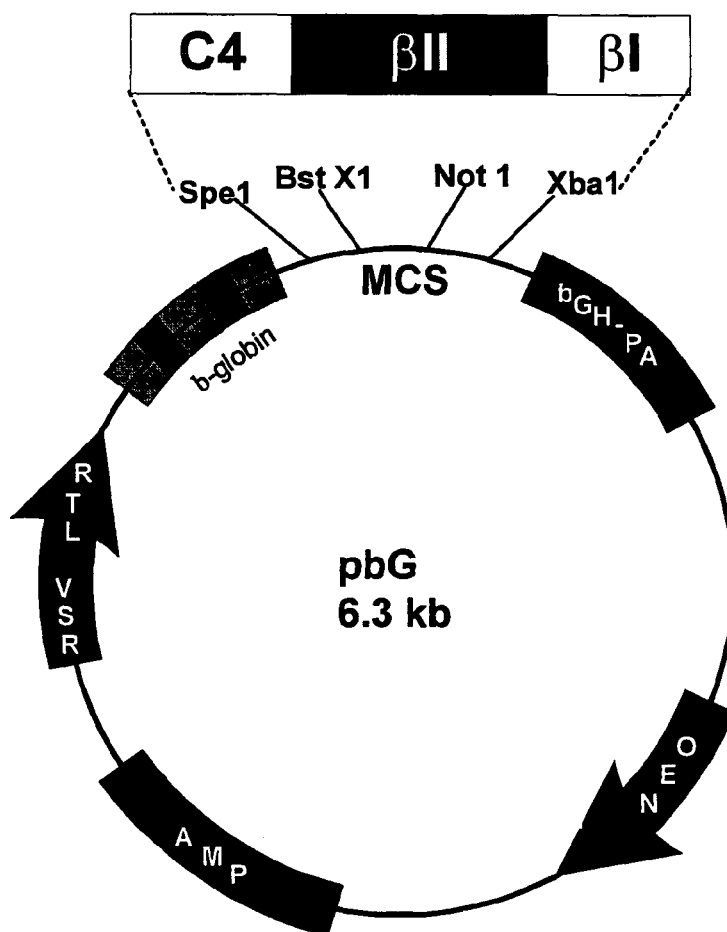


FIG. 34

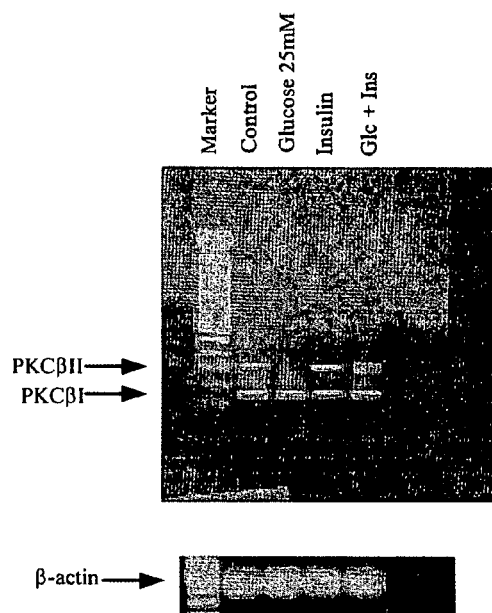


FIG. 29

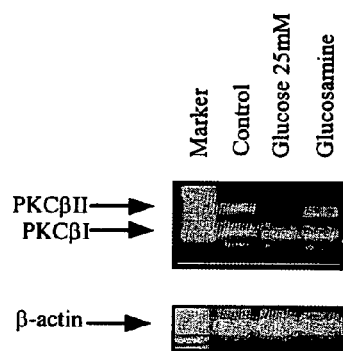


FIG. 30

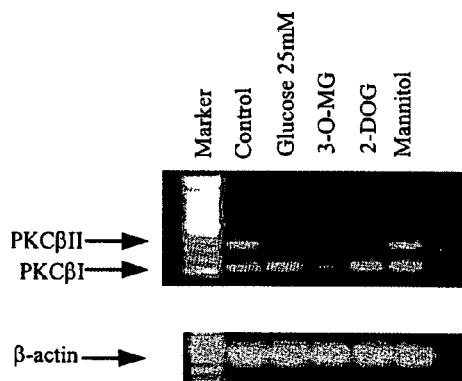


FIG. 31

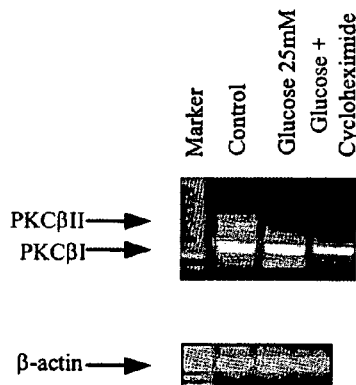


FIG. 32

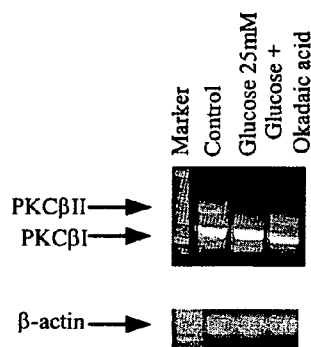


FIG. 33

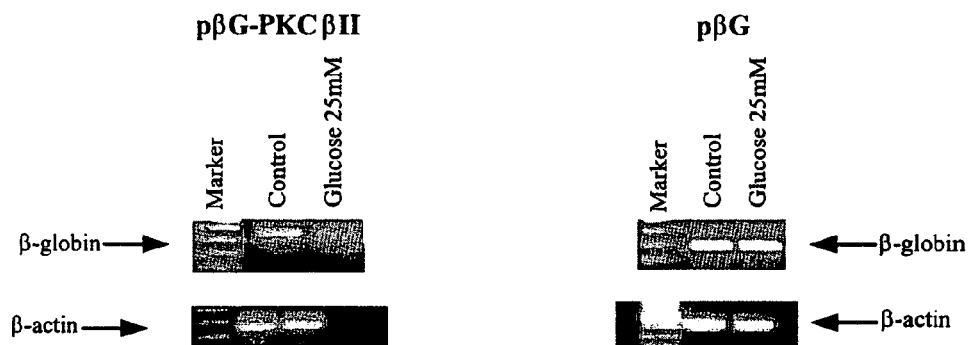


FIG. 35A

FIG. 35B

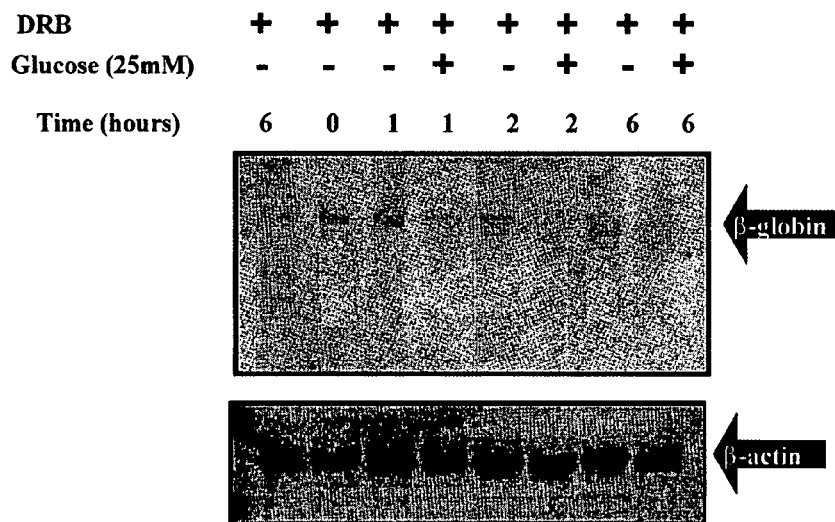


FIG. 36A

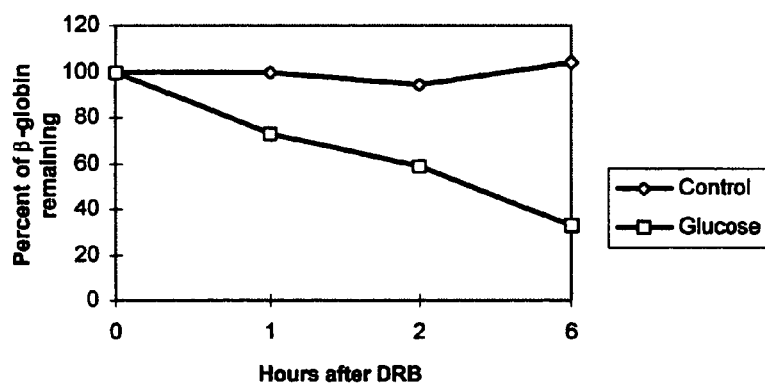


FIG. 36B

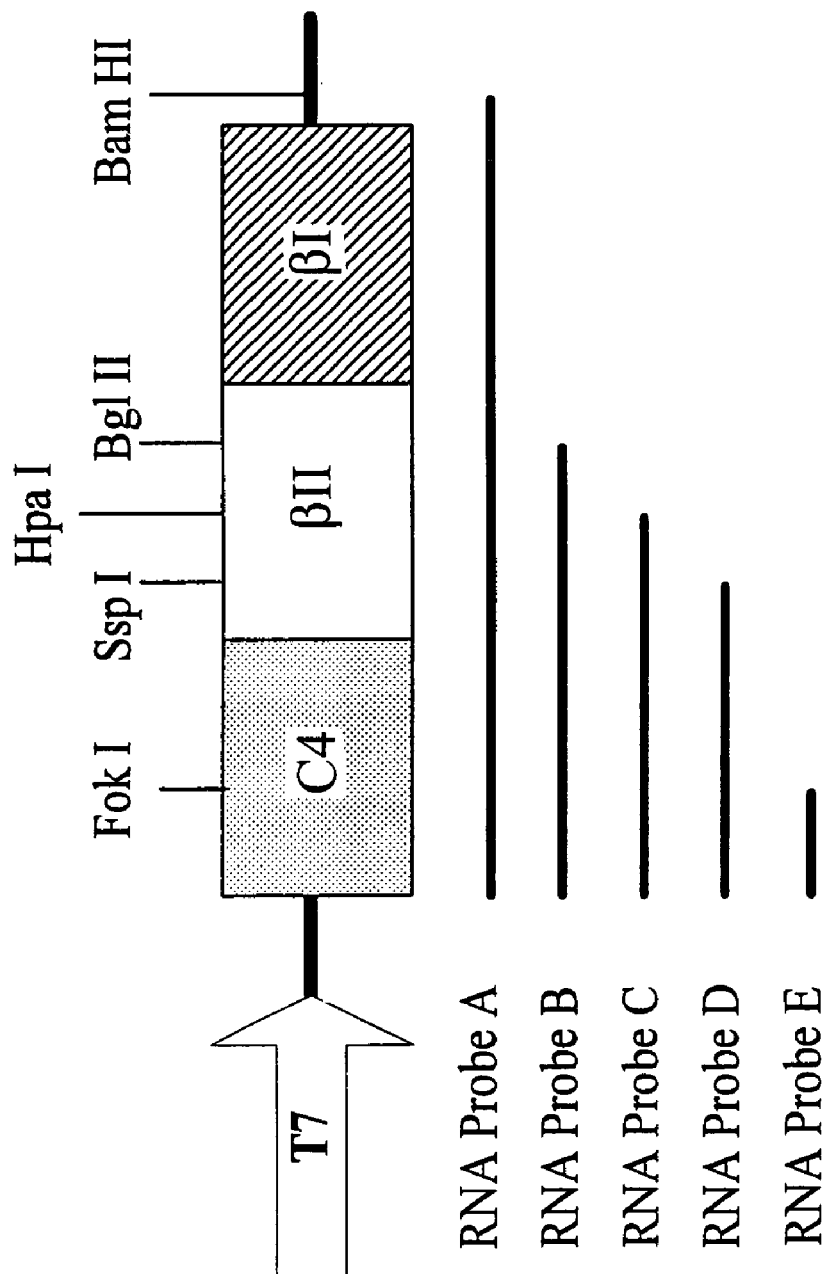


FIG. 37

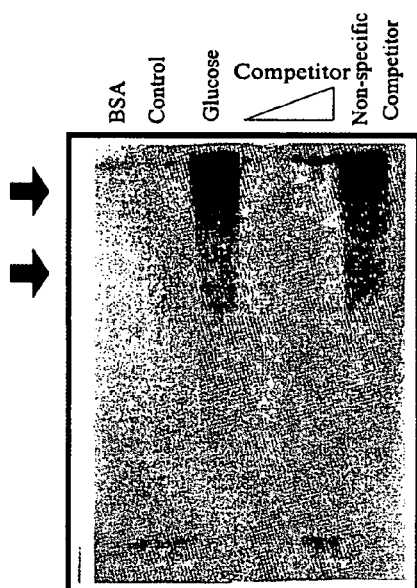


FIG. 38

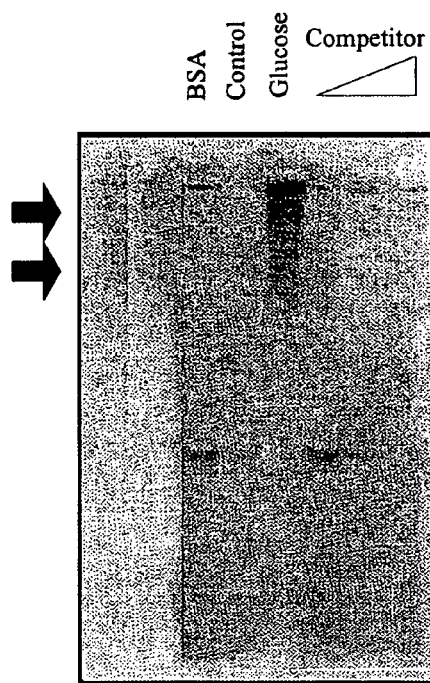


FIG. 39

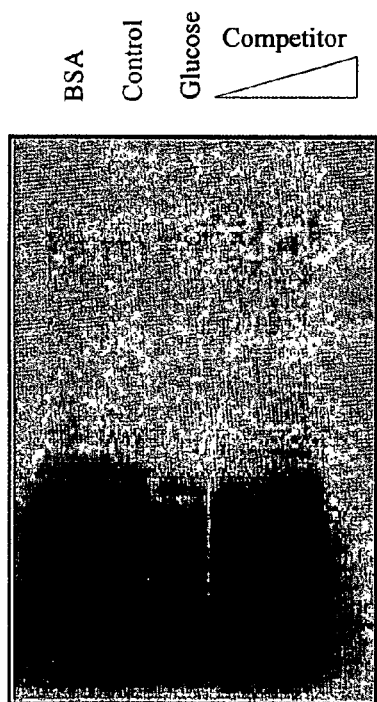


FIG. 40

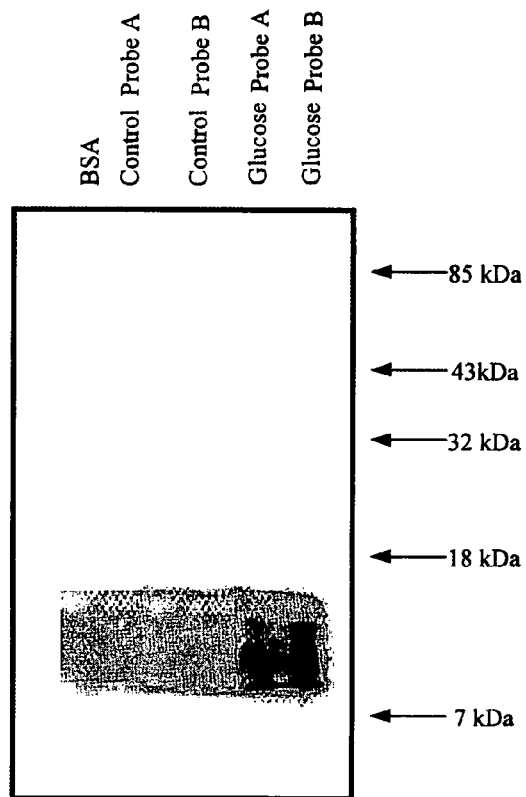


FIG. 41

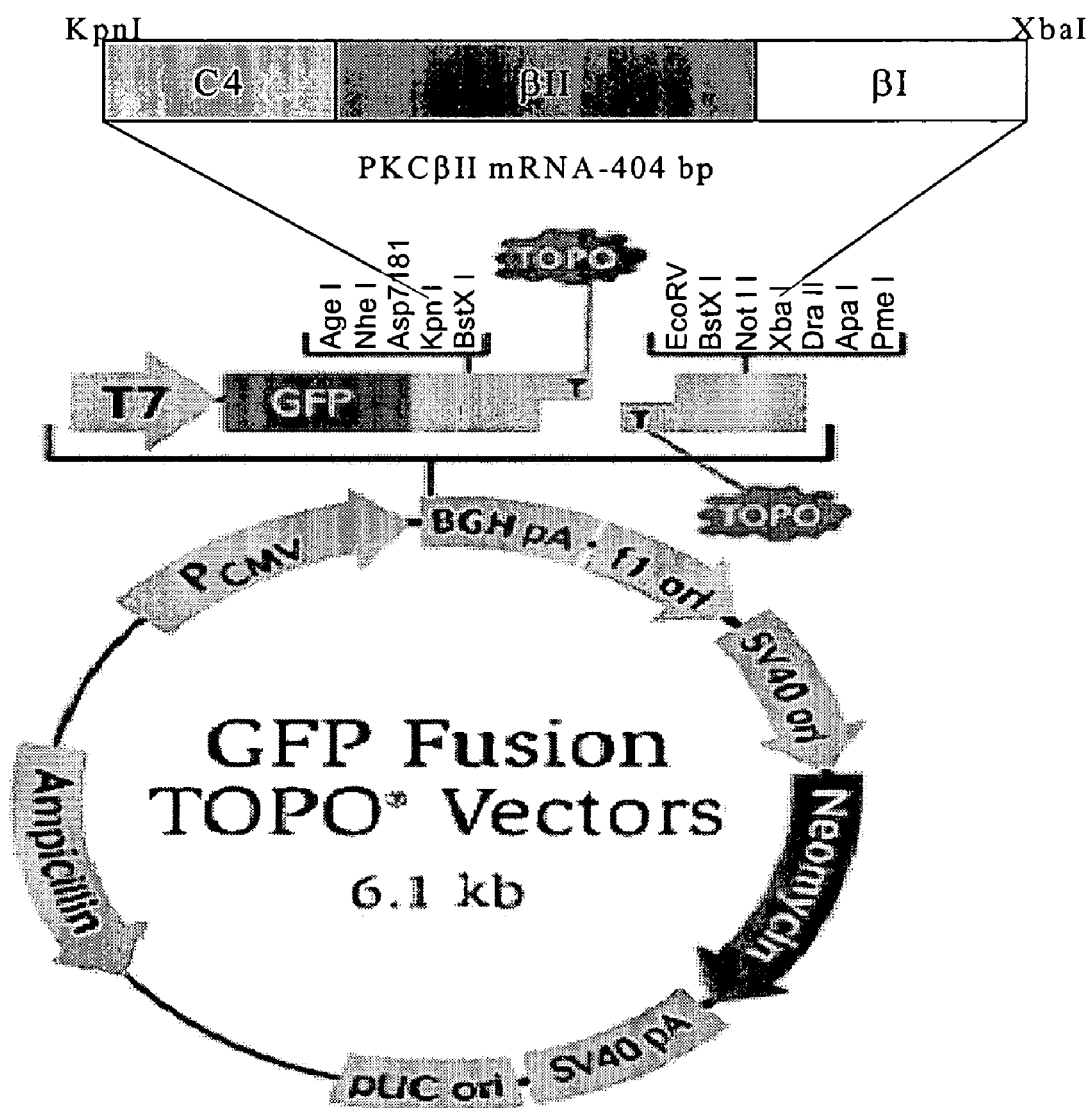


FIG. 42

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COMPOSITIONS AND METHODS FOR DETECTING INTRACELLULAR GLUCOSE AND ANALOGS THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. application Ser. No. 09/435,471, filed Nov. 8, 1999, now U.S. Pat. No. 6,852,529, which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, and drawings.

BACKGROUND OF INVENTION

Multicellular eukaryotic organisms are capable of responding to extracellular signals to regulate or accentuate gene expression through the process of signal transduction. A large number of the components of the signal transduction pathway are comprised of proteins that are covalently modified by ligands such as hormones, growth factors, cytokines or second messengers like camp, Ca^{2+} , diacylglycerol, inositol-3-phosphate and others. This results in the induction of a cascade leading to gene expression in which activation of kinases that phosphorylate tyrosine or serine/threonine residues play central roles. Prominent targets of second messengers are the camp-dependent protein kinase A (PKA) and the Ca^{2+} /phospholipid-activated protein kinase C (PKC). The protein kinase C cascade includes several enzymes that are sequentially phosphorylated in response to external stimuli. PKC established its role in signal transduction when it was demonstrated that diacylglycerol, a product of hormone stimulated phosphatidylinositol hydrolysis, was an activator of the enzyme. Multiple lipid pathways contribute to the production of diacylglycerol, the second messenger for PKC. Phorbol esters, which are potent tumor promoters, activate PKC and this discovery led to further excitement in the field (see for example, Nishizuka, 1995, pp.484-496; Nishizuka, 1992, pp.607-614).

Protein kinase C modulates diverse cellular functions such as cell cycle progression and differentiation, apoptosis, and tumor promotion (Nishizuka, 1986, pp.305-312). PKC regulates gene expression via transcription through the activation of cis-elements. It is a mediator of immune responses, hormone secretion and receptor desensitization. Many of these roles are mediated by accentuating membrane structure events. PKC phosphorylates serine or threonine residues at basic sequences with the motif xRxxS/TxRx. Thus histones H1 and IIIS, and protamine, are efficient substrates for PKC. In addition to catalyzing phosphorylation reactions, PKC autophosphorylates in vitro, (on residues Thr-641 and Ser-660 in PKC β II) by an intramolecular mechanism and this may play a role in its proteolytic activation and degradation.

Together with the fact that activation of PKC isozymes is associated with altered gene expression and its presence in the nucleus, it was expected that PKC could phosphorylate some transcription factors and thereby regulate gene expression. PKC phosphorylates CREB, the camp-responsive element-binding protein and as a result CRE binding is enhanced. NF- κ B, in the cytosol of resting cells, is in a complex with I- κ B. On PKC activation, by phorbol esters, the inhibitory complex dissociates and NF- κ B translocates into the nucleus

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as an active transcription factor. C-jun, a phorbol ester-responsive transcription factor, is also regulated by PKC. Vitamin D₃ receptor, a member of the steroid/thyroid hormone family, binds to vitamin D₃ and associates with its DNA element thereby altering gene transcription. PKC β phosphorylates the vitamin D₃ receptor, and inhibits transcriptional activation by vitamin D₃. TLS/FUS, a DNA-binding nuclear protein, is a regulator of BCR/ABL-mediated leukemogenesis (Perrotti et al., 1998, pp.4442-4455). PKC β II phosphorylates TLS/FUS and regulates its DNA-binding activity in the nucleus.

Historically, Nishizuka et al. discovered protein kinase C in rat brain as the proenzyme for a histone protein kinase (see, for example Takai et al., 1977, pp.7603-7609). This protein kinase M (PKM) was generated as a result of partial proteolysis by trypsin or a calcium-dependent protease. From biochemical studies, it was soon established that the proenzyme, PKC, exhibited kinase activity upon membrane association. It is now well established that protein kinase C, a family of serine/threonine kinase isozymes, translocates from the cytosol to another intracellular compartment like the membrane or nucleus in response to stimuli from neurotransmitters, hormones and growth factors. Most PKCs are activated by phosphatidylserine, an acidic lipid located on the cytoplasmic side of the membrane, diacylglycerol and phorbol esters. Some PKC family members also require Ca^{2+} and other lipid second messengers for optimal activity.

Protein Kinase C Isozyme Family

Biochemical and molecular cloning analysis of protein kinase C revealed a large family of isozymes exhibiting individual characteristics and functions and tissue specific distribution. Protein kinase C isozymes comprise a single polypeptide chain with a 20-70 kDa amino-terminal regulatory domain and an approximately 45 kDa kinase domain at the carboxy-terminus. The PKC isozymes, to date, can be categorized into at least three groups based on their homology and sensitivity to their cofactors (Table 1). The earliest discovered PKC isozymes are the members of convention or classical PKC (cPKC) and include PKCT - α , - β I, - β II (alternatively spliced variants of - β) and - γ . This class of isozymes is activated by diacylglycerol, phosphatidylserine, phorbol esters and Ca^{2+} . The novel PKC (nPKC) subfamily of isozymes include PKC - δ , - ϵ , - η , and - θ and are calcium-independent. The least understood are the atypical PKC isozymes (aPKC) that are insensitive to phorbol esters but are activated by phosphatidylserine and include PKC - ζ and λ /I. A recently described protein kinase C μ (human) and its murine homolog PKD, form a distinct class in that it is activated by phorbol esters and is phospholipid-dependent, but calcium insensitive. It has a kinase core similar to that of calmodulin-dependent kinases and no pseudosubstrate motif has been identified. However, it contains two unique hydrophobic domains in the N-terminal portion (putative transmembrane sequences) of the enzyme, a pleckstrin homology domain and a distinct catalytic domain structure. Another class of PKC, the PKC-related kinases (PRK) consists of three members: PRKs 1, -2 and -3. PRKs are insensitive to Ca^{2+} , DAG and phorbol esters (Palmer et al., 1995c, pp.315-320; Mukai et al., 1994b, pp.897-904). PRK family binds to RhoA, which leads to its activation (Vincent et al., 1997, pp.2247-2256; Amano et al., 1996, pp.648-650; Quilliam et al., 1996, pp.28772-28776; Watanabe et al., 1996, pp.645-648).

TABLE 1

PKC Isozyme Family (Nishizuka, 1986, pp.305-312; Newton, 1997, pp.161-167).		
Class	Molecular Weight	Activators
Conventional PKCs (cPKC)		
α	76.8 kDa	PS, DAG, Ca^{2+} , phorbol esters
βI	76.8 kDa	PS, DAG, Ca^{2+} , phorbol esters
βII	76.9 kDa	PS, DAG, Ca^{2+} , phorbol esters
γ	78.4 kDa	PS, DAG, Ca^{2+} , phorbol esters
Novel PKCs (NpkC)		
δ	77.5 kDa	PS, DAG, phorbol esters
ϵ	83.5 kDa	PS, DAG, phorbol esters
η	78.0 kDa	PS, DAG, phorbol esters
θ	81.6 kDa	PS, DAG, phorbol esters
Atypical PKCs (aPKC)		
ζ	67.7 kDa	PS
λ/ι	67.2 kDa	PS
Protein kinase C μ /PKD	?	PS, phorbol esters

PS, phosphatidyl serine;
DAG, diacylglycerol;
 Ca^{2+} , calcium

Structure of Protein Kinase C

Cloning of the cDNA and extensive biochemical and mutational analysis has indicated that the primary amino acid structure of the PKC isozymes is composed of conserved domains (C1-C4), that serve as functional modules, and are separated by variable domains (V1-V5) that contribute to isozyme specificity and function.

The N-terminal regulatory domain and the C-terminal catalytic domain structure of PKC isozymes are diagrammed in FIG. 1. The N-terminal V1 domain of cPKC isozymes is approximately 20 amino acids while that of the nPKC isozymes is longer and may contribute to regulate the functions of the conserved domains. The autoinhibitory domain contains a pseudosubstrate site (at amino acids 22 to 28) that cannot be phosphorylated and blocks the catalytic site. The C1 domain, that immediately follows the pseudosubstrate sequence, binds to diacylglycerol and phorbol esters. It is present as a tandem repeat of two domains in most PKCs except within the atypical PKC isozymes that have only one copy of this domain. The C1 domain contains a cysteine-rich region that comprises of two zinc-finger motifs each with six Cys-residues, that co-ordinates two Zn^{2+} ions, and a DNA-binding motif. No obvious role has yet been elucidated for the Zn^{2+} ions. The C2 domain contains a binding site for acidic lipids and represents the Ca^{2+} binding domain of the Ca^{2+} dependent PKC isozymes (the novel PKC isozymes notably lack this Ca^{2+} binding domain). The V3 or hinge region separates the regulatory and catalytic domains and becomes proteolytically labile when the enzyme translocates to the membrane on activation. On proteolysis, the pseudosubstrate inhibition is relieved which leads to the generation of the constitutively active kinase domain (protein kinase M). The C3 domain contains an ATP-binding motif, xGxGX₂Gx₁₆Kx, which is conserved in all protein kinases. The C4 domain contains the substrate-binding site and the phosphate transfer region containing the conserved sequence DFG (Kemp et al., 1990, pp.342-346). The V5 region confers specific function and role to the alternatively spliced PKC β forms i.e. PKC - βI and - βII . The V5 region is involved in substrate specificity and

protein trafficking. Protein kinase C μ or PKD has a unique structure in that it contains two hydrophobic domains in the N-terminal (putative transmembrane sequences), a putative pleckstrin homology domain and a distinct catalytic domain.

Tissue, Cellular and Subcellular Localization of PKCs

PKC isozymes show distinct tissue, cellular and subcellular distribution and more than one isoform could be expressed in a cell type (Table 2). PKC α , βI , βII , γ , δ , ϵ , ζ are expressed in the brain. PKC γ is exclusively expressed in the brain and spinal cord. PKC η , θ and λ are abundant in skin and lung, skeletal muscle, and ovary and testis. This preferential expression could imply tissue specific functions while the ubiquitous expression could suggest that the PKCs are essential for general cell function.

Several PKCs are differentially expressed in various regions and cell types of the brain. PKC γ predominates in the dendrites, cell bodies and axons of Purkinje, PKC α , βI , βII γ are contained in the neurons of the brain. Glial cells also selectively express PKC α and βII .

