To the Graduate Council:

I am submitting herewith a thesis written by Susan A. Goodman entitled "Effects of Insulin and the Phorbol Ester Tumor Promoter TPA on Cell Growth and Transcript Levels of the Growth-Associated Enzyme, Ornithine Decarboxylase." 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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EFFECTS OF INSULIN AND THE PHORBOL ESTER TUMOR PROMOTER TPA ON CELL GROWTH AND TRANSCRIPT LEVELS OF THE GROWTH-ASSOCIATED ENZYME, ORNITHINE DECARBOXYLASE

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ABSTRACT

Insulin and a phorbol ester tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were examined for their capabilities to affect the growth of rat hepatoma cells; growth was measured by the incorporation of tritiated thymidine into acid-precipitable DNA. In quiescent cells, insulin induced growth, TPA alone had no effect, and TPA in the presence of insulin caused an inhibition of the insulin-stimulated growth (an effect seen only with the tumor-promoting phorbol esters). Through timed addition and removal of TPA it was discovered that TPA was maximally effective in its inhibitory actions if applied with the first four hours (early G1 phase) of the insulin-stimulated cell cycle. It was proposed that a TPA-sensitive event occurred sometime within this early G1 phase. It was questionable whether insulin and TPA could be acting through a common mechanism. In an attempt to further qualify the observed effects of insulin and TPA on cell growth, the enzyme ornithine decarboxylase was selected as a marker enzyme. Insulin and TPA both increased messenger RNA (mRNA) levels of ornithine decarboxylase and were previously shown to increase the enzyme's activity as well. The time course of induction by each affector molecule was different, but the timing of enzyme activity increases in both cases closely parallelled the increases in mRNA. With the addition of both insulin and TPA, ornithine decarboxylase transcript levels exhibited an additive increase. These increases might be attributed to increased transcription rates or increased mRNA stability. The latter of these possibilities was partially addressed

through experiments using the protein synthesis inhibitor cycloheximide, since the possibility exists that the mRNA may be either degraded or stabilized by a newly synthesized protein. Initial observations appear to indicate that separate post-transcriptional mechanisms are set into motion by insulin and TPA which result in different effects on ornithine decarboxylase mRNA.

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CHAPTER I

INTRODUCTION

The polypeptide hormone insulin has been shown to stimulate a growth response in certain quiescent cells (Dicker and Rozengurt, 1981; Koontz and Iwahashi, 1981). Cells that are quiescent are considered to be in a "resting" or nondividing stage of the cell cycle. When treated with appropriate growth factors, quiescent cells can be stimulated to reenter the cell cycle. Growth is measured by the extent of incorporation of tritiated thymidine into acid-precipitable DNA during the DNA-synthesis stage (S-phase) of the cell cycle. In cells in which insulin acts as a growth factor, increased DNA synthesis occurs . The intracellular mechanisms involved in mediating the effects of insulin are not completely known. It is known that insulin binds to specific cell-surface receptors (reviewed by Cheng and Larner, 1985; Ebina et al., 1985; Koontz, 1984). The insulin receptor possesses a tyrosine kinase activity which, upon insulin binding, is activated (Gammeltoft and Van Obberghen, 1986; Kasuga et al., 1982). One proposed mechanism of insulin action involves a phosphorylation cascade, to control the activities of various proteins (Chu and Granner, 1986; Hunter and Cooper, 1985). Another possible mechanism involves the stimulation of the metabolism of a novel glycolipid-containing phospholipid whose breakdown results upon treatment with insulin (Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986), perhaps

generating the proposed second messenger (Jarett and Seals, 1979) which would mediate actions observed upon insulin binding. The insulin receptor may translocate to the nucleus and bind to specific DNA sequences to influence transcription (Podlecki et al., 1987; Smith and Jarett, 1987).

The KRC-7 clone of the H-35 rat hepatoma cell line is used in this research. These cells become quiescent upon treatment with serum-free medium (SFM) for three days. Addition of physiological concentrations of insulin results in a growth response. Since the G1 phase of these cells is approximately eight hours long, followed by S phase which continues for an additional 10 hours (Koontz and Iwahashi, 1981) the incorporation of tritiated thymidine is measured for the half hour from 15.5 to 16 hours following stimulation to assure that the measurements will be made during peak S phase (Fig. 1*).

A report from the laboratory of Ronald Kahn (Takayama et al., 1984) implied that the tumor-promoting phorbol ester 12-0-tetradecanoyl-phorbol-13-acetate (TPA) caused cells to become refractory to certain effects of insulin. In order to test this, TPA was applied to the rat hepatoma KRC-7 cell system. TPA is considered a second-stage tumor promoter in its effects on mouse epidermal tissue previously treated with an initiator substance (Cerutti, 1985). The hydrophobic nature of TPA allows its penetration of the lipid bilayer of the cell membrane (Driedger and Blumberg, 1980) with subsequent binding to the intracellular calcium- and phospholipid-dependent protein kinase C,

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*All figures may be found in the appendix.

thereby bringing about protein kinase C activation, which results in the phosphorylation of certain intracellular substrates. TPA has structural analogy with diacylglycerol (Brockerhoff, 1986) which has been implicated in intracellular signalling events, and is the endogenous activator of protein kinase C. It has not been conclusively demonstrated that TPA-induced phosphorylation is the causitive phenomena behind its tumor-promoting abilities . TPA, described as a membrane-active agent, may also perturb membrane conformation (Rozengurt, 1980) resulting in increased susceptibility of membrane lipids to autooxidation (Cerutti, 1985).

After obtaining results of insulin and TPA treatment on the growth response of cells, an enzyme already seen to be affected by both insulin and TPA was selected as a marker enzyme. The enzyme selected, ornithine decarboxylase, has a molecular weight of 51,000-54,000 daltons, encoded by a 2-2.6 kb mRNA of which their may be several species (Denhardt, 1980). Ornithine decarboxylase is the rate-limiting enzyme in the biosynthesis of polyamines, which are molecules that have been shown to increase in concentration in almost all actively growing cells, and their presence is considered essential for optimal cell growth (Grillo, 1985; Tabor and Tabor, 1984). Analysis of the rate-limiting enzyme in polyamine synthesis was thought to be pertinent to the further evaluation of growth response characteristics in the H35 cells. The activity of ornithine decarboxylase has been previously shown to increase in the presence of insulin, TPA, and

a combination of the two compounds (Goodman, Esau and Koontz, manuscript in preparation). The ornithine decarboxylase mRNA levels were examined as a potential level of control in the enzyme's regulation.

CHAPTER II

MATERIALS AND METHODS

Materials

Insulin was purchased from ELANCO Products Company, Indianapolis, IN. TPA was from Pharmacia, PL Biochemicals, Milwaukee, WI. Other phorbol esters and gentamycin were purchased from Sigma, St. Louis. MO. Tritiated thymidine was obtained from ICN, Irvine, CA. Calf serum and fetal bovine serum were products of M.A. Bioproducts, Walkersville, Maryland. Cell culture dishes (60mm and 24-well) were from Corning Glass Works, Corning, NY. Fungizone and Dulbecco's modified Eagle's medium were products of Gibco, Grand Island, NY. GeneScreen nylon transfer membrane was produced by New England Nuclear, Boston, MA. The cDNA probe, pODC934, was generously provided by Gordon Watson, University of California, Berkley, CA. A kit for nick translation was obtained from Bethesda Research Laboratories, Gaithersburg, MD.

Cell Culture

We used the KRC-7 clone of H-35 rat hepatoma cells, grown in monolayer culture in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 5% calf serum, gentamycin (.05mg/ml) and fungizone (2.5ug/ml). The environment was maintained at 37°C with 10% CO_2 and 90% air.

Cell Treatment

Prior to cell confluency, the serum-containing medium on the cells is replaced with serum-free medium on which the cells remain for three days preceding treatment.

Insulin stock solution, stored at 4°C, is prepared by dissolving solid insulin in 0.1N hydrochloric acid (HCl) to the desired concentration of 1mg/ml. Precise concentration of the solution is determined spectrophotometrically at a wavelength of 276nm; 1mg/ml gives an absorbance reading of 1.05 at this wavelength (Ron Chance, personal communication).

The stock solution is diluted with .001 N HCl. Except in the insulin dose response experiments, a final concentration of 10^{-9} M insulin was customarily used, which is within the physiological range of action of the molecule.

Stock solution of TPA was prepared by dissolving the ampule contents (5mg) in 5ml of 95% ethanol. The concentration was determined by E_{5400} measuring A_{232} (Windholz, Martha, ed., 1983). Dilutions made in 95% ethanol were added to cell media in a ratio of 1:1000 to a final concentration of ethanol not greater than 0.1%. For most experiments a final concentration of 1μM TPA was used.

Incorporation of Tritiated Thymidine

After insulin and/or TPA have been in contact with cells for 15.5 hours cells, [^{3}H]-thymidine is added to a final concentration of 1μCi/ml, and growth continued for thirty minutes. Then the media is removed and the cells are washed with ice-cold phosphate-buffered

saline (PBS) (0.9% NaCl, 20mM K_2HPO_4, pH 7.4), which essentially stops any further incorporation. The PBS is aspirated, then cold 5% trichloroacetic acid is added and followed by swirling for 15 minutes to allow precipitation of proteins and nucleic acids. The TCA is then aspirated as completely as possible, followed by the addition of 0.3 M NaOH to solubilize the TCA precipitate. Vigorous pipetting with a pasteur pipette assures dissolution of the TCA precipitate. Aliquots are taken from each sample to be assayed for both the tritium counts into DNA and the relative protein concentrations. Aliquots for tritium counting are transferred into scintillation vials followed by the addition of an aqueous scintillation fluor (Beckman Ready-Solv, MP) shaking, and counting in a Beckman LS-230 scintillation counter. Protein concentrations are determined by the method of Lowry (1951). Data was calculated as counts per minute per milligram of protein (cpm/mg). Data presented represents the average of triplicate determinations. All experiments were repeated.

Isolation of RNA

With slight modifications, the method of Chirgwin et al. (1979) was employed in the isolation of RNA from cultured cells grown on 150mm or 100mm culture plates. After treatments had been applied for various lengths of time, the media was aspirated and the cells were rinsed with ice-cold PBS three times. After the final thorough aspiration of PBS, 3mls of a 4M guanidine thiocyanate solution (4M guanidine thiocyanate, 0.5% sodium N-lauroylsarcosine, 25mM sodium citrate, pH 7.0, and 0.1M β-mercaptoethanol) was added to each culture plate

and swirled to assure the solution's contact with all cells. The plates were scraped with a rubber spatula and the cell-containing guanidinium solution was layered over 1.5ml of a 5.7M cesium chloride solution in 50mm x 12mm ultracentrifuge tubes. Tubes were spun at 36,000 rpm in an SW50.1 rotor for 20 hours in a Beckman L3-50 or L8-80 ultracentrifuge. After centrifugation, the supernatant was aspirated, the sides of the tube were wiped with a sterile cotton applicator, taking care not to disrupt the pellet. The pellet was then resuspended in 400µl diethylpyrocarbonate (DEPC)-treated water. Absorbance readings at 260 nm were made of a 1:50 dilution of the samples to determine the content of RNA in each, using an absorbance reading of 1.00 equal to 40 µg/ml of RNA (Maniatis, 1982).

Fractionation of RNA

RNA aliquots of equivalent to 15µg were ethanol-precipitated, according to Maniatis, 1982. This involves adding one-tenth volume of 4M NaCl to the RNA aliquot, then adding 2.5 times the aliquot volume of 100% ethanol, vortexing, then storing overnight at -20°C. Samples are spun in a Beckman Microfuge 12 at 12,000 rpm for 15 minutes after which the supernatant is poured away from the pellet. Fractionation of the RNA is performed following the method of Maniatis, 1982. After allowing the pellets to dry in a vacuum dessicator for 10 minutes, they are resuspended in 7.5µl of buffer (40mM Na_2HPO_4 , pH 7.3, 12% formaldehyde), then placed in a water bath at 60°C (to denature the RNA), then the RNA is quickly cooled on ice. At this time RNA dye (0.4% xylene cyanol, 0.4% bromophenol blue, 1mM EDTA, in

glycerol) is added (3.5µl) and the samples are loaded onto a 1% agarose gel immersed in a running buffer composed of (20mM Na_2HPO_4, pH 7.3, 0.6% formaldehyde). Electrophoresis is carried out at 70 V for approximately 3.5 hours. The gel is stained for 20 minutes in a volume of running buffer to just cover the gel, containing 10µl of an ethidium bromide solution (10mg/ml). The stained gel is then transferred to water to destain for at least an hour. At this point the integrity of the RNA can be examined by placing the gel on an ultraviolet transilluminator. The appearance of two distinct major bands without intermediate streaking indicates the intact RNA of the 28S and 18S ribosomal subunits (Fig. 2), the most predominant RNA within a cell, and implies that the RNA is not degraded.

