

To the Graduate Council:

I am submitting herewith a thesis written by Jong H. Kim entitled "Phorbol Ester mediated Inhibition of Tyrosine Aminotransferase Gene Expression." 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 Biochemistry.

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**PHORBOL ESTER MEDIATED INHIBITION
OF
TYROSINE AMINOTRANSFERASE
GENE EXPRESSION**

**A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville**

**Jong H. Kim
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ABSTRACT

We have investigated the effect of a tumor promoting phorbol ester, TPA, on the expression of the gene for tyrosine aminotransferase. It had been reported that exposure of cells to TPA inhibits insulin mediated induction of TAT activity. We subsequently reported that TPA inhibits not only the insulin mediated induction of TAT activity, but also the induction caused by glucocorticoids and cAMP analogs. To determine the basis for this inhibition of induction caused by three different hormones which are believed to act through separate mechanisms, we investigated the effect of TPA on TAT mRNA levels. TPA causes a time and concentration dependent loss of TAT mRNA from the cells. There is no change in the first hour after addition of 1.6 μ M TPA. At two hours, there is a dramatic decrease in the transcript. The level then remains below the original basal level up to 8 hours. The maximal effect is observed at a concentration of 1 μ M. The EC₅₀ is approximately 7 nM. In the presence of the dexamethasone, TAT mRNA was increased by 6 fold. In the same experiment TPA did not inhibit dexamethasone -mediated induction of the TAT mRNA level, rather TPA inhibited TAT mRNA basal levels. Results of TAT enzyme assays indicated that TPA did inhibit dexamethasone-induced TAT enzyme activity. These studies suggest that TPA may regulate TAT gene expression post-transcriptionally.

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CHAPTER 1

Introduction

Hormonal regulation of gene expression is a primary mechanism by which eukaryotic organisms integrate cellular responses to environmental and metabolic conditions. Many of the hormonally regulated genes studied to date have been demonstrated to be controlled by alterations in the level of transcription. Best understood is the mechanism of regulation for genes whose expression is controlled by steroid hormones, in which hormone receptor complex binds to defined regions of the gene stimulating the efficiency of initiation from linked promoters (1). To date, very little is known of the mechanism by which non-steroid hormones regulate gene transcription or how multiple hormones regulate the transcription of a single gene. The objective of this research is to examine and elucidate the molecular mechanism by which phorbol esters, activators of the phospholipid and Ca^{2+} dependent serine/threonine protein kinase, Protein kinase C (PKC), mediates its effects on gene expression at the level of transcription. The gene that encodes the liver-specific enzyme tyrosine aminotransferase (TAT) is an extensively investigated, well characterized, hormonally regulated enzyme that will serve as the model for studies of transcriptional regulation.

Receptor-mediated hydrolysis of inositol phospholipids was recently realized to be a common mechanism for transducing various extracellular

signals into the cell, such as those from certain hormones, neurotransmitter, antigens, some growth factors, and many other biologically active substances. In 1953, the response of inositol phospholipids to the stimulation of cell surface receptors was recognized by Hokin and Hokin (2), who first showed that acetylcholine induces a rapid incorporation of ^{32}P into phosphatidyl inositol (PI) and phosphatidic acid. It became evident later that this incorporation resulted from the enhanced breakdown and resynthesis of inositol phospholipids occurring in many stimulated cells (3,4). Durell et al (5) suggested a potential role of the PI response in receptor functions, and Michell (3) subsequently postulated that the phospholipid breakdown might open a Ca^{2+} gate. In 1983, Berridge and his co-workers (6) demonstrated that inositol-1,4,5-triphosphate (I-1,4,5- P_3), one of the earliest products of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, serves as a mediator of Ca^{2+} mobilization from an internal store, probably in the compartment of the endoplasmic reticulum (7). Nishizuka (8) provided evidence that 1,2-diacylglycerol, the other product of PIP_2 hydrolysis that remains in membranes, initiates the activation of a specialized protein kinase, protein kinase C, and that the signal pathway through this protein phosphorylation is separate from, and often synergistic to, the Ca^{2+} signaling pathway for the control of various cellular functions and proliferation (9).

Protein kinase C was identified in 1977 as a proteolytically activated protein kinase (10), and is now known to be ubiquitous in tissues and organs (11). In most tissues other than brain (12), this enzyme is recovered mainly from the soluble fraction as an inactive form, and is apparently

translocated to membranes in a Ca^{2+} -dependent fashion when cells are stimulated (13). However, the precise intracellular topography of this protein kinase is unknown, since the enzyme is usually extracted in the presence of high concentrations of a Ca^{2+} chelator to prevent proteolysis by Ca^{2+} -dependent protease, calpain. Recent immunocytochemical analysis using monoclonal antibodies against protein kinase C indicates that intracellular localization of this enzyme varies with cell types, but that the enzyme appears to be absent or poorly represented in the nucleus (14). But recent studies from Malviya et al (15) demonstrated that its location in the rat liver nuclei. A number of nuclear proteins have been shown to be phosphorylated by protein kinase C (16,17), including, most prominently, topoisomerase II (18). Recent data demonstrate the involvement of protein kinase C in the regulation of transcription via phosphorylation of a transcription factor (19) and also in the expression of proto-oncogenes (20). In rat liver, protein kinase C activity in the nucleus is manifested at higher calcium concentrations. In fact, the protein kinase C activity at two optimal calcium concentrations has been observed by Malviya's group in the rat liver homogenate itself (15). Therefore, the clear demonstration of nuclear localization of protein kinase C may lead to an understanding of the molecular pathway involved in signal transduction from the plasma membrane to the nucleus.

Protein kinase C requires Ca^{2+} and phospholipid, particularly phosphatidyl serine, for its activation (21). Diacylglycerol dramatically increases the affinity of this enzyme for Ca^{2+} and thereby renders it fully active without a net increase in the Ca^{2+} concentration (22). Thus, the

activation of this protein kinase is thought to be biochemically dependent on Ca^{2+} , but under some conditions physiologically independent of Ca^{2+} increases. However, the enzyme can also be activated by the synergistic action of an increase in Ca^{2+} concentration and the formation of diacylglycerol (8).

Diacylglycerols containing a 1,2-*sn* configuration, with various fatty acids in different chain length, are capable of activating protein kinase C, with those having an unsaturated fatty acid being most active (23). However, two other stereoisomers, 2,3-*sn* -diacylglycerol and 1,3-diacylglycerol, neither activate nor inhibit the enzyme, suggesting that a highly specific lipid-protein interaction is needed for this enzyme activation (25). From experiments with a Triton X-100 mixed micellar assay system, it has been postulated that about one molecule of diacylglycerol can activate one molecule of protein kinase C in the presence of greater than four but less than ten molecules of phosphatidyl serine (24). Nevertheless, this 1:1 stoichiometry does not necessarily indicate that diacylglycerol binds directly to the enzyme through a ligand-ligand interaction, and the precise mechanism of this enzyme activation is not yet fully understood (25).

Protein kinase C can also be activated by limited proteolysis with calpain. The smaller component is enzymatically active totally independently of Ca^{2+} , phospholipid, or diacylglycerol. Membrane-bound protein kinase C is more susceptible to this proteolysis (27), which has been suggested to occur in intact platelets and neutrophils (28). However, the physiological significance of this proteolytic activation is not known.

Although tumor promoting activity provided the initial motivation for

studying the mechanism of the phorbol esters, as people began to examine the effects of the phorbol esters in different *in vitro* systems, it turned out that the phorbol esters had profound effects on a wide variety of biological systems (1). These effects included induction or inhibition of differentiation, the partial mimicry in normal cells of the transformed phenotype, stimulation of secretory responses in a variety of cell types, modulation of many membrane activities, and generation of active oxygen species. The range of these effects suggested early on that the phorbol esters might be functioning through stimulation of some central regulatory pathway within cells.

Tumor promoting phorbol esters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), have a structure very similar to diacylglycerol and activate protein kinase C directly both *in vivo* and *in vitro* (26). Like diacylglycerol, phorbol esters dramatically increase the affinity of this enzyme for Ca^{2+} , resulting in its full activation at physiological Ca^{2+} concentrations (8). Several lines of evidence provided by this and other laboratories support the notion that protein kinase C is the receptor of tumor promoters (26).

However, phorbol esters may sometimes be unsuitable for studies of the physiological activation of protein kinase C, since diacylglycerol is present only transiently in membranes, while phorbol ester is hardly degraded. Therefore, phorbol ester may extend a usually limited phase of cellular response, thereby distorting the normal sequence of events (8). At an early phase of cellular responses, this enzyme probably acts synergistically with Ca^{2+} as part of a positive response, but more frequently it acts as a negative

feedback control, as in the down-regulation of some receptors, which occurs immediately upon stimulation (29). There is no proof at present for protein kinase C being the sole target of tumor promoters, particularly at their higher concentrations. Therefore, it should not be surprising if slightly different effects are obtained from stimulation of living cells with diacylglycerol as opposed to phorbol ester. For instance, HL-60 leukemia cells have been reported to undergo differentiation in response to phorbol ester but not in response to permeable diacylglycerol (30).

Under appropriate conditions, the two signal pathways, protein kinase C activation and Ca^{2+} mobilization, can be induced selectively and independently by the application of a permeable diacylglycerol or phorbol ester for the former and a Ca^{2+} ionophore such as A23187 or ionomycin (29) for the latter. It became possible to show with this procedure that the two signal pathways are both essential and often act synergistically to elicit full cellular responses. Such a potential role of protein kinase C in signal transduction at the cell surface has also been extrapolated to neural functions, particularly transmitter release in both peripheral and central nervous systems, as well as modulation of membrane ion conductance (14). In fact, experiments with monoclonal antibodies have confirmed the presence of a protein kinase C-positive immunoreactive material in many regions of the cytoplasm, including axons and dendrites of neuronal cells (14).

The two signal pathways, protein kinase C activation and Ca^{2+} mobilization, may also play roles in long-term responses such as gene expression and cell proliferation. For example, the two pathways are both

essential and act synergistically to cause DNA synthesis in macrophage-depleted human peripheral lymphocytes (32). However, in order to stimulate rapid cell proliferation, some growth factor is still needed, indicating that an additional signal pathway is involved in eliciting the full activation of cell proliferation (8). For instance, interleukin-1 presumably utilizes both Ca^{2+} -and protein kinase C-dependent signal pathways, and induces T lymphocyte proliferation by initiating the gene expression of interleukin-2 receptor and by facilitating the release of interleukin-2 itself (8).

In biological systems positive signals are normally followed by immediate negative feedback control to prevent overshoot, and to allow responses to subsequent signals. A major function of protein kinase C appears to be intimately related to such feedback control of cell surface receptors, termed down-regulation. The stimulation of alpha 1-adrenergic receptor is well known to induce inositol phospholipid breakdown (9). Recent evidence obtained for hepatocytes (33) and smooth muscle (34) strongly suggests that protein kinase C exerts negative feedback control on the receptors of epinephrine (alpha 1 agonists) and angiotensin II. A similar feedback control by protein kinase C over the receptors that are coupled to inositol phospholipid breakdown has been suggested for many other cell types, including platelets (14), neutrophils (35), PC 12 cells (36), and pituitary cells (37). The biochemical mechanism of this negative feedback control is a subject of great interest.

The feedback control or down-regulation by protein kinase C also extends to the receptors of other signaling systems. Protein kinase C

phosphorylates the EGF receptor with a concomitant decrease in both its tyrosine specific protein kinase and growth factor binding activities (38). Phorbol ester markedly reduces the EGF binding in many mitogenically responsive cell types (39). In an analogous fashion, the receptor of insulin (40), somatomedin C (40), transferrin (40), and interleukin-2 (41) may be targets of protein kinase C action. However, the function of phosphorylation of these receptors has not yet been elucidated.

Protein kinase C and cyclic adenosine monophosphate (cAMP) dependent protein kinase usually relay information along different signal pathways within the cell, but these two enzymes sometimes cause apparently similar cellular responses (42). These protein kinases often react with the same phosphate acceptor proteins, and even phosphorylate the same seryl and threonyl residues in a single protein molecule (42). It is unclear why these two signaling systems normally exert different but sometimes similar effects on biochemical processes within a single activated cell. Extensive work by investigators (43,44) has shown that the amino acid sequence in the vicinity of the phosphorylation site is a primary factor for substrate recognition. Although attempts have been made with some synthetic peptides (43,44), information about the substrate recognition of protein kinase C is still limited. The topographical arrangement or subcellular localization of the enzyme and its target proteins appear to be crucial factors for determining substrate specificity.

The pathway responsible for transducing the signal generated by the activation of protein kinase C to the transcriptional machinery is not known. It is plausible, however, that the control regions of TPA-inducible

genes contain a common *cis* element that functions in a manner analogous to the responsive element that confers induction by glucocorticoids (61), heavy metal ions (61), double-stranded DNA (62), and serum (63). If analogous elements exist for TPA induction, they may be recognized by a common trans-acting factor whose activity or abundance is modulated by protein kinase C. To elucidate the mechanisms involved in gene regulation by phorbol esters, Angel's group (66) isolated a number of TPA inducible genes. For this purpose a cDNA library was constructed by them, using RNA extracted from TPA-treated primary human fibroblasts. Differential screening of this library led to identification of a number of cDNA clones corresponding to TPA-inducible mRNA species (66). One of these clones was derived from the human metallothionein II_A gene (hMT II_A), and another clone was derived from the collagenase gene. The transcription of both of these genes are induced not only by phorbol esters, but also by serum factors and agents that damaged DNA such as UV or mitomycin C (66,69). Deletion analysis of the collagenase promoter led to the identification of a TPA-inducible enhancer capable of transferring the response to heterologous promoters (66). Karin's group (64), reported that this enhancer element contains a short nucleotide sequence (9 b.p. motif) that is highly conserved in a number of other TPA-inducible promoters. A synthetic copy of this short sequence is capable of conferring TPA induction upon heterologous promoters, and this sequence is the binding site for a trans-acting factor whose activity is regulated by TPA. The highly conserved nature of this sequence, referred to as the TRE, suggests that it serves as a binding site for a sequence-specific DNA

binding protein. The results of the protein footprinting and competition experiments, done by Karin's group (64), indicate that the TRE-binding factor recognizes the TRE consensus sequence. Interestingly, the TRE consensus is essentially identical to the recognition sequence of the previously described mammalian transcription factor AP-1 (82). To investigate the possibility that the TRE is recognized by AP-1, Karin's group (64) constructed a DNA-affinity column based on the collagenase TRE sequence, and purified the factor that binds to that sequence.