TABLE 2

Tissue Expression of PKCs	
PKC ISOFORMS	TISSUES EXPRESSING HIGHEST LEVELS
Conventional PKCs (cPKC)	
α	Ubiquitous
βI (or $\beta 2$)	Brain, heart, spleen, vascular smooth muscle
βII (or $\beta 1$)	Brain, heart, spleen, vascular smooth muscle
γ	Brain, spinal cord
Novel PKCs (nPKC)	
δ	Brain, myeloid cells
ϵ	Brain
η	Skin, lung, skeletal muscle, ovary, testis
θ	Skin, skeletal muscle
Atypical PKCs (aPKC)	
ζ	Brain
λ/ι	Skin, skeletal muscle

PKC isozymes have been shown to translocate to the nucleus upon activation. Phorbol esters translocate PKC α to 3T3 fibroblast nuclei while PKC β in HL60 cells is translocated to the nuclei by phorbol ester activation. PKC β has also been found in liver nuclei. The presence of PKC isozymes in the nucleus suggests that activation of PKC in the nucleus may be important for regulation of gene expression.

The PKC β Gene

The molecular cloning and biochemical experiments on the genomic structure of the PKC isozymes reveals that they are encoded on distinct chromosomes except PKC βI and - βII which are alternatively spliced products derived from a single PKC β gene. The variable V5 domain of the PKC β gene is alternatively spliced to generate PKC βI or PKC βII mRNAs. The βI and βII cDNAs, from rat or rabbit brains, encode 671 and 673 amino acid sequences respectively. The resulting proteins differ in their carboxyl terminal regions by 50-52 amino acid residues.

Alternative splicing of the PKC β pre-mRNA, shown in FIGS. 3A and 3B, generates the mature PKC βI mRNA and the exon-included PKC βII mRNA that shares a common 3' untranslated region and poly (A) tail with PKC βI mRNA.

This insertion into the coding region introduces a termination codon near the splice site such that the PKCβII sequence is only two amino acids longer than PKCβI polypeptide. As shown in FIG. 4, the PKCβII cDNA is longer than the PKCβI cDNA by means of insertion of a 216 bp independent exon.

The expression of the PKCβ gene is developmentally regulated and shows cell type specificity (Niino, S. et al., 1992, pp.6158-6163). To determine the mechanism for the cell type specificity of transcription of PKCβ gene, Niino et al. (Niino, Y., S., S. Ohno, and K. Suzuki. 1992. Positive and negative regulation of the transcription of the human protein kinase C β gene. *J. Biol. Chem.* 267:6158-6163) demonstrated the presence of multiple trans-acting factors that act on positive and negative cis-acting elements and regulate transcription of the human PKCβ gene, hence providing an explanation for regulation of its cell-type specific expression. Obeid et al. (Obeid, L. M., G. C. Blobe, L. A. Karolak, and Y. A. Hannun. 1992. Cloning and characterization of the major promoter of the human protein kinase C β gene. Regulation by phorbol esters. *J. Biol. Chem.* 267:20804-20810) have cloned the 5' region of the gene for PKCβ and identified its promoter. Although the mRNAs for PKCβI and PKCβII are distinct, the transcription of both the mRNAs is under the regulation of the common PKCβ promoter. The transcriptional initiation site is 197 bp upstream of the translational initiation site. The promoter region for PKCβ is GC-rich (>80%) and the TATA and CAAT elements are found upstream of the transcription start site in the reverse order. Other regulatory cis-acting elements are shown in Table 3.

TABLE 3

Potential trans-acting factors regulating cis-acting elements in the promoter region of the PKCβ gene.		
TRANS-ACTING FACTOR	CIS-ACTING ELEMENT	POSITION IN PKCβ GENE
Oct BP	ATGCAAAAT	-76
Sp1	GGGCGG	-94, -63
E _{12/47}	GCAGGTGG	-110, -26, +18
ChoRE	CACGTG	-318 (CACCCG), -226 (CTCCTG)
AP ₂	CCCCACCCC	-330
CTF/NF-1	GCCAAT	-395
AP ₁	TGAGTCA	-442
TFIID	TATAAA	-530

To study the promoter elements, Obeid et al. (Obeid, L. M., G. C. Blobe, L. A. Karolak, and Y. A. Hannun. 1992. Cloning and characterization of the major promoter of the human protein kinase C β gene. Regulation by phorbol esters. *J. Biol. Chem.* 267:20804-20810) used deletion constructs of PKCβ gene. A 154-bp construct (-111 to +43) that contained an octamer motif, two Sp1 sites and one E box could confer promoter activity. This indicated that the TATA or CAAT motifs were not required for basal promoter activity. They further demonstrated that the phorbol esters transcriptionally up-regulated PKCβ in K562 erythroleukemia cells. A feedback loop exists which acts to induce de novo transcription of PKCβ when phorbol esters activate PKCβ and cause its down-regulation.

Activation of Protein Kinase C

Signaling Pathways that Generate PKC Activators

Stimulation of cell surface receptors activates phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) resulting in the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). Inositol 1,4,5-trisphosphate stimulates the release of Ca²⁺ from intracellular storage (see FIG. 5). Furthermore, DAG is also produced from phosphocholine and results in a sustained elevation of DAG in response to various signals like phorbol esters, growth factors and cytokines. Both DAG and Ca²⁺ mediate the activation of PKC isozymes as shown in FIG. 6. Ca²⁺ binds to C2 domain of cPKC isozymes. Translocation of the enzyme to the membrane enables its activation by DAG and phosphatidylserine, which is a membrane acidic lipid, through binding at its C1 domain.

Model for Regulation and Activation of Protein Kinase C

For protein kinase C to be functionally active, the newly synthesized enzyme is first localized on the cytoskeleton and rendered catalytically competent by phosphorylations. Mass spectroscopy has revealed that PKC is phosphorylated at three positions in vivo. The residues corresponding to these phosphorylations in PKCβII include Thr500 in the activation loop, Thr641 and Ser660 at the carboxyl terminus. This triple phosphorylated mature form is inactive because the pseudosubstrate occupies the substrate-binding site. As diagrammed in the FIG. 7, the next step requires the removal of the autoinhibitory pseudosubstrate domain from the kinase core. Activation of protein kinase C by diacylglycerol, Ca²⁺ or phosphatidylserine, and subsequent translocation to the membrane, renders the pseudosubstrate sensitive to proteolysis by trypsin or endoprotease Arg-C. Maximum binding affinity of PKC to the membrane, mediated by domains C1 and C2, releases the pseudosubstrate inhibition. The exposed V3 domain hinge also, becomes proteolytically labile on membrane binding (independent of pseudosubstrate release).

Localization of PKC Isozymes by Anchoring Proteins

Both active and inactive PKC isozymes are localized to specific intracellular sites due to their binding to specific anchoring molecules. The anchoring proteins for activated PKC isozymes are termed receptors for activated C-kinase (RACKs) while proteins that anchor inactive PKC isozymes are termed receptors for inactive PKC isozymes in a selective and saturable manner. For example, RACK1—a PKCβII-specific RACK, co-localizes with activated PKCβII to the perinucleus in cardiac myocytes (Ron et al., 1994, pp.839-843) and enhances its activity. PKCε binds to F-actin in vitro and in synaptosomes through eRACK and thus F-actin may show characteristics of eRACK (Prekeris et al., 1996, pp.77-90). Blobe et al. (Blobe et al., 1996, pp.15823-15830) have shown that PKCβII, and not PKCβI, binds to F-actin and stimulates PKCβII activity. These observations may suggest that F-actin may have the characteristics of both eRACK (Prekeris et al., 1996, pp.77-90) and βIIRACK (Ron et al., 1994, pp.839-843).

In inactive PKC, a binding site for RICK is exposed. On activation, PKC isozymes bind to RACKs, altering its conformation and exposing the substrate-binding site. As depicted in FIG. 8, RICK binding site is eliminated. Binding of anchoring proteins to PKC isozymes is mediated through phosphatidylserine (PS) or lipid bridges and through direct protein-protein interactions. These anchoring proteins could contribute to another level of regulation of PKC signaling and provide a platform for cross-talk between PKC isozymes.

Carbohydrate Response Element (ChoRE)

Glucose, the preferred energy source for most eukaryotic cells, alters gene expression in diverse cell types. Glucose stimulates insulin gene expression in pancreatic β -cells, IGF-1 expression in glioma cells, liver-type pyruvate kinase (L-PK) and S_{14} gene expression in hepatocytes and alters epidermal growth factor signaling in rat fibroblasts. Towle et al. first described the carbohydrate response element (ChoRE) in the S_{14} gene that is regulated by glucose (Sayeski, P. P. and J. E. Kudlow. 1996. Glucose metabolism to glucosamine is necessary for glucose stimulation of transforming growth factor- α gene expression. *J. Biol. Chem.* 271: 15237-15243) in hepatocytes. A consensus motif comprising of 5' CACGTG 3' is central for the glucose response in both the L-PK and S_{14} genes. CACGTG motif is also the core-binding site for the c-Myc family of transcription factors. Two copies of CACGTG motif (with a 4 bp out 6 bp match) separated by a 5 bp spacer is critical for glucose control of gene expression. The requirement for two CACGTG motifs suggests that two identical or closely related factors may bind to provide the carbohydrate response noted for transcription of the L-PK and S_{14} genes.

Although a carbohydrate response factor has not yet been described, we could speculate the mode of action of such a factor. Glucose or its metabolite could serve as a direct activator by binding to the ChoRE. An example is the peroxisome proliferating-activated receptor (PPAR), a member of the thyroid/retinoic acid nuclear receptor family. PPAR activates genes involved in fatty acid oxidation by binding directly to DNA response elements of these genes (Nakshatri et al., 1998, pp.2491-2499). Alternatively, a covalent modification such as phosphorylation/dephosphorylation, in response to carbohydrate metabolism might modify the carbohydrate response factor.

The Pathogenesis of Atherosclerosis

Vascular smooth muscle cell proliferation is a principal contributor to the development of atherosclerosis and hypertension—the two major forms of vascular disease. Atherosclerosis is the predominant factor in the advent of heart attack, stroke and gangrene of the extremities. It is responsible for approximately 50% of all mortality in the U.S.A., Europe and Japan. Blood vessels begin as endothelial channels and develop into adult arteries by differentiation of smooth muscle cells.

Replication of smooth muscle cells occurs as the initial event in the formation of atherosclerotic lesions, preceding lipid accumulation or endothelial injury. Smooth muscle cells may be stimulated to proliferate and develop atherosclerotic plaques within the intimal thickenings (Orekhov et al., 1984, pp.17-24; Santen et al., 1972, pp.833-843). Plaques consist of focal proliferation of smooth muscle cells and accumulation of foam cells and extracellular lipid within the thickenings (Schwartz et al., 1986, pp.427-444). In the early stages of the atherosclerotic process, smooth muscle cells migrate from the media to the intima of the arterial wall. Here, the smooth muscle cells proliferate and deposit extracellular matrix components forming a lesion.

The advanced lesions of atherosclerosis become a disease when in excess and can occlude the artery. The endothelium and smooth muscle cell walls respond to various forms of injury or trauma with excessive inflammation and proliferation. The major hypothesis explaining smooth muscle cell replication and proliferation in the blood vessel is the "response to injury" hypothesis present by Ross in 1986 (Ross, R. 1986. The pathogenesis of atherosclerosis—an update. *New England Journal of Medicine* 314:488-500). Ross proposed that smooth muscle cells in the wall normally

exist in a quiescent state. The principal source of connective tissue in the arterial wall is the smooth muscle cell. Smooth muscle cells show two different phenotypes, synthetic or contractile, based on the presence and distribution of myosin filaments and secretory protein apparatus, endoplasmic reticulum and Golgi. As diagrammed in FIG. 9, the primary role of the smooth muscle cell in the synthetic phenotype is proliferation whereas in the contractile phenotype, it contracts in response to external stimuli.

Injury to the endothelium can be caused by exposure to agents like oxidized LDL, toxins, and viruses or may result from mechanical stress. In response to the injury, the endothelium releases potent growth-regulatory molecules that stimulate smooth muscle cell movement and proliferation into the arterial wall. Growth factors, cytokines, lipids and molecules like nitric oxide induce cell recruitment and migration, cell proliferation and control lipid and protein synthesis in the process of atherogenesis.

As the process continues, monocytes become macrophages on reaching the arterial surface, and accumulate lipid. It then proceeds to become foam cells and together with accompanying lymphocytes become the fatty streak predominantly at branch points of the artery. Continued cell influx and proliferation of the smooth muscle cells lead to advanced lesions and ultimately to the fibrous plaque. FIG. 10 shows a representation of the Ross model. Likewise, there is a shift from the contractile to the synthetic phenotype. Progression of atherosclerotic lesions is accelerated by increased smooth muscle cell proliferation.

Diabetes Mellitus

The prevalence of atherosclerotic vascular disease among diabetic patients occurs more frequently as compared to normal subjects. Diabetes mellitus is one of the major causes of mortality and disability in all ages. Diabetes mellitus is an endocrine disorder caused by relative or total deficiency of insulin action. Hyperglycemia, abnormally high blood glucose level, is the diagnostic hallmark of diabetes. Insulin-dependent or type I diabetes has early onset and is caused by the destruction of pancreatic β cells such that insulin is no longer produced. Non-insulin dependent or type II diabetes occurs in middle to older aged individuals. Patients have reduced basal or glucose-induced insulin release or show insulin resistance. In either case, insufficient insulin action leads to overproduction and underutilization of glucose. The flux of glucose and its metabolites has diverse effects on many cellular processes. Various complications arise with hyperglycemia involving the cardiovascular tissue, peripheral nerves, retina, glomeruli and cells involved in wound healing. Excess glucose can alter signal transduction pathways via activation of diacylglycerol and protein kinase C isozymes.

TABLE 4

Diabetes Mellitus		
CAUSE		ONSET
Type I	Destruction of pancreatic β cells	Early
Type II	Insulin resistance	Late

Hyperglycemia and PKC Signal Transduction Pathway

Hyperglycemia is responsible for increasing the risk of developing vascular complications by stimulating abnormal proliferation of vascular smooth muscle cells (VSMC). VSMC exhibit increases in proliferation in response to elevated glucose. Protein kinase C has been implicated as a mediator of diabetes-induced vascular proliferation. Hyperglycemia-induced increases in diacylglycerol (DAG) in tis-

sues like aorta and heart and retina from diabetic rats leads to a sustained elevation of PKC. Multiple cellular and functional abnormalities in the diabetic vascular tissues have been attributed to the activation of DAG-PKC pathway. These include angiogenesis, cardiomyopathy, increased contractility in macrovessels, increased vascular permeability and neovascularization and other diabetic and neurological complications.

Glucose is incorporated into the glycerol backbone of DAG as shown by studies using labeled [^{14}C]- or [^3H]-glucose in aortic smooth muscle cells and aortic endothelial cells. The *de novo* synthesis pathway of DAG involves the metabolism of glycolytic intermediates. The fatty acids predominantly incorporated into DAG are palmitate and oleic acid along with glycerol-3-phosphate. The elevation of DAG in the vasculature induced by hyperglycemia or diabetes is maintained chronically. Thus, diabetic hyperglycemia leads to increased activation of PKC. Although, the PKC isozymes β - and δ - are predominantly detected by immunoblotting studies of vascular cells associated with the DAG-PKC pathway, PKC β II levels are preferentially elevated as a result of increased DAG levels in the aorta and heart of diabetic rats. In prior investigations, experiments were carried out using *in vivo* diabetic rat models subjected to chronic glucose exposure for 2 to 4 months, see for example, Shiba et al. (Shiba, T., W. Heath, R. Sportsman, and G. L. King. 1990. Characterization of the activation of protein kinase C isozymes in the retina of diabetic rats. *Diabetes* 39(Supplement 1):31A). PKC activity was examined in non-synchronized VSMC cultured under chronic high glucose conditions for 5 to 10 days. The increase in PKC activation was measured as increased translocation of the cytosolic PKC to the membrane.

Acute hyperglycemic conditions are prevalent even in controlled diabetic patients. It was established that hyperglycemia is linked to PKC β expression in vascular smooth muscle cells but the molecular mechanism was not elucidated. Studies of acute glucose exposure (from 0 to 24 hours) on PKC β gene expression in VSMC was not carried out. Further, the potential level(s) at which glucose could exert its effect on PKC β isozymes gene expression had yet to be determined. In order to extend our knowledge towards the immediate effects of hyperglycemia on vascular cell proliferation and function via the PKC pathway, a mechanistic study was undertaken. Signal Transduction

In order to coordinate growth, differentiation and response to the environment, cells of multi-cellular organisms have an intricate signaling network comprising of soluble signal molecules or direct contacts. Generally, a signaling molecule interacts with a cell surface receptor and initiates a cascade through the cytoplasm leading to the nucleus where the specific genes are regulated. Cell surface receptors such as receptor tyrosine kinase (RTK) or transforming growth factor- β receptors autophosphorylate and phosphorylate the cytoplasmic protein in response to binding of an external ligand. A complex series of intracellular responses results in diverse cellular responses as proliferation, differentiation, cell motility, production of extracellular matrix and transcription of specific genes.

Cytoplasmic signal transduction components include Ras, MAP kinase, PKC and other signaling pathways (FIG. 11). Ras is a small membrane-bound guanine nucleotide-binding protein that acts as a molecular switch linking receptor tyrosine kinase activation to downstream signaling events. Ras cycles between an active GTP-bound form and an inactive GDP-bound form. The activity of Ras is regulated by Sos, a guanidine nucleotide exchange factor (GNEF). The carboxyl-terminal of Sos contains proline-rich motifs that mediate the interaction of Sos with Grb2, an adaptor molecule

comprising of a SH2 domain flanked by two SH3 domains. Following receptor tyrosine kinase activation and autophosphorylation, Sos-Grb2 translocates to the plasma membrane and the vicinity of Ras, thereby activating Ras. The downstream effector of RasGTP is Raf, a serine/threonine kinase that links Ras to the MAP kinase pathway.

Mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) is a serine/threonine kinase that phosphorylates microtubule-associated protein (MAP). MAPK is phosphorylated in response to a variety of mitogens including nerve growth factor (NGF), insulin, insulin-like growth factor II, and phorbol esters. MAP kinase kinase (Mek) activates MAP kinase by phosphorylating both tyrosine and threonine residues. Raf forms a stable complex with Mek and phosphorylates Mek on serine residues *in vitro*. Ribosomal S6 kinase (Rsk) is a component of the eukaryotic 40S ribosomal subunit that is phosphorylated on multiple serine residues in response to mitogen stimulation. Purified MAP kinase from insulin-stimulated cells could activate Rsk and comprise a step in the kinase pathway to transduce mitogenic signals to the nucleus. Thus the MAP kinase pathway (FIG. 11), consisting of Ras, Raf, Mek, MAPK and Rsk spans from the plasma membrane to the nucleus and serves to transduce mitogenic signals downstream from the membrane receptor tyrosine kinases. Several components of this pathway such as Ras and Raf are oncogenic in their constitutively active form. MAP kinases can translocate to the nucleus upon mitogenic stimulation and regulate growth-specific gene expression. Transcriptional factors c-Myc and C/EBP β can be phosphorylated by MAP kinases *in vitro* and hence a spectrum of genes can be regulated by the MAP kinase pathway.

Nuclear responses to external signals can be achieved via the transcription factors or by nuclear receptors such as steroid receptors. In response to cytokines such as interferon α/β , the Jak/STAT pathway is activated to regulate the transcription of genes. The initial step is ligand-dependent dimerization of the receptors that brings the Janus kinases (Jaks) into close proximity. Jaks are constitutively associated with the receptor chain and are tyrosine phosphorylated upon receptor aggregation. The activation of Jak correlates with the tyrosine phosphorylation of the receptor components that recruit the signal transducers and activators of transcription (STATs) through their SH2 domains which, in turn, are phosphorylated at their carboxy-terminal tyrosine residue. Homodimers of the STATs translocate to the nucleus where they interact with the promoter elements or combine with DNA binding motifs.

Phosphoinositide-3-kinase (PI3-kinase) is implicated as a mediator of biological responses such as DNA synthesis, glucose transport, cytoskeletal rearrangements, cell survival, oocyte maturation and receptor internalization. PI3-kinase phosphorylates the inositol ring of phosphatidylinositol (PtdIns) to generate phosphatidylinositol-3-phosphate, PtdIns(3)P and further phosphatidylinositol-3,4-bisphosphate, PtdIns(3,4)P $_2$ and phosphatidylinositol-3,4,5-trisphosphate, Ptd(3,4,5)P $_3$. Further, phosphatidylinositol-3,4-bisphosphate is a substrate for phospholipase C which generates the second messengers Ptd(3,4,5)P $_3$ and diacylglycerol.

PI3-kinase consists of two subunits, p85 α and p110 α . The catalytic activity resides in the 110 kDa subunit while the 85 kDa subunit performs a regulatory role. As diagrammed in FIG. 12, PI3-kinase is activated by several molecules that include cytoplasmic Src-family kinases, receptors with intrinsic tyrosine kinase activity, focal adhesion kinase (FAK), Ras-GTP, Rho and others. The downstream targets of PI3-kinase include the protein kinase C family, p70-S6

kinase, glucose transporter GLUT4 (Hara et al., 1994, pp.7415-7419), serine/threonine kinase Akt/Rac.

Protein Phosphatases

Reversible protein phosphorylation is a critical component of the signal transduction mechanisms in eukaryotes. It is the basis for the control of many biological events triggered by extracellular effectors like hormones, mitogens, oncogenes, cytokines and neurotransmitters. Reversible protein phosphorylation catalyzed by the opposing and dynamic action of protein kinases and phosphatases, is a ubiquitous element of intracellular signal transduction pathways that regulate metabolism, gene expression, cell division, differentiation, development, contraction, transport, cell locomotion, and learning and memory. Abnormal changes in the activities of the protein kinases and phosphatases can lead to severe consequences including immunological defects and cancer.

Protein phosphatases can be classified into three groups according to their substrate specificity.

1. Serine/threonine phosphatases dephosphorylate serine (Ser) and threonine (Thr) residues in proteins and usually require a divalent metal ion to function. They are further divided into two classes: type-1 phosphatase (PP1) preferentially dephosphorylates the β -subunit of phosphorylase kinase whereas the type-2 phosphatases (PP2) preferentially dephosphorylate the α -subunit of phosphorylase kinase. PP2 can be further subdivided into spontaneously active (PP2A), Ca^{+2} - and calmodulin-dependent (PP2B) and Mg^{+2} -dependent (PP2C) classes. Okadaic acid, polyether fatty acid and a potent inhibitor of protein phosphatases is cell permeable and serves as a valuable tool to study the functions of protein phosphatases. It is a potent inhibitor of PP2A ($K_i=0.2$ nM) and PP1 ($K_i=20$ nM) and a less potent inhibitor of PP2B ($K_i=\mu\text{M}$).
2. Tyrosine phosphatases dephosphorylate tyrosine (Tyr) residues in proteins and share a conserved active-site sequence motif Cys-X5-Arg (X=any amino acid residue) [SEQ ID NO:1] and a Asp located in a surface loop. Protein tyrosine phosphatases (PTPs) are characterized by a signature sequence motif of 11 amino acid residues, (Ile/Val)-His-Cys-X-Ala-Gly-X-Gly-Arg-(Ser/Thr)-Gly [SEQ ID NO:2] that is found in most PTPs. The diversity within the PTPs arises from the variable N- or C-terminal sequences attached to the core catalytic domain.
3. Dual-specificity phosphatases dephosphorylate Ser/Thr residues in addition to Tyr residues in proteins. Their signature motif, His-Cys-X-X-Gly-X-X-Arg-(Ser/Thr) [SEQ ID NO:3] is analogous to PTPs but these phosphatases display a restricted substrate specificity.

Cell Cycle and Role of PKC β Isozymes in the A10 Vascular Smooth Muscle Cells.

The process of cellular proliferation in eukaryotes includes the division of a cell into two daughter cells. Cell division involves mitosis in which the nuclear membrane dissolves, the chromosomes condense and separate into two groups, and two nuclear membranes reassemble around the chromosomes. Finally, in the process of cytokinesis, the cell membrane contracts in the middle and two halves separate to form individual cells.

The phases of the cell cycle that occur prior to cell division start with the G1 phase. The S phase follows in which the chromosomes are replicated and the cell then enters the G2 phase where the preparation for mitosis (M phase) occurs. Cell cycle regulation predominantly occurs during the G1/S transition and the G2/M transition.

Vascular smooth muscle cells (VSMC) cultured in plasma-derived medium are unable to proliferate and are arrested in the G0 phase. These quiescent cells progress into the S phase upon addition of growth factors and begin DNA synthesis (Ross et al., 1978, pp.497-508). It has been demonstrated that PKC β I stimulated while PKC β II inhibited the G1/S transition. VSMC proliferation is accelerated when the inhibitory role of PKC β II on proliferation is disrupted. Further, the overexpression of PKC β I shortened the S phase while the overexpression of PKC β II prolonged the S phase suggesting a role for PKC β I and - β II in the entry of G2/M phase. The requirement for PKC β II for the G2/M phase transition of cell cycle has also been shown in HL60 cells.

Levels of Gene Regulation

All cells regulate gene expression in response to changes in the external environment. Nutritional and metabolic signals play an important role in controlling gene expression.

Levels of regulation in eukaryotes is shown in FIG. 13. The central dogma of biochemistry states that the genes are aligned on the chromosomes and are made of DNA which is transcribed into RNA, followed by translation into proteins. RNA also undergoes maturation by post-transcriptional modifications like the 5' methylation and capping, 3' polyadenylation, alternative or differential splicing according to tissue specificity and editing. The processing in eukaryotic mRNA occurs in both the nucleus and cytosol and involves a fine balance between transcriptional and posttranscription levels. The protein undergoes post-translational modification such as glycosylation, farnesylation and addition of signal peptides for maturation.

Post-transcriptional regulation of the mRNA stability establishes the steady state level of the transcript. Although the steady state level of any mRNA depends on both the rate of synthesis and degradation of the mRNA, abundance of many mRNAs is correlated with the longevity of the transcript.

The RNA transcript is initially synthesized as the heterogeneous nuclear RNA by the action of RNA polymerase II on genomic DNA, and is further processed into mature mRNA within the nucleus. It is then translocated into the cytoplasm where its fate is determined. The mature mRNA can undergo localization, degradation or be translated into the protein (see FIG. 14). Messenger RNA degradation in eukaryotic cells is a regulated process that represents a powerful means for controlling gene expression. mRNA decay is not a random process involving degradation by nucleases. Rather, it is a precise process orchestrated by cis elements coordinating the trans-acting factors in controlling gene expression.

The elements involved in the regulation of transcript stability are shown in FIG. 15. mRNAs can contain two or more separated stability determinants, each of which may be involved in a distinct decay pathway or a specific response to a regulatory factor. For instance, multiple elements within the interleukin-2 (IL-2) mRNA modulate its stability in a combinatorial manner. Although the 3'UTR with its AU-rich elements are primarily involved in regulating stability, the 5' UTR and its immediate coding region contain an element that confers c-jun NH₂-terminal kinase (JNK)-mediated stabilization.

1. 5' cap: The mRNA cap is important in preventing the degradation of transcripts. mRNAs without caps are fourfold less stable than capped mRNAs as observed in oocytes and cell-free in vitro decay reactions. Decapping could be a rate-limiting step in mammalian mRNA decay. The mRNA cap may be involved in the translational activity of the transcript that could then modulate its rate of degradation.

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2. 5' untranslated region (5' UTR): The half-life of mRNAs is affected by its 5' UTR and its influence on translational regulation. The presence of a secondary structure, like a stem-loop, can inhibit translation. The ferritin mRNA contains an iron responsive element (IRE) in its 5' UTR that affects its translation. Ferritin is a major intracellular iron storage protein. Under low iron conditions, an iron responsive protein binds to the IRE and represses translation.

The 5'UTR of the long-lived *Escherichia coli* ompA transcript functions as an mRNA stabilizer that prolongs the cytoplasmic lifetime of mRNAs by hindering distinct mRNA pathways for mRNA degradation. Two domains of the 5'UTR are responsible in this stabilizing effect: the 5'-terminal stem loop and the single stranded RNA segment (ss2) that contains a ribosome binding site.

3. Premature stop codons: Nonsense codons lead to abrupt termination of translation. Another function attributed to some early premature stop codons is to decrease the levels of nuclear-associated transcripts, thereby reducing the steady state levels of mature mRNA available for translocation to the cytoplasm. This is also observed in phytohemagglutinin mRNA whose stability is decreased by the presence of premature nonsense codons.
4. Coding region sequences: Sequences within the coding region can also play a role in determining the mRNA half-life. Stability determinants have been demonstrated within the coding regions of c-myc mRNA and c-fos is a rapidly inducible gene involved in immediate early response. It contains three stability determinants—one in the 3' UTR and two in the coding region. One of the coding-region determinants encodes the basic and leucine zipper regions and its structure specifies the instability of mRNA irrespective of the protein binding to it. Another example is that of c-myc mRNA coding region that influences the mRNA half-life via a C-terminal determinant containing a part of the helix-loop-helix and the entire leucine zipper motif (Ross, 1995, pp.423-450). The capsid protein L2 of the human papillomavirus (HPV) type 16 contains cis-acting inhibitory sequences in its coding region that act in an orientation dependent manner to reduce cytoplasmic and nuclear mRNA levels. In the case of *Synechococcus* sp. strain PCC 7972, the 5'UTR acts mutually with the coding-region stability determinant to regulate its stability.

Bernstein, P. L., D. J. Herrick, R. D. Prokipcak, and J. Ross. 1992. Control of c-myc mRNA half-life invitro by a protein capable of binding to a coding region stability determinant. *Genes Dev.* 6:642-654 demonstrated a protein binding within a c-myc coding region that affected its stability. Proteins that bind to mRNA coding region are implicated in determining the decay rate of the mRNA. Proteins binding to c-fos and c-myc coding region stability determinants have been described that influence the mRNA stability. A 70 kDa poly-some-associated protein that binds to the c-myc coding region has been identified and purified (see, for example Prokipcak, R. D., D. J. Herrick, and J. Ross. 1994. Purification and properties of a protein that binds to the C-terminal coding region of human c-myc mRNA. *J. Biol. Chem.* 269: 9261-9269) and serves to protect it from endonucleolytic attack. The urokinase-type plasminogen activator receptor (uPAR) mRNA stability is regulated by a 50-kDa protein binding to a 51-nucleotide fragment in the coding region.

In the case of the β -tubulin mRNA, destabilization is initiated by excess tubulin monomers encoded by the coding-

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region stability determinant. However, at present date, involvement of a protein in this autoregulation has not been demonstrated.

