To the Graduate Council:

I am submitting herewith a thesis written by Richard S. Peterson entitled "Molecular Mechanism of Insulin Regulated Tyrosine Aminotransferase Expression in the Cultured Rat Hepatoma Cell Line, Fao." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

John W. Koontz

John W. Koontz, Major Professor

We have read this thesis
and recommend its acceptance:

Wesley D. Wick

Wm Dougall

Accepted for the Council:

C W Minkel

Vice Provost
and Dean of the Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are permissible, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services, when in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Richard S Peterson

Date July 29, 1988

MOLECULAR MECHANISM OF INSULIN REGULATED TYROSINE AMINOTRANSFERASE EXPRESSION IN THE CULTURED RAT HEPATOMA CELL LINE, FAO

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Richard S. Peterson

August 1988

ABSTRACT

The molecular mechanism(s) of induction of tyrosine aminotransferase (TAT) by insulin was studied in the well-differentiated rat hepatoma cell line Fao. Incubation of Fao with insulin resulted in a 1.5-fold increase in TAT activity. The effect of insulin on TAT activity was maximal at 5 hr. In the presence of the transcriptional inhibitor DRB, insulin-stimulated TAT induction is only partially inhibited. At 4 hr following addition, DRB inhibits insulin-mediated induction of TAT activity 40%. In the same experiment DRB inhibited dexamethasone-mediated induction of TAT activity 95%. Results of Northern blot analyses indicate a 2.6-fold maximal increase in TAT mRNA abundance between 4 - 8 hr following the addition of insulin. At early time points following the addition of db-cAMP and insulin together to the Fao cells, there is a greater increase in TAT mRNA levels than with db-cAMP treatment alone. Treatment of Fao cells with the phorbol ester TPA resulted in a rapid increase in the accumulation of TAT mRNA that is maximal 1 hr following treatment. This was followed by a rapid decay in TAT mRNA abundance to below basal levels at 4 hr with a slow recovery approaching basal levels 8 hr following treatment. Pretreatment of Fao cells for 16 hr with excess TPA did not affect the capability of insulin to induce TAT mRNA levels. These studies suggest that insulin may stimulate TAT activity by affecting post-transcriptional processes. The author hypothesizes that the increased TAT activity in Fao cells following the addition of insulin is primarily a result of a selective stabilization of the TAT transcript combined with increased protein synthesis and decreased degradation of the short half-life aminotransferase.

TABLE OF CONTENTS

CHAPTER PAGE

I. INTRODUCTION....1

Insulin....1

Polypeptide Hormones....1

Hormone Action....2

Insulin Receptor....2

Second Messengers....5

Insulin Receptor Tyrosine Kinase....5

Insulin's Metabolic Effects....7

Growth Factors....7

Molecular Mechanism of Insulin Signaling....8

Inositol-glycans....8

Proteoglycans....9

Protein Kinase C....10

Inositol-1.4.5 triphosphate (IP3)/Ca++ Pathway....11

Regulation of Gene Expression -

The Nucleus as a Target of Insulin Action....12

Experimental Model System Justification....12

The In Vitro Model System

for Studies of Insulin Action....12

Tyrosine Aminotransferase....14

Research Objectives and Strategy....17

Relevance....17

Objectives....17

Strategy....18

II. EXPERIMENTAL PROCEDURES....20

Materials....20

Preparation and Handling of Compounds Evaluated....20

Cell Culture....21

TAT Assay....22

Total RNA Isolation....22

RNA Fractionation and Hybridization....23

Evaluation of the Effectiveness of DRB-Inhibition of Transcription....24

III RESULTS....25

Effect of Insulin on TAT Activity....25

Effect of Transcription Inhibitor DRB on Insulin-Stimulated TAT Induction....26

Effect of Insulin on TAT mRNA Abundance....27

Effect of Insulin on cAMP Mediated Increases in TAT mRNA....27

Effect of the Phorbol Ester TPA on TAT mRNA Abundance....28

IV. DISCUSSION....29

LIST OF REFERENCES....36

APPENDIX....46

VITA....73

LIST OF FIGURES

FIGURE PAGE

1. Fractionation of Total RNA on a 1.1% Agarose Denaturing Gel.....49

2. Typical Autoradiograph of a Northern Blot.....51

3. Insulin Time Course and Dose Response on TAT Activity in DMEM Medium.....53

4. Insulin Time Course and Dose Response on TAT Activity in RPMI Medium.....55

5. Effect of DRB on [3H]-Uridine Incorporation into TCA Precipitable Molecules.....57

6. DRB Inhibition of [3H]-Uridine Incorporation into TCA Insoluble Macromolecules.....59

7. Effect of the Transcription Inhibitor DRB on Insulin-Stimulated TAT Activity.....61

8. Effect of Insulin on TAT mRNA Abundance in Fao over an 8 Hour Period.....63

9. Insulin Dose Response on TAT mRNA Abundance in Fao 6 Hours After Treatment..... 65

10. Effect of Insulin on the cAMP-Mediated Increases in TAT mRNA in Fao Cells.....67

11. Time Course of the Effect of 1.6 uM TPA Treatment on TAT mRNA Abundance in Fao Cells.....69

12. Effect of TPA Pretreatment on TAT mRNA Induction by Known Inducers.....71

LIST OF TABLES

TABLE PAGE

1. Time and Dose Dependent Effects of Insulin on TAT Activity in DMEM Medium....47

2. Time and Dose Dependent Effects of Insulin on TAT Activity in RPMI Medium....48

CHAPTER I

Introduction

I. Insulin

Insulin is a polypeptide hormone that is synthesized and secreted by the beta cells of the islets of Langerhans in the endocrine tissue of the pancreas. Structurally insulin is a small protein with a molecular weight of 5808 (human insulin). The mature hormone is composed of two amino acid chains, containing 21 and 30 residues , connected to each other by disulfide linkages. When the two amino acid chains are split apart the functional activity of the insulin molecule is lost. Target tissues for insulin action include the liver, muscle, and peripheral tissues. Functionally the primary roles of insulin are in metabolic regulation and growth promotion (1).

II. Polypeptide Hormones

The mechanism of insulin action is only partly understood. Like other peptide hormones and neurotransmitters, insulin initiates its characteristic biological effects through interaction with specific receptors located on the plasma membrane of target cells (2). The receptor serves two purposes: the first is to recognize the hormone among all other substances in the blood by binding it with high affinity and specificity; the second is to generate a transmembrane signal that allows intracellular information transduction (3).

III. Hormone Action

There are three general mechanisms of intracellular information transfer by hormones, each characterized by the extent to which the hormone itself can penetrate the plasma membrane and reach the nucleus (4). Thyroid hormones enter the nucleus in a free form (106). Steroid hormones also enter the nucleus but as a hormone-receptor complex (HRC) after an initial interaction with specific receptor proteins located in the cytoplasm (98). The peptide hormones and catecholamines are believed not to enter the target cell (107). They interact with specific receptor proteins on the surface of the cell, and other molecules are required to transmit the hormone's information from the plasma membrane to the nucleus.

Unlike thyroid and steroid hormones, which only appear to exert effects on the nucleus leading to alterations in gene expression, the polypeptide hormones primarily affect general biochemical processes in the cytoplasm and other cell compartments (5). In general, polypeptide hormones are responsible for coordinating very short-term changes in metabolism rather than initiating long-term, complex programs of growth and differentiation. Insulin is apparently unique in that metabolic (hormonal) and growth-promoting (mitogenic) effects are mediated through the high affinity insulin receptor (6).

IV. Insulin Receptor

The insulin receptor from liver cells, as well as from a variety of cell types, has been isolated and studied biochemically. It is composed of two distinct subunits each specialized to perform the two major functions of the receptor. The α-subunit (M_r=135,000) contains the insulin-binding domain (7). In intact

cells it is sensitive to proteolytic cleavage suggesting a major extracellular domain (8). In contrast, the ß-subunit (M_r=95,000) is a transmembrane protein which contains an insulin-sensitive protein kinase believed to be involved in signal transduction across the membrane (see below) (9). Both subunits are glycoproteins with complex carbohydrate side chains. Under nonreducing conditions, the α- and ß-subunits of the receptor form disulfide-linked hetero-oligomers. The most commonly depicted model is an α2ß2 heterotetramer (10). The number of receptors per liver-cell plasma membrane is approximately 250,000-300,000 (108).

Insulin bound to its receptor is rapidly internalized by receptor-mediated endocytosis (11). The role of internalization in insulin action is not clear. Insulin receptors are present on some intracellular organelles including the nucleus (12). Translocation of occupied cell surface insulin receptors to the nucleus may be a mechanism which mediates insulin's long term effects (92). In support of this signaling mechanism, D.S. Miller recently has reported an increase in RNA and protein synthesis in stage IV oocytes from *Xenopus laevis* upon microinjection of insulin into the cytoplasm or treatment of isolated nuclei (14). Most cell endosomes containing the insulin-receptor complex fuse with lysosome structures resulting in the rapid degradation of the peptide (11). The receptor may be degraded or recycled back to the membrane (13). It is likely that the primary function of internalization is for clearing the cell of hormone-receptor complexes but it may also serve to bring the activated insulin receptor kinase, or the peptide hormone itself, to a location where it can act on intracellular substrates.

A characteristic property of plasma-membrane insulin receptors is the low percentage occupancy required to generate a maximal biological response. In general 1-10% occupancy of the insulin receptor is sufficient to trigger short-term metabolic responses (15). This means a full insulin-mediated metabolic response can be triggered by only 2500 molecules of hormone occupied receptor.

V. Second Messengers

Since the polypetide hormones bind only to surface receptors, information transfer to the nucleus and other cell compartments must be mediated by intracellular molecules. Also, since maximal biological responses can be generated when only 1% of the target cell surface receptors are occupied by hormone, some mechanism for amplifying the signal must exist. The concept of an intracellular second messenger, a small molecule that can be synthesized rapidly in large quantities in response to a hormone trigger, became established with the discovery of cyclic AMP (cAMP). Studies of cAMP activation of cAMP-dependent protein kinase have led to an understanding of the physiological importance of reversible covalent modification (eg. phosphorylation) of key regulatory enzymes and reaction cascades in metabolic regulation (16). Protein kinases are enzymes involved in the transfer of phosphate groups from ATP to amino acid residues of proteins. A cascade amplifies the effect of the original signal with each additional step incorporated into a cascade leading to an exponential increase. With the exception of insulin, prolactin, and growth hormone, all of the polypeptide hormones that have been examined use cAMP as a second messenger (17).

In general, extracellular regulators, upon binding to specific membrane receptors, stimulate the formation of second messengers (cAMP, cGMP, Ca, IP_3, diacylglycerol, etc.) or their liberation from intracellular stores. The second messengers then further transduce the extracellular signals by acting on target molecules such as protein kinases. These enzymes, upon activation, catalyze the phosphorylation of a large number of cellular proteins. This process is considered to be one of the most important mechanisms in the control of many cellular functions including metabolism, differentiation, growth, and transformation (18). Several distinct protein kinases are present in all cell systems including several for which no physiological activator has been defined. Two prominent classes are grouped into cyclic nucleotide (cAMP, cGMP) and Ca (Ca-calmodulin, Ca-phospholipid)-dependent enzymes according to the second messenger which controls the activity of the individual protein kinases (19).

VI. Insulin Receptor Tyrosine Kinase

The insulin receptor itself has been found to be an insulin-sensitive tyrosine-specific protein kinase (ATP phosphotransferase) (20). Immediately following insulin binding to the receptor there occurs an autophosphorylation reaction leading to incorporation of phosphate groups onto the ß-subunit of the receptor. The effect is maximal within one minute with a 3- to 10-fold increase in receptor phosphorylation. Addition of anti-insulin antibodies to remove cell-bound insulin results in a rapid dephosphorylation of receptor indicating the cell possesses active phosphatases accessible to the receptor (21). Insulin stimulates *in vitro* tyrosine phosphorylation of receptors purified to near homo-

geneity and the receptor itself possesses an ATP binding site both of which would seem to indicate that the tyrosine phosphorylation is due to a kinase activity intrinsic to the receptor itself (22). In addition, insulin receptor can serve as a substrate for cellular serine- or threonine-specific protein kinases (eg. Protein Kinase C) (23).

Accumulated evidence indicates that activated ß-subunit tyrosine kinase is required for intracellular signaling events mediating a number of insulin's actions. If lysine 1030 in the tyrosine kinase-ATP binding domain is replaced by methionine, arginine, or alanine, tyrosine kinase activity is lost, as is insulin-stimulated 2-deoxyglucose transport, enhanced glycogen synthesis, thymidine incorporation into DNA, and ribosomal polypeptide S6 activation (24). In it's activated state the insulin receptor tyrosine kinase may phosphorylate other serine- and threonine-specific protein kinases thereby activating them, thus providing a potential amplification mechanism as well as phosphorylating other endogenous protein substrates. Phosphorylation of these residues is likely to induce conformational changes in the protein molecule because the phosphate group introduces both charge changes and steric hinderance. Such a conformation-induced change of state is the basis of the action of phosphorylation and other forms of covalent modification (109).

Polypeptides that have been identified with increased phosphorylation on tyrosine residues as a result of insulin action include a 185,000-M_r polypeptide by White et al. (25), a 110,000-M_r polypeptide by Rees-Jones and Taylor (26), and a low molecular weight (15,000-M_r) polypeptide by Bernier et al. (27). It has been suggested that phosphorylation of the 15,000-M_r peptide on tyrosine may control glucose transport (27).

VII. Insulin's Metabolic Effects

Insulin controls carbohydrate, lipid, and protein metabolism in target tissues by regulating the phosphorylation of serine and threonine residues in key target enzymes. For example, acetyl-CoA carboxylase (28), ATP-citrate lyase (29), ribosomal protein S6 (30), and the structural protein microtubule-associated protein 2 (MAP 2) (31) are phosphorylated on serine or threonine as a result of insulin treatment.

Also a specific serine kinase shown to be activated by insulin is ribosomal protein S6 kinase. The work of Trevillyan *et al.* (30) and Cobb and Rosen (32) has clearly demonstrated that multiple phosphorylations of S6 occur and lead to enhanced ribosomal translation.

