4-1996

Effects of Insulin and Insulin-Mimetics on mRNA Levels of *c-fos* and *c-jun* in Primary Rat Hepatocytes in Culture

Guangmin Li

EFFECTS OF INSULIN AND INSULIN-MIMETICS ON MRNA LEVELS OF *C-FOS* AND *C-JUN* IN PRIMARY RAT HEPATOCYTES IN CULTURE

by

Guangmin Li

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
April 1996

ACKNOWLEDGMENTS

I wish to express a special acknowledgment and sincere appreciation to my advisor and committee chairman, Dr. Susan Stapleton, for her invaluable guidance and assistance throughout this study; and to my committee members, Dr. Leonard Beuving and Dr. David Reinhold, for their advice and scientific criticism and critical review of this manuscript.

My deepest thanks and appreciation are extended to my parents and sister for their encouragement and support.

Guangmin Li

EFFECTS OF INSULIN AND INSULIN-MIMETICS ON MRNA LEVELS OF *C-FOS* AND *C-JUN* IN PRIMARY RAT HEPATOCYTES IN CULTURE

Guangmin Li, M.S.

Western Michigan University, 1996

Insulin is capable of regulating cellular and metabolic processes as well as gene expression. Selenate and vanadate are two compounds that have been found to mimic the action of insulin in several tissue and cell types. cFos and cJun are the products of immediate early responsive genes *c-fos* and *c-jun*. They are members of the family of the AP-1 (Activator Protein-1) transcription factors. AP-1 transcription factors bind as cJun.cJun homodimer or cJun.cFos heterodimer to the TRE found in the promoter regions of several genes. AP-1 is characterized by its ability to alter gene expression in response to various peptide hormones, growth factors, cytokines, tumor promoters, neurotransmitters as well as to increased expression of several oncogenes. AP-1 is involved in cellular differentiation, proliferation and neoplasmic transformation.

Our results indicate that, in primary rat hepatocytes in culture, insulin and selenate increase the *c-jun* mRNA level in a similar time dependent fashion; vanadate also induces *c-jun* mRNA level, but in a different and longer time dependent fashion; selenate is the only agent tested that shows an induction of *c-fos* mRNA level and the time course of induction corresponds with the induction of *c-jun*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Transcription

RNA is synthesized on a DNA template by a process known as DNA transcription. Transcription generates the mRNAs that carry the information for protein synthesis, as well as the transfer ribosomal, and other RNA molecules that have structure or catalytic functions. All of these RNA molecules are synthesized by RNA polymerase enzymes, which make an RNA copy from a DNA sequence. In eukaryotes, three kinds of RNA polymerase molecules synthesize different types of RNA. Whereas all genes capable of encoding a protein as well as the genes for some small nuclear RNAs involved in RNA splicing, are transcribed by RNA polymerase II, the genes encoding the 28S, 18S, and 5.8S ribosomal RNAs are transcribed by RNA polymerase I. Those encoding the transfer RNAs and the 5S ribosomal RNA are transcribed by RNA polymerase III. The three key steps in transcription are initiation, elongation, and termination. Initiation of mRNA synthesis is a primary control point in the regulation of gene expression.

RNA polymerases bind to regions of DNA known as promoters. A promoter includes the initiation site, where transcription actually begins, and dozens of nucleotides "upstream" from the initiation site. Certain regions within the promoter are especially important for recognition by RNA polymerases. For example, the RNA polymerase II keys in on a region called the TATA box. TATA boxes are centered at locations that are 25 nucleotides upstream from initiation sites. The TATA box is necessary but usually not sufficient for promoter activity. Additional elements are located between -40 and -110. Many promoters contain a CAAT box and some contain

a GC box. RNA polymerase II can not recognize and bind to a promoter on its own. Other proteins, called transcription factors, aid the polymerase in their search for promoter regions along DNA molecules.

Transcription Factors

Transcription in eukaryotic cells requires the formation of a transcription initiation complex, consisting of several general transcription factors and RNA polymerase II. An initial committed complex is formed by TFIID binding to the TATA element of a promoter. This complex acts as a binding site for TFIIB, which can recruit pol II and TFIIF into the complex. Subsequently, TFIIE and TFIIH associate with the initiation complex. Once the complete complex is assembled, an APT-dependent activation step is necessary for transcription to occur (Buratowski, 1994). The general transcription factors listed above are thought to be required for transcription of all genes by RNA polymerase II.

Each gene in an animal cell has a particular combination of positive and negative regulatory *cis* elements that are uniquely arranged as to number, type, and spatial array. These *cis* elements are binding sites for sequence-specific regulatory transcription factors that activate or repress transcription from the gene. Usually *cis* elements are arrayed within several hundred base pairs from the initiation site, but some elements can exert control over much greater distances. Regulatory transcription factors can influence rate-limiting steps in the assembly of transcription initiation complexes or in the activation of bound RNA polymerases. DNA sequence-specific binding by these factors occurs through a DNA-binding domain (von Hippel PH, 1994). All of the common motifs used by proteins that bind DNA in a sequence-specific manner (e.g., helix-turn-helix, helix-loop-helix, zinc fingers, etc.) can be found in one or another transcriptional regulatory protein.

In addition to DNA-binding domains, regulatory transcription factors contain other functional domains that mediate interactions with other proteins that may not bind DNA in a sequence-specific manner. Activation domains, for example, are operationally defined as those segments of the protein that are required to stimulate transcription. *In vivo* evidence indicates that activation domains make specific contacts with other proteins of the transcription complex (Carey et al., 1990; Chen and Pederson, 1993; Pettersson and Schaffner, 1990). Protein-protein contacts between regulatory and general transcription factors as well as other associated proteins, may stabilize the binding of general transcription factors to DNA and each other, and thereby facilitate assembly of transcription initiation complexes. Other regulatory factors appear to contact RNA polymerase II (Liao et al., 1991) and thereby promote polymerase binding to the initiation complex, or facilitate polymerase activation. Many regulatory transcription factors contain a third functional domain that allows them to multimerize or form heterotypic complexes (Jones, 1990). Some regulatory transcription factors contain yet other domains that function in nuclear localization or allosteric regulation by small molecules such as hormones. Finally, functional domains may be subject to phosphorylation or other modifications that influence function. The flexibility of functional features suggest a means by which the same regulatory factor might either stimulate or repress transcription.

AP-1 Transcription Factors

AP-1(Activator Protein-1) is the collective name for a class of sequence-specific DNA-binding transcription factors. Functional AP-1 consists of dimers of proteins of the Fos and Jun families which are related to retrovirally-transduced oncogenes v-*fos* and v-*jun* respectively (Bohmann et al., 1987; Franza et al., 1988; Chiu et al., 1988; Woodgett, 1990). The Jun family of proteins, cJun, JunB and JunD, can each homo-

dimerize or hetero-dimerize with the other AP-1 proteins (Nakabeppu et al., 1988). In contrast, the Fos family of proteins, cFos, Fra-1, Fra-2 and FosB, require one of the Jun family members for dimerization since they cannot form stable homo- or heterodimers within Fos family. Fos and Jun interact via specific structures, referred to as the leucine zipper, present in both proteins. A hallmark of this structure is the presence of a heptad repeat of leucines which are believed to align laterally with the leucines in a zipper of another protein and to establish complex formation by hydrophobic bonding. Adjacent to the leucine zippers in Fos and Jun are strongly basic regions which have been identified as DNA binding sites. Fos/Jun heterodimers are more stable than Jun homodimers and are consequently more efficient in binding to DNA.

AP-1 transcription factor binds to the TPA-responsive element (TRE) found in the promoter regions of several genes including metallothionein IIA, collagenase, *c-jun, c-fos,* phosphoenolpyruvate carboxykinase, stromelysin, and the adipocyte gene, ap2 (Kim and Khan, 1994). The consensus AP-1 recognition sequence is TGACTCA, but there are variations of this sequence in the promoter regions of target genes. AP-1-like binding sequences are also located in a number of other genes including tyrosine hydroxylase, prodynorphin, proenkephalin, and glial fibrillary acidic protein (Pennypacker et al., 1994).

AP-1 complex is involved in different cellular functions. AP-1 may play a role in the regulation of DNA replication (Murakami, 1991). AP-1 is involved in the control of cell proliferation and differentiation, as well as in neoplasmic transformation (Angel and Karin, 1991).

Regulation of AP-1 Activity

AP-1 activity is induced by a variety of peptide hormones, growth factors,

cytokines and neurotransmitters. These agents activate signalling pathways that are initiated with either stimulation of membrane-associated tyrosine kinases or phospholipid turnover, the latter gives rise to increased PKC activity. AP-1 activity is also elevated in cells that express a variety of transforming oncogenes, whose products act as constitutively activated intermediates in the signal transduction pathway that transmit information from cell-surface tyrosine kinases to the nucleus. Such oncogene products include v-Src, Ha-Ras and v-Raf. Another class of agents that induce AP-1 activity share the common ability to induce oxidative stress (Karin and Smeal, 1992).

Regulation of AP-1 activity is partly due to rapid induction of *fos* and *jun* gene transcription as well as the posttranslational modification of both preexisting and newly synthesized Fos and Jun proteins through their phosphorylation. AP-1 activity is also negatively regulated at the level of *fos* and *jun* transcription. Among Fos and Jun family members, cFos and cJun are major components of the transcription factor AP-1 and the regulation of their transcription and posttranslational modification is best understood and therefore discussed below.

Transcriptional Control of *c-fos* Expression

The *c-fos* gene is 4 kbp long, including the proximal promoter sequences, and is interrupted by three introns. From this gene, a single 2.2-kbp mRNA is transcribed, which is in agreement with the position mapped for the transcriptional start site and the polyadenylation site.

The *c-fos* promoter has been analyzed extensively, and a number of regulatory elements that contribute to induced transcriptional activity of *c-fos* have been identified (Figure 1). Activation of the *c-fos* promoter with serum requires the presence of the serum response element (SRE), positioned at -300 in the promoter (Triesman, 1992). This *cis*-element was originally identified as the binding site of serum response

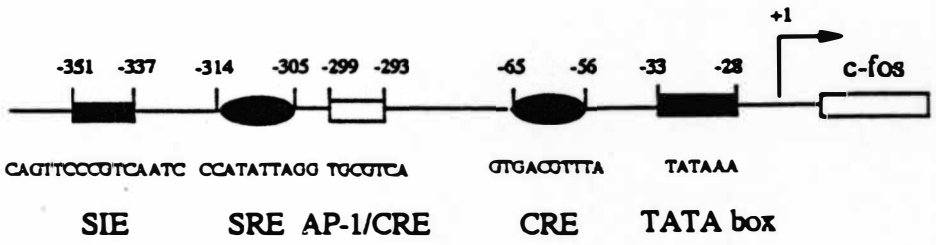

Figure 1. Elements That Mediate Transcriptional Regulation of the Human *c-fos* Gene.

factor (SRF), whose interaction with the SRE appears to be intimately involved in serum stimulation (Gilman, 1988; Triesman, 1985). The SRE mediates promoter stimulation not only by serum but also by phorbol esters, cytokines, growth factors, and even probably UV irradiation and oxidative stress. The SRE is also the target for activated oncogenes like *Ras,* v-*Src, mos,* or *Raf.*

The -60 positioned element contains a sequence resembling a cAMP response element (CRE), which mediates the activation of a number of genes in response to elevated levels of cAMP (Sassone-Corsi et al., 1988b). This element is essential but not fully sufficient for *c-fos* stimulation by both cAMP and increased Ca^{2+}. Conditioned medium from v-*sis* transformed cells, which contains an analog of PDGF β chain, and PDGF itself induce the *c-fos* promoter. This occurs via the v-*sis* conditioned medium induction element (SIE) located upstream of the SRE at -346. On stimulation of cells with a conditioned medium, EGF, or PDGF, the transcription factor SIF (v-*sis*-inducible factor) is activated to bind to this element (Hayes et al., 1987; Wagner et al., 1990). PDGF induction can also be mediated by the SRE (Büscher, 1988). In the wild-type *c-fos* promoter, the two elements probably function together in response to PDGF-triggered signal. In addition to these elements, another element can confer growth factor responsiveness when inserted directly upstream of an unresponsive, truncated *c-fos* gene. It is an AP1/CRE-like element at -295, which is

believed to bind members of the Fos/Jun and CREB families of transcription factors.

In most tissues, *c-fos* expression is tightly controlled. mRNA is expressed at only relatively low levels, but can be rapidly and transiently induced as early as 10 to 15 min after the addition of growth factors, phorbol esters, cytokines, or a number of compounds activating different intracellular pathways. Following a brief peak of expression, mRNA levels are reduced efficiently and kept low in the absence of external stimuli. The very low level of *c-fos* mRNA in quiescent cells is due to a nearly completely repressed state of transcription (Greenberg and Ziff, 1984), combined with a block in the elongation of initiated transcripts (Fort et al., 1987; Bonnieu et al., 1989).

Transcriptional Control of *c-jun* Expression

The first *cis*-acting element in the *c-jun* promoter that was shown to respond to external stimuli was a sequence (between positions -72 and -63bp), which deviates from the consensus AP-1 binding site (TGACTCA) by the insertion of a single nucleotide (5'$_{-71}$TGACATCA$_{-64}$3'). *In vitro* DNA binding experiments confirmed that this sequence is in fact recognized by the AP-1 transcription factor and functions as a high affinity AP-1 binding site (Angel et al., 1988). Another potential AP-1-like sequence was identified further upstream (5'$_{-190}$TTACCTCA$_{-183}$3') (van Dam et al., 1990; Stein et al., 1992). Interestingly, the AP-1 binding sites in the *c-jun* promoter are also occupied under noninduced condition (Herr et al., 1994). Regulation of activity of the binding transcription factors appears to occur while bound to the DNA. Upstream of the first AP-1 recognition sequence, the *c-jun* promoter contains consensus CAAT and GC boxes that correspond to the target sites for the transcription factors CTF/NF-1 and SP-1 respectively (Angel et al., 1988) (Figure 2). Most agents that induce either mitosis, transformation, or differentiation of various cell types lead to the

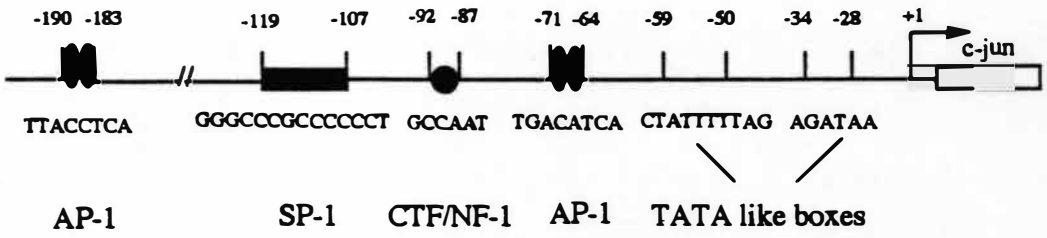

Figure 2. Elements That Mediate Transcriptional Regulation of the Human *c-jun* Gene.

transcriptional activation and increased expression of *c-jun*. These agents include serum, individual growth factors (EGF, PDGF), cytokines, TPA, and UV irradiation (Angel and Karin, 1991). While *c-fos* induction is highly transient and its mRNA is rapidly degraded, in human cells the induction of *c-jun* mRNA by TPA, although transient, lasts considerably longer than *c-fos* induction (Angel et al., 1988 and Schönthal et al., 1988). Part of this difference is probably due to the increased stability of the *c-jun* transcript. Other agents such as TNFα (Brenner, 1989) or TGFβ (Kim et al., 1990) lead to an even longer lasting induction of *c-jun*, while their effect on *c-fos* is as transient as the effect of TPA.