5. 3' untranslated region (3' UTR): The majority of research has focused on the stability determinants that lie in 3' UTR of the mRNAs. The best-characterized stability determinants are in the 3'UTR of the mRNAs for transferring receptor. The transferring receptor imports iron into cells. Post-transcriptional regulation is achieved through a iron responsive element (IRE) which functions by binding an iron regulatory protein (IRP). When intracellular iron concentration is abundant, the IRE-IRP complex does not form and the transferring receptor is unstable. When the concentrations of iron are low, the formation of the IRE-IRP complex stabilizes the transferring receptor. Another example is that of ribonucleotide reductase R2 mRNA which contains a cis-element in the 3' UTR that is regulated by the TGF- β family. Treatment with TGF- β_1 , TGF- β_2 , or TGF- β_3 stabilizes the ribonucleotide reductase R2 mRNA. The GAP-43 mRNA (GAP-43 is a neuronal protein) in undifferentiated PC12 cells contains pyrimidine-rich sequences in its 3'UTR that bind to two proteins and regulate its stability.

6. Adenosine-uridine (AU) rich sequences: AU-rich elements (AUREs or AREs) are found in the 3' UTR of mRNAs that encode proto-oncogenes, nuclear transcription factors and cytokines and represent the most common RNA destability determinants characterized in mammalian cells. The AUREs generally contain one or more copies of the AUUUA pentanucleotide and a high content of uridylate and sometimes adenylate residues. A nonamer with an AUUUA core, UUAUUUA(U/A)(U/A), is suggested to be a better indicator in predicting the destabilizing function of the AU-rich sequences. AUREs facilitate rapid deadenylation as the first step in mRNA degradation. The destabilizing activity of an AURE can be increased or decreased by interactions in cis with other sequences like a U-rich region or interactions with trans-acting regulatory factors like the AU-binding proteins (AUBPs). The AREs can be distinguished into three classes: Class I AUUUA-containing AREs direct the synchronous shortening of poly A tails to 30 to 60 nucleotides before mRNA decay; Class II AUUUA-containing AREs direct the asynchronous shortening of poly A tails, with intermediate fully deadenylated products; and a novel class of non-AUUUA ARE described in the c-jun proto-oncogene mRNA which are insensitive to blockage of their effects by the addition of transcription inhibitors.

7. 3' terminal stem loop structure: The histone gene transcripts are cell cycle regulated with large number of molecules present during the S phase and a dramatic decline at the end of the S phase. The histone mRNAs are not polyadenylated but the presence of a 3' terminal stem loop structure confers cell cycle regulation of transcript stability. A stem-looping binding protein and histone proteins themselves might be involved in the regulation process.

8. Poly(A) tail: The polyadenylated tail has multiple functions affecting nuclear processing of pre-mRNA, transport to the cytoplasm, translation, and cytoplasmic mRNA stability. The poly(A) tail via binding to the poly(A) binding proteins (PABPs), protects mRNAs from rapid or indiscriminate degradation. Polyadenylation can affect transcript stability in mammalian cells by the poly(A) addition site selection used during process-

ing of hnRNA into mature mRNA as seen in the case of insulin-like growth factor I.

Post-transcriptional regulation of gene expression is a critical step in determination of steady state levels of the mRNA. The stability of mRNA can also be influenced by a variety of exogenous factors such as hormones, ions or nutrients (Ross, 1995, pp.423-450). The processing of mRNA involves cis elements present in the transcript that are recognized by specific RNA-binding proteins and participate in RNA masking, mRNA stabilization, and/or movement of mRNAs among different ribosome populations within the cell.

For some time, the development of sensors for the recognition and analysis of sugars has attracted much attention. Such sensors find useful applications in the food industry and in clinical analysis. Detection and monitoring of glucose is crucial for people with diabetes. The importance of glucose monitoring for in vivo and in vitro (e.g., ex vivo) applications has driven the pursuit of the development of an advanced glucose sensor. The use of enzymes has exhibited some limitations in the development of implantable sensors for continuous glucose monitoring in blood or interstitial tissue. Continuous monitoring of blood glucose level is very important for the long term health of diabetics and could lead to important medical technology such as a blood sugar alarm system and an in vivo control device for an implanted insulin pump.

Large variations in blood glucose level can result in serious medical problems for diabetics, such as cardiovascular disease, neuropathies, and blindness. Non-invasive measurement of blood glucose has been a long-standing research goal and a wide variety of such methods have been describe in the literature, including near-infrared spectroscopy, optical rotation, amperometric, calorimetric, and fluorescence detection (Robinson, M. et al., *Clin. Chem.*, 1992, 38(9): 1618-1622; Heise, H. M et al., *Artif. Organs*, 1994, 18(6): 439-447; Burmeister, J. J. et al., *Photochem. Photobiol.*, 1998, 67(1): 50-55; March, W. F. et al., *Trans. Am. Soc. Artif. Intern. Organ*, 1992, 28: 232-235; Rabinovitch, B. et al., *Diabetes Care*, 1982, 5(3): 254-258; Claremont, D. J. et al., *Diabetologia*, 1986, 29: 817-821; Yokoyama, K. et al., *Anal. Chim. Acta*, 1989, 218: 137-142; Schier, G. M. et al., *Diabetes Res. Clin. Pract.*, 1988, 4:177-181; Clarke, W. et al., *Diabetes Res. Clin. Pract.*, 1988, 4:209-214; Tretnak, W. et al., *Anal. Chim. Acta*, 1989, 221:195-203; Meadows, D. et al., *Talanta*, 1988, 35(2): 145-150; Tolosa, L. et al., *Sensors Actuators B*, 1997, 45:93-99; Tolosa, L. et al., *Anal. Biochem.*, 1999, 267: 114-120; D'Auria, S. et al., *Biochem. Biophys. Res. Comm.* 274: 727-731). Despite some promising results, these methods show limitation as important background with the near infrared (NIR) technique and low optical rotation and important depolarization due to the tissue with the optical rotation technique. Enzymes and proteins are widely used in the research for the development of glucose sensors. At present, the most reliable method to measure blood glucose is by finger stick and subsequent glucose measurement, typically by glucose oxidase. A competitive glucose assay using fluorescence resonance energy transfer between concanavalin A and dextran has been developed and efforts are also underway to develop methods for the use of intrinsic fluorescence changes using thermophilic enzymes. Proteins and enzymes show an affinity constant comparable with blood glucose level, show a great selectivity and are biocompatible. Despite these advantages, they exhibit low stability (to heat and organic solvents), solubility problems and are difficult to modify.

Insulin is normally produced in and secreted by the beta cells of the islets of Langerhans in the pancreas. The glucose responsive release of insulin from the beta cells is a complex

event including gene expression, posttranslational modification and secretion. In normally functioning beta cells, insulin production and release is affected by the glycolytic flux. Glucokinase and glucose transporter 2 (GLUT-2) are two proteins that are believed to be involved in sensing changes in glucose concentration in beta cells. A reduction in GLUT-2, which is involved in glucose transport, is correlated with decreased expression of insulin; loss of glucokinase activity causes a rapid inhibition of insulin expression.

Autoimmune destruction of pancreatic beta cells causes insulin-dependent diabetes mellitus or Type I diabetes. As a consequence of partial or complete loss of beta cells, little or no insulin is secreted by the pancreas. Most cells, with the exception of brain cells, require insulin for the uptake of glucose. Inadequate insulin production causes reduced glucose uptake and elevated blood glucose levels. Both reduced glucose uptake and high blood glucose levels are associated with a number of very serious health problems. In fact, without proper treatment, diabetes can be fatal.

In treating diabetic patients, the aim is to tightly regulate the plasma glucose level within the normal physiological range (80-120 mg/dL), so that diabetic adverse effects can be avoided. As an aid to diabetes therapy, continuous monitoring of blood glucose concentrations in vivo has long been recognized as a major objective as a future tool in the fight against diabetes. People with diabetes have increased risk of cardiovascular disease as well as retinopathy and neuropathy. It has been shown that tight control of glucose levels in the diabetic population to normoglycemic or slightly hyperglycemic levels results in delayed onset and slowed progression of retinopathy, nephropathy, and neuropathy (See DCCT study group, *The New England Journal of Medicine*, 1993, 341: 1306:1309).

During the past decade, intense effort has been directed toward the development of glucose monitoring biosensors as an aid to diabetes therapy. Development of an implantable glucose sensor that is specific to glucose and sensitive enough to precisely measure glucose levels in vivo would be a significant advance in the treatment of diabetes. Such ability to more closely control blood glucose levels would also be useful in insulin delivery system responsive to glucose levels in diabetic patients. Glucose biosensor systems have recently been described which employ glucose binding molecules attached to a polymeric hydrogel for example (See e.g., U.S. Pat. No. 6,475,670).

For several decades, fluorescence spectroscopy has been widely use for the detection and analysis of different analytes (D'Auria, S. et al., *Biochem. Biophys. Res. Comm.*, 2000, 274: 727-731; B. Valeur, *Topics in Fluorescence Spectroscopy*, J. R. Lakowicz ed.; Plenum Press, New York, 1994, pp. 21-48; M. Poenie, C. -S. Chen, *New Fluorescence Probes for Cell Biology*, B. Herman and J. J. Lemasters ed.; Academic Press, New York, 1993, pp. 1-25). Wavelength-ratiometric, fluorescence lifetime based sensing and polarization assays are some techniques available for the detection and analysis of analytes by fluorescence spectroscopy (Smacinski, H., J. R. Lakowicz, *Topics in Fluorescence Spectroscopy*, J. R. Lakowicz ed.; Plenum Press, New York, 1994, pp. 295-334; Smacinski, H., et al., *Sensors and Actuators B*, 1995, 29:15; Lakowicz, J. R. et al., *Anal. Biochem.*, 1999, 267:397). Fluorescence techniques for glucose recognition have been predominantly used with enzymes and proteins. Despite some promising results, enzymes and proteins show some stability problems against organic solvents and heat.

Maintenance of normal blood glucose levels is important for nutrition of certain tissues (e.g., brain and other nervous system tissues and gonadal germinal epithelium) that are

substantially incapable of metabolizing other energy sources such as fatty acids or amino acids. Lipid and protein metabolism can be undesirable, in that such metabolism depletes bodily stores of lipids and proteins, and in that the by-products of such metabolism (e.g., certain lipoprotein-containing particles) can cause or contribute to pathological conditions (e.g., deposition of lipoprotein plaque in arteries). Thus, in addition to providing nutrition to tissues that metabolize glucose almost exclusively, maintenance of normal blood glucose levels prevents physiologically inappropriate reliance of the body on non-carbohydrate catabolic routes.

In diabetic patients, in whom aberrantly diminished secretion of insulin leads to defects in carbohydrate metabolism, fat metabolism is abnormally increased, leading to greater-than-normal levels of circulating fatty acids, which in turn cause greater-than-normal deposition of cholesterol and other plaque materials in arteries. Indeed, abnormalities in fat and protein metabolism are common in diabetics, and account for much of the morbidity and mortality experienced by such patients, including acidosis, arteriosclerosis, coronary artery disease and other circulatory disorders, and wasting disease conditions (i.e., attributable to aberrant protein degradation).

In view of the above, there exists a great need in the art for a rapid, convenient, and economical method for routine and early detection of disorders of glucose metabolism.

One conventional treatment for diabetes involves periodic administration of injectable exogenous insulin. This method has extended the life expectancy of millions of people with the disease. However, blood glucose levels must be carefully monitored to ensure that the individual receives an appropriate amount of insulin. Too much insulin can cause blood glucose levels to drop to dangerously low levels. Too little insulin will result in elevated blood glucose levels. Even with careful monitoring of blood glucose levels, control of diet, and insulin injections, the health of the vast majority of individuals with diabetes is adversely impacted in some way. As the treatment of diabetes, a diet therapy, a kinesitherapy, a remedy for obesity, or the like are mainly carried out in mild cases, an oral medicament for diabetes (for example, an agent for promoting insulin secretion such as sulfonylureas) may also be administered when symptoms become severe and, as indicated above, an insulin preparation is administered in serious cases (Ryuzo Abe and Masato Kasuga, "An Approach to EBM on the Treatment of Diabetes Mellitus", Nankodo, 1997; Richard A. Harrigan et al., *Annals of Emergency Medicine*, 2001, 38(1): 68-78; and Japan Diabetes Society, "Tounyoubyou chiryou gaido 2000 (Treatment of diabetes mellitus, Guide 2000)", Bunkodo, 2000).

Sulfonylureas stimulate pancreatic β cells and promote insulin secretion. However, the timing of insulin secretion and an amount of insulin secreted are decided by the timing of a medicament administration and its dose, regardless of a blood glucose level. Therefore, hypoglycemia caused by maintenance of the medicament activity, as a side effect, sometimes occurs. Further, symptoms in the digestive system such as loss of appetite occur. Furthermore, sulfonylureas are contraindicated for patients with a hepatic or renal dysfunction or severe ketosis (Richard A. Harrigan et al., *Annals of Emergency Medicine*, 2001, 38(1): 68-78). Insulin preparations decrease blood glucose; however, they must be administered by injection, and they sometimes cause hypoglycemia (McCrimmon R. J. et al., *Diabete. Metab.*, 1994, 20(6):503-512).

Type II diabetes includes the most prevalent form of diabetes, which results from insulin resistance with an insulin secretory defect. Pharmacological treatments such as metformin and rosiglitazone have an ameliorating effect on insu-

lin resistance and are believed to increase the effectiveness of endogenous insulin and thereby contribute to the lowering of elevated blood glucose levels in type II diabetes patients. Research has shown that the current class of insulin sensitizers that are used to treat type II diabetes, the thiazolidinediones, may act by increasing glucose transport activity of cells (Petersen et al., *Diabetes*, 2000, 49:827-831), and several screening assays for potential type II diabetes drugs measure glucose uptake as a measure of efficacy. However, these assays do not provide further information regarding glucose dynamics that are important in type II diabetes, such as whether the glucose that is taken up by the cell accumulates or is metabolized. It would be advantageous to have available an assay that could be used to complement glucose uptake measurements to provide more complete information concerning glucose dynamics during drug screening.

As described above, conventionally used agents for promoting insulin secretion and insulin preparations have these problems. Therefore, agents capable of advanced control of blood glucose, i.e., agents not simply decreasing blood glucose but capable of controlling blood glucose within a normal range, are desired.

The uptake of glucose by a number of transporters is a fundamental process of cells. Basal glucose uptake is a constitutive process and insulin-stimulated glucose uptake (ISGT) is responsible for glucose uptake as mediated by the signaling cascade involving sequential phosphorylation of the insulin receptor substrate 1, phosphoinositol 3-kinase (PI3K), phosphoinositide-dependent kinase 1, and its targets, PKB/Akt and the protein kinase C isozymes. To measure ISGT, most cell based assays require cells to be treated with insulin in the presence or absence of various inhibitors or drugs followed by another incubation with labeled {3H-1, 2}2-deoxy-D-glucose in the presence or absence of cytochalasin B. Following a rapid rinse in ice-cold buffer, cells are then lysed with NaOH and the lysate is transferred to a scintillation vial and associated radioactivity is determined in a scintillation counter.

Existing products for measuring intracellular glucose are enzymatic assays and radiolabeled glucose, which are time-consuming. Furthermore, these products do not permit direct, live cell, glucose assessments since they require cell lysis, pipetting of samples, and transfer to other mediums to detect glucose import. Therefore, it would be advantageous to have available live cell assays that are adaptable for high-throughput screening of candidate compounds for potential glucose uptake regulators, or for the simultaneous measurement of multiple processes (such as side effects of a potential therapeutic drug on cell apoptosis).

Since there is an increasing prevalence of diabetes and there are high costs associated with diabetes diagnosis, care and management to the healthcare industry, there will be an increased reliance of pharmaceutical companies on biotechnology research tool manufacturers. Development of cell lines equipped with a glucose sensor that provides information as to intracellular glucose levels and that utilizes a real-time sensing scheme would be of interest to major pharmaceutical companies developing drugs to treat type-2 diabetes.

The value of such cell lines may be increased by the utilization of new life science tools and technologies to screen new drugs. The prevalence of type 2 diabetes in an aging and obese population will continue to drive the pharmaceutical industry to develop new compounds. There are many rate-limiting aspects to the development of new compounds, such as solubility, toxicity, etc. Having available a live cell assay would allow for the co-screening of toxicity, for example, by measuring apoptosis in the same cells.

Regulating gene expression by modulating the turnover of mRNA is an important post-transcriptional mechanism to ensure a rapid cellular response to appropriate stimuli (Mitchell P. et al., *Curr. Opin. Genet. Dev.*, 2000, 10:193-198). Stability of RNA transcripts provides a mode of regulating gene expression. While many transcripts are present at low steady-state levels in the cytoplasm due to their inherent lability, transient stabilization under appropriate conditions leads to their rapid accumulation and expression (Guhayinogi J. et al., *Gene*, 2001, 265:11-23). Conventional methods of detecting transcript stabilization are based on blocking transcription with a transcription inhibitor such as actinomycin D, and harvesting the RNA at appropriate intervals. The RNA is then resolved by gel electrophoresis, transferred to membranes, and probed for the transcript of interest by northern hybridizations. Alternatively, the levels of a transcript can be detected by quantitative PCR. Stability is then assessed by the decay or persistence of the transcript over the time-course following actinomycin D addition. These methods are labor intensive and time consuming, making them difficult to adapt for the rapid screening of large numbers of compounds for their ability to influence mRNA turnover rates. Recently, Benjamin et al. reported the application of a green fluorescent protein (GFP)-based cellular assay wherein the GFP coding transcript is rendered unstable by fusion to the IL-3 3'-UTR, which contains a canonical class II adenosine-uridine (AU)-rich element (ARE) that is responsible for destabilizing the native IL-3 transcript (Benjamin D. et al., *Nucleic Acids Research*, 2004, 32(11):e89). Changes in transcript stability were mirrored by altered GFP levels, which could be directly measured by FACS analysis. Using this reporter system, the investigators identified okadaic acid as an mRNA stabilizing compound, and investigated the upstream signaling pathways leading to stabilization.

PKC β II, a member of the conventional PKC family, is implicated in insulin-stimulated glucose transport (Cooper, D. R. et al. *Archives of Biochemistry and Biophysics*, 1999, 372(1):69-79). It has previously shown that in presence of high glucose (25 mM), PKC β II mRNA is destabilized and is thus manifested as "switching off" PKC β II expression (Patel, N. A. et al. *FASEB Journal*, 1999, 13:103-113). It has been demonstrated that glucose-induced post-transcriptional destabilization of PKC β II message is mediated via a nuclease activity present in the cytosol. The specificity of glucose-induced post-transcriptional destabilization of PKC β II mRNA was confirmed via reverse transcriptase-polymerase chain reaction (RT-PCR), in both A10 cells and primary cultures from human aorta, while not significantly altering PKC β I mRNA (Patel, N. A. et al. 1999). An instability element in the PKC β II mRNA, which is regulated by high glucose concentrations, has been recently described. A 38 bp sequence was identified to contain the glucose-regulated instability element (Patel, N. A. et al. *J Biol Chem*, 2003, 278:1149-1157). The present invention utilizes a glucose-regulated mRNA instability element within the PKC β II mRNA as a sensor of intracellular glucose.

BRIEF SUMMARY OF THE INVENTION

Complications involved in cardiovascular tissue injury recovery are aggravated with episodes of acute hyperglycemia. Protein kinase C (PKC) has been implicated as a mediator of diabetes-induced vascular proliferation. Diabetic conditions, especially acute hyperglycemia, may provoke the excessive formation of atherosclerotic lesions that may ultimately lead to the formation of the fibrous plaques. This study elucidates molecular events occurring after initiation of the

cell cycle in quiescent vascular smooth muscle cells following exposure to acute hyperglycemic conditions.

PKC β II is the predominant isoform detected in quiescent smooth muscle cells although these cells also express PKC α , - β I, - δ and - ϵ . Our previous studies indicate that PKC β I and PKC β 2II regulate the vascular smooth muscle cell cycle. In A10 cells (a clonal cell line of VSMC) transfected to overexpress PKC β II, DNA synthesis was attenuated. This suggested that PKC β II might function as a cell cycle mediator during G1/S phase transition in VSMC. It was demonstrated that PKC β II protein expression was decreased and the percentage of A10 cells entering the S phase was increased in VSMC in the presence of acute high extra-cellular glucose concentrations. Northern blot studies indicated that the steady-state levels of PKC β mRNA were decreased by high glucose. Based on these findings, it was hypothesized that the hyperglycemia-induced proliferation of VSMC may be related to the regulation of PKC β gene expression by glucose.

The present invention utilizes an element that regulates messenger RNA (mRNA) stability in response to a metabolite such as glucose or a glucose analog. This glucose-regulated mRNA instability element has been mapped to the protein kinase C β II (PKC β II) mRNA that was found to decrease in the presence of elevated glucose levels. When cloned into a reporter vector, the region of PKC β II containing the mRNA instability element imparts glucose-sensitive instability to the mRNA that is transcribed, thereby down-regulating the expression of the reporter gene when glucose is elevated.

Reporter vectors with the glucose-regulated mRNA instability element can be used as a research tool to indicate how much glucose is present in a cell. Because the glucose sensitive mRNA instability element destabilizes the mRNA of the reporter gene in the presence of elevated glucose levels, the reporter mRNA level or the reporter polypeptide level can serve as an inverse indicator of intracellular glucose levels. Other glucose-based mechanisms for regulating gene expression typically first require metabolism of glucose to other compounds before glucose can function as a regulator of expression. However, using the glucose-regulated mRNA instability element, glucose only has to be phosphorylated to affect mRNA instability. Thus, the element is a more direct sensor of glucose levels than other glucose-based mechanisms.

In one aspect, the present invention includes a reporter vector comprising a PKC β II mRNA instability element and a polynucleotide encoding a reporter molecule (such as green fluorescent protein (GFP)). In another aspect, the present invention includes host cells genetically modified with a reporter vector of the present invention, and methods for detecting intracellular glucose using the host cells of the present invention. In another aspect, the method for detecting intracellular glucose is used to identify an agent or treatment that modulates glucose uptake in a cell (such as a mammalian cell), the method comprising contacting a host cell with a candidate agent, wherein the host cell comprises a vector comprising a PKC β II mRNA instability element and a polynucleotide encoding a reporter molecule (such as green fluorescent protein (GFP)); and determining whether expression of the reporter molecule is modulated. The method can include determining whether the candidate agent or treatment increases or decreases glucose uptake in the cell.

Thus, reporter vector, host cells, and methods of the present invention may be used to measure intracellular glucose concentrations, and as a tool to indirectly measure processes that affect intracellular glucose levels, such as glucose transport or glucose metabolism. The recent emergence of fluorescence-based high-throughput screening systems suggest that this

technology has have particular commercial potential as an intracellular one-step glucose sensor.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Schematic representation of the PKC isozymes domain structure. V1 to V5 are the variable regions while C1 to C4 are the common (or conserved) regions. The regulatory domain comprises of the pseudosubstrate site, and one or two of the membrane-targeting motifs, namely C1 present in all PKCs and C2 present in cPKCs and nPKCs. The regulatory domain is separated from the catalytic domain by the hinge (or V3) region. The catalytic domain consists of the ATP-binding and substrate binding sites, the C3 and C4 regions respectively.

FIG. 2. A schematic representation of PKC β sequence as deduced from the cDNA analysis. The V5 region of alternative splicing for PKC β I and PKC β II is indicated. The resulting amino acids for PKC β I and PKC β II consist of 671 amino acids and 673 amino acids respectively.

FIGS. 3A and 3B. Alternative splicing of PKC β pre-mRNA. In FIG. 3A introns are represented by lines while the boxes represent exons. The approximate sizes of the introns and exons of the 3' end of PKC β gene are indicated. In FIG. 3B, the polyadenylation site contains the hexanucleotide AUUAAA. Splicing of the last common exon C4 to β IV5 exon produces the mature PKC β I mRNA whereas the PKC β II mRNA has the β IV5 exon included. Both PKC β I mRNA and PKC β II mRNA have a common 3' untranslated region and poly(A) tail.

FIG. 4. PKC β II mRNA is generated via exon inclusion in the alternative splicing of PKC β . The 5' end of PKC β cDNA contains 684 nucleotides of untranslated sequence followed by ATG (initiation site) at 685 bp position in the C4 region. PKC β I mRNA is generated by splicing of the β I exon at position 2547. The PKC β II mRNA is generated via inclusion of a 216 bp exon at position 2547. This alternative splicing introduces a STOP codon (TAA) at position 2763 in PKC β II mRNA. The coding regions of PKC β I and PKC β II cDNA are followed by 504 nucleotides and 714 nucleotides, respectively in the 3'-untranslated region. The resulting proteins differ in their carboxyl terminal regions by 50-52 amino acid residues (One et al., 1987, pp 1116-1120).

FIG. 5. Generation of PKC activators. Ligand binding to the receptor activates phospholipase C (PLC) which generates diacylglycerol (DAG) and Ca⁺². PtdIns(4,5)P₂: phosphatidylinositol 4,5-bisphosphate; PtdIns(1,4,5)P₃: phosphatidylinositol 1,4,5-trisphosphate.

FIG. 6. Activation of PKC by diacylglycerol and Ca⁺². Ligand binding to the receptor activates phospholipase C (PLC) which hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) to diacylglycerol (DAG) and Ca⁺². Phospholipase A₂ (PLA₂) is also activated which hydrolyses phosphocholine (PC) to lysophosphatidylcholine (Lyso PC) and free fatty acids (FFA). This results in increased levels of DAG thereby prolonging PKC activation for sustained cellular responses.

FIG. 7. Model for activation of PKC. Newly synthesized PKC is bound to the cytoskeletal. Upon phosphorylation, it translocates towards the membrane but is still inactive due to the pseudosubstrate binding. Diacylglycerol, Ca⁺² and phosphatidylserine activate PKC which binds to the membrane via its C1 and C2 domains and is released from the pseudosubstrate inhibition.

FIG. 8. Localization of PKC isozymes by anchoring proteins. Both active and inactive PKC isozymes are localized to their specific intracellular sites due to their binding to the

specific anchoring molecules. Receptors for inactive C-kinase (RICKs) bind to the inactive PKC isozymes in the cell cytoskeleton in a phosphatidylserine-dependent manner. Receptors for active C-kinase (RACKs) bind to the activated PKC isozymes in a selective and saturable manner at a site distinct from the substrate-binding site.

FIG. 9. Arterial smooth muscle cell phenotypes. In the contractile phenotype, the smooth muscle cells show a heterochromatic nucleus and abundant actin and myosin filaments. In the process of atherogenesis, the smooth muscle cell structure modifies to a synthetic phenotype that has a euchromatic nucleus, prominent ER and golgi complex and a decrease in the content of myofilaments. Cells in the synthetic phenotype produce extracellular matrix components that aid in proliferation.

FIG. 10. Representation of the response-to-injury hypothesis of atherosclerosis proposed by Ross. Exposure to agents like oxidized LDL, toxins and viruses causes an injury to the endothelium and results in the release of growth factors, cytokines and nitric oxide. The smooth muscle cells migrate from the media to the arterial intima where they proliferate and deposit extracellular matrix components thereby forming a lesion.

FIG. 11. The MAP kinase pathway transduces the signal from the membrane to the nucleus. Receptor tyrosine kinase (RTK) phosphorylation upon ligand binding activates Sos-Grb2 which further activates Ras. PKC is also an activator of Ras. The downstream target of Ras is Raf which can activate Mek. Mek phosphorylates MAP kinase which together with its target, ribosomal S6 kinase (Rsk) can translocate to the nucleus and activate transcriptional factors (TF) to regulate gene expression.

FIG. 12. The role of phosphoinositide 3-kinase (PI3 kinase) in signal transduction. Growth factor receptors with intrinsic tyrosine kinase activity recruit and activate PI3 kinase. The upstream activators and downstream targets of PI3 kinase are shown.

FIG. 13. Levels of eukaryotic gene regulation. DNA is transcribed to RNA which upon post-transcriptional modification is translated to proteins. The pool of nucleotides and amino acid is refurbished by mRNA and protein degradation, respectively.

FIG. 14. The fate of mRNA in mammalian cells. RNA polymerase II synthesizes the heteronuclear RNA (hnRNA) transcript form the genomic DNA that undergoes post-transcriptional modifications before the mature mRNA is translocated into the cytoplasm. According to the cellular signals, the mRNA is either translated to protein or it can undergo degradation. Localization of mRNA to free, cytoskeletal-bound or membrane-bound polysomes also takes place in the cytoplasm.

FIG. 15. Representation of the structural elements involved in regulating mRNA stability. The cis-elements to which trans-acting factors bind to modulate the half-life of the mRNA are depicted.

FIGS. 16A and 16B. PKC β I protein levels remain unaltered in the presence of high glucose. In FIG. 16A, A10 cells were synchronized by culturing the cells in DMEM containing 0.5% FBS for 48 hours. To re-initiate the cell cycle, A10 cells were incubated in DMEM with 10% FBS containing 5.5 mM glucose (control cells) or 25 mM glucose (hyperglycemic condition) for indicated periods up to 18 hours. Western blot analysis was carried out on cell lysates using anti-PKC β I as primary antibody. Proteins were detected using enhanced chemiluminescence (ECL, AMERSHAM) as described in methods. Densitometric scanning was used to quantify PKC β I bands from the blots. FIG. 16B depicts PKC β I protein

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levels in the presence of 25 mM glucose plotted as the percent of the total PKC β I levels present in the respective control (5.5 mM glucose) cells. Data are representative of at least 5 separate experiments.

FIGS. 17A and 17B. PKC β II protein levels decreased by 55% in the presence of glucose. In FIG. 17A, A10 cells were synchronized by culturing the cells in DMEM containing 0.5% FBS for 48 hours. To re-initiate the cell cycle, A10 cells were incubated in DMEM with 10% FBS containing 5.5 mM glucose (control cells) or 25 mM glucose (hyperglycemic condition) for indicated periods up to 18 hours. Western blot analysis was carried out on cell lysates using (a) anti-PKC β I and (b) anti-PKC β II as primary antibodies. Proteins were detected using enhanced chemiluminescence (ECL; AMERSHAM) as described in methods. Densitometric scanning was used to quantify PKC β I and PKC β II bands from the blots. FIG. 17B depicts PKC β I and PKC β II protein levels in the presence of 25 mM glucose plotted as the percent of the total PKC β I and PKC β II levels present in the respective control (5.5 mM glucose) cells. Data are representative of at least 5 separate experiments.