Additionally, there are at least five proteins where the extent of phosphorylation of serine and theronine is decreased with insulin action (33). In general, biosynthetic enzymes such as glycogen synthase and pyruvate dehydrogenase are activated, whereas degradative or gluconeogenic enzymes such as triglyceride lipase, phenylalanine hydroxylase, and pyruvate kinase are inactivated by dephosphorylation. Also, phosphoprotein phosphatase inhibitor 1 is dephosphorylated and becomes inactive. The result is a more active phosphoprotein phosphatase (34).

VIII. Growth Factors

The exact role of the tyrosine kinase activity of the insulin receptor in mediating insulin action is still currently under investigation. Much data has been accumulated on the functional consequences of enhanced receptor

tyrosine kinase activity, but there is no evidence thus far linking this activity with the cascade of serine and threonine phosphorylation/dephosphorylation reactions described above. Several factors suggest that activation of the receptor tyrosine kinase is the initial event in insulin action. Aside from insulin and ATP binding, the tyrosine kinase activity is the only known intrinsic activity of the receptor. The activity appears on an intracellular domain of the receptor directly following insulin-receptor interaction. The kinetics of phosphorylation and dephosphorylation of the receptor are consistent with the time course of insulin action (21). Also, tyrosine protein kinase activity is a relatively rare event in cells, being a property of proteins that are important in regulation of cellular growth or metabolism. The growth-promoting action of insulin is important during fetal growth and organogenesis, and tissue repair and regeneration. To date the only known proteins with tyrosine kinase activity are receptors for other growth factors including epidermal growth factor (EGF) (35), platelet-derived growth factor (PDGF) (36), insulin-like growth factor I (IGF-I) (37), and the gene products of several of the transforming RNA viruses (38).

IX. Molecular Mechanism of Insulin Signaling:

Inositol-glycans. Recently a novel mechanism by which proteins are anchored to membranes has been elucidated. Involved is a covalent linkage from the protein to an oligosaccharide which is in turn glycosidically linked to phosphatidylinositol. The resulting class of membrane glycophospholipids, termed glycosyl-phosphatidylinositols, have been implicated in the transmembrane signaling function of insulin (39). In BC3H1 cells, insulin-receptor interaction leads to a rapid, dose-dependent activation of a distinct and

selective phosphatidylinositol-phospholipase C (PI-PLC) that hydrolyzes the glycosyl-phosphatidylinositol with formation of an inositol-glycan and 1,2-diacylglycerol (40). While the precise molecular events involved in the activation of the hormone-sensitive PLC are unknown, evidence suggests a role for a guanine-nucleotide binding protein (G-protein) as an intermediate in coupling of the insulin receptor to the PLC. In support of this putative mechanism insulin-stimulated [^{3}H]myristate diacylglycerol production can be completely blocked by treatment of BC3H1 cells with pertussis toxin (41).

The inositol-glycan regulates the activities of several insulin-sensitive enzymes in cell-free assays and intact adipocytes (42). Included is the activation of a high-affinity cAMP-phosphodiesterase (43) and pyruvate dehydrogenase (44), and the inhibition of phospholipid methyltransferase (45), adenylate cyclase (44), and catecholamine-stimulated lipolysis (46). Based on accumulated evidence it seems likely that the inositol-glycans are involved in mediating a subset of the pleiotropic actions of insulin.

Proteoglycans. While the existence of glycosylated species of phosphatidylinositol on the inner leaflet of the plasma membrane has clear ramifications in insulin action (see above), utilization of similar lipids on the outer cell surface may confer upon a covalently linked protein unique physical properties or susceptibility to hormonal regulation. M.G. Low and A.R. Saltiel recently published a comprehensive review on the structural and functional roles of glycosyl-phosphatidylinositol-anchored proteins in membranes which includes a proposed role in insulin action (47).

The glycosyl-phosphatidylinositol-anchored proteins would not be subject to the physical constraints of most cell-surface proteins which require

anchoring by a short, hydrophobic transmembrane region linked to a cytoplasmic domain. By contrast, the glycosyl-phosphatidylinositol anchor is located entirely within the outer leaflet of the bilayer. Functionally they would allow increased lateral mobility since there is no cytoplasmic domain and cytoskeleton interaction (47).

Evidence indicates the involvement of endogenous, anchor-specific phospholipases in the release of glycosyl-phosphatidylinositol-anchored proteins into the extracellular environment (48). Once released, the proteins could behave as paracrine or autocrine factors. Studies indicate a placenta-derived 34-kD polypeptide with autocrine growth factor properties is initially anchored to the membrane via phosphatidylinositol (49). Similarly, heparin sulfate proteoglycan was released from a hepatocyte line in response to exogenously applied PI-PLC or insulin (50). The released form of the heparin sulfate proteoglycan behaved as an autocrine growth factor in the hepatocyte line as a result of its specific internalization, which occurs at binding sites recognizing the inositol-phosphate moiety of the released proteoglycan. Accumulated evidence is consistent with a general scheme of release, binding, and internalization of proteoglycan (51, 52).

Protein Kinase C. A consequence of the hydrolysis of glycosyl-phosphatidylinositols is the generation in the plasma membrane of 1,2-diacylglycerols (by phospholipase C digestion) or phosphatidic acid (by phospholipase D digestion). 1,2-diacylglycerols are endogenous activators of the calcium- and phospholipid-dependent protein kinase C. The activities of kinase C are known to have direct affects on cellular metabolism, differentiation, and growth (53).

The glycosyl-phosphatidylinositols involved in insulin action appear to have fatty acid compositions that are different from free or protein- anchoring phosphatidylinositols (54). Distinctive 1,2-diacylglycerol species result from the PLC-catalyzed hydrolysis of distinct substrates and it may be that these provide differential regulation of protein kinase C with respect to the extent of activation, substrate specificity, compartmentalization, or susceptibility to proteolytic activation, or are specifically directed to one of the isoforms of the enzyme (55). These possibilities might explain the perplexing relationship between protein kinase C and insulin action reported by P.J. Blackshear et al. (56, 57), in which protein kinase C activators such as phorbol esters mimic some, but not all, of the effects of insulin.

Inositol-1,4,5 triphosphate (IP3)/Ca++Pathway. An insulin mediated phospholipase C hydrolysis of polyphosphoinositides can result in the transient appearance of inositol-1,4,5 triphosphate (IP_3) (53). The generation of intracellular IP_3 results in a transient increase in the intracellular concentration of Ca^{++} ions. This increase is generated by IP_3 mediated release of Ca^{++} from intracellular pools (eg. endoplasmic reticulum) and increased Ca^{++} flux across the plasma membrane. Calmodulin is a calcium-binding protein sensitive to changes in calcium concentration. The increased binding of calcium to calmodulin results in a conformational change in the molecule thereby increasing it's affinity for a number of intracellular protein targets. A number of proteins, many of them protein kinases, have been shown to be activated by calcium-calmodulin. The ability of calcium-calmodulin to activate a rat liver nuclear protein kinase, casein kinase II, provides this second-messenger system with the ability to effect changes in gene expression (58).

X. Regulation of Gene Expression - The Nucleus as a Target of Insulin Action

Recent investigations with cDNA probes have demonstrated that binding of insulin to its receptor enhances or decreases the transcription of specific mRNAs. A rat liver-specific mRNA coding for a polypeptide termed p33 has been shown to increase 5- to 10-fold in a time- and concentration-dependent manner following insulin treatment (59). As little as 1 pM insulin was effective in 30 min, suggesting the effect was mediated through the high-affinity insulin cell surface receptor. Studies of the p33 mRNA stability demonstrated a short half-life of 20-40 min, which was not detectably altered by insulin. The effect of insulin was completely blocked by two RNA synthesis inhibitors, actinomycin D and 5,6-dichloro-1-ß-D-ribofuranosyl benzimidazole (DRB). The accumulated data indicates that insulin acts to increase transcription, i.e., mRNA synthesis or processing.

Insulin has also been demonstrated to negatively regulate the transcription of specific genes. Treatment of cultured H4-II-E rat hepatoma cells with insulin causes a large, specific decrease in cytoplasmic serum albumin mRNA (110). The effect is observed at low doses of insulin (ED_{50} = 2 pM), consistent with the effect being mediated by interaction of insulin with high affinity insulin receptors. Insulin reduces transcription of the albumin gene 4.7-fold, as measured by nuclear transcription assays (110).

XI. Experimental Model System Justification:

The *In Vitro* Model System for Studies of Insulin Action. Studies in our laboratory are performed on rat hepatoma cell lines descended from the Reuber

H35 chemically-induced rat hepatoma. The Reuber H35 rat hepatoma has proven to be a valuable tissue culture system in which to study hormone action. The H35 cell line possess several insulin-responsive functions including insulin binding, insulin-stimulated receptor phosphorylation, glycogen synthesis, tyrosine aminotransferase induction, amino acid transport, increased RNA synthesis, and cell growth (60).

A subline of H35 cells, KRC7, has been characterized for insulin responsiveness. Under serum-deprived conditions KRC7 cells become quiescent, arresting in the early G_1 phase of the cell cycle. Insulin alone at physiological concentrations has been demonstrated to be a potent, specific growth ("progression") factor inducing cell proliferation (6). Further studies have indicated the insulin effects are mediated by the high-affinity insulin receptor and not the IGF-II (MSA) receptor (Note: The IGF-I (somatomedin C) receptor is not present in this clone) (61). The chemically transformed KRC7 appear to be "competent", meaning they do not reside in the G_0 state and therefore have no requirement for a competence factor, i.e. Platelet Derived Growth Factor (PDGF), to enter the cell cycle. Evidence in support of this conclusion is the observed difference for KRC7 and regenerating hepatocytes between the initiating event (i.e. insulin addition or hepatectomy) and the beginning of S-phase. The lag time is much shorter in KRC7 (8 Hrs) than in the liver (15 - 18 Hrs) (6,100). Since the timing of the early response, i.e. c-*myc* induction is identical, this difference suggests the KRC7 may progress through the later phases of G_1 more quickly than liver cells. Thus, deregulation of normal processes in these tumorigenic cells likely occurs during this "progression" phase of the growth cycle.

Treatment of KRC7 with physiological concentrations of insulin inhibits transcription of the tyrosine aminotransferase (TAT) gene in a time- and dose-dependent manner (62). Insulin also inhibits the stimulatory effect of glucocorticoids and cAMP on TAT mRNA levels (62). The KRC7 clone does not synthesize serum albumin; a function associated with the adult, fully differentiated liver. Based on these observations it can be hypothesized the KRC7 subclone represents a fetal phenotype and can serve as a model in studies of the regenerating liver.

The pedigree of hepatoma clones derived from the original Reuber H35 rat hepatoma is well outlined in a report by J. Deschatrette and M.C. Weiss (81). Fao was derived from a population of Faza 967 cells exposed to 2mM ouabain. The Fao clone can grow in glucose-free media, constitutively secretes serum albumin, is positive for four adult liver-specific enzymes, demonstrates insulin stimulation of glycogen synthase and TAT, and glucocorticoid induction of TAT (60). The Fao clone appears to represent a well-differentiated, adult phenotype.

Tyrosine Aminotransferase. Tyrosine aminotransferase (TAT; EC 2.6.1.5) is a liver-specific enzyme that catalyses the rate-limiting step in tyrosine catabolism thereby providing ketogenic and gluconeogenic substrates. Specifically, the enzyme catalyses the transfer of the amino group of tyrosine to a-ketoglutarate with pyridoxal 5'phosphate acting as a cofactor. The product, p-hydroxyphenylpyruvate, is then metabolized to fumarate and acetoacetate through four discrete, enzymatic reactions (63).

To date, no investigator has demonstrated that covalent modification or allosteric effects contribute to the regulation of TAT. Therefore, alterations in catalytic specific activity always parallel changes in the amount of enzyme.

Data accumulated thus far indicate the activity of TAT is regulated solely through changes in rate of synthesis and/or degradation (64).

The level of TAT can be altered by a variety of hormones including insulin, adrenal glucocorticoids, growth hormone, and glucagon and catecholamines (the latter two using cAMP as their intracellular second messenger) (4). Production of the enzyme and its' mRNA is low throughout gestation; increasing abruptly after birth (65). Both the enzyme and its mRNA undergo rapid intracellular turnover (66). Developmental and tissue-specific expression of the aminotransferase gene has been shown to be under control of trans-acting regulatory genes (67). These observations, coupled with the demonstration that lines of tissue culture cells derived from chemically-induced rat hepatomas can serve as valid *in vitro* models for the study of *in vivo* biochemical processes have allowed investigators to gain an increasingly better understanding of the diverse, concerted regulatory mechanisms for TAT gene expression.

The ease with which it can be assayed, coupled with the rapidity and the magnitude of its response to inducers, has made TAT one of the most studied enzymes in animal cells. Examples of phenomena which have been described and which are currently being actively investigated utilizing TAT expression in a rat hepatoma cell culture system as the model include: mechanism of action of hormones, neonatal development and differentiation of a hormone-responsive protein, dedifferentiation as a consequence of malignant transformation, the presence and function of cytoplasmic regulatory factors which affect the synthesis of an enzyme, and the role chromatin modification plays in modulating hormone action on enzyme synthesis (68).

The structural gene encoding rat TAT has been isolated and characterized (70). The gene extends over 11 Kb and is interrupted by 11 introns. The largest exon is a 1.0 Kb segment located at the 3' end. Apparently the gene encodes many untranslated sequences since only 1400 nucleotides of the 2400 base TAT mRNA are required to encode the 53,000 dalton TAT protein. The enzyme exists as a homodimer with subunit molecular weights of 53,000 (70). TAT contains two covalent modifications being both phosphorylated (71) and acetylated (112). The enzyme responsible for the phosphorylation does not appear to be a cyclic AMP-dependent kinase (71). Restriction mapping of independently isolated TAT clones from a genomic library indicate the rat TAT gene is a single copy gene (111). To allow sequence comparisons with other hormonally controlled genes and the design of chimeric genes for functional studies, a region which includes 800 bases upstream of the 5' end of the TAT gene has been sequenced. The start site of transcription has been determined by nuclease S1 mapping (70).

