INSULIN, ADDED TO NUCLEI, STIMULATES TRANSCRIPTION OF SPECIFIC GENES

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CERTIFICATE OF SUPERVISOR

In terms of paragraph eight of "General regulations for the degree of Ph.D." I, as supervisor of the candidate, B.J. Stickells, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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SCOPE OF THIS INVESTIGATION

Insulin regulates cellular gene expression and modulates specific mRNA levels in liver cells. As yet, the mechanism of this control is still unclear.

The effects are initiated following the binding of insulin to the plasma membrane receptor. Although several mediators of the signal from the plasma membrane to the nucleus have been proposed, none has proved capable of eliciting all of the effects of insulin on gene expression.

Therefore, the possibility that insulin itself may directly regulate transcription at the level of the nucleus, was investigated.

SUMMARY

Insulin, added to H4IIE hepatoma nuclei incubated in an *in vitro* transcription assay, was found to have a biological effect. Insulin stimulated total gene expression, as well as the expression of specific insulin-responsive genes. The nuclei were isolated using melittin, a procedure that allows the isolation of pure nuclei, with intact nuclear membranes and good transcriptional activity.

The relative biological activities of various insulin-analogues were examined, with regard to their ability to mimic the effects of insulin in altering the levels of specific mRNA species. These analogues will be used in further studies of the role of insulin at the nucleus.

A preliminary investigation showed that nerve growth factor also increases the expression of c-*fos* when added to PC12 nuclei in an *in vitro* transcription assay. Thus, the demonstrated effects may be representative of a mechanism of gene regulation applicable to peptide hormones in general.

ABBREVIATIONS

ASD	-	2-(*p*-azidosalicylamido)-1,3'-dithiopropionate
BSA	-	Bovine serum albumin
CNBr	-	Cyanogen bromide
DPBSA	-	Dulbecco's Phosphate Buffered Saline without
	-	calcium and magnesium
DMEM	-	Dulbecco's Modification of Eagle's Medium
DMSO	-	Dimethyl sulphoxide
DTT	-	Dithiothreitol
EDTA	-	Disodium ethylenediamine-tetraacetate
EGF	-	Epidermal growth factor
EGTA	-	Ethyleneglycobis(β-aminoethyl)ether tetraacetate
EMEM	-	Earle's Modification of Eagle's Medium
HPLC	-	High pressure liquid chromatography
HPRI	-	Human placental ribonuclease inhibitor
kb	-	Kilobase(s)
NGF	-	Nerve growth factor
NSB	-	Nuclei storage buffer
PBS	-	Phosphate buffered saline
PEPCK	-	Phosphoenolpyruvate carboxykinase
PK	-	Pyruvate kinase
PMSF	-	Phenylmethylsulphonyl fluoride
ODC	-	Ornithine decarboxylase
RIA	-	Radioimmunoassay
SASD	-	Sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-
	-	1,3'-dithiopropionate
SEM	-	Standard error of the mean
TAT	-	Tyrosine aminotransferase

CHAPTER 1

INTRODUCTION

Insulin is an anabolic hormone with a wide range of effects on metabolism in many different cell types. Its complex regulatory mechanisms include the biosynthesis of carbohydrate, protein, lipid and nucleic acid. In spite of the fact that insulin's structure has long been determined and its metabolic effects are well documented, the molecular mechanism of action of insulin is yet to be established.

The steps involved in the short and medium term cellular responses to insulin affecting such processes as membrane transport and the regulation of enzyme activity, have been studied extensively and are beginning to be elucidated (1). However, essentially nothing is known at the molecular level about the mechanism(s) by which insulin regulates the long term responses to the hormone, and particularly its role in the regulation of gene expression.

The binding of insulin to its receptor on the plasma membrane is considered the initial event in all the responses of the target cell to the hormone (2,3), but the mediator/s of the signal from the plasma membrane to the nucleus of the cell resulting in the control of gene expression, has/ve as yet to be identified.

1.1 POSSIBLE MECHANISMS FOR REGULATION OF GENE EXPRESSION BY INSULIN

1.1.1 THE ROLE OF THE PLASMA MEMBRANE RECEPTOR

Peptide hormones interact with target cells initially by binding to a specific receptor on the plasma membrane. The receptor thus serves as the recognition signal for the circulating hormone, binds the hormone and then aids in the transduction of the hormonal signal to the various sites of action - intracellularly or at the membrane. The receptor also facilitates the internalization of the hormone (4).

In the case of insulin, the plasma membrane receptor is an integral membrane glycoprotein consisting of two extracellular α subunits (M_r 135 000) and two transmembrane β subunits (M_r 95 000). The cysteine-rich region of the α subunit contains the insulin binding site (5), while the β subunit is a transmembrane protein with tyrosine-specific protein kinase activity (6). The number of insulin binding sites per receptor is not clear; attempts to determine the stoichiometry have yielded a figure of 1,48 mol of insulin bound per mol of receptor (7). The structure and function of the insulin receptor have been reviewed by Gammeltoft and Van Obberghen (8), Goldfine (9), and Kahn and White (10).

Experimental evidence has supported the binding of insulin to its plasma membrane receptor as the initial event in the regulation of the effects of insulin on gene expression. Insulin inhibits phosphoenolpyruvate carboxykinase (PEPCK) gene transcription; the binding affinity of insulin, proinsulin or guinea pig insulin for the receptor, correlates well with the ability of that particular insulin to decrease PEPCK mRNA levels in H4IIE hepatoma cells (11).

Thus, it is accepted that the plasma membrane receptor is the means whereby insulin locates target cells and initiates responses, including regulation of gene expression, through binding to the receptor. However, the question as to how the signal is transmitted to the nucleus, ultimately resulting in the regulation of the expression of specific genes, still remains unanswered.

1.1.2. POSSIBLE MEDIATORS OF THE SIGNAL FROM THE PLASMA MEMBRANE TO THE NUCLEUS

Several mediators or second messengers of the effects of insulin within the cell have been proposed. These include protein kinases, cAMP, glycosyl-phosphatidylinositol and some unidentified protein mediators. Each one of the suggested second messengers has been proved insufficient in some way in mediating all of the biological effects of insulin, and in particular, in mediating the effects of the hormone on gene expression.

The intriguing alternative, that insulin itself is internalized and has a direct effect on gene expression, although considered unlikely by most research workers, may provide the ultimate answer.

1.1.2.1 INSULIN RECEPTOR PROTEIN KINASE

The plasma membrane insulin receptor is a tyrosine-specific protein kinase which autophosphorylates the cytoplasmic domain of its own β subunit on tyrosine residues following the binding of insulin to the receptor (6,12). This autophosphorylation results in the activation of the receptor kinase towards other cellular substrates and thus provides a means for the internalization and amplification

of the hormonal signal (3,13). Receptor aggregation also plays a role in the activation of the insulin receptor kinase (14-16). The tyrosine kinase activity of the receptor persists even in the absence of receptor associated insulin (13).

The substrates phosphorylated *in vivo* by the activated receptor kinase include many unidentified proteins (8,9,10), as well as a cytoskeleton associated protein (17), calmodulin (18), a protein involved in signal transmission to the glucose transport system (19) and a novel serine kinase - one of the substrates of which is the β subunit of the receptor itself (20). The exact significance of these phosphorylated proteins is unknown. Each is, however, a possible mediator of the hormonal response or a possible generator of a phosphorylation cascade, resulting ultimately in the biological effects of the hormone (3,8,9). A specific phosphoprotein involved in mediating the effects of insulin on transcription, has not been identified.

Receptor kinase activation has been shown to be important in the generation of many of insulin's metabolic and mitogenic effects. The insulin-induced stimulation of 2-deoxyglucose uptake, glycogen synthesis, amino acid transport, S6 kinase activity, as well as thymidine incorporation into DNA, are impaired when tyrosine kinase activity is altered. Experiments to demonstrate this have included: the microinjection of tyrosine kinase inhibiting antibodies (21); site-directed mutagenesis of the receptor resulting in kinase-defective receptors (22,23,24); the lack of insulin effects following receptor dephosphorylation (25); impaired insulin receptor kinase activity in insulin-resistant states (26); increased receptor phosphorylation induced by insulin-mimetic agents (27); and treatment with a selective insulin receptor tyrosine kinase inhibitor (28). The transmembrane receptors for epidermal growth factor, platelet-derived

growth factor and insulin-like growth factor-I stimulate phosphorylation on tyrosine residues of their membrane receptors suggesting that this may be significant in cellular growth control (29).

However, the absolute requirement for receptor tyrosine kinase activity in mediating these responses to insulin is not certain, as anti-receptor antibodies can exert a wide range of insulin-like metabolic effects without the requirement for kinase activation (30,31). Also, a natural inhibitor of the receptor kinase antagonized the growth promoting action of insulin in Fao hepatoma cells, but did not affect hormone mediated increase in amino acid transport and tyrosine aminotransferase induction in these cells (32).

The activity of the kinase is also required for the stimulation of gene expression by insulin. The ability of insulin to increase the levels of c-*fos* in Chinese hamster ovary (CHO) cells is severely impaired in cells transfected with a mutant insulin receptor lacking kinase activity (33). However, the intermediate signalling mechanisms that bridge the gap between kinase activation and gene activation or inactivation are still unknown.

A possible route for the hormonal signal to reach the interior of the nucleus, is via the phosphorylation/ dephosphorylation of nuclear proteins. Insulin treatment has been shown to stimulate the phosphorylation of nuclear lamins A and C (34), increase the activity of nuclear protein kinases (35) and cause a dephosphorylation of nuclear envelope proteins (36). Although none of these events has been shown to be directly involved in the regulation of gene expression, they do corroborate a role for biologically significant insulin-induced protein phosphorylation/dephosphorylation reactions at the nucleus.

Most evidence suggests that signal transmission occurs through a chain of phosphorylation reactions. Two alternative possibilities have been proposed. Firstly, the autophosphorylation of the β subunit of the receptor results in conformational changes in its cytoplasmic domain. These could alter its interaction with other cellular proteins and so mediate signal transmission (37). Secondly, the receptor is rapidly internalized following insulin treatment and kinase activation, thus transferring an active kinase from the cell surface to topographically discrete cellular locations (38). In this way, insulin-dependent phosphorylations are maintained and/or initiated intracellularly. Thus, kinase translocation may provide a means for the phosphorylation of nuclear proteins or the activation of nuclear protein kinases. Such a mechanism could also accommodate a role for the internalized hormone on gene expression.

1.1.2.2 CYCLIC AMP

There is considerable evidence that several hormones which are derivatives of amino acids, polypeptides or catecholamines, can exert their effects on target cells by altering levels of cyclic nucleotides (39). Intracellular cAMP levels can be modulated via altered activity of adenylate cyclase, altered cAMP phosphodiesterase activity or changes in the rate of efflux of cAMP from the cell (40).

Insulin can inhibit the activity of adenylate cyclase and stimulate the activity of certain cAMP phosphodiesterases (41). These effects are mediated via specific guanine nucleotide regulatory proteins (42,43). Insulin can also decrease the activity of cAMP-dependent protein kinase by decreasing the affinity of the kinase for cAMP (44).

Many of the acute actions of insulin are antagonized by intracellular cAMP. Certain of the sites undergoing insulin-induced dephosphorylation are substrates for cAMP-dependent protein kinase. However, there is no definite correlation between the changes in cAMP levels, and the rapid biological effects induced by insulin (45).

Knowledge of the mechanism responsible for the induction of gene expression by cAMP in eukaryotes is very limited in comparison to knowledge about the induction of gene expression by cAMP in prokaryotic systems. However, the promoter-regulatory regions of many cAMP-responsive genes have recently been isolated and characterized, and specific cAMP regulatory elements (CREs) identified. These include the genes for somatostatin, PEPCK, the α-subunit of human chorionic gonadotropin, proenkephalin and vasoactive intestinal peptide (46). The exact mechanism of the cAMP-mediated regulation of mRNA turnover is not known. CREs can regulate transcription from promoters in a manner independent of position or orientation (46). A cAMP-dependent nuclear factor has been detected that interacts with the CRE of the PEPCK gene and is thus a possible mediator of the transcriptional control exerted by cAMP (47). Extended studies of the PEPCK promoter-regulatory region have revealed a total of four sites to which a CRE-binding protein binds (48).

Regulatory control by cAMP and insulin may be linked at the level of gene expression, as many of the genes regulated by cAMP are also regulated by insulin at a transcriptional level. These include PEPCK (49), TAT (50) and c-*fos* (33,51). As yet, no interaction between these two regulatory pathways has been observed, but the possibility does exist that insulin affects cAMP metabolism in a manner still to be elucidated, resulting in the observed regulation of the expression of these genes.

1.1.2.3 GLYCOSYL-PHOSPHATIDYLINOSITOL

Recently the role of inositol triphosphate and diacylglycerol as second messengers in mediating cellular responses to extracellular signals such as hormones, oncogenes, growth factors and antigens, has been investigated. These two lipid-derived second messengers are the products of a cascade of reactions elicited in target cells following the binding to, and activation of, the relevant plasma membrane receptor. The role of inositol triphosphate and diacylglycerol as second messengers has been reviewed by Berridge (52) and Low and Saltiel (53).

Several lines of evidence have suggested that insulin may regulate certain of its biological effects by increasing the production of inositolphosphate-glycans and diacylglycerol (54 and references therein). It is proposed that the binding of insulin to target cells activates the plasma membrane receptor which in turn activates a specific membrane-bound phospholipase C (55). A G-protein may be involved as an intermediate in the coupling of the insulin receptor to the phospholipase C (53). The activated phospholipase C catalyzes the hydrolysis of a novel glycosyl-phosphatidylinositol molecule to diacylglycerol and a novel inositol phosphate-glycan (IP-gly)(containing glucosamine) - two proposed second messengers of insulin action (56,57).

IP-gly can mimic many of the effects of insulin including the regulation of glucose utilization and the modulation of enzyme activity (53,56,58).

Diacylglycerol is always associated with the plasma membrane and is responsible for the activation of protein kinase C, a cAMP independent kinase requiring calcium and phosphatidylserine for full activity (52). In intact cells the insulin-dependent activation of protein kinase C results in the

phosphorylation of the insulin receptor, decreased insulin binding and reduced protein-tyrosine kinase activity (59). Phorbol esters can also activate protein kinase C and elicit insulin-like effects on glucose transport, glucose oxidation and lipogenesis (53).

Chu et al. (54) have shown that phorbol esters and diacylglycerol can mimic the effects of insulin in decreasing the expression of PEPCK in rat H4IIE hepatoma cells. It is therefore possible that diacylglycerols generated in the plasma membrane can act as intracellular signals that regulate specific gene expression. Stumpo et al. (60) found that insulin or the phorbol ester, phorbol 12-myristate 13-acetate (PMA), can stimulate c-*fos* expression in 3T3-L1 fibroblasts and adipocytes. Taub et al. (61) showed similarly, that in Reuber H-35 cells, insulin or PMA induce a rapid increase in the steady state levels of c-*fos* and c-*myc* mRNAs. However, the mechanism of the induction of by PMA appears to be different from the induction by insulin in both instances, as pretreatment of the cells with PMA blocks the PMA-mediated but not the insulin-mediated induction.

Thus, under certain conditions, insulin may interact with phospholipids, but this pathway does not account for all of the actions of insulin and is not a specific mediator of its effects on gene expression.

1.1.2.4 OTHER POSSIBLE MEDIATORS

Other alternatives for the mediating of insulin's effects on gene expression include cGMP (40), intracellular Ca^{2+} (62), hydrogen peroxide (27), as well as membrane hyperpolarization (63), intracellular pH changes (64) or specific peptide fragments (65). Some compounds originally thought to be small peptides are now proposed to be the polar head

groups of the membrane glycolipids discussed in 1.1.2.3 (53). No known small intracellular molecule has been identified that can account for all of the diverse actions of insulin (9).

Sato et al. (66), have reported that insulin may control nuclear transcription of p33 mRNA in hepatoma cells through a putative insulin metabolic mediator, cAMP-dependent protein kinase/adenylate cyclase inhibitory mediator. The mediator stimulates the accumulation of p33 mRNA to the same extent as insulin, but apparently by a different mechanism, as the action of the putative mediator is inhibited by cycloheximide while the action of insulin itself is not. Possible explanations for this difference may be that insulin controls nuclear transcription by multiple signalling mechanisms, that the added putative mediator does not enter the cell in the presence of cycloheximide or alternatively, that the mediator is inactive as such, and must first be converted to an active species by a step requiring protein synthesis (66).

On reviewing the many and often conflicting theories that have arisen from different laboratories regarding a mediator for the diverse effects of insulin on gene expression in target cells, it is apparent that none of these mediators provides the answer. Thus, the possibility of insulin as its own mediator will now be discussed.

1.1.3 SUPPORT FOR A DIRECT EFFECT OF INSULIN

1.1.3.1 INTERNALIZATION OF INSULIN

It is a generally accepted fact that receptor bound insulin is internalized in target cells. The biological significance

of the endocytosis of insulin and its receptor is assumed to be the removal of the signal molecule at the plasma membrane and the modulation of the responsiveness of the cell to the hormone. That endocytosed insulin elicits a biological response, and that endocytosis may therefore be part of the mechanism of action of insulin, cannot however be excluded.

The basic steps involved in the internalization and degradation of receptor bound insulin, have been comprehensively reviewed by Sonne, although many of the specific pathways involved have not been established (67). The general model proposed is that receptors binding insulin aggregate into clathrin-coated pits and are internalized to endosomes. The pH within the endosome is acidified through an ATP-dependent proton pump. The acidic environment may facilitate receptor-ligand dissociation. The majority of receptors are then recycled to the plasma membrane for further use (68), or in some cases, degraded (69). It is proposed that most of the endocytosed insulin is cleaved to small products which are released into the medium, although a proportion may be recycled to the plasma membrane and released intact (70). A second pathway of internalization may involve uncoated endocytotic vesicles, where the activated receptor faces the cytoplasmic environment, allowing its possible interaction with intracellular structures (9).

The exact mechanism of insulin degradation, the enzymes involved and the products that are formed are not known. The process of degradation may involve interaction of the endocytotic vesicle with other organelles such as the Golgi apparatus and the lysosome (67).

If insulin itself is responsible for affecting changes in gene expression within the cell, it is necessary to investigate the possibility of an alternative fate for the

internalized hormone. Such a pathway would require that endocytosed insulin be delivered to the nucleus and still retain full biological activity. A degradation product of insulin may be active, however, insulin associated with the nucleus has been isolated and found to be intact (71,72).

Although an alternative to the endosome as a means for the internalization of insulin has not been identified, it is possible that the intracellular routing of the endocytosed hormone may vary, as much evidence has accumulated which shows the translocation of endocytosed insulin to the nucleus of target cells.