Transfer of RNA to Nylon Membrane

RNA from agarose gels was blotted onto nylon membrane by a method similar to that of Thomas (1980). In a glass 9" x 11" baking dish were placed two inverted 100mm petri dish bottoms, on top of which was placed a glass plate (8" x 6"). The dish was filled with 20xSSC (3M NaCl, 0.3M trisodium citrate) to just below the plate. A piece of 3mm paper was cut so as to cover the glass plate, and a piece of nylon membrane, cut to the same size as the gel and presoaked in distilled water, was placed on top of the gel. Next, two pieces of 3mm paper, also gel-size, were placed on top of the membrane. Brown paper towels, also cut exactly the same size as the gel, were placed on top. Another glass plate was placed atop the paper towels, and the blotting apparatus was covered with plastic wrap. Weights were distributed

evenly across the top glass plate. Gels were allowed to blot overnight. Placing the blotted nylon membrane on a UV transilluminator allowed visualization of the transferred ethidium-bromide-stained RNA. A five minute ultraviolet exposure causes the RNA to become bound to the membrane.

Nick Translation of cDNA Probes

One microgram of the plasmid pODC934 was nick-translated using a nick translation kit from Bethesda Research Laboratories, according to their instructions. Approximately 1-5 x 10^6 cpm were added to each hybridization reaction. The insert size of the probe used, pODC934, is 1.4 kb long (Berger et al., 1984). The mRNA species which bind to this are 2.0 kb and larger (pODC934 is not a full-length probe).

Hybridization of RNA to Specific cDNA Probes

The hybridization scheme used was a modification (Amasino, 1986) of the procedure of Church and Gilbert (1984). Nylon membrane with bound RNA was prehybridized in sealed plastic pouches at 65°C for at least four hours in 20 ml of a solution containing 0.5M sodium phosphate (pH 7.2), 2% SDS, 1% BSA, and 1mM EDTA. Next, a [^{32}P]-labelled nick-translated cDNA probe (pODC934) was added to the prehybridization solution, the pouch resealed, and incubation at 65°C was carried out overnight. After aspirating the hybridization solution, the membrane was removed from the pouch and washed in a succession of four washes of increasing stringencies at 65°C, for 20 minutes each. The first wash contained 0.5 M sodium phosphate (pH 7.2), 1% SDS, and 1mM

EDTA. In the three subsequent washes, II, III, and IV, the sodium phosphate was decreased to 0.25 M, 0.1 M, and 0.05 M, respectively, while the SDS and EDTA concentrations remained identical to those in the first wash. The damp blot was then autoradiographed by enclosing in plastic wrap, and exposing it to Kodak X-AR film at -70°C using a Cronex intensifying screen. After development of the film, the relative intensities of the resulting bands (Fig. 3) were quantitated by densitometry, using a GS 300 Scanning Densitometer by Hoefer Scientific. This instrument, zeroed against general background, scans a one-dimensional pass through an aligned point. The densities of the autoradiogram marks are displayed as peak areas. Peak areas are expressed as they compare to the serum-free control (assigned a value of 1).

CHAPTER III

RESULTS

Dose Response of Insulin-Stimulated Increases in Cell Growth

Since TPA had been reported to alter the effects of insulin (Takayama, 1984), we wished to determine what effect TPA might have on the insulin-stimulated growth response. Insulin concentrations were varied (1pM to 0.1μM) to determine whether TPA could demonstrate an effect at higher or lower concentrations of insulin. TPA was used at a final concentration of 1μM which was shown to be active in affecting other systems (Dicker and Rozengurt, 1981) (Fig. 4). In these experiments 1uM TPA caused inhibition of the insulin-stimulated growth response with the greatest inhibition (35%) observable with an insulin concentration of 100pM to 1nM (Fig. 5). Insulin at a concentration of 1nM was chosen for use in further experiments, not only because this would allow the most readily observable response to TPA, but also because this is within the range of physiological concentrations of insulin.

Dose Response of TPA-Induced Inhibition of Insulin-Stimulated Cell Growth

To insure that we would indeed be dealing with a concentration of TPA which would yield a readily observable response, TPA dose response experiments were performed in the presence of 1nM insulin (Fig. 5). Applying concentrations of TPA from 1pM to 1μM resulted in an increasing inhibition of [^{3}H]-TdR incorporation with increasing TPA

concentration. These concentrations agreed with the concentrations of TPA seen to be active in other systems (Byus et al., 1984; Vaartjes and de Haas, 1985). Half maximal inhibition was found to occur between 1nM and 10nM TPA (Fig. 5). A concentration of 1μM TPA gave the most significant response of all concentrations tested and was therefore selected as the concentration for further use.

Phorbol esters which possess less or no tumor promoting activity were also tested and resulted in either less potent inhibition, as in the case of the weaker tumor promoter, phorbol dibutyrate (Dicker and Rozengurt, 1981), or no inhibition , by phorbol diacetate and 4-α-phorbol -- non-tumor-promoting phorbol esters (Driedger and Blumberg, 1980). These observations indirectly link the inhibition of the insulin-stimulated growth response by TPA to its tumor promoting capacity and not to its general phorbol ester structure.

Effect of Varying the Timing of TPA Additions

With the dose responses on the growth effect established, the next task was to attempt to identify a specific portion within the insulin-stimulated cell cycle in which the tumor promoters might be exerting their influence on the cells. If there was some event that was strongly affected by TPA, narrowing down a time for its occurrence might contribute to the discovery of the event. After the cells were initially stimulated with insulin, 1μM TPA was added to different cultures at two hour intervals. The insulin and phorbol ester were left on the cells with the treatments until they were harvested and assayed for incorporation of tritiated thymidine at sixteen hours. The

results of these experiments showed maximal inhibition by TPA when applied within the first four hours of the insulin-stimulated progression into the cell cycle. Additions of TPA made at the six hour time point gave less than maximal inhibition and later additions of TPA did not have any effect on the insulin-stimulated growth (Fig. 8).

Effect of Varying the Timing of TPA Removal

To observe the timing of the TPA effect in another manner, growth arrested cells were treated with both insulin and TPA at zero time, then TPA was removed at two hour intervals by aspirating the medium, rinsing the cells with serum-free medium, then adding back serum-free medium with insulin. These experiments indicated that if TPA were removed early in the cell cycle there was little or no inhibition of the growth response. Removing TPA at progressively later times resulted in greater growth inhibitions. Do these experiments indicate that TPA exerts its effects on some particular event in the first four hours of G1 which leads to an inhibition of growth? It might be pointed out that the cells exposed to TPA the longest were the cells which showed growth inhibition. Is the length of time of cells' exposure to TPA the critical factor for its inhibitory effects in our system? To clarify whether there was a special moment within the cell cycle which was permissive for TPA to exert some effect, or whether the inhibitory effect seen with TPA was a factor of the duration of TPA's presence, time course additions of TPA were made accompanied by TPA removals. In these experiments growth-arrested cells were treated with insulin, then TPA was added at two

hour time intervals as before, but this time TPA was removed from all cells at a time point 10 hours after insulin stimulation (early S phase), shortening the maximum exposure to TPA to 10 hours (in previous TPA addition experiments cells exposed to TPA at the six hour time point remained in contact with TPA for 10 hours and did not result in maximum inhibition of growth). The data from these 10-hour TPA removal experiments indicated maximal inhibition of growth when TPA was applied during the first four hours of insulin-stimulated cell cycle progression, tilting the evidence in favor of a TPA-sensitive event beginning sometime within the first four hours of G1 phase (Fig. 9).

Testing the Possibility that TPA Causes a Delay of S Phase Initiation

In contemplating possible explanations for TPA's effect of lowering the growth response stimulated by insulin, the possibility that TPA could be causing a delay of S phase initiation was considered. If TPA caused a delay of the initiation of S phase, the DNA-synthesizing phase, and cells were being harvested at a time which fell before the peak of this supposedly delayed S phase, this might result in a lowered value for DNA synthesis. To test this notion cells were subjected to another TPA time course then harvested and assayed for tritiated thymidine incorporation at two hour intervals starting at 8 hours after insulin stimulation (Fig. 10). The results demonstrate an insulin-stimulated S phase beginning at 10 hours, DNA synthesis increasing rapidly then levelling off at 14 to 16 hours. The insulin-stimulated cells treated with TPA appeared to enter S

phase at the same time but the magnitude of thymidine incorporation was lessened for all times of TPA application. Again, TPA applied at two and four hour time points caused the greatest growth inhibition at the time of the final assay. This experiment supports a TPA-sensitive event in a prereplicative phase (G1) of the cell cycle.

Effects of Insulin and TPA on Ornithine Decarboxylase mRNA

We elected to further probe G1 by looking at the effects of insulin and TPA on the growth-associated enzyme, ornithine decarboxylase. The rate-limiting step in polyamine biosynthesis, ornithine decarboxylase enzyme activity has been shown to be induced by mitogenic factors, including insulin and TPA. TPA induces the enzyme activity of ornithine decarboxylase marginally, and insulin induces ornithine decarboxylase significantly, but the two together appear to have an additive effect on the induction of the enzyme (Goodman et al., manuscript in preparation). This fact did not appear to be in agreement with the observed inhibition of growth by TPA. Initially it was thought that TPA would inhibit the insulin-induced activity of this enzyme, which would have nicely paralleled the growth response data.

To scrutinize the effect of TPA and insulin on ornithine decarboxylase, the mRNA levels of the enzyme were examined. The cDNA probe used, pODC934, hybridizes to two species of ornithine decarboxylase mRNA, one of approximately 2.2 kb and the other about 2.7 kb (Berger et al., 1984). The dissimilar sizes are thought to be due to heterogeneity in their 3' noncoding regions (Hickok et al., 1986; Pegg, 1986); possibly this reflects two polyadenylation sites

(Pegg, 1986). Both insulin and TPA induced an increase in ornithine decarboxylase mRNA. The insulin time course demonstrated a maximal effect within eight hours of insulin addition (Fig. 11), a time parallel to that observed for insulin induction of enzyme activity. Also the concentrations of insulin necessary to maximally increase ornithine decarboxylase mRNA (Fig. 12) appeared to be equivalent to those necessary to maximally induce the activity of the enzyme (1nM). Dose responses for TPA also resulted in maximum ornithine decarboxylase transcript increases at a dosage also active in inducing enzyme activity, that is, between 0.1μM and 1μM TPA (Fig. 13). The time course for TPA induction of ornithine decarboxylase mRNA shows a peak occurring between three to five hours after TPA addition (Fig 14). When both agonists were added together, a large increase in ornithine decarboxylase mRNA occurs, much greater than in the presence of either agonist alone.

The time course data contributed to the idea that insulin and TPA were acting through different mechanisms to increase ornithine decarboxylase mRNA. Pursuing this idea, a question arose as to whether one would see a further increase in ornithine decarboxylase mRNA by TPA if the cells were already maximally stimulated by insulin; that is, if cells were treated with insulin alone for eight hours, the time of maximal ornithine decarboxylase mRNA induction by insulin, would TPA addition made at this 8h time point still be able to cause an additive increase in the transcript? Performing this experiment it was discovered that not only could TPA induce the maximal ornithine

decarboxylase induction by insulin even further, but the timing of the increased message was equivalent to the time course of addition of TPA alone with peak induction occurring three to five hours after the addition of TPA (Fig. 15).

Effects of Cycloheximide on Insulin and TPA Induction of Ornithine Decarboxylase mRNA

At this point it seemed possible that insulin and TPA were acting through different intracellular mechanisms to induce the mRNA for this particular growth-associated enzyme. The next logical step seemed to be to attempt to further qualify their respective actions. Is the ornithine decarboxylase mRNA increase dependent upon the synthesis of some insulin- or TPA-induced protein, which could either interact with the mRNA to affect stability, or interact with DNA to alter transcription? By treating cells with cycloheximide, a protein synthesis inhibitor, it could be determined whether protein synthesis was required for induction of the ornithine decarboxylase transcript levels. To perform this experiment cells were treated with insulin, TPA, or a combination of the two, in the presence and absence of cycloheximide, for intervals that would fall before, during, and after the time of the maximal ornithine decarboxylase mRNA levels induced by insulin and TPA. Effects of cycloheximide alone were examined at the separate time points as controls, and resulted in little if any induction above the serum-free control. The results indicated that when cycloheximide was present with TPA for four hours (the time of maximal ornithine decarboxylase mRNA increase by TPA) the signal for ornithine decarboxylase mRNA was increased even further. Repeating the

TPA/cycloheximide treatment for a 9h duration, a time by which the ornithine decarboxylase mRNA increase by TPA has been seen to be returning to basal levels, an even more marked increase above the TPA alone treatment was observed (Fig. 16).