Posttranslational Control of AP-1 Activity

The function of AP-1 is controlled in part through the posttranslational modification (e.g., phosphorylation, glycosylation, redox regulation, association with proteins, and degradation), of its two components, Jun and Fos. Phosphorylation of transcription factors is an important posttranslational modification, since many extracellular stimuli affecting gene expression activate protein kinases (Hunter and Karin, 1992).

Unstimulated cells contain low, but detectable amounts of cJun protein. In this

state, cJun is phosphorylated constitutively at Thr231, Ser243, Ser249, sites close to its C-terminal DNA binding domain. Phosphorylation in this region markedly reduces DNA binding of Jun *in vitro* and *in vivo* (Boyle et al., 1991; Lin, et al., 1992). *In vitro*, one or more of these "C-terminal" sites can be phosphorylated by several protein kinases including glycogen synthase kinase 3 (GSK-3), casein kinase II (CKII), ERT (which is likely to be identical to ERK 1, one of the MAP/ERK kinases), and cyclin B/p34^{cdc2}. Phosphorylation at these sites inhibits DNA binding by cJun homodimers but not by cJun·cFos heterodimers (Boyle et al., 1991). Hypophosphorylation of the DNA binding domain of cJun occurs upon stimulation of cells with agents that activate cJun's transactivation potential (e.g., TPA, UV-C irradiation and transforming oncogenes), thereby increasing the affinity of Jun for its DNA recognition elememt (Smeal et al., 1991; Devary et al., 1992; Radler-Pohl, et al., 1993). The N-terminal part of cJun is also subject to complex phosphorylation events upon stimulation of resting cells with TPA, UV-C, growth factors, or oncoproteins. In resting cells, cJun is phosphorylated at low levels at Ser63 and Ser73. All stimuli that lead to increased transactivating potential of Jun cause hyperphosphorylation at these sites (Smeal et al., 1991; Adler et al., 1992). Phosphorylation of cJun at Ser63 and Ser73 potentiates its ability to activate transcription as either a homodimer (Pulverer et al., 1991; Smeal et al., 1991) or a heterodimer with cFos (Deng and Karin, 1994). These residues which do not affect DNA binding activity, are phosphorylated by the newly discovered members of the MAPK family, the Jun kinase or JNKs (Hibi et al., 1993). So far, the JNKs are the only protein kinases found to efficiently phosphorylate the N-terminal sites of cJun. Therefore, regulation of Jun's transactivation potential by phosphorylation takes place at two different levels: increased phosphorylation in transactivation domain causes enhanced transcriptional activity and enhanced binding to DNA is due to dephosphorylaion in the DNA binding region.

Protein kinase A, protein kinase C, and $p34^{cdc2}$ can phosphorylate cFos *in vitro* at several unique sites. These sites were located within two regions that were known to reduce the transcriptional activity *in vitro* (Abate et al., 1991). Interestingly, the sequence surrounding the N-terminal phosphoacceptors of cJun is also conserved in the C-terminal activation domain of cFos. Phosphorylation at Thr232, the homology of Ser73 of cJun, potentiates cFos transcriptional activity (Sutherland et al., 1992). Despite however the similarity between the two phosphoacceptor sites, Thr232 of cFos is not phosphorylated by either JNK1 or JNK2 but by a novel 88-kDa MAPK termed FRK (Fos regulating kinase) (Deng and Karin, 1994). Like the ERKs and the JNKs, FRK is a proline-directed kinase, whose activity is rapidly stimulated in response to Ha-Ras activation by growth factors.

Negative Control of *c-jun* and *c-fos* Expression

AP-1 activity is also negatively regulated at the level of *c-jun* and *c-fos* transcription. This negative regulation is important for normal cellular function. Because of its ability to positively autoregulate its own transcription, *c-jun* is at the risk of being permanently activated or overexpressed. Overexpression of *c-jun* is particular risky because it can lead to neoplastic transformation (Schütte et al., 1989a). Thus, most animal cells, or at least fibroblasts, must employ negative regulatory mechanism to curtail the amount of cJun expression. One such mechanism may operate at the translational level. The *c-jun* mRNA has one of the longest 5' untranslated regions known thus far (Hattori et al., 1988). It was shown that although TPA leads to a 15 fold increase in the level of *c-jun* mRNA, the rate of cJun protein synthesis is increased only 3-4-fold (Angel et al., 1988). However, at the present time, it is not clear whether this translational efficiency is an intrinsic property of *c-jun* mRNA sequences residing

in its long 5' untranslated region or whether it is due to an interaction of a regulatory protein with the *c-jun* transcript.

The AP-1 binding sites are potential targets for negative *c-jun* regulation. In addition to cJun homodimers, this site is also recognized by homodimers of JunB and JunD. While cJun is an efficient activator of the *c-jun* promoter, transient cotransfection experiments indicate that JunB and JunD are not (Chiu et al., 1989; Yang-Yen et al., 1990; Hirai et al., 1989). In fact, JunB acts as negative regulator and a mere 4-fold excess of JunB over cJun inhibits transactivation of AP-1-dependent indicator genes by cJun (Chiu et al., 1989; Schütte et al., 1989b). Because cJun and JunB have similar DNA binding activities, both proteins might compete for binding to the AP-1 site. As a consequence of enhanced JunB binding the "active" member of the AP-1 complex might get replaced by a less active factor resulting in a net decrease in transcription of the target gene. Another possibility, suggested by Schütte et al. (Schütte et al., 1989b), is the formation of cJun·JunB heterodimer which may be of lower efficiency in activating transcription than the corresponding cJun homodimers. Other elements of the *c-jun* promoter may be important for the attenuation of its activity. Deletion of the CTF and SP-1 sites in a short *c-jun* promoter decreases the basal and TPA-mediated human *c-jun* expression (Angel et al., 1988). Through an unknown mechanism, CTF and SP-1 sequences would then negatively influence *c-jun* transcription.

The *c-fos* gene is also subject to negative regulation. These include rapid degradation of *c-fos* transcripts or cFos protein and repression of the c-*fos* promoter. The rapid turnover of *c-fos* mRNA (app. 8min) depends on the presence of a conserved AU-rich sequence in the 3' untranslated sequence, found in other unstable transcripts including *c-jun* as well as an element present within the *c-fos* protein-coding region (Shaw and Kamen, 1986; Bohmann et al., 1987; Shyu et al., 1989).

In contrast to the negative regulation of *c-jun*, down-regulation of *c-fos* transcription is a function of its own product. Overexpresssion of cFos leads to a rapid decrease in both the basal and serum induced levels of transcription (Sassone-Corsi et al., 1988a; Schönthal et al., 1988). Several reports indicate the cFos transrepression is effected through the SRE. It appears unlikely that a direct interaction of Fos with SRE DNA sequences is responsible for this effect, since no binding of cFos to the SRE or its associated proteins has been observed. Although the mechanism is still unclear, some evidence implicates SRF binding as crucial for cFos autoregulation (Leung and Miyamoto, 1989; Shaw et al., 1989; Rivera et al., 1990). In addition to the SRE, other *cis*-elements in the *c-fos* promoter have been postulated to mediate autoregulatory transrepression, including AP-1-like binding sites at -295 and -60 (Wilson and Triesman, 1988).

Insulin and Diabetes

Insulin is a major anabolic hormone, which is produced by the beta cells of pancreatic islets. It is necessary for (a) transmembrane transport of glucose and amino acids, (b) glycogen formation in the liver and skeletal muscles, (c) glucose conversion to triglycerides, (d) nucleic acid synthesis, and (e) protein synthesis. Its principal metabolic function is to increase the rate of glucose transport into certain cells in the body. These are the striated muscle cells, including myocardial cells, fibroblasts, and fat cells. The most important stimulus that triggers insulin release is glucose, which also initiates insulin synthesis. Other agents, including intestinal hormones and certain amino acids (leucine and arginine), as well as sulfonylureas, stimulates insulin release but not synthesis.

Diabetes mellitus is a chronic disorder of carbohydrate, fat, and protein metabolism. A defective or deficient insulin secretory response which translates into

impaired carbohydrate (glucose) use, is a characteristic feature of diabetes mellitus, as is the resulting hyperglycemia. There are two major forms of diabetes with very different causes. Type I diabetes mellitus (insulin-dependent) is an autoimmune disorder, in which the immune system mounts an attack on the cells of the pancreas, destroying the individual's ability to produce insulin. Type II diabetes mellitus (non-insulin-dependent) is characterized either by a deficiency of insulin , or more commonly, by reduced responsiveness in target cells due to some change in insulin receptors.

Insulin Signal Transduction Pathway

It has been well established that the initial event mediating insulin action is binding of insulin to the α subunit of the cell surface insulin receptors, which results in the transmembrane activation of the cytoplasmic β subunit intrinsic tyrosine kinase domain. The major proximal intracellular target for the kinase-activated insulin receptor has been identified as a 185-KDa protein, termed IRS-1 for insulin receptor substrate-1 (Sun et al., 1991). More recently, a second target, composed of three related proteins (46, 52 and 66KDa) termed Shc has been identified (Pronk et al., 1993). These proteins contain insulin receptor-specific tyrosine phosphorylation sites responsible for their association with various downstream effector molecules. In the case of IRS-1, these include binding sites for the SH2 domains of phosphatidylinositol 3-kinase, protein tyrosine-specific phosphatase Syp, and the small adapter proteins Grb2 and Nck. In contrast, tyrosine phosphorylation of the Shc proteins has only been shown to directly induce the association with Grb2 (Skolnik et al., 1993; Tobe et al., 1993; Kuhne et al., 1993; Lee et al., 1993; Rozakis-Adcock et al., 1992). Grb2 is thought to act as an "adapter molecule" that links the guanine nucleotide exchange factor for $p21^{ras}$ termed SOS (Chardin et al., 1993). The Grb2/SOS complex thus may activate $p21^{ras}$

by stimulating GTP binding. Ras has been shown to bind directly to Raf-1, a serine-threonine kinase, which in turn activates MAP kinase by phosphorylation and activation of the MAP kinase kinase (Crews, 1993). Insulin can also stimulate the S6 kinase, but it is thought that insulin stimulates pp90^{rsk} through the MAP kinase cascade, whereas it stimulates pp70^{s6k} through a distinct cascade involving IRS-1 (Erikson, 1991). PI 3-kinase may also be an upstream regulator of pp70^{s6k} (Figure 3).

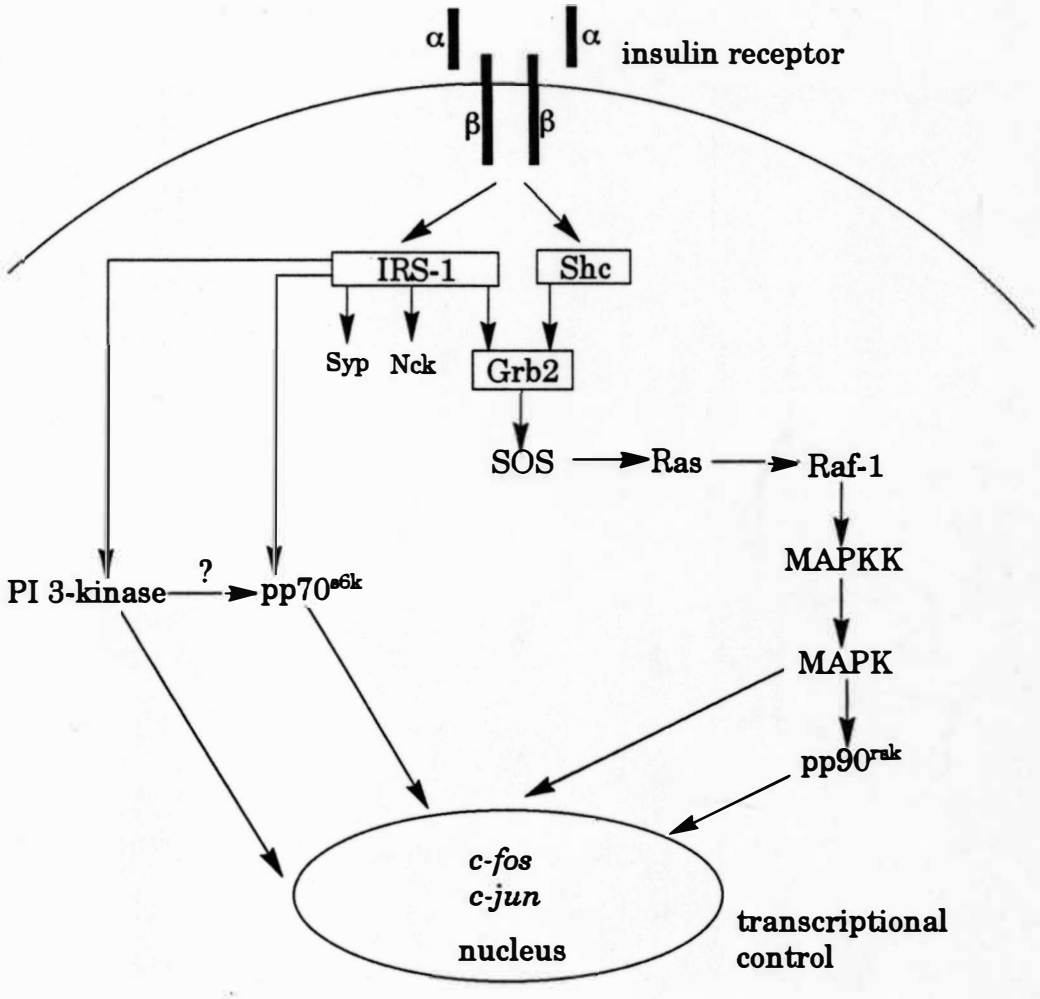

Figure 3. A Potential Network of Insulin Signal Transduction Pathway.