1.1.3.2 EVIDENCE FOR THE TRANSLOCATION OF INSULIN TO THE NUCLEUS

1.1.3.2.1 CELL FRACTIONATION STUDIES

Initial data regarding the accumulation of insulin in the nucleus was derived from cell fractionation studies. In early experiments, rats were injected with radioactively labelled hormone (73). This method was later adapted to the treatment of whole cells, either freshly isolated or grown in culture. The results of all these experiments revealed the accumulation of insulin in many intracellular compartments and particularly in the nucleus (73).

A specific example is the study conducted by Goldfine et al., where [^{125}I]insulin was used to determine cellular uptake and nuclear binding in intact lymphocytes. Purified nuclei retained 7% of the total cellular radioactivity. Uptake and binding were inhibited by increasing concentrations of unlabelled hormone. Extraction of the nuclear radioactivity showed it to be intact insulin (71).

1.1.3.2.2 AUTORADIOGRAPHICAL STUDIES

By employing both light and electron microscopic autoradiography with [^{125}I]insulin, Goldfine et al. were able to study the fate of internalized insulin in lymphocytes. These results revealed insulin binding to the plasma membrane, entrance into the cytosol and binding to the endoplasmic reticulum and to the nucleus (71).

Ten minutes following the injection of radiolabelled insulin into the portal vein of fasted rats, insulin was found to accumulate in the endoplasmic reticulum and the nuclear membrane of liver cells. The Golgi apparatus and some small vesicles also contained some of the label (74).

Although data has gathered showing a nuclear location for internalized insulin (71,73,74), there are also contradictory reports. For example, Carpentier et al. (75) found internalized [^{125}I]insulin to be located mainly in the lysosome and Golgi apparatus.

A certain amount of caution is called for in the interpretation of these early studies with [^{125}I]insulin, as the degree of iodination and the biological activity of the labelled hormone, were not well established. There are also inherent difficulties with electron microscopic autoradiography employing labelled insulin. The use of fluorescent derivatives of insulin can overcome these problems.

1.1.3.2.3 STUDIES WITH FLUORESCENT INSULIN DERIVATIVES

Several fluorescent derivatives of insulin have been used in the study of the localization of internalized insulin.

The fluorescent derivatives, fluorescein isothiocyanate-labelled and tetramethylrhodamine-labelled insulin were used to study the intracellular binding of insulin in a variety of cell types (76). The results showed insulin binding sites in the nuclear envelope, nucleoplasm, nucleoli, mitochondria and rough endoplasmic reticulum. The binding fulfilled the requirements of a biologically active hormone receptor, being rapid, reversible, saturable and temperature and pH dependent. Binding was inhibited by an excess of natural insulin, but not by other hormones.

Monomeric ferritin-labelled insulin, a biologically active electron dense marker of occupied insulin receptors, gave further ultrastructural evidence for the accumulation of insulin in the nuclei of intact 3T3-L1 adipocytes by an insulin-receptor mediated process (77). Small amounts of ferritin-insulin were associated with the nucleus after 30 minutes, increasing until 90 minutes. The ferritin-insulin particles were found mainly near the nuclear pores, associated with the periphery of the condensed chromatin. The association of insulin with the periphery of the condensed chromatin may be significant in that this region has been proposed as the site for gene transcription (78).

1.1.3.2.4 IMMUNOELECTRON MICROSCOPIC STUDIES

Immunoelectron microscopic studies of thin sections of H35 hepatoma cells and rat fibroblast cells transfected with human insulin receptor gene, have shown insulin associated with the heterochromatin near nuclear pores (72). Thompson et al. (79) extended these observations by demonstrating that insulin specifically associates with the nuclear matrix, either as intact insulin or as a higher molecular weight complex - probably representing insulin tightly associated with a matrix protein.

1.1.3.3 EVIDENCE SUPPORTING THE EXISTENCE OF A NUCLEAR RECEPTOR FOR INSULIN

The evidence that insulin can accumulate in the nuclear fraction of a cell prompted further experiments to establish the nature of the binding of insulin, as well as the possible existence of a nuclear receptor for the hormone.

1.1.3.3.1 BINDING STUDIES

The binding of [^{125}I]insulin to isolated nuclei has been observed in preparations from rat liver (80,81,82), mouse liver (83) and bovine thyroid (84) tissue. Horvat (80), determined the binding of natural insulin to nuclei by immunofluorescent techniques. Although initial studies on the nuclear binding of insulin lacked evidence that the nuclei used were entirely free of contamination by plasma membranes (85), the studies reported above employed rigorous controls, and the results obtained cannot be accounted for by contamination with plasma membranes.

The nuclear binding sites were found to be localized primarily to the nuclear membrane as insulin binding could be demonstrated using purified nuclear membranes (81,82). No direct binding of insulin to DNA, histones or non-histone proteins has been observed (71).

The binding of [^{125}I]insulin to nuclei was found to be rapid, reversible and directly proportional to the number of nuclei used. The binding was specific for insulin, since unlabelled pro-insulin and desoctapeptide-insulin showed decreased inhibition of binding, in proportion to the decreased biological potencies of these molecules. Also, other peptide hormones were without effect (80). The number of nuclear binding sites is regulated by plasma insulin

concentration in a manner similar to that observed with binding sites on the plasma membrane (83,86).

Vigneri et al. (82), found the same total capacity to bind insulin per mg protein in both plasma and nuclear membranes and suggested that the binding was due to the same protein. However, the binding affinities of insulin to the two receptors and the pH optima for binding, were different. The two binding sites were also found to be immunologically distinct (87). These differences were attributed to differences in the membrane environment surrounding the receptors (82).

1.1.3.3.2 ISOLATION OF THE NUCLEAR RECEPTOR

Speculation that the plasma membrane and nuclear membrane receptor proteins are the same or very similar, was confirmed by the isolation of the nuclear membrane receptor by Wong et al. in 1988 (88). The isolated nuclear membrane receptor is virtually identical to the isolated plasma membrane receptor in molecular weight and binding affinity, as well as displaying the same optimal pH for binding and similar reactivities to polyclonal anti-receptor antibodies. The two receptors also showed very similar competition-inhibition curves when labelled insulin was competed for by unlabelled insulin (50% inhibition at 800pM).

The presence of only one type of insulin receptor molecule in target cells is borne out by the fact that only one insulin receptor gene has been identified (9).

Nuclear receptors for the polypeptide growth factors, nerve growth factor (NGF) and epidermal growth factor (EGF) have also been identified in isolated nuclei (89).

1.1.3.4 BIOLOGICAL EFFECTS OF INSULIN ON ISOLATED NUCLEI

Several studies have indicated specific biological effects of insulin on isolated nuclei.

Schumm and Webb (90) identified the ability of insulin to directly increase mRNA efflux from isolated nuclei. Further experiments by the same pair (91), demonstrated that insulin promoted a specific increase in the release of mRNA for $\alpha_{2\mu}$-globulin from isolated nuclei.

The activity of the nuclear enzyme, nucleoside triphosphatase (NTPase)(E.C. 3.6.1.15) is required for mRNA transport from the nucleus. A proposed mechanism for the increase of mRNA efflux following insulin treatment involves an increase in the activity of the nuclear membrane NTPase via a decrease in its phosphorylation state (92). Insulin added directly to nuclear envelopes isolated from the livers of diabetic rats showed a decrease in the phosphorylation of TCA-precipitable proteins (36) and an increase in the activity of the nuclear membrane NTPase (92).

Miller (93) showed that in *Xenopus laevis* oocytes, insulin injected into the cytoplasm, could stimulate RNA and protein synthesis. RNA synthesis could also be stimulated by applying insulin to the nucleus directly.

Thus, there may be at least two cellular mechanisms of insulin action. Undoubtedly, insulin binds to receptors on the plasma membrane and this interaction leads directly to changes in various membrane functions such as transport, electrical activity and the activity of membrane bound enzymes. The intracellular effects, and particularly those relating to gene expression, cannot however, be ascribed

solely to the binding of insulin at the plasma membrane. A possible explanation of how insulin regulates intracellular events is that insulin itself enters the cell and then mediates its own actions.

1.2 EFFECT OF INSULIN ON THE EXPRESSION OF SPECIFIC GENES

Insulin is able to regulate the expression of many genes. The specific genes studied in this thesis will be discussed in detail, while others will be referred to only briefly.

1.2.1 PYRUVATE KINASE

The L-type isozyme of pyruvate kinase (PK)(ATP:pyruvate O^2-phosphotransferase, EC 2.7.1.40), the predominant PK isozyme in liver, is involved in the regulation of carbohydrate metabolism and is controlled by dietary and hormonal stimuli (94,95). Short term regulation occurs through changes in the phosphorylation state of the enzyme (96,97) and long term regulation through changes in the levels of translatable and hybridizable mRNA for the enzyme (98).

Insulin is the primary hormonal stimulator of overall PK activity and is able to stimulate the expression of the gene in diabetic rat liver (99). Glucagon, via cAMP, has a negative effect on the transcription of the PK gene. Glucocorticoids and thyroid hormones are involved at a post-transcriptional level (100).

The stimulation of transcription by insulin in diabetic rat liver is dependent on protein synthesis since cycloheximide inhibits the induction of transcription. This suggests that a protein intermediate might be involved in the action of insulin on PK gene expression. Transcription of the PK gene

is also inhibited by α-amanitin, indicating that RNA polymerase II is the enzyme responsible for synthesis of the PK mRNA (99).

Dietary control of PK activity is also due to modifications in the amount of total specific mRNAs, probably through a transcription control mechanism, as in the liver of fasted rats PK mRNA is undetectable (101). Vaulont et al. (100) confirmed this theory by showing that transcription of the PK gene is stimulated by a high carbohydrate diet. In contrast, fructose has no effect on the transcription rate of the PK gene, suggesting that increases seen in PK mRNA with fructose treatment are due to inhibition of the degradation of the nuclear RNA species (99).

In rat hepatocytes maintained in primary culture, both insulin and glucose are required together for the regulation of the expression of the PK gene. It is suggested that some form of transcriptional activator derived from carbohydrate metabolism and accumulating in the presence of insulin, is required for the expression of the PK gene. Insulin/glucose also increase the stability of the mRNA product (102).

The L- and R-type isozymes of PK are produced from the same structural gene with the use of different promoters. The R-type is produced exclusively in erythroid cells and is not responsive to insulin (103). Mounting evidence indicates that the 5'-flanking sequence of a gene contains *cis*-acting DNA elements responsible for tissue specific and hormonal regulation of gene expression (104). Thus the region upstream of the cap site of the PK gene may contain the DNA sequence/s allowing differential regulation of gene expression as a result of insulin treatment (103).

1.2.2 TYROSINE AMINOTRANSFERASE

Tyrosine aminotransferase (TAT)(L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) is a gluconeogenic enzyme and is synthesized exclusively by the parenchymal cells of the liver (105). The activity of TAT is controlled by several hormones including insulin, glucagon (via cAMP) and glucocorticoids (50).

Increased TAT activity following glucocorticoid treatment is a consequence of a direct effect on the expression of the TAT gene. Two glucocorticoid response elements (GREs) located 2.5 kb upstream of the transcription initiation site of the TAT gene have been found. These GREs are able to activate transcription in a cooperative manner. The proximal GRE has no inherent capacity to stimulate transcription, but when present in conjunction with the distal GRE, is able to enhance glucocorticoid induction of gene expression (106).

cAMP increases TAT activity through increasing the rate of transcription of the TAT gene (107), as well as increasing the turnover rate of TAT mRNA (108). The mechanism of this regulation is not known. No cAMP responsive element has, as yet, been found in the 5'-flanking sequence of the TAT gene.

In contrast to the well documented induction of TAT activity at the transcriptional level by glucocoticoids and cAMP, the mechanism of the induction of TAT activity by insulin is still not clear.

Studies using adrenalectomized rats, showed that insulin caused an increase in the amount of TAT mRNA in the liver (109). This increase in TAT mRNA was later proven to be the result of an increased transcription rate of the TAT gene (110). In HTC hepatoma cells, the increase in TAT mRNA has been ascribed to the fact that insulin selectively slows the

degradation rate of TAT mRNA (111). In a recent study using the Fao hepatoma cell line, Crettaz et al., have shown that insulin stimulates TAT activity by at least two distinct mechanisms. Insulin alone increases TAT activity by increasing TAT mRNA due to increases in the rate of transcription of the TAT gene. However, in the presence of glucocorticoids, insulin increases TAT activity, but has no additional effect on the level of TAT mRNA. These findings suggest a transcriptional, as well as a posttranscriptional, site of insulin action (112).

The mechanisms of insulin action involved in the regulation of TAT activity appear complex and still remain to be clarified. A binding site for insulin on the TAT gene, that is in some way similar to a GRE, has not been found.

1.2.3 PHOSPHOENOLPYRUVATE CARBOXYKINASE

Cytosolic phosphoenolpyruvate carboxykinase (PEPCK) (GTP:oxaloacetate carboxylyase, E.C. 4.1.1.32) is the rate limiting enzyme in hepatic gluconeogenesis and its activity is subject to multihormonal regulation. (See review by Granner et al. (113)).

Glucocorticoids and cAMP increase the rate of hepatic gluconeogenesis by increasing the synthesis of PEPCK (114). This increased synthesis is the result of increased levels of PEPCK mRNA caused by an increase in the rate of transcription of the PEPCK gene (49,115,116). Glucocorticoids also increase the stability of the PEPCK mRNA product (117).

The effects of insulin on gluconeogenesis and PEPCK activity oppose those of glucocorticoids and cAMP. Insulin inhibits PEPCK gene transcription (118), thus resulting in the

observed reduced levels of PEPCK mRNA (115) and the decreased PEPCK enzyme synthesis (114). Insulin decreases the levels of PEPCK mRNA in rat liver and kidney (115), primary cultures of rat hepatocytes (119), as well as in rat hepatoma cells (118).

The combined result of insulin, glucocorticoid and cAMP administration to the H4IIE rat hepatoma cell line is a decrease in the transcription of the PEPCK gene. Insulin thus exhibits the dominant effect (116). Insulin decreases PEPCK mRNA and PEPCK gene transcription by a mechanism that is independent of cAMP (120).

In contrast to the above results obtained in a hepatoma cell line, the combined effects of hormone treatment in primary rat hepatocyte cultures show glucagon to be the dominant hormone for the expression of the PEPCK gene (119). This discrepancy could be due to the dedifferentiation of the hepatoma cell lines and indicates that caution should be exercised in generalizing results obtained in experiments using whole animals, primary cultures or stable cell lines.

The multihormonal regulation of the PEPCK gene is via *cis*-acting response elements adjacent to the transcription start site of the PEPCK gene (121,122).

Initially, two glucocorticoid response elements and a single cAMP regulatory element were identified in the promoter region of the PEPCK gene (123,124). Subsequently, additional multiple protein binding domains have been identified in the PEPCK promoter using DNase I footprinting studies with nuclear extracts from various rat tissues. These include three more sites to which the CRE-binding protein binds (48). A cAMP-dependent nuclear factor has been detected that interacts with the cAMP-responsive element of the PEPCK gene

and is thus a possible mediator of the transcriptional control exerted by cAMP (47).

Transiently expressed chimeric PEPCK genes are regulated in the same way as the endogenous PEPCK gene in H4IIE hepatoma cells - glucocorticoids and cAMP stimulate gene expression and these effects are opposed by insulin (122). In contrast, a PEPCK-thymidine kinase or PEPCK-neo, gene containing regions of the PEPCK promoter, was not responsive to insulin when stably transfected into FTO-2B cells (121). However, retroviral transfer of chimeric genes containing the PEPCK promoter has localized the insulin regulatory element to within -547 to +73 bp of the promoter regulatory region (125).

The factors that interact with this sequence in an insulin-responsive way, have not been identified. As yet, only one protein that binds to a fragment of DNA following insulin treatment has been described. This is a protein associated with the insulin and serum induction of the c-fos gene (33).

1.2.4 c-*fos*

The proto-oncogene c-*fos* is induced rapidly by a variety of mitogenic and differentiation specific agents and its expression is of import in cell proliferation and differentiation. How c-*fos* is targeted by activated signal transduction pathways for such an efficient transcriptional response is thought to lie in the nature of the promoter and in the transcription factors that interact with it (126).

Several factors involved in the serum responsiveness of c-*fos* have been identified including p67, the serum response factor and p62, a protein that forms a ternary complex with the serum response element and p67 (127). The serum-induced

transcription of c-*fos* is under negative feedback regulation mediated by the fos protein. This feedback mechanism is only operational when c-*fos* is induced by serum. c-*fos* serum-inducible promoter elements are thus involved (128).

Characterization of the protein components involved in the formation of a transcriptional complex is an important step towards understanding gene regulation. There is evidence to suggest that the Fos protein, together with the protein products of other early response genes such as c-*jun*, may be involved in mediating the hormone/growth factor-induced transcription of specific genes through *trans* activation of the particular genes involved. Also, Fos contains sequences that might be involved in the recognition of specific promoter elements (129).

Insulin administration to 3T3-L1 fibroblasts and adipocytes causes a rapid and transient increase in the level of c-*fos* expression (60). A similar stimulation was shown in H35 rat hepatoma cells (61). Pretreatment of the cells with phorbol esters to deplete them of protein kinase C decreased, but did not eliminate, the ability of insulin to stimulate an increase in c-*fos* mRNA. Two independent pathways for the stimulation of c-*fos* by insulin may therefore exist. The normal insulin receptor is required for the insulin induction of c-*fos* and four bases (-320 to -299) of the sequence corresponding to the serum response element (SRE) are required for induction of c-*fos* transcription by both insulin and phorbol ester (33). The responsive cells were also found to contain a nuclear factor, thought to be a protein, which binds specifically to this sequence of the c-*fos* gene. This factor along with p67 and p62, may play a role in the regulation of c-*fos* expression by insulin and phorbol esters (33).

1.2.5 OTHER PROTEINS INFLUENCED BY INSULIN AT THE LEVEL OF GENE EXPRESSION

The concentrations of many mRNAs for insulin-regulated proteins have been quantitated and responses to insulin observed. In several instances these changes have been identified as being regulated at the level of transcription. Some *cis*-acting regulatory elements have been identified.

Aside from insulin's effects on PK, TAT, PEPCK and c-*fos*, insulin also regulates the levels of other mRNAs in the liver. These include the mRNAs for malic enzyme (130), fatty acid synthetase (131), apolipoprotein AI and AIV (132), glucose-6-phosphate dehydrogenase (133), albumin (134), the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (135), the glucose transporter (136), carbamoyl-phosphate synthetase (137)(decreased) and $\alpha_{2\mu}$-globulin (138). Insulin controls the expression of the genes for tryptophan 2,3-dioxygenase (139)(inhibits induction), gene 33 which codes for an unknown 53 kDa protein (140,141), glucokinase (142,143) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(144), in the liver. The inductive effect of insulin on human GAPDH expression is mediated through *cis*-acting sequences located between -487 and +20 of the human GAPDH gene (144).