In the case of ornithine decarboxylase message induction by insulin, a 9h (usually peak) treatment with cycloheximide resulted in a slightly decreased mRNA level. The same results were observed for the 13.5h insulin-plus-cycloheximide time point, which is a time point during which the insulin-induced effect would be on the decline (Fig. 16).

CHAPTER IV

DISCUSSION

The results have consistently shown that 1μM TPA inhibits the insulin-stimulated growth response. Although the degree of inhibition may vary, usually growth inhibition by TPA will fall in the 30% range. Between separate experiments the cpm readings often vary greatly, though proportionately that are similar. Since the same activity of tritiated thymidine is added to each experiment, and the same concontration of the insulin and TPA treatments are added in the repeated experiments, these factors are not suspected to be the major causes of the differences. It has been observed that the degree of cell confluency seems to affect the amount of DNA being synthesized. Cells that are very confluent do not usually display high counts in the tritiated TCA precipitates. Cells that are less confluent are visibly bigger and tend to have higher counts per milligram of protein.

The results also indicate that the timing of the TPA addition is important in achieving inhibition of growth. Although TPA's presence within the first four hours of insulin stimulation is most effective in growth inhibition, this does not prove the existence of a TPA-sensitive event during this time. As demonstrated with the TPA induction of ornithine decarboxylase mRNA, certain effects triggered by TPA may by delayed in their actual expression. The expression of such a

previously stimulated effect may be what actually caruse the growth inhibition.

In the TPA removal experiments, in which at various times TPA-containing media was replaced with media not containing TPA, it appeared that the timing of the TPA removal did affect the extent of growth inhibition by TPA. These TPA removal experiments lent support for there being a particular time during the cell cycle when the presence of TPA is most effective. It has been previously observed that TPA, due to its extremely hydrophobic nature, is not easily removed from cells. In this instance, in which even a one hundred fold dilution of TPA has little effect on growth (Fig. 5), TPA probably would not have to be totally removed in order to discontinue the growth inhibitory effect. Therefore, even though the extent of removal of TPA from the cells is not known, it is suggested that the removal experiments give valid data to assist in the discovery of a TPA-sensitive portion of the cell cycle.

In trying to determine what effect TPA might have on the timing of S phase, the experiment portrayed in Figure 10 seems to indicate that at least the initiation of S phase does not seem to be altered by TPA. However, within the margin of error, it can not be definitely stated for all the TPA-treated cells that S phase is underway by the 12 hour time point. Since at the last time point assayed, none of the TPA-treated cells have reached a plateau, it could be that a peak will come at some later time point in these cells. It can be concluded from these results that at the time of the assay for growth (16 hours after insulin stimulation) the TPA-treated cells do have lower values

for tritiated thymidine incorporation than the cells treated with insulin alone. If the data were extended to include later time points, one might determine whether TPA is prolonging S phase.

In interpreting any of these growth response experiments, it should also be kept in mind that the term "growth" is defined in terms of tritiated thymidine incorporation. Although frequently used as an assay for growth, the possibility exists that inhibition of incorporation of tritiated thymidine may be the result of less of it getting into the cell. Another possibility is that there is an increase in the synthesis of endogenous thymidylate which would dilute the concentration of tritiated thymidine incorporated into DNA. Neither of these alternative explanations was tested for this system.

The results suggest that the intracellular mechanisms of insulin and TPA actions may intercept at certain points to bring about such effects as TPA's inhibition of the insulin-stimulated growth response, and the additive increase in ornithine decarboxylase mRNA by insulin and TPA. At the same time, the observation that TPA alone has no effect on cell growth and the fact that the time courses of ornithine decarboxylase induction by insulin and TPA differ support the conclusion that the mechanisms through which insulin and TPA exert their effects certainly are not identical. Some possibilities exist as potential sites of interaction between the TPA- and insulin-stimulated pathways.

Insulin and TPA additions to cells result in increased activity of separate kinases. The binding of insulin to its receptor stimu-

lates a tyrosine kinase on the β subunit of the receptor which may be capable of phosphorylating specific intracellular substrates as well as phosphorylating itself (Kasuga et al., 1982). Tyrosine-specific protein kinase activity is known to be associated with ligand-activated growth factor receptors (Hunter and Cooper, 1985), as well as with oncogene products of the retroviral σθψ gene family (Akiyama et al., 1987; Hunter and Cooper, 1985). It is possible that there is an important role of tyrosine phosphorylation in cell proliferation (Akiyama et al., 1987). Protein kinase C, which is an intracellular serine- and threonine-specific protein kinase, is activated by TPA (Castagna et al., 1981; Niedel et al., 1983). It has been shown that TPA can alter the phosphorylation of the insulin receptor (Takayama et al., 1984; Hachiya et al., 1987) by phosphorylating specific serine and threonine residues of the β subunit; and it has been suggested (Takayama et al., 1984) that through this mechanism phorbol esters may modulate early events in the pathway of insulin action. It has also been observed that TPA increases the already rapid internalization of the occupied insulin receptor (Hachiya et al., 1987). Neither of these reports provided conclusive evidence that altered insulin receptor phosphorylation was the cause of the effects that were observed. It is possible, however, that TPA's effects on insulin receptor phosphorylation may be involved in mediation of the TPA inhibition of the insulin-stimulated growth response. It has been reported that TPA induces internalization of unoccupied epidermal growth factor (EGF) receptors, but these TPA-affected receptors were not subsequently targeted for degradation. Without TPA, EGF-bound receptors are de-

graded (Hachiya et al., 1987; Beguinot et al., 1985). Since it has been shown that TPA increases internalization of the insulin receptor (Hachiya et al., 1987), it could be, although not yet shown, that TPA causes a change in the pattern of insulin receptor degradation. This could feasibly have an effect on insulin-stimulated cell growth. A decrease in insulin-plus receptor degradation caused by TPA could seemingly result in decreased induction of insulin-stimulated growth if growth were stimulated by degradation products.

A recently reported and currently favored candidate for a second messenger of insulin-mediated actions is a novel glycolipid-containing phospholipid, present in low quantities, which is released from the plasma membrane immediately after the binding of insulin to its receptor (Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986). This novel phospholipid has been proposed to stimulate some specific C kinase bringing about specific substrate phosphorylations which would in turn bring about the pleiotropic effects (Horuk and Olefsky, 1985) seen upon insulin's binding to its receptor. Again phosphorylation exists as a possible mechanism which could be interfaced by TPA's stimulation of calcium- and phospholipid-dependent protein kinase C.

To examine another possible avenue for TPA actions, one might consider TPA's effects on the plasma membrane which could compromise the release of the insulin second messenger, especially if this second messenger is a plasma membrane component. The chaotropic effect of TPA on membrane conformation leads to the increased vulnerability of certain membrane lipids to autooxidation (Cerutti, 1985). This could

complicate the effect of the insulin second messenger proposed by Saltiel (1986a, 1986b).

In attempting to associate insulin-stimulated growth and its inhibition by TPA with the observed activities of the marker enzyme, ornithine decarboxylase, one might consider how super stimulation of ornithine decarboxylase activity by insulin plus TPA at the critical pre-S-phase induction point, might manifest itself in a growth inhibition. If polyamines are synthesized above that amount required for growth, the excess, in the form of spermidine, has been shown to be acetylated and excreted (Pegg and McCann,1982). Since putrescine is rapidly converted to spermidine, and spermine is known to undergo conversion to spermidine, all forms could eventually be excreted, reducing the entire intracellular polyamine content. The metabolic significance of spermidine excretion is uncertain. It is possible that this is a defense mechanism to prevent intracellular build-up of polyamines. The highly basic nature of polyamines could alone wreak intracellular havoc in the event of an excess; at physiological pH values, the amino groups of the polyamines are largely charged and can participate in electrostatic interactions with anionic sites on ribosomes, DNA, and membranes (Beninati et al., 1985). Additionally, there exists exists within the cell certain diamine and polyamine oxidases which degrade their substrates to yield such cytotoxic compounds as acrolein, aminoaldehydes, hydrogen peroxide, and ammonia (Kimes and Morris, 1971; Andersson et al., 1980; Bolkenius and Seiler, 1981). Exogenous addition of oxidation products of polyamines to bacteria (which also produce polyamines) has been shown to cause inhibition of

DNA synthesis (Kimes and Morris, 1971); oxidation products of spermidine and spermine have also been shown to be toxic to a variety of eukaryotic cells. The enzymes responsible for synthesis of the oxidases have been shown to be induced by certain cell toxins (Pegg and McCann, 1982); perhaps TPA would fall in this cell toxin category. Analysis of diamine and polyamine oxidases during the cycling insulin- and TPA-treated cells could test whether there was indeed an increase in these enzymes. Another possible test would be to use either N^1-methyl-N^2-(2,3-butadienyl)-1,4-butadiamine, or N^1,N^2-bis-(2,3-butadienyl)-1,4-butadiamine, specific inhibitors of polyamine oxidase (Bolkenius and Seiler, 1981; Pegg, 1986), or aminoguanidine, a diamine oxidase inhibitor (Andersson et al., 1985), to determine if cell growth could still be inhibited by TPA in their presence. Experiments have been performed which indicate that extracellular addition of polyamines results, by uncertain means, in cell death (Tabor and Tabor, 1972). Experiments in which polyamine additions (5mM) made to the hepatoma cells in culture did result in the death of the cells (data not shown), but this was not pursued in depth. Measurement of spermidine levels in the cell media could indicate whether spermidine was being excreted, and if so, whether the resulting concentration might be high enough to result in growth inhibition. The complete function of polyamines are still not understood, but they have been observed to have some effect on cell membranes (Cerutti, 1985), possibly due to the fact that they are readily acetylated, making them more lipophilic (Bolkenius and Seiler, 1981). Polyamines are also thought

to have the capabilities for interacting with anionic surface proteins (Tabor and Tabor, 1972; Beninati et al., 1985), and there is evidence that di- and polyamines are in vivo substrates for transglutaminases which incorporate them into proteins. Either of these characteristics could conceivably result in some alteration of some intracellular properties. Taking into account the results accumulated, and incorporating some known characteristics of polyamines, an hypothesis was formulated. Suppose insulin induced certain growth mechanisms, in a dose-dependent manner, which allowed for a certain concentration of polyamines which would be co-effectors of the response. Insulin itself would induce polyamines to a certain level which would saturate other growth mechanisms synchronously turned on. In keeping with this theory, TPA added in addition to insulin would result in a polyamine level which would exceed the insulin-induced growth mechanisms, and since TPA does not induce a series of putative growth mechanisms in these cells to accompany the increased ornithine decarboxylase increase there would be a polyamine excess. The excess would be either excreted or degraded by polyamine oxidases (assuming one or both of these occurences could bring about an inhibition of DNA synthesis). A second hypothesis has to do with the diversity between the timing of the ornithine decarboxylase induction by insulin and TPA. Induction of ornithine decarboxylase at 8h falls at approximately the G1/S phase border, a time when ornithine decarboxylase is normally increased in normally dividing cells (Grillo, 1985; Heby et al., 1976, Heby, 1980). The TPA induction of ornithine decarboxylase is maximal at 4h. If insulin and TPA are added together, the early induction of ornithine

decarboxylase might absorb much of the cells' resources for polyamine production, then at the time of ornithine decarboxylase induction critical for maximal DNA synthesis, i.e. at the G1/S phase border, there would a shortage of polyamines. It has been previously demonstrated through the use of polyamine synthesis inhibitors, that decreases in polyamine concentrations result in a decreased level of DNA synthesis (Boynton et al., 1980; Holtta, 1985; Fillingame et al. 1975).

The ornithine decarboxylase mRNA induction experiments performed in the presence of the protein synthesis inhibitor cycloheximide presented support for separate mechanisms of induction by insulin and TPA. In the case of insulin, the preliminary results suggest that ornithine decarboxylase mRNA might be stabilized by an insulin-induced factor which upon cessation of protein synthesis would not come into existence. TPA, on the other hand, might be stimulating a proteolytic enzyme as well as causing an increase in the transcription of the ornithine decarboxylase message. Together, TPA and insulin would then induce ornithine decarboxylase to a level beyond either alone. Transcriptional run-offs, and subsequent hybridization of the resulting [^{32}P]-labelled mRNA to ornithine decarboxylase cDNA, would be helpful in determining whether insulin or TPA altered the transcription of ornithine decarboxylase mRNA.