Insulin's Regulation of *c-fos* and *c-jun* Expression

Insulin regulates the transcriptional expression of a number of cellular genes. Expression of distinct genes may be either stimulated or inhibited by the action of insulin. Insulin can induce changes in the expression of *c-fos* in defined cellular systems. In rat H4-IIE hepatoma cells which proliferate in response to insulin, addition of the hormone results in an increased rate of *c-fos* gene transcription and accumulation of *c-fos* mRNA (Messina, 1990). This accumulation is dose dependent and reaches a maximum at 30 min. Induction of *c-fos* transcription in H4-IIE cells occurs as early as 5 min after the addition of insulin. This is the most rapid effect of insulin yet known on the induction of gene expression. Insulin treatment of H35 rat hepatoma cells that become quiescent under serum-deprived conditions or by treatment with dexamethasone increases DNA synthesis; and this induction is associated with an increase in the level of expression of *c-fos* gene transcript (Taub et al., 1987).

Insulin has also been shown to increase *c-fos* mRNA expression in 3T3-L1 fibroblasts and adipocytes (Stumpo and Blackshear, 1986), L6 rat skeletal muscle cells (Ong et al., 1987) and Chinese hamster ovary cells transformed with high levels of the insulin receptor gene (Stumpo et al., 1988) but apparently has no effect in murine fibroblasts (Greenberg and Ziff, 1984), PC 12 rat pheochromocytoma cells (Kruijer et al., 1985) and adult rat hepatocytes (Kruijer, 1986). The reason for these differences is unclear, but may in part reflect a requirement for additional tissue-specific transcription factors whose expression varies between cells.

Transient transfection experiments using *c-fos*/CAT fusion genes have shown that the *c-fos* promoter sequence corresponding to the serum response element (SRE) is required for induction of *c-fos* transcription by insulin. Studies with two different Chinese hamester ovary cells lines indicated that the presence of normal insulin

receptors was required for the insulin induction of *c-fos* mRNA. Induction did not occur in cells with similar numbers of truncated insulin receptors, which exhibit similar amounts of insulin binding but essentially no insulin-stimulated receptor tyrosine kinase activity (Stumpo et al., 1988). This result demonstrates that regulation of *c-fos* by insulin is mediated by the insulin receptor, and suggests that the kinase activity of the receptor is essential for this regulation.

Recently, one complete pathway linking the insulin receptor to the transcriptional activation of the *c-fos* SRE has been identified. This pathway requires Ras activation as an upstream mediator of the Raf/MAPKK/MAPK, resulting in the phosphorylation and activation of several transcriptional factors (TCF and SRF) necessary for SRE responsiveness (Yamauchi and Pessin, 1994). It was demonstrated that IRS-1 and Shc compete for a limited cellular pool of Grb2, and insulin activation of MAP kinase and *c-fos* transcription predominately occurs through the Shc-Grb2 signal pathway (Yamauchi and Pessin, 1994).

In Reuber H-35 cells, *c-jun* achieves a higher peak level of expression with insulin stimulation, and the level of expression remains elevated for a longer period of time. There appears to be a secondary peak of *c-jun* expression at 16 h, which may be because of the continued cycling of the cells (Mohn et al., 1990). NIH 3T3 fibroblasts expressing the wild-type receptor show a 4-5-fold induction of *c-jun* in response to insulin, but the cell lines expressing mutant receptors show an impairment in the ability of insulin to stimulate *c-jun* expression on a percentage base. This demonstrates the importance of insulin receptor to this signal transduction pathway (Quon et al., 1992).

Insulin-Mimetics Vanadate and Selenate

Most tissues of higher animals contain intracellular vanadium at concentrations varying between 0.1 and 1 μM. Vanadium seems to be an essential nutritional trace

element that is required for normal growth and development and is also necessary for the growth and survival of mammalian cells in culture (Macara, 1980). Selenium is also recognized as an essential element. Selenium's exact function is not fully understood; however, it is required for the activity of glutathione peroxidase (Rotruck et al., 1973) and is also believed to act as an antioxidant (McKeehan et al., 1976), and lack of which results in the development of cardiomyopathy (Chen et al., 1980). There is a growing interest in vanadium and selenium in the field of insulin action and diabetes, since they mimic the actions of insulin in various insulin-responsive cells and tissues.

Insulin-like Actions of Vanadate and Selenate on Cell Regulation

In vitro insulin-like actions of vanadate on cell regulaton include: the stimulation of glucose uptake and oxidation and inhibition of lypolysis in adipose tissue (Tolman et al., 1979; Shechter et al., 1980; Dubyak et al., 1980); stimulation of glucose utilization and of glycogen synthesis and the inhibition of gluconeogenesis in the liver (Gil et al., 1988); and stimulation of glucose uptake, glucose utilization, and glycogen synthesis in skeletal muscle (Challis et al., 1987). Vanadate is also shown to mimic insulin in isolated rat hepatocytes by increasing levels of fructose-2,6-bisphosphate, a key regulatory substance in glucose metabolism (Miralpeix, 1989). *In vivo*, vanadate has been reported to lower or even normalize blood glucose concentration in various animal models of diabetes (Malabu et al., 1994; Berg et al., 1995). Vanadate stimulates autophosphorylation of the tyrosine kinase domain of insulin receptors. Incubation of intact rat adipocytes with vanadate stimulates tyrosine phosphorylation of the protein tyrosine kinase of the insulin receptor. Similar findings are observed in intact hepatoma Fao cells and cultured NIH 3T3 fibroblasts (Stern et al., 1993). Stapleton et al. have also demonstrated that vanadate is capable of

stimulating phosphorylation of the insulin receptor in primary rat hepatocytes in culture and 3T3-L1 adipocytes (Stapleton et al., unpublished results).

In vitro studies have demonstrated the selenate also possesses insulin-like actions. Sodium selenate, when incubated with rat adipocytes, markedly stimulated glucose transport and also stimulated insulin-sensitive cAMP phosphodiesterase and ribosomal S6 protein phosphorylation but did not stimulate insulin-receptor kinase or inhibit phosphotyrosine phosphatase (Ezaki, 1990). Stapleton et al. have demonstrated however that selenate is capable of stimulating phosphorylation of the insulin receptor in primary rat hepatocytes in culture and 3T3-L1 adipocytes (Stapleton et al., unpublished results). Selenate has been shown to stimulate tyrosine phosphorylation of the epidermal growth factor (EGF) receptor in A431 cells and enhance the tyrosine phosphorylation of endogenous proteins in response to EGF in A431 cells and insulin in NIH3T3 HIR3.5 cells (Pillary and Makgoba, 1992). Selenate also has insulin-like effects when administered *in vivo*. Treatment of streptozotocin (STZ)-induced diabetic rats with sodium selenate resulted in a decrease in plasma glucose, food intake, and water intake to control or near control levels (McNeill et al., 1991; Berg et al., 1995).

Insulin-like Actions of Vanadate and Selenate on Gene Expression

In addition to the ability to mimic insulin with regard to regulation of cellular processes, several recent investigations have shown vanadate and selenate also regulate gene expression in a fashion similar to insulin. In streptozotocin-induced diabetic animals, vanadate increases pancreatic amylase mRNA (Johnson et al., 1990); restores glucokinase, L-type pyruvate kinase and phosphoenolpyruvate carboxykinase activity and mRNA levels (Brichard et al., 1993; Valera et al., 1993); and stimulates liver 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphophatase mRNA and protein levels (Inoue et al., 1994). In a variety of cell types in culture, vanadate inhibits expression

of transfected phosphoenolpyruvate carboxylase gene (Bosch et al., 1990), stimulates mRNA accumulation and transcription of gene 33 (Weinstock and Messina, 1992), induces the expression of L-type pyruvate kinase and glucokinase gene (Miralpeix et al., 1991) and Glut-1 mRNA (Mountjoy and Flier, 1990).

In STZ-induced diabetic rats, both vanadate and selenate can normalize mRNA levels of glucose-6-phosphate dehydrogenase (G6PDH) and fatty acid synthase (FAS) in a similar fashion to insulin (Berg et al., 1995). G6PDH and FAS mRNA levels were also induced by vanadate and selenate in rat hepatocytes in culture (Stapleton et al., unpublished results). There is no other evidence concerning selenate's regulation of gene expression in a manner similar to insulin.

Objective of the Study

Vanadate and selenate have been reported to be potent insulin-mimetic agents in many cells. Our lab has demonstrated that G6PDH mRNA level was induced similarly when either STZ-induced diabetic rats (Berg et al., 1995) or primary rat hepatocytes in culture (Stapleton et al., unpublished results) were treated with either insulin, vanadate or selenate. Rank et al. (Rank et al., 1994) reported that there are three putative AP-1 binding sites in the promoter region of the gene G6PDH. The goal of my study is to determine whether or not the enhancement of G6PDH expression by insulin and insulin-mimetics is associated with the expression of *c-fos* and *c-jun*. *c-fos* and *c-jun* mRNA levels will be detected by Northern blot analysis.

Significance of the Study

Vanadate and selenate have been proposed to replace or to be used in conjunction with insulin in treatment of diabetes. It is important to establish if these compounds truly mimic insulin and to determine their mechanism. Understanding how

these compounds regulate gene expression may help us understand the mechanism of action of mimetics and the action of insulin. Information acquired from these kinds of studies could ideally lead to insulin-mimetics as potential therapeutic agents in the management of diabetes.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats were obtained from Harlen Sprague-Dawley (Kalamazoo, MI). Waymouth's MB 752/1 medium, restriction enzymes, gentamicin were purchased from Gibco BRL (Grand Island, NY). [α-^{32}P] dCTP (3000 Ci/mmol) and GeneScreen membrane were obtained from Dupont/NEN (Boston, MA). Random Primer Labeling Kit was from Amersham (Arlington Heights, IL). Insulin was a gift from Eli Lilly Corp.(Indianapolis, IN). Collagenase D, DNase-free RNase were from Boehringer Mannheim biochemicals (Indianapolis, IN). Rat tail collagen (type IV) was from Collaborative Research Incorporated (Bedford, MA). Phenol was purchased from Amresco (Solon, OH). All other reagents were obtained from Sigma Chemical Corp. (St.Louis, MO).

Isolation and Maintenance of Hepatocytes

Hepatocytes were isolated using collagenase and hyaluronidase perfusion as described by Elliget and Koloja (Elliget and Koloja, 1983), and modified by Stapleton et al. (Stapleton et al., 1993). Male rats weighing 150-200 g were fasted 48 hr prior to the isolation procedure. After perfusion and digestion, the liver was excised and forced through four layers of sterile gauze. The cells were washed two times with cold Waymouth's MB 752/1 medium supplemented with 5% BSA, and pelleted for 3 min at 4^0C and 50xg. The pellet was gently resuspended in culture medium and an aliquot of cells counted with a hemocytometer. Cell viability was determined by trypan blue dye exclusion and a cell population with a viability greater than 85% was plated. Collagen-

coated 60-mm tissue culture dishes were incubated with $3.0x10^6$ cells/plates. The cells were incubated in Waymouth's MB 752/1medium supplemented with 5% BSA and gentamicin (10 μg/ml) under a humidified atmosphere of 5% CO_2 and 95% air at 37^0C.

Cell Treatment

After 4 h in culture, the cells were washed with Waymouth's MB 752/1 without BSA, and fresh media was applied. Hepatocytes were treated with either nothing (control), 160 nM insulin, 500 μM sodium orthovanadate, or 500 μM sodium selenate at times indicated in the figure and table legends.

RNA Isolation

After removing the medium, total RNA was isolated from the cells by the guanidinium thiocyanate/phenol/chloroform method (Chromczynski and Sacchi, 1987). All solutions were prepared with diethypyrocarbonate (DEPC)-treated water. The cells were homogenized directly in extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarkosyl, 0.1 M 2-mercaptoethanol); followed by acid phenol-chloroform extraction and isopropanol precipitation. The final RNA preparation was dissolved in water treated with DEPC. Extracted RNA was quantified by absorbance at 260 nm. The ratio between the readings at 260 nm and 280 nm provides an estimate for the purity of the isolated RNA.

Extraction and Purification of Plasmid DNA

The bacterial cells were harvested at 6000 rpm for 20 min at 4^0C. The bacterial pellet was lysed by alkali method modified from Birnbiom and Doly (Birnbiom and Doly, 1979) and Ish-Horowicz and Burke (Ish-Horowicz and Burke, 1981). The bacterial pellet was resuspended in solution I (50 mM glucose, 25 mM Tris·Cl [pH

8.0], 10 mM EDTA [pH 8.0], 5 mg/ml lysozyme), and additionally incubated in solution II (0.2 N NaOH, 1% SDS) and solution III (3 M potassium acetate and glacial acetic acid). The bacterial lysate was centrifuged at 10,000rpm for 30 min at 4^0C. Nucleic acids were precipitated by isopropanol. Plasmid DNA was purified by incubation with DNase-free RNase (2000 U/ml activity), followed by phenol and chloroform extraction. The plasmid DNA was recovered by adding ammonium acatate (final concentration 2 M) and 100% ethanol. The concentration and purity of plasmid DNA were determined by absorbance at 260 nm and 280 nm.

DNA Probes

The following probes were used in Northern blot analyses: the 1.8 kb EcoRI rat *c-jun* cDNA subcloned into pGEM-4 (gift from Dr. Curran, Roche Institute of Molecular Biology), the 1352 bp EcoRI/Xho I rat *c-fos* cDNA subcloned into pBluescript II (gift from Dr. Curran, Roche Institute of Molecular Biology), the 1.8kb HindIII chicken *β-actin* cDNA subcloned into pBR322 (gift from Dr. Cleveland, the Johns Hopkins University). The *β-actin* cDNA was used as an internal control. The cDNA inserts were purified by digestion with the appropriate restriction endonucleases, electrophoresis on 1% agarose gels and electroelution by low salt (0.1 M LiCl, 10 mM Tris [pH 7.5], 1 mM EDTA [pH 8.0]) wash and high salt (20% ethanol, 1 M LiCl, 10 mM Tris [pH 7.5], 1 mM EDTA) elution. The cDNA fragments were radiolabelled with [α-^{32}P] dCTP to about 10^8 cpm/μg using random primer labeling kit. The labeled cDNA probes were separated from [α-^{32}P] dCTP by centrifugation through G50 spun columns with NETS (100 mM NaCl, 1 mM EDTA [pH 8.0], 10 mM Tris·Cl, 0.1% SDS) solution. The radioactivities of the purified probes were determined by using liquid scintillation counter.