Insulin also controls the level of expression of many genes in other tissues. In the pancreas, insulin controls amylase synthesis at the level of transcription. Experiments with a hybrid amylase/CAT gene in transgenic mice localized the determinants of pancreas specificity and insulin dependence, to the region between -208 and +19 of the mouse pancreatic amylase gene (145). Insulin also decreases the mRNA levels of its own receptor in pancreatic acinar cells (146).

Thyroglobulin gene transcription is increased in FRTL-5 thyroid cells following insulin treatment (147).

Insulin also plays a role in regulating the accumulation of casein mRNA in mouse mammary epithelial cells (148), myosin heavy chain and creatine phosphokinase mRNAs in rat skeletal muscle (149), δ-crystallin mRNA in cultures of chicken lens epithelial cells (150) and an amplified glutamine synthetase gene in Chinese hamster ovary cells (151).

Insulin is the major hormone regulating endogenous fatty acid synthesis and esterification into triglycerides. Insulin inhibits the transcription of the adipsin gene and stimulates the transcription of the glycerol-3-phosphate dehydrogenase (GPDH) gene which is coupled to increased stability of the GPDH mRNA (152). Insulin also causes the accumulation of lipoprotein lipase mRNA (153).

In GH_3 pituitary cells, insulin suppresses the expression of the growth hormone gene. *cis*-Acting regulatory sequences in the 5'-flanking region of the gene are a requirement for the response to the insulin signal (154). Other studies showed that insulin stimulates prolactin mRNA accumulation (155) and transcription (156) in GH_3 cells. The latter study found growth hormone to be unresponsive to added insulin, and attributed these discrepancies to either clonal variations in the cell line or the fact that the first experiments (154), were conducted in the presence of serum.

The changes in mRNA levels induced by insulin could result from an altered rate of transcription, processing of transcripts, transport of mRNA from the nucleus to the cytoplasm, or a change in the rate of mRNA turnover. The results of the studies quoted have indicated that although regulation may occur at more than one level, a definite effect of insulin on transcription is observed.

It is the intention of this thesis to investigate whether insulin itself is capable of acting directly on the nucleus to regulate gene expression.

1.3 THE RAT HEPATOMA MODEL

Insulin plays a major role in regulating metabolism in the liver. It controls the synthesis of the key enzymes involved in gluconeogenesis and glycolysis. This synthesis is controlled at the level of the expression of the genes coding for these enzymes and the effects are either stimulatory or inhibitory. The liver thus represents a suitable tissue for the study of the mechanism whereby insulin modulates gene expression.

The H4IIE rat hepatoma cell line was selected as the model of choice in this study of insulin action. These cells were adapted to culture from the Reuber H35 transplantable hepatoma by Pitot et al. in 1964 (157). H4IIE cells have retained most of the characteristics of normal liver tissue cells and have normal insulin receptors (158).

Although it may be argued that the immortalizing of a cell alters it such that it no longer represents a true image of the normal cell, the advantages of using a clonal cell line are considerable. The administration of insulin to whole animals results in insulin-induced hypoglycemia which alters the state of the cell through changes in the availability of various substrates, and results in the release of other hormones to counter these effects. Diet and exercise also play a role. A tissue culture system allows the study of the effects of insulin in isolation, and is particularly useful in the study of the long term effects of the hormone on gene expression.

CHAPTER TWO

REGULATION OF TRANSCRIPTION BY INSULIN IN H4IIE HEPATOMA CELLS

As has been discussed in the introduction to this thesis, the H4IIE hepatoma cell line was selected as a model system for the study of the mechanism by which insulin regulates gene expression. However, much of the reported data regarding the effect of insulin on the transcription of specific genes has been the result of experiments conducted using rat liver (109), primary hepatocyte cultures (102) or hepatoma cell lines other than the H4IIE cell line kept in this laboratory (112).

As this H4IIE hepatoma has its own peculiar characteristics (157), the experiments that follow were conducted in order to verify that the transcriptional control exerted by insulin in these other systems, is also operative in the H4IIE hepatoma cell line.

The cells were therefore exposed to insulin at different concentrations for varying time intervals. The changes in the total transcriptional activity of the cells, as well as in the levels of mRNAs for specific genes were monitored.

2.1 EFFECT OF INSULIN ON TOTAL TRANSCRIPTIONAL ACTIVITY

The effect of insulin on the total transcriptional activity of H4IIE hepatomas was determined by measuring the incorporation of radiolabelled uridine into the cells in the presence and absence of insulin.

2.1.1 TIME COURSE OF [^{3}H]URIDINE INCORPORATION

H4IIE hepatoma cells were plated in 4,5 cm^2 multi-well dishes and grown to 80-100% confluency using the routine culturing procedures described in 8.2.1.1. The cells were transferred from growth in normal medium to growth in serum-free medium 20 h prior to the addition of insulin in fresh serum-free medium. The cells were incubated for 0, 0,5, 1, 2, 3 or 6 h with 1 nM insulin. [^{3}H]Uridine was added at 2 µCi/ml together with the hormone. Following incubation, the cells were rinsed with Dulbecco's phosphate buffered saline without calcium and magnesium (DPBSA), to remove all excess radioactivity, and then detached from the flasks (8.2.1.2). The incorporation of [^{3}H]uridine into TCA-precipitable material was determined by precipitation onto glass-fibre filters and counting in a liquid scintillation counter. A detailed experimental protocol is given in 8.4.1.1.

After a lag phase of 30 min in both the insulin treated and untreated cultures, [^{3}H]uridine incorporation increased with time. Following 3 h of insulin administration, the level of incorporation in the insulin treated cultures was 73 dpm/µg DNA as opposed to 47 dpm/µg DNA in the untreated control cultures, representing a 1,55-fold increase. The time course of the stimulation of [^{3}H]uridine incorporation by insulin, is detailed in Figure 2.1.

2.1.2 DETERMINATION OF OPTIMUM INSULIN CONCENTRATION

Serum-starved H4IIE hepatoma cells were treated with insulin at concentrations ranging from 0,1 nM to 100 nM for 3 h, according to the experimental protocol described in 2.1.1 above, and the incorporation of [^{3}H]uridine into TCA-precipitable material determined as described in 8.4.1.1.

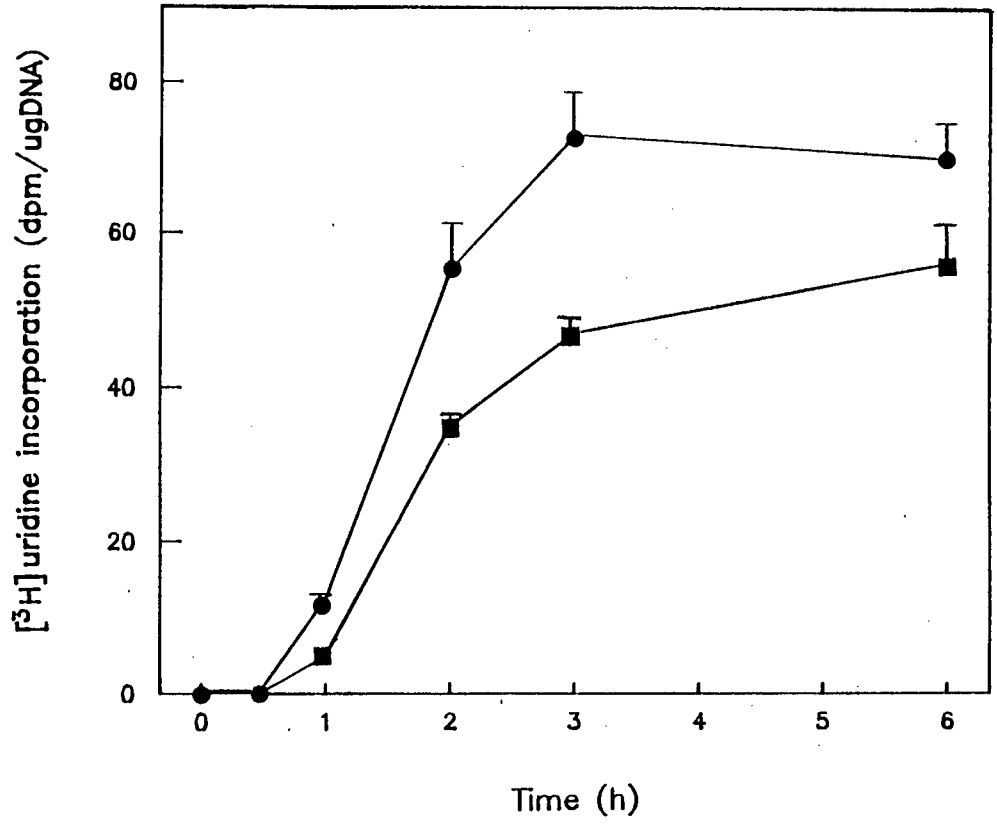

Figure 2.1 Stimulation of [³H]uridine incorporation into H4IIE hepatoma cells as a function of time. Serum starved cells were incubated with (●) or without (■) 1 nM insulin for 0, 0,5, 1, 2, 3 or 6 h. Each point is the mean of duplicate results from two separate experiments ± SEM.

The incorporation of [^{3}H]uridine by the cells was dependent on the insulin concentration in the growth medium. Maximal stimulation occurred at 5-10 nM and half-maximal stimulation at 0,2-0,5 nM insulin, under the described conditions. High concentrations of 100 nM insulin, were found to elicit a reduced response. The incorporation at 10 nM insulin represented an increase of 1,7-fold above the control.

The relationship between insulin concentration and [^{3}H]uridine incorporation, is shown in Figure 2.2.

2.2 EFFECT OF INSULIN ON SPECIFIC mRNA LEVELS

The effect of insulin on the production of mRNA coding for certain proteins and liver specific enzymes was examined in H4IIE hepatoma cells. As has been discussed in the introduction to this thesis, insulin is able to cause an increase in the transcriptional activity of certain genes while simultaneously decreasing the activity of others.

In this study, the specific probes used to monitor the effect of insulin on mRNA levels, were the labelled cDNAs to genes known to be under the transcriptional control of insulin:

Pyruvate kinase (PK) - Positive control (100,102),
Tyrosine aminotransferase (TAT) - Positive control (112),
Phosphoenolpyruvate carboxykinase (PEPCK) - Negative control (116,118),
c-*fos* - Positive control (33)

α-Tubulin is a house-keeping gene and was used as a neutral control in all experiments (142).

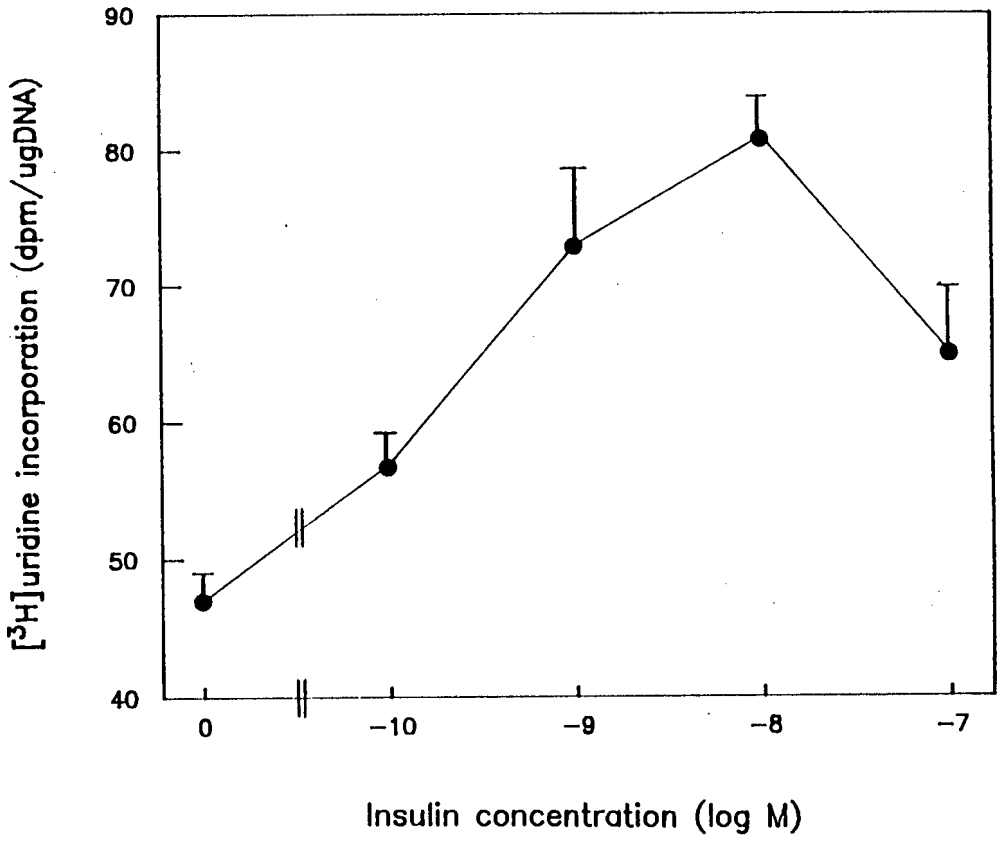

Figure 2.2 Stimulation of [^{3}H]uridine incorporation into H4IIE hepatoma cells as a function of insulin concentration in the growth medium. Serum starved cells were incubated with insulin over a concentration range of 0,1 nM to 100 nM for 3 h. Each point is the mean of duplicate results from two separate experiments ± SEM.

The experimental procedures applied to study specific mRNA accumulation, namely: the treatment of the H4IIE cells with insulin; the isolation of the RNA; and the hybridization and detection techniques, are all described in detail in "Materials and methods" (8.4.1.2).

Briefly, previously serum-starved cells were incubated with varying concentrations of insulin in serum-free medium for a range of time intervals. The concentration dependence of the response of PK, TAT or PEPCK was monitored after 3 h of insulin treatment (112,118). c-*fos* Induction was monitored after 30 min of insulin treatment, as it is rapidly induced in all systems studied to date (61). The time dependence of the responses was determined at 10 nM insulin, the concentration that resulted in maximal incorporation of [^{3}H]uridine by the cells (see 2.2).

Immediately following incubation with insulin, the cells were removed from the flasks, pelleted and guanidinium thiocyanate stock solution was added to stop any further reaction. The total cellular RNA was isolated from the cells by the guanidinium thiocyanate/CsCl gradient ultracentrifugation method of Chirgwin et al. (159) (8.3.2). Following ethanol precipitation of the RNA, the concentration of the resuspended total RNA product was determined by measuring the optical density (OD) at 260nm. An OD_{260} to OD_{280} ratio of greater than 2:1 was always ensured.

Equal amounts of total RNA for each variation in insulin concentration were denatured using glyoxal, dot blotted on a nylon membrane, cross-linked to the filter by UV irradiation, and hybridized according to standard procedures using cDNA probes labelled by nick-translation. pBR322 was also labelled and used as a probe, but did not hybridize at all to the isolated H4IIE hepatoma RNA. The results of the experiments are expressed as "arbitrary units", which

reflect changes in the densitometer readings of the autoradiograms obtained for each sample (8.4.2.1). The results of the addition of insulin to the cells as outlined above, will be described in detail for each individual cDNA probe used.

2.2.1 EFFECT OF INSULIN ON THE LEVELS OF PK mRNA

The addition of insulin to H4IIE hepatomas resulted in a 3-fold increase in the amount of PK mRNA in the cells. This was determined by using a labelled 1,8 kb cDNA probe to the 5'end of the gene (161). Maximal stimulation by insulin occurred after 1 h of treatment and at a concentration of 1 nM. Figure 2.3 illustrates the stimulation of PK gene expression by insulin.

The effect of insulin on PK mRNA levels has previously been observed in studies with whole animals or primary hepatocytes. The mechanism of this induction was found to be at the level of transcription (98,99,102).

The levels of α-tubulin mRNA remained constant following insulin administration as determined by using the labelled full-length 2,1 kb α-tubulin cDNA, pHFαT-1 (8.1.2). The result of a typical dot blot using α-tubulin as probe, is shown in Figure 2.4.

2.2.2 EFFECT OF INSULIN ON THE LEVELS OF TAT mRNA

The results of the experiment showed that TAT mRNA levels in starved H4IIE hepatoma cells are increased in the presence of insulin.

When the cells were treated with insulin for 3 h, the effect

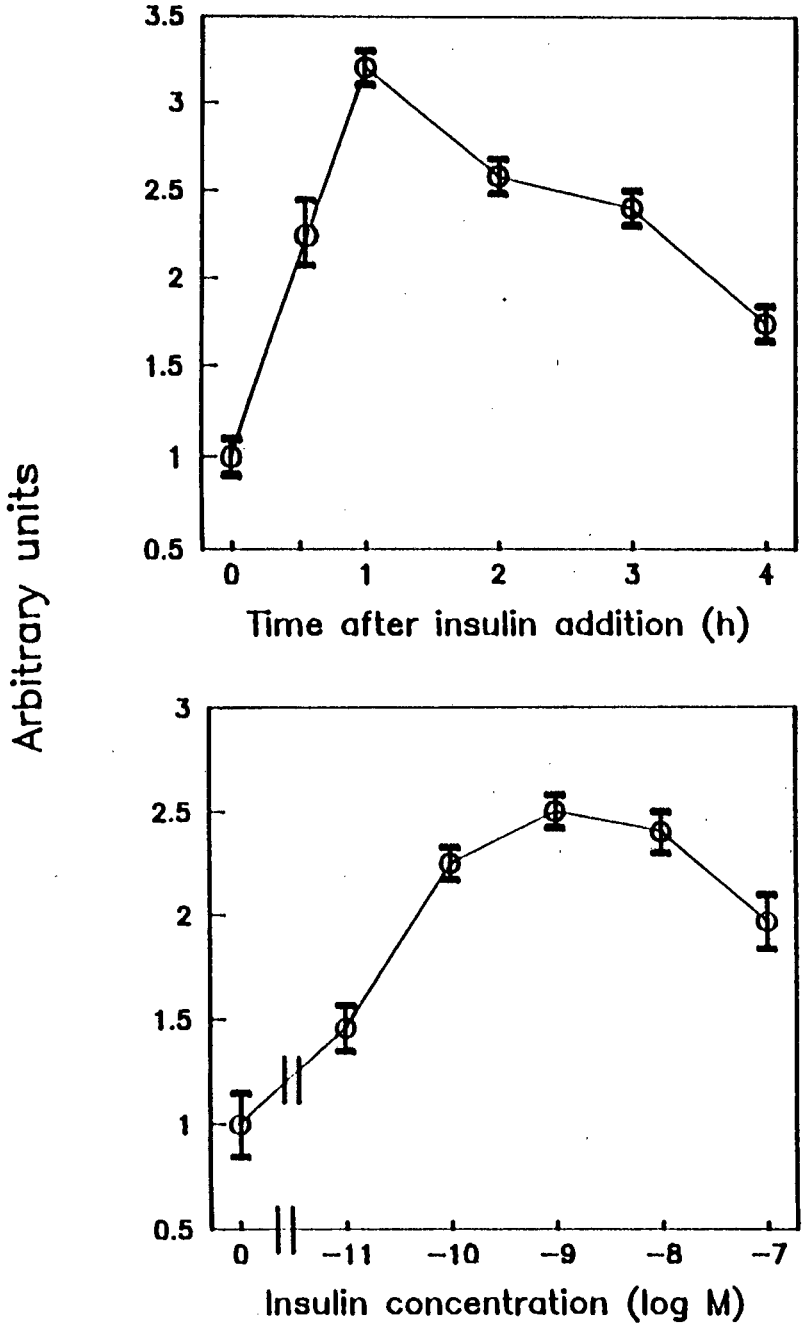

Figure 2.3 Stimulation of PK mRNA production by insulin. Cells were incubated with insulin at 10 nM for 0-4 h to determine the time dependence, and at 0,01 nM to 100 nM insulin for 3 h to determine the concentration dependence, of the response. Each value is the mean of three determinations ± SEM.