There appears to be a family of ornithine decarboxylase genes, with as many as 12 existing gene copies for ornithine decarboxylase distributed among as many as six chromosomes (Berger et al. 1986).

Apparently, the regulation of ornithine decarboxylase is accomplished through multiple mechanisms, including changes in the rate of protein synthesis and turnover, posttranslational modifications, RNA synthesis, and binding of macromolecular inhibitors (Berger et al., 1984; McCann, 1980). Synthesis of ornithine decarboxylase has also been reported to be affected at the level of transcription of the ornithine decarboxylase gene (Pegg, 1986). These observations emphasize the importance of the role of this enzyme and its products.

LIST OF REFERENCES

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Akiyama, Tetsu, Ishida, Junko, Nakawaga, Suguru, Ogawara, Hiroshi, Watanabe, Shun-ichi, Itoh, Noriki, Shibuya, Masabumi and Fukami, Yasuo (1987) J. Biol. Chem. 262, 5592-5595

Amasino, R.M. (1986) Anal. Biochem. 152, 304-307

Andersson, Anne-Charlotte, Henningsson, Stig, and Rosengren, Elsa in Polyamines in Biomedical Research (Gaugas, J.M., ed.) pp. 273-283, John Wiley and Sons, Inc., New York

Beguinot, Laura, Hanover, John A., Ito, Seiji, Richert, Nancy D., Willingham, Mark C. and Pastan, Ira (1985) Proc. Natl. Acad. Sci. USA 82, 2774-2778

Beninati, Simone, Piacentini, Mauro, Argento-Ceru, Maria-Paola, Russo-Caia, Salvatore, and Autuori, Francesco. (1985) Biochim. Biophys. Acta 841, 120-126

Berger, Franklin G., Szymanski, Paul, Read, Evan, and Watson, Gordon (1984) J. Biol. Chem. 259, 7941-7946

Berger, Franklin, G., Loose, David, Meisner, Herman, and Watson, Gordon (1986) Biochemistry 25, 1170-1175

Blackshear, Perry J., Nemenoff, Raphael A., Hovis, John G., Halsey, David L., 'Stumpo, Deborah J., and Huang, Jenq-Kuen (1987) Mol. Endo. 1, 44-52

Bolkenius, Frank N., and Seiler, Nikolaus (1981) Int. J. Biochem 13, 287-292

Boynton, A.L., Whitfield, J.F. and Walker, P.R., (1980) in Polyamines in Biomedical Research (Gaugas, J.M., ed.) pp. 63-79, John Wiley and Sons, Inc., New York

Brockerhoff, Hans (1986) FEBS Lett. 201, 1-4

Buckley, Arthur R., Putnam, Charles W. and Russell, Diane H. (1985) Biochem. Biophys. Acta 841, 127-130

Byus, Craig V., Trevillyan, James M., Cavit, Leslie J., and Fletcher, William H., (1983) Cancer Res. 43, 3321-3326

Castagna, Monique, Takai, Yoshimi, Kaibuchi, Kozo, Sano, Kimihiko, Kikkawa, Ushio, and Nishizuka, Yasutomi (1982) J. Biol. Chem. 257, 7847-7851

Cerutti, Peter A. (1985) Science 227, 375-381

Chance, Ron; personal communication

Cheng, Kang and Larner, Joseph (1985) Ann. Rev. Physiol. 47, 405-424

Chirgwin, John M., Przybyla, Alan E., MacDonald, Raymond J., and Rutter, William J., (1979) Biochemistry 18, 5294-5299

Chu, David T.W. and Granner, Daryl K. (1986) J. Biol. Chem. 261, 16848-16853

Church, G.M., and Gilbert W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991-1995

Denhardt, David T., Edwards, Dylan R. and Parfett, Craig L.J. (1986) Biochem. Biophys. Acta 865, 83-125

Dicker, Phillip, and Rozengurt, Enrique (1981) J. Cell Physiol. 109, 99-109

Dircks, Lori, Grens, Ann, Slezynger, Thelma C., and Scheffler, Immo E. (1986) J. Cell Physiol. 126, 371-378

Driedger, Paul E., and Blumberg, Peter M. (1980) Proc. Natl. Acad. Sci. USA 77, 567-571

Ebina, Yousuke, Ellis, Leland, Jarnagin, Kurt, Edery, Marc, Graf, Laszio, Clauser, Eric, Ou, Jing-hsiung, Masiarz, Frank, Kan, Y.W., Goldfine, I.D., Roth, Richard A., and Rutter, William J. (1985) Cell 40, 747-758

Fillingame, Robert H., Jorstad, Caroline M., and Morris, David R. (1975) Proc. Natl. Acad. Sci. USA 72, 4042-4045

Gammeltoft, Steen, and Van Obberghen, Emmanuel (1986) Biochem. J. 235, 1-11

Gilmour, Susan K., Verma, Ajit K., Madara, Thomas and O'Brien, Thomas (1987) Cancer Res. 47, 1221-1225

Goodman, Susan A., Esau, Brian and Koontz, John W. manuscript in preparation

Grillo, M. A., (1985) Int. J. Biochem. 17, 943-948

Hachiya, Harumi L., Takayama, Sumiko, White, Morris F. and King, George L. (1987) J. Biol. Chem. 262, 6417-6424

Heby, Olle and Andersson, Gunnar (1980) in Polyamines in Biomedical Research (Gaugas, J.M., ed.) pp. 17-34, John Wiley and Sons, Inc., New York

Heby, Olle, Gray, Joe W., Lindl, Patricia A., Marton, Laurence J., and Wilson, Charles B. (1976) Biochem. Biophys. Res. Commun. 71, 99-105

Hickok, Noreen J., Seppanen, Pauli J., Kontula, Kimmo K., Janne, Pasi A., Bardin, C. Wayne., and Janne, Olli A., (1986) Proc. Natl. Acad. Sci. USA 83, 594-598

Holtta, Erkki (1975) Biochim. Biophys. Acta 399, 420-427

Horuk, Richard and Olefsky, Jerrold M., (1985) Molecular and Cellular Endocrinology 42, 1-20

Hunter, Tony, and Cooper, Jonathan A. (1985) Ann. Rev. Biochem. 54, 897-930

Jarett, L. and Seals, J.R. (1979) Science 206, 1407-1408

Jetten, Anton M., Ganong, Barry R., Vandenbark, G.R., Shirley, J.E., and Bell, Robert M. (1985) Proc. Natl. Acad. Sci. USA 82, 1941-1945

Kallio, Arja, Poso, Hannu, Scalabrino, Giuseppe, Janne, Juhani (1977) FEBS Lett. 73, 229-234

Kasuga, Masato, Zick, Yehiel, Blith, Diana L., Karlsson, F. Anders, Haring, Hans U. and Kahn, C. Ronald (1982) J. Biol. Chem. 257, 9891-9894

Kathuria, Satish, Hartman, Steven, Grunfeld, Carl, Ramachandran, J., and Fujita-Yamaguchi, Yoko (1986) Proc. Natl. Acad. Sci. USA 83, 8570-8574

Kimes, Brian W. and Morris, David R. (1971) Biochem. Biophys. Acta 228, 223-244

Koontz, John W. (1984) Mol. Cell. Biochem. 58, 139-146

Koontz, John W., & Iwahashi, Mieko, (1981) Science 211, 947-949

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

McCann, Peter P. (1980) in Polamines in Biomedical Research (Gaugas, J.M., ed,) pp. 109-124, John Wiley and Sons, Inc., New York

Morisaki, Mobuhiro, Tomei, L. David, Milo, George E., and Cornwell, David G. (1985) Lipids 20, 602-610

Niedel, James E., Kuhn, Linda J., and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. USA 80, 36-40

O'Brien, Thomas G., (1980) in Polyamines in Biomedical Research (Gaugas, J.M., ed.) pp. 237-253, John Wiley and Sons, Inc., New York

Pegg, Anthony E. (1986) Biochem. J. 234, 249-262

Pegg, Anthony E., and McCann, Peter P. (1982) Am. J. Physiol. 243 (Cell Physiol. 12) C212-C221

Podlecki, David A., Smith, Robert M., Kao, Marilee, Tsai, Phoebe, Hueckstead, Thomas, Brandenburg, Dietrich, Lasher, Robert S., Jarett, Leonard, and Olefsky, Jerrold M. (1987) J. Biol. Chem. 262, 3362-3368

Rozengurt, Enrique, (1980) Current Topics in Cellular Regulation 17, 59-88

Saltiel. Alan R., and Cuatrecasas, Pedro (1986a) Proc. Natl. Acad. Sci. USA 83, 5793-5797

Saltiel, Alan R., Fox, Judith A., Sherline, Peter, and Cuatrecasas, Pedro, (1986b) Science 233, 967-972

Shipley, Gary D., Childs, Christy B., Volkenant, Mary E. and Moses, Harold L. (1984) Cancer Res. 44, 710-716

Smith, Robert M., and Jarett, Leonard (1987) Proc. Natl. Acad. Sci. USA 84, 459-463

Tabor, Celia White, and Tabor, Herbert (1984) Ann. Rev. Biochem. 53, 749-790

Tabor, Herbert, and Tabor, Celia (1972) Adv. Enzymol. 36, 203-268

Takayama, Sumiko, White, Morris F. Lauris, Vilma, and Kahn, C. Ronald (1984) Proc. Natl. Acad. Sci. USA 81, 7797-7801

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

Vaartjes, W.J. and de Haas, C.G.M. (1985) Biochem. Biophys. Res. Commun. 129, 721-726

Verma, Ajit K., Erickson, Deborah, and Dolnick, Bruce J. (1986) Biochem. J. 237, 297-300

Windholz, Martha (ed.) (1983) Merck Index, 7720, Merck and Company, Inc., Rahway, N.J.

Wu, Vincent S. and Byus, Craig V. (1981) Life Sciences 29, 1855-1863

Wu, Vincent S., Donato, Nicholas J. and Byus, Craig V., (1981) Cancer Res. 41, 3384-3391

APPENDIX

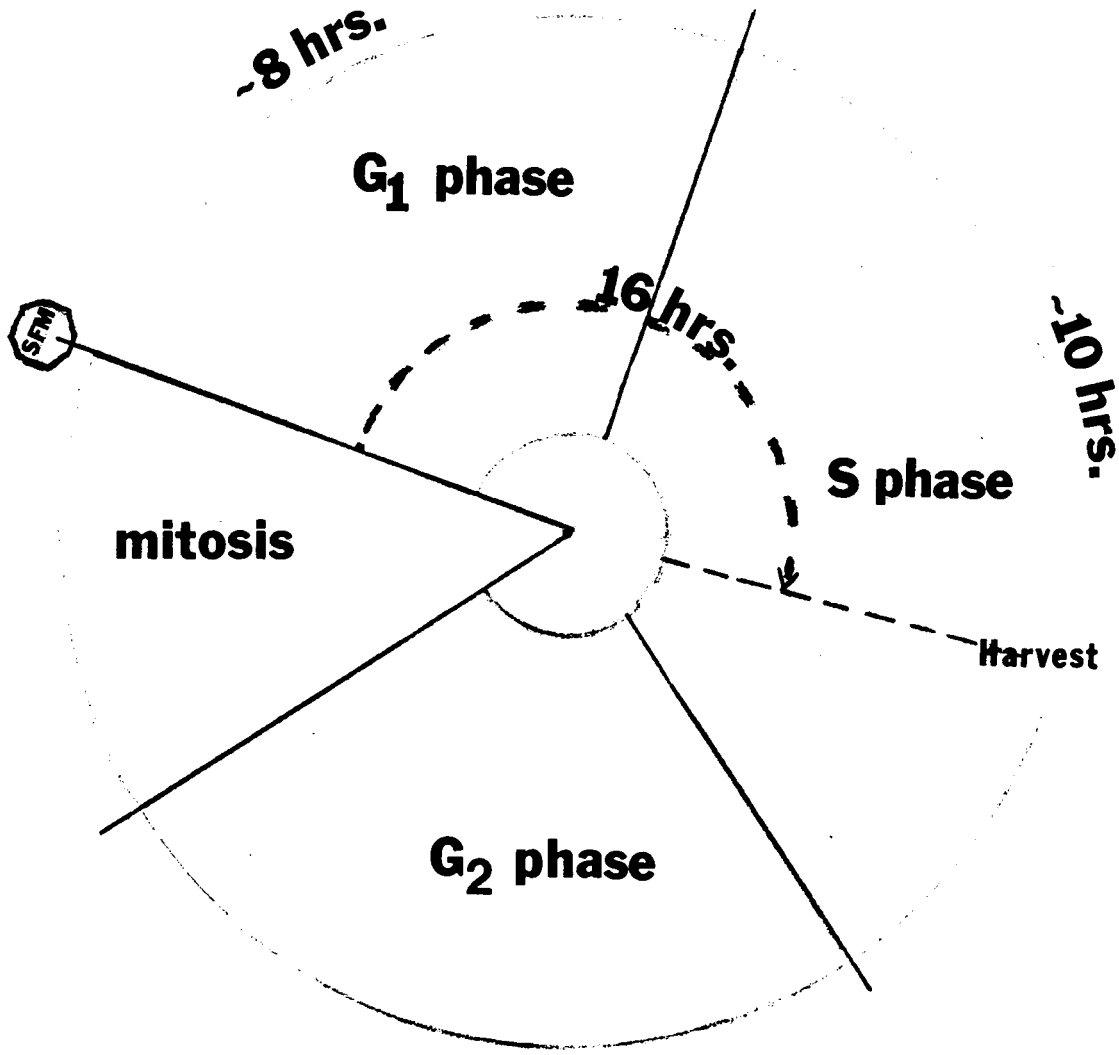


Fig. 1 Cell cycle of the KRC7 clone of the Reuber H35 rat hepatoma cell line.