FIGS. 18A and 18B. Down regulation of PKC β (I+II) mRNA by glucose. In FIG. 18A, total RNA was extracted from A10 cells that were synchronized in culture with DMEM containing 0.5% FBS for 48 hours, then re-initiated to proliferate with DMEM +10% FBS, and incubated for 15-18 hours in medium (DMEM +10% FBS) containing 5.5 mM glucose (lane 1), 25 mM glucose (lane 2) or 25 mM mannitol (lane 3) as indicated. RNA (10 μ g) was fractionated on 1.2% agarose-formaldehyde gel, 28S and 18S rRNA were visualized to ensure equal loads of RNA (lower panel), capillary transferred to Hybond membrane (AMERSHAM) and probed with a 32 P-labeled PKC β cDNA probe that would detect PKC β (I+II) mRNA as described in methods. Following exposure to X-ray film, the autoradiogram was analyzed densitometrically. In FIG. 18B, a 60-75% decrease in PKC β (I+II) mRNA (3.5 Kb) was observed under high glucose conditions. Data is representative of an experiment repeated with similar results on at least 4 occasions.

FIG. 19. Map of PKC β promoter and lengths of deletion constructs. 5' deletion mutants of PKC β gene cloned in the PKC β -CAT plasmid were obtained from Dr. S. Ohno (Yokohama College of Medicine). The positions of cis-acting regulatory elements on the promoter region of PKC β gene are indicated. Construct A (-4.1 kb to +179 CAT), construct C (-674 to +179 CAT), construct D (-411 to +179 CAT) and construct E (-234 to +179 CAT) were transiently transfected into synchronized A10 cells.

FIG. 20. Effect of high glucose on PKC β deletion constructs. Constructs A, C, D, and E as designated above, were transiently transfected into A10 cells synchronized for 48 hours in DMEM+0.5% FBS, re-initiated to proliferate in DMEM+10% FBS, using calcium phosphate-DNA precipitate along with β -galactosidase (1 μ g) to normalize transfection efficiency. After overnight transfection, the cells were washed with DPBS and placed in fresh medium (DMEM+10% FBS) containing either 5.5 mM or 25 mM glucose. Cells were extracted after 6 hours and relative CAT/ β -galactosidase activity was measured in control (5.5 mM glucose) and glucose (25 mM)-treated cells. Construct D showed maximum quenching of PKC β promoter activity. This graph represents relative CAT/ β -galactosidase activity for 3 individual experiments performed in duplicate.

FIG. 21. Construct D quenches PKC β promoter activity at 10 h post-synchronization corresponding to S phase. Construct D, as described in FIG. 19, was transiently co-transfected with β -galactosidase into synchronized A10 cells. Pro-

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motor activity in control (5.5 mM glucose) and glucose (25 mM)-treated cells following 6-24 hours of treatment was calculated as relative CAT/ β -galactosidase units. Maximum quenching by high glucose of PKC β promoter construct D (-411/+179CAT) was observed at 10 hours which is the peak of S phase as determined by 3 [H]-thymidine uptake studies as described in the description. Error bars indicate \pm S.E.M. Data shown is the mean of \pm SE for triplicate determinations of an experiment that was repeated on three separate occasions with similar results. (* indicates significance at $p < 0.05$).

FIGS. 22A and 22B. Northern blot analysis of PKC β II mRNA in A10 cells treated with actinomycin D in the presence or absence of high glucose. In FIG. 22A, A10 cells were synchronized by maintaining them in DMEM+0.5% FBS for 48 hours, followed by re-initiation of cell proliferation in DMEM+10% FBS. Synchronized A10 cells were pre-treated with actinomycin D (5 μ g/ml) for 30 minutes in DMEM+10% FBS. At time zero, glucose was added such that the medium contained 5.5 mM glucose (Con) or 25 mM glucose (Glc). Total RNA was extracted from 0 to 6 hours, and then 10 μ g of RNA was fractionated on 1.2% agarose-formaldehyde gels, 28S and 18S rRNA were visualized to ensure equal loads of RNA (lower panel), capillary transferred to Hybond membrane (AMERSHAM), and probed with a 32 P-labeled PKC β cDNA probe that detects PKC β (I+II) mRNA as described in methods. Images of the 3.5 kb transcript were quantitated using Molecular Dynamics Phosphorimaging system and are representative of three individual experiments. FIG. 22B depicts PKC β (I+II) mRNA levels, in the presence of 25 mM glucose plotted as the percent of the total PKC β (I+II) mRNA levels present in the control (5.5 mM glucose) cells. A 60% decrease (* indicates significance at $p < 0.05$) in PKC β (I+II) mRNA is seen at 6 hours with glucose-treated cells.

FIG. 23. In vitro assay for RNA stability. Post-nuclear extracts were prepared from A10 cells treated with either 5.5 mM glucose (control cells) or 25 mM glucose (glucose-treated cells) for 2, 6, 18, or 24 hours. Total RNA (previously extracted from synchronized A10 cells) was incubated for 30 minutes at 4 $^{\circ}$ C. with 2.7% (vol/vol) post-nuclear extracts from control (5.5 mM glucose) or 25 mM glucose-treated A10 cells in a 50 μ l total reaction volume. Northern blot analysis for PKC β (I+II) mRNA was then performed on the RNA as described in methods. Images of the 3.5 kb bands were quantitated by phosphorimaging and are representative of four individual experiments with similar results. The graph shows the total PKC β (I+II) mRNA levels remaining after incubation with post-nuclear extracts from glucose-treated cells plotted as a percent of the total PKC β (I+II) mRNA in the respective control incubations with post-nuclear extracts from cells treated with 5.5 mM glucose. Protein concentrations of control (5.5 mM glucose) post-nuclear extracts (control PNE) and 25 mM glucose-treated post-nuclear extracts (glucose PNE) at 2 hours were 2.5 mg/ml and 2.23 mg/ml respectively; at 6 hours, protein levels were 1.34 mg/ml and 1.34 mg/ml respectively; at 18 hours, levels were 1.3 mg/ml and 1.25 mg/ml respectively; and at 24 hours, levels were 1.22 mg/ml and 1.27 mg/ml.

FIG. 24. In vitro assay for RNA stability in the presence of EDTA. No degradation of total RNA by glucose-treated post-nuclear extract was observed when 50 mM EDTA was added to the reaction mix as described above. Similar results were obtained in three separate experiments.

FIG. 25. A schematic representation of the PKC β as deduced from cDNA sequence analysis. The shaded blocks C1-C4 represent the conserved regions while V1-V5 blocks represent the variable regions. The regulatory and the catalytic domains are separated by V3, which serves as a hinge

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region. PKC β I and - β II, the alternatively spliced products of PKC β pre-mRNA, diverge in their C-terminal sequence by 50 or 52 amino acids respectively. The enlarged area represents the genomic structure of the alternatively spliced V5 region. Introns are represented by lines and exons are represented by blocks. The arrows indicate the regions that correspond to the sense primer (the last common region (C4) for PKC β I and - β II) and antisense primer (to the β IV5 region) used for amplification of both PKC β I and - β II cDNAs simultaneously.

FIGS. 26A and 26B. High glucose destabilizes PKC β II mRNA. RT-PCR was performed on total RNA isolated from A10 cells synchronized by serum starvation for 48 hours or from primary cultures of AoSMC from humans synchronized by serum starvation for 72 hours. Cells were re-initiated to proliferate by serum introduction to the basal medium and incubated with 5.5 mM glucose (control) or 25 mM glucose (glucose-treated) for 4 hours. The first strand cDNA synthesized was amplified using a sense primer to the common, C4, domain and antisense primer to the PKC β I exon. The PCR product size was 187 bp for PKC β I; 404 bp for PKC β II and 550 bp for β -actin. PKC β II mRNA was decreased >95% by 25 mM glucose treatment while PKC β I mRNA remained unchanged. No change was observed in the β -actin PCR products both in 5.5 mM and 25 mM glucose treated cells. Results are representative of an experiment repeated on five occasions.

FIG. 27. PKC β II cDNA (350 bp) sequence [SEQ ID NO:8].

FIG. 28. PKC β II cDNA (350 bp) restriction sites map.

FIG. 29. Effect of glucose and insulin on PKC β II mRNA in aorta smooth muscle cells. RT-PCR was performed on total RNA isolated from primary cultures of AoSMC from humans incubated with 5.5 mM glucose (control), 25 mM glucose (glucose-treated), 100 nM insulin or 25 mM glucose+100 nM insulin for 4 hours. The first strand cDNA synthesized was amplified using a sense primer to the common, C4, domain and antisense primer to the PKC β I exon. The PCR product size was 187 bp for PKC β I; 404 bp for PKC β II and 550 bp for β -actin. In control cells (lane 1), both PKC β I and - β II mRNA were detected; PKC β II mRNA was decreased >95% by 25 mM glucose treatment while PKC β I mRNA remained unchanged (lane 2); 100 nM insulin (lane 3) did not significantly affect either PKC β I or - β II mRNA; 25 mM glucose+100 nM insulin (lane 4) PKC β II mRNA was decreased by 60%. No change was observed in the β -actin PCR products both in 5.5 mM and 25 mM glucose treated cells. Results are representative of an experiment repeated on five occasions.

FIG. 30. Glucosamine does not affect PKC β II mRNA stability. RT-PCR was performed on total RNA isolated from synchronized A10 cells incubated with 5.5 mM glucose (control), 25 mM glucose (glucose-treated) or 10 mM glucosamine for 4 hours. The first strand cDNA synthesized was amplified using a sense primer to the common, C4, domain and antisense primer to the PKC β I exon. The PCR product size was 187 bp for PKC β I; 404 bp for PKC β II and 550 bp for β -actin. Glucosamine did not destabilize PKC β I or - β II mRNA.

FIG. 31. Effect of glucose metabolites on PKC β II mRNA stability. Synchronized A10 cells upon re-initiation of the cell cycle by DMEM containing 10% FBS were treated with 5.5 mM glucose (lane 1, control), 25 mM glucose (lane 2, glucose), 25 mM 2-deoxyglucose (lane 3, 2-DOG), 25 mM 3-O-methylglucose (lane 4, 3-O-MG) or 5.5 mM glucose+19.5 mM mannitol (lane 5) for 4 hours. Total RNA was extracted and 2 μ g of RNA was used to perform RT-PCR analysis. The first strand cDNA synthesized was amplified using a sense primer to the common, C4, domain and antisense primer to

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the PKC β I exon. The PCR product size was 187 bp for PKC β I; 404 bp for PKC β II and 550 bp for β -actin. 2-DOG and 3-O-MG destabilized PKC β II mRNA while mannitol, the osmotic control did not.

FIG. 32. Effect of cycloheximide on glucose-induced PKC β II destabilization. Synchronized A10 cells upon re-initiation of the cell cycle with DMEM containing 10% FBS were pre-incubated for 30 minutes with 20 mM cycloheximide followed by the addition of glucose. Total RNA was isolated and RT-PCR was performed on 2 μ g of RNA. The first strand cDNA synthesized was amplified using a sense primer to the common, C4, domain and antisense primer to the PKC β I exon. The PCR product size was 187 bp for PKC β I; 404 bp for PKC β II and 550 bp for β -actin. Lane 1: 5.5 mM glucose, control cells; lane 2: 25 mM glucose and lane 3: 25 mM glucose+cycloheximide. Cycloheximide did not block the ability of glucose to destabilize PKC β II mRNA.

FIG. 33. RT-PCR analysis of PKC β I and - β II mRNA after okadaic acid treatment. Synchronized A10 cells upon re-initiation of the cell cycle with DMEM containing 10% FBS were pre-incubated for 30 minutes with 20 nM okadaic acid followed by the addition of glucose. Total RNA was isolated and RT-PCR was performed on 2 μ g of RNA. The first strand cDNA synthesized was amplified using a sense primer to the common, C4, domain and antisense primer to the PKC β I exon. The PCR product size was 187 bp for PKC β I; 404 bp for PKC β II and 550 bp for β -actin. Lane 1: 5.5 mM glucose, control cells; lane 2: 25 mM glucose and lane 3: 25 mM glucose+okadaic acid. Okadaic acid interfered with the ability of glucose to destabilize PKC β II.

FIG. 34. The p β G-PKC β II chimeric minigene. The parent p β G vector (6.3 kb) contains a chimeric β -globin/growth hormone gene. The promoter derived from the Rous sarcoma virus long terminal repeat (RSV-LTR) is followed by the transcription start site, the coding sequence (open boxes), and two introns (closed boxes) from the rabbit β -globin genomic DNA. The multi-cloning site (MCS) contains four unique restriction sites into which the PKC β II cDNA was subcloned in frame with the β -globin coding region, at the Spe I and Xba I sites. The 3' untranslated region and polyadenylation site of the bovine growth hormone cDNA (bGH-pA) is maintained. It also contains genes that direct eukaryotic cell resistance to neomycin (Neo) and bacterial resistance to ampicillin (AMP).

FIGS. 35A and 35B. The p β G-PKC β II chimeric minigene is destabilized by high glucose. The parent vector p β G and p β G-PKC β II minigene were transfected into human aorta smooth muscle cells. Following acute exposure of three hours with high glucose, total RNA was extracted. RT-PCR was then performed on 2 μ g of RNA using sense and antisense primers for the β -globin coding region. PCR results for p β G-PKC β II minigene and p β G vector are shown in FIGS. 35A and 35B, respectively.

FIGS. 36A and 36B. Half-life analysis of p β G-PKC β II mRNA. In FIG. 36A, the p β G-PKC β II minigene was stably transfected into A10 cells. 50 μ g/ml DRB in 95% ethanol was added to the plates and the 0-hour RNA sample was isolated. 25 mM glucose was added to the cells or the cells were maintained in 5.5 mM glucose. RNA samples were isolated at 1 hour, 2 hours, and 6 hours. 10 μ g of total RNA was analyzed by Northern blotting. FIG. 36B depicts the mRNA levels remaining at various times after addition of DRB. Blot and graph represents an experiment repeated on 5 different occasions.

FIG. 37. Schematic of the RNA probes used for the RNA EMSAs. RNA probes A to E and the restriction enzyme cleavage sites used for their generation by T7 RNA poly-

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merase are indicated. Probe A represents the 404 bp PKC β II exon included mRNA and was prepared by linearization with BamH I. Probe B was linearized at 175 bp with Bgl II, probe C was linearized at 137 bp with Hpa I, probe D was linearized at 102 bp with Ssp I while probe E was linearized at 45 bp with Fok I.

FIG. 38. RNA electrophoretic mobility shift assay using full length PKC β II mRNA probe. ³²P labeled RNA probe A was incubated with 3 μ g of cytoplasmic extracts from control cells or glucose-treated cells. The complexes were separated on a 10% polyacrylamide gel, dried and exposed to a Molecular Dynamics Phosphoimaging Screen. RNA-protein binding complexes were observed (black arrows) with glucose-treated A10 cells which was competed out by specific excess unlabeled competitors. Non-specific competitor did not compete for the binding. No binding was observed with BSA. The experiment is representative of results obtained on five separate occasions.

FIG. 39. A cytosolic factor binds to a glucose-regulated element present in the PKC β II coding region. ³²P labeled RNA probes B were incubated with 3 μ g of cytoplasmic extracts from control cells or glucose-treated cells. The complexes were separated on a 10% polyacrylamide gel, dried and exposed to a Molecular Dynamics Phosphoimaging Screen. RNA-protein binding complexes were observed (black arrows) with glucose-treated A10 cells using probe B, which was competed out by specific excess unlabeled competitors. No binding was observed with BSA. The experiment is representative of results obtained on five separate occasions.

FIG. 40. No RNA protein binding observed using RNA probe C.

FIG. 41. UV cross-linking analysis of the RNA-protein binding complex. ³²P labeled RNA probes A and B were incubated with 3 μ g cytoplasmic extracts from control cells or glucose-treated A10 cells. The unbound RNA was digested with RNase T1 and then exposed to UV radiation. The proteins were resolved by electrophoresis on a SDS-polyacrylamide gel. Gels were dried and exposed to Molecular Dynamics Phosphoimaging Screen. A 10-14 kDa protein was observed to bind to probes A and B in glucose-treated extracts. The experiment is representative of results obtained on five separate occasions.

FIG. 42. A vector map of a reporter vector of the subject invention (pcDNA3.1/NT-GFP-TOPO vector).

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 is the amino acid sequence of tyrosine phosphatase conserved active-site motif.

SEQ ID NO: 2 is the amino acid sequence of tyrosine phosphatase signature sequence motif.

SEQ ID NO: 3 is the amino acid sequence of dual-specificity phosphatase signature sequence motif.

SEQ ID NO: 4 is the nucleotide sequence of PKC β I and PKC β II upstream sense primer.

SEQ ID NO: 5 is the nucleotide sequence of PKC β I downstream anti-sense primer.

SEQ ID NO: 6 is the nucleotide sequence of β -globin sense primer.

SEQ ID NO: 7 is the nucleotide sequence of β -globin anti-sense primer.

SEQ ID NO: 8 is the nucleotide sequence of 350 basepairs of PKC β II cDNA.

SEQ ID NO: 9 is the nucleotide sequence of the human and rat glucose-responsive mRNA instability element within PKC β II cDNA.

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SEQ ID NO: 10 is the nucleotide sequence of PKC β II mRNA.

SEQ ID NO: 11 is the partial nucleotide sequence of PKC β II mRNA.

SEQ ID NO: 12 is the partial nucleotide sequence of PKC β III mRNA.

SEQ ID NO: 13 is the amino acid sequence of protein kinase ATP-binding motif.

SEQ ID NO:14 is the nucleotide sequence of the mouse/rabbit/bovine metabolite responsive instability element within PKC β II cDNA.

SEQ ID NO:15 is the nucleotide sequence encoding the rat glucose-responsive mRNA instability element, with flanking sequences:

SEQ ID NO:16 is the nucleotide sequence encoding the mouse/rabbit/bovine glucose-responsive mRNA instability element, with rat flanking sequences

SEQ ID NOs:17-18 are primers.

SEQ ID NO:19 is the rat PKC β II mRNA 404 bp, including flanking sequences.

SEQ ID NO:20 is the human 404 base pair sequence encoding the human glucose-responsive mRNA instability element, including flanking sequence. The sequence was generated by splicing the C4 exon to betaII exon to betaI exon.

DETAILED DISCLOSURE OF THE INVENTION

Regulatable vectors for gene expression are often not reliable since they can be "leaky". They usually consist of systems where the gene of interest is cloned downstream of a minimal viral promoter fused to copies of the tetracycline operator. The promoters used with these repressor sequences are, however, not totally repressed in many cells. By engineering a construct that is also regulated at the post-transcription level, full repression of such a promoter could be achieved in a wider variety of cells. We propose that by inserting instability sequences downstream of an inserted cDNA, genes can be further regulated using high extracellular concentrations of glucose or non-metabolized analogues. Both Tet and Retroviral Tet Systems (CLONTECH) show promise for regulating transcription of genes in cells. The ecdysone-inducible expression system derived from *Drosophila* is also commercially available (INVITROGEN) and may have even lower basal activity in mammalian cells. Basal activity of the tetO.HCMC IE promoter is highly variable when tested in several cell lines. Although the promoter was repressed in HeLa and PC12 cells, basal levels were 10-30 fold higher in BHK cells. This high basal activity limits the use of the tetracycline-repressed promoter. If a construct could be engineered that was also regulated at the post-transcriptional level, full repression of such a promoter could be achieved in a wider variety of cells. There are several inducible promoters available; however, no one has developed a system to regulate gene expression at the post-transcriptional level. The post-transcriptional regulation of mRNA destabilization of a gene is another possibility for designing regulatable gene expression constructs. If the cDNA can be destabilized by a non-toxic nutrient or analogue, then expression can be stabilized when the analogue is withdrawn.

Protein kinase C (PKC) has been implicated as a mediator of diabetes-induced vascular proliferation. This study investigated the regulation of PKC β gene expression following acute glucose exposure in human vascular smooth muscle cells and in A10 cells, a rat aortic smooth muscle cell line. Western blot analysis showed that PKC β II protein levels decreased with high glucose (25 mM) while PKC β I level were unaltered. PKC β mRNA levels were depleted by

60-75% in hyperglycemic conditions. Quenching of PKC β promoter activity by glucose suggested involvement of a carbohydrate response element in the 5' promoter region. Simultaneous cell cycle studies indicated an increase in the percentage of cells going into S phase in high glucose implying that quenching of PKC β transcription may be related to cell cycle progression. It was demonstrated that glucose induced post-transcriptional destabilization of PKC β II message via a nuclease activity present in the cytosol. The specificity of glucose to post-transcriptionally destabilize PKC β II, but not the PKC β I, isoform was confirmed in both A10 cells and primary cultures from human aorta.

To further elucidate the intracellular signaling mechanisms, glucose analogs were used to study the pathways by which glucose acted to destabilize PKC β II mRNA. Glucose-induced destabilization of PKC β II mRNA is independent of the hexosamine or hexokinase pathways. Cycloheximide did not block destabilization of PKC β II mRNA by high glucose indicating that the process is independent of translation. Glucose may act via PKC signaling pathways and may be regulated by serine/threonine phosphorylation/dephosphorylation.

A heterologous chimeric minigene encoding PKC β II cDNA subcloned into the p β G expression vector comprising the coding sequences of β -globin genomic DNA and 3'UTR and polyadenylation site of bovine growth hormone cDNA was constructed. Half-life analysis indicated a rapid glucose-induced destabilization of β -globin mRNA in p β G-PKC β II transfected cells. Mobility shift analysis indicated the presence of a glucose-responsive instability element within the exon included in the mature PKC β II mRNA in VSMC. UV cross-linking analysis showed a small protein (~10-14 kDa) binding near a stem-loop structure within the PKC β II-specific exon. This is a novel finding of a instability element within the PKC β II mRNA coding region that is regulated by glucose in aorta smooth muscle cells.

ABBREVIATIONS

AoSMC Aorta smooth muscle cells
aPKC Atypical PKC
ARE Adenosine-uridine rich element
Ca⁺² Calcium
CAT Chloramphenicol acetyltransferase
cDNA Complementary DNA
ChoRE Carbohydrate Response element
cPKC Conventional PKC
CRE cAMP-responsive element
CREB cAMP-responsive element-binding protein
DAG Diacyl glycerol
DMEM Dulbecco's modified eagle's medium
DPBS Dulbecco's phosphate-buffered saline
DRB 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole
DTT Dithiothreitol
EMSA Electrophoretic mobility shift assays
ERK Extracellular signal-regulated kinase
FBS Fetal bovine serum
FFA Free fatty acids
HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPV Human papillomavirus
IGF Insulin-like growth factor
IL-2 Interleukin-2
IP₃ Phosphatidylinositol 1,4,5-trisphosphate
IRE Iron responsive element
IRP Iron regulatory protein
JAK Janus kinase

JNK c-jun NH₂-terminal kinase
L-PK L-pyruvate kinase
Lyso PC Lysophosphosphotidyl choline
MAPK Mitogen-activated protein kinase
5 Mek MAP kinase kinase
MOPS 3-(N-morpholino)propanesuphonic acid
mRNA Messenger RNA
NaCl Sodium chloride
NGF Nerve growth factor
10 nPKC Novel PKC
oxLDL Oxidized low density lipoprotein
PC Phosphocholine
PI3 kinase Phosphoinositide-3-kinase
PIP₂ Phosphatidylinositol 4,5-bisphosphate
15 PKC Protein kinase C
PLA₂ Phospholipase A₂
PLC Phospholipase C
PMSF Phenylmethylsulfonyl fluoride
PNE Post-nuclear extract
20 PP1 Type-1 phosphatase
PP2 Type-2 phosphatase
PPAR Proliferating-activated receptor
PRK PKC-related kinases
PS Phosphatidyl serine
25 PTP Protein tyrosine phosphatases
RACK Receptor for activated PKC
RICK Receptor for inactive PKC
Rsk Ribosomal S6 kinase
RTK Receptor tyrosine kinase
30 SmGM Smooth muscle growth medium
STATs Signal transducers and activators of transcription
TF Transcriptional factors
TPA 12-O-tetradecanoylphorbol-13-acetate
UPAR Urokinase-type plasminogen activator receptor
35 UTR Untranslated region
VSMC Vascular smooth muscle cells
Zn⁺² Zinc

To measure intracellular glucose (and glucose analog) levels, the present invention utilizes a glucose-sensing element that can be cloned downstream of a polynucleotide encoding a reporter polypeptide, such as a fluorescent polypeptide. The signal emitted from the cells via the reporter polypeptide changes according to the intracellular glucose (and glucose analog) concentrations. Insulin promotes glucose uptake. 40 Therefore, in the presence of high glucose (and glucose analog), the reporter's signal decreases. It will be possible to "read" the intracellular concentration based on the degree of signal (e.g., fluorescence) detected.

In one aspect, the present invention includes a reporter 50 vector including a first polynucleotide encoding a glucose-responsive mRNA instability element and a second polynucleotide encoding a reporter polypeptide. The second polynucleotide is also referred to herein as the "reporter gene"). Both the first polynucleotide encoding the glucose-responsive mRNA instability element and the second polynucleotide encoding the reporter polypeptide are operably-linked to a promoter sequence. As used herein, the terms "first" and "second" are merely used as a convention for sake of clarity and not intended to be limiting in terms of orientation within the reporter vector of the invention. Advantageously, the glucose-sensing element of the present invention is specific in that other hexoses such as mannitol and fructose do not destabilize the element.

In one embodiment, the vector utilizes a glucose instability 65 element cloned with a reporter gene encoding a fluorescently tagged protein. Preferably, the fluorescently tagged protein is green fluorescence protein (GFP). The glucose instability

element can be cloned downstream of the reporter gene in a vector such as the NT-GFP-TOPO vector (INVITROGEN LIFE SCIENCES). Since the glucose instability element destabilizes mRNA when glucose levels exceed normal (e.g., approximately 5.5 mM) concentrations, the reporter molecule's signal will be decreased within minutes of high glucose exposure. Insulin-responsive cells, such as L6 myoblasts and 3T3-L1 fibroblasts, can be transfected with the vector and selected to stably express the GFP containing construct.

Reporter vectors having more than one glucose-responsive mRNA instability element cassette (e.g., multimers polynucleotides encoding glucose-responsive mRNA instability elements, polynucleotides encoding reporter polypeptides, and operably-linked promoters) can be used to enhance the glucose-sensing ability of the cells.

Clones with high expression can be used for experiments by plating them on multi-well plates (e.g., 96-well or 100-well plates) that can be read in a plate reader with the appropriate fluorescence filter. As a live cell assay, cells can then be exposed to treatments or agents (e.g., a condition or a potential drug) that may alter glucose transport, and subsequently treated with insulin. Agents that increase the ability of cells to transport glucose would have less reporter gene expressed after treatment. In the basal state, reporter protein levels would be sustained at an equilibrium level. Since this is a live cell assay, other dynamic processes can also be followed during the same time course. The cells can be plated in multi-well plates, thus enabling the technology to be used by pharmaceutical companies to screen potential therapeutic drugs that lower blood glucose.

Shown in Table 6 is a sequence alignment of the nucleotides encoding the glucose-responsive mRNA instability element, produced using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) server. The sequence comparison demonstrates that the nucleic acid sequence of the exemplified mRNA instability element is conserved across species (e.g., human, mouse, rat, rabbit, bovine).

TABLE 6

1. Human	A A C T C T G A A T T T T T T A A A C C C G A A G T C A A G A G C T A G T A
PKCβII: (SEQ ID NO: 9)	
2. Rat	A A C T C T G A A T T T T T A A A C C C G A A G T C A A G A G C T A G T A
PKCβII: (SEQ ID NO: 9)	
3. Mouse	A A C T C T G A A T T T T T A A A C C T G A A G T C A A G A G C T A G T A
PKCβII: (SEQ ID NO: 14)	
4. Rabbit	A A C T C T G A A T T T T T A A A C C T G A A G T C A A G A G C T A G T A
PKCβII: (SEQ ID NO: 14)	
5. Bovine	A A C T C T G A A T T T T T A A A C C T G A A G T C A A G A G C T A G T A
PKCβII: (SEQ ID NO: 14)	

The NCBI accession numbers, citations, parameters, and results corresponding to the BLAST search are as follows:

1. *Homo sapiens* protein kinase C beta-II type (PRKCB1) mRNA, complete cds; NCBI accession number M13975.1; Coussens, L. et al., *Science*, 1986, 233 (4766):859-866, incorporated herein by reference in its entirety:

Score=69.9 bits (35), Expect=2e-10

Identities=35/35 (100%)

Strand=Plus/Plus

2. *Rattus norvegicus* mRNA for protein kinase C type II; NCBI accession number X04440; Ono, Y. et al., *FEBS Lett.*, 1986, 206(2):347-352, incorporated herein by reference in its entirety:

Score=69.9 bits (35), Expect=2e-10

Identities=35/35 (100%)

Strand=Plus/Plus

3. *Mus musculus* mRNA for protein kinase C beta-II; NCBI accession number X53532.1; Tang, Y. M. and Ashendel, C. L., *Nucleic Acids Res.*, 1990, 18(17):5310, incorporated herein by reference in its entirety:

Score=61.9 bits (31), Expect=4e-08

Identities=34/35 (97%)

Strand=Plus/Plus

4. *Oryctolagus cuniculus* mRNA for protein kinase C alpha (PKC-alpha); NCBI accession number X04793; Ohno, S. et al., *Nature*, 1987, 325(7000):161-166, incorporated herein by reference in its entirety:

Score=61.9 bits (31), Expect=4e-08

Identities=34/35 (97%)

Strand=Plus/Plus

5. *Bos taurus* beta type protein kinase C mRNA complete cds; NCBI accession number M13974; Coussens, L. et al., *Science*, 1986, 233(4766):859-866, incorporated herein by reference in its entirety:

Score=61.9 bits (31), Expect=4e-08

Identities=34/35 (97%)

Strand=Plus/Plus

Thus, polynucleotides encoding the PKCβII mRNA instability element listed in Table 6, or homologs thereof, can be used in the reporter vector, host cells, and methods, in accordance with the subject invention.