Footprinting showed that this factor protects the sequences centered around the TRE not only of collagenase, but also of hMTIIA and SV40. These footprints were identical to those produced by AP-1 purified by its ability to bind to the hMTIIA (82). Furthermore, TRE-binding factor contains a major protein species that migrates in a gel system as a polypeptide of 44,000 mol. wt., identical in its mobility to the polypeptide identified by Lee, et al. (82) as AP-1. Several lines of other evidence suggested that AP-1 is indeed the TRE recognition factor whose abundance or activity is increased after treatment of HepG2 (or HeLa tk-) cells with TPA. Single, synthetic TREs confer TPA inducibility upon heterologous promoters and compete efficiently for the binding of AP-1 to TPA-inducible enhancers. A point mutation that abolishes TRE activity strongly interferes with AP-1 binding (64). These results strongly suggest that binding of AP-1 to the TRE is responsible for activation of this *cis* element.

Because the activity of AP-1 and the TRE is regulated by phorbol esters that activate protein kinase C and not by inactive phorbol esters, such as 4 alpha-phorbol-12,13,-didecanoate, these elements may serve to transmit the

growth response signals elicited by the activation of this enzyme to the transcriptional machinery (29). This hypothesis is supported by the observations that expression of hMTIIA (65), collagenase (66), and stromelysin (67) are induced not only by TPA but also by growth factors and oncogenic transformation. However, insulin, which stimulates growth of HepG2 cells and is a potent inducer of hMTIIA gene expression in these cells, does not activate the TRE or increase the level of activity of AP-1 (64). This suggests the existence of multiple growth-stimulatory signal transduction pathways in the cell.

The exact mechanism responsible for the increase in TRE activity shortly after TPA treatment remains to be determined, although the corresponding increase in AP-1 activity suggests that two may be directly related. We cannot yet rule out the unlikely possibility that additional TPA-induced steps are also involved. The increase in AP-1 activity could reflect either elevated synthesis, decrease turnover, or posttranslational modification of the factor. The resistance of the TPA induction response to cycloheximide (66) and the rapid increase in AP-1 activity within 1 hr, which is also resistant to cycloheximide, suggest that the mechanism is posttranslational. TPA does not seem to affect the intracellular compartmentation of the factor because similar results were obtained using either whole-cell or nuclear extracts (64). The intracellular half-life of AP-1 is quite long, as its level does not decrease significantly even after incubation of HeLa cells for 12 hr in the presence of cycloheximide (69). Therefore, it is unlikely that TPA acts to block AP-1 degradation. Two explanations for the increase in its activity are possible because TPA is

known to exert its biological effects via the activation of protein kinase C (29). First, site-specific phosphorylation of AP-1 may be responsible for its increased activity. Second, protein kinase C mediated phosphorylation may inactivate an inhibitory protein whose role is to regulate AP-1 activity. The elucidation of the exact biochemical basis for modulation of AP-1 activity is to be seen. Recently, Sen and Baltimore (68) showed that NF-kB, a factor that binds to a site in the k-immunoglobulin enhancer which does not resemble the TRE, is strongly induced by TPA in the cells that do not transcribe immunoglobulin light chain genes. This observation reiterates the complexity of the transcriptional response to phorbol esters. It remains to be tested whether NF-kB is involved in the activation of other TPA-inducible genes.

To identify additional transcriptional factors that may have a role in mediating the effects of other environmental and hormonal stimuli that do not involve protein kinase C activation on hMTIIA transcription, Karin's group (69), isolated another transcription factor AP-2, which binds to that gene. The binding site for AP-2 forms a cell-type-specific enhancer element whose activity is increased in response to treatment of cells with phorbol esters and agents that elevate cAMP levels. Among the cellular and viral genes recognized by AP-2 are hMTIIA, c-myc, SV40, BPV, and hGH. In contrast, the binding site for the previously characterized transcription factor AP-1 (66) is responsive to phorbol esters but not cAMP. These results indicate that AP-2 is a transcription factor that mediates the effects of two distinct signal-transduction systems known to be involved in growth control, one activated by phorbol esters and involving

protein kinase C, and the second activated by cAMP and involving protein kinase A.

Recently, studies on oncogenes encoding nuclear proteins have united with those concerning the regulation of eukaryotic gene transcription. A solid link to a bona fide transcription factor was made by Vogt et al (70) who reported a convincing similarity (40 %) between a region of the protein encoded by jun oncogene, which is responsible for induction of fibrosarcomas in chickens by avian sarcoma virus 17 (71), and the DNA-binding domain of the yeast transcriptional activator GCN4 (72). GCN4 induces transcription of several genes involved in amino acid biosynthesis and binds as a dimer to the target sequence ATGA(C/G)TCAT (72). The consensus binding site of GCN4 is closely related to that of the mammalian transcription factor AP-1 (66). Similarities between Jun and GCN4 stimulated an investigation of the relationship between Jun and AP-1 (73). Antisera raised against two v-jun peptides, PEP1 (amino acid 209-225) and PEP2 (amino acid 73-87) (74), were used to examine affinity purified preparations of AP-1. In immunoblot assays both antisera recognized a single major polypeptide (73). Furthermore, sequencing of AP-1 tryptic peptides revealed that several were identical to portions of the predicted amino acid sequence of Jun. These data, together with the finding that a Jun polypeptide expressed in E.coli binds to the AP-1 site (73), provided compelling evidence identifying Jun as AP-1. However, AP-1 preparations contain several proteins (74), and many of the AP-1 peptides sequenced are unrelated to Jun. Indeed, some of the AP-1 peptides have now been identified in the predicted protein sequence of jun-B, a jun-related gene

(83). Thus, Jun is only one of the proteins that contribute to AP-1 activity and it should not be referred to as transcription factor AP-1. In fact, it is unclear how many cellular proteins regulate transcription via AP-1 sequence elements.

Several other proteins covered by the term AP-1 have subsequently been identified as a result of studies of another oncogene product, Fos, which is present in complexes that recognize a specific nucleic acid regulatory element. Its normal cellular homolog, c-fos, encodes a nuclear protein (Fos) that undergoes extensive posttranslational modification and participates in protein complexes with a 39,000 dalton protein (p39) (75). Fos-p39 complexes are associated with chromatin in isolated nuclei and bind to DNA cellulose *in vitro* (75). The observation by Distel et al (76) of a protein-DNA complex whose formation was inhibited by anti-Fos antibodies suggested that Fos interacts with a regulatory element of a gene specific for adipocyte differentiation. A combination of studies involving mutagenesis (77), competition analysis (77), and DNA-affinity precipitation (77) revealed that Fos binds to the AP-1 consensus recognition sequence. Indeed, variations of the AP-1 site were found to bind several other proteins, including Fos-related antigens and p39. While these studies established a connection between Fos, Jun, and AP-1, they did not establish the nature of connection that is, whether Fos and Fos-related proteins bind to the AP-1 site directly, indirectly by way of association with p39, or by interaction with Jun or other AP-1 polypeptides.

Growing evidence from studies in both prokaryotic and eukaryotic systems supports a model in which transcription factors interact as dimers

with half-sites of palindromic recognition sequences. Heterodimeric complexes may have altered affinities for one of these half-sites. In this way a single recognition element could interact with a hierarchy of heterodimeric factors with differing activities. Cell-surface stimulation results in increased synthesis of several Fos and Jun-related proteins that associate with AP-1 sites. These proteins appear and are turned over with different kinetics. Thus, the rate of transcriptional initiation of specific target genes may depend upon the concentrations and relative affinities of these proteins for homo or hetero-dimeric protein complex formation. The association of Fos, Jun, and AP-1 sites in the nucleus is analogous to cell-surface interactions of ligands and receptors. AP-1 elements may be regarded as nuclear receptors with different affinities depending upon exact nucleotide sequence, chromatin structure, associated proteins, and structural modifications. Fos, Jun, and related proteins would constitute a class of ligands with multiple and variable subunits that interact with genomic receptors containing the AP-1 site, thereby modulating gene transcription. The affinities, specificities, and activities of these ligands may depend upon subunit composition, posttranslational modifications, competing ligands, and flanking nucleotide sequences or overlapping binding sites.

Phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) are potent tumor promoters, capable of potentiating the effect of a subcarcinogenic dose of an initiating carcinogen (45). TPA and other phorbol esters activate protein kinase C which affects a number of processes including gene expression (46), phospholipid and protein

synthesis (47), ornithine decarboxylase activity and polyamine biosynthesis (48), DNA synthesis (49), phosphorylation of specific cellular proteins (50), binding of insulin and epidermal growth factor to their respective receptors (51), and cellular differentiation and proliferation (49).

The insulin-like effects of TPA are of particular interest. For example, TPA stimulates the phosphorylation of the same sites on ribosomal protein S6 in H4IIE hepatoma cells as does insulin (52), and both insulin and TPA activate the S6 kinase in 3T3-L1 adipocytes (50). TPA also stimulates hexose transport in chicken embryo fibroblasts (53), BC3H-1 myocytes (54), and rat adipocytes (55), and it enhances amino acid uptake and pyruvate dehydrogenase activity in BC3H-1 myocytes (54). Finally, TPA increases lipogenesis in rat adipocytes (56) and stimulates phosphorylation of the insulin receptor in IM-9 lymphocytes (40) and H4IIE cells (57).

Phorbol esters do not mimic all the actions of insulin; indeed TPA added simultaneously with insulin results in inhibition of several effects of the hormone. Notable examples include the stimulation of hexose transport in adipocytes (56), the phosphorylation of insulin receptors in Fao hepatoma cells (57) and BC3H-1 myocytes (54), the activation of glycogen synthase activity and induction of tyrosine aminotransferase (TAT) in cultured hepatoma cells (57), and the stimulation of lipogenesis in adipocytes (56). Furthermore, insulin binding to its receptor is inhibited by TPA in IM-9 lymphocytes (40) and rat adipocytes (55). These anti-insulin effects of TPA have been suggested to play a role in the negative feedback regulation of the insulin action (56). Thus, phorbol esters can at times be insulin-like, and in other instances they can antagonize insulin's

action.

The regulation of tyrosine aminotransferase (TAT) has served as an excellent model system for the study of hormonal and developmental regulation of gene expression ever since the hormonal regulation of this mammalian enzyme was first shown by Lin and Knox in 1957. TAT is the rate-limiting enzyme for tyrosine catabolism and prevents the accumulation of toxic level of tyrosine as well as providing ketogenic and gluconeogenic substrates (78). TAT exists solely in liver; the ability of mitochondrial aspartate aminotransferase to transaminate tyrosine accounts for reports of tyrosine aminotransferase activity in other tissues (79). Current data indicate that the activity of tyrosine aminotransferase is regulated solely through changes in its rate of synthesis and/or degradation. TAT is one of a cluster of enzyme activities that first appears at birth, when overall liver metabolism is shifted toward gluconeogenesis. TAT catalytic activity is virtually absent in fetal rat liver up to the 20th day of gestation; detectable amounts of catalytic activity have been reported on the 21st day of gestation (80). Enzyme activity begins to rise dramatically shortly after birth, reaches a maximum of at least twice the adult levels by 12hr, and returns to basal adult levels by 2 days after birth (80). In the period of transition from late fetus to newborn, the fetal plasma concentration of a number of hormones, such as epinephrine, glucagon, hydrocortisone, and insulin, changes dramatically. During the time that this transition has been studied, each of the first three of these hormones, acting singly or in combination with the others, has been proposed as the primary inducer responsible for the dramatic increase in TAT catalytic activity seen within

a few hours of birth (81).

It has been reported that in adult rat liver, insulin increases the amount of functional TAT mRNA and increases in RNA synthesis. According to Lee et al, an increase in transcription accounts for the increase in TAT mRNA and activity. In our laboratory, studies of insulin's effects on TAT expression in KRC-7 showed an increase in TAT enzyme activity with a decrease in transcription of TAT mRNA(102). This was shown in three different experiments. First, 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB), an inhibitor of RNA synthesis was used to stop RNA synthesis and study the effect of insulin on TAT enzyme activity. Results indicated that TAT enzyme activity is still induced by insulin, indicating that insulin works through a post-transcriptional pathway. Second, a cDNA probe was used to determine the amount of TAT mRNA. After treatment with insulin, the activity of TAT enzyme increased but the amount of TAT mRNA decreased. Finally, using nuclei from insulin treated cells, the rate of TAT mRNA synthesis was measured. The results showed a decrease in the rate of TAT mRNA synthesis after insulin treatment.

TAT enzyme activity can also be induced by glucocorticoids or cAMP analogs. It is believed that glucocorticoids, cAMP analogs, and insulin act through separate pathways. The tumor promoting phorbol esters have been reported to stimulate phosphorylation of the insulin receptor and thereby decrease the ability of insulin to induce TAT. Recently, our lab reported that increases in TAT activity stimulated by insulin, glucocorticoid, or cAMP analogs are inhibited by TPA. The mechanism of insulin action remains largely unexplained despite intensive efforts. It is

known that insulin binds to a heterodimeric cell surface receptor that contains a hormonally responsive tyrosine kinase activity in its β -subunit (58). The regulation by insulin of enzymes controlling intermediary metabolism is often the result of changes in the state of phosphorylation of the enzyme (59). What remains unknown is the precise biochemical link between the activated receptor and regulated enzyme. Several substances have been proposed to act as second messengers for insulin although each has proved inadequate to account for the action of the hormone (60). It is very important to understand the mechanism by which insulin exerts its diverse biological responses in a target cell. Studies utilizing phorbol esters will facilitate these investigations since they may prove to mediate part of the insulin response or they may share a common post receptor distal converging pathway.