TAT represents only about 0.03% of the total hepatic cytosolic protein content, and after maximal induction is no greater than 0.5% (72). The enzyme is degraded with a half-life of 2-3 hours. It is, therefore, quite labile when compared to other cytosolic proteins, whose average half-life is about 48 hours (101). In general, the time required for transcription and processing of poly (A) containing mRNAs in eukaryotic cells has been estimated to be approximately 30 minutes. The TAT transcript half-life is 2 hours. The ribosomal transit time for TAT in H35 hepatoma cells in culture is 5-8 minutes (69).

XII. Research Objectives and Strategy:

Relevance. A question of fundamental importance is how the insulin-mediated nuclear signaling mechanism affects expression of specific genes. The pathway responsible for transducing the signal generated by the activated insulin receptor tyrosine kinase to the transcriptional machinery within the nucleus is presently unknown. It is plausible, however, that the control regions of insulin-modulated genes contain a common *cis* -acting DNA element (enhancer) that acts in a manner similar to the responsive elements that confer induction by glucocorticoids (73), heavy metal ions (74), double-stranded RNA (75), serum (76), phorbol esters (77), and cAMP (78). Enhancers are *cis* -regulatory elements composed of a modular arrangement of short DNA sequence motifs each with a specific function in conferring inducibility, tissue specificity, or a general enhancement of transcription (105). These motifs are binding sites for nuclear proteins (*trans* -acting factors). The result of this specific interaction is a significant potentiation of transcription from RNA polymerase II-transcribed promoters. It is postulated that one mechanism by which enhancers serve to regulate gene expression at the level of transcription is by inducing a conformational modification in chromatin-nuclear matrix topology. The result is helix destablization exposing single-stranded DNA for which RNA polymerases have a high affinity. If insulin-responsive *cis* -control elements exist, they may be recognized by a common *trans* -acting effector whose activity or abundance is modulated by insulin-receptor interaction.

Objectives. The goal of my research was to determine the molecular mechanism of insulin induction of TAT activity in the Fao subclone. This information will be valuable to the goals of our laboratory. If, in the Fao clone,

TAT activity is increased by a transcriptional activation; we will have in our hands two rat hepatoma clones derived from the same tumor that diverge in some postreceptor step(s) which may ultimately prove useful in dissecting the pathways of insulin action.

Studies were carried out to investigate the effects of cAMP on insulin action by following effects on TAT mRNA production over time. Also evaluated were the effects of a potent activator of protein kinase C, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), on TAT activity and mRNA. These experiments were designed to investigate if insulin's action on gene expression in Fao is mediated, in part, by PK-C.

Strategy. The strategy included the characterization of the insulin induction of TAT enzyme activity. Time course and dose response relationships for insulin action have been determined . The use of the transcription inhibitor, 5,6 Dichloro-1-ß-D-Ribofuranosyl Benzimidazole (DRB) together with insulin, allowed determination of the requirement for concomitant transcription for induction of TAT activity. Northern blot analysis of an experiment examining insulin time course and dose response relationships, using a full-length cDNA probe, has allowed quantitation of insulin's effect on abundance of TAT mRNA.

To determine if cAMP modifies insulin action on TAT in Fao cells, we have studied the effect of a cAMP analog alone and in the presence of insulin on TAT activity and TAT mRNA. Insulin was either added together with cAMP or at designated times after cAMP treatment of cells.

Experiments were carried out to examine the effects of the phorbol ester TPA, a known activator of a potential mediator of insulin (i.e. Protein Kinase C), on TAT mRNA levels for comparison to that of insulin-mediated effects.

Investigated was the possibility that insulin mediates its affects on the nucleus through Protein Kinase C (PK-C). Fao cells were first desensitized to activation of PK-C by a 16 hr pretreatment in excess TPA. Cells were then treated with insulin or TPA and TAT mRNA levels were examined.

CHAPTER II

EXPERIMENTAL PROCEDURES

I. Materials

Porcine insulin (lot # 7RH87D) was obtained from Elanco Products Co., Indianapolis, IN. Cell culture medium, Dulbecco's modified Eagle's medium (DMEM), was from Gibco, Grand Island, NY. Calf serum and fetal bovine serum were products of M.A. Bioproducts, Walkersville, Ma. Fe-supplemented calf serum was obtained from Hyclone Laboratories, Inc., Logan, Utah. Cell culture dishes were from Corning Glass Works, Corning, NY or Costar, Cambridge, MA. Guanidine thiocyanate was purchased from Research Organics, Cleveland, Ohio, agarose from FMC Corp., Rockland ME, cesium chloride from Bethesda Research Laboratories (BRL), Bethesda, Ma., formamide from Fisher Scientific, Fairlawn, NJ, [γ-32P] dCTP from ICN Radiochemicals, Irvine, CA. All other chemicals were of analytical grade and were obtained from Sigma Chemicals or Fisher Scientific.

II. Preparation and Handling of Compounds Evaluated

Insulin was prepared as a 1 mg/ml stock in 0.1 M HCL, quantitated at OD_{276} where an absorbance of 1.05 = 1 mg/ml Insulin, and stored at 4°C. Immediately prior to treatment, a 1uM stock solution was prepared in 1.0 mM HCl.

Dexamethasone was prepared as a 100 uM solution in serum-free DMEM medium and stored in 1 ml aliquots at -20°C. Final concentration used in the induction experiments was 1 uM.

Dibutyryl - cAMP was prepared as a 20 mM stock solution in serum-free DMEM medium and stored at -20°C. The final concentration used for induction was 500 uM (71).

Due to its limited solubility, 5,6 Dichloro-1-ß-D-ribofuranosyl benzimidazole (DRB) was prepared at its treatment concentration, 35 uM, in serum-free medium. 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was prepared as a 1.6 mM stock solution in 95% ethanol. The maximal final treatment concentration with TPA was 1.6 uM. In these experiments, control dishes received 0.095% ethanol.

III. Cell Culture

Fao is a well-differentiated subclone derived from the Reuber H35 rat hepatoma (81). Fao cells were from Dr. Kahn's laboratory, being obtained through Dr. Rebecca Taub at the Elliot P. Joslin Research Laboratory, Harvard Medical School, Boston, MA. The cells were stored in liquid nitrogen, being thawed and placed into culture immediately prior to the initiation of these experiments.

The cells were maintained by weekly serial passage in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and 5% calf serum or 10% Fe-supplemented calf serum. HEPES buffer was added to a final concentration of 25 mM, pH 7.4. The cells were maintained at 37°C in a humidified atmosphere enriched with 10% CO_2. For enzyme analyses, cells were cultured on 20, 35, or 60 mm dishes. For total RNA preparation, cells were cultured on 100 mm dishes. Upon reaching confluency serum-containing medium was removed by aspiration and replaced with serum-

free medium (SFM) for at least 16 hr and no greater than 23 hr prior to addition of the various compounds.

IV. TAT Assay

Cells were harvested by first washing twice with ice-cold phosphate buffered saline (PBS) (0.9% NaCl, 0.2% KCl, 20 mM K_2HPO_4, pH 7.2) and then scraped directly into 1 ml of a harvest buffer (85 ml PBS, 10 ml 0.1 M α-ketoglutarate, 4 ml (40 ug/ml) pyridoxal phosphate (B_6), and 0.1% NP-40). The cell suspension is throughly mixed and then centrifuged at 4°C for 10 min at 15000 xg. An aliquot of the supernatant fraction was taken for the measurement of TAT activity by the method of Diamondstone (82). One unit of TAT activity catalyzed the formation of 1 uM of p-hydroxyphenylpyruvate/min at 37°C. Protein was determined according to the method of Lowry (83).

V. Total RNA Isolation

Total RNA was prepared by the guanidinium thiocyanate-cesium chloride method of Chirgwin (84). Treated cells were washed 3 x with ice-cold PBS and dissolved in 4 M guanidinium thiocyanate. The suspension was layered over a 5.7 M cesium chloride cushion and centrifuged for 18 hr at 36,000 rpm at 20°C. The resulting total RNA pellet was dissolved in 0.1% diethylpyrocarbonate (DEPC)-H_2O, ethanol/salt precipitated, and dissolved in DEPC-H_2O for storage at -20 C. RNA was quantitated by measuring OD_{260} of the solution using an absorbance of 1.000 as equal to 40 ug/ml RNA.

VI. RNA Fractionation and Hybridization

Fifteen micrograms of total RNA was separated by electrophoresis in a 1.1% agarose gel containing 6% formaldehyde and capillary blotted onto nitrocellulose paper using 20X SSC (175.3 g NaCl/liter, 88.2 g NaCitrate/liter, pH 7.2) as the transfer buffer (85). Visualization was achieved by staining with ethidium bromide, destaining and illumination by short-wavelength ultraviolet irradiation. The nitrocellulose blots were visually examined for RNA transfer, 28 and 18 S ribosomal bands were delineated for future reference (Figure 1), the blots were air-dried and baked in a vacuum oven at 80°C for 1-1.5 hours to ensure irreversible RNA binding (113).

The blots were incubated overnight in a rotary oven at 42°C in a prehybridization solution containing 50% formamide, 10X Denhardt's (0.2 % ficoll, 0.2 % polyvinylpyrrolidone, 0.2 % BSA Pentax fraction V), 5X SSPE (0.1875 M NaCl, 6.425 mM NaH_2PO_4, 1.375 mM EDTA, pH 7.4), 100 ug/ml denatured salmon sperm DNA, and 0.1% SDS (85). The full-length cDNA probe, pTAT 123 (86), was nick-translated, using a nick translation kit obtained from Promega Biotec, to a specific activity of $2.5 \times 10^7 - 10^8$ cpm/ug with [γ-32P]CTP. The denatured labeled probe was then added to the blot in the prehybridization solution and allowed to hybridize for an additional 16 hours. The blot was then put through a series of washes of increasing stringency to eliminate any non-specific binding of the probe. Washes 1 and 2 were with 2x SSC and 0.1% SDS for 30 min at room temperature. Washes 3 and 4 (if necessary) were with 0.1 X SSC and 0.1% SDS for 30 min at 55°C (85). The damp blot was prepared for autoradiography by being wrapped in plastic wrap, sandwiched between Kodak XRP film and a Cronex intensifying screen, and stored in a casette at -70°C. The film was developed and analyzed as de-

scribed below. If necessary the blot was put back on film and stored for a different length of time to attain greater or less exposure. See Figure 2 for a typical autoradiograph.

The relative TAT mRNA levels were quantitated by scanning desitometry of autoradiographs, basal mRNA levels being arbitrarilly set at 1 unit. An LKB model 2222-010 Ultroscan XL laser densitometer was used for analysis. To ensure a linear relationship between densitometery values and quantities of TAT mRNA varying amounts (ranging from 2 - 16 ug) of total RNA from cells treated with known TAT mRNA inducers were analyzed by Northern dot-blot analysis. Multiple exposures of each autoradiograph were performed to ensure linearity of response.

VII. Evaluation of the Effectiveness of DRB-Inhibition of Transcription

Prior to treatment Fao cells were placed on SFM for 16 hr. Cells were treated with DRB at varying concentrations. After 1 hr, 5 uCi [^{3}H]-uridine was added to each well and incubated an additional 30 min. Medium was removed by aspiration and the cells were then washed 2x with cold PBS. 1 ml of 5% trichloroacetic acid (TCA) was then added and mixed on a gyro shaker for 15 min. Aliquots were then removed for counting acid-soluble radioactivity. After removal of the TCA-soluble fraction, acid-insoluble macromolecules remaining in the culture dish were dissolved in 1 ml 0.3 M NaOH and an aliquot was removed for counting. A 100 ul aliquot from each fraction was added to 3 ml of a triton-toluene scintillation cocktail for counting.

CHAPTER III

RESULTS

I. Effect of Insulin on TAT Activity

Time course and dose response relationships for the effect of insulin on TAT activity and mRNA levels in the Fao rat hepatoma were established first (Table 1 and Figure 3). Insulin stimulated TAT activity maximally at 5 hr (Figure 3). At early times a concentration-dependent response can be observed. The higher insulin concentrations (10 nM and 100 nM) result in a rapid induction while the response with 1.0 nM insulin is seen to lag. The lowest insulin concentration that gave a maximal response was 10 nM. This is in contrast to a previous report by Crettaz, *et al.* (80), where 100 nM insulin concentration was required for maximum response. 100 nM insulin gave no greater response than 10 nM insulin. Also, Crettaz,*et al.* reported a 2-fold induction in TAT activity (80) however, we observed a 1.5-fold increase with our culture conditions. As indicated below, we investigated this discrepancy by attempting to duplicate the previous investigator's culture conditions.

To duplicate these conditions we grew the Fao cells in the less-rich RPMI medium. These conditions resulted in lower basal TAT activities (compare the zero insulin values in Tables 1 and 2). We observed maximal TAT activity with 10 nM insulin (Figure 4). Higher concentrations of insulin did not accelerate the rate of increase or give a greater maximal response. This agrees with our observations using DMEM as the growth medium. Consistent with both culture conditions is the early dose-dependent response. The higher insulin con-

centrations (10 nM and 100 nM) resulted in maximal induction between 3 and 4 hr following treatment while 1.0 nM insulin gave maximal induction 4 - 5 Hr following treatment (Figure 4). Crettaz, *et al.* report a maximal 2.0-fold induction however, in our hands, insulin only gives a 1.5-fold induction in the RPMI medium.

Comparison of the results in DMEM medium vs. RPMI medium indicates that the induction of TAT in RPMI medium is apparently more transient than in DMEM medium. The molecular basis for this difference remains to be established.

II. Effect of Transcription Inhibitor DRB on Insulin-Stimulated TAT Induction

5,6-Dichloro-1-ß-D-Ribofuranosyl-Benzimidazole (DRB) is an inhibitor of RNA synthesis that acts by increasing the rate of premature chain termination (114). In Fao cells 35 uM DRB inhibited the incorporation of ^{3}H-uridine into acid precipitable material by greater than 70% (Figures 5 and 6). In the presence of 35 uM DRB insulin-stimulated TAT induction is only partially inhibited (Figure 7). At 4 hr following addition, 35 uM DRB inhibits the insulin-mediated induction of TAT activity by 40%. In this same experiment DRB was seen to inhibit dexamethasone-mediated induction of TAT activity 95%. From this result it can be concluded that insulin-stimulated increases in TAT activity are mediated, at least in part, through a post-transcriptional mechanism. This observation is consistent with earlier reports that provide evidence for an insulin-mediated post-transcriptional increase in TAT activity (80, 110). Since, in the presence of DRB, there is no lag in the insulin-mediated TAT induction; it

can be concluded that a post-transcriptional mechanism is responsible for the initial induction of TAT activity.