Northern Blot Analysis

Denaturation and Size Fractionation of RNA

15 µg of total RNA was heated to 65^{0}C for 2 min after addition of 2 µl ethidium bromide (0.1 mg/ml), and loaded onto a submarine gel. For gel electrophoresis, agarose (1% final) was melted in H_2O and cooled to 60^{0}C, and MOPS electrophoresis buffer and formaldehyde were added to final concentrations of 1x (0.02 M 4-morpholinepropanesulfonic acid, 0.005 M sodium acatate, 0.001 M EDTA) and 2.2 M, respectively. The RNA was separated electrophoretically for 3 h at 55 V (gels 7 cm wide, 9 cm long), using 1xMOPS as the electrophoresis buffer.

Blotting of RNA Onto "GeneScreen" Membrane

After electrophoresis was complete, the gel was photographed using a UV source to determine the positions of the 28S and 18S ribosomal RNA bands. Then the gel was placed onto a vacuum-blotting device (VacuGene, Pharmacia LKB, Bromma, Sweden) and a vacuum of 40 mbar was applied for at least 6 h using 10xSSC (1xSSC is 0.15 M NaCl, 0.015 M Na_3-citrate [pH 7.0]) as blotting buffer.

Hybridization and Autoradiography

The blot was prehybridized in the prehybridized solution (50% deionized formamide, 0.25 M $NaHPO_4$ [pH 7.2], 0.25 M NaCl, 1 mM EDTA, 100 µg/ml denatured salmon sperm DNA, and 7% SDS) at 42^{0}C overnight. The labeled cDNA probe was first denatured by incubation in boiling water for 5 min and then added to prehybridized solution. Hybridization was performed at 42^{0}C overnight. The hybridized membrane was rinsed twice in 2xSSC/0.1% SDS for 20 min at 48^{0}C, and

once in 1xSSC/0.1%SDS for 20 min at 52^0C.

The blot was then air-dried briefly and exposed at -70^0 to Kodak X-Omat AR film using Dupont lighting-plus intensifying screens. The mRNA bands corresponding to *c-fos* and *c-jun* were visualized by autoradiography, and quantitated by densitometric scanning. The blots were stripped in 95^0C water for 5 min. The blots were then hybridized to ^{32}P-labeled β-actin, a control probe which was used to quantitate total RNA.

Statistical Analysis

The results are expressed as the means±S.E. The differences between non-treated and treated hepatocytes were evaluated by one-tailed Student's *t*-test. Significance was tested at $P < 0.05$.

RESULTS

Visualization of RNA After Gel Electrophoresis

Visualization of RNA by ethidium bromide staining after gel electrophoresis is very important in Northern analysis, because it provides a way of evaluating the integrity of the RNA (Figure 4). Degradation can be recognized by smearing and disappearance of high-molecular-weight RNA bands. At the same time, the stained size-separated RNA allows for determination of the length of the eventually hybridized mRNA relative to the migration distance of the ribosomal 28S and 18S, which serve as internal molecular weight markers (Kroczek and Siebert, 1990).

Restriction Digestion of Plasmids

The plasmids were isolated as described in " Materials and Methods". The plasmids containing *c-fos* cDNA (Figure 5), *c-jun* cDNA (Figure 6) or *β-actin* cDNA (Figure 7) were cut by appropriate restriction enzymes and subject to 1% agarose gel electrophoresis. DNA fragments can be visualized under UV light with ethidium bromide staining (Figure 8). Elution of DNA fragments from gel and random primer labeling of fragments for Northern analysis were as described in "Materials and Methods".

Effect of Insulin on *c-jun* Gene Expression

Insulin was reported to increase *c-jun* gene expression in Reuber H-35 cells and NIH 3T3 fibroblasts (Mohn et al., 1990; Quon et al., 1992). To examine the effect of insulin on *c-jun* gene expression in our model system, rat hepatocytes in culture were

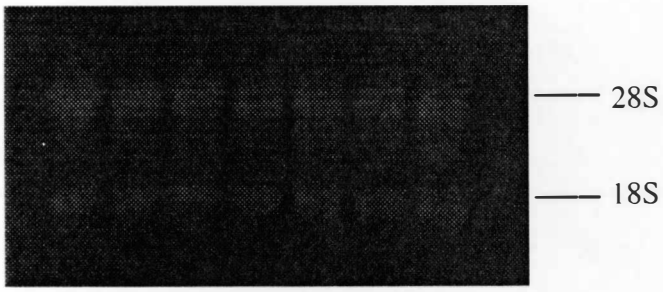

Figure 4. Total RNA (15 µg per lane) from Primary Rat Hepatocytes in Culture was Size-Separated on an Agarose Gel as Described Under "Materials and Methods".

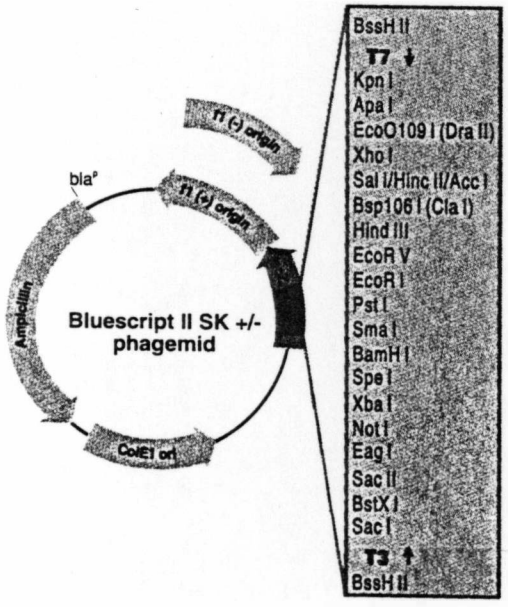

Figure 5. pBluescript II Vector Circle Map. 1352bp Rat *c-fos* cDNA was Subcloned into the EcoRI/XhoI Sites of pBluescript Vector.

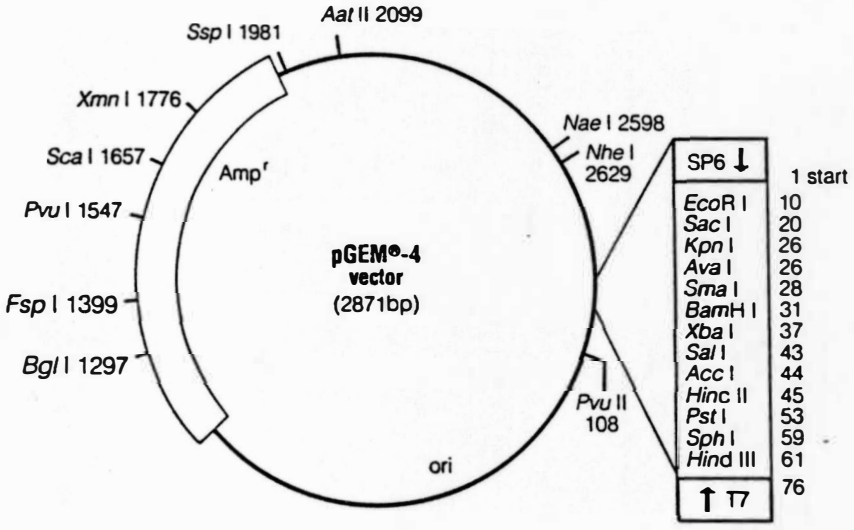

Figure 6. pGEM-4 Vector Circle Map. 1.8kb Rat *c-jun* cDNA was Subcloned into the EcoRI Site of pGEM-4 Vector.

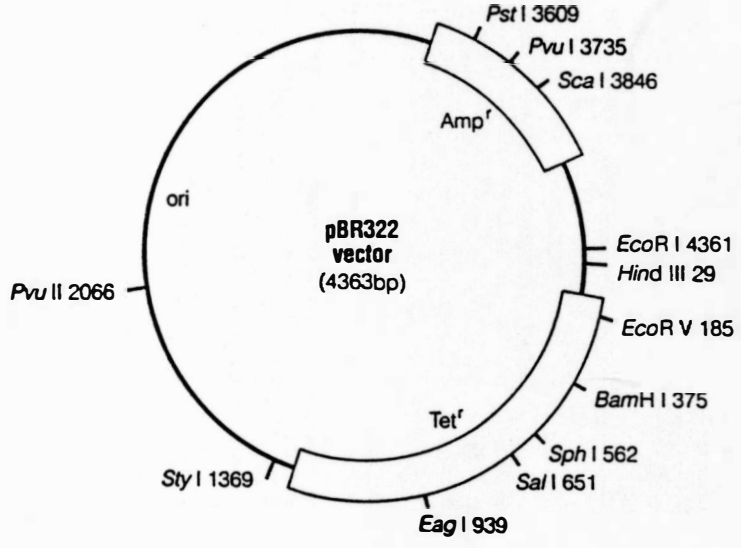

Figure 7. pBR322 Vector Circle Map. 1.8kb β-*actin* cDNA was Subcloned into the HindIII Site of pBR322 Vector.

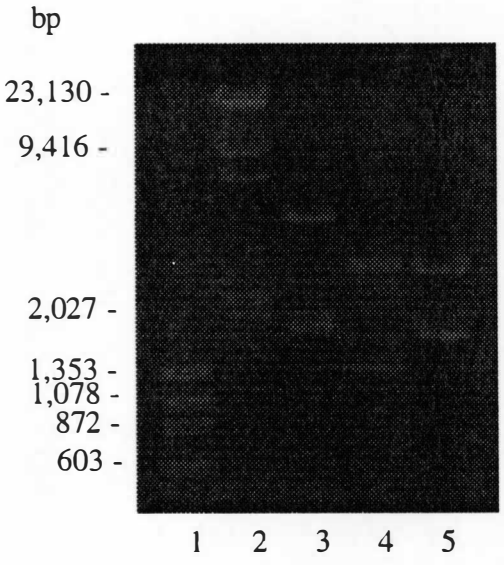

Figure 8. Restriction Digestion of Plasmids.

Lane 1: ϕX174 RF DNA/HaeIII fragments
Lane 2: λDNA/HindIII fragments
Lane 3: pBR322 with *β-actin* cDNA insert cut by HindIII
Lane 4: pBluescript II with *c-fos* cDNA insert cut by EcoRI and XhoI
Lane 5: pGEM-4 with *c-jun* cDNA insert cut by EcoRI

treated with insulin (160 nM) for different periods of time, and levels of *c-jun* mRNA after treatment by insulin were detected by Northern analysis. As shown in Figure 9, *c-jun* mRNA has two mRNAs, one at 2.8 kb, and the other at 3.2kb. This observation was compatible with previous reports (Gurland et al., 1990; Webb et al., 1990). Both mRNAs are transcripts from the same gene, but the small transcript is derived from the use of one of the additional polyadenylation sites (Ryseck et al., 1988). Note that the expression of both mRNAs are similarly controlled. Quantitation of the relative changes in *c-jun* mRNA level between control and insulin-treated cells was determined by densitometric scanning (Table 1). Control cells were incubated in serum-free medium only. The quantitative data were corrected for uneven loading of RNA by stripping membranes that were hybridized with radiolabeled *c-jun* probe and

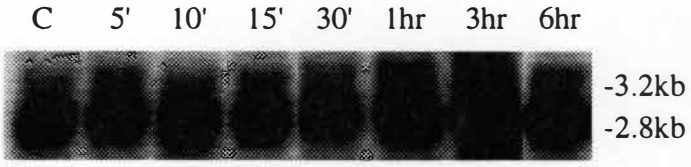

Figure 9. Northern Blot Analysis of *c-jun* mRNA Level in Primary Rat Hepatocytes in Culture Treated with Insulin.

Total cytoplasmic RNA from primary rat hepatocytes in culture that either received no treatment (control) or were treated with insulin (160 nM) for the designated time was separated on a formaldehyde-agarose gel, blotted onto "GeneScreen" membrane, and probe with a [α-^{32}P]CTP-labeled *c-jun* cDNA. Control cells were incubated in serum-free medium.

Table 1

Effect of Insulin on *c-jun* mRNA Level in Primary Rat Hepatocytes in Culture

Treatment	*c-jun* mRNA level as % of control
control	100
Ins 5'	90.0 ± 7.4 (2)
Ins 10'	95.0 ± 2.8 (2)
Ins 15'	90.7 ± 4.8 (3)
Ins 30'	123.0 ± 8.7* (10)
Ins 1hr	126.0 ± 10.6* (7)
Ins 3hr	141.7 ± 9.1* (10)
Ins 6hr	102.5 ± 9.8 (4)

* Significantly different from control, $P < 0.05$.

Primay rat hepatocytes in culture either received no treatment (control) or were treated with insulin (Ins) (160 nM) for the designated time. Total cytoplasmic RNA was prepared and Northern blot analysis was performed under "Materials and Methods". The control of each experiment was arbitrarily set to 100 and the change by insulin treatment was expressed compared to the control value. Data shown are the means ± S.E. for the number of experiments shown in parentheses.

rehybridized with a radiolabeled cDNA probe for the constitutively expressed protein, β-actin. As shown in Figure 10, insulin increased the expression of *c-jun* mRNA in a time-dependent manner. There was no observable increase in the level of *c-jun* mRNA within 15 min of insulin addition, but induction did occur after 30 min of insulin treatment. *c-jun* mRNA levels were increased approximately 1.2-fold and 1.3-fold by 30 min and 1hr, respectively, reaching maximal increase by 3 hr and representing an increase of 1.4-fold over that seen in unstimulated cells. By 6 hr, this induction declined to basal levels. Serum was reported to be a stimulator of *c-jun* expression in mouse 3T3 Balb/c and NIH 3T3 fibroblasts (Ryseck et al., 1988; Lamph et al., 1988). In our model system, serum was also found to increase levels of *c-jun* mRNA and the maximal induction occurred at 30 min, which was 1.8 fold compared to control (data not shown).