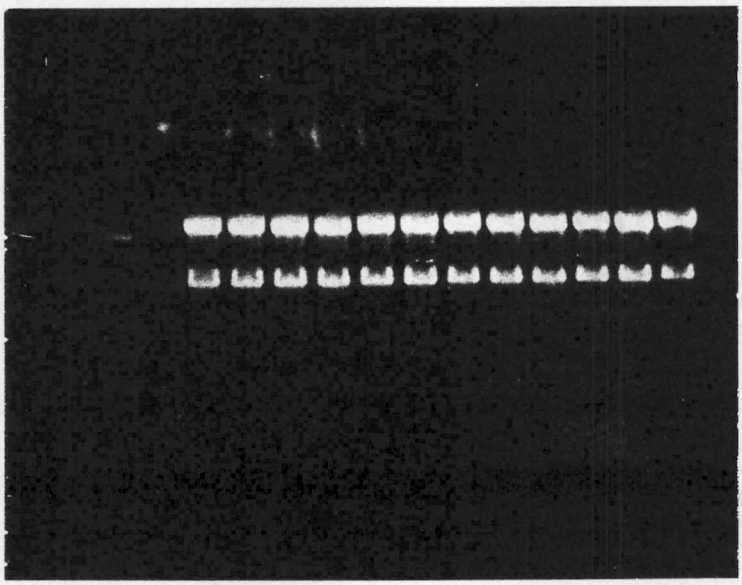


Fig. 2 Typical RNA gel. The upper and lower bands indicate the 28S and 18S RNA from the ribosomal subunits. The distinctness of these two bands suggests that the RNA is intact.

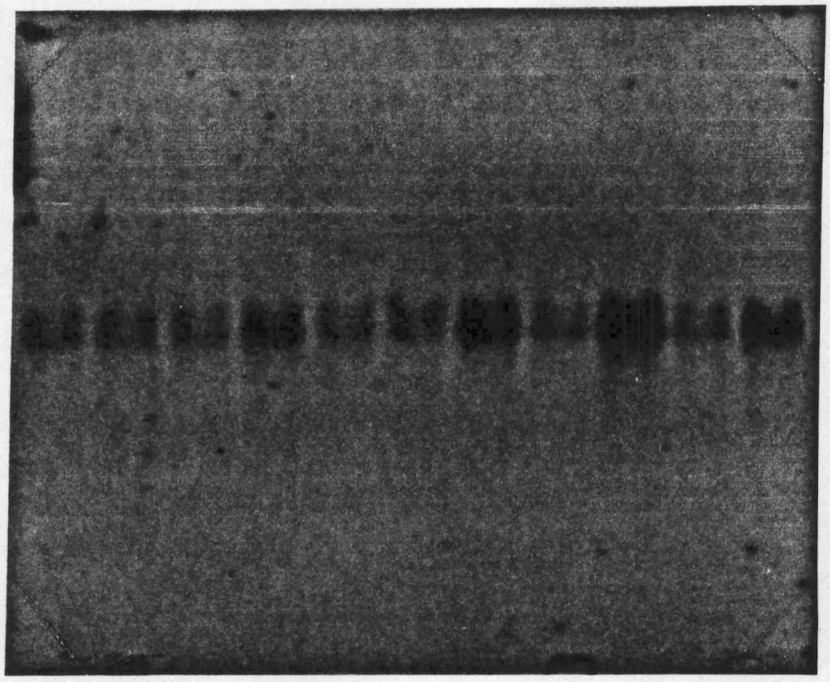


Fig. 3 Autoradiogram of a Northern blot. Total cellular RNA, which had been fractionated on a 1% agarose gel, was probed with [^{32}P]-labelled probe for ornithine decarboxylase, pODC934. The bands demonstrate the 2.7kb and 2.2kb forms of the mRNA for the enzyme which hybridize to the pODC934 probe.

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100% COTTON FIBRE

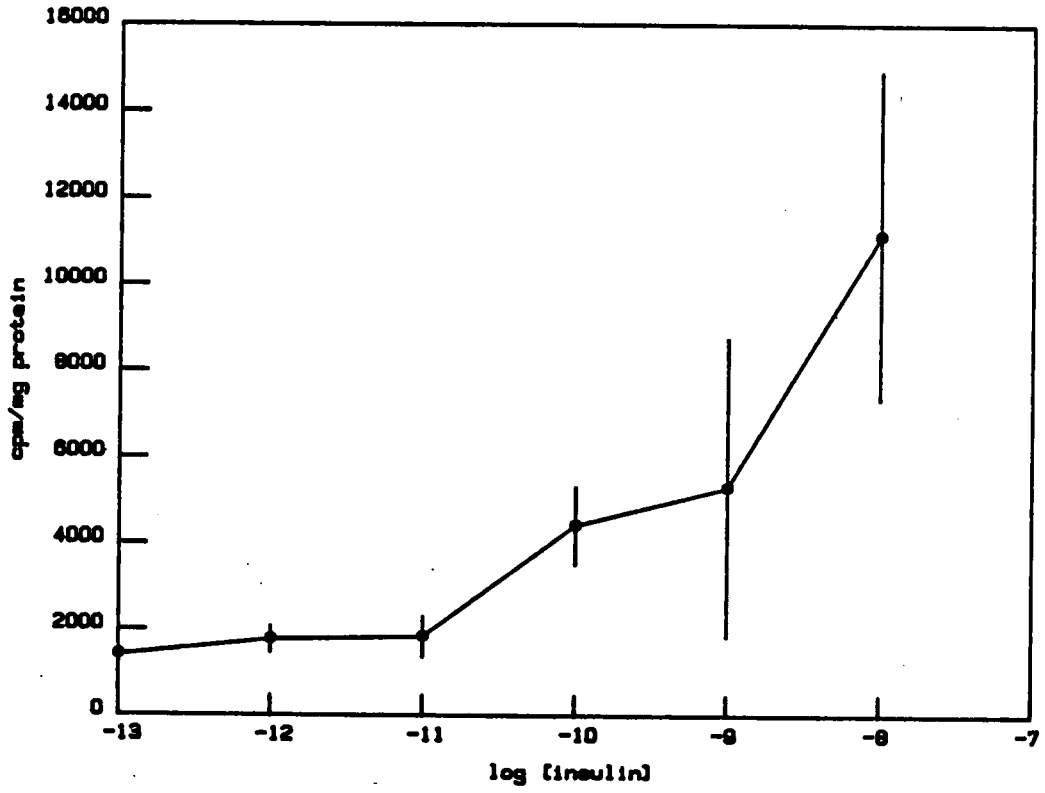


Fig. 4 Dose response for insulin stimulation of growth. Insulin treatments remained in contact with the cells for 16h, when all cells were harvested, presumably during mid-S-phase. The points represent an average of triplicate cell wells. The error bars represent one standard deviation.

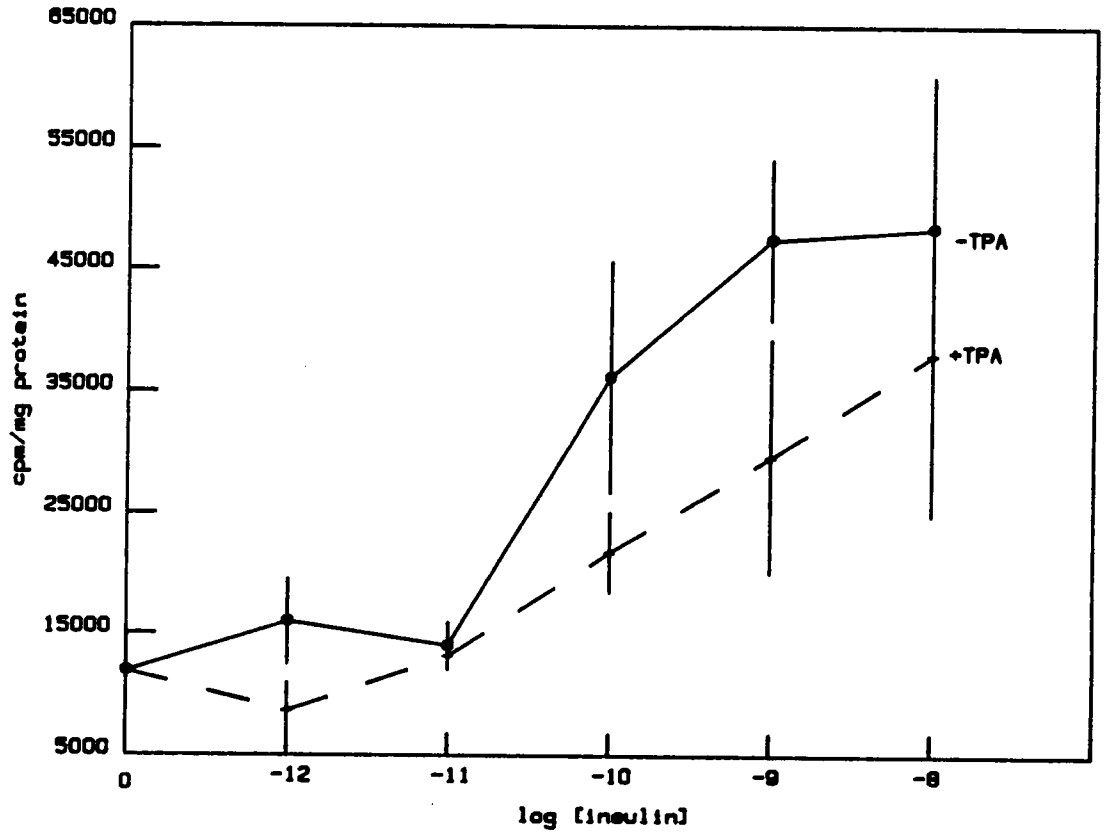


Fig. 6 Effects of TPA on a dose response of insulin-stimulated growth. Cells were treated with 10^{-9}M insulin without (o——o) or with (*---*) TPA (10^{-6}M). All cells were harvested at 16h and assayed for incorporation of tritiated thymidine. Each data point represents the average of triplicate determinations.