In one embodiment of the reporter vector, the polynucleotide encoding the glucose-responsive mRNA instability element comprises the nucleotide sequence of SEQ ID NO:9. In another embodiment of the reporter vector, the polynucleotide encoding the glucose-responsive mRNA instability element comprises the nucleotide sequence of SEQ ID NO:14. In another embodiment, the polynucleotide encoding the glucose-responsive mRNA instability element further includes flanking sequences to increase mRNA instability in the presence of intracellular glucose or glucose analog. In a specific embodiment, the polynucleotide encoding the glucose-responsive mRNA instability element, including flanking sequences, comprises the nucleotide sequence of SEQ ID NO: 15, SEQ ID NO:16, or SEQ ID NO:20. In another embodiment, the polynucleotide encoding the glucose-responsive mRNA instability element comprises the nucleotide sequence of SEQ ID NO: 15, SEQ ID NO:16, or SEQ ID NO:20, and further comprising a number of nucleotides of the glucose-responsive mRNA instability element's flanking sequence selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262,

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263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, and 366 nucleotides.

The flanking sequence may be the sequence of the same species of organism as the glucose-responsive mRNA instability element or a different species.

The nucleotide sequence encoding the human glucose-responsive mRNA instability element, with flanking sequences:

ACAACGTAGCCTATCCCAAGTCTATGTC-
CAAGGAAGCTGTGGCCATCTGCAAAG GGCTGAT-
GACCAAACACCCAGGCAAACGTCTGGGT-
TGTGGACCTGAAGGTGAAC
GTGATATCAAAGAGCATGCATTTTCCG-
GTATATTGATTGGGAGAACTGAACG CAAA-
GAGATTACAGCCCCCTTATAAGC-
CAAAAGCTTGTGGGCGAAATGCTGAAAA
CTTCGACCGATTTTACCCGCCATC-
CACCAGTCCTAACACCTCCTGACCAGGAA GTCAT-
CAGGAATATTGACCAATCAGAATTC-
GAAGGATTTTCTTTGTTAACTCTG
AATTTTAAAAACCCGAAGTCAAGAGCTAAGTAGA
TGTGTAGATCTCCGTCTCTTCA TTTCTGTCAT-
TCAAGCTCAACGGCTATTGTGAGAGA-
CAAGAGAGACACCTCCAAC TTCGACAAAGAGT-
TCACCAGACAGCCTGTGGAAGTACCCCCACTGAT
AAATC TTCATCATGAACCTTGACCAAAAAT-
GAATTTGCTGGCTTCTCTTATACTAACCCAG
AGTTTGTCTAATATGTG (SEQ ID NO:20; the underlined
portion is the 38 base pairs encoding glucose-regulated insta-
bility element itself)

The nucleotide sequence encoding the rat glucose-respon-
sive mRNA instability element, with flanking sequences:

TTCCGGTATATCGACTGGGAGAACTC-
GAACGCAAGGAGATTAGCCACCTTAT AAAC-
CAAAAGCTTGTGGGCGAAACGCT-
GAAAACCTCGACCGGTTTTTACCCGC
CATCCACCAGTCCTAACACCTCCGAC-
CAGGAAGTCATCAGGAATATTGACCAAT CAGAAT-
TCGAAGGATTTTCTTTGTT
AACTCTGAATTTTTAAAACCCGAAGTCAA
GAGCTAAGTAGATCTGTAGACCTCCGTCTCATTT
CTGTCAITCAAGCTCAACA GCTATCATGAGAGA-
CAAGCGAGACACCTCCAACCTTCGA-
CAAAAGTTTACCAGGC AGCCTGTGGAAGTACTC-
CCACTGACAAACTCTGTGCGACTAGAATGCCCTGAA
TT CTGCAGATATCCATCACAAGTGC (SEQ ID NO: 15;
the underlined portion is the 38 base pairs encoding glucose-
regulated instability element itself)

The nucleotide sequence encoding the mouse/rabbit/bo-
vine glucose-responsive mRNA instability element, with rat
flanking sequences:

TTCCGGTATATCGACTGGGAGAACTC-
GAACGCAAGGAGATTAGCCACCTTAT AAAC-
CAAAAGCTTGTGGGCGAAACGCT-
GAAAACCTCGACCGGTTTTTACCCGC
CATCCACCAGTCCTAACACCTCCGAC-
CAGGAAGTCATCAGGAATATTGACCAAT CAGAAT-
TCGAAGGATTTTCTTTGTT
AACTCTGAATTTTTAAAACCTGAAGTCAA
GAGCTAAGTAGATCTGTAGACCTCCGTCTTCATTT

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CTGTCATTCAAGCTCAACA GCTATCATGAGAGA-
CAAGCGAGACACCTCCAACCTTCGA-
CAAAAGTTTACCAGGC AGCCTGTGGAAGTACTC-
CCACTGACAAACTCTGTGCGACTAGAATGCCCTGAA
TT CTGCAGATATCCATCACAAGTGC (SEQ ID NO:16;
the underlined portion is the 38 base pairs encoding glucose-
regulated instability element itself)

In another aspect, the method for detecting intracellular
glucose is used to identify an agent or treatment that modu-
lates glucose uptake in a cell (such as a mammalian cell), the
method comprising contacting a host cell with a candidate
agent, wherein the host cell comprises a vector comprising a
first polynucleotide encoding a glucose-responsive mRNA
instability element (such as the PKC β II mRNA instability
element) and a second polynucleotide (also referred to herein
as a reporter gene) encoding a reporter polypeptide (such as
green fluorescent protein (GFP)); and determining whether
expression of the reporter gene is modulated, wherein a
decrease in reporter gene expression indicates that the con-
centration of intracellular glucose has increased, and wherein
an increase in reporter gene expression indicates that the
concentration of intracellular glucose has decreased. The
method can include determining whether the candidate agent
or treatment increases or decreases glucose uptake in the cell.
Thus, the method can optionally include an additional step of
comparing glucose uptake by the cell in the presence of the
candidate agent or treatment, with glucose uptake by a cell in
the absence of the candidate agent or treatment.

The contacting step in the assays (methods) of the inven-
tion can involve combining or mixing the test agent and the
cell in suitable receptacle, such as a reaction vessel, microves-
sel, tube, microtube, well, or other solid support. Host cells
and/or candidate agents may be arrayed on a solid support,
such as a multi-well plate. "Arraying" refers to the act of
organizing or arranging members of a library, or other collec-
tion, into a logical or physical array. Thus, an "array" refers to
a physical or logical arrangement of, e.g., library members
(candidate agent libraries). A physical array can be any "spa-
tial format" or physically gridded format" in which physical
manifestations of corresponding library members are
arranged in an ordered manner, lending itself to combinato-
rial screening. For example, samples corresponding to indi-
vidual or pooled members of a candidate agent library can be
arranged in a series of numbered rows and columns, e.g., on
a multiwell plate. Similarly, host cells can be plated or other-
wise deposited in microtitered, e.g., 96-well, 384-well, or-
1536 well, plates (or trays). Optionally, host cells may be
immobilized on the solid support.

A "solid support" (also referred to herein as a "solid sub-
strate") has a fixed organizational support matrix that prefer-
ably functions as an organization matrix, such as a microtiter
tray. Solid support materials include, but are not limited to,
glass, polycryloylmorpholide, silica, controlled pore glass
(CPG), polystyrene, polystyrene/latex, polyethylene, poly-
amide, carboxyl modified teflon, nylon and nitrocellulose and
metals and alloys such as gold, platinum and palladium. The
solid support can be biological, non-biological, organic, inor-
ganic, or a combination of any of these, existing as particles,
strands, precipitates, gels, sheets, tubing, spheres, containers,
capillaries, pads, slices, films, plates, slides, etc., depending
upon the particular application. Other suitable solid substrate
materials will be readily apparent to those of skill in the art.
The surface of the solid substrate may contain reactive
groups, such as carboxyl, amino, hydroxyl, thiol, or the like
for the attachment of nucleic acids, proteins, etc. Surfaces on
the solid substrate will sometimes, though not always, be
composed of the same material as the substrate. Thus, the

surface can be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials.

Detection of intracellular glucose and other assays carried out on the host cells before, during, and/or after detection of intracellular glucose, may be carried out in an automated fashion, in a high-throughput format.

Measurement of reporter gene expression can be carried out using RT-PCR, for example. However, it is preferable that the reporter gene encodes a fluorescent polypeptide such as GFP, which allows detection in live host cells, in real time, without the need to lyse the host cells. Commercially available fluorescence plate readers can be used to assess expression of the reporter gene in this case. Screening of candidate agents or treatments (e.g., determination of reporter gene expression and, hence, intracellular glucose levels) can be performed in a high-throughput format using combinatorial libraries, expression libraries, and the like.

Additional assays can be carried out on the host cells before, during, and/or after determination of reporter gene expression. For example, assays directed at determination of channel excitation and/or oxidative process could be carried out on the host cells before, during, and/or after determination of reporter gene expression. A cytotoxicity assay may be carried out on the host cells using standard apoptosis detection protocols, such as DNA laddering and TUNEL assay, before, during, and/or after determination of reporter gene expression. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) is an in situ method for detecting the 3'-OH ends of DNA exposed during the internucleosomal cleavage that occurs during apoptosis. Incorporation of biotinylated dUTP allows detection by immunohistochemical procedures. The labeled apoptotic cells may be visualized, for example, by light microscopy (Gavrieli et al., *J. Cellular Bio.*, 1992, 119:493-501). In accordance with the invention, the TUNEL assay and other apoptosis assay methods, cytotoxicity assay methods, cell proliferation/viability assay methods, and special applications of cell death and cell proliferation methods may be carried out on the host cells before, during, and/or after intracellular glucose detection. Such methods are described in *Roche Molecular Biochemicals*, Apoptosis and Cell Proliferation, Second Revised Edition, which is incorporated herein by reference in its entirety. Depending on the particular assay utilized, the apoptosis assay methods, cytotoxicity assay methods, and cell proliferation/viability assay methods may be carried out on the host cells while they are alive and intact (unlysed) or on lysed cells, where necessary or desired.

DNA laddering is a technique used to identify DNA cleavage that occurs during apoptosis (Rosl F., *Nucleic Acids Res.*, 1992, 20:5243; Smith M. L. et al., *Mutat. Res.*, 1996, 340: 109-124; Nagata S., *Exp. Cell Res.*, 2000, 256:12-18, which are incorporated herein by reference in their entirety). By way of example, pellets containing, for example, 1×10^6 cells from the attached and floating cell population are washed in PBS and resuspended in 20 μ l of Solution I (10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% (w/v) SDS) plus Proteinase K (20 mg/ml stock, used at 0.5 mg/ml). Samples are incubated at 50° C. for 1 hour before adding 10 μ l of 0.5 mg/ml RNaseA and incubating at 50° C. for 1 hour. The samples are then heated rapidly to 70° C., supplemented with 10 μ l of Solution II (10 mM EDTA, 1% (w/v) low melting agarose, 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue), and immediately loaded onto a 2% agarose gel containing 0.1 μ g/ml of ethidium bromide (stock=10 mg/ml). The gel is then cooled

to 4° C. for approximately 5 minutes to allow the samples to set in the wells, and then run in Tris-acetate buffer at 40V until the dye front migrates 4-5 cm. The DNA is then observed using UV transillumination and photographed.

For recognition of apoptotic nuclei in vitro, a NEURO-TACS In Situ Apoptosis Detection Kit (R&D SYSTEMS) can be used, for example. Freshly fixed cultures are first permeabilized with NeuroPore reagent and endogenous activity will be quenched using H₂O₂. DNA fragmentation in individual apoptotic cells will be visualized by detection of biotinylated nucleotides incorporated into the free 3'-hydroxyl residues of these DNA fragments. A streptavidin-conjugated horseradish peroxidase bound to the biotinylated DNA fragments generates brown precipitates in the presence of diaminobenzidine (DAB). Blue counterstaining will be used for easier identification of cells. The positive controls are generated by brief treatment of cells with nuclease prior to labeling in order to generate DNA strand breaks in virtually all cells. Negative controls consist of slides in which terminal deoxynucleotidyl transferase (tdt) will be omitted from the reaction mixture. The number of apoptotic nuclei versus total number of cells will be determined from three independent culture platings for every RA exposure and time point (1 and 5 DIV). The number of apoptotic and non-apoptotic cells will be counted using a 20 \times objective placed randomly over two randomly selected non-overlapping sites per well (4 wells/plating, 3 platings in total). Percentages are determined as a ratio of apoptotic cells/total number of cells multiplied by 100. The mean values \pm S.E.M. from 0, 3, 4 and 5 weeks RA exposures at each time point are compared using Student's t-test.

An agent or treatment that shows desirable therapeutic characteristics may include one that increases glucose uptake by the cell, increases glucose accumulation within the cell, and/or increases glucose metabolism within the cell. An agent or treatment that shows desirable characteristics with regard to modulating intracellular glucose (such as an insulin sensitizer, e.g., rosiglitazone) can be further tested in an assay of insulin stimulated glucose uptake in differentiated L6-K1 cells or other skeletal muscle cells, muscle tissue biopsies, adipocytes or adipocyte cell lines. A therapeutic agent will preferably be of such nature that it is suitable for oral administration (e.g., when combined with a pharmaceutically acceptable carrier), but any route of administration, such as intravenous, suppository, or parenteral routes can be considered.

In another aspect, the present invention includes a method for the manufacture of a medicament (also referred to herein as a pharmaceutical composition) for the treatment and/or prevention of a medical condition connected with elevated levels of blood glucose, such as diabetes type I, diabetes type II, and gestational diabetes. In this context, the terms "prevent" and "treat" are not intended to exclusively mean the complete abolishment of the disease or condition, but is meant that there is complete or some amelioration, so that an improvement over the expected symptomology is clinically observed. An example of one such criterion could be the lowering of blood glucose levels by more than 25%. In accordance with the method of manufacture, once an agent has been identified using the intracellular glucose assay (method) of the present invention, the agent can be combined or otherwise brought into association with a pharmaceutically acceptable carrier.

The pharmaceutical compositions of the subject invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Formulations are described in a number of sources which are well known and

readily available to those skilled in the art. For example, *Remington's Pharmaceutical Sciences* (Martin E. W., 1995, Easton Pa., Mack Publishing Company, 19th ed.) describes formulations which can be used in connection with the subject invention. Formulations suitable for parenteral administration include, for example, aqueous sterile injection solutions, which may contain antioxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the condition of the sterile liquid carrier, for example, water for injections, prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powder, granules, tablets, etc. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the subject invention can include other agents conventional in the art having regard to the type of formulation in question.

Host Cells

The terms "recombinant host cells", "host cells", "genetically modified host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, immaterial of the method by which the DNA is introduced into the cell or the subsequent disposition of the cell. The terms include the progeny of the original cell that has been transfected. Cells in primary culture can also be used as recipients. Host cells can range in plasticity and proliferation potential. Host cells can be differentiated cells, progenitor cells, or stem cells, for example.

Host cells can be genetically modified with the vectors of the present invention, which may be a cloning vector or an expression vector, for example. The vector may be in the form of a plasmid, a virus, (e.g., a retrovirus or other virus), a viral particle, a phage, etc. The genetically modified host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants/transfectants or amplifying the subunit-encoding polynucleotide. The culture conditions, such as temperature, pH and the like, generally are similar to those previously used with the host cell selected for expression, and will be apparent to those of skill in the art.

In one embodiment, the host cell is a human cell. In another embodiment, the host cell is a non-human mammalian cell. Preferably, the host cell is a human or non-human mammalian muscle cell or adipocyte. In one embodiment, the host cell is a mammalian cell of a pancreatic beta cell line. In another embodiment, the host cell is a mammalian cell that differentiates into insulin-responsive cells. For example, in a specific embodiment, the host cell is an L6 rat myoblast or murine 3T3-L1 adipocyte or preadipocyte (Negrel R. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1978, 75:6054-6058; Yaffe D. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1968, 61:477-483). Other host cells include L8 myoblasts, C2C12 myoblasts, BRIN-BDII cells, INS-1 cells, RIN-m5F cells, RIN-1046 cells, and HIT cells.

Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences (e.g., promoter sequences) that are compatible with the designated host are used. For example, among prokaryotic hosts, *Escherichia coli* may be used. Also, for example, expression control sequences for prokaryotes

include but are not limited to promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts can be derived from, for example, the plasmid pBR322 that contains operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, that also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Commonly used prokaryotic control sequences include but are not limited to the lactose operon system (Chang et al. *Nature* 198: 1056, 1977), the tryptophan operon system (reported by Goeddel et al. *Nucleic Acid Res.* 8:4057, 1980) and the lambda-derived P1 promoter and N gene ribosome binding site (Shimatake et al. *Nature* 292:128, 1981), the hybrid Tac promoter (De Boer et al. *Proc. Natl. Acad. Sci. U.S.A.* 292: 128, 1983) derived from sequences of the trp and lac UV5 promoters. The foregoing systems are particularly compatible with *E. coli*; however, other prokaryotic hosts such as strains of *Bacillus* or *Pseudomonas* may be used if desired.

Eukaryotic hosts include yeast and mammalian cells in culture systems. *Pichia pastoris*, *Saccharomyces cerevisiae* and *S. carlsbergensis* are commonly used yeast hosts. Yeast-compatible vectors carry markers that permit selection of successful transformants by conferring prototrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Yeast compatible vectors may employ the 2- μ origin of replication (Broach et al. *Meth. Enzymol.* 101:307, 1983), the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences that will result in incorporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors are known in the art and include but are not limited to promoters for the synthesis of glycolytic enzymes, including the promoter for 3-phosphoglycerate kinase. (See, for example, Hess et al. *J. Adv. Enzyme Reg.* 7:149, 1968; Holland et al. *Biochemistry* 17:4900, 1978; and Hitzeman *J. Biol. Chem.* 255:2073, 1980). For example, some useful control systems are those that comprise the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, terminators also derived from GAPDH, and, if secretion is desired, leader sequences from yeast alpha factor. In addition, the transcriptional regulatory region and the transcriptional initiation region which are operably linked may be such that they are not naturally associated in the wild-type organism.

Host cells useful for expression of the polynucleotides of the present invention may be primary cells or cells of cell lines. The host cells may be tumor cells or non-tumor cells. Mammalian cell lines available as hosts for expression are known in the art and are available from depositories such as the American Type Culture Collection. These include but are not limited to HeLa cells, human embryonic kidney (HEK) cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and others.

The number of host cells used in a particular assay will vary with the objectives of the assay, the solid support used to support or contain the cell(s), etc. Thus, in some protocols, the host cell may be a single cell. In other protocols, a plurality of host cells will be used.

In accordance with the invention, the polynucleotide encoding the glucose-responsive mRNA instability element and the polynucleotide encoding the reporter polypeptide are operably linked to the same promoter sequence. Suitable promoters sequences for mammalian cells also are known in the art and include viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV) and cytomegalovirus

(CMV). Mammalian cells also may require terminator sequences and poly A addition sequences; enhancer sequences which increase expression also may be included, and sequences which cause amplification of the gene also may be desirable. These sequences are known in the art. Vectors suitable for replication in mammalian cells may include viral replicons, or sequences which ensure integration of the appropriate sequences including the glucose-responsive mRNA instability element and/or reporter gene into the host genome. An example of such a mammalian expression system is described in Gopalakrishnan et al. *Eur. J. Pharmacol.-Mol. Pharmacol.* 290: 237-246, 1995).

Candidate Agents and Treatments

Candidate agents and treatments that may be tested by the assays (methods) of the present invention include polypeptides, non-peptide small molecules, biological agents, and any other source of candidate agents potentially having the ability to modulate intracellular glucose (or glucose analog) levels. Candidate agents and treatments may be useful for the treatment of medical conditions involving reduced glucose uptake, such as type 2 diabetes. Candidate agents can be virtually any substance and can encompass numerous chemical classes, including organic compounds or inorganic compounds. A candidate agent may be substance such as genetic material, protein, lipid, carbohydrate, small molecules, a combination of any of two or more of foregoing, or other compositions. Candidate agents may be naturally occurring or synthetic, and may be a single substance or a mixture. Candidate agents can be obtained from a wide variety of sources including libraries of compounds. A candidate agent can be or include, for example, a polypeptide, peptidomimetic, amino acid, amino acid analog, polynucleotide, polynucleotide analog, nucleotide, nucleotide analog, or other small molecule. A polynucleotide may encode a polypeptide that potentially modulates glucose levels within the cell, or the polynucleotide may be a short interfering RNA (siRNA), antisense oligonucleotide, ribozyme, or other polynucleotide that targets an endogenous or exogenous gene for silencing of gene expression and potentially modulates glucose levels within the cell. Candidate treatments may include exposure of the host cells to any conditions that potentially modulate intracellular glucose (or glucose analog) levels within the host cells. The treatment may involve exposing the cells to an energy source, for example.

Reporter Polypeptides

The present invention provides a reporter vector including a polynucleotide encoding a glucose-responsive mRNA instability element and a polynucleotide encoding a reporter polypeptide (also referred to herein as the "reporter gene"), and host cells genetically modified with the reporter vector, which are useful as a sensor of intracellular glucose or glucose analogs. Reporter polypeptides such as Beta-globin, CAT, luciferase, and beta-gal may be used. Preferably, the reporter polypeptide is one whose production can be detected (and, optionally, measured qualitatively, quantitatively, and/or semi-quantitatively) in living, intact cells. More preferably, the reporter polypeptide is one whose production can be detected (and, optionally, measured qualitatively, quantitatively, and/or semi-quantitatively) in living, intact cells in a single step (e.g., without the need for further chemical reactions, for example). Examples of such reporter polypeptides include fluorescent polypeptides (also referred to herein as fluorescent proteins (FP)) such as the green fluorescent proteins (GFP), and variants of GFP such as yellow fluorescent proteins (YFP), etc., for example, PS-FP (Yang F. et al., *Nat. Biotechnol.*, 1996, 10:1246-1251; Cubitt A. B. et al., "Understanding Structure-Function Relationships in the *Aequorea*

victoria Green Fluorescent Protein, in *Methods in Cell Biology*, Vol. 58, Green Fluorescent Protein, Academic Press, 1999:19-29; Kain S. R., "Enhanced Variants of the Green Fluorescent Protein for Greater Sensitivity, Different Colours and Detection of Apoptosis", in *Fluorescent and Luminescent Probes*, 2nd Edition, 1999, Chapter 19:284-292; Tsien R. Y., *Annu. Rev. Biochem.*, 1998, 67:509-544; Eisenstein, M., *Nature Methods*, January 2005, Research Highlights, 2(1):8-9; each of which are incorporated herein in their entirety). As used herein, "variants of GFP" include, but are not limited to, polypeptides known in the art as green fluorescent protein-like proteins, GFP-like chromoproteins, green fluorescent protein fragments, red fluorescent proteins, and orange fluorescent proteins.

In addition to the exemplified GFP fusion TOPO expression vector (INVITROGEN; e.g., catalog nos. K4810-01 and K4820-01) described herein (Buechler C. et al., *J. Biol. Chem.*, 2002, Nov. 1; 277(44):41307-10), another example of commercially available GFP vectors includes Mammalian LUMIO GATEWAY vector (INVITROGEN; e.g., catalog nos. 12589-016, 12589-024, and 12589-032; see, for example INVITROGEN life technologies Instruction Manual, Version C, 7 December 2004; Tour O. et al., *Nat. Biotechnol.*, 2003, 21(12):1505-1508, which is incorporated herein by reference in its entirety), useful for site-specific fluorescence labeling and detection of proteins in live mammalian cells. The LUMIO recognition sequence is a small, six-amino acid sequence (Cys-Cys-Pro-Gly-Cys-Cys (SEQ ID NO:21)). This unique sequence rarely appears in endogenous proteins, providing precise detection of proteins with this fusion tag. The LUMIO detection reagents bind this sequence with high specificity and affinity, resulting in a bright fluorescent signal. A number of LUMIO vectors are available from INVITROGEN, allowing a variety of applications in multiple host systems.

Therefore, it will be possible to "read" the intracellular concentration of glucose based on the degree of fluorescence detected in living, intact (non-lysed) cells, which will be an inverse relationship. Cloning and in vitro transcription of GFP fusion constructs is well known in the art and may be used to carry out the present invention (Oancea E. et al., *J. Cell Biology*, 1998, 140(3):485-498, which is incorporated herein by reference in its entirety).

In addition, the reporter vector of the present invention may optionally include another marker gene such as an antibiotic resistance gene and the fluorescent protein is used here as a visualization marker gene for example, FP/PS1/Ble, to aid visualization and fluorescent quantitation of the protein. Many FPs, originally isolated from the jellyfish *Aequorea Victoria* (for example, GFP) retain their fluorescent properties when expressed in heterologous cells, thereby providing a powerful tool as fluorescent recombinant probes to monitor cellular events or functions (see, for example, Chalfie et al., *Science*, 1994, 263(5148):802-805; Prasher, *Trends Genet.*, 1995, 11(8):320-3; and PCT publication no. WO 95/07463, each of which are incorporated herein by reference in their entirety).

Several spectral and mutational variants of GFP proteins have since been isolated, for example, the naturally occurring blue-fluorescent variant of GFP (Heim et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1994, 91(26):12501-4; U.S. Pat. No. 6,172,188, both of which are incorporated herein by reference), the yellow-fluorescent protein variant of GFP (Miller et al., *J. Mol. Biol.*, 1999, 288:975-987; Weiss, et al., *Proc. Natl. Acad. Sci. U.S.A.*, 2001, 98(26):14961-62001; Majoul, et al., *Dev. Cell.*, 2001, 1(1):139-53; Laird et al., *Microsc. Res. Tech.*, 2001;52(3):263-72; Daabrowski et al., *Protein Expr. Purif.*,

1999, 16(2):315-23, and more recently the red fluorescent protein isolated from the coral *Discosoma* (Fradkov et al., *FEBS Lett.*, 2000, 479(3):127-30; Miller et al., *J. Mol. Biol.*, 1999, 288:975-987), which allows the use of fluorescent probes having different excitation and emission spectra permitting the simultaneous monitoring of more than one process. GFP proteins provide non-invasive assays that allow detection of cellular events in intact, living cells. The skilled artisan will recognize that the invention is not limited to the fluorescent polypeptides explicitly described herein and one may use any other spectral or mutational variant or derivative in accordance with the present invention.

Host cells of the invention may be transduced with an additional vector (in addition to the reporter vector of the present invention. For example, the reporter vector of the invention can be used in conjunction with an FP-linked oxygen sensor (see, for example, Metzen E. et al., *Journal of Cell Science*, 2003, 116:1319-1326, which is incorporated herein by reference in its entirety). Preferably, in the case where the reporter polypeptide is a fluorescent polypeptide, the two fluorescent polypeptides (the fluorescent polypeptide encoded by the reporter gene and the fluorescent polypeptide encoded by the oxygen sensor) are of different colors (e.g., different detectable wavelengths). Thus, in addition to detection of intracellular glucose, intracellular oxygen may also be detected within the host cell before, during, or after detection of intracellular glucose is carried out.

Several methods to identify and quantitate cells that are fluorescently tagged with fluorescent gene products are well known in the art and may be used in the context of the present invention. One example is the use of fluorescent activated cell sorting (FACS), flow cytometry or flow microfluorometry provides the means of scanning individual cells for the presence of a fluorescent protein. The method employs instrumentation that is capable of activating, and detecting the excitation emissions of cells that express a fluorescent marker in a liquid medium. FACS is unique in its ability to provide a rapid, reliable, quantitative, and multi-parameter analysis on either living or fixed cells in culture or in vivo. Other methods to measure fluorescent markers are also well known.

The present invention also includes an antisense nucleotide sequence that is complementary to the nucleic acid sequence of SEQ ID NO:9 or SEQ ID NO:14. In one embodiment, the antisense nucleotide sequence is complementary to at least 15 consecutive bases of SEQ ID NO:9 or SEQ ID NO: 14. The present invention also includes primers useful for detection of nucleic acid sequences encoding glucose-responsive mRNA instability elements or detection of the instability elements themselves. The primers are capable of recognizing and binding (amplifying) the nucleic acid sequence encoding the glucose-responsive mRNA instability element or the mRNA instability element itself. In one embodiment, the primer is capable of recognizing and binding (amplifying) the nucleotide sequence comprising SEQ ID NO:9 or SEQ ID NO:14. The present invention also includes a nucleic acid probe comprising a nucleotide sequence having affinity for the nucleotide sequence encoding a glucose-responsive mRNA instability element or having affinity for the glucose-responsive mRNA instability element itself. In one embodiment, the nucleic acid probe hybridizes with a nucleotide sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:20.

Various degrees of stringency of hybridization can be employed. The more stringent the conditions, the greater the complementarity that is required for duplex formation. Severity can be controlled by temperature, probe concentration, probe length, ionic strength, time, and the like. Preferably,

hybridization is conducted under stringent conditions by techniques well known in the art, as described, for example, in Keller, G. H., M. M. Manak (1987) *DNA Probes*, Stockton Press, New York, N.Y., pp. 169-170. Low-stringency hybridization is the preferred method when a larger gene fragment is used.

As used herein "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ³²P-labeled gene-specific probes was performed by standard methods (Maniatis, T., E. F. Fritsch, J. Sambrook [1982] *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). In general, hybridization and subsequent washes are carried out under stringent conditions that allow for detection of target sequences with homology to the exemplified polynucleotides encoding glucose-responsive mRNA instability elements. For double-stranded DNA gene probes, hybridization was carried out overnight at 20-25° C. below the melting temperature (T_m) of the DNA hybrid in 6×SSPE, 5× Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz, G. A., K. A. Jacobs, T. H. Eickbush, P. T. Cherbash, and F. C. Kafatos, *Methods of Enzymology*, 1983, R. Wu, L. Grossman and K. Moldave [eds.] Academic Press, New York 100:266-285).

$T_m = 81.5^\circ \text{C.} + 16.6 \text{ Log}[\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.61(\% \text{ formamide}) - 600/\text{length of duplex in base pairs.}$

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1×SSPE, 0.1% SDS (low stringency wash).