Studies in our laboratory are performed on *in vitro* rat hepatoma cell lines descended from the Reuber H35 chemically induced rat hepatoma. Reuber H35 rat hepatoma cells are one of the few cell-line in culture known to respond to physiologic concentrations of insulin as a growth factor (84), and have been shown to possess a number of liver like functions (85).

KRC7 is a rat hepatoma cell line derived from the Reuber H35 clone that has been characterized for insulin responsiveness. Insulin, at physiological levels, has been demonstrated to be a potent growth factor inducing cell proliferation in KRC7 cells (84,86). Treatment of KRC7 cells with insulin inhibits transcription of the TAT gene in a time and dose dependent manner (87). Insulin also inhibits the stimulatory effects of glucocorticoids and cAMP on TAT mRNA levels (87).

I. Research Objectives and Experimental Design

The goal of the research outlined in this thesis was to gain further insight into the mechanisms by which second messengers mediate their effects on eukaryotic gene expression at the level of transcription. The regulation of the gene that encodes the liver specific enzyme Tyrosine Aminotransferase (TAT) will be examined. Assessed will be the role of protein kinase C (PKC), a phospholipid and Ca^{2+} -dependent serine/threonine protein kinase, which is known to be activated by the intracellular second messenger, diacylglycerol (DAG). The tumor promoting phorbol esters activate PKC in a manner analogous to DAG. Unlike DAG, phorbol esters are not rapidly metabolized and are more potent in activating PKC. They therefore serve as an efficient tool in studies of the second messenger role of DAG. In previous studies, the tumor promoter phorbol esters have been reported to stimulate phosphorylation of the cytoplasmic domain of the insulin receptor at specific serine and threonine residues thereby inhibiting the ability of insulin to induce TAT activity (91). The research described here will expand our understanding of this attenuation.

Studies were carried out to investigate the effect of TPA on TAT mRNA levels. Time course and dose response relationships for TPA action were determined, using Northern blot analysis of TAT mRNA. A full length cDNA probe, TAT 123, allowed quantitation of the effect of TPA on abundance of TAT mRNA.

To determine if TPA and insulin modify dexamethasone's action on TAT, we studied the effect of TPA and insulin alone, and in the presence of dexamethasone on TAT mRNA. Dexamethasone was added together with insulin and TPA.

Next, cycloheximide, an inhibitor of protein synthesis, was added alone or in the presence of TPA to KRC7 cells to determine if ongoing protein synthesis is required for the effect of phorbol ester on TAT mRNA levels. The effect on TAT enzyme activity of dexamethasone, cAMP, and insulin alone and in the presence of TPA was also examined.

CHAPTER II

Methods and Materials

I. Materials

Phorbol ester was purchased from Sigma company, St. Louis, MO. Insulin was purchased from Elanco products company, Indianapolis, IN. Cell culture medium, Dulbecco's modified Eagle's medium (DMEM), was from GIBCO, Grand Island, N.Y. Calf serum and fetal bovine serum were products of M.A. Bioproducts, Walkersville, Ma. Cell culture dishes were from Corning Glass Works, Corning, N.Y. Cesium chloride was purchased from BRL, Bethesda, Ma. Guanidine thiocyanate was purchased from Research Organics, Cleveland, OH. ^{32}P -dCTP for nick-translations was from ICN, Irvine, Ca. All other chemicals were obtained from Sigma Chemicals or Fisher Scientific.

II. Preparation of Compounds

12-O-tetradecanoyl-phorbol-13-acetate (TPA) was prepared as a 1.6 mM stock solution in 95% ethanol or 5 % DMSO. The maximal final concentration of TPA treatment was 1.6 μM . As a control, 95 % ethanol was added to the cells to a final concentration of 0.1 %. Insulin was prepared as a 1 mg/ml stock in 0.1 M HCl, quantitated at OD276 where an absorbance of 1.05 = 1 mg/ml insulin. Dexamethasone was prepared as a 100 μM solution in serum-free DMEM medium. Dibutyryl-cAMP was prepared as a 20mM stock solution in serum-free DMEM medium.

III. Cell Culture

KRC7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% calf serum or 10% Fe-supplemented calf serum. 35 mm dishes were used to grow cells for enzyme assays. Each enzyme assay was done on 3 biological replicates per treatment and each experiment was repeated 3 times. 100 mm dishes were used for RNA preparation. Each Northern blot analysis was repeated 3 times with different RNA preparation. Upon reaching confluency serum-containing medium was removed and replaced with serum-free medium for at least 18 hr before addition of various inducing agents.

IV. Preparation of Cell Extracts For Enzyme Assays

Cells were washed with 0.9 % NaCl once and then scraped directly into 1 ml of cold phosphate buffered saline harvest buffer (PBS - 0.9% NaCl, 0.2% KCl, 20 mM K₂HPO₄, pH 7.2 and 0.1 % NP-40). The cell suspension was thoroughly mixed and then centrifuged at 4⁰C 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 (88). Protein was determined according to the method of Lowry (89).

V. RNA Isolation

Total cellular RNA was isolated by the method of Chirgwin (90). Treated cells were washed with 1X PBS twice and dissolved in 4M guanidinium thiocyanate, layered over a 5.7M cesium chloride cushion, and centrifuged for 18 hrs at 36,000 rpm at 20⁰ C. The RNA pellet was dissolved in 0.1% diethylpyrocarbonate (DEPC) treated water, and was

precipitated with ethanol. Next, the pellet was dissolved in DEPC treated water for storage at -20°C . RNA was quantitated by OD₂₆₀, using an absorbance of 1 equal to 40 ug/ml RNA.

VI. RNA Fractionation and Hybridization

10 ug of total RNA was fractionated on 1.1% agarose gels containing 6% formaldehyde and transferred to nitrocellulose using 20X SSC (175.3 g NaCl/liter, 88.2 g Na Citrate/liter, pH 7.2) as the transfer buffer, according to Thomas (101). Visualization of the RNA in the gel was done by staining with ethidium bromide, and destaining with water. The nitrocellulose membranes were visually examined under UV light for uniform RNA transfer, and blots were air-dried and baked in a vacuum oven at 80°C for 1 hr. The blot was incubated overnight at 42°C in pre-hybridization solution mix (50 % formamide, 10X Denhardt's (Ficoll 5g, polyvinylpyrrolidone 5g, BSA 5g, and H₂O to 500 ml), 5X SSPE (NaCl 174g, NaH₂PO₄-H₂O 27.6g, EDTA 7.4g and adjust pH to 7.4 in 800 ml H₂O), 100 ug/ml denatured salmon sperm DNA, and 0.1 % SDS) (109). The probe pTAT 123 (kindly provided by Dr. G. Schutz) was nick-translated to a specific activity of 2.5×10^7 - 10^8 cpm/ug, added to the pre-hybridization solution and allowed to hybridize overnight at 42°C . The blot was then washed with a series of washes of increasing stringency. Washes 1 and 2 were with 2X SSC and 0.1 % SDS for 30 minutes at room temperature. Washes 3 and 4 were with 0.1X SSC and 0.1 % SDS for 30 min at 55°C . If extra washes were needed, distilled water was added to the blot and washed for 10 to 15 min at room temp. The damp blot was then wrapped in plastic wrap and exposed to Kodak XRP film at -80°C using a

Cronex intensifying screen. As an internal control, the pR β .3 plasmid construct (a cDNA probe to rat β -tubulin kindly provided by Dr. Farmer) was used to measure β -tubulin mRNA level for all Northern blots. Relative intensities of the bands were quantitated by densitometry. As for standardization of the intensities of the bands, different amounts of total RNA (1 ug, 5 ug, 10 ug, 15 ug, and 20 ug) were fractionated on each gel (Figure 1)* and intensity of these bands were quantitated. The intensities of the bands increased linearly with increasing amounts of total RNA (Figure 2). Once the data was obtained, statistical analysis was performed on each treatment according to F test and t test (104). F test and t test are designed to indicate whether there is a significant difference between two experiments based on their standard deviations.

* all figures may be found in the Appendix.

CHAPTER III

Results

I. Time Course of TPA Mediated Decreases in TAT mRNA

Koontz and Goodman (91) have shown that TPA inhibits induction of TAT enzyme activity by glucocorticoids, cAMP, and insulin. Because these three agonists act through different mechanisms, it was believed that TPA must be affecting some step that would be common to each of these agonists. Since changes in transcript levels may result in increases in activity, the effect of TPA on TAT mRNA levels was examined. Initially, the time course of the TPA effect on TAT mRNA was investigated using KRC7 cells which had been incubated in a serum-free medium for 18 hours. 1.6 uM final concentration TPA was added for various periods of time. Half hour treatment with TPA caused a slight increase in TAT mRNA abundance. TPA had no apparent effect on TAT mRNA abundance at 1 hour. TPA caused a decrease in TAT mRNA at 2 hour (90 % inhibition), and TAT mRNA abundance increased slowly with longer periods of incubation (Figure 3).

II. Dose Response of TPA Mediated Decreases in TAT mRNA

To further characterize TAT mRNA levels after TPA treatment the effect of various concentrations of TPA on TAT mRNA levels was measured. A 2 hour incubation of the cells with TPA was used because that is the time at which maximal inhibition of TAT mRNA levels was

observed. Increasing concentrations of TPA gave decreasing levels of TAT mRNA (Figure 4). Maximal inhibition was observed at 0.16 μ M with no greater inhibition observed at 1.6 μ M TPA. Half- maximal inhibition was obtained with approximately 10 nM TPA. Thus, the loss of TAT mRNA is both dose and time dependent.

III. Time Course of TPA Effect on Dexamethasone Mediated Changes in TAT mRNA within One Hour.

The TPA time course experiment (Figure 3) suggests that TPA has no effect on TAT mRNA at 1 hour. To determine if TPA modifies the action of dexamethasone on TAT mRNA in KRC7 cells during the 1st hr, the cells were treated with TPA plus dexamethasone, and TAT mRNA levels were measured. After incubation of KRC7 cells in the serum-free medium for 18 hours, dexamethasone and TPA were added and cells were harvested after 15 min, 30 min, and 60 min. Dexamethasone increased the TAT mRNA level by 3 fold over the basal level within 60 minutes of treatment (Figure 5). As expected, TPA alone had no effect on TAT mRNA abundance at 1 hour (Figure 5). When TPA and dexamethasone were added simultaneously, TPA had no effect on dexamethasone's ability to induce TAT mRNA during the 1st hr (Figure 5).

IV. Effect of TPA and Insulin on Hybridizable TAT mRNA and Dexamethasone Mediated Increases in TAT mRNA.

Previously, our laboratory demonstrated that when insulin is added simultaneously with dexamethasone, TAT mRNA levels do not increase to the level obtained with dexamethasone alone (102). It was also observed

that when insulin is added to cells that have been treated with dexamethasone for 4 hours, the level of TAT mRNA drops by 50 % within 2 hours of insulin addition (102). This suggests that insulin either causes increased degradation of the existing or newly synthesized TAT mRNA or inhibits the transcription of TAT mRNA. Therefore, I compared the effect of TPA with that of insulin as it relates to dexamethasone mediated increases in TAT transcript.

Increases in TAT mRNA after glucocorticoid or cAMP induction have been reported before by Scherer et al. (92). 3 hour treatment of KRC7 cells with dexamethasone increased TAT mRNA levels 6 fold above basal levels (Figure 6). Both insulin and TPA inhibited accumulation of TAT mRNA about 50 % at 3 hour of treatment (Figure 6). When TPA or insulin were added simultaneously with dexamethasone, TPA had no effect on dexamethasone's ability to induce TAT mRNA (statistical analysis shows no significant differences between dexamethasone 3 hours and dexamethasone plus TPA 3 hours), but insulin inhibited dexamethasone-induced TAT mRNA by 55 % (Figure 6).

V. Effect of Preincubation with TPA on Dexamethasone induced TAT mRNA.

We have demonstrated that TPA does not cause a decrease in TAT mRNA basal level at 1 hour, but it does decrease it's basal level at later times. We have also shown that TPA does not inhibit the dexamethasone induced TAT mRNA at 1 hour or 3 hours. To address the question of whether TPA inhibits the basal level thus lowering dexamethasone induced TAT mRNA, we pre-incubated the KRC7 cells with TPA for 1 or 2 hours

and then added dexamethasone for 4 hours. 4 hour treatment of KRC7 cells with dexamethasone increased TAT mRNA levels 2.5 fold above basal levels (Figure 7). Interestingly, dexamethasone increased TAT mRNA level 3 fold at 1 hour (Figure 4) and TAT mRNA level was increased 6 fold by dexamethasone at 3 hour (Figure 6). This figure shows that dexamethasone induced TAT mRNA only 2.5 fold. There is a discrepancy among the different experiments with respect to the inducibility of the TAT mRNA level by dexamethasone. However, this is not a surprising result. It has been demonstrated that dexamethasone has 3 to 6 maximal fold induction at 2 to 4 hours of treatment (95, 102, 105). Perhaps, I observed maximal induction by dexamethasone at one experiment (Figure 6) and observed minimal induction by dexamethasone in other experiment (Figure 7).

TPA treatment inhibited basal accumulation of TAT mRNA about 60 % at 5 or 6 hours of treatment (Figure 7). When dexamethasone was added for 4 hours after pre-incubation with TPA for 1 or 2 hours, TPA did not inhibit dexamethasone induced TAT mRNA, rather TPA inhibited dexamethasone-induced TAT mRNA by lowering TAT mRNA basal levels (Figure 7).