Theis conclusion is dependent upon how rapidly DRB affects transcription. DRB was added immediately prior to insulin addition. If the onset of action of DRB was delayed we may observe no effect for some time. However, it has been reported that DRB is effective within 2 min of addition (114).

III. Effect of Insulin on TAT mRNA Abundance

Further studies were performed to determine if the insulin stimulation of TAT activity requires a concomitant increase in abundance of TAT mRNA. Our results using Northern blot analysis indicate a 2.6-fold maximal increase in TAT mRNA abundance between 4 - 8 hr following the addition of 10 nM insulin (Figure 8). The effect of insulin on TAT activity appears to precede the increase on TAT mRNA by approximately 60 minutes. Time points beyond 8 hr were not investigated.

The dose response relationship for insulin increasing TAT mRNA abundance was investigated next. The effect of a 6 hr incubation of Fao cells in the presence of insulin resulted in an increase in TAT mRNA abundance with increasing concentrations (Figure 9). 10 nM - 100 nM insulin was sufficient for maximal induction.

IV. Effect of Insulin on cAMP Mediated Increases in TAT mRNA

Either the cAMP analog db-cAMP or insulin can induce increased levels of TAT mRNA (78). At early time points following the addition of db-cAMP and

insulin together to the Fao cells there was a greater increase in TAT mRNA levels than with db-cAMP alone (Figure 10). Within 3 - 4 hr following the addition of insulin the TAT mRNA levels were approximately the same as in the presence of db-cAMP alone These observations provide further support for a post-transcriptional mechanism for insulin action as will be discussed.

V. Effect of the Phorbol Ester TPA on TAT mRNA Abundance

Treatment of Fao cells with the phorbol ester TPA resulted in a rapid increase (maximal 1 hr following treatment) in the accumulation of TAT mRNA (Figure 11). This is followed by a rapid decay to below basal levels (4 hr after treatment) and a slow return toward basal levels 8 hr after treatment.

Incubation of Fao cells for 16 hr with excess TPA has been reported to down-regulate the TPA receptor (i.e. PK-C) (56). Under these conditions insulin is still able to increase TAT mRNA (Figure 12). These results indicate that the pathway for insulin-mediated effects on gene expression is independent of PK-C.

CHAPTER IV

Discussion

The molecular mechanism(s) involved in the insulin-mediated induction of TAT activity in Fao cells appears to be complex. Our studies indicate that insulin may stimulate TAT activity by affecting both transcriptional and post-transcriptional processes.

Insulin treatment alone induces the accumulation of TAT mRNA in a time- and dose-dependent manner (Figure 8). Three mechanisms can account for this insulin-mediated increase in TAT mRNA abundance. These include an increase in the transcription rate of the TAT gene, an increase in TAT mRNA stability, and factors such as hn-RNA processing that result in increased steady-state levels of the TAT transcript.

Other investigators have reported that insulin stimulates increases in TAT mRNA. Lee *et al.* (87) concluded that the induction of TAT by insulin in adrenalectomized, adult male Sprague-Dawley rats was primarily a consequence of a selective increase in the rate of transcription of the aminotransferase gene. They found that in response to insulin TAT mRNA increased quickly, reaching a maximum of 4-fold above basal levels within 1 hr following treatment and declining to basal level at 3 hr (87). This correlated with a 4-fold increase in the rate of transcription of the TAT gene which preceded the increase in mRNA levels by 15 - 30 min. Transcription then returned to the uninduced rate by 2 hr.

Crettaz, *et al.* (80) conclude that insulin increases TAT activity in Fao cells by at least two distinct mechanisms. Evidence for the first mechanism was that insulin appeared to increase TAT activity and TAT mRNA by a stimulation of the rate of transcription of the TAT gene (80). Nuclei isolated from cells treated for 2 hr with 100 nM insulin exhibited a 1.5 - 1.7-fold higher transcriptional rate of the TAT gene when compared to nuclei from control cells (80). We have not tested this. Unlike the experiments of Lee, *et al.* (87), Crettaz, *et al.* (80) neglected to include an internal standard, a constitutively expressed gene that is not induced in the presence of insulin, in their experiments on the rate of transcription. Consequently, they did not eliminate the possibility of an insulin-mediated rapid and transient increase in generalized transcription. The evidence for a second mechanism was that in the presence of glucocorticoids insulin increases TAT activity but not TAT mRNA, suggesting an insulin effect at a post-transcriptional level.

Our results are consistent with the hypothesis that insulin exerts at least some of its positive effects on TAT mRNA levels in Fao cells by increasing its stability. In support of our observations recent investigations with L_6A_1 myoblast cells demonstated an insulin-mediated selective stabilization of the hormone-induced creatine phosphokinase (CK) mRNA (89). Upon removal of the hormone (de-induction), a selective and rapid degradation of CK mRNA occured. If during this de-induction period insulin was added back to the cultures CK mRNA levels began to rise again. The effect of adding cycloheximide was the same as adding back insulin during de-induction. The transcriptional inhibitor actinomycin D also caused the arrest of CK mRNA degradation. The investigators suggest that "superinduction" and "de-induction"

are two facets of the same phenomenon of specific mRNA stablization. Their findings suggest the involvement of short-lived protein(s) in regulating the stability of induced mRNAs.

The very high degree of specificity associated with a selective degradation of only a few mRNAs may be determined by the information within the mRNA itself, since a repeated AU-rich motif specified by the sequence UUAUUUAU has been identified in the 3' untranslated region of mRNAs coding for a number of proteins involved in the inflammatory or proliferative responses (102). Such an AU-rich sequence has been identified in the 3'-untranslated region of the TAT mRNA (103).

The resulting greater increase in TAT mRNA levels in Fao cells when treated with db-cAMP and insulin together as compared to db-cAMP treatment alone (Figure 10) provides evidence in support of a post-transcriptional mechanism for the insulin-mediated enhanced abundance of TAT mRNA in Fao cells. cAMP is known to increase TAT mRNA levels by selectively increasing the rate of transcription of the TAT gene (116). Insulin, when added together with db-cAMP, greatly enhances the db-cAMP-induced levels of TAT mRNA 1 - 3 hr following treatment (Figure 10). This effect is apparent up to 3 hr following treatment after which the TAT mRNA levels in the presence of either db-cAMP alone or db-cAMP and insulin together are approximately the same. These results suggest that the insulin-mediated enhancement of TAT mRNA levels may be through a different mechanism then the transcriptional activation known to take place in the presence of db-cAMP.

In the presence of DRB, insulin-stimulated TAT induction is only partially inhibited (Figure 7). This result is consistent with the idea that insulin-stimulated

increases in TAT activity are mediated, at least in part, through a post-transcriptional mechanism(s). This observation is also consistent with earlier reports that provide evidence for an insulin-mediated post-transcriptional increase in TAT activity (80,91). Since, in the presence of DRB, there is no lag in the insulin-mediated induction of TAT activity; it suggests that a post-transcriptional mechanism(s) is responsible for this initial increase. This is corroborated by the studies on TAT mRNA transcript levels. As indicated earlier, these levels do not increase significantly until 3 - 4 hr after the addition of insulin, at a time when activity is almost maximally elevated.

There are many post-transcriptional processes that may be positively effected as a result of insulin treatment. Insulin action may lead to accelerated HnRNA processing to mature mRNA. Transport of the transcript from the nucleus to the site of protein synthesis may be facilitated. Studies demonstrate an insulin-stimulation of a nuclear envelope nucleoside triphosphatase (NTPase) that provides the energy for mRNA effux into the cytoplasm (92). The translatability of selected transcripts may be affected. Measurements of ribosomal transit times for TAT mRNA indicate that this process is selectively increased by insulin treatment (93).

The phorbol ester TPA is a stable structural analog of diacylglycerol that is often used to study the relationship of Protein Kinase C (PK-C) to a given cellular function. TPA has been shown to exert several insulinomimetic actions suggesting a common activation of one or more distal pathways. Several observations indicate that the initially independent signaling pathways of insulin and activators of PK-C may ultimately converge at the level of gene expression. Both TPA and insulin stimulate increases in ornithine decarboxylase mRNA (57)

and a rat liver-specific mRNA coding for a polypeptide termed p33 (59) while also inhibiting cAMP-induced increases in phosphoenolpyruvate carboxykinase activity and mRNA (104). Also, although they appear to act by different initial mechanisms and their responses are additive in 3T3-L1 cells, insulin and TPA required the same intact sequences in the c-*fos* serum response element (SRE) (bases -299 to -320, a region of dyad symmetry with properties of a viral enhancer) in order to induce c-*fos* transcription (94). Insulin-stimulated c-*fos* transcription is the earliest transcriptional response to insulin reported. The insulin induction of c-*fos* may be the first physiological response in the nucleus of target tissues. c-*fos* may then affect later insulin-stimulated transcriptional responses. Data identifying the c-*fos* protein as a *trans* -acting regulator of gene transcription are fairly conclusive (95). It may be that the *trans* -acting effectors(s) involved in the insulin-stimulated and PK-C-mediated transcription of c-*fos* are identical.

Interestingly, a sequence from -351 to -337 in the human c-*fos* gene has been shown to bind a Platelet Derived Growth Factor (PDGF)-inducible factor in BALB/c-3T3 cells (96). This factor is not induced by insulin or TPA. Serum is a complex mixture of potentially inducing compounds which includes PDGF and insulin . Whereas insulin activates c-*fos* only through a PK-C- and cAMP-independent pathway, PDGF activates c-*fos* expression by PK-C-dependent and -independent pathways as well as by cAMP-dependent pathways (115).

A recent report provides evidence for the accumulation of autocrine factor(s) that are released extracellularly by Reuber H35 or Fao hepatoma cells when maintained in serum-free culture. An autocrine factor partially purified from conditioned medium was demonstrated to stimulate DNA synthesis and

activate acetyl-coenzyme A carboxylase in both H35 and Fao cells (97). The structure of this autocrine factor is consistent with a glycan structure with strong resemblance to insulinomimetic factors generated in BC3H1 myocytes and H35 cells in response to insulin and on digestion of membranes with a phosphatidylinositol-specific phospholipase C. The data suggests that serum-free media might contain an autocrine insulin-like factor(s) that increases in concentration during serum deprivation, thereby masking effects of added insulin.

If this is found to be the case, during the 16 hr serum-free pretreatment prior to inducer additions, Fac cells may be constitutively stimulated by a putative insulin mediator that affects TAT enzyme levels. This would account for the exceedingly low 1.5-fold maximum induction of TAT activity when Fao cells are treated with insulin. Signal transduction to the nucleus, leading to affects on gene expression, may require activation of the tyrosine kinase activity of the insulin receptor, and therefore, only occur in the presence of insulin.

One can speculate that the KRC7 subclone is deficient for the pathway required for the production of the autocrine factor(s). A deficiency of this pathway may prove to be a contributing factor for the potent proliferative response and induction in TAT activity (2.5 - 3.5-fold increase) observed upon insulin treatment (6).

Any effector that mediates its response through an initial interaction at the plasma membrane, and has been demonstrated to increase TAT mRNA levels through an increase in the rate of transcription, appears to follow a very similar time course (64,79,87, 116, and the author's results - Figure 11). Observed is a rapid increase in the accumulation of TAT mRNA that is maximal 1 - 2 hr

following treatment. This is followed by a rapid decay to below basal levels approximately 4 hr after treatment and a slow return toward basal levels 8 hr after treatment. Our time course for insulin-stimulation of TAT mRNA levels (Figure 8) and that of Crettaz, *et al.* (80) is not consistent with that of previous investigators (64,79,87,116) in that the enhanced TAT mRNA levels as a result of insulin treatment are sustained at maximal ievels for at least 3 hr.

It seems probable that the insulin stimulated TAT induction in Fao cells is not related to the mitogenic effect known to be mediated by insulin nor is there an involvement of an insulin-stimulated *cis* -regulatory element. The author hypothesizes that the increased TAT activity in Fao cells following the addition of insulin is primarily a result of a selective stabilization of the TAT transcript combined with increased protein synthesis and decreased degradation of the short half-life aminotransferase. Puzzling is the fact that the fold induction of TAT activity (maximal 1.5-fold induction) never reaches that of the TAT transcript (maximal 2.6-fold induction). It may be that a portion of the insulin-induced TAT mRNA levels, quatitated through Northern analyses, represents a non-functional pool of mRNA or represents a TAT mRNA species with reduced translatability.

LIST OF REFERENCES

REFERENCES

1. R. Walker (1983). *In* The Molecular Biology of Enzyme Synthesis - Regulatory Mechanisms of Enzyme Adaptation. John Wiley & Sons. pp. 223 - 224.

2. C.R. Kahn, *et al.* (1981). Rec. Prog. Horm. Res. 37: 477 - 538.

3. M. Czech, *et al.* (1984). Biochemical Actions of Hormones. Vol. XI - Mechanisms of Biological Signaling by the Insulin Receptor. Academic Press. pp. 94 - 125

4. R. Walker (1983). *In* The Molecular Biology of Enzyme Synthesis - Regulatory Mechanisms of Enzyme Adaptation. John Wiley & Sons. pp. 227 - 240.

5. I.D. Goldfine (1981). Biochemical Actions of Hormones. Vol. VIII - Effects of Insulin on Intracellular Functions. Academic Press. pp. 274 - 280.