(2) Once at T_m-20° C. for 15 minutes in 0.2×SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization was carried out overnight at 10-20° C. below the melting temperature (T_m) of the hybrid in 6×SSPE, 5× Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T_m for oligonucleotide probes can be determined by the "nearest-neighbor" method. See Breslauer et al., "Predicting DNA duplex stability from the base sequence," *Proc. Natl. Acad. Sci. U.S.A.*, 83 (11): 3746-3750 (June 1986); Rychlik and Rhoads, "A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA," *Nucleic Acids Res.*, 17 (21): 8543-8551 (Nov. 11, 1989); Santa Lucia et al., "Improved nearest-neighbor parameters for predicting DNA duplex stability," *Biochemistry* 35 (11): 3555-3562 (Mar. 19, 1996); Doktycz et al., "Optical melting of 128 octamer DNA duplexes. Effects of base pair location and nearest neighbors on thermal stability," *J. Biol. Chem.*, 270 (15): 8439-8445 (Apr. 14, 1995). Alternatively, the T_m can be determined by the following formula:

$$T_m (^\circ \text{C.}) = 2(\text{number } T/A \text{ base pairs}) + 4(\text{number } G/C \text{ base pairs}) \text{ (Suggs, S. V., T. Miyake, E. H. Kawashime, M. J. Johnson, K. Itakura, and R. B. Wallace [1981] ICN-UCLA Symp. Dev. Biol. Using Purified Genes, D. D. Brown [ed.], Academic Press, New York, 23:683-693).}$$

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes 1×SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1×SSPE, 0.1% SDS (moderate stringency wash).

The nucleotide sequences of the subject invention can also be used as primers for PCR amplification. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and

commonly used by those skilled in this art (see Mullis, U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki, Randall K., Stephen Scharf, Fred Faloona, Kary B. Mullis, Glenn T. Horn, Henry A. Erlich, Norman Arnheim "Enzymatic Amplification of β -Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia," *Science*, 1985, 230:1350-1354). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are oriented with the 3 ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5 ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, which is isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated.

The present invention further includes a method for controlling expression of a gene in a host cell, comprising introducing a polynucleotide encoding a metabolite responsive instability element into a gene of the host cell, whereby post-transcriptional stability of mRNA is controlled by the presence of intracellular glucose or an analog thereof. In one embodiment, the polynucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:20.

The present invention further includes a method for treating a disease in a human or non-human mammalian patient, comprising administering a vector to cells of the patient in vivo or ex vivo, wherein the vector comprises a first polynucleotide encoding a glucose responsive mRNA instability element; a second polynucleotide encoding a therapeutic polypeptide for treatment of the disease; and a promoter sequence operably linked to said first polynucleotide and said second polynucleotide; wherein post-transcriptional stability of said first polynucleotide and said second polynucleotide is controlled by the presence of glucose or an analog thereof. In one embodiment, the therapeutic polypeptide comprises insulin. In another embodiment, the disease is selected from the group consisting of vascular disease, cancer, hypertension, and atherosclerosis.

The present invention further includes a method of screening for mutations of the metabolite responsive instability element, comprising: obtaining a DNA sample from a patient; sequencing said metabolite responsive instability element; and detecting mutations within said metabolite responsive instability element.

The present invention further includes a kit for detecting a nucleic acid sequence encoding a metabolite responsive instability element, comprising multiple containers wherein each of the separate containers comprise: a set of primers for PCR detection of said metabolite responsive instability element; and optionally, a positive control comprising the metabolite responsive instability element DNA.

As used herein, the term "glucose" is intended to include glucose analogs, such as glucose metabolites. Examples of glucose metabolites include 3-O-methylglucose and 2-deoxyglucose.

The term "genetic modification" as used herein refers to the stable or transient alteration of the genotype of a cell of the subject invention by intentional introduction of exogenous nucleic acids by any means known in the art (including for example, direct transmission of a polynucleotide sequence from a cell or virus particle, transmission of infective virus particles, and transmission by any known polynucleotide-bearing substance) resulting in a permanent or temporary alteration of genotype. The nucleic acids may be synthetic, or naturally derived, and may contain genes, portions of genes, or other useful polynucleotides in addition to the glucose-responsive mRNA instability element and the reporter gene. A translation initiation codon can be inserted as necessary, making methionine the first amino acid in the sequence. Host cells of the invention include those cells that have been transfected with reporter vectors of the present invention and the progeny of those host cells.

The term "vector" is used to refer to any molecule (e.g., nucleic acid, plasmid, or virus) usable to transfer coding sequence information (e.g., the glucose-responsive mRNA instability element and reporter gene), such as to a host cell. A vector typically includes a replicon in which another polynucleotide segment is attached, such as to bring about the replication and/or expression of the attached segment. The term includes expression vectors, cloning vectors, and the like. Thus, the term includes gene expression vectors capable of delivery/transfer of exogenous nucleic acid sequences into a host cell. The term "expression vector" refers to a vector that is suitable for use in a host cell (e.g., a patient's cell, tissue culture cell, cells of a cell line, etc.) and contains nucleic acid sequences which direct and/or control the expression of exogenous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present. Nucleic acid sequences can be modified according to methods known in the art to provide optimal codon usage for expression in a particular expression system. The vector may include elements to control targeting, expression and transcription of the nucleic acid sequence in a cell selective manner as is known in the art. It should be noted that often the 5'UTR and/or 3'UTR of the gene may be replaced by the 5'UTR and/or 3'UTR of the expression vehicle. The vector can include a promoter for controlling transcription of the exogenous material and can be either a constitutive or inducible promoter to allow selective transcription. The expression vector can also include a selection gene.

A "coding sequence" is a polynucleotide sequence that is transcribed into mRNA and/or translated into a polypeptide. For example, a coding sequence may encode a glucose-responsive mRNA instability element or a reporter polypeptide. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, mRNA, cDNA, and recombinant polynucleotide sequences. Variants or analogs may be prepared by the deletion of a portion of the coding sequence, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art (See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, 1989; *DNA Cloning*, Vols. I and II, D. N. Glover ed., 1985). Optionally, the polynucleotides of the present invention, and composition of matter and methods of the invention that utilize such polynucleotides, can include non-coding sequences.

As used herein, the term “polypeptide” refers to any polymer comprising any number of amino acids, and is interchangeable with “protein”, “gene product”, and “peptide”.

As used herein, the term “nucleoside” refers to a molecule having a purine or pyrimidine base covalently linked to a ribose or deoxyribose sugar. Exemplary nucleosides include adenosine, guanosine, cytidine, uridine and thymidine.

The term “nucleotide” refers to a nucleoside having one or more phosphate groups joined in ester linkages to the sugar moiety. Exemplary nucleotides include nucleoside monophosphates, diphosphates and triphosphates.

The terms “polynucleotide”, “nucleic acid molecule”, and “nucleotide molecule” are used interchangeably herein and refer to a polymer of nucleotides joined together by a phosphodiester linkage between 5' and 3' carbon atoms. Polynucleotides used in the vectors and methods (assays) of the present invention include, for example, those comprising a glucose-responsive mRNA instability element and/or a nucleotide sequences encoding a reporter molecule such as a fluorescent polypeptide. Polynucleotides may also be candidate agents that potentially modulate intracellular glucose levels within host cells. Such polynucleotides may encode a polypeptide which, when expressed, modulates intracellular glucose levels. However, the polynucleotide may also be short interfering RNA (siRNA), antisense nucleic acids (antisense oligonucleotides), aptamers, ribozymes (catalytic RNA), or triplex-forming oligonucleotides (i.e., antigene), for example.

As used herein, the term “RNA” or “RNA molecule” or “ribonucleic acid molecule” refers generally to a polymer of ribonucleotides. The term “DNA” or “DNA molecule” or “deoxyribonucleic acid molecule” refers generally to a polymer of deoxyribonucleotides. DNA and RNA molecules can be synthesized naturally (e.g., by DNA replication or transcription of DNA, respectively). RNA molecules can be post-transcriptionally modified. DNA and RNA molecules can also be chemically synthesized. DNA and RNA molecules can be single-stranded (i.e., ssRNA and ssDNA, respectively) or multi-stranded (e.g., double stranded, i.e., dsRNA and dsDNA, respectively). Based on the nature of the invention, however, the term “RNA” or “RNA molecule” or “ribonucleic acid molecule” can also refer to a polymer comprising primarily (i.e., greater than 80% or, preferably greater than 90%) ribonucleotides but optionally including at least one non-ribonucleotide molecule, for example, at least one deoxyribonucleotide and/or at least one nucleotide analog.

As used herein, the term “nucleotide analog” or “nucleic acid analog”, also referred to herein as an altered nucleotide/nucleic acid or modified nucleotide/nucleic acid refers to a non-standard nucleotide, including non-naturally occurring ribonucleotides or deoxyribonucleotides. Preferred nucleotide analogs are modified at any position so as to alter certain chemical properties of the nucleotide yet retain the ability of the nucleotide analog to perform its intended function. For example, locked nucleic acids (LNA) are a class of nucleotide analogs possessing very high affinity and excellent specificity toward complementary DNA and RNA. LNA oligonucleotides have been applied as antisense molecules both in vitro and in vivo (Jepsen J. S. et al., *Oligonucleotides*, 2004, 14(2): 130-146).

As used herein, the term “RNA analog” refers to a polynucleotide (e.g., a chemically synthesized polynucleotide) having at least one altered or modified nucleotide as compared to a corresponding unaltered or unmodified RNA but retaining the same or similar nature or function as the corresponding unaltered or unmodified RNA. As discussed above, the oligonucleotides may be linked with linkages which result

in a lower rate of hydrolysis of the RNA analog as compared to an RNA molecule with phosphodiester linkages. Exemplary RNA analogues include sugar- and/or backbone-modified ribonucleotides and/or deoxyribonucleotides. Such alterations or modifications can further include addition of non-nucleotide material, such as to the end(s) of the RNA or internally (at one or more nucleotides of the RNA).

The term “operably-linked” is used herein to refer to an arrangement of flanking control sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking control sequence operably-linked to a coding sequence may be capable of effecting the replication, transcription and/or translation of the coding sequence under conditions compatible with the control sequences. For example, a coding sequence is operably-linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence, and the promoter sequence can still be considered “operably-linked” to the coding sequence. Each nucleotide sequence coding for a polypeptide will typically have its own operably-linked promoter sequence. The reporter vectors of the invention include, but are not limited to, a first polynucleotide encoding a glucose-responsive mRNA instability element; a second polynucleotide encoding a reporter polypeptide; and a promoter sequence operably linked to said first polynucleotide and said second polynucleotide; wherein post-transcriptional stability of said first polynucleotide and said second polynucleotide is controlled by the presence of glucose or an analog thereof.

The terms “transfection”, “transformation”, and “introduction”, and grammatical variations thereof, are used interchangeably herein to refer to the insertion of an exogenous polynucleotide (e.g., a nucleic acid sequence encoding a glucose-responsive instability element and/or a reporter gene) into a host cell, irrespective of the method used for the insertion, the molecular form of the polynucleotide that is inserted, or the nature of the cell (e.g., prokaryotic or eukaryotic). The insertion of a polynucleotide per se and the insertion of a plasmid or vector comprised of the exogenous polynucleotide are included. The exogenous polynucleotide may be directly transcribed and translated by the cell, maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be stably integrated into the host genome. Thus, host cells of the invention include those that have been transfected with reporter vectors of the invention.

The phrases “isolated” or “biologically pure” refer to material that is substantially or essentially free from components which normally accompany the material as it is found in its native state.

An “isolated polynucleotide” that encodes a particular polypeptide refers to a polynucleotide that is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include functionally and/or structurally conservative mutations as defined herein.

The terms “cell” and “cells” are used interchangeably herein to refer to a single cell or plurality of cells (i.e., at least one cell). Typically, host cells used in the methods of the invention are isolated. However, tissues, and genetically modified or transgenic animals may also be utilized.

The terms “comprising”, “consisting of” and “consisting essentially of” are defined according to their standard mean-

ing. The terms may be substituted for one another throughout the instant application in order to attach the specific meaning associated with each term.

As used in this specification, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a cell" includes more than one such cell. Reference to "an instability unit" includes more than one such instability unit. Reference to "a reporter gene" or "reporter polypeptide" includes more than one such reporter gene or reporter polypeptide, and the like.

The practice of the present invention can employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, electrophysiology, and pharmacology, that are within the skill of the art. Such techniques are explained fully in the literature (see, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); DNA Cloning, Vols. I and II (D. N. Glover ed. 1985); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Transcription and Translation* (Hames et al. eds. 1984); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller et al. eds. (1987) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); *Scopes, Protein Purification: Principles and Practice* (2nd ed., Springer-Verlag); and *PCR: A Practical Approach* (McPherson et al. eds. (1991) IRL Press)).

Materials and Methods

Cell culture. The vascular smooth muscle cell line (A10, ATCC CRL 1476), derived from rat aorta, was grown in Dulbecco's modified Eagle's media (DMEM with 5.5 mM glucose) containing 10% fetal bovine serum (FBS) and 100 units penicillin G and 100 µg streptomycin sulfate/ml at 37° C. in a humidified 5% CO₂, 95% air atmosphere in either 6-well plates (for promoter studies, Western blot analysis, [³H]-thymidine uptake assay) or 100-mm plates (for Northern blot analysis, RNA stability assay). Cells were grown to >90% confluency and media was changed every 4 days. Cell synchronization was achieved by serum deprivation (0.5% FBS) for 48 hours (see, for example, Ross, R., C. Nist, B. Kariya, M. J. Rivest, E. Raines, and J. Callis. 1978. Physiological quiescence in plasma-derived serum: Influence of platelet derived growth factor on cell growth in culture. *J. Cell. Physiol.* 97:497-508).

Primary cultured human aortic smooth muscle cells (AOSMC) (CLONETICS, San Diego, Calif.) were grown in SmGM (CLONETICS) medium containing 5.5 mM glucose, 5% FBS, 10 ng/ml human recombinant epidermal growth factor (hEGF), 390 ng/ml dexamethasone, 50 µg/ml gentamicin, and 50 ng/ml amphotericin-B at 37° C. in a humidified 5% CO₂, 95% air atmosphere. Cells were grown to >90% confluency and medium was changed every 5 days.

The isotopes [α -³²P]dCTP and [α -³²P]UTP (specific activity 3000 Ci/mmol) were purchased from ICN Biochemicals. Oligo probe labeling kit (Prime-a-Gene) was purchased from Promega. Riboscribe T7 RNA Probe Synthesis kit and Ampliscribe T7 Transcription kit were purchased from Epicenter Technologies. RNase A, RNase T1, heparin, RNase inhibitor were purchased from Sigma. All other biochemicals were purchased from the usual vendors as noted. Inhibitors for the signaling pathways were used as described in the results. Cycloheximide (20 mM), PD98059 (20 µM), Okadaic acid (20 nM), AG490 (5 µM), rapamycin (1 mM), herbimycin A (1 µM) and LY 294002 (20 µM) were obtained from Cal-

biochem. CGP41251 (5 µM) was a gift from Dr. Dorian Fabbro, CIBA-GEIGY (Basel, Switzerland).

Calcium-phosphate transfections. For promoter studies, A10 cells were grown and synchronized in 6-well plates. Three hours prior to transfections, fresh medium (DMEM containing 10% fetal bovine serum) was substituted on A10 cells. pβG or pβG-PKCβII expression vectors were transfected with the calcium-phosphate-DNA precipitate. Following overnight incubation, calcium phosphate-DNA precipitate was washed off with 1× Dulbecco's phosphate buffered saline (DPBS) and replaced with fresh medium. For stably-transfected cell selection, 0.7 mg/ml G-418 was added to the media. It was changed every 4 days and 10-14 days later, the colonies were pooled and maintained in DMEM (10% FBS) with 0.2 mg/ml G-418.

mRNA half-life determination. A10 cells were pre-treated with actinomycin D (5 µg/µl) for about 30 minutes, followed by the addition of glucose (25 mM) to the glucose-treated plates. RNA samples were isolated from control (5.5 mM glucose) and glucose-treated (25 mM glucose) dishes at 0 h, 2 h, 4 h, or 6 h. Northern blot analysis was then performed on about 10 µg of the total RNA as described below.

In separate experiments, pβG-PKCβII stable transfectants were plated into 100 mm dishes. 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (50 µg/ml) dissolved in 95% ethanol was added to the plates and the 0h RNA sample was isolated. Glucose (25 mM) was added to the glucose-treated plates. RNA samples were isolated from control (5.5 mM glucose) and glucose-treated (25 mM glucose) pβG-PKCβII plates at 2 h, 4 h, and 6 h. In a separate control, an equivalent amount of 95% ethanol was added.

Isolation of RNA and Northern Blot Analysis. Total cellular RNA was isolated from 100-mm plates using Tri-Reagent (Molecular Research Center, Inc.). RNA samples (10 µg) were prepared in formamide, formaldehyde and 1×3-(N-morpholino)propanesulfonic acid (MOPS), and fractionated on 1.2% agarose-formaldehyde gels. Ethidium bromide was added in the loading buffer for visualization and quantitation of 18S and 28S RNA. After fractionation, the integrity and loading of RNA was assessed under UV light (23,24). The size-fractionated RNA was then capillary transferred to Hybond membranes (AMERSHAM), and cross-linked to membranes by baking at 80° C. in a vacuum oven for 2 hours. Membranes were hybridized overnight at 42° C. with 2×10⁷ cpm of the full length PKCβ cDNA probe or β-globin probe (labeled with [³²P] dCTP by nick translation as described (25)) per ml of hybridization buffer (5× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, 50% (v/v) formamide). Membranes were washed with high stringency conditions (1×SSC, 0.1% SDS for 15 minutes at 42° C. followed by 0.1× SSC, 0.1% SDS for 15 minutes at 42° C.), and quantitated using Molecular Dynamics Phosphorimaging System. In experiments, where mentioned, membranes were exposed to X-ray film and the autoradiograms were quantitated densitometrically.

RNA stability assay. To prepare post-nuclear extracts (see, for example, Wager, R. E. and R. K. Assoian. 1990. A phorbol ester-regulated ribonuclease system controlling transforming growth factor BI gene expression in hematopoietic cells. *Mol. Cell. Biol.* 10:5983-5990), synchronized A10 cells, re-initiated to proliferate with DMEM+10% FBS were treated with either 5.5 mM or 25 mM glucose for 2 h to 24 h, then washed and collected in ice-cold 1× DPBS. The cellular pellet was re-suspended in extract buffer containing 0.01M Tris-HCl, 0.15M NaCl, 0.5% Nonidet P-40, leupeptin (10 µg/ml) and aprotinin (10 µg/ml) incubated on ice for 10 minutes and carefully layered over extract buffer containing 24% sucrose.

Samples were centrifuged over a sucrose gradient at 10,000xg for 20 minutes at 4° C. Post-nuclear extract was separated, stored on ice and aliquots taken for protein concentration using Bradford protein assay (see Bradford, M. M. 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). Total RNA (10 µg) from A10 cells, was incubated with 2.7% (vol/vol) of the post-nuclear extracts for 30 minutes at 4° C. in 50 µl (final volume) of extract buffer. When specifically noted, 50 mM EDTA was added to the reactions as a control to inhibit nuclease activity. The reactions were terminated by phenol-chloroform extraction, supplemented with 10 µg yeast tRNA as carrier and precipitated with ethanol. RNA was fractionated on 1.2% formaldehyde-agarose gels and Northern blot analysis was carried out with full length PKC, cDNA probe. The assay and analyses was repeated at least three times with separate post-nuclear extracts to assess and confirm reproducibility.

Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was isolated from control (5.5 mM glucose) or glucose-treated (25 mM glucose) A10 cells and 2 µg was used to synthesize first strand cDNA using on Oligo(dT) primer and Superscript II reverse transcriptase (LIFE TECHNOLOGIES Pre-amplification Kit). The upstream sense primer corresponded to the C4 kinase domain common to both PKCβI and PKCβII (5' CGTATATGCGGCCGCGTTGTGGGCCTGAAGGGG 3') [SEQ ID NO:4] and the downstream antisense primer was specific for PKCβI (5' GCATTCTAGTCGACAAGAGTTTGTCTAGTGGGAG 3') [SEQ ID NO:5] (Chalfant et al., 1995, pp.13326-13332). These primers detect inclusion of the PKCβII exon in the mature mRNA as well as PKCβI mRNA. Sense and antisense primers for β-actin (#5402-3) were obtained from Clontech. PCR was performed using ampliTaQ Gold DNA polymerase from PERKIN ELMER (#N808-0240) on 10% of the reverse transcriptase reaction product. Following 35 cycles of amplification in a BIOMETRA Trioblock thermocycler (PKCβI and -βII: 95° C., 30 sec; 64° C., 2 min for 35 cycles; and for β-actin: 94° C., 1 min; 58° C., 1 min; and 72° C., 3 min for 35 cycles), 25% of the PCR reaction was resolved on a 1.2% agarose gel. Bands were observed under UV light and photographed.

For the stability reporter system, β-globin primers were designed. The sense primer was (5' GCATCTGTCCAGTGAGGAGAA 3') [SEQ ID NO:6] while the antisense primer for β-globin was (5' AACAGCACGTTGCCAGGAG 3') [SEQ ID NO:7]. PCR was performed using ampliTaQ Gold DNA polymerase from PERKIN ELMER (#N808-0240) on 10% of the reverse transcriptase reaction product. Following 25 cycles of amplification in a BIOMETRA Trioblock thermocycler (94° C., 1 min; 58° C., 1 min; and 72° C., 3 min for 25 cycles), 25% of the PCR reaction was resolved on a 1.2% agarose gel. Bands were observed under UV light and photographed. The expected size of the amplified produce was 320 bp.

Synthesis of pCR-blunt-PKCβII. Total RNA was isolated from A10 cells and 10 µg of the RNA was used in the RT-PCR reaction (as described above). The amplified products were separated on 1.2% agarose gel. The PKCβII-specific band of 404 bp was cut from the gel and purified using QIAquick gel extraction kit (Qiagen). The PKCβII cDNA was cloned into the vector pCRblunt (purchased from INVITROGEN, Inc.) and used in the sequencing reaction. By sequence analysis, it was confirmed that the PKCβII mRNA resulted from the inclusion of the 216 bp PKCβII specific exon via alternative splicing of the PKC, pre-mRNA.

β-globin cDNA probe preparation. The pRSV-βG vector (obtained from N. P. Curthoys, Colorado State University) (Hansen et al., 1996, pp. F126-31) was restricted with HindIII and BglII to obtain a 507 bp fragment, which was purified from agarose gel using QIAquick gel extraction kit (Qiagen). 25 ng of the probe was labeled with [α-³²P]dCTP using Prime-a-Gene (Promega) to a specific activity of 10⁹ d.p.m.

Synthesis of pβG-PKCβII chimeric minigene. The parent vector, pβG, was obtained from N. P. Curthoys (see Hansen W. R., Barsic-Tress N., Taylor L., and Curthoys N. P. The 3'-nontranslated region of rat renal glutaminase mRNA contains a pH-responsive stability element. [Journal Article] *American Journal of Physiology.* 271(1 Pt 2):F126-31, 1996 Jul), Colorado State University. The pβG contains the strong viral promoter derived from the long terminal repeat of the Rous sarcoma virus followed by the transcriptional start site, the 5'-nontranslated region, the entire coding sequence, and two introns from the rabbit cloning gene; a multicloning site containing four unique restriction sites; and the 3'-nontranslated region and polyadenylation site of the bovine growth hormone. PKCβII cDNA was cloned earlier into the pCR-Blunt vector (INVITROGEN) and to expedite the procedure, it was digested with SpeI and XbaI, extracted from the gel (Qiagen's QIAquick gel extraction kit) and purified. The PKCβII cDNA was subcloned in frame with the β-globin reading frame into the SpeI and XbaI sites of the multicloning region of pβG vector (FIG. 34). The 3'-nontranslated region and polyadenylation site of the bovine growth hormone were maintained in the resultant pβG-PKCβII minigene. The construct was verified by restriction mapping and dideoxynucleotide sequencing.

Transcription vectors. The 404 bp PKCβII product was obtained by PCR amplification using sense primer to the upstream PKCβ common C4 domain (5' CGTATATGCGGCCGCGTTGTGGGCCTGAAGGGG 3') [SEQ ID NO:4] and anti-sense primer to βIV5 domain (5' GCATTCTAGTCGACAAGAGTTTGTCTAGTGGGAG 3') [SEQ ID NO:5] such that the exon-included PKCβII mRNA was amplified. This PKCβII cDNA piece was cloned into the pCR-Blunt vector (INVITROGEN) such that sense transcripts could be generated from the upstream T7 RNA polymerase promoter. A 410 bp β-globin segment cloned into the pCR-Blunt vector was used as a non-specific competitor probe.

In vitro transcription. The RNA probes were generated by consecutive restriction digestion of the pCR-Blunt-PKCβII vector. Probe A was the full length PKCβII insert linearized with BamHI; probe B was PKCβII linearized at 175 bp with BglII within PKCβII exon such that the PKCβI-specific exon was eliminated; probe C was linearized at 137 bp with HpaI which cuts within the PKCβII-specific exon was eliminated; probe D was linearized at 102 bp with SspI which cuts within the PKCβII-specific exon; probe E was linearized at 45bp with FokI which cuts within the C4 exon. After digestion, the probes were purified and their sizes and linearity were confirmed on agarose gels and further used for in vitro transcription with Ampliscribe kit (Epicenter) for competitor unlabeled probes or with Riboscribe kit (Epicenter) for transcribing labeled RNA probes according to the manufacturer's instructions.

Cytoplasmic extract (S100) preparation for RNA Electrophoretic Mobility Shift Assay. In brief, A10 cells incubated in 5.5 mM glucose (normal) or 25 mM glucose (high glucose) for 4 h were washed with 1x DPBS, scraped, collected and centrifuged at 3000 rpm for 10 min. The packed cells were re-suspended in hypotonic buffer (10 mM HEPES, pH7.9 at 4° C.; 1.5 mM MgCl₂; 10 mM KCl; 0.2 mM PMSF; 0.5 mM DTT; 10 µM leupeptin; 10 µM antipain), allowed to swell for

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10 min on ice, and homogenized in a Dounce homogenizer with 60 strokes using a type B pestle. The nuclei was pelleted by centrifuging at 3300× g for 15 min, and the cytoplasmic extract was mixed with 0.11 vol of cytoplasmic buffer (0.3M HEPES, pH7.9 at 4° C.; 1.4M KCl; 0.03M MgCl₂). After centrifugation for 1 h in Beckman type 50 rotor at 40,000 rpm, the supernatant was aliquoted, frozen in liquid nitrogen and stored at -80° C. An aliquot was used to determine the protein concentrations using the Bradford method (Bradford, M. M. 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) for protein assay.

RNA Electrophoretic Mobility Shift Assay (EMSA). With modifications to methods described by Brewer, G. and J. Ross. (1989. Regulation of c-myc mRNA stability in vitro by a labile destabilizer with an essential nucleic acid component. *Mol Cell Biol* 9:1996-2006; Amara, F. M., J. Sun, and J. A. Wright. 1996. Defining a Novel cis-Element in the 3'-Untranslated Region of Mammalian Ribonucleotide Reductase Component R2 mRNA. *The Journal of Biological Chemistry* 271, No. 33:20126-20131) and Amara, F. M., G. M. Smith, T. I. Kushak, T. L. Takeuchi, and J. A. Wright (1996. A cis-trans interaction at the 3'-untranslated region of ribonucleotide reductase mRNA is regulated by TGF-β1, TGF-β2 and TGF-β3. *Biochemical and Biophysical Research Communications* 228 (2): 347-351) cytoplasmic extracts from A10 cells (normal and high glucose) containing 3 μg protein were incubated with 3 μg yeast tRNA, 10 units of RNase inhibitor in a final volume of 10 μl RNA shift buffer (12 mM HEPES, pH7.9, 10 mM KCl, 10% glycerol, 5 mM EDTA, 5 mM DTT, 5 mM MgCl₂) for 10 min at room temperature. 100 to 300 fold excess specific cold competitors or 100-fold excess non-specific cold competitor were added to the binding-reactions and incubated for 5 min at room temperature. ³²P labeled RNA probes A, B, C or D (described above) to a specific activity of ~1×10⁶/μg were added and incubated for 20 min at room temperature. 100 U of RNaseT1 was added and further incubated for 15 min at room temperature, followed by the addition of 5 mg/ml heparin to reduce nonspecific binding, for 10 min on ice. Samples were separated on a 10% polyacrylamide gel in 0.5×TBE buffer. Gels were dried and exposed to Molecular Dynamics Phosphorimaging Screen.

UV cross-linking of RNA-protein complexes. RNA-protein binding reactions were carried out as described above for the RNA EMSA. Following heparin addition, the samples were transferred to a 96-well plate, irradiated for 10 min in Stratalinker (Stratagene) on ice. Laemmli's buffer was added to the sample which was then boiled for 5 min and electrophoresed in a 10% SDS-polyacrylamide gel. Gels were dried and exposed to Molecular Dynamics Phosphorimaging Screen.

EXAMPLES

Example 1

PKCβII Protein Levels Decreased when A10 Cells Were Exposed to 25 mM Glucose

To better understand the biochemical mechanism underlying the acute effects of high glucose exposure on PKCβ expression in aortic vascular smooth muscle cells, protein levels of PKCβI and βII were determined in synchronized A10 cells. Cell growth synchronization was achieved by serum deprivation for 48 hours as described in methods, and following synchronization, medium on A10 cells was

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replaced with DMEM containing 10% FBS to re-initiate cell proliferation and incubated with high (25 mM) or normal (5.5 mM) glucose. Over a twenty-four hour period, protein was extracted at various time points from cells re-initiated by serum addition to proliferate. It appeared that as cells progressed through the cell cycle, the expression of PKCβII appeared to be cell cycle associated, with highest levels of protein expression observed at 6 and 18 hours and lowest levels occurring at 14 hours. PKCβII protein levels in cells treated with high glucose decreased by 55% within first 6 hours and remained low following 14 hours of continuous high glucose treatment and began to increase at 18 hours (FIGS. 17A and 17B). No significant changes in PKCβI protein levels were detected between high and normal glucose (FIGS. 16A and 16B). To check the specificity of high glucose to down-regulate PKCβII, western blot analysis was repeated using primary antibodies to PKCα and PKCε. Again, no significant changes in PKCα or -ε protein levels were detected between high and normal glucose treatments (data not shown). Thus, the down-regulation by acute glucose appeared specific for the PKCβII isoform and did not affect the other PKC isozymes.