Effect of Selenate on *c-jun* Gene Expression

Early studies on the biochemical function of selenium focused on its role as an antioxidant, and as an essential constituent of glutathione peroxidase (McKeehan et al., 1976; Rotruck et al., 1973). Recently, selenate has been found to be a potent insulin-mimetic agent in isolated rat adipocytes (Ezaki, 1990) and *in vivo* (McNeill et al., 1991, Berg et al., 1995). In the present study, I have explored the effect of selenate on *c-jun* gene expression in primary rat hepatocytes in culture to see if it is an effective insulin-mimetic agent with regard to regulation of *c-jun* expression. As shown in Figure 11 and Table 2, selenate elevated *c-jun* mRNA level in a time-dependent manner. The steady state levels of *c-jun* mRNA began to increase at 30 min, peaked by 3 hr, and then decreased, reaching pre-stimulation level by 12 hr. The maximal increase is about 1.6-fold. Selenate was able to stimulate *c-jun* mRNA levels in a fashion similar to insulin (Figure 10). This increase in *c-jun* mRNA was observed

over a longer period of time than insulin. These results are in agreement with other effects on signal proteins that we have observed with the mimetics.

Effect of Vanadate on *c-jun* Gene Expression

In the presence of oxidizing agents near neutral pH, vanadium ions at micromolar concentration exist as the hydrated monomer of vanadate (HVO_4^{2-} or $H_2VO_4^-$). Vanadate begins to polymerize at concentrations greater than 0.1mM at

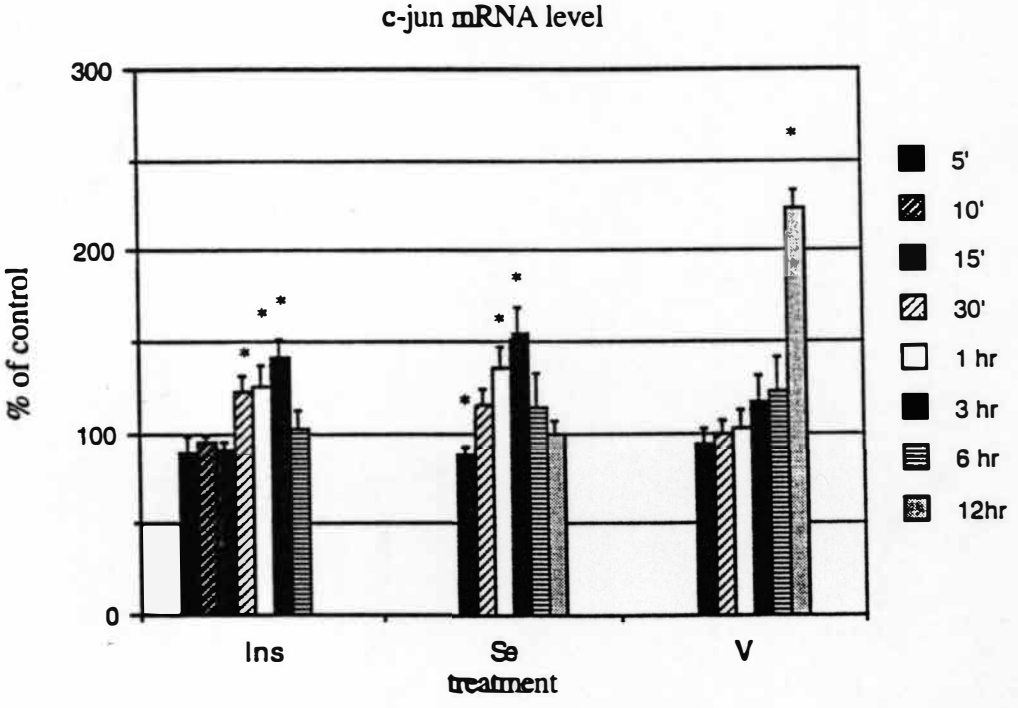

Figure 10. Effects of Insulin (Ins), Selenate (Se) and Vanadate (V) on *c-jun* mRNA Level in Primary Rat Hepatocytes in Culture.

Primary rat hepatocytes in culture were treated with nothing (control), insulin (160 nM), selenate (500 μM) and vanadate (500 μM) for designated time. Total cytoplasmic RNA was prepared and Northern blot analysis was performed under "Materials and Methods". The control of each expriment was arbitrarily set to 100 and the changes by different treatments were expressed compared to the control value. Values represent the means±S.E. Starred treatments were significantly different from control ($P < 0.05$).

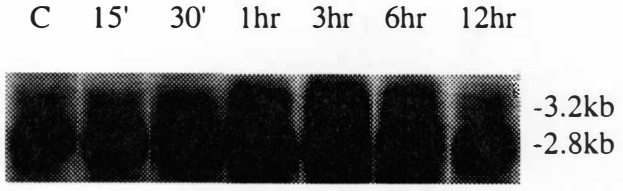

Figure 11. Northern Blot Analysis of *c-jun* mRNA Level in Primary Rat Hepatocytes in Culture Treated with Selenate.

Total cytoplasmic RNA from primary rat hepatocytes in culture that either received no treatment (control) or were treated with selenate (500 μM) for the designated time was separated on a formaldehyde-agarose gel, blotted onto "GeneScreen" membrane, and probed with a [α-^{32}P]CTP-labeled *c-jun* cDNA. Control cells were incubated in serum-free medium.

Table 2

Effect of Selenate on *c-jun* mRNA Level in Primary Rat Hepatocytes in Culture

Treatment	*c-jun* mRNA level as % of control
control	100
Se 15'	87.7 ± 4.8* (5)
Se 30'	115.6 ± 8.8 (7)
Se 1hr	135.1 ± 11.8* (7)
Se 3hr	155.0 ± 13.3* (10)
Se 6hr	114.6 ± 18.5 (3)
Se 12hr	98.8 ± 7.7 (3)

* Significantly different from control, $P < 0.05$.

Primay rat hepatocytes in culture either received no treatment (control) or were treated with selenate (Se) (500 μM) for the designated time. Total cytoplasmic RNA was prepared and Northern blot analysis was performed under "Materials and Methods". The control of each experiment was arbitrarily set to 100 and the change by selenate treatment was expressed compared to the control value. Data shown are the means ± S.E. for the number of experiments shown in parentheses.

neutral pH. In this study, both monomeric and polymeric forms of vanadate were used to treat cells, but no difference was found in the induction of *c-jun* mRNA level. No elevation of *c-jun* mRNA level was observed within 1 hr. There was a small increase by 3 hr, reaching a maximal induction of 2.2-fold by 12 hr (Table 3). The time course of induction of *c-jun* expression by vanadate was therefore slower than that by insulin and selenate (Figure 10).

Effect of Insulin on *c-fos* Gene Expression

Insulin stimulated *c-fos* gene transcription in H4IIE cells (Messina, 1990). Insulin treated 3T3-L1 fibroblasts and adipocytes (Stumpo and Blackshear, 1986)

Table 3

Effect of Vanadate on *c-jun* mRNA Level in Primary Rat Hepatocytes in Culture

Treatment	*c-jun* mRNA level as % of control
control	100
V 15'	94.1 ± 8.1 (5)
V 30'	100.1 ± 6.2 (9)
V 1hr	102.8 ± 9.4 (8)
V 3hr	116.9 ± 14.1 (8)
V 6hr	123.2 ± 18.4 (2)
V 12hr	223.4 ± 10.8* (2)

* Significantly different from control, $P < 0.05$.

Primay rat hepatocytes in culture either received no treatment (control) or were treated with vanadate (V) (500 μM) for the designated time. Total cytoplasmic RNA was prepared and Northern blot analysis was performed under "Materials and Methods". The control of each experiment was arbitrarily set to 100 and the change by vanadate treatment was expressed compared to the control value. Data shown are the means ± S.E. for the number of experiments shown in parentheses.

and Chinese hamester ovary cells (Stumpo et al., 1988) also showed rapid accumulation of *c-fos* mRNA. In contrast, insulin treated murine fibroblasts (Greenberg and Ziff, 1984) and PC12 rat pheochromocytoma cells (Kruijer et al., 1985) showed no effect on *c-fos* mRNA levels. Increase of *c-fos* mRNA level was also not found in primary cultures of rat hepatocytes obtained from Fisher/344 rats (Krüijer et al., 1985). In the present study, rat hepatocytes in culture were prepared from Sprague-Dawley rats. Control, untreated cells incubated in serum-free medium did not express any detectable *c-fos* mRNA. This result was in agreement with a previous report (Etienne et al., 1988). When rat hepatocytes were incubated with 160 nM insulin for 5', 10', 15', 30', 1 hr, 3 hr and 6 hr, *c-fos* mRNA was still undetectable. This suggests insulin is not an effective stimulator of *c-fos* gene expression in our model system.

Effect of Selenate on *c-fos* Gene Expression

Since insulin showed no effect on levels of *c-fos* mRNA, and since selenate is an effective insulin-mimetic agent, we expected that selenate would not have any effect on the expresion of *c-fos*. Surprisingly, we found selenate was able to induce *c-fos* mRNA levels in a time-dependent manner. *c-fos* expression was not detectable in control cells maintained in serum-free medium, and it was hardly detectable at 15', 30' and 1 hr after 500 μM selenate treatment. It did, however, become detectable at 3 hr after selenate treatment, and remained so after 6 hr, only decreasing to an undetectable level after 12 hr. Unlike most inducers of *c-fos* expression, the effect of selenate on *c-fos* expression is not transient, as it lasted up to 6 hr. These data suggest that selenate regulates *c-fos* gene expression in a different fashion from insulin. As shown in Figure 12, *c-fos* mRNA was observed as a single band of 2.2kb on Northern blot. The mRNA size was similar to those reported previously (Greenberg and Ziff, 1984).

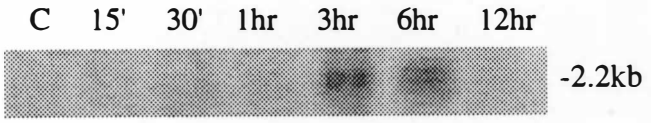

Figure 12. Northern Blot Analysis of *c-fos* mRNA Level in Primary Rat Hepatocytes Treated with Selenate.

Total cytoplasmic RNA from primary rat hepatocytes in culture that either received no treatment (control) or were treated with selenate (500 μM) for the designated time was separated on a formaldehyde-agarose gel, blotted onto "GeneScreen" membrane, and probed with a [α-^{32}P]CTP-labeled *c-fos* cDNA. Control cells were incubated in serum-free medium.

Effect of Vanadate on *c-fos* Gene Expression

Vanadate was shown to stimulate transient expression of *c-fos* in BC_3H1 mouse fibroblasts (Wice et al., 1987) and stimulate *c-fos* mRNA level in C2 myoblasts (Montarus et al., 1988). These studies linked expression of *c-fos* to differentiation and proliferation. In order to evaluate the effect of vanadate on *c-fos* gene expression in our model system, rat hepatocytes in culture were incubated with 500 μM vanadate for 15', 30', 1 hr, 3 hr, 6 hr and 12 hr. Similar to insulin, *c-fos* mRNA was undetectable after treatment of vanadate for the different time points analyzed.

DISCUSSION

Insulin is capable of regulating cellular and metabolic processes as well as gene expression. In recent years, the enthusiam for investigating the effects of insulin-mimetics on cellular processes and regulation of gene expression has increased due to the possible implications in the design of future treatments for diabetes. In fact, vanadate is currently being studied as an orally active replacement for insulin in a NIH-sponsored human clinical trial (Borman, 1995). Berg et al. (Berg et al., 1995) reported that G6PDH mRNA level was induced similarly when streptozotocin-induced diabetic rats were treated with either insulin, selenate or vanadate. These agents have also been shown to induce G6PDH enzyme activity and mRNA level in primary rat hepatocytes in culture (Stapleton et al., unpublished results). The mechanism by which this induction occurs, however, is unknown. There exist three putative AP-1 binding sites in the promoter region of the gene for G6PDH (Rank et al., 1994). *c-jun* and *c-fos* are "immediate-early" genes, whose transcription is rapidly induced, independently of *de novo* protein synthesis, following cell stimulation. Extracellular stimuli include TPA, serum, growth factors, cytokines,and UV irradiation (Angel and Karin, 1991). We hypothesized that the induction of G6PDH by insulin and insulin-mimetics may be possible through these AP-1 binding sites. To examine this hypothesis, the effects of insulin and insulin-mimetics on *c-jun* and *c-fos* mRNA levels in primary rat hepatocytes in culture were determined.

Insulin has been shown to induce changes in the expression of *c-jun* and *c-fos* in defined cellular systems. Insulin can stimulate *c-jun* gene expression in Reuber H-35 cells (Mohn et al., 1990) and NIH 3T3 fibroblasts (Quon et al., 1992). In the present study, we also find insulin induces the level of *c-jun* mRNA. *c-jun* is not

expressed in normal rat liver (Hatayama et al., 1991). However, in rat hepatocytes in culture, an increase in the abundance of *c-jun* mRNA was found within an hour of plating. At 2 hr postplating, *c-jun* expression decreased, and then rised slightly to a plateau and remained at this level throughout a 3-day culture period (Rana et al., 1994). In our study, rat hepatocytes were maintained in serum-free medium (control) for 24 to 48 hours depending on conditions tested. We found that *c-jun* was always minimally expressed in these control cells. Previously it was demonstrated that insulin increased *c-fos* mRNA expression in 3T3-L1 adipocytes and fibroblasts (Stumpo and Blackshear, 1986), Reuber H35 (Taub et al., 1987) and H4IIE (Messina, 1990) rat hepatoma cells, as well as Chinese hamester ovary cells transformed with high levels of the insulin receptor gene (Stumpo et al., 1988). Other reports, however, indicated that insulin failed to stimulate *c-fos* mRNA accumulation in primary cultures of adult rat hepatocytes (Krüijer, 1986) or PC12 rat pheochromocytoma cells (Krüijer et al., 1985). Insulin also failed to stimulate *c-fos* gene transcription in murine fibroblasts (Greenberg and Ziff, 1984). *c-fos* is also not expressed in normal rat liver (Hatayama et al., 1991). But Etienne et al. found that the *c-fos* gene was expressed at a low level 4 hours after rat hepatocytes were plated. *c-fos* transcript disappeared thereafter and was not detectable during the culture time (Etienne et al., 1988). Our results showed that *c-fos* expression was undetectable in hepatocytes maintained in serum-free medium (control) up to 48 hours. We also did not observe stimulation of *c-fos* mRNA accumulation by insulin in our model system. This suggests that the transcriptional programs regulated by cFos are likely to be cell-specific.