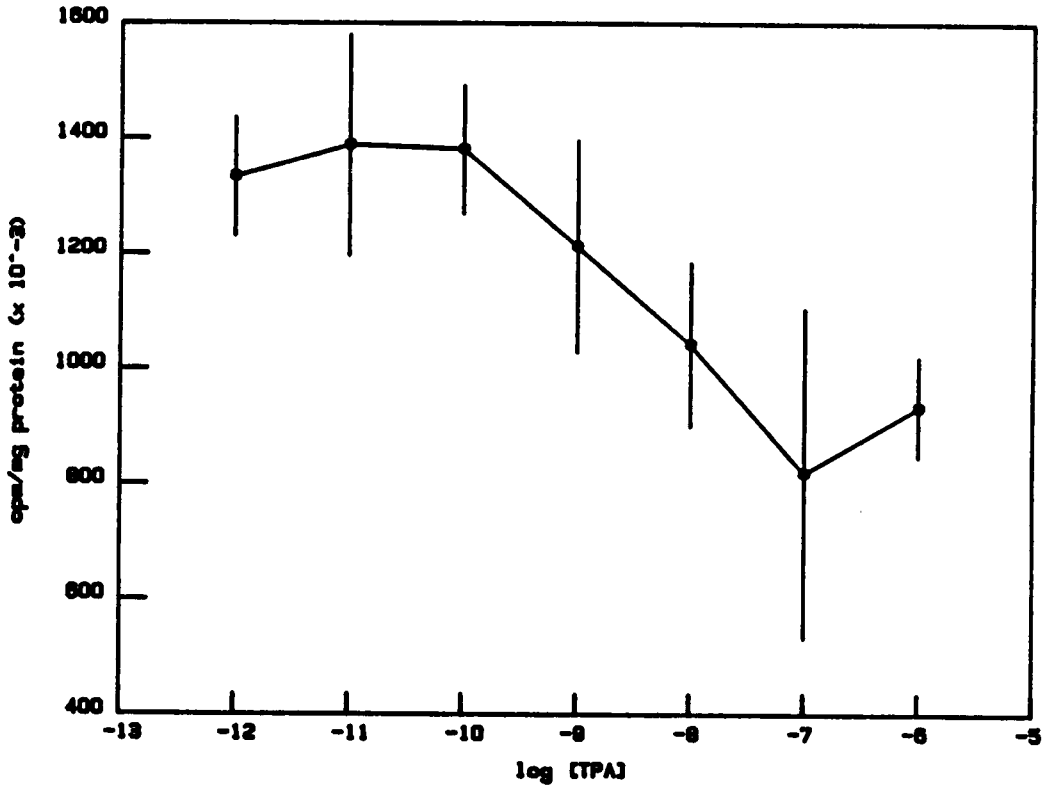


Fig 5 Dose response for TPA inhibition of insulin-stimulated growth. The points are obtained as an average of triplicates. Error bars represent one standard deviation.

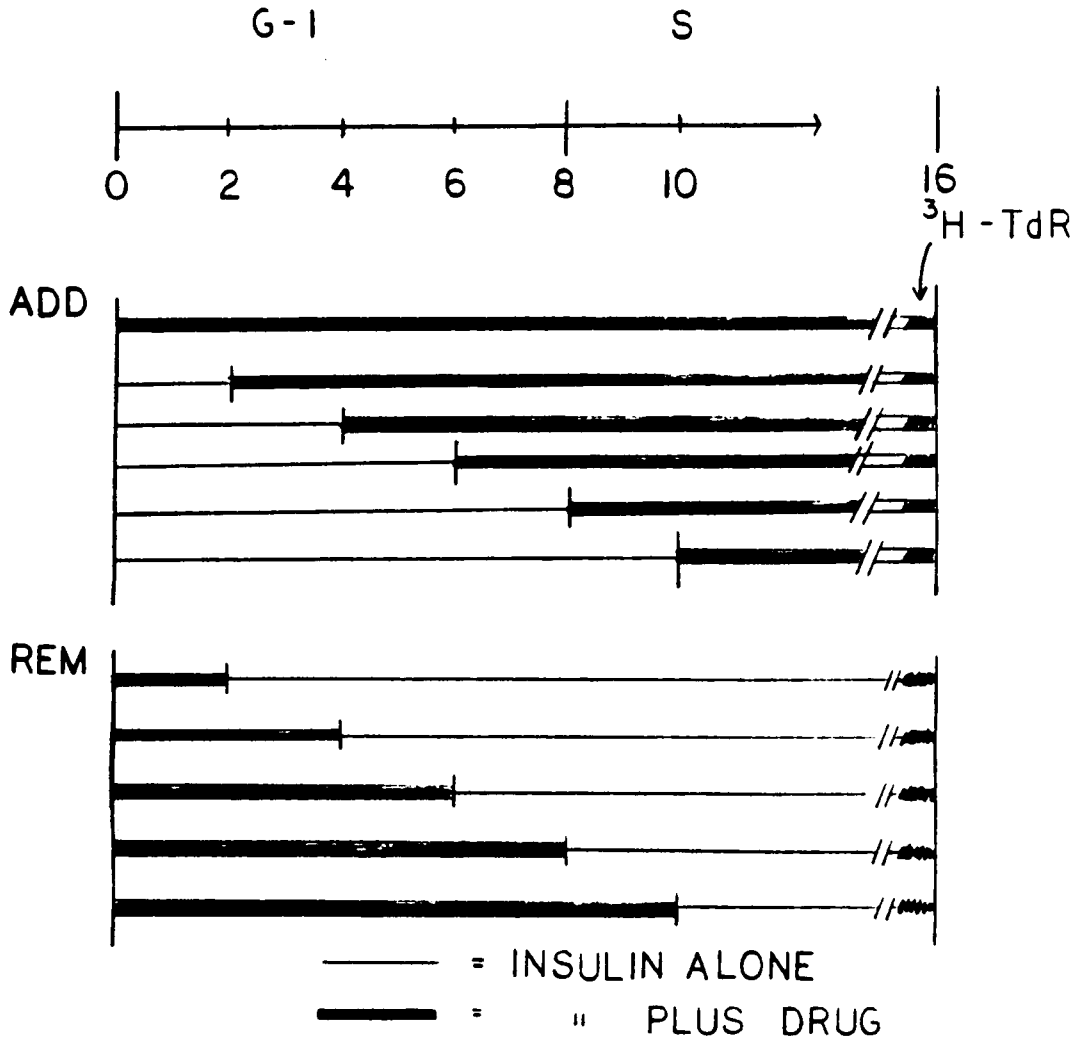


Fig. 7 Depiction of the TPA addition and removal scheme.

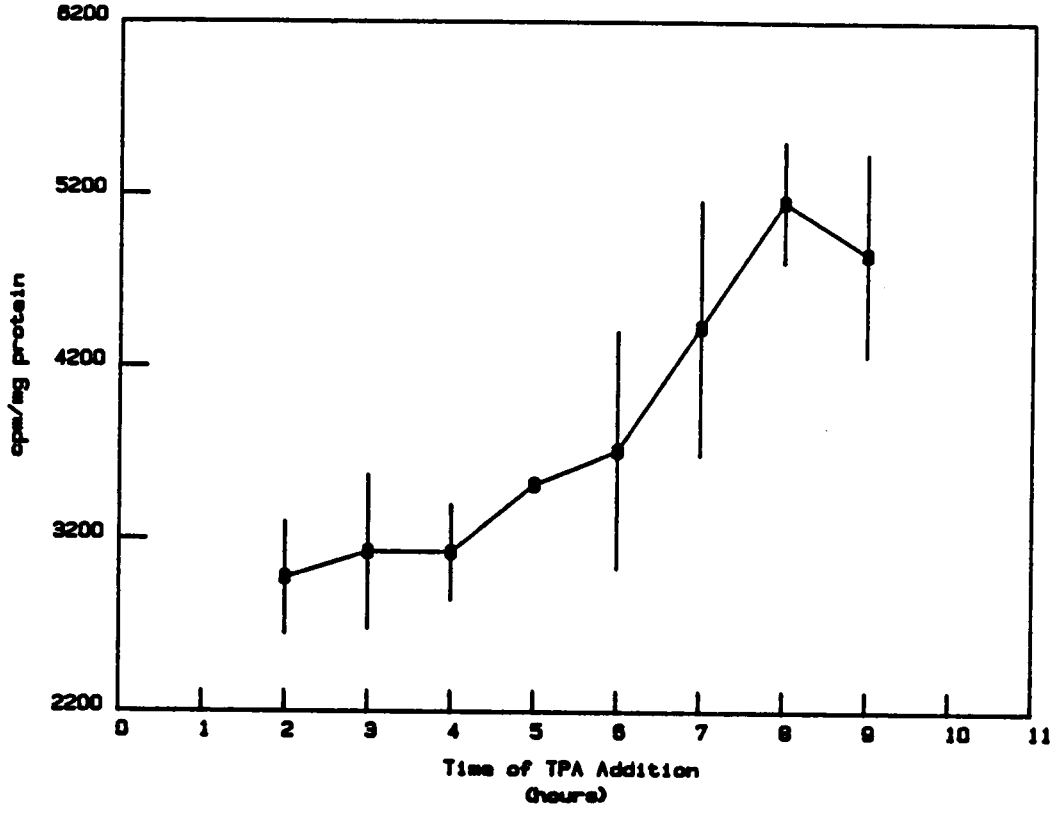


Fig. 8 Time course for TPA additions. Insulin (10^{-9}M) was added to all cells. At the times indicated, TPA (10^{-6}M) was added. Cells were harvested at 16h and subsequently assayed for tritiated thymidine incorporation. All treatments were made in triplicate and error bars represent one standard deviation.

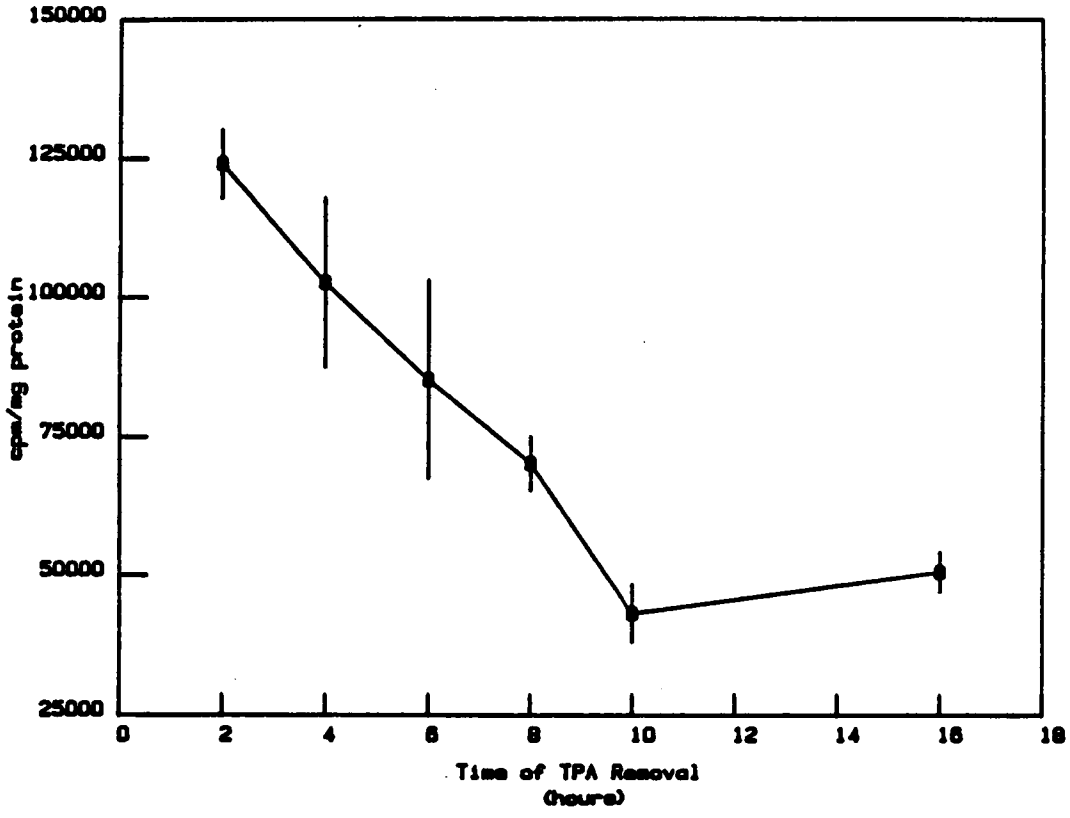


Fig. 9 Time course of TPA removal. All cells initially treated with insulin (10^{-9}M) and TPA (10^{-6}M). At the times indicated the media was removed and replaced with media containing insulin only. Each treatment was performed in triplicate. Error bars represent one standard deviation.