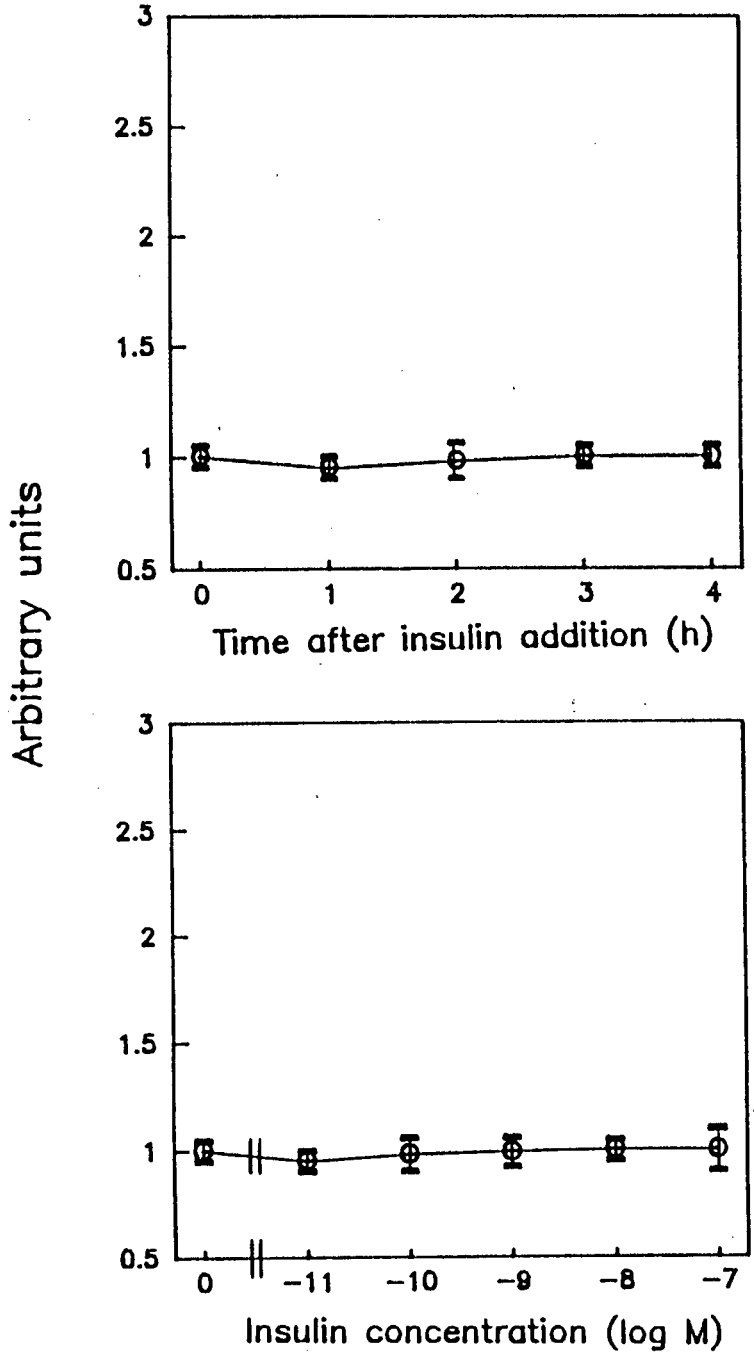

Figure 2.4 Effect of insulin on α-tubulin mRNA production. Cells were incubated with insulin at 10 nM for 0-4 h to determine the time dependence and at 0,01 nM to 100 nM insulin for 3 h to determine the concentration dependence, of the response. Each value is the mean of three determinations ± SEM.

was maximal at 0,1 nM insulin and half-maximal at approximately 0,02 nM. The levels of TAT mRNA increased 2,7-fold above that of the control without insulin. The time dependence of the response was maximal after 2 h of insulin treatment. The probe used in the hybridization studies was the 0,60 kb TAT cDNA clone, pcTAT-3 constructed by Scherer et al. (160)(8.1.2).

The effects of time and concentration of insulin addition on TAT mRNA production are depicted graphically in Figure 2.5.

The increased amount of TAT mRNA in the cells after insulin treatment has been shown by Crettaz et al. (112), using Fao hepatoma cells, to be the result of an increase in the rate of transcription of the TAT gene.

α-Tubulin mRNA remained constant following insulin administration. A typical result is shown in Figure 2.4.

2.2.3 EFFECT OF INSULIN ON THE LEVELS OF PEPCK mRNA

Insulin inhibited the production of PEPCK mRNA in H4IIE hepatomas. The decrease was concentration dependent, being almost undetectable at 10 nM insulin after 4 h of treatment.

These specific values for time and concentration dependence differ from those reported in the literature, where PEPCK mRNA in H4IIE hepatomas decreased to minimum levels after 2 h at 1 nM insulin. However, the overall trend is in agreement (118). These differences could be due to the specific state of the cells at the time of the experiment.

The PEPCK probe used, pPCK2, is a cloned 0,59 kb cDNA to the PEPCK gene and was isolated by Cimbala et al. (115)(see 8.1.2). The results are shown in Figure 2.6.

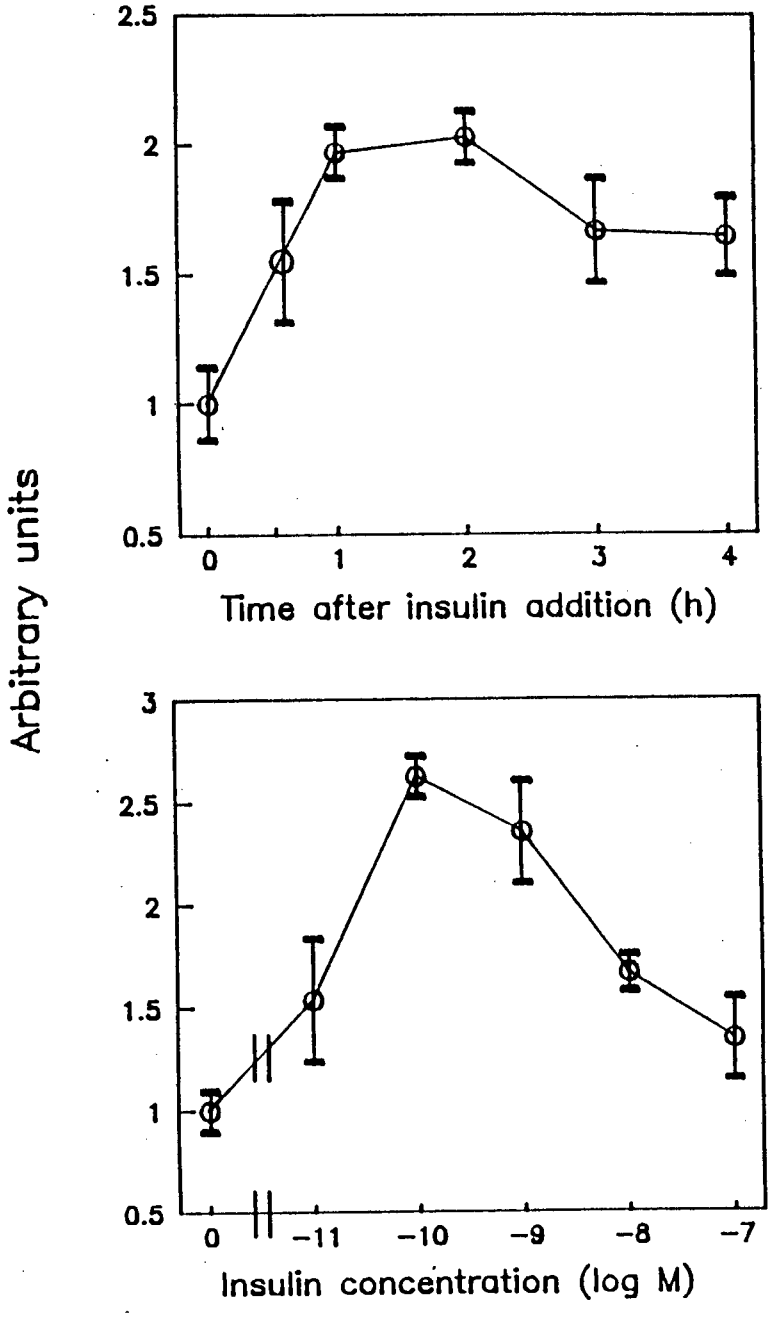

Figure 2.5 Stimulation of TAT mRNA production by insulin. Cells were incubated with insulin at 10 nM for 0-4 h to determine the time dependence, and at 0,01 nM to 100 nM insulin for 3 h to determine the concentration dependence, of the response. Each value is the mean of three determinations ± SEM.

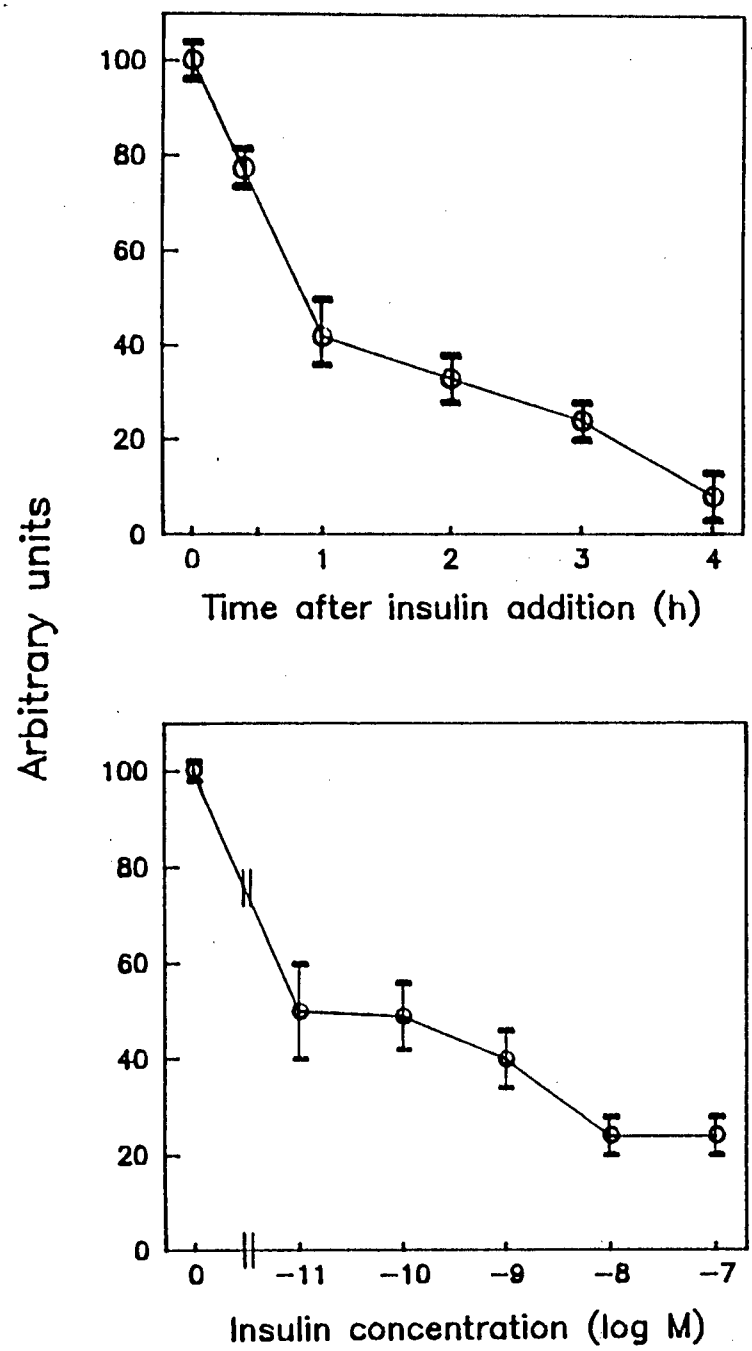

Figure 2.6 Inhibition of PEPCK mRNA production by insulin. Cells were incubated with insulin at 10 nM for 0-4 h to determine the time dependence, and at 0,01 nM to 100 nM insulin for 3 h to determine the concentration dependence, of the response. Each value is the mean of three determinations ± SEM.

Once again the levels of α-tubulin mRNA were used as a control and remained constant. Refer to Figure 2.4.

2.2.4 EFFECT OF INSULIN ON THE LEVELS OF c-*fos* mRNA

The stimulation of c-*fos* gene expression was found to be transient. A rapid increase in c-*fos* mRNA occurred within 30 min of insulin treatment, being maximal at 1 h and returning to basal levels within 2 h. This stimulation was effective in the concentration range of 0,1-100 nM, being maximal at 0,1 nM insulin. Thus c-*fos* was induced at very low concentrations of insulin and did not exhibit the same degree of concentration dependence as that observed for the other genes studied. The induction of c-*fos* is shown in Figure 2.7.

The c-*fos* cDNA was cloned by te Water Naude (unpublished result) and is a 1,2 kB fragment of the original pc-fos(human)-1 cDNA cloned by van Straaten et al. (162). A similar result of insulin induction of c-*fos* activity has recently been reported using the H-35 rat hepatoma cell line (61).

No increase in the levels of α-tubulin mRNA were found. Refer to Figure 2.4.

2.3 EFFECT OF INSULIN ON THE LEVELS OF SPECIFIC mRNAs IN CELLS MAINTAINED IN INSULIN-FREE SERUM

Cells maintained in serum-free medium prior to an experiment are deprived of the nutrients and growth factors that would be available to a cell *in vivo*. This allows the observation of responses directly due to insulin without the possibility that other hormones may be involved and also enhances the

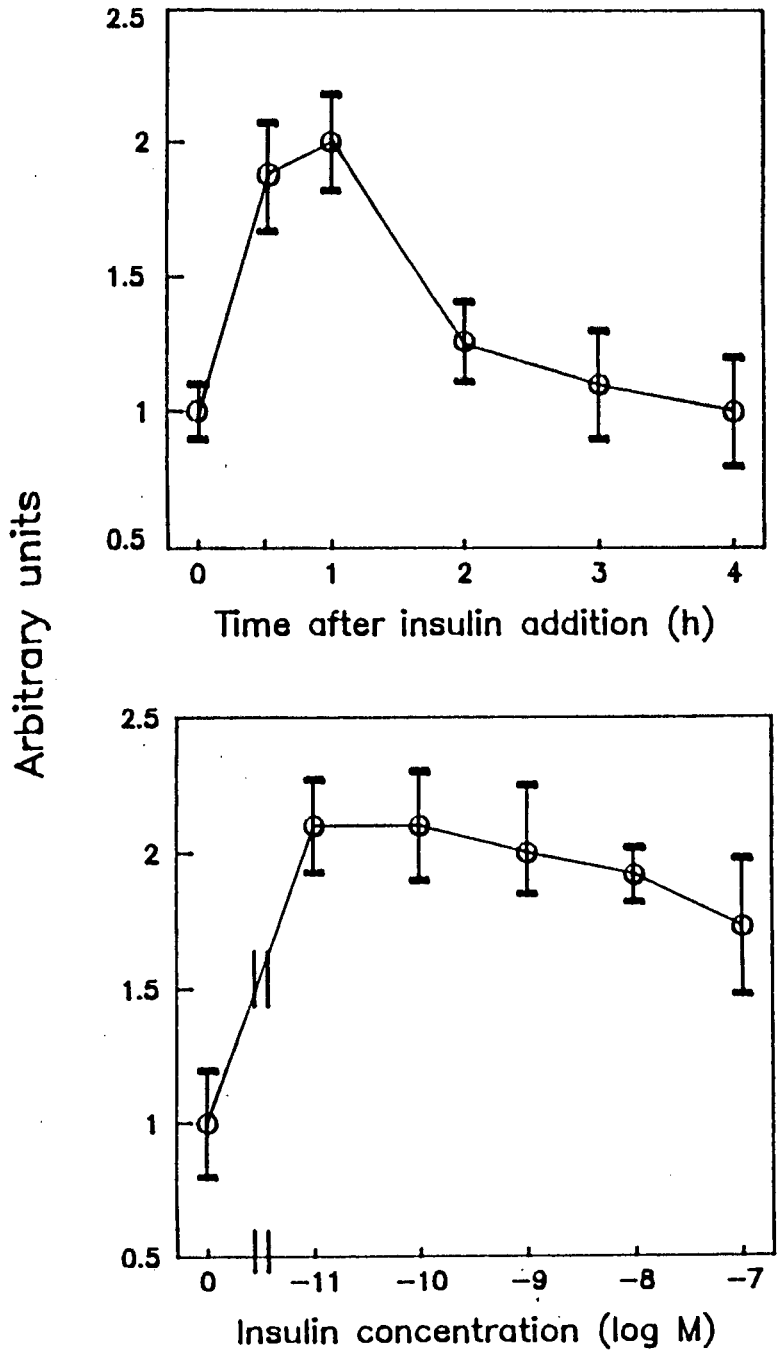

Figure 2.7 Stimulation of c-*fos* mRNA production by insulin. Cells were incubated with insulin at 10 nM for 0-4 h to determine the time dependence, and at 0,01 nM to 100 nM insulin for 30 min to determine the concentration dependence, of the response. Each value is the mean of three determinations ± SEM.

response of the cells to insulin. The response of starved cells to added insulin may, however, not be a good reflection of a natural response to the hormone. In order to retain the cells in the most "normal" environment possible, and yet be able to distinguish responses directly due to insulin, the removal of insulin from the fetal calf serum was investigated. The effect of insulin on the levels of specific mRNAs, isolated from cells maintained in such serum, were determined.

2.3.1 PREPARATION OF INSULIN-FREE SERUM

Insulin was removed from the fetal calf serum used for the routine maintenance of the cells in culture by affinity chromatography. The serum was passed over an anti-insulin antibody affinity column and the initial and final insulin concentrations were determined.