VI. Effect of Translation Inhibitor Cycloheximide on TPA Stimulated TAT mRNA.

Cycloheximide (CHO) is a widely used inhibitor of protein synthesis. It was reported that CHO and other protein synthesis inhibitors were capable of increasing TAT mRNA in rat liver (93). It was suggested that CHO might block the synthesis of a short-lived protein repressor of

TAT gene transcription (93). Alternatively, CHO might inhibit turnover of the mRNA template by inhibiting the synthesis of a labile protein necessary for TAT mRNA degradation. If the decrease in the transcript of TAT caused by TPA is mediated by modification of an existing short-lived protein, then CHO might block TPA-mediated decreases in TAT mRNA.

To test this hypothesis, an experiment was performed to determine the effect of CHO on the ability of TPA to cause a decrease in TAT mRNA. After incubation of KRC7 cells in a serum-free medium for 18 hours, 10 μ M CHO was added to the cells. 10 μ M CHO elevated TAT mRNA levels 2 fold within the 1st hour, and 2.5 fold in the next hour of treatment (Figure 8). As a control, TPA was added to the cells for 1, 2, or 4 hours. As expected, TPA alone does not cause a decrease in TAT mRNA for the 1st hr, but shows significant inhibition at 2 hour and 4 hour of treatment (Figure 8). When CHO and TPA are added simultaneously, the TAT mRNA level was almost the same as with CHO alone at 1 hour (Figure 8). The effect was similar at 2 and 4 hours (Figure 8). These results are consistent with the possibility that TPA decreases the level of TAT mRNA by modification of an existing short-lived protein. This short-lived protein might be causing an increased rate of degradation or a decreased rate of synthesis of TAT mRNA. My results don't distinguish these two possibilities.

VII. Effect of TPA on Dexamethasone Mediated Increases in TAT Enzyme Activity.

Next, the level of TAT enzyme activity was measured to examine whether the effect of TPA on TAT mRNA levels is reflected in decreased

activity of the enzyme. TPA had no effect on TAT activity at 1 hour, but its activity began to decrease slowly during the next 3 hours (Figure 9). By comparing Figures 3 and 9, it can be seen that the TAT mRNA and TAT activity curves correlate well with each other. While TAT mRNA and enzyme activity were not affected by TPA at 1 hour, both curves showed inhibition by TPA at later times. Therefore, it is reasonable to assume that a decrease in TAT activity is caused by a decrease in its mRNA levels.

When KRC7 cells were treated with dexamethasone, TAT activity increased slightly during the 1st hour, but increased greatly at a later time points (Figure 9). By 4 hours, TAT activity was increased 3.5 fold by dexamethasone. When TPA and dexamethasone were added simultaneously, TPA had no effect on dexamethasone-induced TAT activity at 1 hour, but TPA inhibited dexamethasone-induced TAT activity by almost 50% at later times (Figure 9). Interestingly, if one calculates the fold induction for dexamethasone plus TPA using the TPA treated values as the baseline, the values are the same as for induction by dexamethasone alone. This indicates that the inhibitory effect of TPA on dexamethasone induced TAT enzyme activity is probably due to a decrease in basal TAT enzyme activity and not on dexamethasone induction.

VIII. Effect of Preincubation with TPA on Dexamethasone induced TAT Enzyme Activity.

Figure 6 demonstrated that pre-incubation of the cells with TPA for 1 or 2 hours did not cause a decrease in dexamethasone induced TAT mRNA levels. Therefore, the level of TAT enzyme activity was measured to

examine whether the effect of TPA on TAT mRNA levels is reflected in activity of the enzyme. When KRC7 cells were treated with TPA for 5 or 6 hours, TAT activity decreased 55% lower than its basal level (Figure 10). When dexamethasone alone was added for 4 hours, TAT activity was increased 7 fold (figure 10). When dexamethasone was added for 4 hours after pre-incubation with TPA for 1 or 2 hours, TPA inhibited dexamethasone induced TAT activity by almost 32% and 25% respectively (figure 10). Once again, by using TPA alone as the basal values, the induction by dexamethasone in the presence of TPA is equal to or greater than that in the absence of TPA. Therefore I conclude that the effect of TPA on basal TAT mRNA levels is reflected in decreased basal enzyme activity but it does not affect dexamethasone induction of TAT mRNA or enzyme activity.

IX. Effect of TPA on cAMP Mediated Increases in TAT Enzyme Activity.

In addition to being regulated by glucocorticoids, TAT is also induced by cAMP. As a further test of the specificity of the effects of TPA on TAT induction, the ability of TPA to affect cAMP stimulated increases in enzyme activity was measured. cAMP increased TAT enzyme activity by almost 2 fold at 1 hour, and by 3 fold at the end of 4 hours (Figure 11). As expected, TPA showed no effect at 1 hour, but inhibited TAT activity at later times (Figure 11). When TPA and cAMP were added simultaneously, TPA did inhibit cAMP induced TAT activity at 3 and 4 hours but it did not inhibit its activity in the first hour (Figure 11). When the fold induction for TPA plus cAMP was calculated using the activity from TPA treated cells as basal, the induction due to cAMP was essentially identical to that

obtained with cAMP alone. This indicates that inhibitory effect of TPA on cAMP induced TAT enzyme activity is due to the decrease in basal enzyme activity.

X. Effect of TPA on Insulin Mediated Increases in TAT Enzyme Activity.

As another means of determining the specificity of the effect of TPA, the induction of TAT activity by insulin was measured. Recently, our laboratory reported that insulin decreases TAT mRNA abundance in KRC7 cells but increases the TAT enzyme activity (102). No other cell line has shown increased mRNA levels and decreased activity. Therefore, it was of interest to determine the effect on TAT activity when insulin and TPA were added together, since both insulin and TPA inhibit TAT mRNA, but show opposite effects on TAT activity.

It has been demonstrated that excess TPA in treated hepatoma cells causes inhibition of PEPCCK transcription within the first few hrs, but PEPCCK transcription increases slowly during next 12 hours and virtually no effect is seen at 20 hours after treatment (94). This has been attributed to down-regulation of protein kinase C (94). TPA treatment had similar effects on TAT activity (Figure 12). TPA caused significant inhibition at 4 hours but TAT activity went up slowly during next 8 hours and had returned to basal level at the end of 20 hours (Figure 12). Insulin alone caused induction of TAT activity that continues to increase for up to 12 hours. After that enzyme activity decreased and maintained 3 fold induction even after 20 hours of insulin incubation (Figure 12). When TPA is added simultaneously with insulin, TPA also inhibits the induction of TAT activity by insulin at early time points by lowering TAT basal

activity, but TPA does not inhibit insulin induced TAT activity at later times (Figure 12). These data demonstrate that TPA does not inhibit insulin induction of the TAT enzyme. Rather TPA inhibits the TAT basal activity and lowers insulin induced TAT activity. Longer-term exposure of cells to TPA causes down regulation of the TPA receptor and thereby loss of the TPA response.

CHAPTER IV

Discussion

The results of this study demonstrate that TPA provokes an insulin-like action in that it, too, reduces the amount of TAT mRNA in KRC7 cultured rat hepatoma cells. This inhibitory effect of TPA is concentration- and time-dependent. However, it does not override the stimulatory effect of glucocorticoid analogs. TPA does not mimic insulin-like action on TAT enzyme activity in KRC7 cells. Unlike insulin, TPA inhibits basal TAT enzyme activity but does not inhibit the stimulatory effects of cAMP analogs, a glucocorticoid analog, or insulin.

Previously, our laboratory observed that insulin increased the activity of TAT enzyme while inhibiting the transcription of its gene in KRC7 cells. In the fully-differentiated adult rat liver Fao cell line, insulin increases TAT enzyme activity and TAT mRNA level (95). It was proposed that in both cell lines, insulin has a post-transcriptional effect and that regulation of TAT by insulin is not through the same mechanism in all cell types. The insulin effects can be mimicked by anti-insulin receptor antibodies, indicating that insulin acts on TAT via binding and activation of the insulin receptor, causing an accumulation of TAT mRNA (95). Kahn's group also found that an increase in TAT mRNA is associated with an increase in the transcription rate of the TAT gene. They also characterized the effect of TPA on insulin receptors and insulin action in Fao cells. They

observed that TPA did not affect insulin binding to receptors either acutely or chronically, but it inhibited insulin activation of glycogen synthase and TAT activity (57). This insulin-resistant state was produced within 30 min by TPA and occurred in a dose-dependent manner. They also found that TPA stimulated the phosphorylation of insulin receptors in intact hepatoma cells at serine and threonine residues, and that pretreatment of cells with TPA resulted in a decrease in the insulin-induced phosphorylation of receptors, particularly at tyrosine residues. They proposed that TPA, acting through protein kinase C, can stimulate serine and threonine phosphorylation of the insulin receptor, altering insulin receptor autophosphorylation and insulin action.

In KRC7 cells, the TPA-mediated effect on TAT indicates that the decrease in enzyme activity can be attributed to a decrease in mRNA for the enzyme in a time- and dose-dependent manner. There is no change in the first hour after addition of 1.6 μ M TPA. At two hours, there is a dramatic decrease in the transcript. It is possible that this decrease in the transcript levels is due to inhibition of transcription by TPA.

Previously, it has been reported that the tumor promoting phorbol esters stimulate phosphorylation of the insulin receptor and thereby decrease the ability of insulin to induce TAT. Our laboratory demonstrated that TPA will not only inhibit the insulin stimulated increase in TAT activity, but also inhibits induction of the enzyme activity by glucocorticoids or by cAMP. Does TPA inhibit the glucocorticoid mediated induction of TAT mRNA and enzyme activity? Our results suggest that it is not necessary to postulate that TPA inhibits the dexamethasone mediated pathway; rather TPA lowers the basal mRNA

abundance and enzyme activity. Each point in Figure 6 and 7 was normalized to the amount of β -tubulin mRNA. When we compare the fold induction by dexamethasone in figures 6 and 7, they show no inhibition of TPA on dexamethasone induced TAT mRNA; rather TPA increased fold induction of dexamethasone induced TAT mRNA. But TPA did inhibit basal TAT mRNA level. In Figure 6, dexamethasone inducibility of TAT mRNA basal abundance is 6.25X. However, dexamethasone inducibility of TAT mRNA in the presence of TPA is 12X. This result indicates that TPA shows an inductive, rather than inhibitory effect. Figure 7 shows the same result as figure 4. Dexamethasone inducibility of basal abundance is 2.5X. Dexamethasone inducibility after pre-treatment of the cells with TPA is about 5X. Interestingly, TPA inhibited basal abundance of TAT mRNA by 50 % in both figures 6 and 7. From these results, we can conclude that TPA does not inhibit dexamethasone induced TAT mRNA abundance. Rather, from the above result, one can conclude that it enhances induction of TAT mRNA abundance in the presence of dexamethasone. More work have to be done to verify the above conclusion.

Figures 9 and 10 show the similar effect by TPA on dexamethasone induced TAT enzyme activity. In figure 9, dexamethasone induced TAT enzyme activity was increased 2.8 fold at 3 hours and 3.5 fold at 4 hours. When TPA and dexamethasone were added simultaneously, TPA inhibited dexamethasone-induced TAT activity by almost 50 % at that time. If we calculate the fold induction for dexamethasone plus TPA using the TPA treated values as the baseline, the values are almost the same as for induction by dexamethasone alone. Figure 10 shows the same effect as figure 9. Dexamethasone increased TAT enzyme activity 7 fold above

basal enzyme activity. When dexamethasone was added for 4 hours after pre-incubation with TPA for 1 or 2 hours, TPA inhibited dexamethasone induced TAT enzyme activity by 32 % and 25 % respectively. If one calculates the fold induction for dexamethasone plus TPA using TPA treated values as the baseline, the induction by dexamethasone in the presence of TPA is equal to or greater than that in the absence of TPA. These results indicate that TPA inhibition of dexamethasone induced TAT enzyme activity is probably due to a decrease in basal TAT enzyme activity and not to inhibition of dexamethasone induction.

When we compare our dexamethasone induced TAT mRNA results and enzyme activity results, we conclude that TPA regulates gene expression post-transcriptionally. In Figure 6 and 7, TPA did not inhibit dexamethasone induced TAT mRNA levels. However, in Figures 9 and 10, TPA inhibition of dexamethasone induced TAT enzyme activity is great. There is a significant discrepancy. This can be attributed most likely to a post-transcriptional effect by TPA.

Interestingly, when I compare basal TAT mRNA results and enzyme activity results, I can conclude that TPA regulates gene expression transcriptionally. In Figures 3 and 9, TPA inhibition of TAT basal mRNA (75 - 80 %) and enzyme activity (60 - 70 %) do correlate with each other.

In addition to being regulated by dexamethasone, TAT enzyme activity is also induced by cAMP, and cAMP induced TAT enzyme activity can be inhibited by TPA. Our results suggest that TPA also does not inhibit the cAMP mediated pathway; rather TPA lowers the basal enzyme activity only. In figure 11, cAMP increased TAT enzyme activity by 2 fold at 1 hour, and by 3 fold at the end of 4 hours. When TPA and cAMP were

added simultaneously, TPA inhibited cAMP induced TAT enzyme activity at 3 and 4 hours. If one measures the fold induction for TPA plus cAMP using the TPA treated TAT enzyme activity as basal, the induction due to cAMP is identical to that with cAMP alone. This analysis also suggests that the decrease in basal TAT enzyme activity is responsible for the inhibition by TPA on cAMP induced TAT enzyme activity.

Interestingly, TPA increased the TAT activity slightly within 30 min and had no or little effect on TAT activity and its mRNA abundance at 1 hr in KRC7 cells. But at 3 hours, TPA decreased TAT activity and its mRNA abundance. In Fao cells, however, Kahn's group demonstrated that TPA inhibits insulin induced TAT activity within 30 min. One possibility is that the rate of action by TPA depends on the state of cell differentiation. Fao cells represent a fully-differentiated adult rat liver phenotype but KRC7 cells represent neither a fully-differentiated adult type nor fetal phenotype; their differentiated state has not been determined. The rate of PKC activation by TPA may be different in the two cell types.