A concomitant increase in *c-fos* and *c-jun* mRNA has been shown in response to the addition of EGF to rat-1 cells (McDonnell et al., 1990) and growth hormone to 3T3-F442A cells (Gurland et al., 1990). Our results did not show a parallel increase in *c-jun* and *c-fos* mRNA in cells responding to insulin. The interaction between cJun

and cFos plays an important role in cellular regulation. In the majority of the cells analyzed so far the cFos protein and mRNA have shorter half-lives than the cJun protein and mRNA. Therefore, the composition of the AP-1 complex changes from predominantly Jun homo- and heterodimers (e.g., cJun:JunD), before induction, to mostly Jun:Fos heterodimer immediately after induction, followed by Jun homo- and heterodimer after the Fos proteins have decayed (Angel and Karin, 1988). Since we did not find changes in *c-fos* mRNA after insulin administration, this suggests cFos and cJun may not function in combination in our cells responding to insulin.

Insulin regulates a variety of metabolic actions as well as cell growth and differentiation. Insulin signalling is initiated by activation of the insulin receptor kinase, autophosphorylaton of the receptor β-subunit, and stimulation of phosphorylation of intracellular receptor substrate (Kasuga et al., 1982; White et al., 1985; Rothenberg et al., 1991; Sun et al., 1991). These events are coupled to a series of cytoplasmic protein serine/threonine kinases, such as MAP kinase, S6 kinase and phosphatidylinositol 3-kinase (Ray and Sturgill, 1988; Erikson and Maller, 1989; Bacher et al., 1992). Phosphorylation of transcription factors is an important post-translational modification, since many extracellular stimuli affecting gene expression activate protein kinases (Hunter and Karin, 1992). Insulin stimulates phosphorylation of cJun, cFos and Fos related proteins in adipocytes. This is associated with changes in AP-1 mediated gene expression *in vivo*, suggesting that AP-1 phosphorylation by insulin plays a role in insulin-regulated gene expression (Kim and Kahn, 1994). TRE is the major *cis*-element in the *c-jun* promoter that mediates its induction in response to extracellular stimuli. This TRE differs from the consensus TRE sequence by 1-base pair insertion (Angel et al., 1988). Due to this subtle change, it is more efficiently recognized by cJun·ATF2 heterodimers than by conventional AP-1 complexes (van Dam et al., 1993). Unlike cJun, ATF2 is a constitutively expressed protein. However,

despite its inducible expression, most cell types contain some cJun protein prior to their stimulation. The *c-jun* TRE is constitutively occupied *in vivo* (Rozek and Pfeifer, 1993). Following exposure to stimuli that activate members of the JNK group of MAPKs (Derijard et al., 1994), both cJun and ATF2 are rapidly phosphorylated. JNK is the only protein kinase found to efficiently phosphorylate the N-terminal sites of cJun. Phosphorylation of cJun and ATF2 stimulate their ability to activate transcription, thereby leading to *c-jun* induction. Insulin might activate protein kinases in the signal transduction pathway, which will stimulate phosphorylation of cJun and ATF2, and then lead to induction of *c-jun* expression.

What might be the function of insulin-induced increase in *c-jun* mRNA level in primary rat hepatocytes in culture, which are capable of neither further differentiation or division? The product of the *c-jun* gene encodes a transcription factor thought to be involved in cell proliferation and differentiation (Angel and Karin, 1991). Insulin has growth factor activity (Koontz and Iwahashi, 1981) but also has many effects on fully differentiated tissues (Dentons, 1986). In both cases, it is postulated that the stimulation by insulin of *c-jun* gene expression may mediate insulin's effect on the expression of other genes.

Selenate has been shown to exhibit insulin-like properties in several cell types. In insulin responsive tissues such as rat adipocytes in culture, selenate affects glucose transport and translocation of glucose transporters. But selenate has not been previously shown to stimulate insulin-receptor kinase or inhibit phosphotyrosine phosphatase activity (Ezaki, 1990). In our laboratory, however, preliminary work indicates selenate does increase overall tyrosyl phosphorylation, activate MAP kinase and increase phosphorylation of IRS-1 and the β-subunit of the insulin receptor similarly to insulin. In the present study, selenate was found to induce both *c-jun* and *c-fos* mRNA levels, but the changes in the abundance of these mRNA were somewhat

different. These results may indicate the diference in the turnover rate of the two mRNAs. The maximal increase of *c-jun* with selenate was comparable to the increase we observed with insulin. Previous studies from our lab also showed that the time course of activation of signal proteins by selenate in the insulin signal cascade is slower than that of insulin. Our present data demonstrate that the kinetics of *c-jun* stimulation were identical when cells were treated with either insulin or selenate, suggesting that insulin and selenate share a common pathway to elicit their effects on *c-jun*. Stapleton et al. (Stapleton et al., unpublished results) found that the maximal induction of G6PDH mRNA level occured at 12 hr after treatment by insulin or selenate. The time course of *c-jun* activation by insulin or selenate precedes the maximal induction of G6PDH making it a possible step in the signal transduction pathway to activate gene expression.

Selenate is known as a catalyst of the oxidation of SH groups (Tsen and Tappel, 1958). It is conceivable that the oxidation of SH groups of the insulin receptor causes aggregation of the receptor or conformational changes of the receptor, and this leads to mediate insulin-like effects. Debant et al. (Debant et al., 1989) have reported that receptor cross-linking restores the insulin metabolic effect altered by mutation of the kinase domain of the receptor suggesting the importance of receptor aggregation in signal transduction.

Vanadate has been shown to exert insulin-like effects both *in vitro* and *in vivo*. The mechanism by which vanadate exerts its insulin-like effect is not clearly understood. In adipocytes, vanadate has been shown to enhance glucose transport and glycogen synthesis by stimulating insulin receptor kinase (Tamura et al., 1984; Bernier et al., 1988). Autophosphorylation and tyrosine kinase activity of the isolated insulin receptor can be stimulated by vanadate *in vitro* (Gherzi et al., 1988). However, some investigators have suggested that vanadate could act at the post-insulin receptor level.

In adipocytes and diaphragm it mimics insulin action without having any effect on insulin receptor (Strout et al., 1989; Green, 1986). Blondel et al (Blondel et al., 1990) using diabetic rats did not observe any *in vivo* effect of vanadate on insulin receptor kinase. These data suggest that vanadate and insulin may not share a common pathway in exerting their physiological effects. It was however demonstrated that vanadate phosphorylates and stimulates the two isoforms of MAP kinases in the absence of insulin receptor β-subunit tyrosyl phosphorylation (D'Onofrio et al., 1994). Our data show vanadate to induce a prolonged increase in the amount of *c-jun* mRNA, which reaches maximal levels at 12 hr after treatment. Since vanadate is a potent phosphotyrosine phosphatase inhibitor (Swarup et al., 1982), the mechanism of vanadate action might be due to inhibition of cellular phosphotyrosine phosphatase and activation of specific protein kinases. But whether the tyrosine kinase activity of the insulin receptor is required in the signalling pathway leading to the vanadate-mediated action of *c-jun* expression remains unknown. Other preliminary data from our lab indicate vanadate does stimulate phosphorylation of β-subunit of insulin receptor and IRS-1 and activate MAP kinase in primary rat hepatocytes in culture. The time course of activation of *c-jun* expression by vanadate is slower than that of insulin suggesting perhaps a different mechanism of action.

Vanadium does induce cell proliferation and differentiation. This function is broadly considered a growth factor mimic effect (Stern et al., 1993). In cultured embryonic chicken osteoblasts, insulin-like growth factor I and orthovanadate together stimulate greater [^{3}H]thymidine incorporation than insulin-like growth I alone (Lao et al., 1988). Vanadium also stimulates Na^+, H^+-exchange at micromolar concentration, corresponding to the doses which stimulate DNA synthesis and cell proliferation in human fibroblasts (Jamieson et al., 1988; Cassel et al., 1984), embryonic chicken bone cells (Lau et al., 1988), interleukin-3-dependent mast cells (Tojo et al., 1987) and

Swiss mouse 3T3 cells (Smith, 1983). Vanadate induces transformation in BALB/3T3 cells (Sabbioni et al., 1991), hamester embryo cells (Rivedal et al, 1990), and bovine papilloma virus DNA-transfected $10T_{1/2}$ cells (Kowalski et al., 1992). Since vanadium displays some of the biochemical behavior of growth factors, it would not be surprising that it also modulates protooncogene expression. In BC_3H1 mouse fibroblasts, it stimulated the transient expression of *c-fos* gene (Wice et al., 1987), and it increased the expression of *c-myc* gene in actively dividing humans ovary carcinoma cells (Itkes et al., 1990). The increased *c-jun* expression by vanadate that we observe may also be related to its capacity to mimic mitogenic growth factors. Similarly to insulin, induction of *c-jun* expression by vanadate was not shown in our study.

Although selenium and vanadium are trace elements, the effects of selenate and vanadate we observed were only in the presence of pharmacological concentrations. However, the precise mechanism by which toxic levels of selenuim and vanadium interfere with cellular function is poorly understood.

In conclusion, our results show that, in primary hepatocytes in culture, insulin and selenate increase the *c-jun* mRNA level in a similar time dependent fashion; vanadate also induces *c-jun* mRNA level, but in a different and longer time dependent fashion; selenate is the only agent tested that shows an induction of *c-fos* mRNA level and the time course of induction corresponds with the induction of *c-jun*.

Appendix A

Abbreviations

Abbreviations: AP-1, activator protein-1; ATF2, activating transcription factor 2; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; CREB, cAMP response element binding protein; EGF, epidermal growth factor; ERK, extracellular stimulus responsive kinase; FRK, Fos regulating kinase; Glut-1, glucose transporter 1; G6PDH, glucose-6-phosphate dehydrogenase; Grb2, growth factor receptor bound protein 2; IRS-1, insulin receptor substrate; JNK, Jun N-terminal kinase; MAP, mitogen activated protein kinase; MAPKK, mitogen activated protein kinase kinase; PDGF, platelet-derived growth factor; PI 3-kinase, phosphatidynositol 3-kinase; PKC, protein kinase C; rsk,90KDa ribosomal S6 kinase; S6K, 70KDa ribosomal S6 kinase; SIE, v-*sis* conditioned medium induction element; SIF,v-*sis* inducible factor; SOS, son of sevenless; SRE, serum response element; SRF, serum response factor; STZ, streptozotocin; TCF, ternary complex factor; TFIIB, transcription factor polymerase II B; TFIID, transcription factor polymerase II D; TFIIE, transcription factor polymerase II E; TFIIF, transcription factor polymerase II F; TFIIH, transcription factor polymerase II H; TGF β, transforming growth factor β; TNF α, tumor necrosis factor α; TPA, 12-tetradecanoyl-phorbol-13-acatate; TRE, TPA (12-tetradecanoyl-phorbol-13-acetate) responsive element.

BIBLIOGRAPHY

Abate, C., Marshak, D.R., & Curran, T. (1991). Fos is phosphorylated by $p34^{cdc2}$, cAMP dependent protein kinase and protein kinase C at multiple sites clustered within regulatory regions. Oncogene, 6, 2179-2185.

Adler, V., Franklin C.C., & Kraft, S. (1992). Phorbol esters stimulate the phosphorylation of c-jun but not v-jun: regulation by the N-terminal delta domain. Proceedings of the National Academy of Sciences USA, 89, 5341-5345.

Angel, P., Hattori, K., Smeal, T., & Karin, M. (1988). The *jun* proto-oncogene is positively autoregulated by its own product, Jun/AP-1. Cell, 55, 875-885

Angel, P., & Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochimica et Biophysica Acta, 1071, 129-157.

Backer, J.M., Myer, M.G., Shoelson, S.E., Chin, D.J., Sun, X.Y., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J., & White, M.F. (1992). Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. EMBO Journal, 11, 3469-3474.

Berg, E.A., Wu, J.-Y., Campbell, L., Kagey, M., & Stapleton, S.R. (1995). Insulin-like effects of vanadate and selenate on the expression of glucose-6-phosphate dehydrogenase and fatty acid synthase in diabetic rats. Biochimie (in press).

Bernier, M., Laird, D.M., & Lane, M.D. (1988). Effect of vanadate on the cellular accumulation of pp15, an apparent product of insulin receptor tyrosine kinase action. Journal of Biological Chemistry, 263, 13626-13634.

Birnboim, H.C., & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research, 7, 1513-1519

Blondel, O., Simon, J., Chevalier, B., & Portha, B. (1990). Impaired insulin action but normal insulin receptor activity in diabetic rat liver: effect of vanadate. American Journal of Physiology, 258, E 459-E 467.

Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., & Tjian, R. (1987). Human proto-oncogene *c-jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science, 238, 1386-1392.

Bonnieu, A., Rech, J., Jeanteur, P., & Fort, P. (1989). Requirement for *c-fos* mRNA downregulation in growth stimulated murine cells. Oncogene, 4, 881-888.

Borman, S. (1994). C&EN News, Feb. 21, 35-36.

Bosch, F., Hatzoglou, M., Park, E.A., & Hanson, R.W. (1990). Vanadate inhibits expression of the gene for phosphoenolpyruvate carboxykinase (GTP) in rat hepatoma cells. Journal of Biological Chemistry, 265, 13677-13682.

Boyle, W.J., Smeal, T., Difize, L.H.K., Angel, P., Woodgett, J.R., Karin, M., & Hunter, T. (1991). Activation of protein kinase C decreases phosphorylation of c-Jun at site that negatively regulate its DNA-binding activity. Cell, 64, 573-584.

Brenner, D.A., O'Hara, M., Angel, A.P., Chojkier, M., & Karin, M. (1989). Prolonged activation of jun and collagenase genes by tumor necrosis factor α. Nature, 337, 661-663.

Brichard, S.M. Desbuquis, B., & Girand, J. (1993). Vanadate treatment of diabetic rats reversed the impaired expression of genes in hepatic glucose metabolism-effects on glycolytic and gluconeogenic enzymes, and on glucose transporter GLUT2. Molecular and Cellular Endocrinology, 91, 91-97.

Buratowski, S. (1994). The basics of basal transcription by RNA polymerase II. Cell, 77, 1-3.