The affinity column was prepared as described in detail in 8.2.2.1. The anti-insulin antibody was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. Determination of the ratio of protein-bound to protein-free, yielded a coupling efficiency of 99%. The binding capacity was determined by adding increasing known amounts of insulin to the column together with a tracer amount of ^{125}I-insulin (163). By this method, the column was capable of binding 99% of the expected maximum of 1 µg insulin, of which 96% could be recovered from the column by elution with glycine pH 2,5. The amount of insulin removed by the column from insulin standards was also 98-100%, as determined by radioimmunoassay (RIA) (8.2.2.2). The insulin was loaded in phosphate buffered saline (PBS), as well as in serum, with no difference in the overall binding efficiency.

The concentration of insulin present in fetal calf serum before and after passage over the column was also determined by RIA. The levels of insulin present in the serum were very low, being less than the accurate limit of detection of the assay. However, triplicate readings gave reproducible results and are therefore reported in Table 2.1.

Table 2.1 Insulin concentrations in serum with or without the addition of extra insulin, before and after passing sample over anti-insulin antibody affinity column. The insulin concentrations were determined in triplicate by RIA. The removal of insulin from samples loaded in phosphate buffered saline is also shown.

Sample	Insulin concentration Before	After	% Insulin removed
Fetal calf serum	16 pM*	0 pM*	100
Insulin in serum	1 nM	10 pM*	99
	10 nM	90 pM	99,1
Insulin in buffer	1 nM	10 pM*	99
	10 nM	110 pM	98,9

* Values are below 70 pM, the accurate limit of detection of the assay.

2.3.2 EFFECTS OF INSULIN ON PK, TAT AND PEPCK mRNA LEVELS

Cells were changed from growth in normal medium to growth in medium containing insulin-free serum 20 h prior to the addition of insulin. A range of insulin concentrations was added to the cells in fresh insulin-free serum medium. The

effects of insulin treatment on the levels of PK, TAT and PEPCK mRNAs were determined as described for starved cells in 2.2 and 8.4.1.2.

The levels of the different mRNAs in the cells with respect to variations in insulin concentration, as well as the time course of response to insulin, were the same as those reported for starved H4IIE cells in Figures 2.3 to 2.5. The results are given in Table 2.2.

Table 2.2 Comparative results of the effect of insulin on mRNAs for PK, TAT and PEPCK when the cells are maintained in serum-free medium (SF) or in medium containing insulin-free serum (S-ins), with respect to the time and concentration of exposure to insulin. Values represent arbitrary units based on densitometer readings.

Insulin Concentration	PK		TAT		PEPCK	
	SF	S-ins	SF	S-ins	SF	S-ins
Blank	1,00	1,00	1,00	1,00	100	100
10 pM	1,46	1,40	1,54	1,84	50	57
100 pM	2,25	2,33	2,63	2,53	49	45
1 nM	2,5	2,6	2,36	2,15	40	40
10 nM	2,4	2,45	1,67	1,58	24	28
100 nM	1,97	1,8	1,35	1,56	23	20
Time						
0 h	1,00	1,00	1,00	1,00	100	100
0,5 h	2,4	2,6	1,55	1,24	75	73
1 h	3,2	3,0	1,97	1,87	42	50
2 h	2,58	2,58	2,03	2,00	33	35
3 h	2,4	2,45	1,67	1,58	24	28
4 h	1,74	2,0	1,65	1,63	8	15

2.4 CONCLUSIONS

Insulin has the ability to cause an increase in total RNA production in the H4IIE hepatoma cell line, as well as to control the levels of mRNAs for specific genes. Although the cumulative effect of insulin regulation results in increased total cellular RNA synthesis, insulin is able to specifically increase the levels of PK, TAT and c-*fos* mRNA, decrease those for PEPCK mRNA and leave the levels of α-tubulin mRNA unchanged.

The changes in mRNA levels could be due to changes in the transcriptional activity of the genes, alterations in the stability of the mRNA product or to changes in the degradation of the mRNA. The levels of the mRNAs studied, namely PK, TAT, PEPCK and c-*fos* , have been shown by other research workers, to be regulated at the level of the transcription of the gene (102,112,118,33).

The response to insulin is concentration dependent and is maximal in the 1 nM concentration range. The peak of induction by insulin occurs most often after approximately 2 h of exposure of the cells to insulin. The induction of c-*fos* is transient, being induced after 1 h and returning to base level after 2 h of insulin treatment.

Cells in medium containing serum void of insulin, display the same response to added insulin as do cells starved of serum. Serum starvation and the absence of possible permissive factors do not alter insulin-induced changes in mRNA levels. Studies on the long term effects of insulin on the cells, may thus be conducted without hesitation, on cells kept in serum-free medium prior to the experiment.

2.5 DISCUSSION

The stimulatory effect of insulin on total RNA synthesis has been reported by other investigators in rat liver and Hep G-2 hepatoma cells (164-166), as well as in *Xenopus laevis* oocytes (93). The limited nature of the increase found in total [^{3}H]uridine incorporation following insulin treatment was expected, in the light of the fact that insulin causes the down regulation of many genes, as well as having a stimulatory effect on others.

The decrease in PEPCK mRNA is in keeping with that described in the literature for this particular hepatoma cell line (118). Stimulation by insulin of PK, TAT or c-*fos* mRNA production has not been previously reported for the H4IIE cell line. However, increased levels of mRNAs following insulin treatment have been reported for TAT in Fao hepatoma cells (112) and for c-*fos* in H-35 hepatoma cells (61). An increase in the levels of PK mRNA has previously been observed in hepatocytes maintained in primary culture (102), however, no response to insulin was found in the MH_1C_1, Fao or Faza hepatoma cell lines (167). This lack of response could be due to the degree of dedifferentiation of the cell lines resulting in differences in the specific genes expressed. The H4IIE cell line exhibits insulin responsiveness in accordance with liver tissue. Thus, these cells provide a suitable model for the further study of the mechanism of the regulation of gene expression by insulin.

The decreased transcriptional activity of the cells following treatment with high concentrations of insulin could be ascribed to the well documented fact that exposure of cells to high concentrations of insulin causes a down-regulation of insulin receptors (168). The transcriptional response is mediated via the plasma membrane receptor (11). A decreased receptor number could diminish the signal to the

genes and decrease the transcriptional activity of the cells. However, the effects of receptor down-regulation in cells that are derived from the Reuber hepatoma are minimized, as down-regulation is accompanied by an increase in receptor affinity (169). Alternatively, some unknown intracellular mechanism of regulation may occur, perhaps at the level of the nuclear membrane or alternatively at the gene itself.

The function of the Fos protein is still unclear, but seems to form part of the transcription initiation complex (126). The rapid induction of c-*fos* and production of Fos, could be in order to facilitate the effect of insulin on the transcription of the liver specific enzymes. The lack of a pronounced concentration effect in the case of c-*fos* could be due to the fact that the Fos protein down-regulates the activity of c-*fos* and the cell thus has an inherent control system preventing the over-expression of the gene (128).

CHAPTER THREE

ISOLATION OF H4IIE HEPATOMA NUCLEI

Nuclei suitable for the study of the effects of insulin added directly to an *in vitro* transcription system need to fulfill four basic requirements:

Be pure;
Have intact nuclear membranes;
Be isolated in high yield;
Retain transcriptional activity.

Existing methods for the isolation of nuclei from tissue culture cells can be divided into three main categories:

Those employing the addition of a detergent;
Those using osmotic shock;
Those using homogenization only.

These methods were evaluated on the basis of the stated requirements, and found to be unsuitable for the following reasons:

Pure nuclei are isolated in high yield from cells exposed to detergents, but these nuclei do not have intact nuclear membranes (170). Intact nuclear membranes were required in order to ensure that the purified nuclei resemble *in vivo* nuclei as closely as possible. Any interaction of insulin with a nuclear membrane component, or any insulin-mediated nuclear membrane effect, would therefore not be affected adversely by the isolation process.

Osmotic shock results in a high percentage of nuclei that are impure and retain tags of intracellular membranes. Homogenization only, results in pure nuclei with intact nuclear membranes, but in low yield, as the extensive homogenization required to lyse the cells results in increasing destruction of nuclei already released (171).

A method had been developed in this laboratory for the isolation of nuclei from Friend erythroleukemia cells using melittin, the lytic polypeptide from bee venom (172).

The structure of melittin resembles that of an ionic detergent, in that the first 20 amino acids are predominantly hydrophobic while the remainder are hydrophilic in nature. Melittin partitions into the plasma membrane of the cell in an irreversible fashion and causes the destabilization of the membrane structure (172).

The minimal concentration of melittin required for the perturbation of the Friend cell nuclei was titrated. At these low concentrations, all added melittin was found to be absorbed by the plasma membrane, thus preventing exposure of intracellular membranes to melittin. Pure nuclei were isolated in high yield and found to have intact nuclear membranes. Full transcriptional activity of the nuclei was retained (172).

This melittin isolation procedure, therefore, fulfilled all the requirements for the production of nuclei suitable for studies relating to the effects of insulin on transcription *in vitro*, and was adapted for use with the H4IIE hepatoma cell line.

3.1 ISOLATION PROCEDURES

3.1.1 NUCLEI ISOLATION USING MELITTIN

All operations were carried out as aseptically as possible using RNase-free solutions, glassware and plasticware (8.1.3).

H4IIE hepatoma cells were grown routinely as described in 8.2.1.1. The cells were removed from the flasks using DPBSA (Dulbecco's Phosphate Buffered Saline without calcium and magnesium) (8.2.1.2) and pelleted at 1 500g for 7 min in a bench top centrifuge. The cells were rinsed once with DPBSA and again pelleted. Following the harvesting of the cells, all procedures were carried out at 4°C.

The cells were resuspended in a small volume of DPBSA (approximately 5 ml per 10^8 cells) and melittin added at a concentration of 1 mg per 10^8 cells, while vortexing. The cells were again pelleted at 1 500g for 7 min. The DPBSA supernatant containing the excess melittin was poured off and the cells resuspended in 0,34 M sucrose solution in Hewish and Burgoyne Buffer A (15 mM NaCl, 65 mM KCl, 0,15 mM spermine, 0,5 mM spermidine, 15 mM Tris, pH 7,4, 15 mM β-mercapto- ethanol, 0,1 mM PMSF, 0,2 mM EDTA, 0,2 mM EGTA) (173) at a concentration of 10^8 cells per ml.

The suspension was homogenized for 5 to 10 strokes in a 5 ml hand-held glass Dounce homogenizer with a tight fitting pestle. The degree of cell breakage was monitored under the fluorescence microscope using Rhodamine 123 as the fluorescent stain (see 3.2.2).

9 Volumes of 2.3 M sucrose in Buffer A were added to the broken cell suspension, mixed thoroughly, but gently, and the nuclei pelleted by centrifugation at 50 000g for 45 min

in a Beckman SW65 Ti or SW28 rotor, depending on the quantity of cells being processed.

The interface layer of unbroken cells and debris was carefully removed and the supernatant poured off. The sides of the tubes were wiped and the nuclear pellet resuspended in nuclei storage buffer (NSB) (12,5% (v/v) glycerol in Hewish and Burgoyne Buffer A) to a DNA concentration of 1 mg/ml as determined by OD_{260} in 4 M NaCl.

The nuclei were quick-frozen in liquid nitrogen and stored at -70°C.

The purity of the nuclei was determined routinely by examination of the preparation under the fluorescence microscope using the fluorescent dye, Rhodamine 123. Electron microscopy was employed in the establishment of the method (8.5.3). A purity of greater than 99% nuclei was achieved.

Yields of nuclei ranged between 80 and 100%.

3.1.2 NUCLEI ISOLATION USING TRITON X-100

For comparative purposes, nuclei were isolated from H4IIE hepatoma cells using the non-ionic detergent Triton X-100.

Cells were treated in the same way as described in 3.1.1 except that Triton X-100 was substituted for melittin, and added to a final concentration of 0,5% (w/v) (174).

The purity of the isolated nuclei was greater than 99% as established by fluorescence and electron microscopy.

The yield of nuclei was from 80-100%.

3.1.3 NUCLEI ISOLATION USING HOMOGENIZATION ONLY

Nuclei were isolated from cells without the prior treatment of the cells with melittin or a detergent. These nuclei served as a control in electron microscopic evaluations, as well as in transcription studies, relating to the effects of the exposure of cells to melittin or Triton X-100. The nuclei isolation procedure was the same as that described in 3.1.1 without the addition of melittin. The cells required at least 30 strokes of homogenization in order to achieve a high proportion of cell breakage.

The purity of the nuclei isolated using homogenization only, was greater than 99% as established by fluorescence and electron microscopy.

The yield of nuclei was 50-70%. The excessive homogenization required to break a high percentage of cells resulted in damage to existing nuclei and thus to decreased yields.

The comparative yields of nuclei and the degree of homogenization required to achieve those yields using either melittin, Triton X-100 or homogenization only, are depicted in Figure 3.1.

3.2 ANALYSIS OF ISOLATED NUCLEI

3.2.1 ELECTRON MICROSCOPY

Nuclei were isolated according to the methods already described in 3.1.1 to 3.1.3 using melittin, Triton X-100 and homogenization only.

The purity and morphology of the isolated nuclei were examined using electron microscopy (EM). The EM procedures

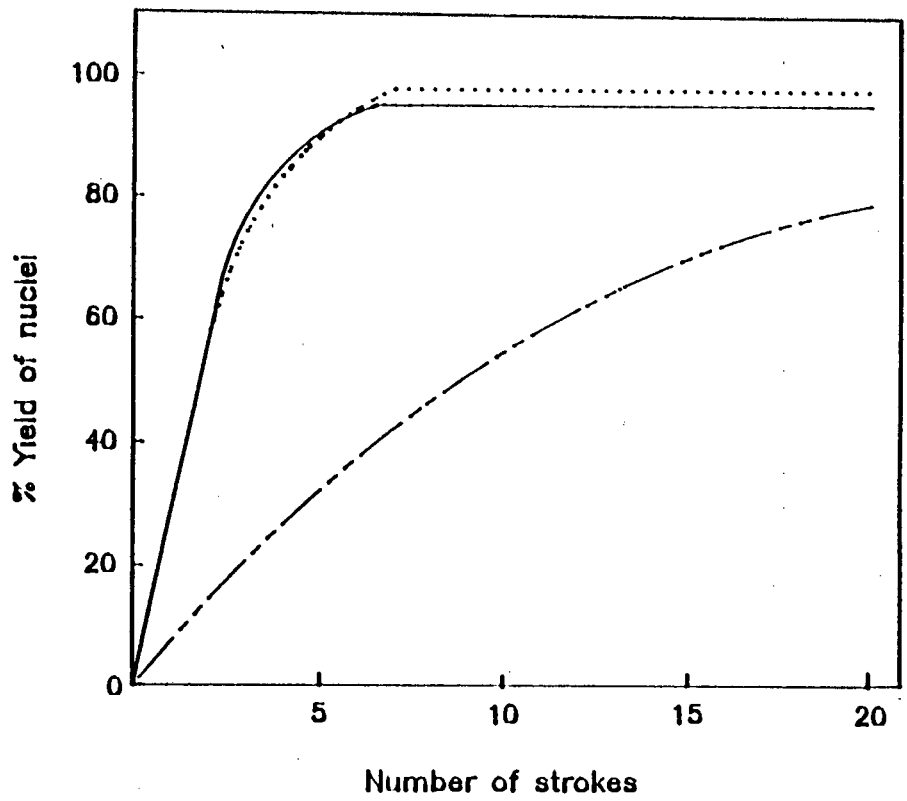

Figure 3.1 The extent of homogenization required to achieve a maximum yield of nuclei using homogenization only (- - - - - -), Triton X-100 (————) or melittin (·····).

and methods employed are described in 8.5.3.

Normal whole cells, as well as those treated with melittin or Triton X-100 were also examined under EM so that the condition of the nuclei *in vivo* prior to isolation procedures could be assessed.

The EM photographs appear in Figure 3.2.

Nuclei isolated using melittin retain intact double nuclear membranes. These are continuous and clearly visible. In contrast, nuclei isolated using Triton X-100 have no clear membrane structure at all surrounding them, although a more dense border area is apparent. Nuclei isolated by homogenization only, have the same double membrane structure as do the melittin isolated nuclei.

These effects are also evident when the whole cell is still intact. After treatment with Triton X-100, the clear delineation of nuclei in the cell disappears, though it is still possible to isolate nuclei.