Besides the TAT gene, regulation of other genes by TPA has been studied as well by other investigators. The enzyme PEPCK is an excellent model for studies of multihormonal regulation of specific gene expression. PEPCK, which catalyzes the rate-limiting conversion of oxaloacetate to phosphoenolpyruvate in hepatic gluconeogenesis, is subject to positive and negative regulation. Glucocorticoids and cAMP increase transcription of the PEPCK gene in H4IIE cells, which represent the fully differentiated adult phenotype rat liver hepatoma cell. These results are due to an increased level of PEPCK mRNA, increased rate of synthesis of the enzyme, and an enhanced rate of gluconeogenesis (96). Conversely, insulin

inhibits transcription of the PEPCK gene and thus results in reductions of PEPCK mRNA, the rate of enzyme synthesis, and the rate of gluconeogenesis (96). Granner and his group demonstrated that TPA provokes an insulin-like action in that it too reduces the amount of PEPCK mRNA in cultured rat hepatoma H4IIE cells (97). This inhibitory effect of TPA is concentration and time-dependent, and it overrides the stimulatory effects of potent cAMP and glucocorticoid analogs (97).

Blackshear's group (98) investigated the effect of insulin and TPA on the ornithine decarboxylase gene in H4IIE cells. Both insulin and TPA increased its activity and its mRNA abundance. Our laboratory also studied insulin and TPA effects on ornithine decarboxylase in KRC7 cells. We observed that both insulin and TPA increased its activity (103). Larner's group (99) studied the p33 gene in H4IIE cells. Both insulin and TPA increased its mRNA level. From the above results, it can be speculated that unlike insulin, which does not act through the same mechanism using in all cell-types, TPA appears act through PKC pathway in all cell-types but its resultant action depends on the type of enzyme. Whatever the cell phenotype might be, TPA inhibits TAT and PEPCK genes, and increases p33and ODC genes.

The mechanism by which TPA is able to enhance gene expression and transcription remains unknown. However, it is generally accepted that changes in the activity of protein kinase C mediated by TPA may initiate a cascade of events such as phosphorylation and dephosphorylation of various cellular components that may ultimately affect the action of specific transcription factors. The pathway for the transduction of a signal triggered by TPA has not been fully charted. However, the occurrence of

TPA-induced changes in patterns of transcription even in the absence of protein synthesis suggest that a likely mode of action may be through posttranslational alterations of preexisting transcription factors. Thus it is reasonable to propose that TPA-induced phosphorylation or other posttranslational modification directly or indirectly modulates the activity of transcription factors in the cell. In the case of AP-1, the data presented by Angel et al. suggest that both the DNA-binding activity *in vitro* and transcriptional properties *in vivo* are enhanced after treatment with TPA. In other cases, however, changes of transcriptional activity have been observed without concomitant modulation of DNA-binding properties of specific transcriptional factors (100), suggesting that mechanisms other than altered binding activity may be used. At present, there is no direct evidence for specific covalent modifications of transcription factors that influence either DNA binding or transcriptional activation. Interaction of DNA and protein which influence transcription appears to be complex and our current molecular techniques may not be equal to the task of unveiling true mechanism of gene expression. However, by using antibodies to study potential protein-protein interactions that contribute to the specificity of promoter recognition and the mechanism of transcriptional activation, we can understand how eukaryotic cells are regulated.

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APPENDIX

FIGURE 1

Typical Northern Blot Gel Picture and Autoradiogram of the Blot.

Gel picture and autoradiogram were taken as described in Materials and Methods. First 5 bands from the left were different amounts of total RNA (1 ug, 5 ug, 10 ug, 15 ug, and 20 ug) to test linearity of the intensities of the bands.

285»
185»



FIGURE 2

A Linear Increase in Intensity with Increasing RNA Amounts

Intensities of the bands (1 ug, 5 ug, 10 ug, 15 ug, and 20 ug of total RNA) were measured as described in Materials and Methods.

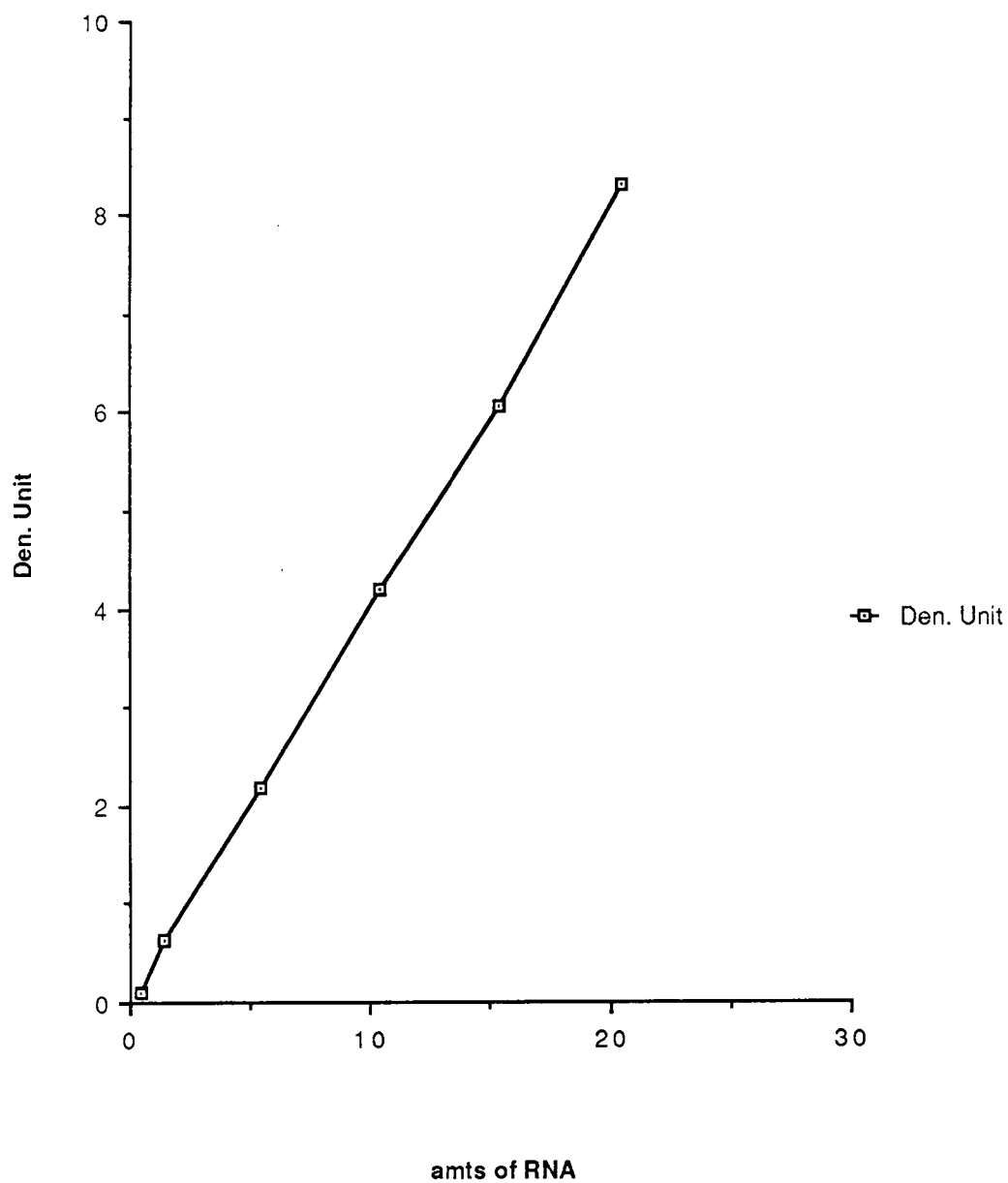


FIGURE 3

Time Course of TPA Mediated Decreases in TAT mRNA.

Northern blot analysis of the time course of the decrease in Tyrosine Aminotransferase mRNA in response to TPA. Quiescent cells were incubated with TPA (1.6 μ M) for the incubated times. Cells were harvested and RNA was extracted as described in Materials and Methods. As a control, β -tubulin mRNA was measured from the same blots using pR β T.3. probe. The graph represents quantitation of the autoradiograph by densitometry. Each data point represents the mean of three different replicates plus standard error measurement (SEM).

A) Data is expressed as a densitometry units of the particular mRNA.

B) The ratio of TAT to β -tubulin mRNA for these experiments.

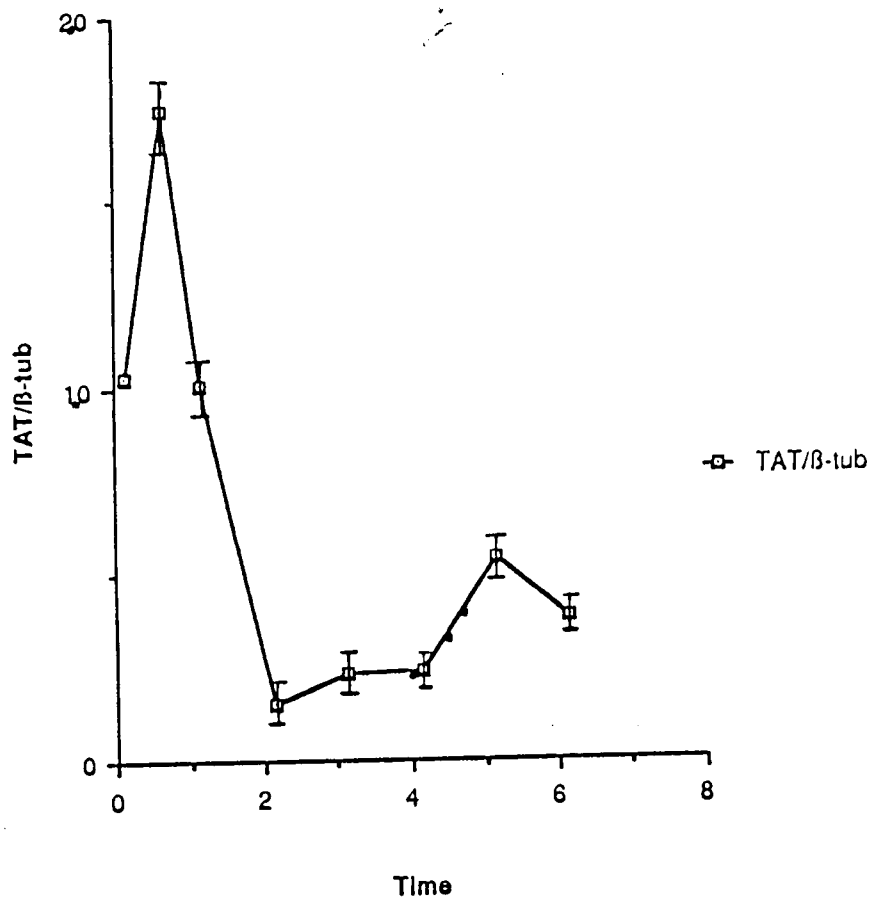
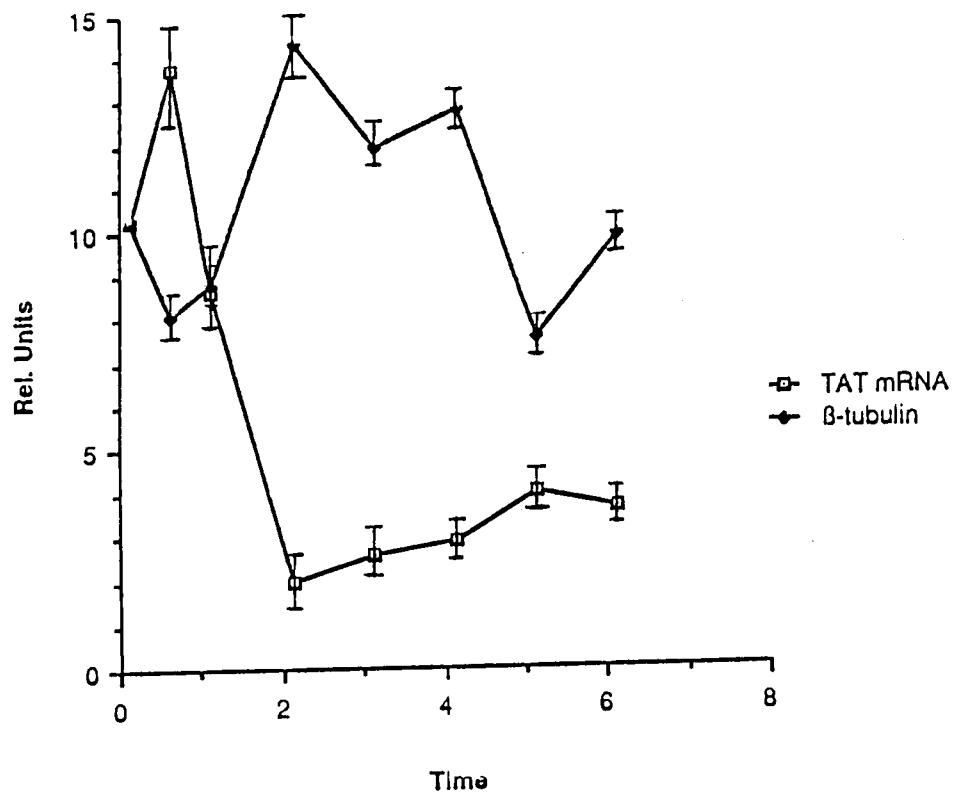


FIGURE 4

Dose Response of TPA Mediated Decreases in TAT mRNA.

Northern blot analysis of the dose response for TPA-stimulated decrease in Tyrosine Aminotransferase mRNA. Cells were made quiescent as described in Materials and Methods. Quiescent cells were incubated with the indicated concentration of TPA for 2 hrs. Following this incubation, cells were harvested, RNA was extracted, and gels were run, blotted, and probed as described in Materials and Methods. As a control, β -tubulin mRNA was measured from the same blots using pR β T.3. probe. The graph represents quantitation of the autoradiograph by densitometry. Each data point is expressed as the mean of three different replicates plus standard error measurement (SEM).

- A) Data is expressed as a densitometry units of the particular mRNA.
- B) The ratio of TAT to β -tubulin mRNA for these experiments.