6. J.W. Koontz and M. Iwahaski (1981). Science 211: 947 - 949.

7. C.C. Yip, C. Yeung, *et al.* (1980). Biochemistry 19: 70 - 76.

8. M. Kasuga, C.R. Kahn, *et al.* (1982). J. Biol. Chem. 257: 10392 - 10399.

9. J.A. Hedo, S.W. Cushman, and I. Simpson (1982). Diabetes 31 (Suppl. 2): 2A.

10. J. Massaque, M.P. Czech, *et al.* (1982). J. Biol. Chem. 256: 3182 - 3190.

11. B.I. Posner, *et al.* (1981). Rec. Prog. Horm. Res. 37: 539 - 578.

12. I.D. Goldfine, *et al.* (1977). Nature 269: 698 - 700.

13. M. Fehlmann, *et al.* (1982). Proc. Natl. Acad. Sci. USA 79: 5921 - 5925.

14. D.S. Miller (1988). Science 240: 506 - 508.

15. R. Walker (1983). *In* The Molecular Biology of Enzyme Snnthesis - Regulatory Mechanisms of Enzyme Adaptation. John Wiley & Sons. pp. 240 - 241.

16. E.W. Sutherland (1972). Science 177: 401 - 408.

17. R.H. Herman and O.D. Tauton (1980). *In* Principles of Metabolic Control in Mammalian Systems. Plenum Press, NY. pp. 535 - 630.

18. S.M. Lohmann and U. Walter (1984). Adv. Cyclic Nucleotide Res. 18: 63 - 117.

19. Y. Nishizuka (1984). Recent Prog. Horm. Res. 40: 301 - 345.

20. M. Kasuga, C.R. Kahn, *et al.* (1983). Proc. Natl. Acad. Sci. USA 80: 2137 - 2141.

21. H. Haring, C.R. Kahn, *et al.* (1984). Biochemistry 23: 3298 - 3306.

22. R. Roth and D.J. Cassell (1983). Science 219: 299 - 301.

23. M. Kasuga, H.U. Haring, *et al.* (1982). J. Biol. Chem. 257: 9891 - 9894.

24. Y. Ebina, *et al.* (1987). Proc. Natl. Acad. Sci. USA 84: 704 - 708.

25. M.R. White, C.R. Kahn, *et al.* (1985). Nature 318: 183 - 186.

26. R.W. Rees-Jones and S.I. Taylor (1985). J. Biol. Chem. 260: 4461 - 4467

27. M. Bernier, D.M. Laird, and M.D. Lane (1987). Proc. Natl. Acad. Sci. USA 84: 1844 - 1848.

28. R.W. Brownsey and R.M. Denton (1987). *In* The Enzymes 3rd ed. Vol 18. Academic Press, NY. pp. 123 - 146.

29. J. Avruch, P.J. Blackshear, *et al.* (1985). *In* Molecular Basis of Insulin Action. Plenum Press NY. pp. 67 -94.

30. J.M. Trevillyan, *et al.* (1985). J. Biol. Chem. 260: 3041 - 3044.

31. L.B. Ray and T.W. Sturgill (1987). Proc. Natl. Acad. Sci. USA 84: 1502 - 1506.

32. M. Cobb and O.M. Rosen (1983). J. Biol. Chem. 258: 12472 - 12481.

33. J. Larner (1988). Diabetes 37: 262 - 275.

34. J. G. Foulkes, P. Cohen, *et al.* (1980). FEBS Lett. 112: 21 -24.

35. S. Cohen, *et al.* (1982). J. Biol. Chem. 257: 1523 - 1531.

36. B. Ek and C.H. Heldin (1982). J. Biol. Chem. 257: 10486 - 10492.

37. S. Jacobs, J.J. VanWyk, *et al.* (1983). J. Biol. Chem. 258: 9581 - 9584.

38. R. Weiss, *ed.* (1982). RNA Tumor Viruses, Cold Spring Harbor Symposium. New York: Cold Spring Harbor Lab.

39. A.R. Saltiel and P. Cuatrecasas (in press). Am. J. Physiol.

40. A.R. Saltiel, P. Cuatrecasas, *et al.* (1986). Science 233: 967.

41. L.M. Luttrell and A.D. Rogal (1987). Endocrinology (suppl.) 120: 240.

42. P. Stalfors, S. Alemany and J. Mato (1987). Nautre 330: 77 - 79.

43. A.R. Saltiel and P. Cuatrecasas (1986). Proc. Natl. Acad. Sci. USA 83: 5793.

44. A.R. Saltiel (1987). Endocrinology 120: 967.

45. K.L. Kelly, L. Jarett, *et al.* (1986). FEBS Lett. 209: 238.

46. K.L. Kelly, L. Jarett, *et al.* (1987). Proc. Natl. Acad. Sci. USA 84: 6404 - 6407.

47. M.G. Low and A.R. Saltiel (1988). Science 239: 268 - 275.

48. M.G. Low, I. Silman, *et al.* (1986). Trends Biochem. Sci. 11: 212.

49. S. Roy-Choudhury, M.G. Low, *et al.* (in press). Proc. Natl. Acad. Sci. USA.

50. M. Ishihara, H.E. Conrad, *et al.* (1987). J. Biol. Chem. 262: 4708.

51. J.R. Levy, J.M. Olefsky, *et al.* (1986). Endocrinology 119: 1786.

52. A.R. Saltiel and L. Sorbara-Cazan (1987). Biochem. Bioplys. Res. Commun. 149: 1084.

53. M.J. Berridge (1987). Ann. Rev. Biochem. 56: 159 - 193.

54. M.A.J. Ferguson and G. A. M. Cross (1984). J. Biol. Chem. 259: 3011.

55. J.L. Kopf, *et al.* (1986). Cell 46: 491.

56. P.J. Blackshear, *et al.* (1987). Mol. Endo. 1: 44 - 52.

57. D.T.W. Chu, P.J. Blackshear, D.K. Granner, *et al.* (1987). Mol. Endo. 1: 53 - 59.

58. P.R. Walker, J.F. Whitfield (1980). Biochem. Biophys. Res. Commun. 93: 1196 - 1203.

59. J.L. Messina, J. Larner, *et al.* (1987). Endo. 121: 1227 - 1232.

60. M. Crettaz and C.R. Kahn (1983). Endo. 113: 1201 - 1209.

61. J.W. Koontz (1984). Mol. and Cell. Bio. 58: 139 - 146.

62. P.S. Moore (1986). MS Dissertation University of Tennessee: .M669.

63. B.N. LaDu and L.R. Gjessing (1978). In The Metabolic Basis of Inherited Disease. McGraw Hill, NY pp. 256 - 278.

64. D.K. Granner and J.L. Hargrove (1983). Mol. and Cell. Bio. 54: 113 - 128.

65. S.T. Perry, K.L. Lee, F.T. Kenney, *et al.* (1983). J. Cell. Biochem. 21: 47 - 61.

66. J.M. Nickol, F.T. Kenney, *et al.* (1978). J. Biol. Chem. 253: 4009 - 4015.

67. A.M. Killery and R.E.K. Fournier (1985). Mol. and Cell. Bio. 67: 2491 - 2494.

68. R. Walker (1983). The Molecular Biology of Enzyme Synthesis - Regulatory Mechanisms of Enzyme Adaptation. John wiley & Sons, Inc.

69. M.D. Roper and W.D. Wicks (1978). Proc. Natl. Acad. Sci. USA 75: 140 - 144.

70. T. Shinomiya, G. Schutz, *et al.* (1984). Proc. Natl. Acad. Sci. USA 81: 1346 - 1350.

71. W.D. Wicks and J.L. Su (1978). J. Cyclic. Nuc. Res. 4: 113 - 122.

72. D.K. Granner, *et al.* (1970). J. Biol. Chem. 245: 1472 - 1478.

73. M. Karin, *et al.* (1984). Nature 308: 513 - 519.

74. Fujita, *et al.* (1987). Cell 41: 489 - 496.

75. J. Ryals, C. Weissmann, *et al.* (1985). Cell 41: 497 - 507.

76. R. Treisman (1986). Cell 46: 567 - 574.

77. P. Angel, M. Karin, *et al.* (1987). Cell 49:729 - 739.

78. M.R. Montminy and L.M. Bilezikjian (1987). Nature 328: 175 - 178.

79. R.E. Hill, F.T. Kenney, *et al.* (1981). J. Bio. Chem. 256: 1510 - 1513.

80. M. Crettaz, D. Muller-Wieland, and C.R. Kahn (1988). Biochem. 27: 495 - 500.

81. M.C. Weiss and J. Deschatrette (1974). Biochimie 56: 1603 - 1611.

82. T.I. Diamondstone (1986). Endocrinology 119: 1741 - 1747.

83. O.H. Lowry, *et al.* (1951). J. Biol. Chem. 193: 5294 - 5299.

84. J. Chirgwin, *et al.* (1979). Biochemistry 18: 5294 - 5299.

85. P. Thomas (1980). Proc. Natl. Acad. Sci. USA 77: 5201 - 5205.

86. R. Pictet, *et al.* (1985). J. Mol. Biol. 184: 347 - 350.

87. K. Lee, F. Kenney, *et al.* (1986). Archives of Biochem. and Biophysics 248: 597 - 603.

88. W.E.G. Muller, *et al.* (1987). EMBO 6: 3939 - 3944.

89. A. Pontecorvi, J. Robbins, *et al.* (1988). EMBO 7: 1489 - 1495.

90. I.M. Kerr, M. Knight, *et al.* (1982). Phil. Trans. R. Soc. Lond. B. 299: 59 - 67.

91. C.J. Spencer, T.D. Gelehrter, *et al.* (1978). J. Biol. Chem. 253: 7677 - 7682.

92. G.A. Clawson, I.D. Goldfine, *et al.* (1982). Science 216: 1005 - 1007.

93. D. Granner, *et al.* (1982). J. Biol. Chem. 257: 2386 - 2390.

94. D.J. Stumpo, P.J. Blackshear, *et al.* (1988). J. Biol. Chem. 263: 1611 - 1614.

95. B.S. Rosen, B.M. Speigelman, *et al.* (1987). Cell 49: 835 - 844.

96. T.E. Hayes, B.H. Cochran, et al. (1987). Proc. Natl. Acad. Sci. USA. 84: 1272 - 1276.

97. L.A. Witters and T.D. Watts (1988). J. Biol. Chem. 263: 8027 - 8036.

98. C.D. Green (1980). In Biochemistry of Cellular Regulation. Vol I. Gene Expression. CRC Press, Boca Raton, Florida pp. 59 - 83.

100. J. Chirgwin, *et al.* (1979). Biochem. 18: 5294 - 5299.

101. A.L. Goldberg and A.C. St.. John (1976). Ann. Rev. Biochem. 45: 747 - 803.

102. G. Shaw and R. Kamen (1986). Cell 46: 659 - 667.

103. T. Grange, *et al.* (1985). J. Mol. Biol. 184: 347 - 350.

104. P.J. Blackshear, D.K. Granner, *et al.* (1987). Endo. 1: 53 - 59.

105. E. Serfling, W. Schaffner, *et al.* (1985). TIG 1: 224 - 230.

106. J.H. Oppenheimer (1979). Science 202: 971-979.

107. R. Walker (1983). *In* The Molecular Biology of Enzyme Synthesis-Regulatory Mechanisms of Enzyme Adaptation. John Wiley & Sons. pp. 227 - 260.

108. B.H. Ginsbery (1979). The Insulin Receptor: Properties and Regulation. *In* G. Litwack (Ed.) Biochemical Action of Hormones,

Vol. 4. Acad. Press NY. pp. 313 - 349.

109. E.G. Krebs and J.A. Beavo (1979). Ann. Rev. Biochem. 48: 923 - 959.

110. D.S. Straus and C.D. Takemoto (1987). J. Biol. Chem. 262: 1955 - 1960.

111. G. Scherer, G. Schutz, *et al.* (1982). Proc. Natl. Acad. Sci. USA 79: 7205 - 7208.

112. J.L. Hargrove and D.K. Granner (1981). J. Biol. Chem. 256: 8012 - 8017.

113. T. Maniatis, *et al.* (1982). In Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory.

114. I. Tamm, *et al.* (1976). J. Cell. Biol. 69: 229 - 240.

115. D.J. Stumpo and P.J. Blackshear (1987). Proc. Natl. Acad. Sci. USA 83: 9453 - 9457.

116. C. Spielholz, D. Schlichter, and W.D. Wicks (1988). Mol. Endo. 2: 344 - 349.

APPENDIX

TABLE 1

Time and Dose Dependent Effects of Insulin on TAT Activity* (Units/mg Protein) in DMEM Medium

Ins Conc. (nM)	Time 1 Hr	2 Hr	3 Hr	4 Hr	5 Hr	6 Hr
0.0	50.0	55.1	58.7	54.5	56.6	55.2
SEM	3.1	0.2	2.9	0.4	1.3	0.6
1.0	56.3	59.2	67.7	72.8	71.4	76.9
SEM	1.5	2.4	1.4	2.5	1.1	1.0
10.0	61.2	71.2	72.9	79.4	80.6	78.1
SEM	0.6	0.7	0.6	1.3	0.8	1.2
100.0	61.5	70.9	78.7	73.5	75.6	75.6
SEM	2.4	1.5	2.4	0.3	0.6	0.7

Fao cells were grown on DMEM medium + 10% Fe-supplemented Calf Serum. At confluency and prior to treatment the cells were incubated for at least 16 hr in SFM. Insulin was added at zero time and cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowery method.

* - Data is expressed as the mean of three replicates ± SEM.

TABLE 2

Time and Dose Dependent Effects of Insulin on TAT Activity* (Units/mg Protein) in RPMI Medium

Ins Conc. (nM)	Time 1 Hr	2 Hr	3 Hr	4 Hr	5 Hr	6 Hr
0.0	25.0	30.7	29.4	27.7	28.8	26.3
SEM	2.3	1.5	0.3	0.5	0.6	0.3
1.0	28.3	31.0	33.5	32.9	34.8	30.5
SEM	0.9	1.2	1.2	0.5	0.6	1.8
10.0	32.2	37.4	41.3	41.1	41.2	40.1
SEM	0.7	1.4	0.9	0.3	0.9	0.5
100.0	34.3	37.8	39.9	39.2	38.5	35.3
SEM	0.5	0.7	0.3	1.2	0.9	0.5

Fao cells were grown on RPMI medium + 10% Fe-supplemented Calf Serum. At confluency and prior to treatment the cells were incubated for at least 16 hr in SFM. Insulin was added at zero time and cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowery method.

* - Data is expressed as the mean of three replicates ± SEM.

FIGURE 1

Fractionation of Total RNA on a 1.1 % Agarose Denaturing Gel.