3.2.2 FLUORESCENCE MICROSCOPY: STAINING WITH RHODAMINE 123

The nuclei of the H4IIE hepatoma cells are very large, making it difficult to distinguish free nuclei from whole cells. A method was developed to overcome this problem using the fluorescent dye, Rhodamine 123, which is a mitochondrial specific stain (175). DNA-specific stains were unsuitable as both whole cells and free nuclei fluoresced to the same extent. With the use of Rhodamine 123, whole cells are seen as shadow centres surrounded by a bright ring of fluorescence. In contrast, nuclei do not fluoresce at all, and are discerned only as dark shadows. Thus, in a

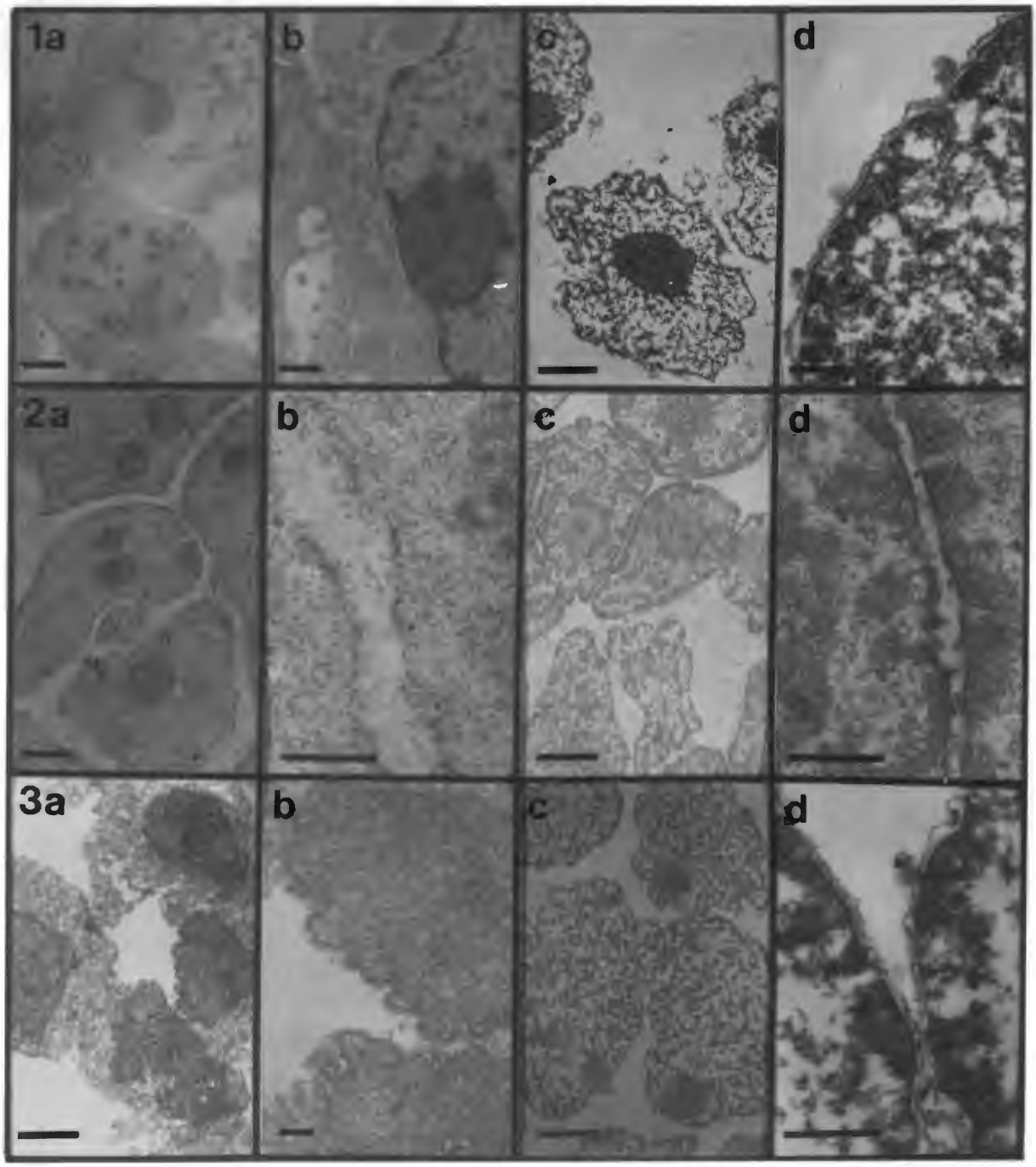

Figure 3.2 Micrographs of hepatoma cells or isolated nuclei

1a. Normal hepatoma cells (bar: 2 µm)
b. Normal hepatoma cells (bar: 0,5 µm).
c. Nuclei isolated using homogenization only (bar: 2 µm)
d. Nuclei isolated using homogenization only (bar: 0,5 µm)

2a. Triton X-100 treated whole cells (bar: 2 µm)
b. Triton X-100 treated whole cells (bar: 0,5 µm)
c. Triton X-100 isolated nuclei (bar: 2 µm)
d. Triton X-100 isolated nuclei (bar: 0,5 µm)

3a. Melittin treated whole cells (bar: 2 µm)
b. Melittin treated whole cells (bar: 0,5 µm)
c. Melittin isolated nuclei (bar: 2 µm)
d. Melittin isolated nuclei (bar: 0,5 µm)

preparation of nuclei, any contaminating cells are readily visible.

A photograph illustrating the differential use of Rhodamine 123 is shown in Figure 3.3. The staining method is given in detail in 8.5.4.

3.2.3 TRANSCRIPTIONAL ACTIVITY OF ISOLATED NUCLEI

The transcriptional activity of the nuclei isolated using melittin, Triton X-100 or homogenization only, was determined. The *in vitro* transcription assay is given in 8.4.1.3.

Melittin isolated nuclei incorporated labelled nucleotide to the same extent as nuclei isolated using homogenization only. Triton X-100 isolated nuclei had transcriptional activity reduced to 40% of that of the other nuclei. Melittin, therefore, does not interfere with the transcriptional apparatus of the nuclei. The low transcriptional activity of the nuclei isolated using Triton X-100 was not unexpected considering the damage to the nuclei apparent in the EM photographs.

The result of the comparative *in vitro* transcription assays is shown in Figure 3.4.

3.2 CONCLUSION

The use of melittin to lyse the cells is the best method for the production of nuclei suitable for the purposes of studying the effects of insulin on the transcriptional activity of the isolated nuclei. This method allows the isolation, in good yield, of pure nuclei with intact nuclear membranes and a high level of transcriptional activity.

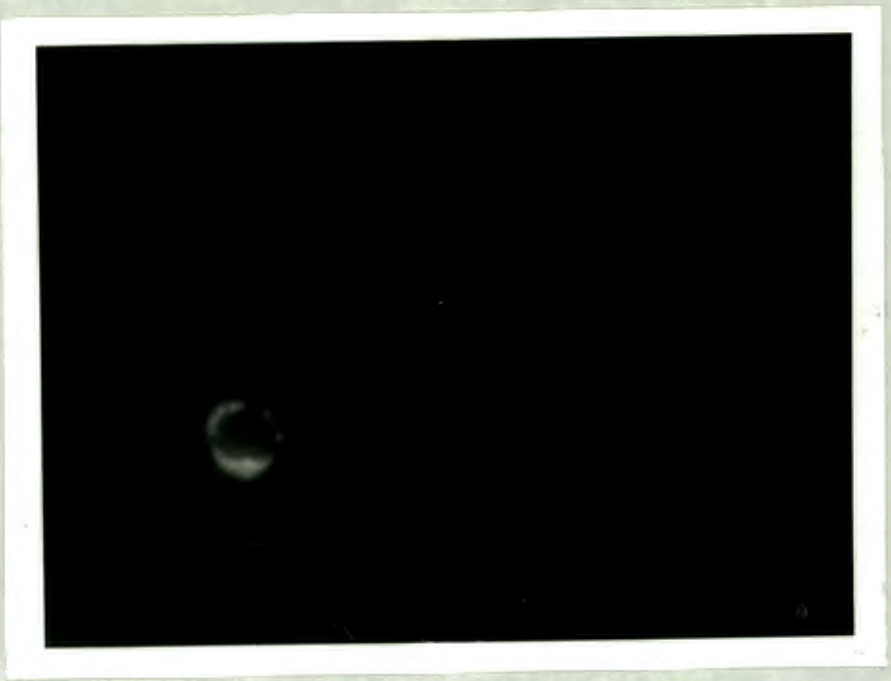

Figure 3.3 Detection of a whole cell present in a preparation of H4IIE nuclei. The sample is stained with Rhodamine 123, a fluorescent dye which is selectively taken up by mitochondria. The cell is seen as a ring of fluorescence surrounding a dark centre whereas the nuclei are seen only as dark shadows.

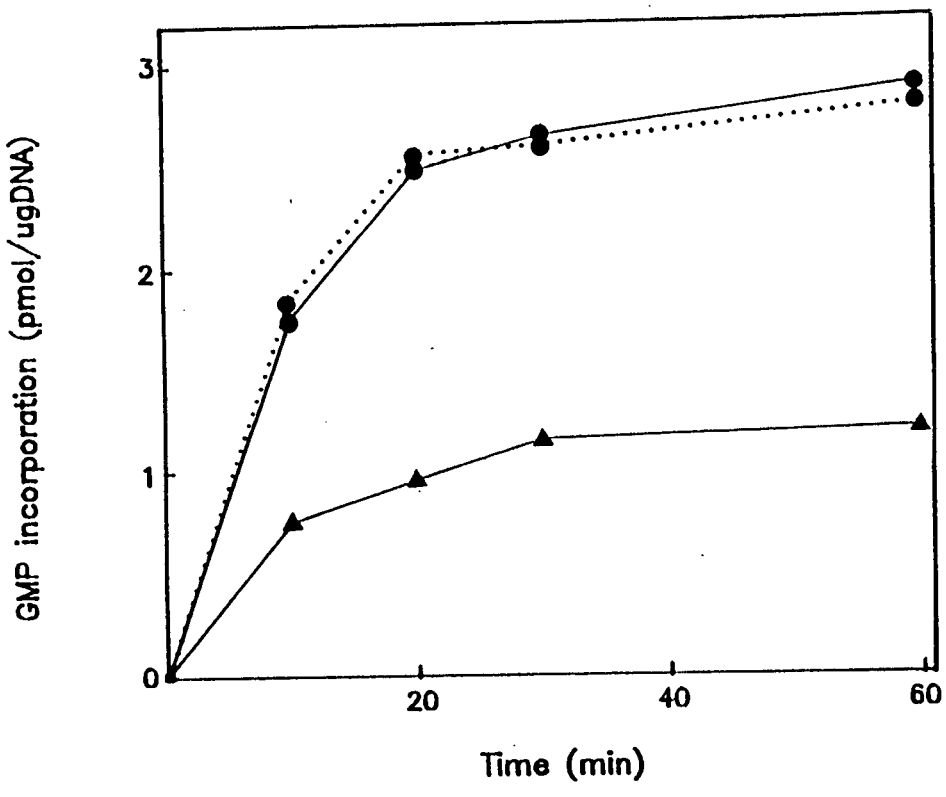

Figure 3.4 Transcriptional activity of nuclei isolated using homogenization only (●·····●), Triton X-100 (▲——▲), or melittin (●——●).

3.3 DISCUSSION

In whole tissue, brief homogenization is generally sufficient to allow complete breakage of cells and release of nuclei. This is the result of the shearing forces created by the fibrous connective tissues. However, nuclei isolation from tissue culture cells with this method is less efficient. Without connective tissue to aid the shearing, diminished yields of nuclei are obtained, either because the cells never break or because excessive homogenization causes damage to freed nuclei. For this reason, the use of alternative methods to facilitate the release of nuclei from these cells is required.

The Hewish and Burgoyne buffer system was employed in the isolation procedure as it does not contain magnesium. Magnesium is required for the activity of nucleases and as the isolated nuclei were ultimately being used for the study of transcription, spermine and spermidine rather than magnesium, were used to stabilize the nucleus (173).

The melittin isolated nuclei fulfill the stated requirements of purity, activity, good yield and intact nuclear membranes. Therefore, all further investigations reported here in the study of insulin's action at a nuclear level, will use nuclei isolated with this method.

CHAPTER FOUR

INSULIN ADDED DIRECTLY TO ISOLATED H4IIE HEPATOMA NUCLEI STIMULATES TRANSCRIPTION

Insulin regulates cellular gene expression and modulates specific mRNA levels in several cells. As yet, the mechanism of this induction is still unclear. It is accepted that the ability of insulin to regulate the expression of specific genes is triggered by the binding of insulin to its receptor on the plasma membrane. How this signal is transmitted from the plasma membrane to the nucleus, is the primary question remaining unanswered.

Two main possibilities exist: firstly, insulin enters the cell and is itself responsible for the induction of gene expression; alternatively, insulin elicits its effects via a series of second messengers, leading to the regulation of the expression of the certain genes known to be under the transcriptional control of insulin. As no second messenger capable of regulating all the effects of insulin on gene expression has been identified, the possibility that insulin itself may regulate transcription in liver tissue at the level of the nucleus, was investigated. This hypothesis is substantiated by recurrent reports in the literature claiming that insulin binds to the nucleus, both to the nuclear membrane and to the interior of the nucleus. Also, insulin is known to be internalized in insulin responsive cell lines, although the presence of this insulin is normally ascribed to degradative processes.

Thus, the effect of insulin on total gene expression, as well as on the levels of mRNAs for specific insulin responsive genes, was studied when insulin was added

directly to isolated H4IIE hepatoma nuclei in an *in vitro* transcription system.

4.1 OPTIMIZATION OF *IN VITRO* TRANSCRIPTION ASSAY

The basic method employed in the *in vitro* transcription assay was that of Marzluff and Huang (176). This method creates conditions suitable for RNA synthesis by RNA polymerases I, II and III in mammalian nuclei. However, different cell types have different requirements for salts (KCl, $MgCl_2$, $MnCl_2$) in the reaction mix, and the method was, therefore, optimized for use with H4IIE hepatoma nuclei. The final protocol is given in 8.4.1.3.

Nuclei were isolated according to the method developed in Chapter 3 and summarized in 8.3.1., using melittin. Purified nuclei were incubated at a concentration of 1mg DNA/ml (as determined by diphenylamine method, 8.5.2) at 26°C in a reaction mix containing 25 mM Tris/HCl pH 8,00, 7,5 mM $MgCl_2$, 90 mM KCl, 1 mM $MnCl_2$, 0,4 mM each of unlabelled nucleoside triphosphates, 0,04 mM of radioactively labelled nucleoside triphosphate (1 Ci/mmol), 1 mM PMSF, 10 mM β-mercaptoethanol, 12,5% glycerol and 100 U/ml HPRI (human placental ribonuclease inhibitor).

Aliquots were removed from the incubation mix at various time intervals from 0 to 60 min and added to 9 volumes of stop buffer (1% SDS/10 mM EDTA pH 7,0). This aliquot was then TCA-precipitated on a glass-fibre filter, washed and the incorporation of radiolabelled nucleotide into TCA-precipitable material was determined. Incorporation was linear for the first 20 min and maximal after 40-60 min.

Determination of requirement for manganese

The activity of RNA polymerase II is reported to be greatly stimulated by manganese ions when studied using DNA as a template *in vitro*. However, in isolated nuclei, manganese ions at 1 mM inhibit transcription by all three polymerases by 50% (176). In order to verify these findings in H4IIE hepatoma nuclei, manganese ($MnCl_2$) concentrations ranging from 0-3 mM were included in the *in vitro* transcription assay (8.4.1.3). The time course of incorporation of [^{32}P]GMP into TCA-precipitable material was determined.

The incorporation of [^{32}P]GMP was found to decrease with increasing concentrations of manganese. Total transcription was inhibited by 78% at 3 mM $MnCl_2$. 50% Inhibition of total transcription occurred at approximately 1,8 mM $MnCl_2$. Manganese is thus not a requirement for *in vitro* transcription in H4IIE hepatoma nuclei. The effect of manganese on the incorporation of GMP per µg DNA after 60 min of incubation is shown in Figure 4.1.

Determination of optimal KCl concentration

KCl concentrations ranging from 60-200 mM were used in the *in vitro* transcription assay (8.4.1.3). The time course of incorporation of [^{32}P]GMP into TCA-precipitable material was determined. RNA synthesis is expressed as pmol GMP incorporated per µg DNA per 60 min.

The results of this experiment, by extrapolation of the data, show the optimal KCl concentration for *in vitro* transcription in these nuclei to be 105 mM. The incorporation of radioactivity is given graphically in Figure 4.1.

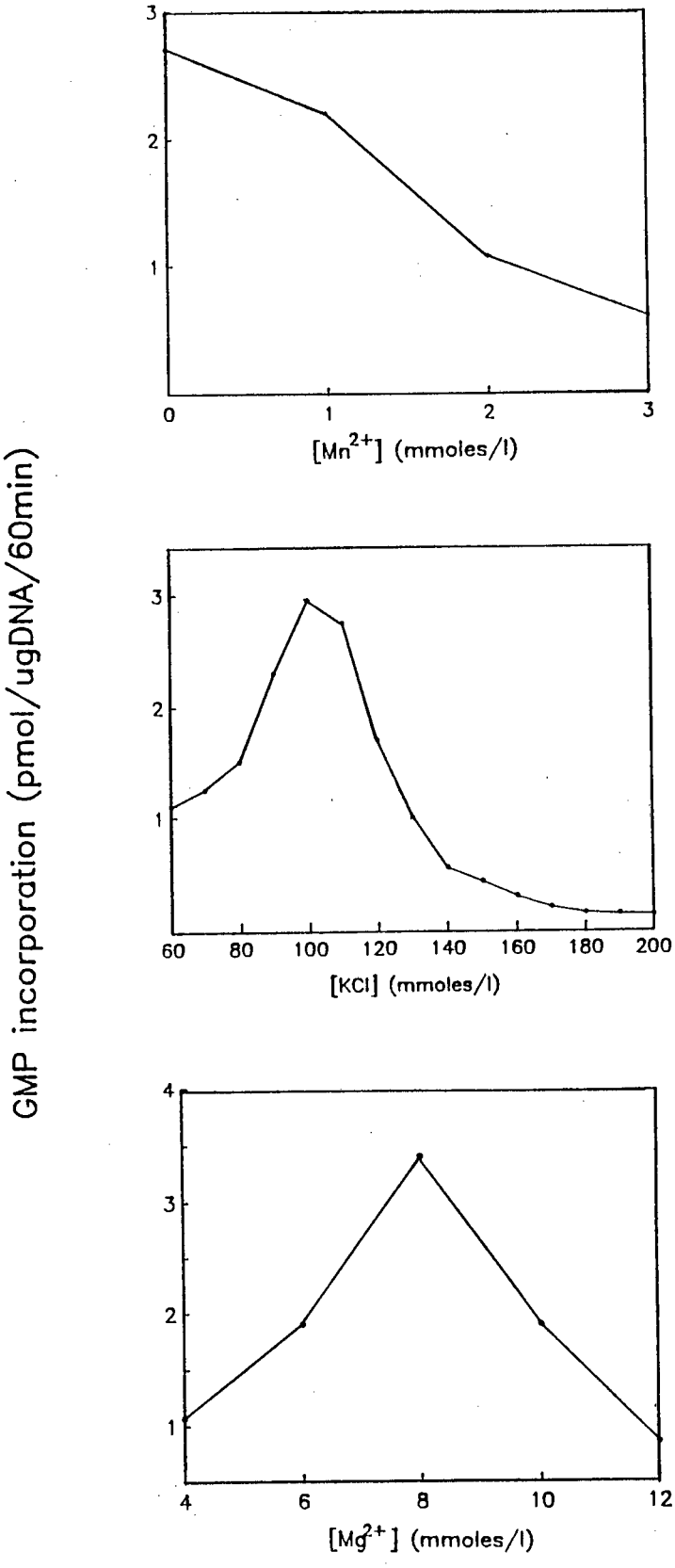

Figure 4.1 Determination of optimum Mn^{2+}, KCl and Mg^{2+} concentrations for maximal *in vitro* transcriptional activity of isolated H4IIE hepatoma nuclei.

Determination of optimal magnesium concentration

Magnesium ($MgCl_2$) concentrations ranging from 4-12 mM were used in the *in vitro* transcription assay (8.4.1.3). The time course of the incorporation of [^{32}P]GMP into TCA-precipitable material was determined, and RNA synthesis expressed as pmol GMP incorporated per µg DNA per 60 min.

The optimal magnesium concentration for *in vitro* transcription in H4IIE nuclei was found to be 8 mM. The results are shown in Figure 4.1

4.2 EFFECT OF INSULIN ON TOTAL TRANSCRIPTIONAL ACTIVITY

4.2.1 EFFECTS OF INSULIN ON TRANSCRIPTION IN NUCLEI ISOLATED FROM STARVED CELLS

Nuclei were isolated according to the method described in 8.3.1 using melittin. They were, thus, of the highest purity and retained full transcriptional activity. Most importantly, the nuclei had intact nuclear membranes allowing maximal opportunity for insulin to interact with the nuclear membrane insulin receptor - should this be part of a putative mechanism of the action of insulin on the nucleus.

The optimal *in vitro* transcription assay for H4IIE hepatoma nuclei developed in 4.1 and summarized in 8.4.1.3, was used. Insulin was added at final concentrations ranging from 0,01-100 nM. The time course of incorporation of [^{3}H]UMP was monitored at 10 min intervals from 0 to 60 min. RNA synthesis is expressed as pmol UMP incorporated per µg DNA per unit time.