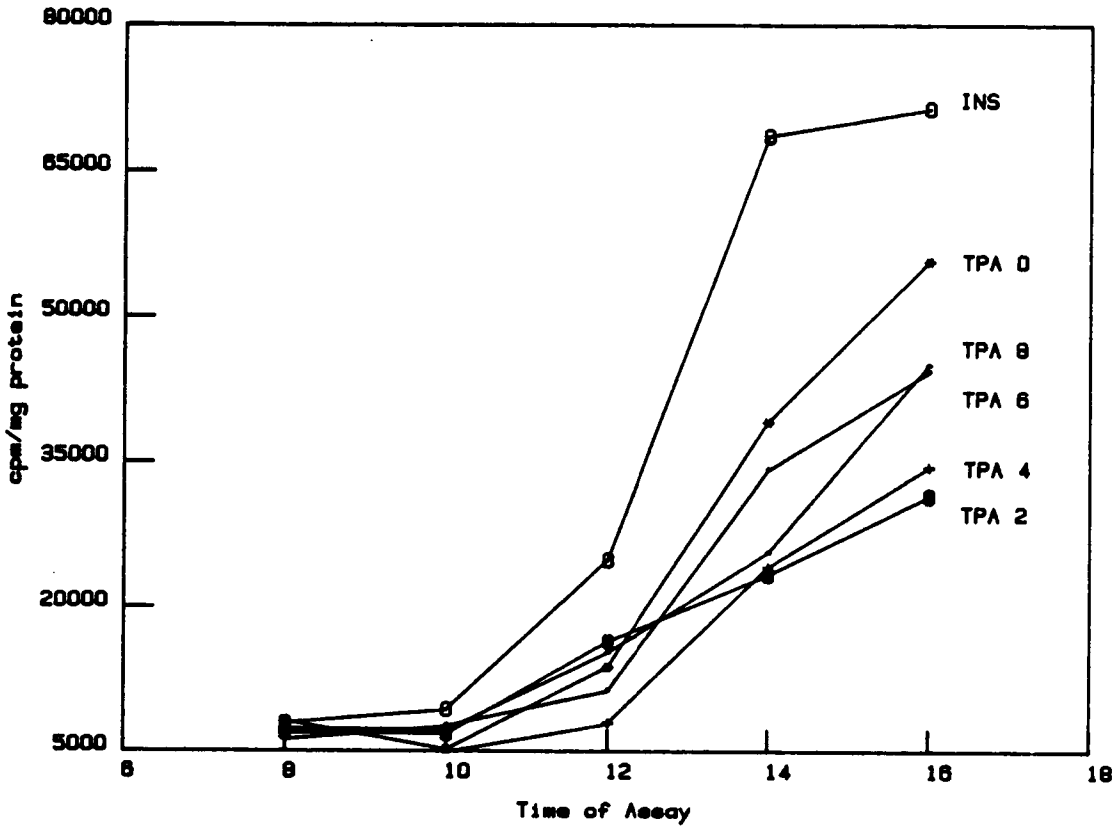


Fig. 10 Analysis for potential delay of S phase initiation by TPA. The times along the x-axis indicate the times at which the cells were harvested after all had been treated with insulin at zero time. The figure legends (TPA 0-8) indicate the times after insulin addition that TPA was added. The treatment of insulin alone (INS) shows the most induction.

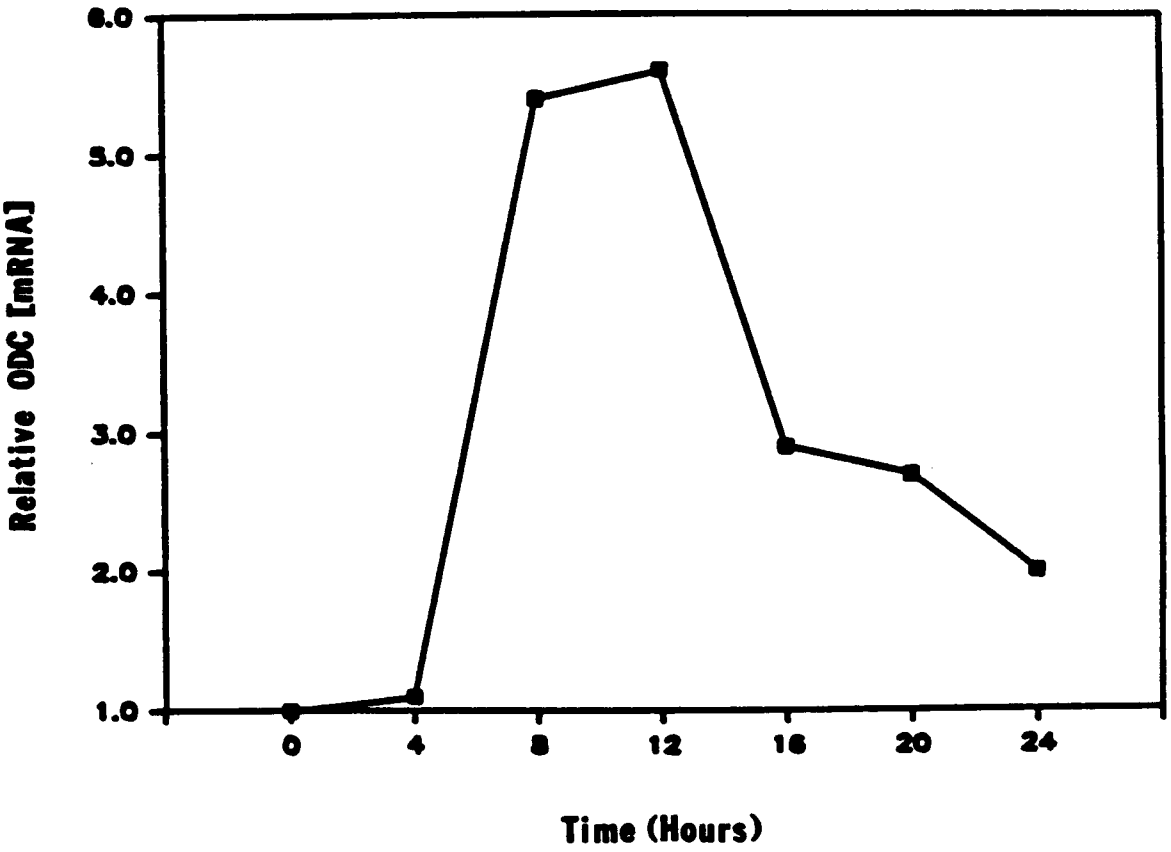


Fig. 11 Time course of insulin stimulation of ornithine decarboxylase mRNA. Insulin (10^{-9}M) was added to the cells at zero time, and the cells were harvested and assayed for ornithine decarboxylase mRNA at the times indicated. The peak area for the serum-free control was divided into the peak areas for all treatments, giving the control a value of 1, and others expressed relative to that.

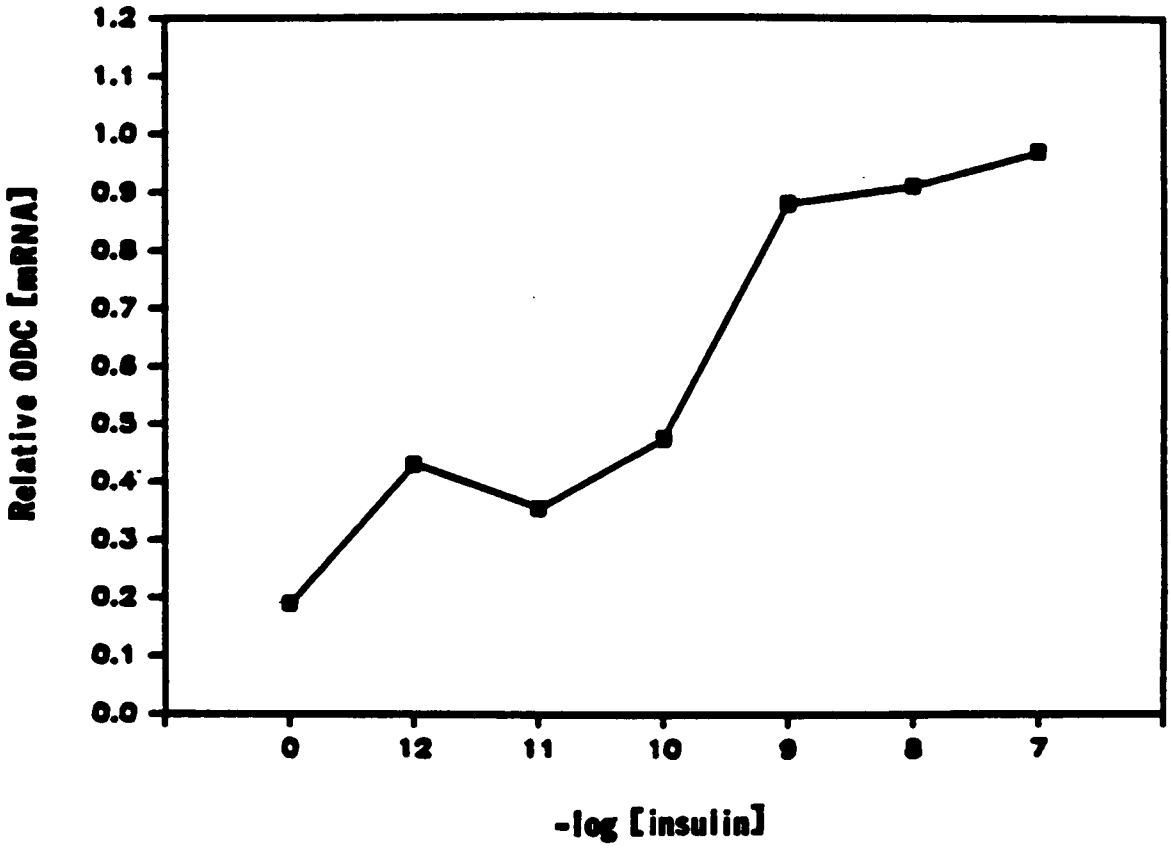


Fig. 12 Dose response of insulin on levels of ornithine decarboxylase mRNA. Cells were harvested after an 8h exposure to insulin. The first data point is the peak area obtained for the serum-free control. Other data points are actual peak areas obtained from analysis of densitometric scans.

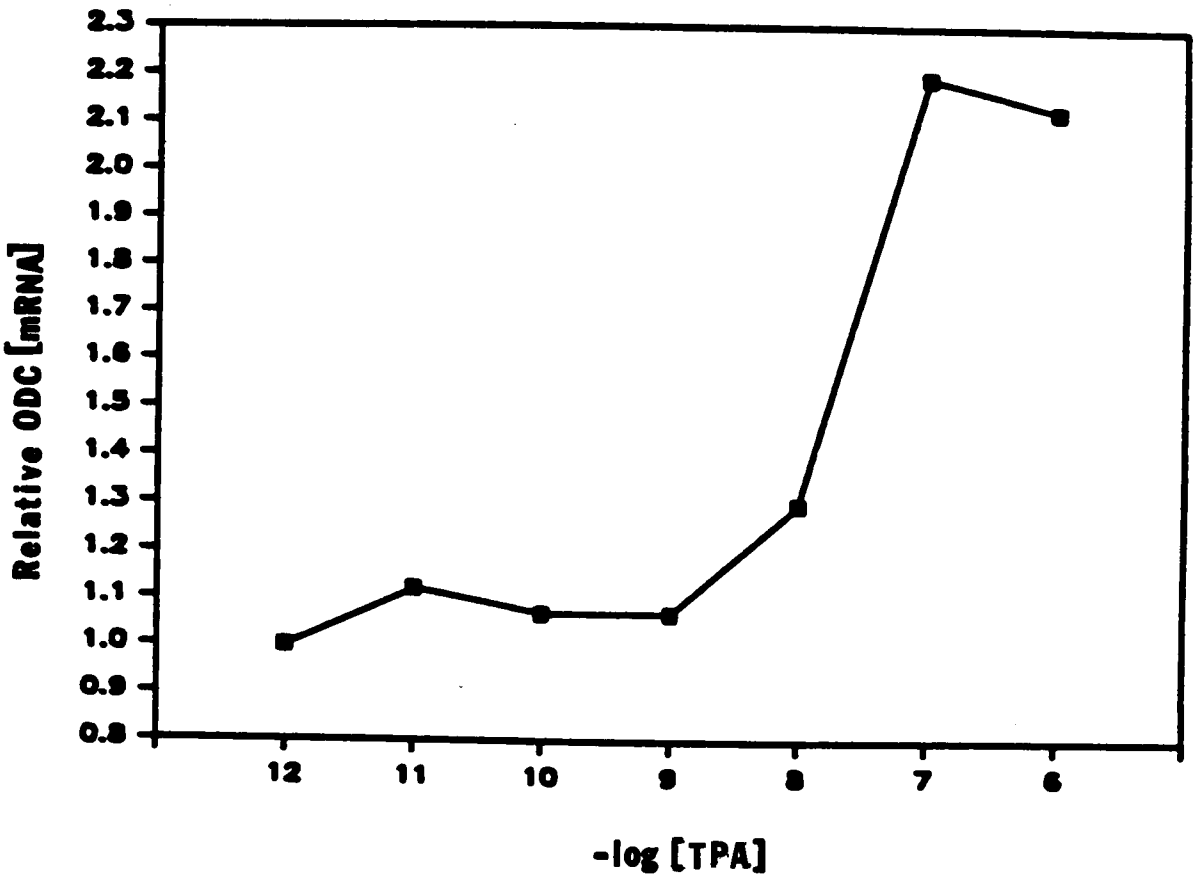


Fig. 13 Dose response of TPA additions on ornithine decarboxylase mRNA. Cells were harvested after a 4h exposure to the various concentrations of TPA. Data points were obtained as in Fig. 12.