Total RNA is fractionated on a 1.1 % agarose denaturing gel. Each lane has been loaded with 15 ug of total RNA. The gel is stained with ethidium bromide, destained, and illuminated with short wavelength UV light for visualization.

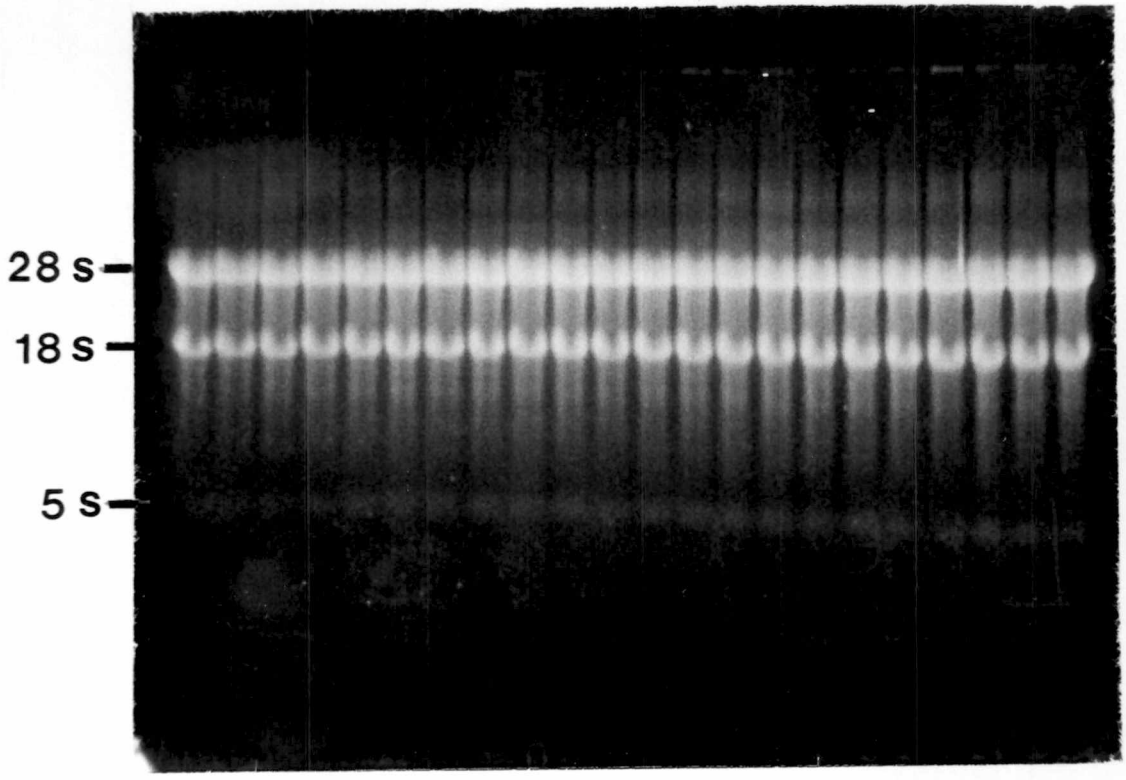

28 s
18 s
5 s

FIGURE 2

Typical Autoradiograph of a Northern Blot.

Northern blot analysis and autoradiography were done as described in Experimental Procedures. Lane treatments are as indicated on figure heading.

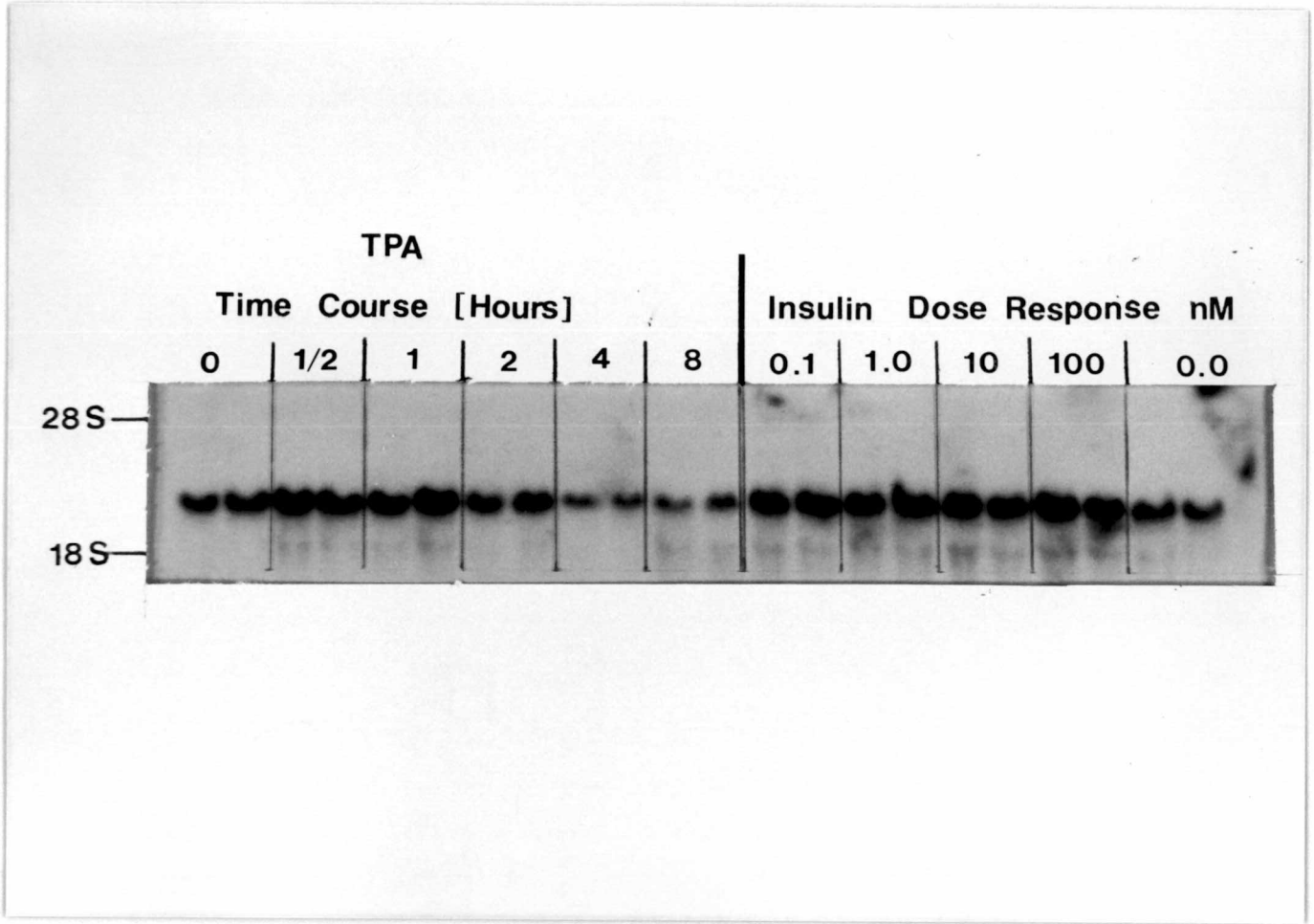

TPA
Time Course [Hours]
0
1/2
1
2
4
8
Insulin Dose Response nM
0.1
1.0
10
100
0.0
28 S
18 S

FIGURE 3

Insulin Time Course and Dose Response on TAT Activity in DMEM Medium.

Fao cells were grown on DMEM medium + 10% Fe-supplemented Calf Serum. At confluency and prior to treatment the cells were incubated for at least 16 hr in SFM. Insulin was added at zero time and cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowery method.

Each data point is expressed as the mean of three replicates ± SEM.

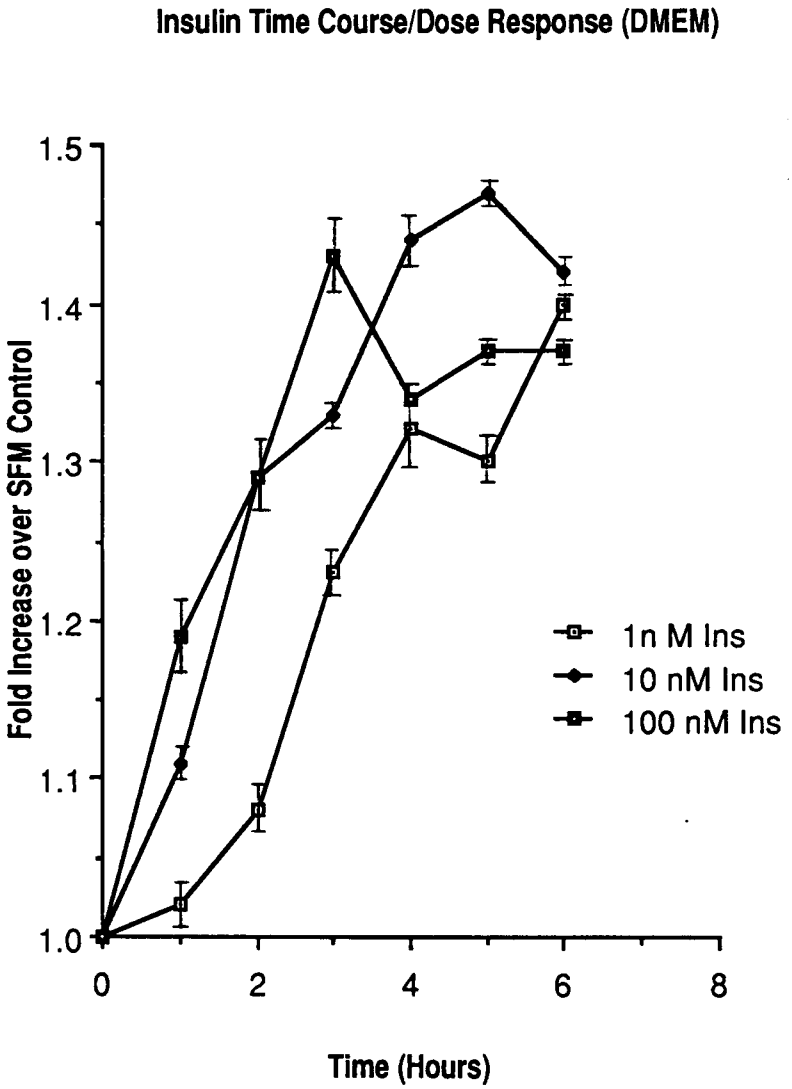

Insulin Time Course/Dose Response (DMEM)
Fold Increase over SFM Control
1.5
1.4
1.3
1.2
1.1
1.0
0
2
4
6
8
Time (Hours)
1n M Ins
10 nM Ins
100 nM Ins

FIGURE 4

Insulin Time Course and Dose Response on TAT Activity in RPMI Medium.

Fao cells were grown on RPMI medium + 10% Fe-supplemented Calf Serum. At confluency and prior to treatment the cells were incubated for at least 16 hr in SFM. Insulin was added at zero time and cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowery method.

Each data point is expressed as the mean of three replicates ± SEM.

Insulin Time Course/Dose Response (RPMI)

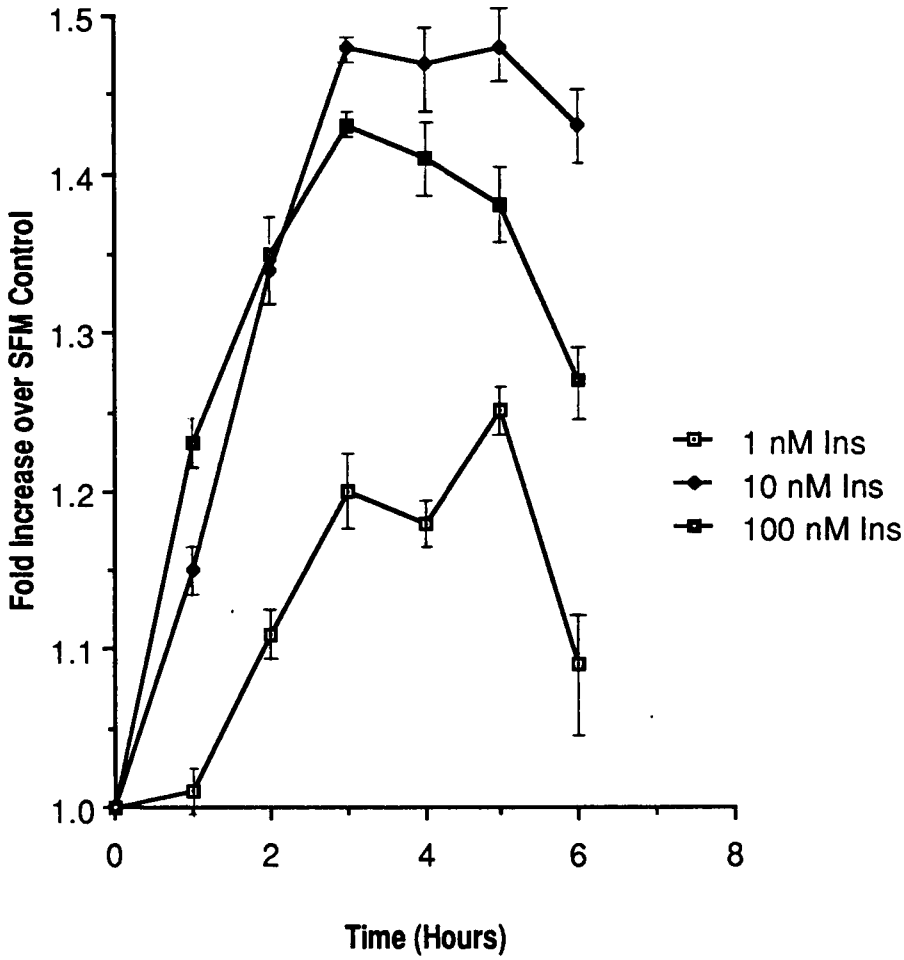


FIGURE 5

Effect of DRB on [3H]-Uridine Incorporation into TCA Precipitable Molecules. Prior to treatment Fao cells were placed on SFM for at least 16 hrs. Cells were incubated with the indicated concentrations of DRB for 1.5 hr. For the final 30 min 5 uCi [3H]-uridine was added to each well. At the end of this incubation cells were harvested and assayed for TCA-precipitable activity, TCA-soluble activity, and total protein. The data was corrected for [3H]-uridine uptake in the TCA-soluble fraction.