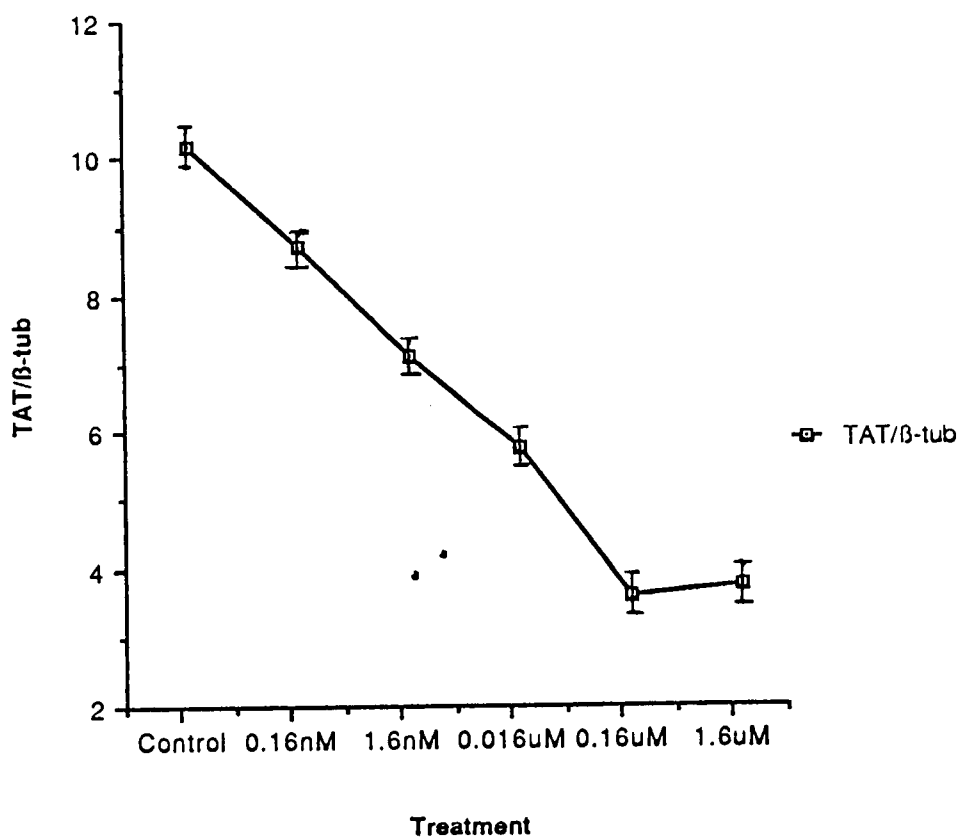
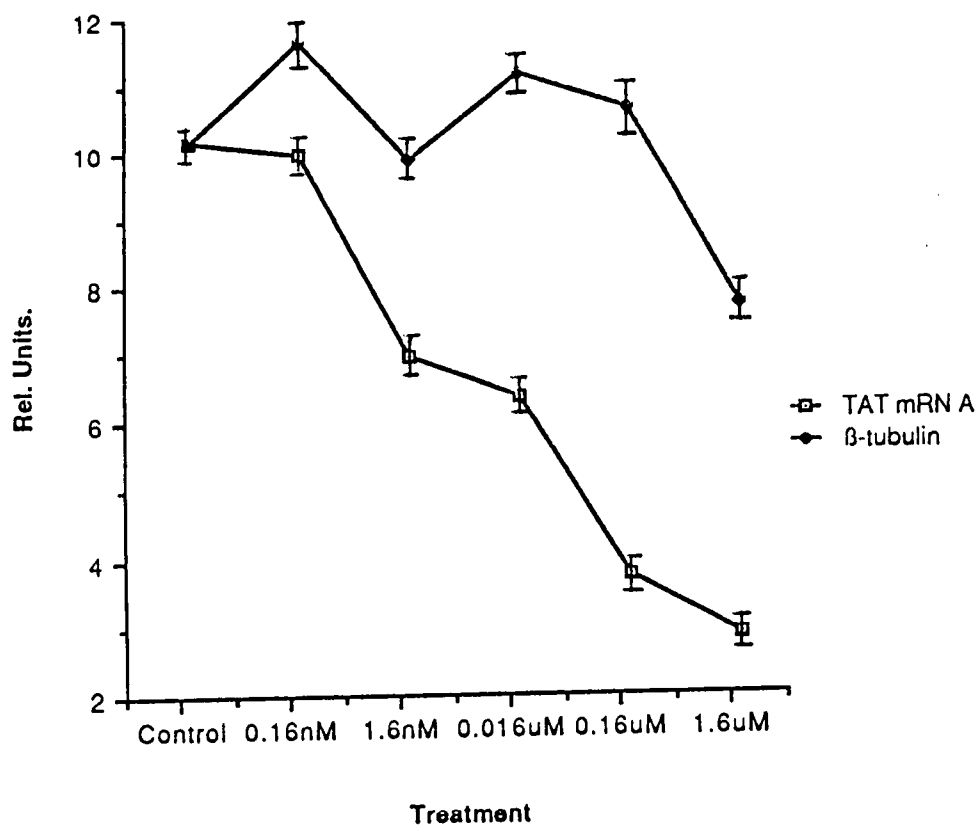


FIGURE 5

Time Course of TPA Effect on Dexamethasone Mediated Changes in TAT mRNA Within One Hour.

Cells were grown and prepared for treatment as described in Materials and Methods. TPA (1.6 μ M), Dexamethasone (1 μ M), or the two in combination were added to the culture medium and the cells were harvested at the indicated times. RNA was extracted as described under Materials and Methods. The line graph represents quantitation of the autoradiograph by densitometry. As a control, β -tubulin mRNA was measured from the same blots using p β RT.3 probe. Each data point is normalized by using β -tubulin mRNA values, and is expressed as the mean of three different replicates plus standard error measurement (SEM).

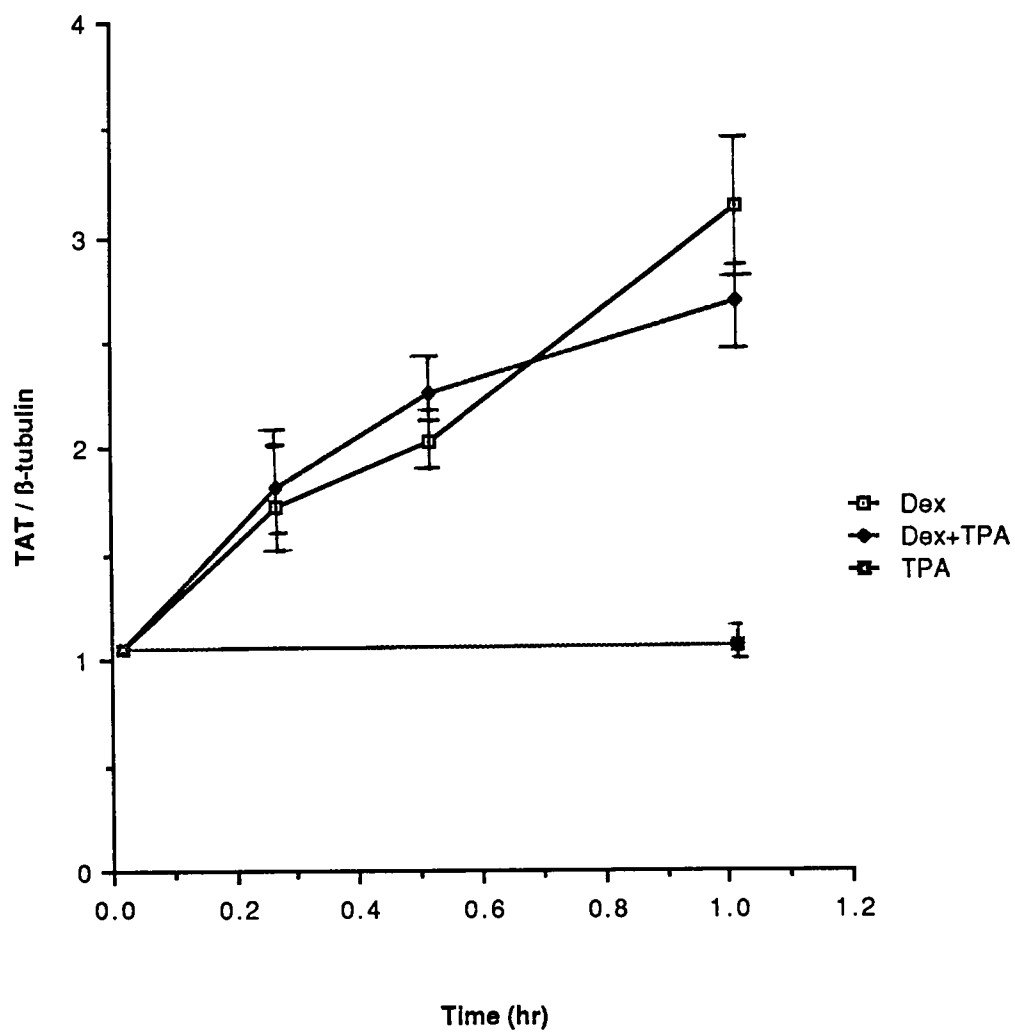


FIGURE 6

Effect of TPA and Insulin on Hybridizable TAT mRNA and Dexamethasone Mediated Increases in TAT mRNA.

The effect of TPA and Insulin on induction of Tyrosine Aminotransferase mRNA by Dexamethasone was measured in this experiment. Cells were made quiescent as described under Materials and Methods. Insulin (10 nM), Dexamethasone (1 μ M), TPA (1.6 μ M), or two in combination were added to the culture medium and the cells were incubated for 3 hours. Cells were harvested and RNA was extracted and analyzed as described in Materials and Methods. As a control, β -tubulin mRNA was measured from the same blots using p β RT.3 probe. Each bar is expressed as the mean of three different replicates plus standard error measurement (SEM).

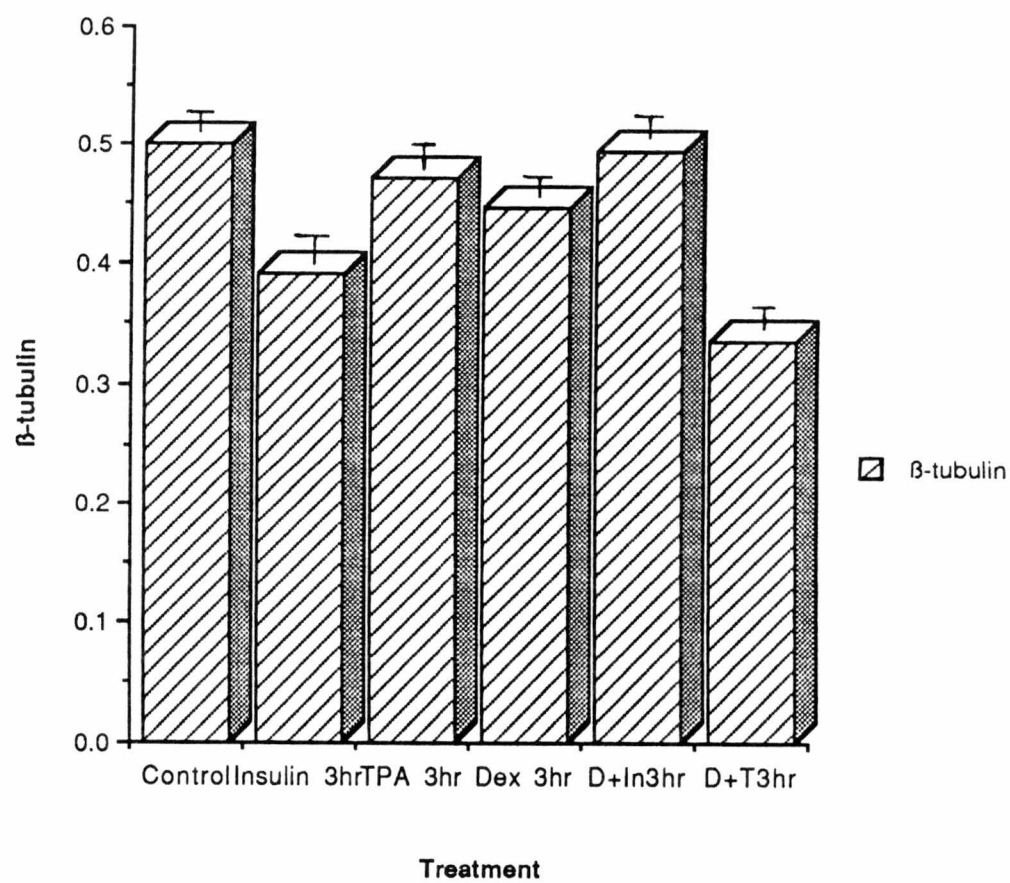
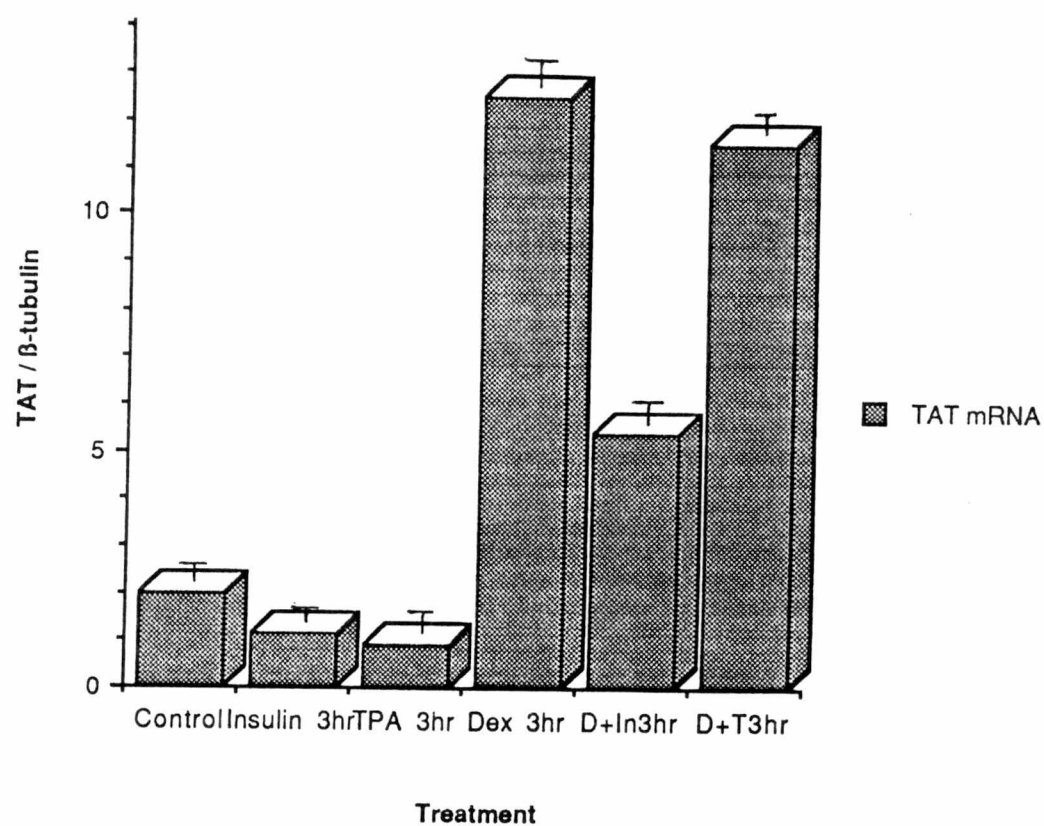


FIGURE 7

Effect of Preincubation with TPA on Dexamethasone Induced TAT mRNA.