Büscher, M., Rahmsdorf, H.J., Litfin, M., Karin, M., & Herrlich, P. (1988). Activation of the *c-fos* gene by UV and phorbol ester: different signal transduction pathways converge to the same enhancer element. Oncogene, 3, 301-311.

Carey, M., Lin, Y., Green, M.R., & Ptashne, M. (1990). A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. Nature, 345, 361-364.

Cassel, D., Zhuang, Y.X., & Glase, L. (1984). Vanadate stimulates Na^+/H^+ exchange activity in A431 cells. Biochemical and Biophysical Research Communication, 118, 675-681.

Challis, K.A.J., Leighton, B., Lozeman, F.T., Budohoski, L., & Newsholme, E.A. (1987). Effects of chronic administration of vanadate to the rat on the sensitivity of glycolysis and glycogen synthesis in skeletal muscle to insulin. Biochemical Pharmacology, 36, 357-361.

Chardin, P., Camonis, J.H., Gale, N.W., Van Aelst, L., Schlessinger, J., Wigler, M.H., & Raesagi, D. (1993). Human Sos1: a guanine nucleotide exchange factor for Ras that binds to Grb2. Science, 260, 1338-1343.

Chen, J., Pederson, D.S. (1993). A distal heat shock element promotes the rapid response to heat shock of the HSP26 gene in the yeast *Saccharomyces cerevisiae*. Journal of Biological Chemistry, 68, 7442-7448.

Chen, Y., Yang, G., Chen, Y., Chen X., Wen, Z., Ge, X. (1980). Studies of selenium and keshan disease. Biological Trace Element Research, 2, 91-107.

Chiu, R., Angel, P., & Karin, M. (1989). Jun-B differs in its biological properties

from, and is a negative regulator of c-Jun. Cell, 59, 979-986.

Chiu, R., Boyle, W.J., Meck, J., Smeal, T., Hunter, T., & Karin, M.(1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell, 54, 541-552.

Chomczynski, P., & Sacchi, N. (1987). Single step method of RNA isolation by guanidium thiocyanate-phenol-chloroform extraction. Analytical Biochemisrty, 162, 156-159.

Crews C.M., & Erikson, R.L. (1993). Extracellular signals and reversible protein phosphorylation: what to mek of it all. Cell, 74, 215-217.

Debant, A., Ponzio, G., Clauser, E., Contrese, J.O., & Rossi, B. (1989). Receptor cross-linking restores an insulin metabolic effect altered by mutation on tyrosine 1162 and tyrosine 1163. Biochemistry, 28, 14-17.

Deng, T., & Karin, M. (1994). c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. Nature, 371, 171-175.

Denton, R.M. (1986). Early events in insulin actions. Advances in Cyclic Nucleotide and Protein Phosphorylation Research, 20, 293-341.

Derijard, B., Hibi, M., Wu, I.-H., Barret, T., Su, B., Deng, T., Karin, M., & Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell, 76, 1025-1037.

Devary, Y., Gottlieb, R. A., Smeal, T., & Karin, M. (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell, 71, 1081-1091.

D'Onofrio, F., Le, M.Q.U., Chiasson, J.-L., & Srivastava, A.K. (1994). Activation of motogen activated protein (Map) kinase by vanadate is independent of insulin receptor autophosphorylation. FEBS Letters, 340, 269-275.

Dubyak, G.R., & Kleinzeller, A. (1980). The insulin-mimetic effects of vanadate in isolated rat adipocytes: dissociation from effects of vanadate as an (Na^+-K^+) ATPase inhibitor. Journal of Biological Chemistry, 255, 5306-5312.

Elliget, K.A., & Kolaja, G.J. (1993). Preparation of primary cultures of rat hepatocytes suitable for in vitro toxicity testing. Journal of Tissue Culture Methods, 8, 1-6.

Erikson, E., & Maller, J.L. (1989). In vivo phosphorylation and activation of ribosomal protein S6 kinase during *Xenopus Oocyte* Maturation. Journal of Biological Chemistry, 264, 13711-13717.

Erikson, R.L. (1991). Structure, expression and regulation of protein kinase involved in the phosphorylation of ribosomal protein S6. Journal of Biological Chemistry, 266, 6007-6010.

Etienne, P.L., Baffet, G., Desvergne, B., Boisnard-Rissel, M., Glaise, D., & Guguen-Guillouzo, C. (1988). Transient expression of *c-fos* and constant expression of *c-myc* in freshly isolated and cultured adult rat hepatocytes. Oncogene Research, 3, 255-262.

Ezaki, O. (1990). The insulin-like effects of selenate in rat adipocytes. Journal of Biological Chemistry, 265, 1124-1128.

Fort, P., Rech. J., Vie, R., Piechaczyk, M., Bonnieu, A., Jeanteur, P., & Blanchard, J.-M. (1987). Regulation of *c-fos* gene expression in hamster fibroblasts: initiation and elongation of transcription and mRNA degradation. Nucleic Acids Research, 15, 5657-5667.

Franza, B. R., Rauscher , F.J., Joseph, S.F., & Curran, T. (1988). The Fos complex and Fos-related antigen recognize sequence elements that contain AP-1 binding sites. Science, 239, 1150-1153.

Gherzi, R., Caratti, C., Andraghetti, G., Bertolini, S., Montemurro, A., Sesti, G., Bertolini, S., Montemurro, A., Sesti, G., & Cordera, R. (1988). Direct modulation of insulin-receptor protein tyrosine kinase by vanadate and anti-insulin receptor monoclonal antibodies. Biochemical and Biophysical Research Communication, 152, 1474-1480.

Gil, J., Miralpeix, M., Carreras, J., & Bartrons, R. (1988). Insulin-like effects of vanadate on glucokinase activity and fructose 2,6-biphosphate levels in the liver of diabetic rats. Journal of Biological Chemistry, 263, 1867-1871.

Gilman, M.Z. (1988). The *c-fos* serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes and Development, 2, 394-402.

Green, A. (1986). The insulin-like effect of sodium vanadate on adipocyte glucose transporter is mediated at a post-insulin-receptor level. Biochemical Journal, 238, 663-669.

Greenberg, M., & Ziff, E.B. (1984). Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. Nature, 311, 433-438.

Gundula, R., Karin, J., Manfred N., Brüller, H.-J., & Müller, R. (1989). Asymmetrical recognition of the palindromic AP-1 binding site (TRE) by Fos protein complexes. EMBO Journal, 8, 3825-3832.

Gurland, G., Ashcom, G., Cochran, B.H., & Schwartz, J. (1990). Rapid events in growth hormone action. Induction of *c-fos* and *c-jun* transcriprion in 3T3-F442A preadipocytes. Endocrinology, 127, 3187-3195.

Hatayama, I., Yamada, Y, Tanaka, K., Ichihara, A., & Sato, K. (1991). Induction of glutathione S-transferase P-form in primary cultured rat hepatocytes by epidermal growth factor and insulin. Japanese Journal of Cancer Research, 82, 827-814.

Hattori, K., Angel, P., LeBeau, M.M., & Karin, M. (1988). Structure and

Hattori, K., Angel, P., LeBeau, M.M., & Karin, M. (1988). Structure and chromosomal location of the functional intronless human *jun* protooncogene. Proceedings of the National Academy of Sciences USA, 85, 9148-9152.

Hayes, T.E., Kitchen, A.M., & Cochran, B.H. (1987). Inducible binding of a factor to the *c-fos* regulatory region. Proceedings of the National Academy of Sciences USA, 84, 1272-1276.

Herr, I., van Dam, H., & Angel, P. (1994). Binding of promoter associated AP-1 is not altered during induction and subsequent repression of the *c-jun* promoter by TPA and UV iradiation. Carcinogenesis, 15, 1105-1113.

Hibi, M., Lin, A., Smeal,T., Minden, A., & Karin, M. (1993). Identification of an oncoprotein and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain . Genes and Development, 7, 2135-2148.

Hirai, S.I., Ryseck, R.P., Mechta, F., Bravo, R., & Yavin, M. (1989). Characterization of junD: a new member of the jun proto-oncogene family. EMBO Journal, 8, 1433-1439.

Hunter, T., & Karin, M. (1992). The regulation of transcription by phosphorylation. Cell, 77, 375-387.

Inoue, H., Kaku, K., & Matsutani, A. (1994). Insulin-like effects of vanadate on rat liver 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase mRNA and protein inductions in diabetic rat. Endocrine Journal, 41, 75-82.

Ish-Horowicz, D., & Burke, J.F. (1981). Rapid and efficient cosmid vector cloning. Nucleic Acids Research, 9, 2989-2995.

Itkes, A.V., Imamova, L.R., Alexandrova, N.M., Favorova, O.O., & Kisselev, L.L. (1990). Expression of *c-myc* gene in human ovary carcinoma cells treated with vanadate. Experimental Cell Research, 188, 169-171.

Jamieson, G.A., Etscheid, B.G., Muldoon, L.L., & Villereal, M.L. (1988). Effects of phorbol ester on mitogen and orthovanadate stimulated responses of cultured human fibroblasts. Journal of Cellular Physiology, 134, 220-228.

Johnson, T.M., Meisler, M.H., Bennett, M.I., & Willsky, G.R. (1990). Vanadate induction of pancreatic amylase mRNA in diabetic rats. Diabetes, 39, 757-759.

Jones, N. (1990). Transcriptional regulation by dimerization: two sites to an incestuous relationship. Cell, 61, 9-11.

Karin, M., & Smeal, T. (1992). Control of transcription factors by signal transduction pathways: the beginning of the end. TIBS, 17, 418-422.

Kasuga, M., Karlsson, F.A., & Kahn, C.R. (1982). Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science, 215, 185-187.

Kim, S.-J. & Kahn, C.R. (1994). Insulin stimulates phosphorylation of c-Jun, c-Fos, and Fos related proteins in cultures adipocytes. Journal of Biological Chemistry, 269, 11887-11892.

Kim, S.Y., Angel,P., Lafyatis, R., Hattori, K., Kim, K.Y., Sporn, M.B., Karin, M., & Roberts, A.B. (1990). Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. Molecular and Cellular Biology, 10, 1492-1497.

Koontz, J.W., & Iwashashi, M.I. (1981). Insulin as a potent specific growth factor in a rat hepatoma cell line. Science, 211, 947-949.

Kowalski, L.A., Tsang, S.-S., & Davison, A.J. (1988). Vanadate enhances transformation of bovine papilloma virus DNA-transfected C3H/101/2 cells. Cancer Letter, 61, 83-90.

Kroczck, R.A., Siebert, E. (1990). Optimization of Northern analysis by vacuum-blotting, RNA-transfer visualization, and ultraviolet fixation. Analytical Biochemistry, 184, 90-95.

Kruijer, W., Schubert, D., & Verma, I.M. (1985). Identification of the proto-oncogene *fos* by nerve growth factor. Proceedings of the National Academy of Sciences USA, 82, 7330-7334.

Kruijer, W., Skelly, H., Botteri, F., Van der Putten, H., Barber, J.R., Verma, I.M., & Leffert, H.L. (1986). Proto-oncogene expression in regenerating liver is stimulated in cultures of primary adult rat hepatocytes. Journal of Biological Chemistry, 261, 7929-7933.

Kuhne, M.R., Pawson,T., Lienhard, G.R., & Feng, G.-S. (1993). The insulin receptor substrate I associates with the Sh2-containing phosphotyrosine phosphatase Syp. Journal of Biological Chemistry, 268, 11479-11481.

Lamph, W.W., Wamsley, P., Sassone-Corsi, P., & Verma, I.M. (1988). Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature, 334, 629-631.

Lao, K.H.W., Tanimoto, H., & Baylink, D.J. (1988). Vanadate stimulates bone cell proliferation and bone collagen synthesis in vitro. Endocrinology, 123, 2858-2867.

Lee, C.H., Li, W., Nishimura, R., Zhou, M., Batzer, A.G., Myers, M.G.Jr., White, M.F., Schlessinger, J., & Skolnik, E.Y. (1993). Nck associates with the Sh2 domain-docking protein IRS-1 in insulin-stimulated cells. Proceedings of the National Academy of Sciences USA, 90, 11713-11717.

Leung, S., & Miyamoto, N.G. (1989). Point mutational analysis of the human *c-fos* serum response factor binding site. Nucleic Acids Research, 17, 1177-1195.

Liao, S.M., Taylor, I.C., Kingston, R.E., Yong, R.A. (1991). RNA ploymerase II carboxy-terminal domain contributes to the response to multiple acidic activators in vitro. Genes and Development, 5, 2431-2440.

Lin, A., Frost, J., Deng, T., Smeal, T., Al-Alawi, N., Kikkawa, U., Hunter, T., Brenner, D., & Karin, M. (1992). Casein kinase II is a negative regulator of c-jun DNA binding and AP-1 activity. Cell, 70, 777-789.

Macara, I.G. (1980). Vanadium-an element in search of a role. Trends in Biochemical Sciences, 5, 92-92.

Malabu, U.H., Dryden, S., Mccarthy, H.D., Kilpatrick, A., & Williams, G. (1994). Effects of chronic vanadate administration in the STZ-induced diabetic rat. Diabetes, 43, 9-15.

McDonnel, S.E., Ker, L.D., & Matrisian, L.M. (1990). Epidermal growth factor stimulation of stromelysin mRNA in rat fibroblasts requires induction of proto-oncogene *c-fos* and *c-jun* and activation of protein kinase C. Molecular and Cellular Biology, 10, 4284-4293.

McKeehan, W.L., & Ham, R.G. (1976). Selenium is an essential trace nutrient for growth of Wi-38 diploid human fibroblasts. Proceedings of the National Academy of Sciences USA, 73, 2023-2027.

McNeill, J.H., Delgatty, H.L.M., & Battell, M.L. (1991). Insulin-like effects of sodium selenate in streptozotocin-induced diabetic rats. Diabetes, 40, 1678-1678.

Messina, J.L. (1990). Insulin's regulation of *c-fos* gene transcription in hepatoma cells. Journal of Biological Chemistry, 265, 11700-11705.

Miralpiex, M., Decaux, J.-F., Kahn, A., & Bartrons, R. (1991). Vanadate induction of L-type pyruvate kinase mRNA in adult hepatocytes in primary culture. Diabetes, 40, 462-467.

Miralpeix, M., Gil, J., Rosa, J.L., Carreras, J., & Bartrons, R. (1989). Vanadate counteracts glucagon effects in isolated rat hepatocytes. Life Sciences, 44, 1491-1497.