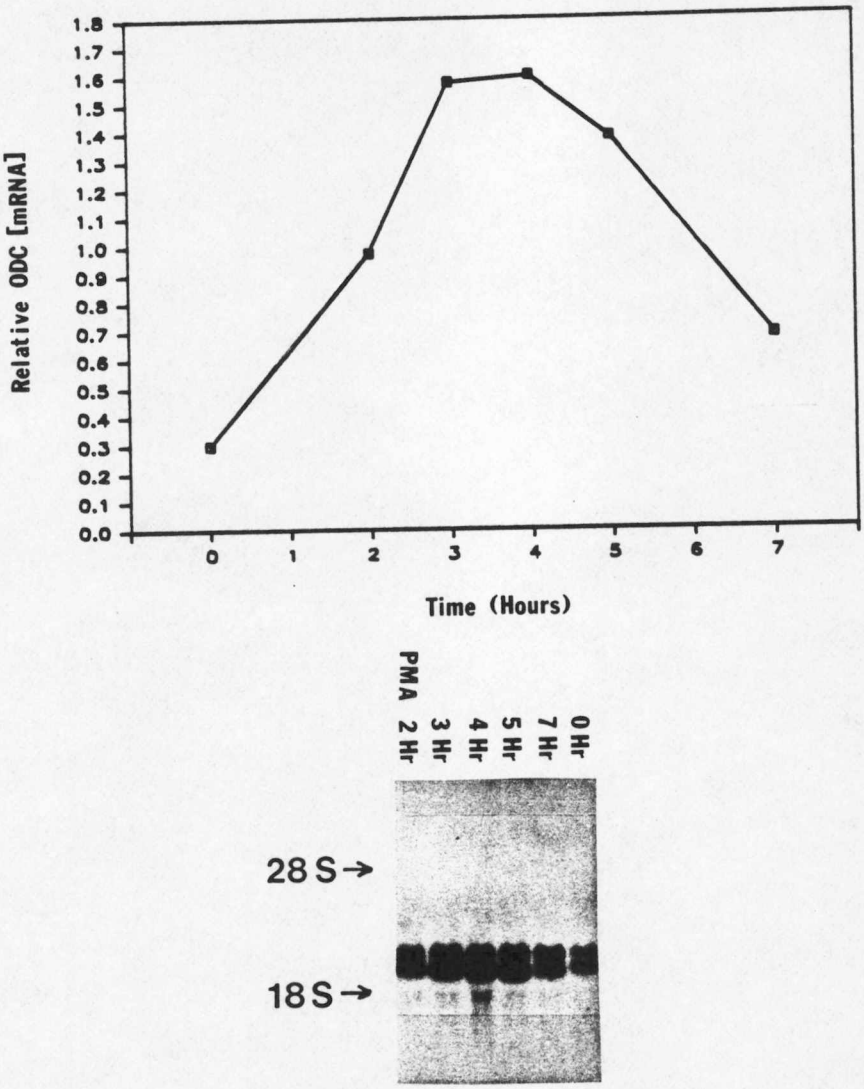


Fig. 14 Time course for induction of ornithine decarboxylase mRNA by TPA. Cells were harvested at the times indicated after exposure to TPA. Data points were obtained as in Fig. 12. The autoradiogram for the data is shown below the graph.

LANCASTER BOND
100% COTTON FIBRE

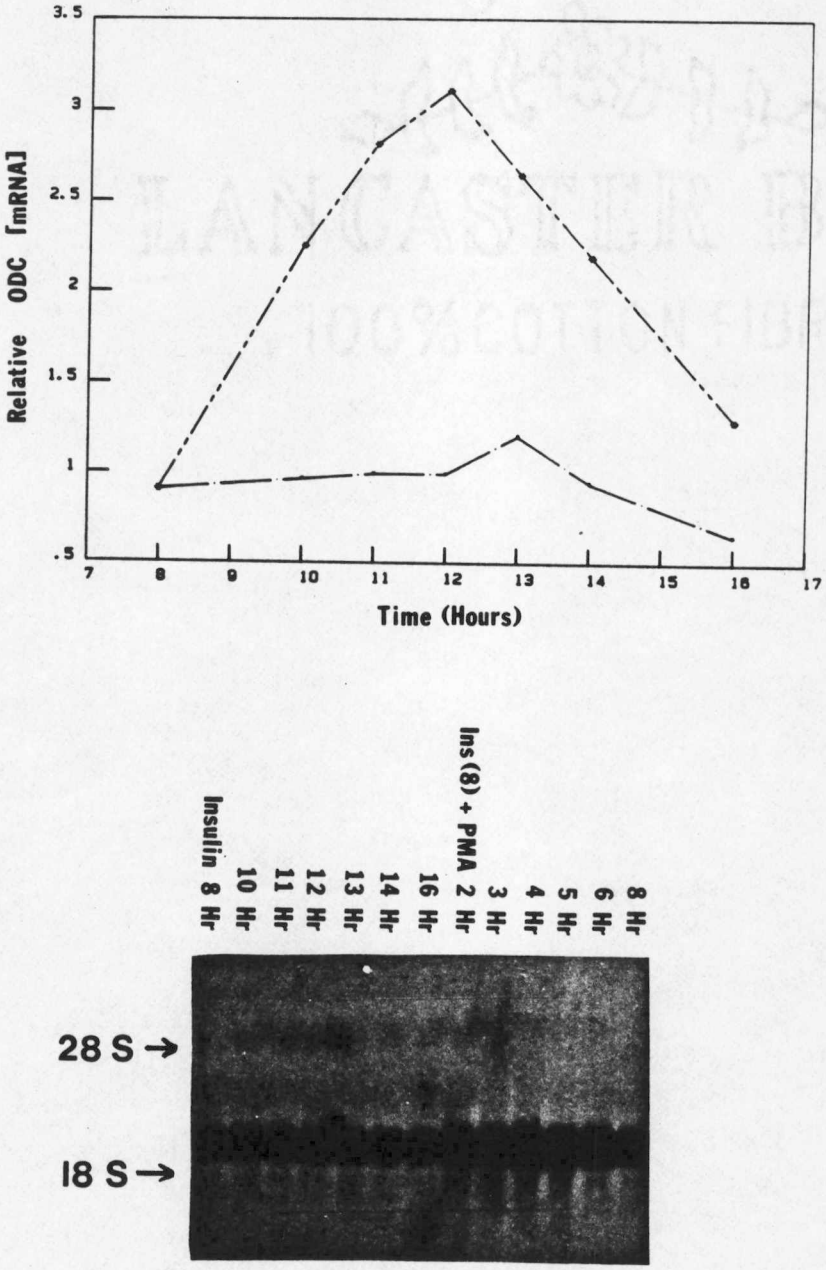


Fig. 15 Effects of TPA on ornithine decarboxylase mRNA at the time of maximal stimulation by insulin. After cells had been in contact with insulin (10^{-9}M) for 8h, a time of maximum insulin stimulation, TPA (10^{-6}M) was added and cells were harvested at the indicated times thereafter. The lower line on the graph indicates that cells treated with insulin maintain a fairly constant level of ornithine decarboxylase mRNA throughout the superinduction by TPA (upper line). Data points were obtained as in Fig. 12.

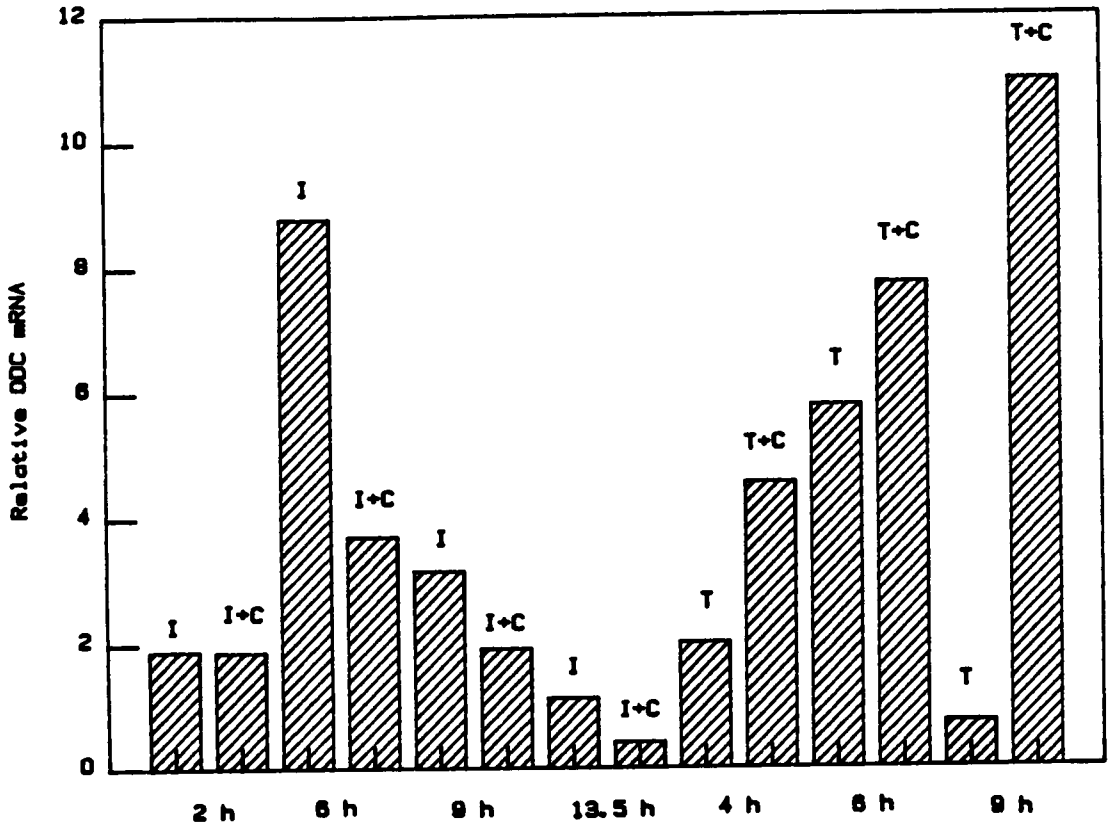


Fig. 16 Cycloheximide effects on induction of ornithine decarboxylase mRNA by insulin or TPA. The time points were selected as those which would fall at various stages of mRNA induction, i.e., pre-peak, peak,and post-peak, for each of the two agonists. The y-axis coordinates were obtained as comparative values to the serum-free control, which was assigned a value of 1 for each experiment. Values were obtained as peak areas of densitometric scans of autoradiograms obtained by probing Northern blots with [^{32}P]-labelled pODC934; the peak area for the serum-free control was divided into all other peak area values, giving the control a value of 1. At all times cycloheximide alone demonstrated little if any stimulation above the serum-free control.

VITA

Susan Austin Goodman was born in Nashua, New Hampshire on October 17, 1956. She attended schools in East Lansing, Michigan, Mt. Clemens, Michigan, Blacksburg, Virginia, and Tifton, Georgia, graduating from Tift County High School, Tifton, GA., in June, 1974. In September 1974 she entered Abraham Baldwin Agricultural College in Tifton, receiving an Associate degree in Pre-Veterinary Medicine in 1976.

In the fall of 1979 she entered The University of North Alabama in Florence, Alabama. In 1980 she entered a co-op program with International Fertilizer Development Center (IFDC) in Muscle Shoals, Alabama. In August, 1982, she received a Bachelor of Science degree in Professional Biology, and was afterwards employed by IFDC.

Susan accepted a teaching assistantship in the spring of 1984 at the University of Tennessee, Knoxville, and began studies towards a Master's degree in Biochemistry. Her research project was under the direction of Dr. John W. Koontz. In August, 1987 she completed the requirements for a Master of Science degree in Biochemistry, officially graduating in December of that year.

Formerly Susan Diane Austin, she married William Robert Goodman in August, 1977. She will be employed by the College of Veterinary Medicine, University of Tennessee, Knoxville beginning in September, 1987.