Prior to treatment KRC7 cells were placed on SFM for at least 16 hrs. TPA (1.6 μ M) was added prior to the addition of dexamethasone and the cells were harvested at the indicated time. RNA was extracted as described under Materials and Methods. The bar graph represents quantitation of the autoradiograph by densitometry. As a control, β -tubulin mRNA was measure by using p β RT.3 probe. Each data point is normalized by using β -tubulin mRNA values, and is expressed as the mean of three different replicates plus standard error measurement (SEM).

T5; TPA treatment for 5 hrs.

T6; TPA treatment for 6 hrs.

T1+D4; pre-treatment of TPA for 1 hr then add dexamethasone for 4 hrs.

T2+D4; pre-treatment of TPA for 2 hr then add dexamethasone for 4 hrs.

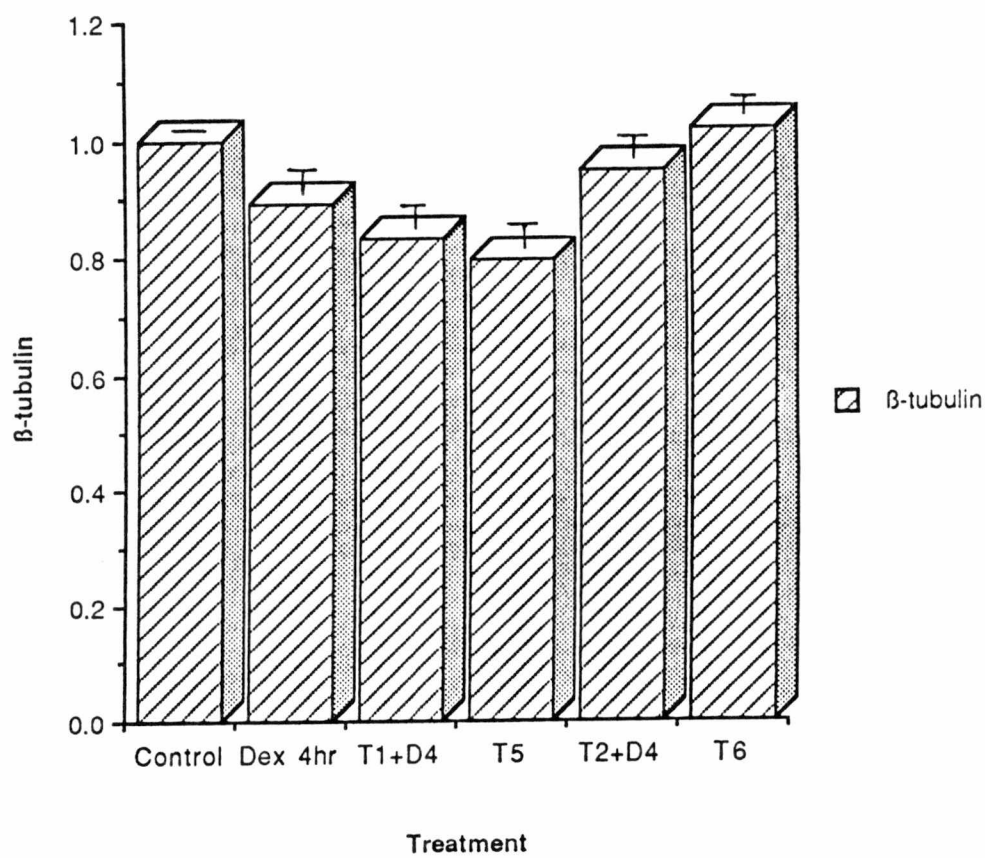
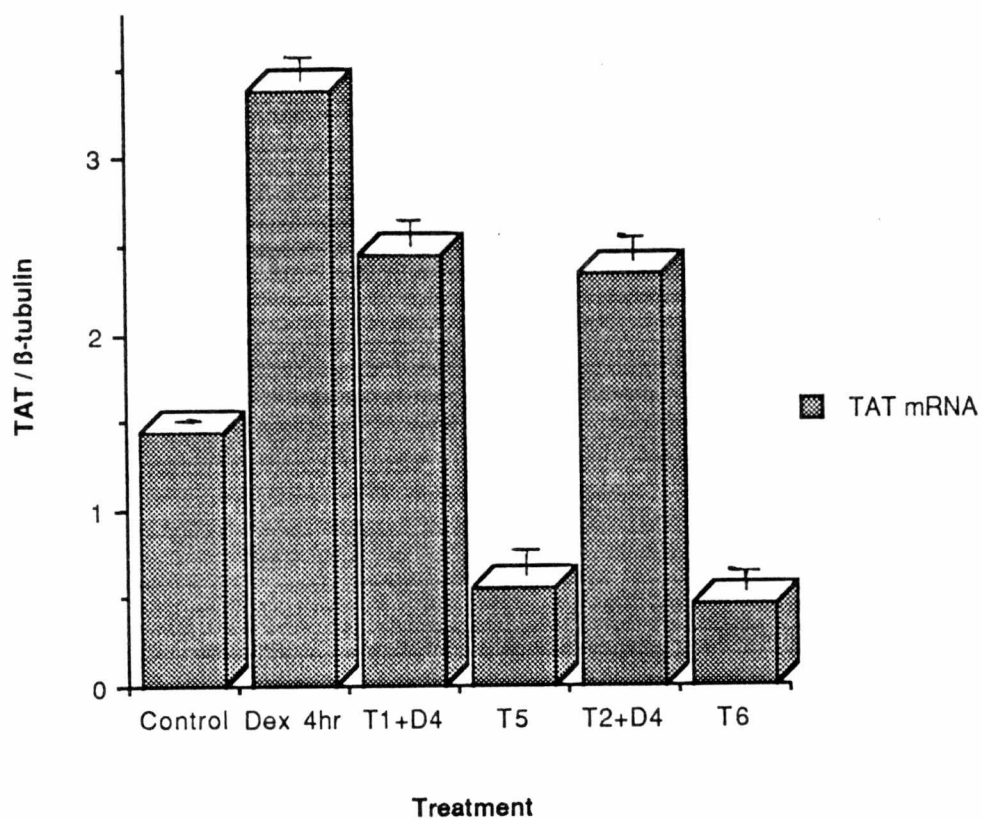


FIGURE 8

Effect of the Translational Inhibitor Cycloheximide on TAT mRNA.

Prior to treatment the KRC7 cells were placed on SFM for at least 16 hrs. TPA (1.6uM), CHO (10uM), or the two in combination were added to the culture medium and the cells were harvested at the indicated times. RNA was extracted as described in Materials and Methods. As a control, β -tubulin mRNA was measured from the same blots using pRBT.3. probe. Each data points are normalized by using β -tubulin mRNA values, and is expressed as the mean of three different replicates plus standard error measurement (SEM).

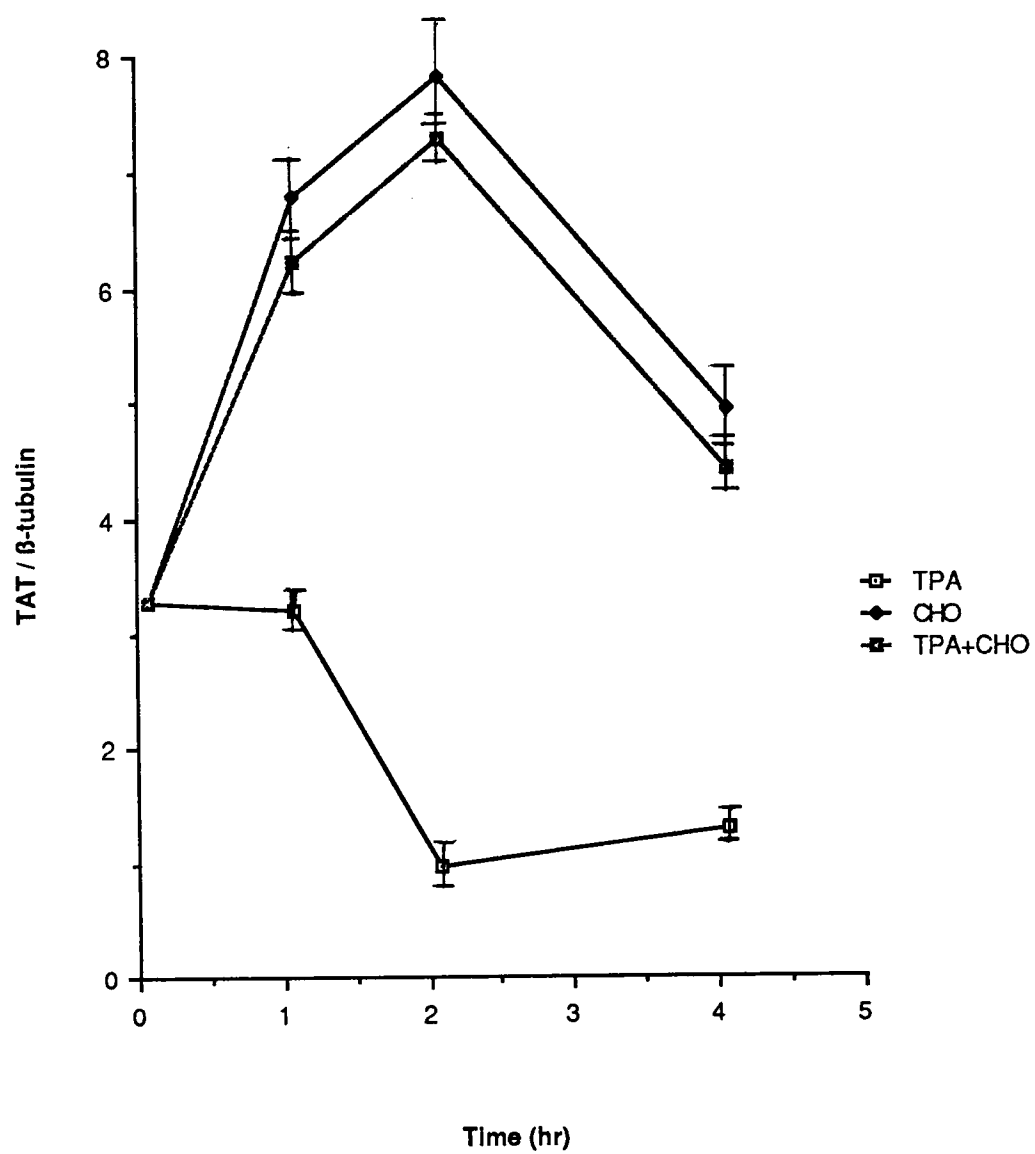


FIGURE 9

Effect of TPA on Dexamethasone Mediated Increases in TAT Enzyme Activity.

Prior to treatment the KRC7 cells were placed on SFM for at least 16 hrs. Cells were treated with 1.6 uM TPA, 1 uM dexamethasone, or both. Cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowry method. Each data point is expressed as the mean of three replicates plus standard error measurement (SEM).