Example 2

Hyperglycemic Conditions Markedly Reduce PKCβ(I+II) mRNA in A10 Cells

To determine the acute effects of hyperglycemia on PKCβ gene expression, steady state levels of PKCβ, mRNA were examined in synchronized A10 cells (by serum deprivation for 48 hours as described in methods) that were re-initiated to proliferate by changing the medium to DMEM containing 10% FBS and incubated for 15-18 hours with either high (25 mM) or normal (5.5 mM) glucose. Cells incubated with 25 mM mannitol were used as osmotic controls. Total RNA was extracted and the mRNA transcript levels were detected using a full length PKCβ cDNA probe which would cross-hybridize with both PKCβI and PKCβII mRNAs. As shown in FIG. 18B, a 60-75% decrease in PKCβ(I+II) mRNA levels were observed in cells treated overnight with high glucose (25 mM). The mRNA levels in the control cells (5.5 mM glucose) and osmotic control cells (mannitol-treated) remained unchanged. This suggested that high glucose down-regulated PKCβ(I+II) gene expression. Since protein analysis following high glucose exposure indicated a decrease only in PKCβII levels, it was assumed that the northern blot analysis reflected a down-regulation of PKCβII mRNA while PKCβI mRNA remained unaltered. Since PKCβII is the product of PKCβ mRNA alternatively splicing, the possibility existed that transcriptional as well as post-transcriptional controls were altered by high glucose.

Example 3

Glucose Quenches PKCβ Promoter Activity

Our studies indicated that PKCβII protein levels "cycled" with synchronized A10 cells during a 24 hour period post re-initiation of the cell cycle with serum (Yamamoto et al., 1998, pp.349-358). We chose first to systematically study the effect of high glucose on PKCβ promoter activity in synchronized A10 cells. Glucose may be exerting its effect on PKCβ gene expression through a response element located in the common promoter upstream of the transcription start site as reported with L-PK and S14 gene expression in hepatocytes. Since PKCβ is a low copy gene, nuclear run-on assays were

not a practical approach to examine transcriptional regulation. Hence, the PKC α -CAT expression plasmids cloned by Dr. Ohno (Yokohama College of Medicine) (Niino, Y., S., S. Ohno, and K. Suzuki. 1992. Positive and negative regulation of the transcription of the human protein kinase C β gene. *J. Biol. Chem.* 267:6158-6163) were used to study transcriptional regulation of PKC β gene expression by glucose. PKC β promoter constructs with successive deletions of the 5' region cloned in front of a promoterless chloramphenicol acetyltransferase (CAT) as the reporter gene were transiently transfected into A10 cells (FIG. 19). pSV- β -Galactosidase was co-transfected with the CAT constructs to normalize the efficiency of transfection. Simultaneous positive and negative controls were carried out as described previously.

Transcriptional repression of PKC β promoter by transcriptional factors in response to high glucose was anticipated. However, in A10 cells incubated with high glucose (25 mM) for six hours, a moderate decrease or quenching in the PKC β promoter activity of constructs A (-4.1 kb to +179CAT), C (-674 to +179CAT) and D (-411 to +179CAT) was observed (FIG. 5). Construct E (-234 to +179CAT) showed no quenching with glucose indicating that a putative carbohydrate response element had been deleted. Construct E contains the basal response elements required for promoter activity and was not affected by glucose concentrations. Thus construct D contained the elements involved in maximum quenching of promoter activity, presumably within a 177 bp region which was deleted in construct E. In parallel experiments, a control with 100 nM TPA increased the promoter activity ten-fold in all constructs. This confirmed the response of PKC β basal promoter element with phorbol esters.

In the case of transcriptional repression, DNA binding is inhibited by repressors blocking the binding site for a factor or forming a non-DNA-binding protein-protein complex resulting in inhibition of transcription. The reduction in promoter activity observed here, however, did not account for the dramatic down-regulating effect of glucose on total PKC β mRNA. If glucose effects were mediated exclusively at the transcriptional level, total repression would have occurred. The reduction in promoter activity observed here is most likely explained as a quenching effect that involves interfering with transcriptional activation by a DNA-bound factor. An example of transcriptional quenching is illustrated by the yeast factor GAL80, which inhibits gene activation by the positively acting GAL4 protein in the regulation of galactose in yeast. Since the extent of quenching of the PKC β promoter by glucose varied depending on the time points examined, the results suggested that as cells progress through the cell cycle, high glucose regulation of PKC β gene expression may be cell cycle mediated. Simultaneous cell cycle studies and promoter activity of construct D (-411 to +179 CAT) in high glucose were determined. The results demonstrated an increase in the percentage of cells going into S phase in high glucose implying that quenching of PKC β transcription may be related to cell cycle progression (FIG. 21).

Example 4

PKC β mRNA is Destabilized by Glucose

Since the quenching of PKC β promoter activity by acute high glucose did not account for the 60-75% reduction in PKC β mRNA levels as observed by northern blot analysis (FIGS. 18A and 18B), the major effect of glucose may be exerted post-transcriptionally. However, high glucose also diminished PKC β II protein levels, suggesting that high glucose could affect the stability of the PKC β II transcript at the

post-transcriptional level. Northern blot analysis of PKC β (I+II) mRNA level (see methods) was performed on total RNA from synchronized A10 cells. Upon re-initiation of proliferation by DMEM+10% FBS, A10 cells were pre-treated with actinomycin D for 30 minutes followed by incubation with normal or high glucose. The mRNA levels were detected using the full-length PKC β II cDNA probe which recognizes both PKC β I and PKC β II mRNA. A10 cells exposed to high glucose showed a decrease in PKC β (I+II) mRNA levels within 2 hours of treatment (FIG. 22B) and levels further decreased to 60% of control by 6 hours. Thus, the results suggested that post-transcriptional destabilization of PKC β II mRNA by glucose was also occurring.

Example 5

In vitro Assay for mRNA Destabilization

To assess the involvement of a nuclease activity in the destabilization of PKC β mRNA by glucose, an in vitro RNA stability assay was performed (see, for example, Wager, R. E. and R. K. Assoian. 1990. A phorbol ester-regulated ribonuclease system controlling transforming growth factor B1 gene expression in hematopoietic cells. *Mol. Cell. Biol.* 10:5983-5990). A10 cells incubated with 5.5 mM or 25 mM glucose for 2 h, 6 h, 18 h and 24 h were used to prepare control post-nuclear extracts (control PNE) or glucose-treated post-nuclear extracts (glucose PNE). Total RNA isolated from untreated A10 cells was incubated with 2.7% (vol/vol) post-nuclear extracts or glucose-treated post-nuclear extracts from A10 cells in 50 μ l total volume of extract buffer. As a positive control, RNA incubated without post-nuclear extracts was used. A reaction with no RNA served as the negative control. Since involvement of divalent cations is a feature of some ribonucleases that regulate mammalian RNA turnover, EDTA was added to post-nuclear extracts to distinguish nuclease activity from cyclizing RNases and acid lysosomal RNase. Northern blot analysis was then performed to detect non-degraded PKC β (I+II) mRNA. Incubation of RNA with post-nuclear extracts from synchronized A10 cells treated with 25 mM glucose for 2 hours and 6 hours showed 45% and 65% decreases, respectively, in PKC β (I+II) RNA levels while incubation for 18 hours and 24 hours showed 8% and 12% decreases, respectively, in PKC β (I+II) RNA levels (FIG. 23). No degradation of RNA was observed in the presence of EDTA, either in the control or high glucose treated post-nuclear extracts (FIG. 24). These results indicate the activation of a divalent cation-dependent nuclease activity by high glucose. The destabilization of PKC β (I+II) mRNA by glucose accounted for a significant down-regulation of PKC β II expression although these observations suggested that destabilization of PKC β II mRNA occurred as an early event in the regulation of mRNA processing by high glucose.

Example 6

Specificity of mRNA Destabilization by Glucose

Since a full length PKC β cDNA probe was used for analysis of PKC β (I+II) RNA levels by the northern blot analysis, RT-PCR was performed on total RNA from A10 cells to determine the specificity of high glucose to down-regulate PKC β II mRNA rather than PKC β I mRNA. Previously, it had been demonstrated that mature PKC β II mRNA results from differential processing resulting in PKC β II exon inclusion into PKC β I mRNA transcript (Chalfant et al., 1995, pp.13326-13332; Ono et al., 1987, pp.1116-1120). Thus, the

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PKC β II transcript contains the PKC β I exon with the poly A tail and a common 3' untranslated region (FIG. 25). Inclusion of the alternatively spliced exon encodes a stop codon such that the PKC β I exon facilitates poly-adenylation but the exon is not translated, thereby generating PKC β II protein. Primers were designed for the upstream common kinase region (C4) (-sense primer) and the PKC β I exon (-antisense primer) for simultaneous amplification of both PKC β I and - β II messages by RT-PCR. In control A10 cells (5.5 mM glucose), both PKC β I and PKC β II PCR products were detected. Although, this assay is semi-quantitative, the ratio of PKC β II to PKC β I mRNA was about 2 to 1. In glucose-treated (25 mM) A10 cells, PKC β II PCR product decreased >90% while PKC β I mRNA levels did not show any significant change. β -actin mRNA levels remained constant in both control and glucose-treated A10 cells (FIG. 26). Thus, PKC β II mRNA was specifically destabilized in response to 25 mM glucose.

To extend the physiological relevance of the down-regulation of PKC β II by high glucose, we repeated the RT-PCR described above using primary cultured aortic smooth muscle cells from humans (AoSMC CC2571, CLONETICS Normal Human Cell System™). In high glucose medium (25 mM glucose), AoSMC showed a >95% decrease in PKC β II mRNA while PKC β I mRNA was not significantly affected. β -actin mRNA levels remained the same in both control (5.5 mM glucose) and glucose-treated (25 mM glucose) AoSMC (FIG. 26). Since cells from human primary culture responded in the same manner, the A10 cells proved to be a reliable model for studying the effects of high glucose on PKC β gene expression in vascular smooth muscle cells.

Example 7

PKC β II mRNA Is Generated Via Exon Inclusion of the Alternatively Spliced PKC β pre-mRNA in VSMC

To verify that the amplified product was PKC β II cDNA, the 404 bp band corresponding to the PKC β II cDNA was excised from the gel and cloned into the pCR-blunt vector.

Dideoxy sequencing and restriction digestion confirmed that the mature PKC β II mRNA was a result of a 216 bp exon inclusion via alternative splicing of the PKC β mRNA. The sequence for up to 350 bp is shown in FIG. 27 while the restriction sites map is depicted in FIG. 28.

Example 8

PKC β II mRNA Was Destabilized When AoSMC Cells Were Incubated with 25 mM Glucose

Insulin activates PKC and regulates the activity of glucose transporters (GLUT4) which are essential for regulated glucose uptake in quiescent smooth muscle cells. Since efficient transport into muscle cells is critical for glucose metabolism, we determined if insulin affected the stability of PKC β isozymes by high glucose. AoSMC were synchronized by serum starvation as described in methods and upon re-initiation of the cell cycle with SmGM containing 5% FBS, were treated with either 5.5mM glucose (normal glucose), 25 mM glucose (high glucose), 100 nM insulin or 25 mM glucose+ 100 nM insulin for 4 hours. Total RNA was isolated and products quantified as percent PKC β II present in the total PKC (β I+ β II) mRNA. Insulin did not destabilize PKC β I or PKC β II mRNA (FIG. 29) but in the presence of high glucose and insulin, PKC β II mRNA was diminished by 60%. Thus insulin-stimulated PKC activation and glucose uptake was

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not associated with high glucose-induced destabilization of PKC β II mRNA. Insulin enables glucose uptake by the cells and results in the metabolism of glucose at a higher rate. This suggested that the buildup of free glucose in the cytosol may be essential for the destabilization of PKC β II mRNA by high glucose.

Example 9

Metabolism of Glucose to Glucosamine is Not Required

Glucosamine, a metabolite of glucose and a product of the hexosamine pathway, has been implicated in the regulation of transforming growth factor- α gene expression in VSMC (Sayeski et al., 1996, pp.15237-15243; McClain et al., 1992, pp.8150-8154.) and other transcriptionally regulated genes. Although, glucosamine effects were primarily associated with transcriptional regulation, we investigated whether glucose also mediated its regulation of the PKC β II transcript stability via glucosamine. Synchronized A10 cells were treated with 5.5 mM glucose (normal glucose), 25 mM glucose or glucosamine (10 mM in the presence of 5.5 mM glucose) for 4 hours prior to RNA isolation and analysis (FIG. 30). Glucosamine did not affect either PKC β I or - β II mRNA. Thus, further metabolism of glucose by the hexosamine biosynthetic pathway was not involved in the destabilization of PKC β II transcript by glucose.

Example 10

Glucose Analogs Mimic Its Effect

To further explore whether glucose is required to be metabolized in order to down-regulate PKC β II mRNA, we studied the effects of glucose analogs on PKC β I and - β II mRNA levels. A10 cells were synchronized by serum starvation as described supra and upon re-initiation of the cell cycle with DMEM containing 10% FBS, were treated with 5.5 mM glucose (normal glucose), 25 mM glucose, 25 mM 2-deoxyglucose, 25 mM 3-O-methylglucose or 5.5 mM glucose+19.5 mM mannitol (osmotic control) for 4 hours. 2-deoxyglucose (2-DOG), is transported into the cell and phosphorylated to 2-deoxyglucose-phosphate but cannot be metabolized further (See, for example, Patel, N. A., C. E. Chalfant, M. Yamamoto, J. E. Watson, D. C. Eichler, and D. R. Cooper. 1999. Acute hyperglycemia regulates transcription and post-transcriptional stability of PKC β II mRNA in vascular smooth muscle cells. FASEB J. 13). As seen in FIG. 31, 2-DOG (25 mM) destabilized PKC β II mRNA.

This suggested that the metabolism of glucose beyond the glucokinase step is not required for the down-regulation of PKC β II mRNA. Next, we studied 3-O-methylglucose (3-OMG), an analog which can be transported into cell but not metabolized further. 3-O-Methylglucose (25 mM) also destabilized PKC β II mRNA and, to a lesser extent, PKC β I mRNA. These results suggested that further metabolism by the hexose mono-phosphate shunt was not involved. Furthermore, glucose need not be phosphorylated to bring about its effect, thereby eliminating the regulation by hexokinase in PKC β II gene expression. The levels of PKC β I and - β II mRNA remained unchanged with mannitol treatment, the osmotic control.

Example 11

New Protein Synthesis Is Not Required for Destabilization of PKC β II mRNA by Glucose

Even though glucose effects on PKC β II destabilization were detected in 2 hours, it was still possible that a new

protein was expressed in response to glucose. To investigate this, synchronized A10 cells (achieved by serum starvation as described in methods) upon re-initiation of the cell cycle with DMEM containing 10% FBS, were treated with 5.5 mM glucose (normal glucose), 25 mM glucose, or pre-incubated with 20 mM cycloheximide for 30 min followed by the addition of 25 mM glucose. High glucose destabilized PKCβII mRNA in the presence of cycloheximide indicating that glucose did not signal the synthesis of a new protein but likely activated a protein already present in the cytosol (Table 5) (FIG. 32).

An inhibitor of translation such as cycloheximide induces a rapid stabilization of the message (Ross, 1995, pp.423-450). Since cycloheximide did not alter the glucose-induced destabilization by glucose, it suggests that this process is independent of translation.

Example 12

Kinase Inhibitors Implicate a PKC-dependent Pathway

Inhibitors of various kinases were used to elucidate the cascade of events involved in the glucose-mediated destabilization of PKCβII mRNA. The choice of concentrations used and the incubation times were based on previous observations. Phosphatidylinositol 3-kinase (PI 3-kinase) is a mediator of transmitting signals from upstream activators to downstream targets like PKC family (Palmer et al., 1995a, pp.22412-22416), p70-S6 kinase (Chung et al., 1994, pp.71-75), glucose transporter GLUT4 (Hara et al., 1994, pp.7415-7519), serine/threonine kinase Akt/Rac (Franke et al., 1998, Ridley et al., 1992, pp.401-410). To determine the possible involvement of PI 3-kinase, synchronized A10 cells were pre-incubated for 30 minutes with LY 294002 (20 μM), a specific inhibitor of PI 3-kinase prior to high glucose indicating the possible involvement of a PI 3-kinase-dependent downstream kinase pathway (Table 5).

TABLE 5

Effect of Inhibitors of several signaling pathways.			
INHIBITOR	TARGET	N VALUE	PERCENT OF PKCβII mRNA POST-GLUCOSE TREATMENT
None (5.5 mM glucose control)	NA	6	100%
None + Glucose (25 mM)	NA	6	5%
Glucose + Cycloheximide (20 mM)	Protein synthesis	5	10%
Glucose + LY 294002 (20 μM)	PI 3-kinase	6	90%
Glucose + CG 41251 (5 μM)	PKC isozymes	5	100%
Glucose + PD 98059 (20 μM)	MEK	5	5%
Glucose + AG-490 (5 μM)	JAK	5	5%
Glucose + Rapamycin (1 μM)	p70/85 S6 kinase	5	5%
Glucose + Herbimycin A (1 μM)	Tyrosine kinase	5	90%

To further target downstream pathways involved in PKCβII destabilization by high glucose, synchronize A10 cells were pre-incubated with CG 41251 (5 μM), a PKC inhibitor, PD 98059 (20 μM), a MAP kinase kinase (MEK) inhibitor, AG-490 (5 μM), a Janus kinase (JAK) inhibitor, Rapamycin (1 μM), inhibitor of p70/85 S6 kinase signaling pathway or herbimycin A (1 μM), a tyrosine kinase inhibitor. Following re-initiation of the cell cycle with DMEM contain-

ing 10% FBS, inhibitors were added to synchronized A10 cells 30 minutes prior to the addition of 5.5 mM glucose (control) or 25 mM glucose (glucose-treated) or 25 mM glucose in addition to the inhibitors. The PKC inhibitor CGP 41251, blocked the action of glucose on PKCβII mRNA destabilization. Hence, PKC could phosphorylate the components involved directly or the process involved protein kinases that require phosphorylation by PKC. Results also indicated the potential involvement of a tyrosine kinase-dependent pathway since herbimycin also prevented PKCβII mRNA destabilization by high glucose. The process was unlikely to be dependent of the MAPK cascade, JAK kinase or p70/85 S6 kinase pathways (FIG. 11) since their putative inhibitors did not block the effects of high glucose.

Example 13

Involvement of a Reversible Phosphorylation Process

Extracellular effectors regulate a large number of biological processes by protein phosphorylation-dephosphorylation as an important component of signal transduction cascades. Regulation of cellular functions like gene expression, cell proliferation and differentiation is controlled by a balance between protein kinases (that phosphorylate the substrate) and protein phosphatases (that dephosphorylate) (Jia, 1997, pp.17-26; Barford, 1996, pp.407-412). To further test if phosphatases played a role in this process, okadaic acid (20 nM), a potent inhibitor of protein phosphatase PP-2A and PP-1, was added to synchronized A10 cells 30 minutes prior to the addition of either 5.5 mM or 25 mM glucose. Okadaic acid interfered with ability of glucose to destabilize PKCβII mRNA indicating that the process may be regulated by phosphorylation/dephosphorylation (FIG. 33).

Example 14

Expression of a pβG-PKCβII Stability Reporter System

To demonstrate whether PKCβII specific exon encoded crucial elements that could confer instability in response to high glucose to other genes, a heterologous chimeric minigene was established. The 404 bp PKCβII cDNA amplified by RT-PCR, using the sense primer to the upstream PKCβ common C4 domain and the antisense primer to the βIV5 domain, resulted from the 216 bp βIV5 exon inclusion. This PKCβII cDNA was cloned into the pβG vector (kindly provided by N. P. Curthoys, Colorado State University) that encodes a chimeric β-globin/growth hormone mRNA (see FIG. 34). The β-globin coding sequence within the parent pβG vector was followed by a multi-cloning site containing four unique restriction sites, into which the PKCβII cDNA was subcloned in frame at the SpeI and XbaI sites thereby maintaining the 3'UTR and poly(A) tail of the growth hormone.

The resulting chimeric minigene pβG-PKCβII was transiently transfected into human aorta smooth muscle cells. The parent vector, pβG, produced a β-globin mRNA whose levels remained unaltered by glucose concentrations as determined by RT-PCR analysis (FIGS. 35A and 35B). Following acute exposure of 3 hours with high glucose (25 mM), an 80% decrease in β-globin mRNA in the AoSMC cells transfected with pβG-PKCβII minigene as compared to incubation with normal (5.5 mM) glucose was observed. As demonstrated by the RT-PCR analysis, FIGS. 35A and 35B, the levels of β-actin mRNA remained constant in both control and glucose-

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treated AoSMC and A10 cells transfected with p β G-PKC β II stability reporter system. Thus, the introduction of the PKC β II cDNA in a reporter system was sufficient for its down-regulation by exogenous high glucose.

Example 15

Acute Exposure to High Glucose Destabilizes p β G-PKC β II Minigene

To further characterize the instability element, half-life determinations were carried out in the presence of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) to inhibit transcription via RNA polymerase II. A10 cells stably transfected with the p β G-PKC β II stability reporter system and the parent vector, p β G were incubated with DRB and incubated with normal or high (5.5 mM or 25 mM) glucose over a period of 6 hours. Northern blot analysis indicated that the β -globin mRNA of p β G-PKC β II transfected A10 was destabilized within the first 6 hours by high glucose exposure. No changes in the β -actin mRNA levels were observed between normal and high glucose as seen in FIGS. 36A and 36B. In the presence of 25 mM glucose it appears that the mRNA destabilization involves shortening of the mRNA presumably via deadenylation.

Example 16

Cytoplasmic Extracts from Glucose-treated A10 Cells Contain a Protein that Binds to the PKC β II mRNA Causing Its Destabilization

Our earlier work with RNA stability assays, had indicated that a nuclease activity involved in PKC β II mRNA destabilization, was present in the cytosolic extract of glucose-treated A10 cells. To ascertain if cytoplasmic extracts from glucose-treated A10 cells contain trans-acting factors which could bind to the cis-elements of PKC β II mRNA, an in vitro transcription vector was produced by cloning PKC β II cDNA into the pCR-Blunt vector (INVITROGEN), upstream of a T7 promoter (FIG. 38). A full length transcript of PKC β II cDNA was in vitro transcribed as described in methods and used in the band shift assays. RNA-protein complex was observed when the labeled RNA probes were incubated with cytoplasmic extracts of glucose-treated A10 cells, binding reaction carried out as described in methods. No complex formation was observed with the control cytoplasmic extracts as shown in (FIG. 39).

To determine if this RNA-protein complex showed specific binding, excess cold competitor RNA probes were added. Binding was eliminated by the specific RNA probes while the non-specific RNA probe did not compete for protein binding.

Example 17

Glucose-regulated Instability Element Is Present within the PKC β II Exon

To further target the site of sequences involved in the glucose-induced destabilization of PKC β II mRNA, restriction digestion of the PKC β II transcript cloned into pCR-blunt vector was performed to generate deletion constructs as described in FIG. 38. RNA gel shift analyses were carried out with probes B, C, D or E incubated with glucose-treated or control cytoplasmic extracts from A10 cells.

RNA-protein binding was observed in glucose-treated cytoplasmic extracts from A10 cells using probe B (FIG. 39)

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which was linearized at 175 bp with BglII within PKC β II exon such that the PKC β I-specific exon was eliminated. This RNA-protein binding was competed out with excess of the cold RNA probe B.

No complex formation was observed with RNA probes C (which was linearized at 137 bp with HpaI which cut within the PKC β II-specific exon) incubated with cytoplasmic extracts from A10 cells (FIG. 40). Probes D (linearized at 102 bp with SspI which cut within the PKC β II-specific exon) and E (linearized at 45 bp with FokI which cut within the C4 exon) did not show complex formation (data not shown).

This indicated that the putative glucose-regulated instability element was deleted in probes C, D and E, and hence the sequence with protein-mRNA binding activity may lie between the BglII site (at position 175) and HpaI site (at position 137) within the PKC β -specific coding region.

Example 18

UV Cross-linking Detected Associate of Proteins

To provide an insight to the proteins that bind to the PKC β II mRNA in response to high glucose exposure, ultra-violet cross-linking experiments were performed using probes A and B that demonstrated specific binding. The binding reaction was set up as described for the RNA shift assays and subjected to UV light to generate covalent bonds between RNA and the associated proteins. The samples were digested with RNase A and separated by SDS-polyacrylamide gel electrophoresis. A small (~10-14 kD) protein-RNA binding complex was observed (FIG. 41) in glucose-treated cytoplasmic extracts from A10 cells.

In this study, several molecular mechanisms by which glucose may regulate PKC β I and - β II gene expression in VSMC were investigated. The elucidation of this regulatory phenomenon is essential since the depletion of PKC β II levels in response to acute hyperglycemia can accelerate cellular proliferation, which may be directly involved in the development of occlusive atherosclerotic lesions. This may occur in addition to the well studied phenomenon of PKC β II activation associated with chronic exposure (10 days to 4 months) of hyperglycemia.

Glucose regulation of PKC β gene expression in VSMC may occur at transcriptional, post-transcriptional, translational, or post-translational levels. The potential sites of regulation included repression of transcription, improper nuclear export of PKC β II mRNA, post-transcriptional mRNA destabilization, decreased translation or multiple effects culminating in decreased PKC β II protein levels. PKC β II protein levels declined sharply by 6 hours of high glucose exposure while PKC β I protein levels did not change significantly between high and normal glucose conditions. Since protein levels often reflect the amount of mRNA transcribed, steady state levels of PKC β mRNA were determined. A decrease in PKC β (I+II) mRNA levels observed in A10 cells exposed to high glucose was likely due to the down-regulation of PKC β gene expression.

Since PKC β I and PKC β II share a common DNA promoter, transcriptional processing of PKC β in VSMC was examined to determine if high glucose down-regulated PKC β gene expression by repressing promoter activity. Transactivating factors, which interact on the cis-elements located in the common promoter region of PKC β gene, could positively or negatively regulate the PKC β pre-mRNA splicing in response to high glucose. It was possible that glucose affected trans-activators to preferentially process PKC β pre-mRNA splicing to PKC β I mature mRNA. Transcriptional processing of

PKC β in VSMC was first examined to determine if high glucose down-regulated PKC β II mRNA levels by repressing promoter activity. Hyperglycemic conditions, however, only quenched activities of PKC β promoter constructs rather than repress promoter activity. An example is quenching exerted by the yeast factor GAL80, which inhibits gene activation by the positively acting GAL4 protein in the galactose regulation of yeast.

This transcriptional effect accounted for only a fraction of the effect of glucose on PKC β I and PKC β II protein levels. Although promoter activities of construct A (−4.1 kb to +179 CAT) and construct C (−674 to +179CAT) were decreased by high glucose, promoter activity of construct D (−411 to +179CAT) showed maximum quenching in high glucose. Construct E showed no quenching by high glucose indicating that a potential carbohydrate response element was deleted. Quenching involves the inhibitor interfering with the ability of the activator's activation domain to stimulate transcription. Quenching of transcriptional activity accounted for only a fraction of the effect of high glucose on PKC β I and PKC β II protein levels.

Various positive and negative regulatory cis-elements have been identified on the PKC β gene. TATA box (−530) and CAAT box (−395) are found in the reverse order upstream from the transcription start site, with TATA box position towards 5' end. The distant positions of TATA box and CAAT box from the transcription start site indicated involvement of other regulatory elements for promoter activity. Other cis-acting regulatory elements including octamer binding motif, AP $_1$, AP $_2$, SP1 binding sites and E boxes were also identified. The 5' region is also rich in CG bases. Construct E, containing −111 to +43 sequence, was sufficient for basal promoter activity indicating that neither the TATA box nor CAAT box is essential for promoter activity. One possibility of quenching of promoter activity may be through the AP $_1$ binding site, which has been shown to act as a repressor (Benbow et al., 1997, pp.519-526), present at −442 bases upstream of transcription start site. However, construct D (−411 to +179) in which the AP $_1$ binding site has been deleted, showed maximum quenching in the presence of high glucose indicating that some other mechanism is probably involved in the quenching of PKC β promoter by glucose.

It is possible that glucose may be exerting its quenching effect through a response element located upstream of the transcription start site. First, a carbohydrate response element (ChoRE) that mediates its action via glucose is known. Second, the consensus sequence, 5' CACGTG 3', has been described in the promoter region of a number of glucose responsive genes including L-PK and S-14 in hepatocytes. Third, CACGTG motif is also the core-binding site for the c-Myc family of transcription factors. Fourth, multimers of the ChoREs of either L-PK or S-14 genes can function independently to support the glucose response. Fifth, multiple copies of CACGTG motif are found in the region upstream of the transcription initiation site of the PKC β promoter region at positions −318 and −226 with one or two mismatches.

It is therefore likely that glucose plays a role as a quencher of positively acting regulatory factors by binding to or otherwise activating a ChoRE. Construct D contains a ChoRE motif in proximity to the CAAT box and AP $_2$ binding site (TATA box and AP $_1$ binding site have been deleted). The ability of a bound trans-acting factor to stimulate transcription via its activation domain could be inhibited by quenching of the activation domain by glucose which can either bind to trans-acting factors without binding to DNA or bind to a site adjacent to the trans-acting factor (Latchman., 1995, pp.239-

278). This may be a possible explanation for the quenching of PKC β promoter observed in the presence of high glucose.

Since the extent of quenching of the PKC β promoter activity by high glucose varied at different times, simultaneous cell cycle progression studies were performed. Results indicated that PKC β II levels "cycled" with synchronized A10 cells during the 24 hour period of re-initiation of cell proliferation by serum addition. In the presence of high glucose, PKC β promoter activity was down regulated at 14 hours (S phase) compared to the up-regulation of promoter activity following G1 phase in normal glucose. Simultaneous [3 H]-thymidine uptake studies indicated an increase in the percentage of cells going through S phase following high glucose exposure. A possible explanation for this effect is that PKC β II may function as a "gate-keeper" for DNA synthesis, with increased PKC β II levels attenuating DNA synthesis in VSMC. In the presence of high glucose, the down-regulation of PKC β II allows for more cells to enter S phase. This increased rate of DNA synthesis accounts for the acceleration of proliferation of VSMC. Thus, in hyperglycemic conditions, the down regulation of PKC β II coincides with increased proliferation of VSMC as shown here.