The effect of insulin on the incorporation of UMP per µg DNA as a function of time is illustrated in Figure 4.2.

The results of the experiment show that insulin is able to increase transcription in H4IIE hepatoma nuclei when added directly to the nuclei *in vitro*. Addition of insulin causes a rapid increase in transcriptional activity which is evident after 10 min of incubation. The average rate of transcription was 0,054 pmol UMP incorporated/µg DNA/min in control incubations without insulin, and 0,065 pmol UMP incorporated/µg DNA/min in insulin treated incubations, after 40 min. An exact concentration dependence was not apparent. After 20 min of incubation, 100 pM insulin produced maximal incorporation of radioactivity, whereas after 60 min, 10 nM insulin produced the maximum incorporation.

The increase in the transcriptional activity of the nuclei is 17% after 40 min, and although marginal, is reproducible, as the experiment was repeated 3 more times and always yielded incorporations greater in insulin treated nuclei than in control nuclei.

4.2.2 INHIBITION OF TRANSCRIPTION BY α-AMANITIN

α-Amanitin inhibits the activity of RNA polymerase II, the major enzyme involved in the transcription of mRNA for single copy genes in eukaryotic cells. In order to determine what proportion of transcription in H4IIE hepatoma nuclei was due to the activity of RNA polymerase II, nuclei were exposed to α-amanitin at 2 µg/ml for 30 min at 0°C prior to incubation in the *in vitro* transcription assay (8.4.3). The incorporation of [^{3}H]UMP into TCA-precipitable material was monitored at 0, 20, 40 and 60 min.

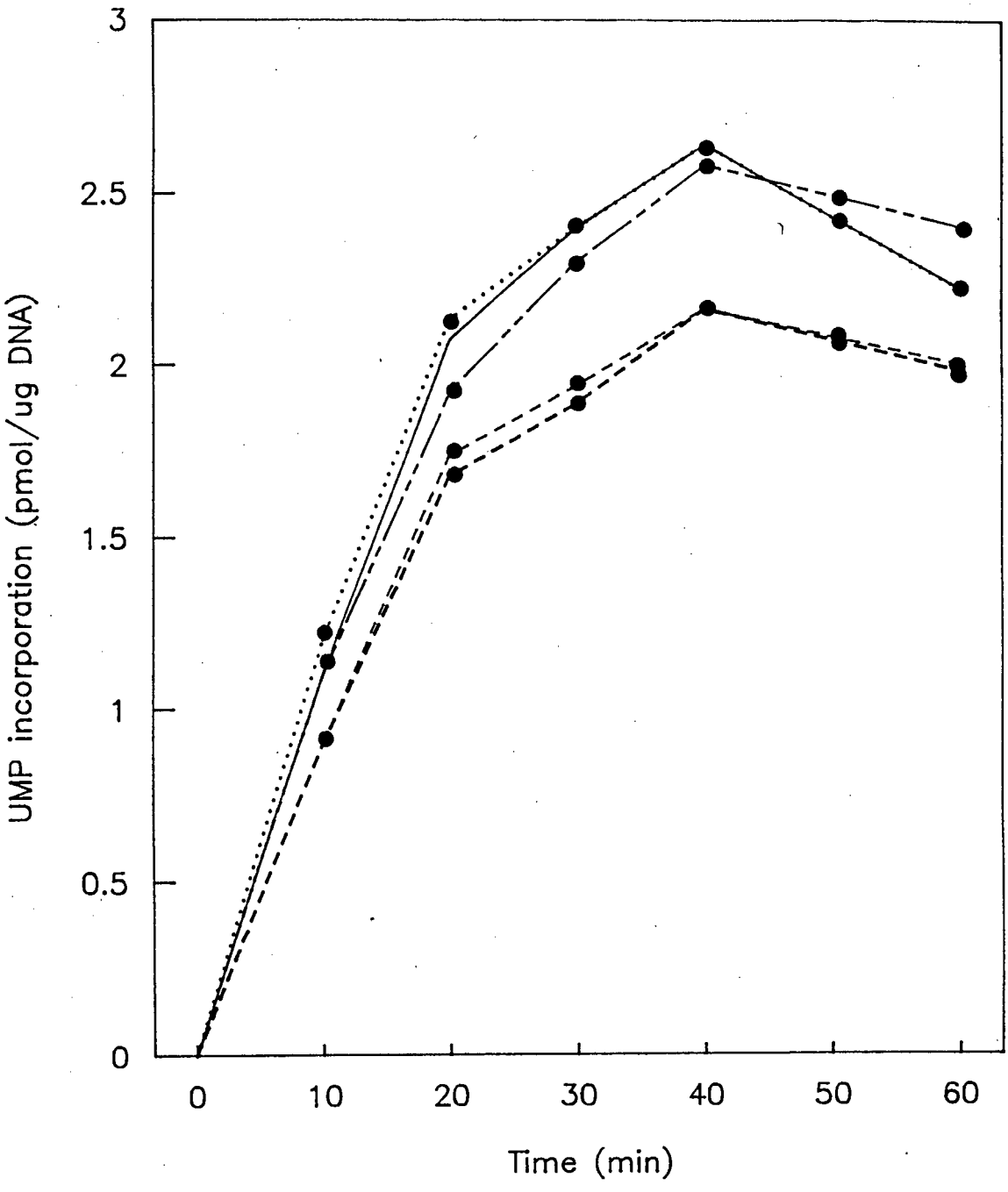

Figure 4.2 Effect of insulin on the total transcriptional activity of isolated H4IIE hepatoma nuclei. RNA synthesis is shown as pmol UMP incorporated/µg DNA for duplicate control samples without insulin (-------), or experimental samples with 100 pM insulin (————), 1 nM insulin (·····) or 10 nM insulin (--———). The values given are the result of a representative experiment. The average SEM for three separate experiments was 0,1.

α-Amanitin inhibited transcription on average by 66%. The result of the experiment is depicted graphically in Figure 4.3. From studies conducted by others, the expected inhibition of RNA polymerase II transcription by α-amanitin in eukaryotic cells grown in tissue culture is in the region of 50% (176).

In order to determine whether the effects of insulin were directly on gene transcription, rather than on mRNA stability, and if they were mediated by RNA polymerase II, nuclei isolated from starved H4IIE cells using melittin, were treated with α-amanitin, in the same way as already described, prior to the addition of insulin and incubation in the *in vitro* transcription assay (8.4.1.3). Insulin was added at final concentrations ranging from 0,01-100 nM. Control incubations contained nuclei with no α-amanitin. The time course of incorporation of [^{3}H]UMP into TCA-precipitable material was monitored at 20 min intervals from 0 to 60 min.

The increased transcriptional activity observed in H4IIE hepatoma nuclei following the addition of insulin to the *in vitro* transcription assay was no longer apparent when the nuclei were preincubated with 2 µg/ml of α-amanitin. The level of RNA synthesis is the same in all incubations whether insulin was added or not. This would indicate that the effect of insulin in increasing transcription in these nuclei, is a specific effect on transcription controlled by RNA polymerase II.

Table 4.1 shows the comparative effects of insulin on transcription in nuclei incubated with or without prior exposure of the nuclei to α-amanitin.

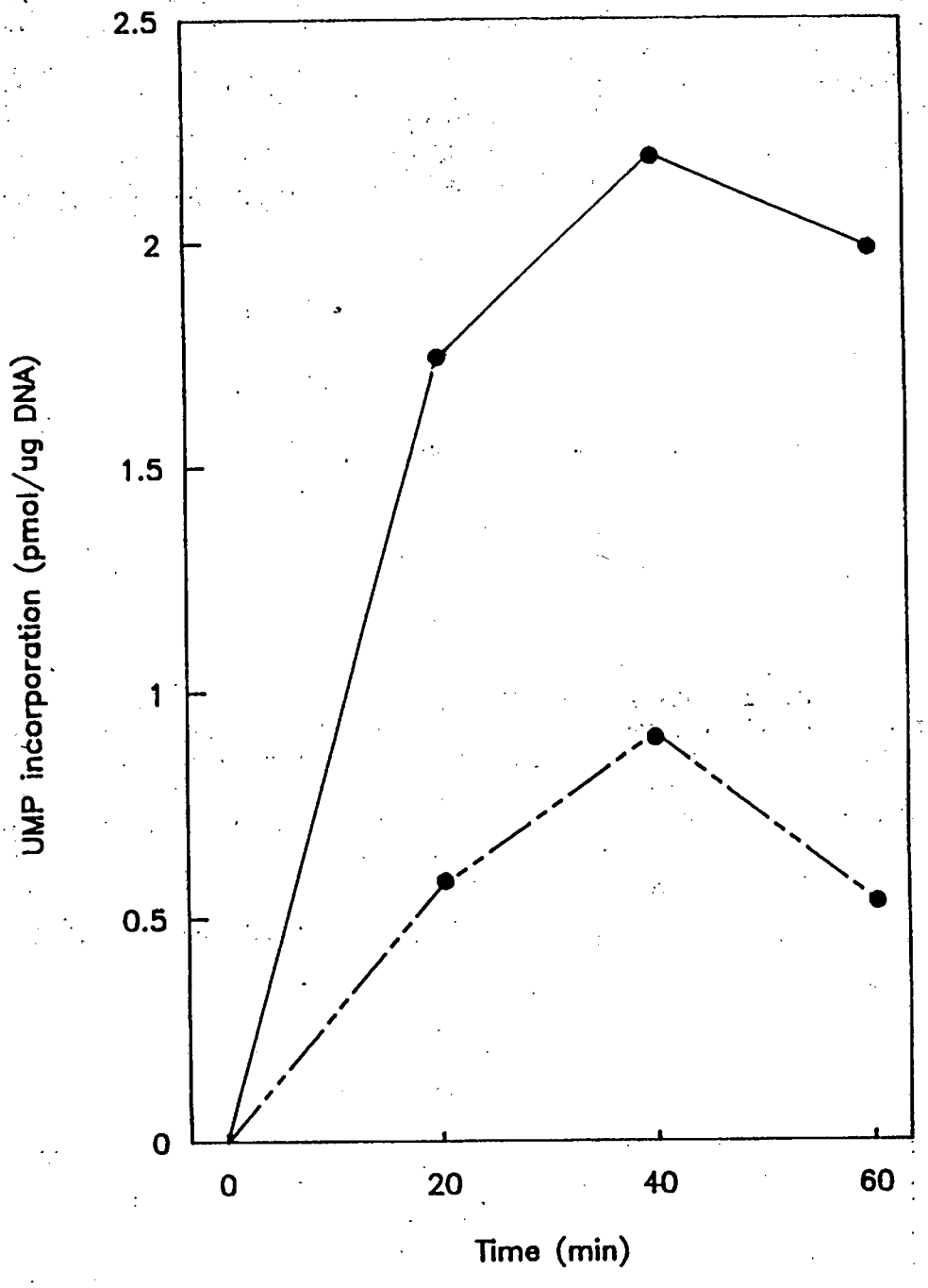

Figure 4.3 Effect of α-amanitin on transcription in isolated H4IIE hepatoma nuclei. Nuclei were preincubated with (- - - -) or without (————) α-amanitin at 2 µg/ml for 30 min at 0°C prior to the addition of salts and nucleotides required for transcription. The inhibitory effect of α-amanitin on the incorporation of nucleotides clearly demonstrates a transcriptional effect, rather than and alteration in mRNA stability or a change in the pool size of nucleotides within the nucleus.

Table 4.1 The effects of the RNA polymerase II inhibitor, α-amanitin, on the ability of insulin to stimulate increased transcription in isolated H4IIE hepatoma nuclei. RNA synthesis is expressed as pmol UMP incorporated/μg DNA. Nuclei were preincubated with α-amanitin for 30 min and incubated in the *in vitro* transcription assay for 40 min.

Insulin concentration (log M)	RNA synthesis	
	- α-amanitin	+ α-amanitin
0	2,16	0,91
-10	2,58	0,90
-9	2,64	0,89
-8	2,58	0,93

4.2.3 EFFECT OF INSULIN ON TRANSCRIPTION IN NUCLEI ISOLATED USING TRITON X-100

The effect of insulin on the transcriptional activity of H4IIE nuclei was investigated in nuclei isolated using the non-ionic detergent, Triton X-100. The effects of Triton X-100 on the nucleus have been discussed in Chapter 3, particularly relating to the effects of the detergent on the stability of the nuclear membrane, the integrity of intranuclear organization and on decreasing the transcriptional activity of the isolated nuclei. As insulin added directly to melittin isolated nuclei increased the transcriptional activity of the nuclei *in vitro*, it was of interest to determine whether these effects of insulin could be mediated in nuclei isolated using Triton X-100.

Nuclei were isolated from starved H4IIE cells as described in 3.1.2 with the addition of 0,5% Triton X-100. The optimal *in vitro* transcription assay for H4IIE hepatoma nuclei

developed in 4.1 and summarized in 8.4.1.3 was used. Insulin was added to the incubation at final concentrations ranging from 0,01-100 nM. The time course of incorporation of [^{3}H]UMP into TCA-precipitable material was monitored at 20 min intervals from 0 to 60 min.

As already documented in 3.1.2, nuclei isolated using Triton X-100 have reduced transcriptional activity. This amounts to approximately 40% of the transcriptional activity in nuclei isolated using melittin or homogenization only. The result of this experiment shows that, in addition to the decreased transcriptional activity of the Triton X-100 isolated nuclei, these nuclei display no response to the addition of insulin. The result of the experiment is portrayed in Figure 4.4.

A possible explanation for such an effect is that the binding of insulin to the nuclear membrane is inhibited in Triton X-100 isolated nuclei. Triton X-100 solubilizes the membrane lipid and a proportion of the nuclear membrane protein, resulting in the collapse of the double membrane structure (170). This could alter the membrane milieu surrounding a nuclear membrane insulin receptor and result in the decreased binding of insulin. If the effects of insulin on transcription are mediated via the binding of insulin to a nuclear membrane receptor, such effects would be difficult to imagine when the membrane structure has collapsed. Alternatively, a nuclear receptor for insulin may be solubilized by the detergent.

Another possible explanation revolves around the general decreased transcriptional activity of the Triton X-100 isolated nuclei and the perturbation of the intranuclear structure in these nuclei. Triton X-100 has been found to solubilize histones in preparations of nuclei (177). This fact, taken together with the possible solubilization of RNA

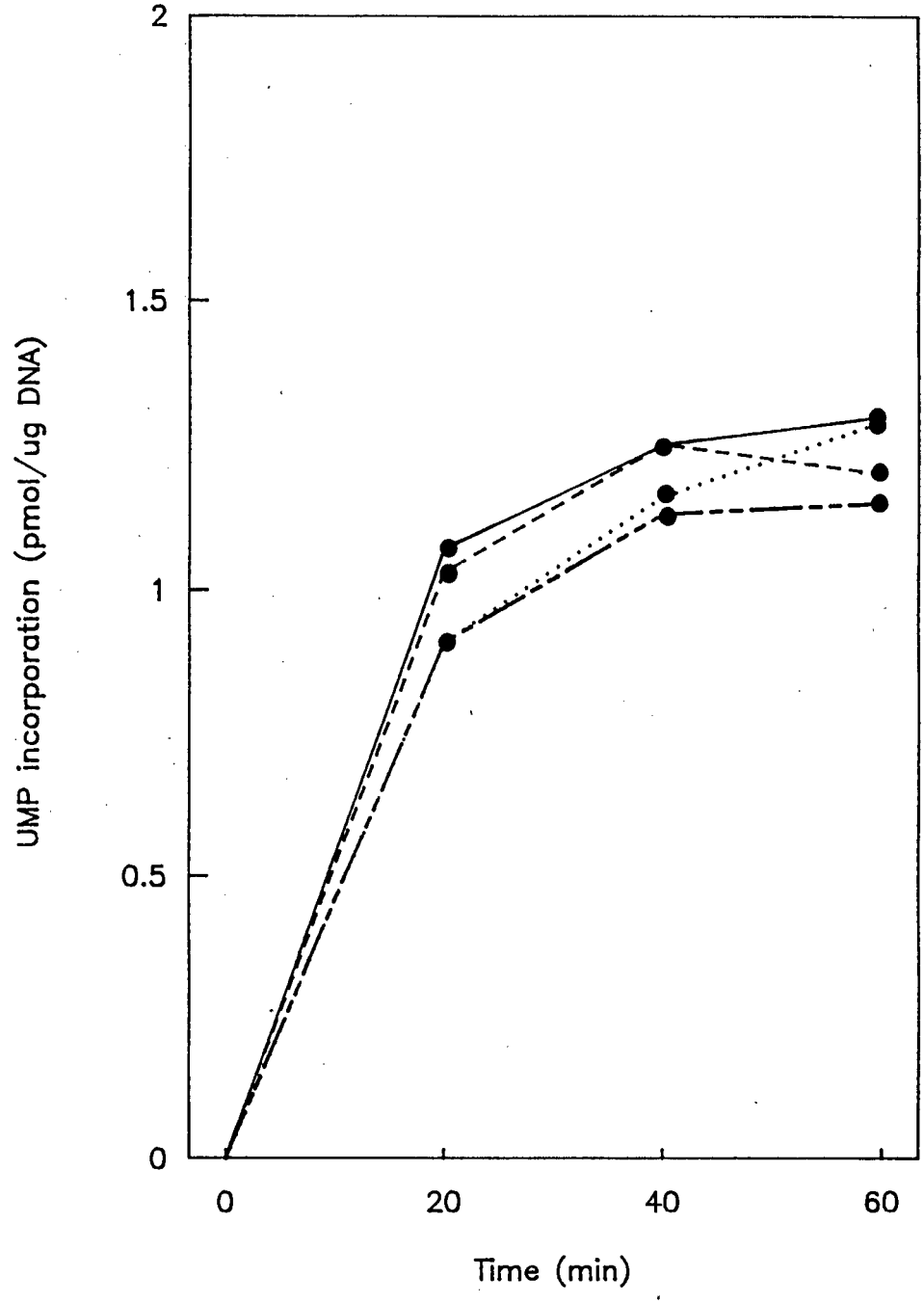

Figure 4.4 Effect of insulin on *in vitro* transcription in nuclei isolated using Triton X-100. RNA synthesis is shown as pmol UMP incorporated/µg DNA for a control sample without insulin (-------), or experimental samples with 100 pM insulin (————), 1 nM insulin (·····) or 10 nM insulin (-- ——). The values given are the average result of triplicate samples from a single experiment.

polymerases required for transcription, and the possible disruption of the transcription complex, could explain the decreased total transcriptional activity of the Triton X-100 isolated nuclei. Triton X-100 may also prevent insulin associating with a transcription factor, nuclear protein or DNA regulatory element that may form part of the mechanism by which insulin regulates transcription.