Mohn, K.L., Laz, T.M., Melby, A.E., & Taub, R. (1990) Immediate-early gene expression differs between regenerating liver, insulin-stimulated H-35 cells, and mitogen-stimulated Balb/c 3T3 cells. Journal of Biological Chemistry, 265, 21914-21912.

Montarras, D., Pinset, D., Dubois, C., Chenevert, J., & Gros, F. (1988). High levels of *c-fos* mRNA accumulation is not obligatory for renewed cell proliferation. Biochemical and Biophysical Research Communications, 153, 1090-1096.

Mountjoy, K.G., & Flier, J.S. (1990). Vanadate regulates glucose transporter (Glut-1) expression in NIH 3T3 mouse fibroblast. Endocrinology, 126, 2778-2787.

Murakami, Y., Satake, M., Yamaguchi-Iwai, Y., Sakai, M., Muramatusu, M., & Ito, Y. (1991). The nuclear protooncogene *c-jun* and *c-fos* are regulators of DNA replication. Proceedings of the National Academy of Sciences USA, 88, 3947-3951.

Nakabeppu, Y., Ryder, K., & Nathans, D. (1988). DNA binding of three murine Jun proteins: stinulation by Fos. Cell, 55, 907-915.

Ong, J., Yamashita, S., & Melmed, S. (1987). Insulin-like growth factor I induces *c-fos* messenger ribonucleic acid in L6 rat skeletal muscle cells. Endocrionology, 120, 353-357.

Pennypacker, K.R., Hong, J.-S., & McMillian, M.K. (1994). Pharmacological regulation of AP-1 transcription factor DNA binding activity. FASEB Journal, 8, 4 75-478.

Pettersson, M., Schaffner, W. (1990). Synergistic activation of transcription by multiple-binding sites for NF-kappa B even in the absence of cooperative factor binding to DNA. Journal of Molecular Biology, 214, 373-380.

Pillary, T.S., & Makgoba, M.W. (1992). Enhancement of epidermal growth factor (EGF) and insulin-stimulated tyrosine phosphorylation of endogenous substrates by sodium selenate. FEBS, 308, 38-42.

Pronk, G.J., McGlade, J., Pelicci, G., Pawson, T., & Bos, J.L. (1993). Insulin-induced phosphorylaiton of the 46- and 52-kDa Shc protein. Journal of Biological Chemistry, 268, 5748-5753.

Puvverer, B.J, Kyriakis, J.M.,Avruch, J., Nikolakaki, E., & Woodgett, J.R. (1991). Phosphorylation of c-Jun mediated by MAP kinases. Nature, 353, 670-674.

Quon, M.J., Cama, A., & Taylor, S.I. (1992). Postbinding characterization of five naturally occuring mutations in the human insulin receptor gene: impaired insulin-stimulated *c-jun* expression and thymidine incorporation despite normal receptor autophosphorylation. Biochemistry, 31, 9947-9945.

Radler-Pohl, A., Sachsenmaier, C., Gebel, S., Auer, H.-P., Bruder, J.T., Rapp, U., Angel, P., Rahmsdorf, H.J. & Herrich, P. (1993). UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-kinase. EMBO Journal, 12, 1005-1012.

Rana, B., Mischoulon, D., Xie, Y.-H., Bucher, N.L.R., & Farmer, S. (1994). Cell-extracellular matrix interactions can regualte the switch between growth and differentiation in rat hepatocytes: reciprocal rexpression of C/EBPα and immediately-early growth response transcription factor. Molecular and Cellular Biology, 14, 5858-5869.

Rank, K.B., Harris, P.K., Ginsberg, L.C., & Stapleton, S.R. (1994). Isolation and sequence of a rat glucose-6-phosphate dehydrogenase promoter. Biochimica et Biophysica Acta, 1217, 90-92.

Ray, L.B., & Sturgill, T.W. (1988). Characterization of insulin-stimulated microtubule-associated protein kinase. Journal of Biological Chemistry, 263, 12721-12727.

Razokis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Eatzer, A., Thomas, S., Brugge, J., Pelicci, P.G., Schlessinger, J., & Pawson, T. (1992). Association of the Shc and Grb2/Sem5 Sh2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinase. Nature, 360, 689-692.

Rivedal, E., roseng, L.E., & Sanner, T. (1990). Vanadium compounds promote the induction of morphological transformation of hamster embryo cells with no effect on gap junctional communication. Cell Biology Toxicology, 6, 303-314.

Rivera, V.M.m, & Greenberg, M.E. (1990). The ups and downs of fos regulation. The New Biologists, 2, 751-757.

Rothenberg, P.L., Lane, W.S., Backer, J.M., White, M.F., & Kahn, C.R. (1991). Purification and partial sequence analysis of pp185, the major cellular substrate of the insulin receptor tyrosine kinase. Journal of Biological Chemistry, 266, 8302-8311.

Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A. B., Hafeman, D.G., & Hoekstra, W.G. (1973). Selenuim: biochemical roles as a component of glutathione peroxidase. Science, 179, 588-590.

Rozek, D., & Pfeifer, G.P. (1993). In vivo protein-DNA interactions at the *c-jun* promoter preformed complexes mediate the UV response. Molecular and Cellular Biology, 13, 5490-5499.

Ryseck, R.P., Hirai, S.I., Yaniv, M., & Bravo, R. (1988). Transcriptional activation of c-jun during the G_0/G_1 transttion in mouse fibroblasts. Nature, 334, 535-537.

Sabbioni,E., Pozzi, G., Pintar, A., Casella, L., & Garrantini, S. (1991). Cellular retention, cytotoxicity and morphological transformation by vanadium (IV) and vanadium (V) in BALB/3T3 cell lines. Carcinogenesis, 12, 47-52.

Sassone-Corsi, P., Sisson, J.C., & Verma, I.M. (1988a). Transcriptional autoregulation of the proto-oncogene *fos*. Nature, 334, 314-319.

Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P.L., & Verma, I.M. (1988b). Inducton of proto-ongene *fos* transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. Genes and Development, 2, 1529-1538.

Schönthal, A., Gebel, S., Stein, B., Ponta, H., Rahmsdorf, H.J., & Herrich, P. (1988). Nuclear oncoproteins determine the genetic program in response to external stimuli. Cold Spring Harbor Symposia on Quantitative Biology, 53, 779-787.

Schütte, J., Minna, J.D., & Birrer, M.J. (1989a). Deregulated expression of human c-jun transforms primary rat embryo cells in cooperation with an activated c-Ha-ras gene and transforms rat-1 cells as a single gene. Proceedings of the National Academy of Sciences USA, 86, 2257-2261.

Schütte, J., Viallet, J., Nace, M., Segal, S., Fedorko, J., & Minna, J. (1989b). *Jun-B* inhibits and *c-fos* stimulates the transforming and trans-activating activities of *c-jun*. Cell, 59, 987-997.

Shaw, P.E., Frasch, S., & Nordhein, A. (1989). Repression of *c-fos* transcription is mediated through $P67^{srf}$ bound to the SRE. EMBO Journal, 8, 2569-2574.

Shaw, G., & Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell, 46, 659-663.

Shecher, Y., Karlish, S.J.D. (1980). Insulin-like stimulation of glucose oxidation in rat adipocytes by vanadyl (IV) ions. Nature, 248, 556-558.

Shyu, A.-B., Greenberg, M. E., & Belasco, J.G. (1989). The *c-fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes and Development, 3, 60-72.

Skar, R. & Serck-Hanssen, G. (1993). IGF-I-induced *c-fos* and *c-jun* expression in bovine chromaffin cells in primary culture. Annals of the New York Academy of Sciences, 692, 305-307.

Skolnik, E.Y., Lee, C.H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Myers, M.G.Jr., Backer, J.M., UUrich, A., White M.F., & Schlessinger, J. (1993). The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS-1 and Shc: implications for insulin control of ras signalling. EMBO Journal, 12, 1929-1936.

Smeal, T., Binetruy, B., Mercola, D., Birrer, M., & Karin, M. (1991). Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serine 63 and 73. Nature, 354, 494-496.

Smith, J.B. (1983). Vanadium ions stimulate DNA synthesis in Swiss mouse 3T3 and 3T6 cells. Proceedings of the National Academy of Sciences USA, 80, 6162-6166.

Stapleton, S.R., Stevens, G.J., Teel, J.F., Rank, K.B., Berg, E.A., Wu, J.Y., Ginsberg, L.C., & Kletzien, R.F. (1993). Effects of acetaldehyde on glucose-6-phosphate dehydrogenase activity and mRNA levels in primary rat hepatocytes in culture. Biochimi, 75, 971-976.

Stein, B., Angel, P., van Dam, H., Ponta, H., Herrich, P., & van der Eb, A. (1992). Ultraviolet irradiation induced *c-jun* gene transcript: two AP-1 like binding sites mediate the response. Photochemistry and Photobiology, 55, 409-415.

Stern, A., Yin, X.-F., Tsang, S.-S., Davison, A., & Moon, J. (1993). Vanadium as a modulator of cellular regulatory cascades and oncogene expression. Biochemistry and Cell Biology, 71, 103-112

Strout, H.V., Vicario, P.P., Saperstein, R., & Slater, E.E. (1989). The insulin-

mimetic effect of vanadate is not correlated with insulin receptor tyrosine kinase activity nor phosphorylation in mouse diaphragm in vivo. Endocrinology, 124, 1918-1924.

Stumpo, D.J., & Blackshear, P.J. (1986). Insulin and growth factor effects on *c-fos* expression in normal and protein kinase C-deficient 3T3-L1 fibroblasts and adipocytes. Proceedings of the National Academy of Sciences USA, 83, 9453-9457.

Stumpo, D.J., Stewart, T., Gilman, M., & Blackshear, P.J. (1988). Identification of *c-fos* sequences involved in induction by insulin and phorbol esters. Journal of Biological Chemistry, 263, 1611-1614.

Sun, X.Y., Rotherberg, P., Kahn, C.R., Backer. J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J., & White, M.F. (1990). Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature, 352, 73-77.

Sutherland, J.A., Cook, A., Bannister,A.J., & Kouzarides, T. (1992). Conserved motifs in Fos and Jun define a new class of activation domain. Genes and Development, 6, 1810-1819.

Swarup, G., Cohen, S., Garbers, D.L. (1982). Inhibition of membrane phosphotyrosine-protein phosphatase activity by vanadate. Biochemical and Biophysical Research Communication, 107, 1104-1109.

Tamura, S., Brown, T.A., Whipple, J.H., Fujita-Yamaguchi, Y., Dubler, R.E., Cheng, K., & Larner, J. (1984). A novel mechanism for the insulin-like effect of vanadate on glucogen synthase in rat adipocytes. Journal of Biological Chemistry, 259, 6650-6658.

Taub, R., Roy, A., Dieter, R., & Koontz, J. (1987). Insulin as a growth factor in rat hepatoma cells: stimulation of proto-oncogene expression. Journal of Biological Chemistry, 262, 10893-10897.

Tobe, K., Matuoka, K., Tametome, H., Ucki, K., Kaburagi, Y., Asai, S., Noguchi, T., Matsuda, M., Tanaka, S., Hattori, S., Fukui, Y., Akanuma, Y., Yazaki, Y., Takenewa, T., & Kadowaki, T. (1993). Insulin stimulates association of insulin receptor substrate-1 with the protein abundant src homology/growth factor receptor-bound protein 2. Journal of Biologcal Chemistry, 268, 11167-11171.

Tojo, A., Kasuga, M., Urabe, A., & Takaku, F. (1987). Vanadate can replace interleukin 3 for transient growth of factor-dependent cells. Experimental Cell Research, 171, 16-23.

Tolman, E.L., Barris, E., Burns, M., Pansini, A., & Partridge, R. (1979). Effects of vanadium on glucose metabolism in vitro. Life Sciences, 25, 1159-1164.

Triesman, R. (1985). Transient accumulation of *c-fos* RNA following serum stimulation requires a conserved 5' element and *c-fos* 3' sequences. Cell, 42, 889-902.

Triesman, R., (1992). The serum response element. TIBS, 17, 423-426.

Tsen, C.C. & Tappel, A.L. (1958). Catalytic oxidation of glutathione and other sulhydryl compounds by selenate. Journal of Biological Chemistry, 233, 1230-1232.

Valera, A., Rodriguez-Gil, J.E, & Bosch.F. (1993). Vanadate treatment restores the expession of genes for key enzymes in the glucose and ketone bodies metabolism in liver of diabetic rats. Journal of Clinical Investigation, 92, 4-11.

van Dam, H., Offringa, R., Meijer, I., Stein, B., Smit, A.M., Herrlich, P., Bos, J.L., & van der Eb, A.J. (1990). Differential effects of the adenovirus E1A on members of the AP-1 transcription factor family. Molecular and Cellular Biology, 10, 5857-5864.

von Hippel PH. (1994). Protein-DNA recognition: a new perspectives and underlying themes. Science, 263,769-770.

Wagner, B.J., Hayes, T.E., Hoban, C.J., & Cochran, B.H. (1990). The SIF binding element confers sis/PDGF inducibility onto the *c-fos* promoter. EMBO Journal, 9, 4477-4484.

Webb, D.K., Moulton, B.C., & Khan, S.A. (1990). Estrogen induced expression of the *c-jun* proto-oncogene in the immatute and mature rat uterus. Biochemical and Biophysical Research Communication, 168,721-726.

Weinstock, K.S, & Messina, J.L. (1992). Vanadate and insulin stimulate gene 33 expression. Biochemical and Biophysical Reseacrh Communication, 189, 931-937.

White, M.F., Maron, R., & Kahn, C.R. (1985). Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. Nature, 318, 183-186.

Wice, B., Milbrandt, J., & Glaser, L. (1987). Control of muscle differentiation in BC_3H1 cells by fibroblast growth factor and vanadate. Journal of Biological Chemistry, 262, 1810-1817.

Wilson, T., & Triesman, R. (1988). Fos-terminal mutations block down-regulation of *c-fos* transcription following serum stimulation. EMBO Journal, 7, 4193-4198.

Woodgett, J.R. (1990). Fos and jun: two into one will go. Seminars in Cancer Biology, 1, 389-397.

Yamauchi, K., & Pessin, J.E. (1994). Insulin receptor substrate-1(IRS-1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signalling. Journal of Biological Chemistry, 269, 31107-31114.

Yang-Yen, H.F., Chiu, R., & Karin,M. (1990). Too many transcription factors positive and negative interactions. The New Biologists, 2, 351-361.