Each data point represents the mean of six replicates ± SEM.

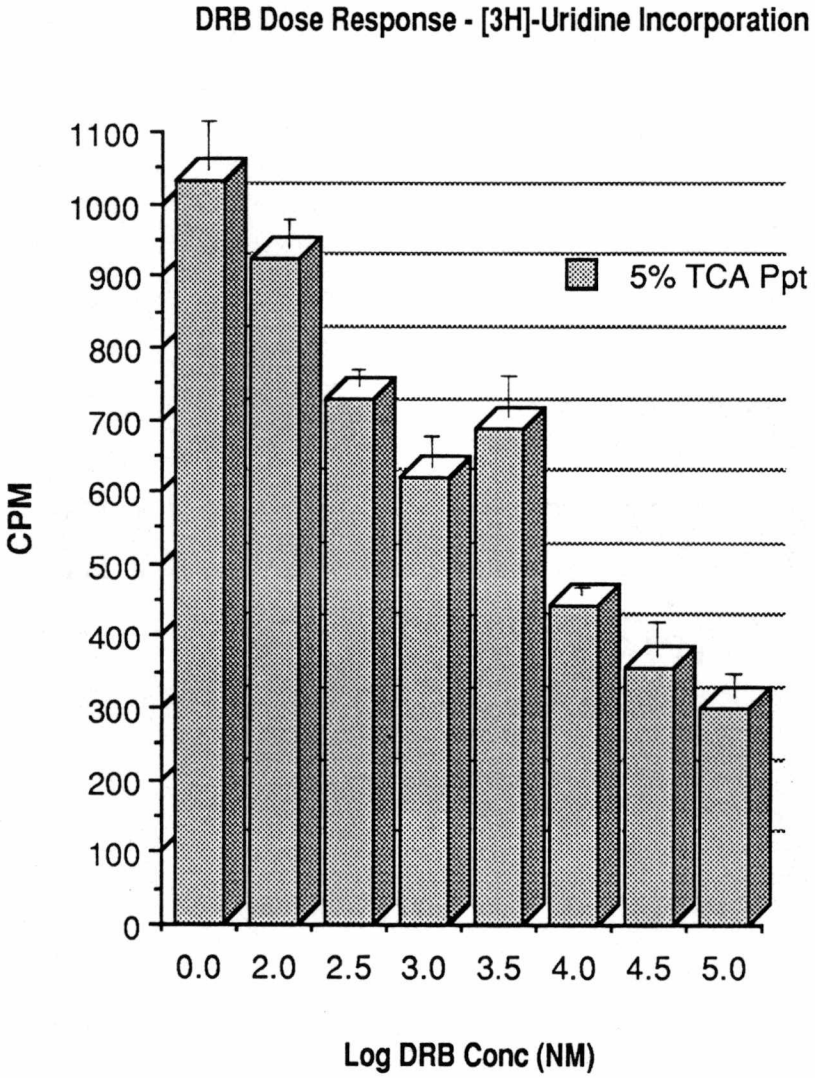

DRB Dose Response - [3H]-Uridine Incorporation
CPM
1100
1000
900
800
700
600
500
400
300
200
100
0
5% TCA Ppt
0.0
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Log DRB Conc (NM)

FIGURE 6

DRB Inhibition of [^{3}H]-Uridine Incorporation into TCA Insoluble Macromolecules.

Prior to treatment Fao cells were placed on SFM for at least 16 hrs. Cells were incubated with the indicated concentrations of DRB for 1.5 hr. For the final 30 min 5 uCi [3H]-uridine was added to each well. At the end of this incubation cells were harvested and assayed for TCA-precipitable activity, TCA-soluble activity, and total protein. % inhibition of [^{3}H]-uridine incorporation for each DRB concentration was determined.

Each data point represents the mean of six replicates ± SEM.

DRB Inhibition of [3H]-Uridine Incorporation Into Macromolecules

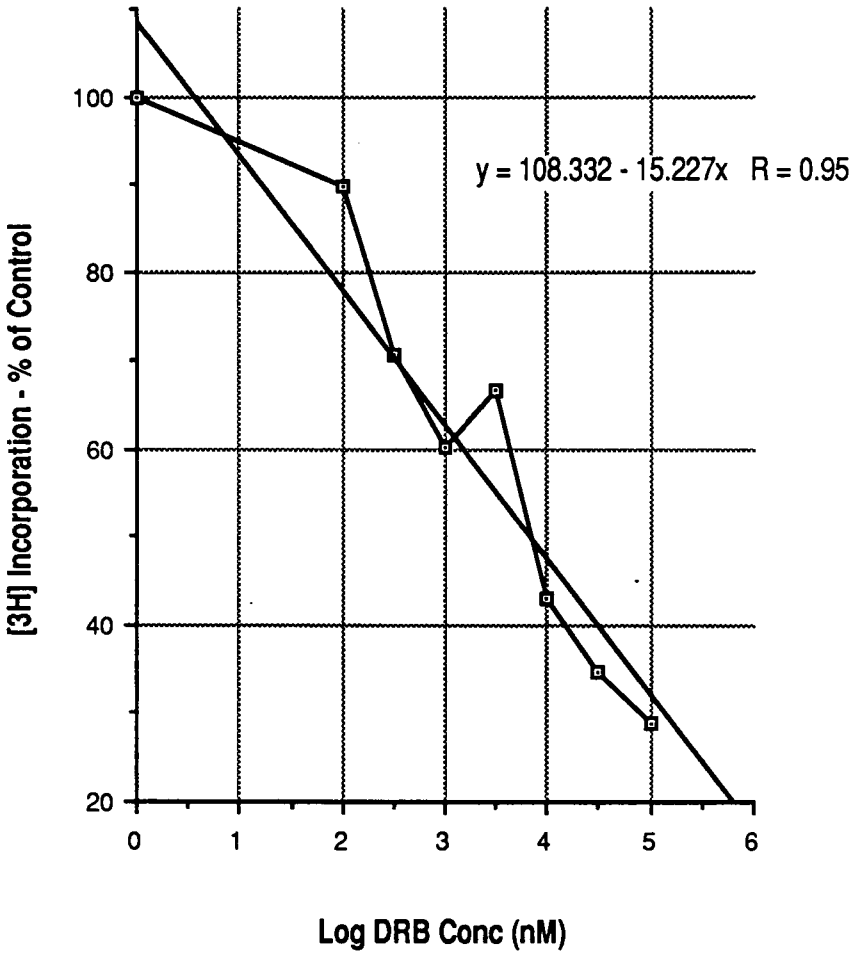


FIGURE 7

Effect of the Transcription Inhibitor DRB on Insulin-Stimulated TAT Activity.

Prior to treatment Fao cells were placed on SFM for at least 16 hr. Due to its limited solubility the DRB was made-up in serum-free medium at a final treatment concentration of 35uM. Prior to insulin treatment the medium was aspirated from those plates to be treated with insulin in the presence of DRB and the medium was replaced with fresh serum-free medium containing 35 uM DRB. This medium had been previously warmed to 37°C. Insulin was added immediately thereafter.

Each data point represents the mean of three replicates ± SEM.

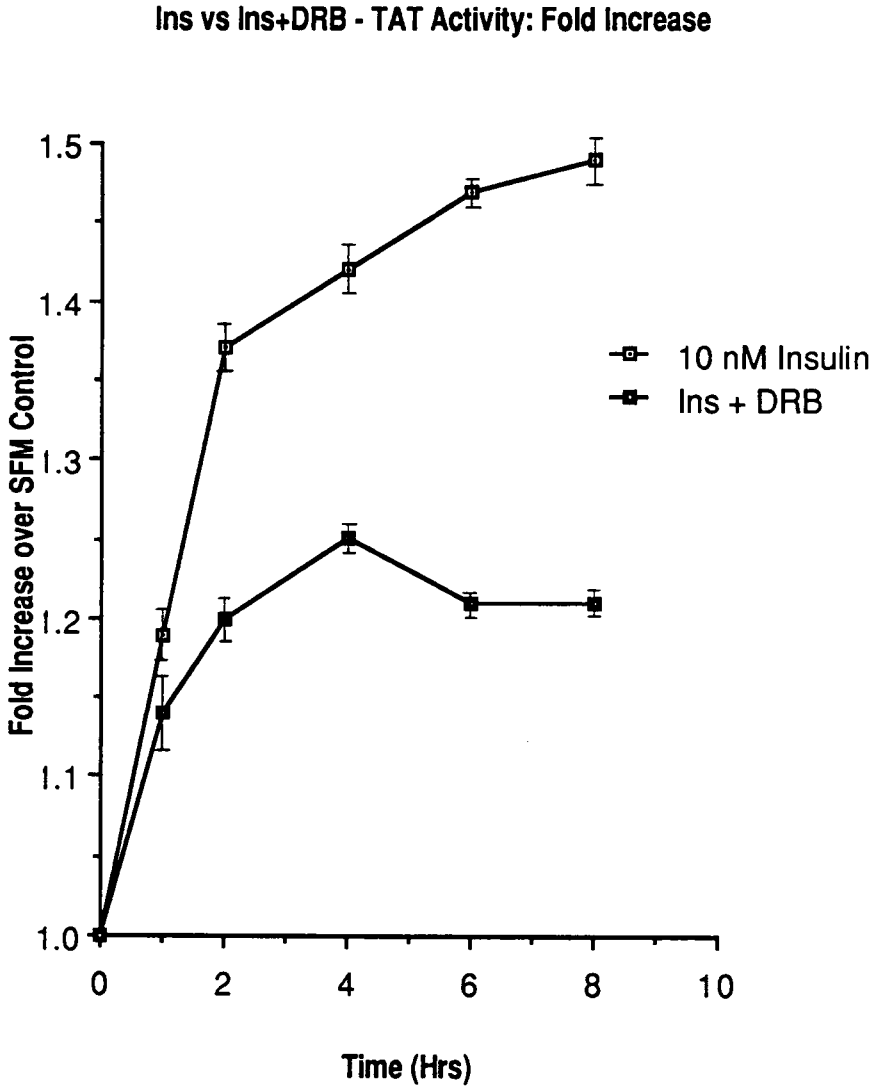

Ins vs Ins+DRB - TAT Activity: Fold Increase
Fold Increase over SFM Control
1.5
1.4
1.3
1.2
1.1
1.0
0
2
4
6
8
10
Time (Hrs)
10 nM Insulin
Ins + DRB

FIGURE 8

Effect of Insulin on TAT mRNA Abundance in Fao over an 8 Hour Period.

Prior to treatment the Fao cells were placed on SFM for at least16 hrs. Cells were treated with 10.0 nM insulin. Cells were harvested at the indicated times. For each time point both insulin-treated and serum-free control cultures were harvested.

All data points, excluding the 6 hr point, represent the mean of four replicates ± SEM. The 6 hr data point represents the mean of six replicates ± SEM.

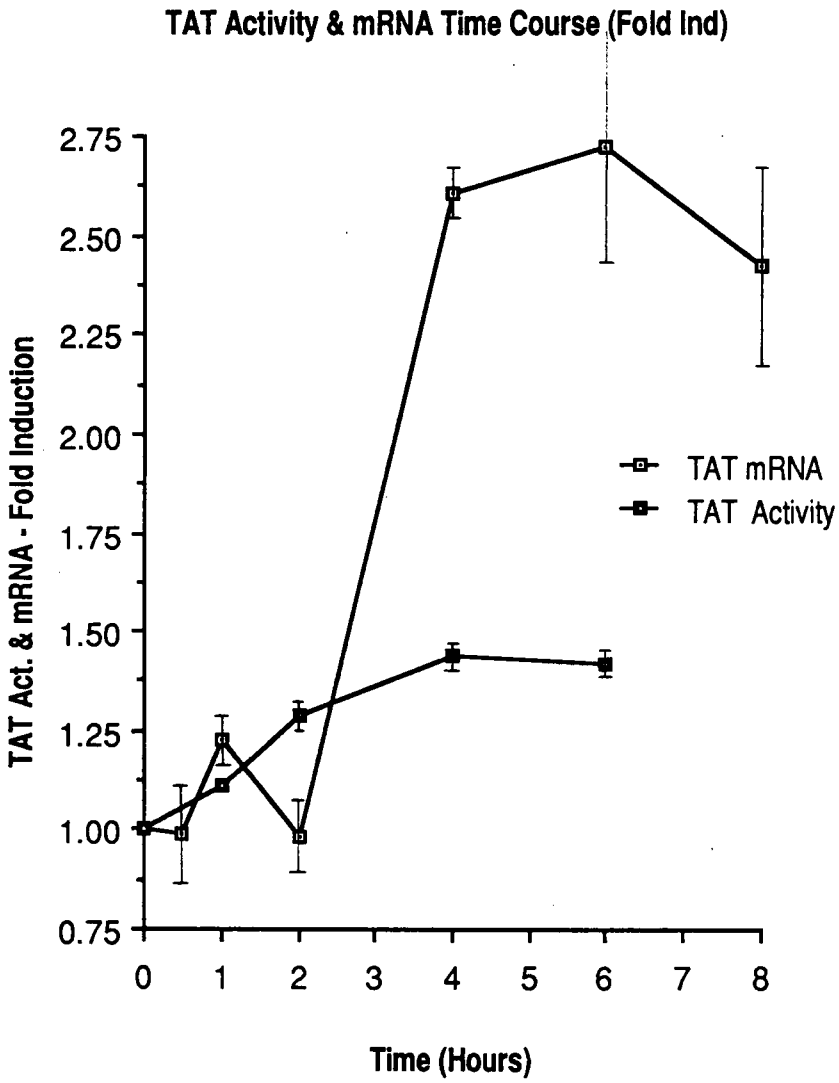

TAT Activity & mRNA Time Course (Fold Ind)
TAT Act. & mRNA - Fold Induction
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0
1
2
3
4
5
6
7
8
Time (Hours)
TAT mRNA
TAT Activity

FIGURE 9

Insulin Dose Response on TAT mRNA Abundance in Fao 6 Hours After Treatment.

Prior to treatment Fao cells were placed on SFM for at least 16 hr. Cells were treated with the various concentrations of insulin for 6 hr.