We further investigated whether high glucose affected the post-transcriptional processing of PKC β transcripts since quenching of PKC β promoter activity, however, did not fully explain the reduction in PKC β mRNA levels. A10 cells exposed to high glucose showed a decrease in PKC β (I+II) mRNA stability within 2 hours in the presence of actinomycin D (an inhibitor of RNA polymerases), indicating an increase in message degradation was also occurring.

Further, the in vitro stability assay demonstrated the induction or activation by glucose of a nuclease activity involved in the destabilization of PKC β II mRNA. This divalent cation-dependent nuclease activity was distinct from cyclizing RNases and acid lysosomal RNase since its activity was inhibited by EDTA. Although it was not possible to exclude the presence of potential lysosomal RNase in the reaction completely, the functional role for this enzyme was eliminated by the observation that the degradation of the transcript required divalent cations. This requirement of divalent cations is the hallmark of ribonucleases that regulate mammalian mRNA turnover.

To confirm that glucose specifically destabilized the PKC β II mRNA isoform, RT-PCR was performed with primers that amplified both PKC β I and PKC β II mRNA simultaneously. High glucose destabilized PKC β II mRNA while PKC β I mRNA was not affected significantly. Since the mature PKC β II mRNA is generated by exon inclusion and both PKC β I and PKC β II transcripts contain the PKC β I exon and share a common 3'UTR, the destabilizing elements appear to be introduced within the PKC β II exon or be introduced around the 5' splice site between the exons. Although stability determinants have been described within the mRNA coding regions of genes like c-myc is the first report of a instability element present within an exon that is regulated by glucose. This is a novel observation since the destabilization elements regulated by glucose occur within the PKC β II exon rather than in the 3'UTR of PKC β II mRNA.

The studies were extended to human aortic smooth muscle cells (AoSMC) and it was demonstrated that glucose induced PKC β II destabilization in primary human cell cultures. The combined effects of high glucose on transcriptional quenching of the PKC β promoter and specific destabilization of the PKC β II transcript account for the 60-75% reduction of PKC β mRNA levels observed under steady state conditions in rat and human proliferating aortic smooth muscle cells. The

human primary cultures further underscore the physiological relevance of acute high glucose effects on vascular smooth muscle cell proliferation.

In other studies, we used CGP53353 a PKC β II-specific inhibitor, to demonstrate that CGP53353 inhibits glucose-stimulated rat VSMC proliferation (Yamamoto et al., 1998b, pp.205-216). These studies confirmed that PKC β II attenuates cell proliferation and predicts that cells expressing PKC β II are more differentiated and less likely to undergo apoptosis. This could provide an explanation towards the observation that vascular smooth muscle cells overexpressing PKC β II are found in the microvasculature of diabetic rats subjected to untreated hyperglycemia for 2 to 4 months.

Here, we studied the effects of high glucose on PKC β I and PKC β II mRNA on synchronized VSMC during the first cell cycle post-synchronization and demonstrated that glucose-induced destabilization of PKC β II mRNA occurs during the course of the cell cycle. Also, instead of PKC β II, the PKC β 2 nomenclature has been used on occasions which would imply that PKC β I was the isoform increased by hyperglycemia.

In none of these studies reflected in the prior art were PKC β mRNA levels examined within acute glucose exposure. Our present studies examined the regulation exerted by acute glucose exposure on the PKC β gene and have shown that glucose acted both transcriptionally and post-transcriptionally, with the latter effect accounting for the majority of the down-regulation of PKC β II mRNA.

It was crucial to investigate the mode of glucose action and elucidate potential pathways involved in glucose-induced destabilization of PKC β II mRNA in VSMC. Insulin regulates the alternative splicing of PKC β II mRNA in rat skeletal muscle cells and hence it was essential to investigate the involvement of insulin signaling in the post-transcriptional regulation of PKC β II mRNA by glucose in VSMC. In human aorta smooth muscle cells, glucose destabilizes PKC β II mRNA and not PKC β I mRNA, while insulin did not destabilize PKC β I or - β II mRNA. This implies that the destabilization of PKC β II mRNA by glucose is distinct from the regulation of insulin-induced alternative splicing of PKC β II mRNA. Since insulin enables the uptake of glucose for efficient metabolism in the cell, the implication is that buildup of free glucose in the cytosol may be essential for glucose-induced destabilization of PKC β I mRNA.

Studies with the PKC inhibitor showed that a PKC-dependent pathway may be associated with the destabilization of PKC β II mRNA by high glucose. Insulin is known to activate PKC in VSMC. The finding that insulin did not destabilize PKC β II mRNA suggests that a different activation mechanism may be involved. The PKC inhibitor used here, CGP41251, is not specific for a single PKC isoform. This could imply the presence of a cross-talk between PKC isoforms similar to that between PKC α and PKC δ . They demonstrated that activated PKC α up-regulated the steady-state levels of PKC δ mRNA, which caused an increase in the PKC δ protein level. Protein kinase C is a mediator of signal transduction pathways that alter gene expression. Activation through a PKC-dependent pathway, of transcription factor like AP-1 can be altered by tumor-promoting phorbol esters like 12-O-tetradecanoylphorbol-13-acetate (TPA). For instance, PKC activation by TPA destabilizes PKC δ mRNA via a PKC-dependent pathway in A20 cells.

Using glucose analogs, we showed that glucose need not be metabolized further by glucokinase nor activate the hexose mono-phosphate shunt or the hexosamine biosynthetic pathway. Glucose could destabilize PKC β II mRNA in VSMC without any modification even though it is predominantly present as glucose-6-phosphate in the cell.

Cycloheximide did not block destabilization of PKC β II mRNA by high glucose indicating that de novo protein synthesis is not required. Further, it is known that mRNA half-life is linked to translation. Inhibition of translation by cycloheximide results in stabilization of mRNA. Since cycloheximide does not interfere with glucose-induced destabilization of PKC β II mRNA, it suggests that the regulation is independent from translation.

Okadaic acid inhibited the destabilization thereby implying that serine/threonine phosphatases like PP-2A and PP-1 are involved in regulating the process. An inhibitor of PI3-kinase blocked the destabilization of PKC β II mRNA by high glucose implying the involvement of a downstream target of PI3-kinase such as PKC. Further, the lack of effect of inhibitors for the MAPK cascade, JAK kinase or p70/85 S6 kinase suggest that these pathways are not involved in high glucose-induced PKC β II mRNA destabilization in VSMC.

Since acute hyperglycemia destabilized PKC β II mRNA and PKC β I mRNA levels remaining unaltered, it was possible that the putative glucose-responsive instability element resided within the exon encoding PKC β II mRNA. The PKC β II cDNA was cloned into the pCR-blunt vector and its sequence was determined. Restriction digestion and sequencing confirmed that the mature PKC β II mRNA was generated by inclusion of the 216 bp exon.

A heterologous stability reporter system was established in vascular smooth muscle cells to demonstrate that PKC β II exon contains a instability element regulated by high glucose. The chimeric p β G-PKC β II minigene comprises of the parent p β G vector into which the PKC β II cDNA was subcloned in frame with the β -globin exon into the multi-cloning site. p β G vector is a chimera a β -globin/growth hormone such that the multi-cloning site is followed by the 3'UTR and polyadenylation site of the bovine growth hormone. This parent vector was an appropriate choice since the 3'UTR and poly (A) tail, which are common to both PKC β I and - β II mRNA, are eliminated as the determinants of stability. β -globin mRNA was destabilized by acute hyperglycemia in the p β G-PKC β II stably transfected human aorta smooth muscle cells while the β -globin mRNA in the p β G stable transfectants were not affected by extra-cellular high glucose concentrations. This has two implications: (i) the PKC β II cDNA contains a glucose-responsive instability element within its exon; and (ii) the 3'UTR and poly(A) tail-the common determinants of mRNA stability, are not involved in glucose-mediated destabilization of PKC β II mRNA in VSMC. This chimeric gene contains the PKC β II cDNA (404 bp exon) subcloned in frame with the β -globin. The introns and other 3' flanking sequences of PKC β II mRNA are not present. These sequences could play an important role in secondary structure and could contain stability determinants that act mutually to influence the mRNA stability. These sequences are omitted in the chimeric minigene (p β G-PKC β II), and hence a lesser extent of destabilization is observed. It may be possible that the PKC β II exon that is not translated (due to the introduction of a STOP codon) in the mature PKC β II mRNA could act as a longer 3'UTR thereby affecting its stability.

Half-life analysis of the β -globin mRNA in the p β G-PKC β II stable transfectants, using DRB, indicated that glucose destabilized β -globin mRNA within 2 hours of exposure. DRB was preferred as the inhibitor of transcription by polymerase II since actinomycin D was reported to inhibit translation and inhibit mRNA degradation in some instances. This rapid destabilizing effect of acute hyperglycemic further strengthens the hypothesis that the glucose effect is conferred by the instability element present within the PKC β II exon.

Although chimeric mRNAs provide valuable information regarding the sequences affecting the stability of the mRNA, truncated chimeric mRNAs may give misleading information regarding mRNA stability since factors such as translation rate, secondary structure, and intracellular localization may influence their stability. Further, more than one stability determinant may be present in the mRNA which could be regulate its turnover. Hence, to identify the instability elements in PKC β II mRNA regulated by high glucose in VSMC, in vitro binding interactions between RNA and proteins present in the cytosol were observed by RNA mobility shift assays. Cytoplasmic extracts from glucose-treated A10 cells were incubated with varying lengths of in vitro transcribed PKC β II labeled probes. A region between 175 bp and 137 bp in PKC β II cDNA was identified to contain an element that bound to a protein present in the cytoplasm of glucose-treated A10 cells. This region containing the 38 nucleotides corresponds to positions 2127 bp and 2165 bp of the PKC β II cDNA and occurs within the PKC β II coding region. Interestingly, its position is immediately before the STOP codon of the PKC β II exon. This binding site was in close proximity to a stem-loop structure in the PKC β II mRNA. Premature stop codons affect mRNA stability as observed in phytohemagglutinin mRNA whose stability is decreased by the presence of premature nonsense codons.

Elimination of RNA-binding by competitor probes demonstrated the specificity of the interaction by the instability element without altering the native RNA. Using UV cross-linking, a 10-14 kD protein was identified as binding to probes A and B. The function of this protein could be similar to that of an endoribonuclease as in the case of transferrin receptor or apolipoprotein or an accessory protein aiding in the endoribonucleic cleavage. For example, recently Canete-Soler et al. (Canete-Soler, R., M. L. Schwartz, Y. Hua, and W. W. Schlaepfer. 1998. Characterization of ribonucleoprotein complexes and their binding sites on the neurofilament light subunit mRNA. *J. Biol. Chem.* 273:12655-12661) described a stability region present within a 68-nt sequence, localized between the 3'UTR and the 3'-coding region of the neurofilament NF-L, that serves as the binding site for a unique ribonucleoprotein complex.

Glucose plays an important regulatory role in the expression of PKC β II in VSMC. Acute high glucose could exert regulatory effects through a putative carbohydrate response element located upstream of the PKC β transcription start site and through glucose-induced post-transcriptional destabilization of PKC β I message via a nuclease activity present in the cytosol. In this case, the rate of mRNA degradation plays an important role in establishing levels of gene expression. Although hormones, growth factors and ions are known to induce changes in mRNA stability, here we determined that PKC β II mRNA levels are regulated primarily by mRNA destabilization in response to glucose, a cellular nutrient. This multi-level regulation of gene expression by glucose suggests that PKC β II may play a pivotal role in vascular smooth muscle cell function.

The physiological implications in diabetic patients that suffer from frequent episodes of acute hyperglycemia are profound. Acute hyperglycemia down-regulates PKC β II levels which results in accelerated proliferation of vascular smooth muscle cells and ultimately leading to increased risk of developing atherosclerosis.

In conclusion, this investigation provides molecular insight into the mechanisms involved in the destabilization of PKC β II by high glucose in vascular smooth muscle cells. To our knowledge, this is the first report of identification of instability element present within the PKC β II coding region

that is regulated by acute high glucose exposure. The ability of glucose, an important cellular nutrient, to influence mRNA turn-over within acute exposure in vascular SMC further emphasizes its role in regulation of gene expression.

The BII-exon should destabilize a cDNA insert in the presence of high extracellular glucose. For our initial studies, the PBglobin (pBG) vector (obtained from Norman P. Curthoys, Colorado State University) was used (2). The chimeric pBG-BII plasmid was constructed by inserting the PKC β II exon and flanking regions as shown below into the vector at a multicloning site. The vector was created by inserting the PvuII-Bgl II fragment of pSVB10, the portions of the first three exons and the two introns of the rabbit B-globin gene, into the HindIII site of the pRc/RSV vector. The B-globin genomic sequence extends from 9 bp upstream of the transcription initiation site to the translation stop codon. pBG contains a strong viral promoter derived from the long terminal repeat of the Rouse sarcoma virus followed by genomic DNA containing the transcriptional start site, the 3' nontranslated region, the full coding sequence and two introns of the rabbit B-globin gene, a MCS with four restriction sites, and the 3' nontranslated region and polyadenylation site of the bovine growth hormone gene. Other chimeric constructs will also be tested such as CAT and luciferase to validate the effect of the BII exon (3). The minimal region of the sequence will be used in tetracycline repressor construct systems (4). The chimeric reporter constructs are tested in a number of cell types. We have found that glucose destabilizes PKC β II mRNA in L6 rat skeletal muscle cells, rat aortic cells, PC12 cells, and other tumorigenic and normal cell lines will also be tested for the ability of glucose to destabilize to chimeric construct.

To further define the minimal boundaries of the cis-elements, a portion of the 3' region of the PKC β II mRNA as diagrammed above was obtained as a 404 bp insert containing the entire 216 bp BII exon and 3' and 5' sequences from the flanking common and BI exon (4). This insert will be restricted by deletion mutagenesis to further limit the amount of mRNA necessary for destabilizing genes of interest.

The cis-acting elements destabilizing mRNA in response to high extracellular glucose were identified by several criteria in the exon encoding the C-terminal 52 amino acids for PKC β II and are shown in FIG. 25. The PKC β II-specific exon is inserted into mature mRNA via alternative splicing of pre-mRNA. The elements inserted may form stem-loop structures providing the secondary structure recognized by destabilizing endonuclease-protein complexes that break A-T bonds (1).

Analysis of PKC β II exon sequences reveals multiple potential cis-acting elements that may be involved in the destabilization of the PKC β II sequence. These elements may form stem-loop structures that are recognized by putative carbohydrate response-acting factors to target the sequence for decay by cytosolic endonucleases (1).

FIG. 28 sets forth an exemplary sequence.

Throughout this specification a variety of references have been cited, each of which is herein incorporated in its respective entirety.

The many features and advantages of the invention are apparent from the detailed specification and thus, it is intended by the appended claims to cover all such features and advantages of the invention which fall within the true spirit and scope of the invention. Further, since numerous modifications and variations will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and operation illustrated and described, and accordingly, all suitable modifications and equivalents

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may be restored to, falling within the scope of the invention. While the foregoing invention has been described in detail by way of illustration and example of preferred embodiments, numerous modifications, substitutions, and alterations are possible without departing from the scope of the invention defined in the following claims.

Example 19

Reporter Vector Design

The glucose-regulated mRNA instability element and its flanking sequences are cloned downstream of a green fluorescent protein (GFP) coding sequence. This vector can be stably transfected into cells to perform live cell assays. In normal glucose concentrations, GFP is expressed at high levels. When glucose concentration increases (such as in diabetes mellitus), destabilization of the mRNA results in low levels of GFP expression. The levels of GFP can be read directly on a plate reader at 510 nm wavelength. This unique system allows the measurement of glucose, a natural metabolite whose varying levels in the body is the cause of the disease, diabetes mellitus. This tool can be used separately or can be stably transfected into an insulin-sensitive cell line such as L6 skeletal muscle or 3T3 adipocytes, and can be invaluable to pharmaceutical companies developing glucose lowering drugs for diabetes.

The PKC β II instability element and its flanking sequences are cloned into the NT-GFP-TOPO vector (INVITROGEN LIFE TECHNOLOGIES) such that the GFP fusion protein is expressed directly into mammalian cells. The instability element is to be cloned downstream of the GFP coding sequences in the multiple cloning site, which is followed by the bovine growth hormone (BGH) polyA tail, thus rendering the fusion protein stable under normal conditions.

Previous data has indicated that 404 base pair (bp) of PKC β II mRNA were required for complete destabilization by high glucose (25 mM) concentrations. In vitro studies indicated the presence of a 38 bp glucose-responsive instability element within the 404 bp.

Protocol for synthesis of the GFP-PKC β II minigene: The rat PKC β II mRNA 404 bp, including flanking sequences (with the 38 nucleotide (nt) instability element underlined) sequence is:

(SEQ ID NO: 19)
 UUCCGGUAUAUCGACUGGGAGAAACUCGAACGCAAGGAGAUUCAGCCACC
 UUAUAAACCAAAGCUUGGGCGAAACGCUGAAACUUCGACCGGUUUU
 UCACCCGCCAUCCACCAGUCCUACACCUCGACCAGGAAGUCAUCAGGA

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-continued

AUAUUGACCAAUCAGAAUUCGAAGGAUUUCCUUGUUAACUCUGAAUUU
UUAAAACCCGAAGUCAAGAGCUAAGUAGAUUCUGAGACCUCGUCUUA
 5 UUUUCUGUCAUUCAGCUCAACAGCUAUCAGAGAGACAAGCGAGACACCU
 CCAACUUCGACAAAAGUUCACCAGGCAGCCUGUGGAACUGACUCCACUG
 ACAAACUCUGUCGACUAGAAUGCCUGAAUUCUGCAGAUAU
 10 CCAUCACACUGCG

This product is amplified using PCR sense primer C4 with KpnI site (underlined): 5' GGTACCGTTGTGGGCCTGAAGGGGAACG 3' (SEQ ID NO:17) and antisense primer β 1V5 with XbaI site (underlined): 5' TGCCTGGTGAACCTTTGTCTCTAGA 3' (SEQ ID NO:18). After size fractionation, it is extracted from the gel (QIAGEN QIAquick gel extraction kit), digested with KpnI and XbaI, and purified. The PKC β II cDNA will be ligated into the KpnI and XbaI sites of the multicloning region of pcDNA3.1/NT-GFP-TOPO vector (INVITROGEN LIFE SCIENCES), as shown in FIG. 42. The minigene GFP-PKC β II will be verified by restriction mapping and sequencing.

Upon translation, the GFP-PKC β II fusion protein will fluoresce and can be detected at 510 nm. In the presence of high glucose, the fusion protein is destabilized and, hence, fluorescence will not be detected. To obtain a higher degree of destabilization by glucose, multiple "cassettes" of PKC β II instability sequence can be inserted sequentially within the multiple cloning region of the pcDNA3.1/NT-GFP-TOPO vector.

Further information regarding glucose-induced PKC β II mRNA destabilization or the PKC β II mRNA instability element can be found in U.S. patent application Ser. No. 09/435, 471, filed Nov. 8, 1999; Patel N. A. et al., *Molecular Endocrinology*, 2004, 18(4):899-911; Patel N. A. et al., *J. Biol. Chem.*, 2003, 278(2):1149-1157; Patel N.A. et al., *J. Biol. Chem.*, 2001, 276(25):22648-22654; Yamamoto et al., *Am. J. Physiol. Cell Physiol.*, 2000, 279:C587-C595; Patel N. A. et al., *FASEB Journal*, 1999, 13:103-113; and Chalfant C. E. et al., *J. Biol. Chem.*, 1998, 273(2):910-916; each of which is incorporated herein in its entirety.

All patents, patent applications, provisional applications, and publications (including accession number sequences) referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

SEQUENCE LISTING

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<222> LOCATION: (1)..(7)

<223> OTHER INFORMATION: Tyrosine phosphatase conserved active-site

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motif
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<223> OTHER INFORMATION: Xaa = any amino acid residue

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<400> SEQUENCE: 6

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gaaggatttc ctttgttaac tctgaatttt taaaaccga agtcaagagc tagtagatct 180

gtagacctcc gtccttcatt tctgtcattc aagctcacag ctatcatgag agacaagcga 240

gacacctcca acttcgacaa aagttcacca ggcagcctgt ggaactgact cccactgaca 300

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cctttgttaa ctctgaattt ttaaaacccg aagtcaagag ctaagtagat ctgtagacct	240
ccgtccttca tttctgtcat tcaagctcaa cagctatcat gagagacaag cgagacacct	300
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 <213> ORGANISM: Homo sapiens

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ctaacacctc cgaccaggaa gtcacagga atattgacca atcagaattc gaaggatattt	180
cctttgttaa ctctgaattt ttaaaacctg aagtcaagag ctaagtagat ctgtagacct	240
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ccaacuucga caaaaguuca ccaggcagcc uguggaacug acucccacug aaaaacucug 360
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acccagagtt tgtcattaat gtc 563
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<213> ORGANISM: Artificial Sequence
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Cys Cys Pro Gly Cys Cys
1 5
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We claim:

1. A vector comprising a first polynucleotide encoding a mammalian protein kinase C β II (PKC β II) glucose responsive mRNA instability element; a second polynucleotide encoding a reporter polypeptide; and a promoter sequence operably linked to said first polynucleotide and said second polynucleotide; wherein said first polynucleotide comprises the sequence shown in SEQ ID NO:9, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:20 and wherein post-transcriptional stability of said first polynucleotide and said second polynucleotide is controlled by the presence of glucose or an analog thereof.

2. The vector of claim 1, wherein said glucose analog comprises 3-O-methylglucose, 2-deoxyglucose, or a combination thereof.

3. The vector of claim 1, wherein said reporter polypeptide is detectable intracellularly.

4. The vector of claim 1, wherein said reporter polypeptide comprises a fluorescent polypeptide.

5. The vector of claim 1, wherein said reporter polypeptide comprises luciferase, or green fluorescent protein (GFP) or a variant thereof.

6. The vector of claim 5, wherein said GFP variant comprises a polypeptide selected from the group consisting of a GFP fragment, red fluorescent protein, and orange fluorescent protein.

7. The vector of claim 1, wherein said vector is a viral vector.

8. The vector of claim 1, wherein said vector is a non-viral vector.

9. The vector of claim 1, wherein said vector is naked DNA.

10. The vector of claim 1, wherein said vector is a plasmid.

11. A genetically modified host cell comprising a first polynucleotide encoding a mammalian protein kinase C β II (PKC β II) glucose responsive mRNA instability element; a second polynucleotide encoding a reporter polypeptide; and a promoter sequence operably linked to said first polynucleotide and said second polynucleotide; wherein said first polynucleotide comprises the sequence shown in SEQ ID NO:9, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:20 and wherein post-transcriptional stability of said first polynucleotide and said second polynucleotide is controlled by the presence of glucose or an analog thereof.

12. The host cell of claim 11, wherein said host cell is a eukaryotic cell.

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13. The host cell of claim 11, wherein said host cell is a prokaryotic cell.

14. The host cell of claim 11, wherein said host cell is a mammalian host cell.

15. The host cell of claim 11, wherein said host cell is a cell of a pancreatic beta cell line.

16. The host cell of claim 11, wherein said host cell differentiates into an insulin-responsive cell.

17. The host cell of claim 11, wherein said host cell is an L6 myoblast, L8 myoblast, C2C12 myoblast, 3T3-L1pre-adipocyte, BRIN-BDII cell, INS-1 cell, RTN-m5F cell, RIN-1046 cell, or HIT cell.

18. The host cell of claim 11, wherein said reporter polypeptide comprises luciferase, or green fluorescent protein (GFP) or a variant thereof.

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19. A nucleic acid probe comprising a nucleotide sequence having affinity for a nucleotide sequence encoding a glucose responsive mRNA instability element, wherein said probe is the nucleotide sequence of SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:20.

20. A construct for post-transcriptional control of expression of a gene encoding a protein, wherein said construct comprises a first polynucleotide encoding said protein and a second polynucleotide encoding a mammalian protein kinase C β II (PKC β II) glucose responsive mRNA instability element, wherein said second polynucleotide sequence comprises the nucleotide sequence shown in SEQ ID NO:9, SEQ ID NO: 14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:20.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 8,008,071 B2
APPLICATION NO. : 11/054024
DATED : August 30, 2011
INVENTOR(S) : Denise R. Cooper and Niketa A. Patel

Page 1 of 5

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 3,

Line 61, ““xGxGX₂Gx₁₆Kx” should read --xGxGX₂Gx₁₆Kx--

Column 4,

Line 20, “βII γ” should read --βII, γ--

Line 32, “Brian” should read --Brain--

Line 33, “Brian” should read --Brain--

Column 6,

Line 21, “cytoskeleton ad” should read --cytoskeleton and--

Column 7,

Line 24, “the made of action” should read --the mode of action--

Column 10,

Line 60, “p85α and p110α” should read --p85α and p110α.--

Column 12,

Line 27, “posttranscription” should read --post-transcription--

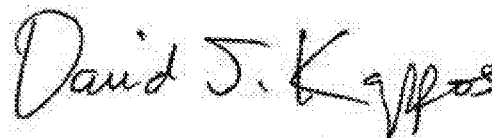
Column 15,

Line 33, “calorimetric,” should read --colorimetric,--

Column 16,

Line 1, “posttranslational” should read --post-translational--

Signed and Sealed this
Twenty-eighth Day of February, 2012

A handwritten signature in black ink that reads "David J. Kappos". The signature is written in a cursive, flowing style with a large initial 'D' and 'K'.

David J. Kappos
Director of the United States Patent and Trademark Office

Column 20,

Line 6, “PKC62II” should read --PKC β II--

Column 21,

Line 1, “has have particular” should read --has particular--

Column 23,

Line 13, “(b) anti-PKC β I” should read --(b) anti-PKC β II--

Line 49, “PKCO” should read --PKC β --

Column 24,

Line 30, “PKC(3(I+II))” should read --PKC β (I+II)--

Line 35, “were prepared form” should read --were prepared from--

Line 46, “PKC(β I+II)” should read --PKC β (I+II)--

Line 48, “PKC(β I+II)” should read --PKC β (I+II)--

Column 25,

Line 38, “404 by for” should read --404 bp for--

Line 39, “bother PKC β I” should read --both PKC β I--

Column 26,

Line 13, “PKCI;” should read -- PKC β I--

Line 38, “subdloned” should read --subcloned--

Column 28,

Line 6, “PKC β III mRNA” should read --PKC β II mRNA--

Lines 66-67, “level were” should read --levels were--

Column 29,

Line 34, “of a instability” should read --of an instability--

Column 30,

Line 53, ““reporter gene”).” should read --“reporter gene”.--

Column 34,

Line 39, “gridded format”” should read --“gridded format”--

Column 39,

Line 11, “*et al. Eur. J. Pharmacol.*” should read --*et al. (Eur. J. Pharmacol.*--

Column 41,

Line 14, “vector (in addition” should read --vector in addition--

Column 43,

Line 62, “and optionally,” should read --and, optionally,--

Column 47,

Line 49, “(AOSMC)” should read --(AoSMC)--

Line 60, “Ampliscribe T7” should read --Ampliscribe T7--

Line 62, “Al Other” should read --All other--

Column 49,

Line 16, “PKC, cDNA” should read --PKC β cDNA--

Line 23, “using on” should read --using an--

Line 67, “PKC, pre-mRNA” should read --PKC β pre-mRNA--

Column 50,

Line 27, “pflG vector” should read --p β G vector--

Line 64, “packed ells” should read --packed cells--

Column 51,

Line 16, “Ross. (1989.” should read --Ross (1989).--

Line 18, “2006; Amara” should read --2006); Amara--

Line 19, “Wright. 1996” should read --Wright (1996--

Line 22, “33:20126-20131) and” should read --33:20126-20131); and--

Column 53,

Line 2, “PKC α -CAT” should read --PKC β -CAT--

Line 18, “PKC#” should read --PKC β --

CERTIFICATE OF CORRECTION (continued)

Page 4 of 5

U.S. Pat. No. 8,008,071 B2

Column 55,

Line 58, “ad described” should read --and described--

Line 61, “100NM” should read --100nM--

Line 62, “100M” should read --100nM--

Column 56,

Line 41, “cannon be” should read --cannot be--

Line 50, “Nest,” should read --Next,--

Column 58,

Line 47, “ β IV5 exon” should read -- β IIV5 exon--

Column 62,

Line 9, “down regulated” should read --down-regulated--

Lines 21-22, “down regulation” should read --down-regulation--

Column 64,

Line 55, “that the PKC β II” should read --that the PKC β I--

Column 65,

Lines 7-8, “which could be regulate” should read --which could regulate--

Column 66,

Line 5, “The BII-exon” should read --The β II-exon--

Line 7, “PB globin (pBG)” should read --P β globin (p β G)--

Lines 8-9, “chimeric pBG-BII plasmid” should read --chimeric p β G- β II plasmid--

Line 9, “PKCBII exon” should read --PKC β II exon--

Line 12, “PvuII-Bgl II” should read --PvuII- β gl II--

Line 13, “B-globin” should read -- β -globin--

Line 14, “Hindul Site” should read --HindII site--

Line 14, “The B-globin” should read --The β -globin--

Line 16, “pBG” should read --p β G--

Column 66,

Line 21, "B-globin" should read -- β -globin--

Line 25, "BII exon" should read -- β II exon--

Line 28, "PKCBII" should read --PKC β II--

Line 34, "PKCBII" should read --PKC β II--

Line 36, "BII exon" should read -- β II exon--

Line 37, "BI exon" should read -- β I exon--

Line 38, "restricted be" should read --restricted by--

Line 43, "PKCBII and" should read --PKC β II and--

Line 43, "The PKCBII-specific" should read --The PKC β II-specific--

Line 49, "Analysis of PKCBII" should read --Analysis of PKC β II--

Line 51, "the PKCBII sequence" should read --the PKC β II sequence--