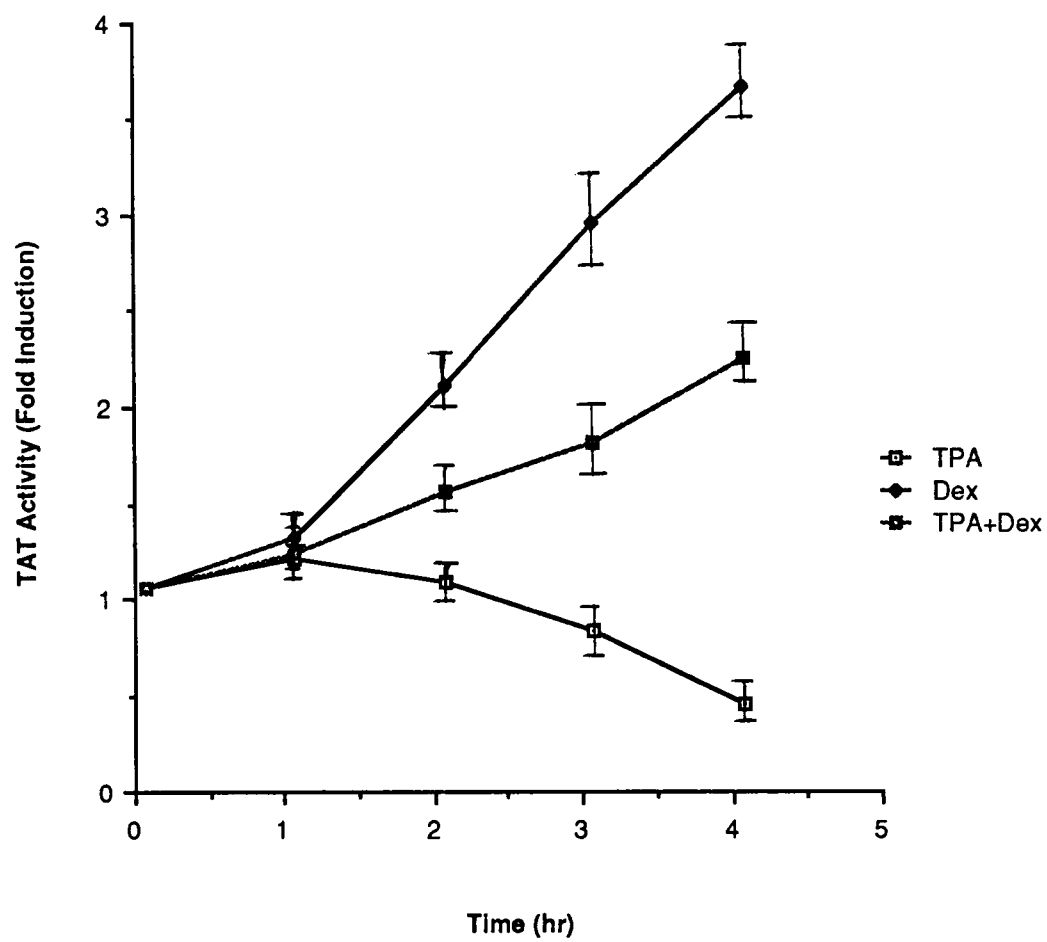


FIGURE 10

Effect of Preincubation with TPA on Dexamethasone Induced TAT Enzyme Activity.

Prior to treatment the KRC7 cells were placed on SFM for at least 16hrs. Cells were treated with 1.6 μ M TPA prior to addition of 1 μ M dexamethasone. Cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowry method. Each data point is expressed as the mean of three replicates plus standard error measurement (SEM).

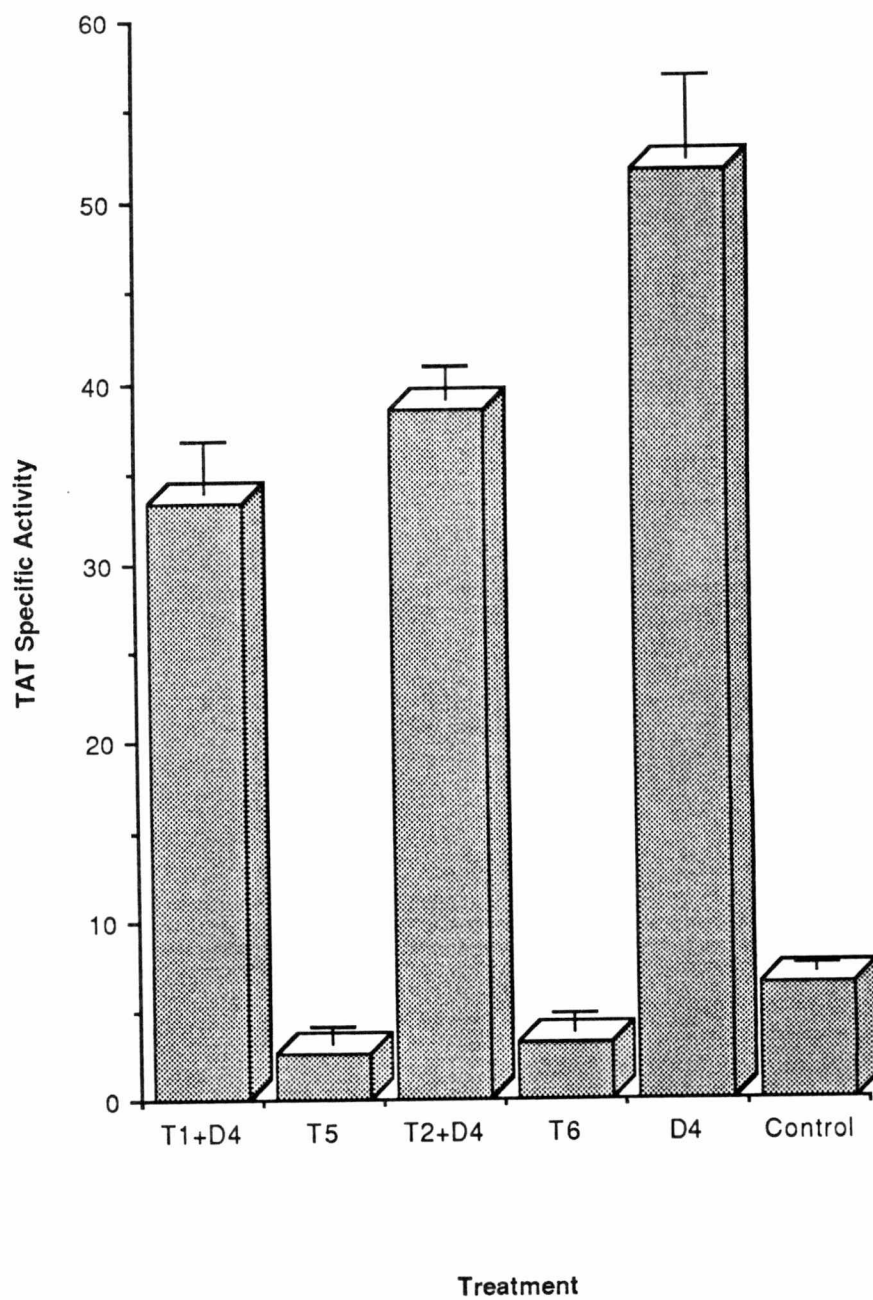


FIGURE 11

Effect of TPA on cAMP Mediated Increases in TAT Enzyme Activity.

Prior to treatment the KRC7 cells were incubated for at least 16 hrs in SFM. Cells were treated with 1.6 μ M TPA , 500 μ M cAMP analog, or both. Cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowry method. Each data point is expressed as the mean of three replicates plus standard error measurement (SEM).

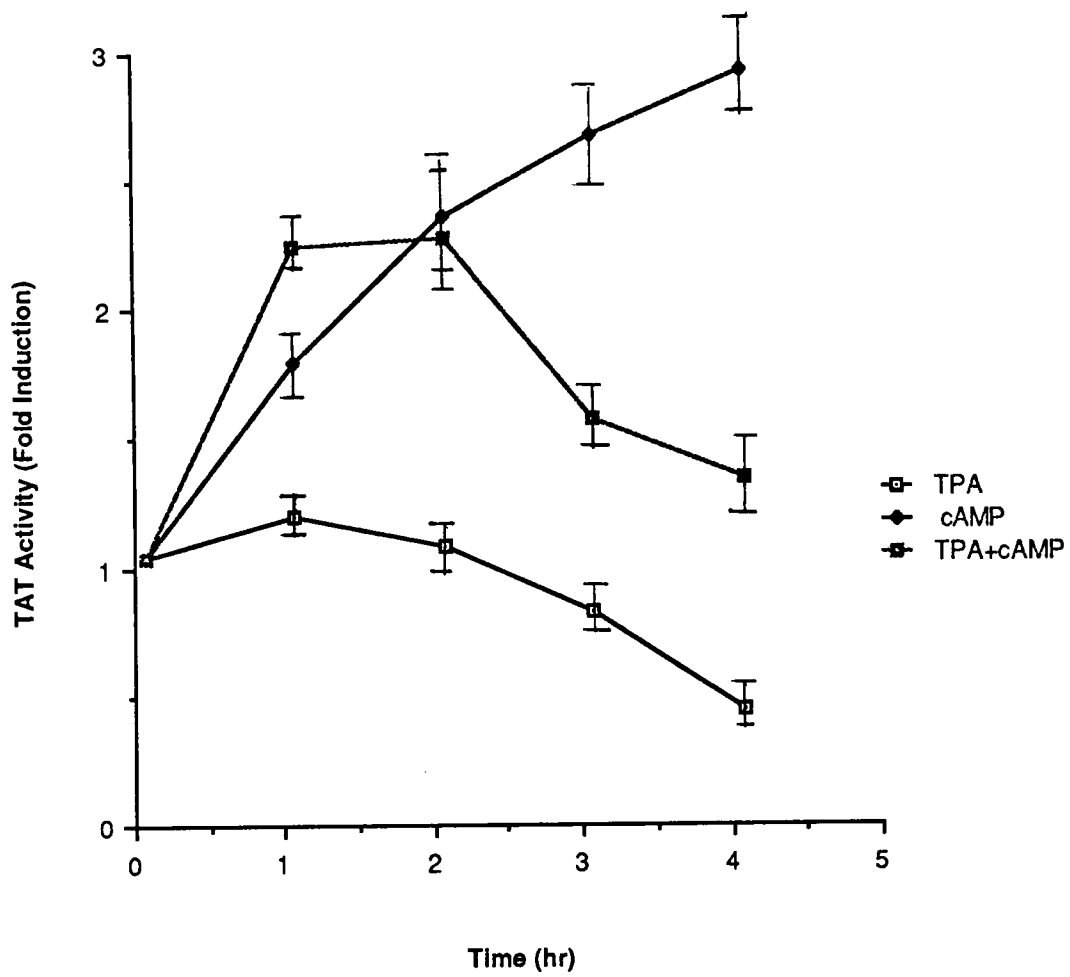
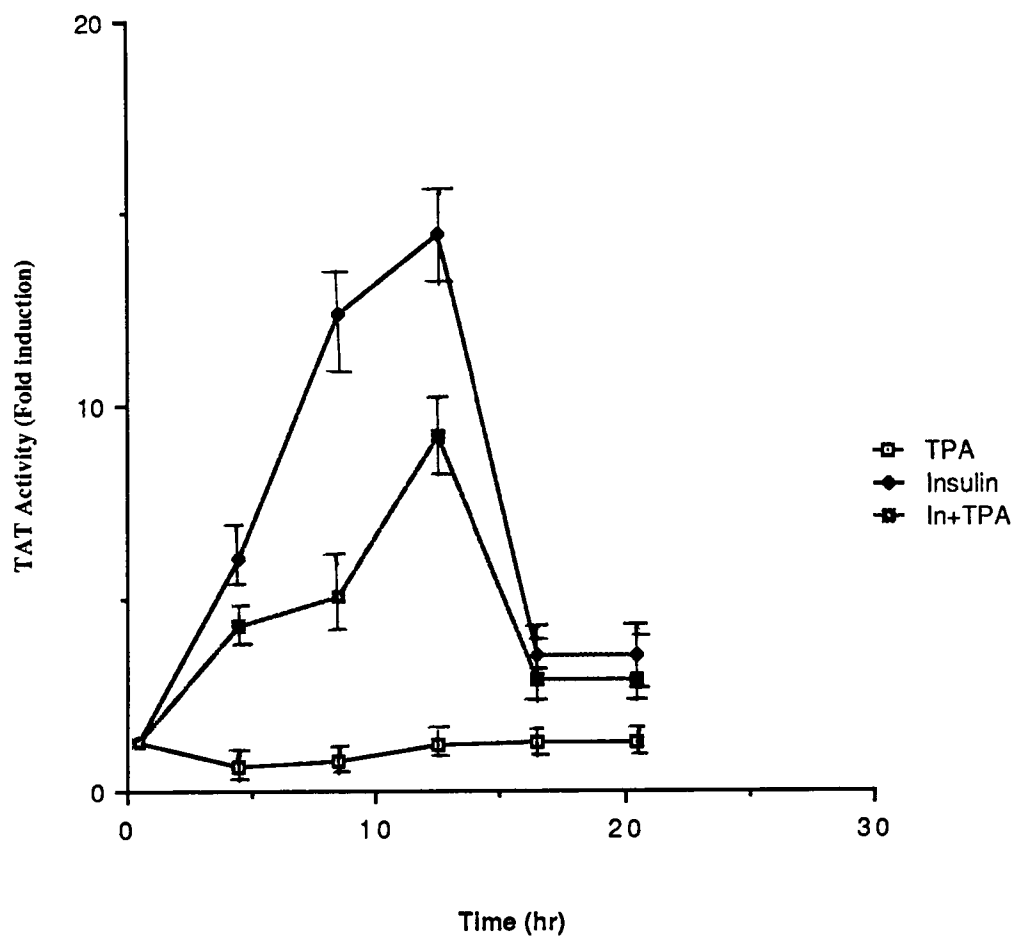


FIGURE 12

Effect of TPA on Insulin Mediated Increases in TAT Enzyme Activity.

Prior to treatment the KRC7 cells were incubated for at least 16 hrs in SFM. Cells were treated with 1.6 μ M TPA, 10 nM Insulin, or both. Cells were harvested at the indicated times. TAT was determined by the method of Diamondstone, total protein by the Lowry method. Each data point is expressed as the mean of three replicates plus standard error measurement (SEM).



VITA

Jong H. Kim was born in Seoul, Korea in January 6 1964. He came to United States in March 1978 and settled in Chicago, Illinois. He enrolled to Daniel Boone Elementary School in same year as 7th grade. In 1979, he went to Stephen Mather High School. While in high school, he was very actively involved in sports particularly in football (4 yr letterman), basketball (2 yr letterman), and track team (2 yr letterman). Upon his graduation from high school, he enrolled to University of Illinois at Urbana-Champaign in 1983. He majored in biochemistry as undergraduate student. During his freshmen year, he joined social fraternity Sigma Tau Gamma (alpha chi chapter). He was very actively involved in greek and campus activity (Student Senate, SGA, and many philanthropy). He graduated in 1987 spring semester and went on to University of Tennessee Graduate School. He received his master during 1990 summer semester. He is planning to go the MBA school in 1991 (probably Northwestern or Illinois). Currently, he is single and hope to stay as single for next couple years, but he is actively dating right now to find Ms. Right.