Each data point reflects the mean of RNA isolations ± SEM from two independent dishes.

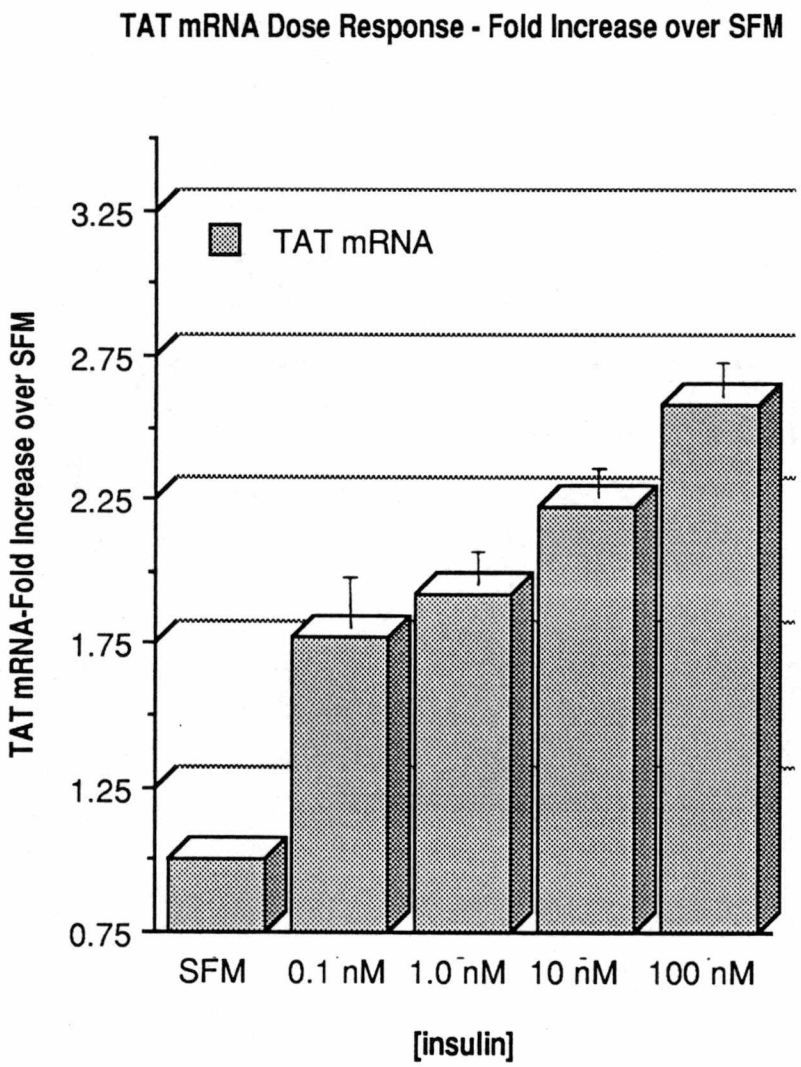

TAT mRNA Dose Response - Fold Increase over SFM
TAT mRNA-Fold Increase over SFM
3.25
2.75
2.25
1.75
1.25
0.75
TAT mRNA
SFM
0.1 nM
1.0 nM
10 nM
100 nM
[insulin]

FIGURE 10

Effect of Insulin on the cAMP-mediated Increases in TAT mRNA in Fao Cells.
Prior to treatment Fao cells were placed on SFM for at least 16 hr. Cells were treated with either 0.2 mM db-cAMP alone, 0.2 mM db-cAMP and 10.0 nM insulin together, or pretreated with 0.2 mM db-cAMP for 1 hr and followed by 10.0 nM insulin treatment.
Each data point represents the mean of two replicates ± SEM.

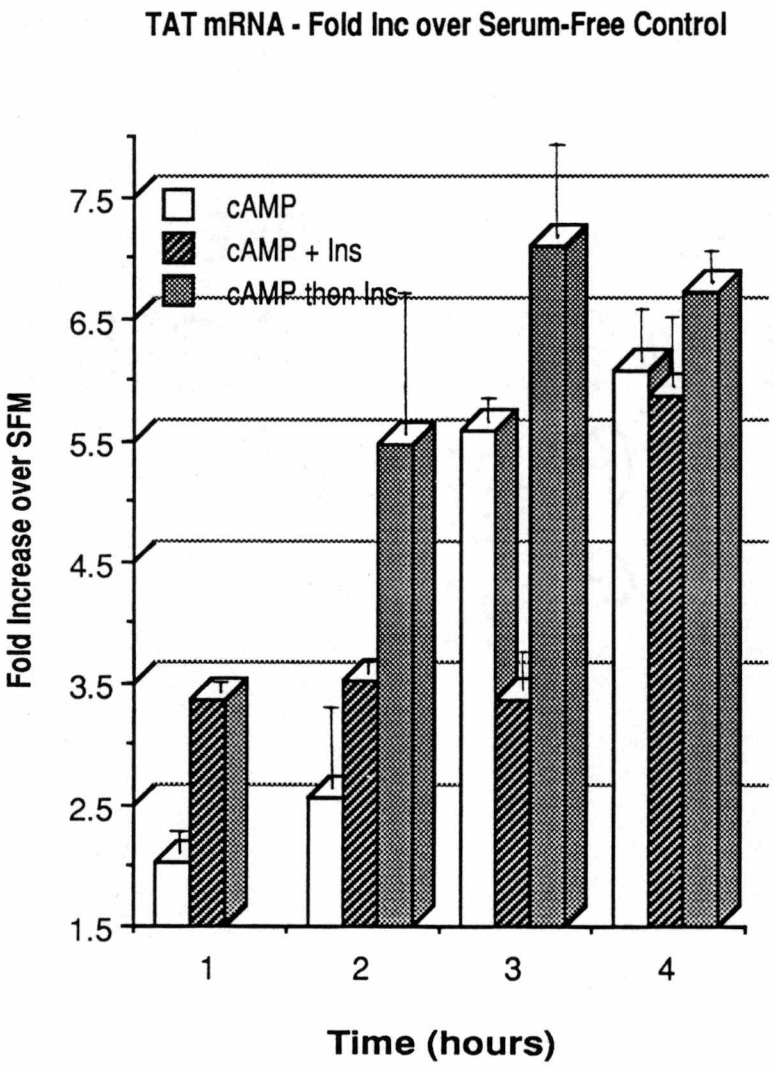

TAT mRNA - Fold Inc over Serum-Free Control
cAMP
cAMP + Ins
cAMP then Ins
Fold Increase over SFM
7.5
6.5
5.5
4.5
3.5
2.5
1.5
1
2
3
4
Time (hours)

FIGURE 11

Time Course of the Effect of 1.6 uM TPA Treatment on TAT mRNA Abundance in Fao cells.

Prior to treatment cells were placed on SFM at least 16 hr. Final TPA concentration was 1.6 uM. Final ethanol-carrier concentration was 0.095 %. Ethanol was added to serum-free controls to a final concentration of 0.095%. This experiment was reproduced four times in two sets of two replicates.

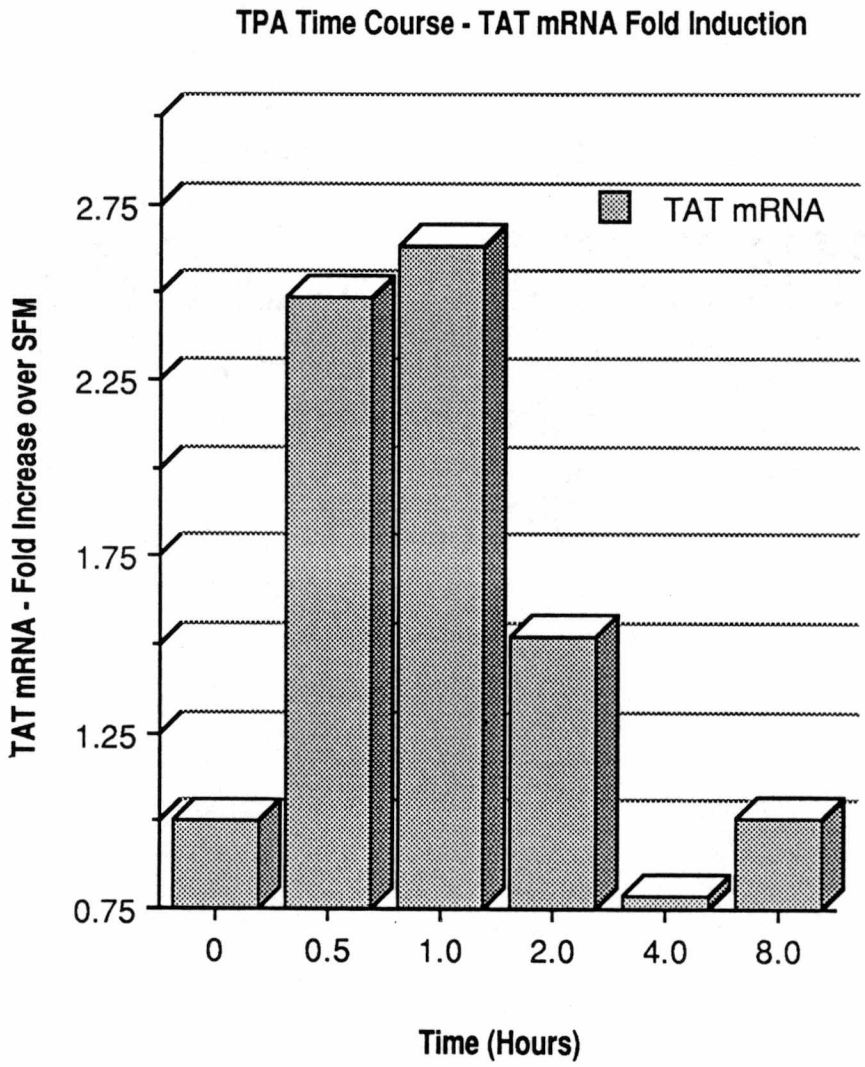
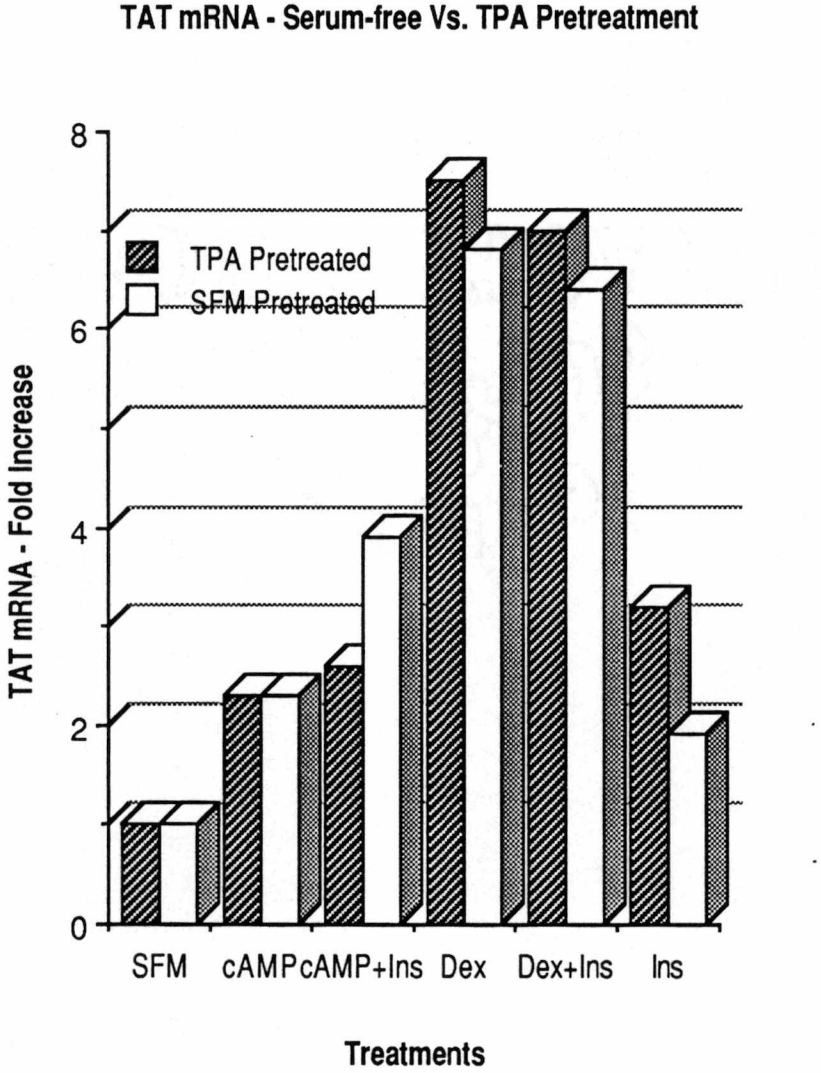

TPA Time Course - TAT mRNA Fold Induction
TAT mRNA
TAT mRNA - Fold Increase over SFM
2.75
2.25
1.75
1.25
0.75
0
0.5
1.0
2.0
4.0
8.0
Time (Hours)

FIGURE 12

Effect of TPA Pretreatment on TAT mRNA Induction by Known Inducers.

Prior to treatment Fao cells were placed on either SFM or SFM containing 16 uM TPA and allowed to incubate for at least 16 hr. The various drugs were then added and the incubation was continued for an additional four hours. Cells were then harvested and RNA analyzed as described in the Experimental Procedures.

The data is from a single, non-replicated experiment.


TAT mRNA - Serum-free Vs. TPA Pretreatment
TAT mRNA - Fold Increase
8
6
4
2
0
TPA Pretreated
SFM Pretreated
SFM
cAMP
cAMP+Ins
Dex
Dex+Ins
Ins
Treatments

VITA

Richard Peterson was born on November 23, 1955 in Syracuse, New York. Richard received a Bachelor of Science degree in Biology in May, 1977 from The State University of New York - College of Environmental Science and Forestry at Syracuse. After a period of employment he entered The University of Tennessee, Knoxville in Fall, 1986. Richard has worked in the laboratory of Dr. John Koontz in the department of Biochemistry. He was awarded the Master of Science degree in Life Sciences in August, 1988.

Richard was married to Judy May Naeye Peterson on December 20, 1986.