4.2.4 EFFECT OF INSULIN ON TRANSCRIPTION IN NUCLEI ISOLATED FROM FED H4IIE CELLS

The experiment was undertaken in order to determine the direct effects of insulin on transcription in nuclei that had been isolated from cells not starved of serum and thus also not of insulin.

Nuclei were isolated using melittin (8.3.1) from H4IIE cells that were grown to 80-100% confluency and then refed fresh medium containing serum and antibiotics, for 20 h prior to harvesting. Insulin was added to the nuclei at final concentrations ranging from 0,01-100 nM and the *in vitro* transcription assay carried out as described in 8.4.1.3. The time course of incorporation of [^{3}H]UMP was monitored at 20 min intervals from 0 to 60 min.

As can be seen from the results reported in Figure 4.5, the increased transcription seen in the starved nuclei is no longer apparent. All samples in the experiment, with or without insulin, displayed the same initial rate of transcription. When compared to the results obtained in 4.2.1, the initial rate of transcription in the starved nuclei is greater than in the fed nuclei. This may suggest an effect of insulin on the initiation of transcription in the nuclei.

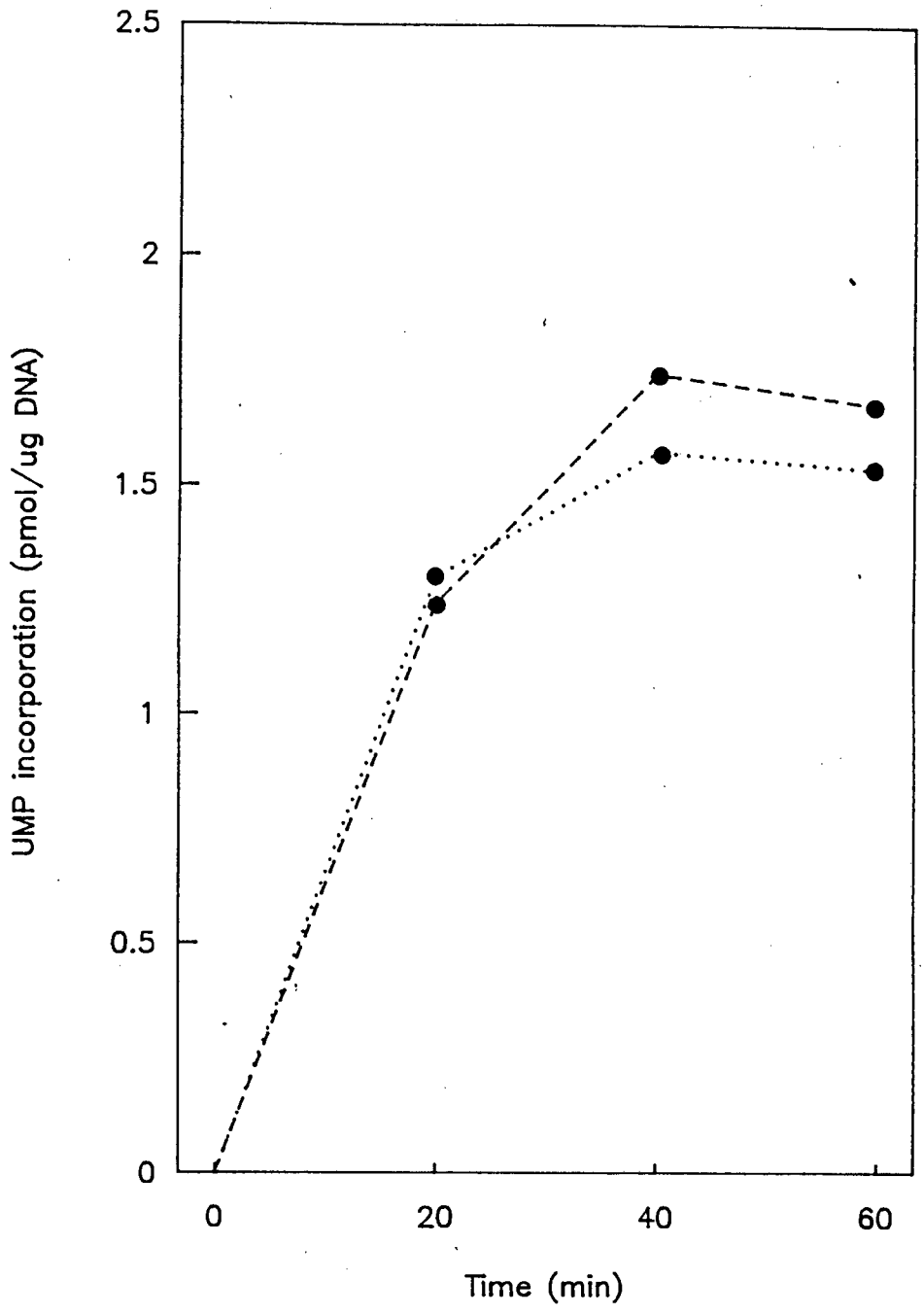

Figure 4.5 Effect of insulin on transcription in nuclei isolated from cells that had not been starved of serum. The incorporation of UMP/µg was monitored in the presence (·····) and absence of 1 nM insulin (------)

4.2.5 EFFECT OF INSULIN ON INITIATION OF TRANSCRIPTION

The ability of insulin to increase transcription when added to isolated hepatoma nuclei *in vitro* is an interesting development in the current understanding of the mechanism by which insulin regulates transcription in target cells.

An experiment was undertaken in order to determine whether any initiation of transcription was taking place in the nuclei *in vitro*, if this initiation could be increased by insulin, and if any differences were observed in nuclei isolated with melittin or with Triton X-100. This was not a comprehensive analytical investigation, but was intended, with the available materials, to make the best comparative assessment possible.

Published reports of *in vitro* transcription assays report that initiation of transcription under the described conditions is minimal (less than 1%)(178). However, these observations were made with nuclei isolated with Triton X-100, a method that may disrupt the transcription initiation complex or solubilize proteins required for the initiation of transcription to take place. Therefore, the effect of insulin on the initiation of transcription of nuclei isolated with melittin was investigated.

The optimal *in vitro* transcription assay for H4IIE hepatoma nuclei (8.4.1.3) was used with [γ-^{32}P]ATP as the labelled nucleoside triphosphate, according to the method of Marzluff (176). Thus, any label incorporated into isolated RNA would be a reflection of a newly initiated molecule, as only such a transcript would retain a label in its 3'end. It is also reported that capping of newly transcribed RNA molecules takes place after 10-15 min *in vitro* (176). Thus, the transcription initiation reaction was stopped after 10 min in order to allow sufficient time for initiation to take

place to a reasonable extent, without the capping process removing all the label. The problem of capping can be overcome by using a nucleotide labelled in the β position or alternatively by using a thiolated nucleotide which is not capped *in vitro* (176). However, [γ-^{32}P]ATP is synthesized routinely in this laboratory and was therefore employed in this preliminary investigation. Insulin was added at final concentrations ranging from 0,01-100 nM.

The data in Table 4.2 summarizes the levels of incorporation of radioactivity into total RNA per 100 µg DNA - a reflection of RNA transcripts initiated *in vitro* on adenosine. This value does not reflect total initiation as no [γ-^{32}P]GTP was used in the experiment.

Table 4.2 Incorporation of radioactivity into total purified RNA, per 100 µg DNA, following *in vitro* transcription to determine the effects of insulin on the initiation of transcription.

Nuclei isolation method	Transcription incubation	cpm in RNA per 100 µg DNA
Melittin	+ insulin	4000
	- insulin	4400
Triton X-100	+ insulin	4500
	- insulin	4800

The purified radiolabelled RNA was further analyzed by digestion with ribonucleases A and T_1 and reprecipitation with ethanol. All of the radioactivity remained in the supernatant, confirming the fact that the radiolabel was present in RNA.

To quantitate the amount of radiolabel incorporated into a specific gene, and thus gain an estimate of the degree of reinitiation of that gene, the following calculations were made:
Specific activity of [γ-^{32}P]ATP is 5 Ci/µmol
11 x 10^{18} cpm = 1 mol ATP
40 000 cpm incorporated into melittin isolated nuclei plus insulin is therefore representative of 3,6 x 10^{-15} mol ATP

If the 66% inhibition of transcription by α-amanitin represents the proportion of mRNA transcribed, then radioactivity incorporated into mRNA represents 2,38 x 10^{-15} mol ATP.

If we assume that the mRNA of a single copy gene is 0,1% of total mRNA, then 2,38 x 10^{-18} mol ATP or 1 432 760 molecules of ATP are incorporated per gene.

The amount of DNA in the transcription incubation was 0,625 mg. If 1 x 10^{8} nuclei contain 1 mg DNA, this represents 6,25 x 10^{7} nuclei and thus also 6,25 x 10^{7} copies of a specific single copy gene.

If we assume that there is only one RNA polymerase II molecule present on a gene at any given time, then 1 432 760 molecules of ATP are incorporated by 6,25 x 10^{7} genes, and 0,023 molecules of ATP are incorporated per gene.

The efficiency of reinitiation of a single copy gene is thus approximately 2,3%.

These results indicate firstly, that a low percentage of initiation of transcription is taking place under the conditions described for the *in vitro* transcription assay. Secondly, initiation is not increased in the presence of added insulin and thirdly, the initiation of transcription

is not improved in nuclei isolated with melittin rather than with Triton X-100.

4.3 EFFECT OF INSULIN ON THE LEVELS OF SPECIFIC mRNAs WHEN ADDED DIRECTLY TO ISOLATED NUCLEI

The ability of insulin to regulate total transcriptional activity in isolated nuclei, combined with its ability to control the accumulation of specific mRNAs in whole cells, led to the investigation of the direct effects of insulin on the levels of specific mRNAs in isolated nuclei. The mRNAs monitored were those for PK, TAT, PEPCK, α-tubulin and c-*fos*. The effect of insulin on the levels of the mRNAs for these genes, in whole H4IIE hepatoma cells, was confirmed in Chapter 2. Insulin has been shown, by other research workers, to regulate these effects at the level of transcription (102,112,118,33).

The *in vitro* transcription assay used, is given in 8.4.1.4. The assay was conducted as described in 4.2 to monitor the total transcriptional activity in response to insulin, however, no labelled nucleotide was added. After 30 or 60 min of incubation at 26°C, guanidinium thiocyanate stock solution was added and the RNA isolated by the guanidinium thiocyanate/CsCl gradient ultracentrifugation method of Chirgwin (159)(8.3.2). The isolated RNA was denatured and hybridized to labelled cDNA probes using the dot blot hybridization procedure (8.4.2.1) or the Northern blot hybridization procedure (8.4.2.2).

In preliminary investigations, RNA was labelled with [α-^{32}P] GTP *in vitro*, isolated, and hybridized to an immobilized cDNA probe. However, due to the limited volume of the transcription assay (8.4.1.4) and the low amounts of radiolabel incorporated per mRNA species, quantitation of

the relative amounts of mRNA by this method was not possible. The use of radiolabelled cDNA probes was thus the method of choice.

The results of the effect of a range of insulin concentrations on the transcriptional activity of specific genes will be given independently for each gene studied. A more detailed investigation of the kinetics of mRNA production would have been outside the scope of this thesis.

4.3.1 EFFECT OF INSULIN ON THE LEVELS OF PK mRNA

The addition of insulin to isolated nuclei results in an increase in the amount of hybridizable mRNA for PK.

An example of a typical dot blot and a Northern blot showing the increased amounts of PK mRNA after the addition of insulin at various concentrations to an *in vitro* transcription assay, is given in Figure 4.6.

A very weak signal with irregularly shaped bands was obtained from the Northern blot, as sufficient sample to achieve a stronger signal could not be loaded on the gel. The varied sizes of the hybridized mRNA is probably the result of uncontrollable nuclease activity during the isolation of the nuclei and the transcription incubation, resulting in the nicking of the DNA template and the degradation of the mRNA. Nevertheless, the effect of insulin in increasing the amount of hybridizable PK mRNA is recognizable. However, for routine analysis of the effects of insulin on the amounts of the various mRNA levels studied, the dot blot was found to give reproducible results. Sufficient RNA could be accumulated on a membrane to allow the production of a strong signal which could easily be quantitated by densitometric scanning (8.4.2.1).

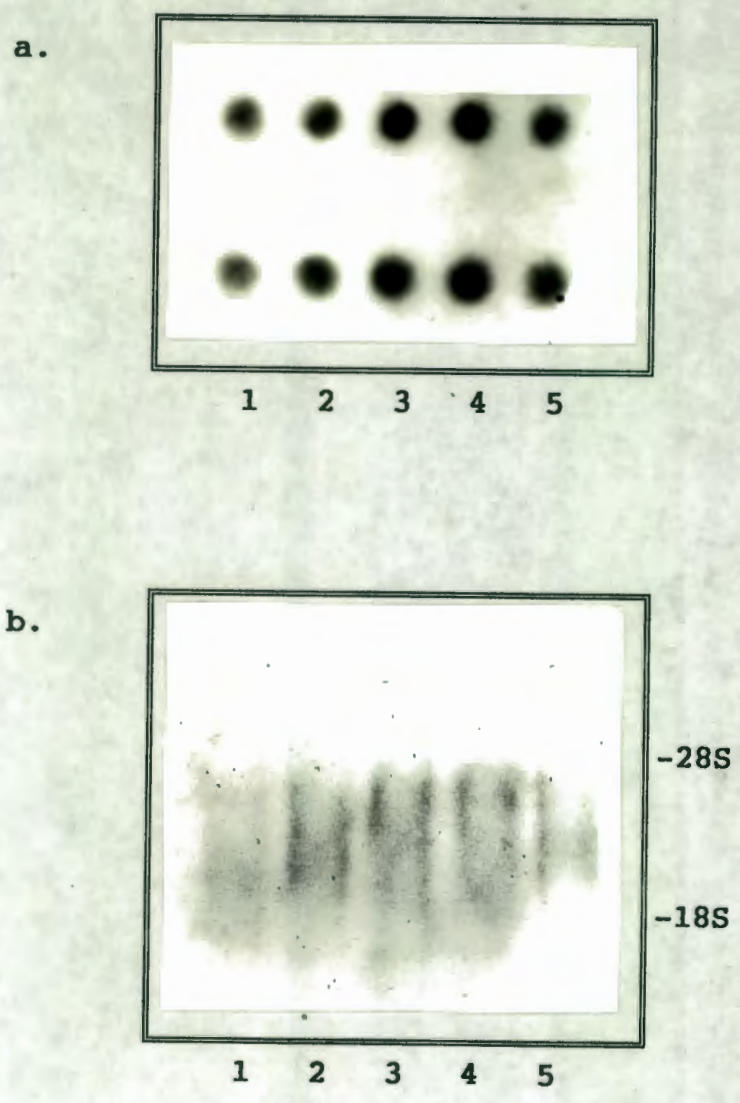

Figure 4.6 Dot blot (a) and Northern blot (b) analysis of PK mRNA in H4IIE hepatoma nuclei, isolated after incubation of the nuclei in an *in vitro* transcription assay. Insulin was added to the nuclei at concentrations of 0,1-100 nM. The dot or lane numbers represent the following insulin concentrations: (1) No insulin; (2) 0,1 nM insulin; (3) 1 nM insulin; (4) 10 nM insulin; and (5) 100 nM insulin.

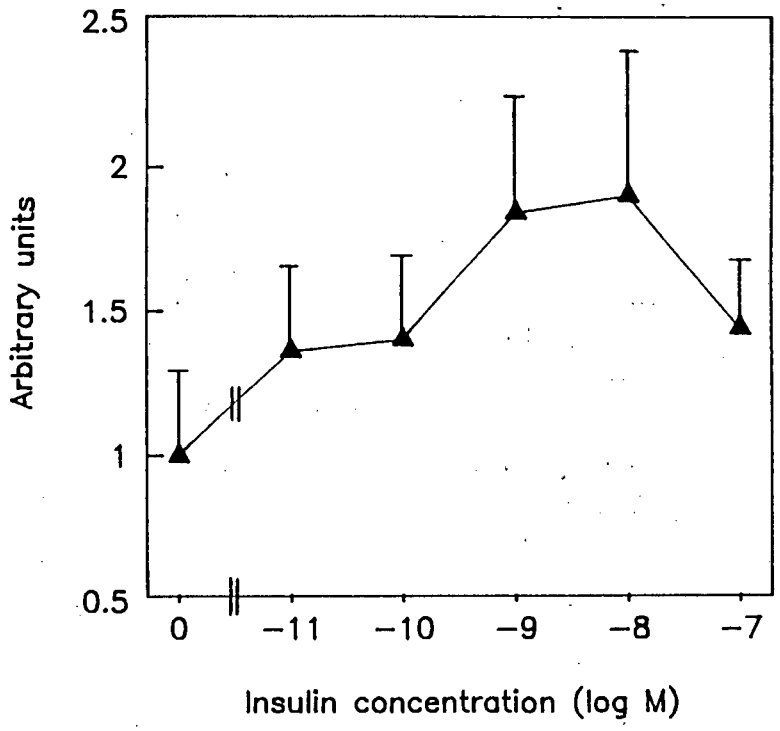

Figure 4.7 Effect of insulin added directly to isolated nuclei on the level of PK mRNA. Nuclei were incubated with insulin at concentrations ranging from 0,01 to 100 nM for 30 min and the total RNA isolated and probed. Each point is the mean result ± SD of three separate experiments. There is a significant increase in the maximum level of PK mRNA in insulin treated and untreated samples ($p < 0,01$). The difference in the maximum level of PK mRNA and the level of α-tubulin mRNA, a house-keeping gene, at corresponding insulin concentrations, is also significant ($p < 0,01$).

The results of the further studies on the effects of insulin on transcription in the isolated nuclei, will be given as the graphical representation of the densitometric scans of the autoradiograms obtained from dot blots.

Insulin addition to isolated nuclei resulted in a 2-fold increase in the amount of PK mRNA after 30 min. This effect was concentration dependent, being maximal at 1-10 nM and half maximal at 10-100 pM insulin. The result is depicted graphically in Figure 4.7.

The amount of hybridizable mRNA for α-tubulin remained constant at all concentrations of insulin administration. The effect of insulin on α-tubulin mRNA is shown in Figure 4.8.

4.3.2 EFFECT OF INSULIN ON THE LEVELS OF TAT mRNA

The addition of insulin to isolated nuclei lead to a 2-fold increase in the amount of hybridizable mRNA for TAT after 30 min. This effect was also concentration dependent being maximal at 100 pM and half maximal at approximately 5-10 pM. This result is shown in Figure 4.9.

The amount of hybridizable mRNA for α-tubulin remained constant at all concentrations of insulin administration (Figure 4.8).

4.3.3 EFFECT OF INSULIN ON THE LEVELS OF PEPCK mRNA

The addition of insulin to isolated nuclei showed no specific effect on the amount of hybridizable mRNA for PEPCK after 30 min at any of the insulin concentrations measured.

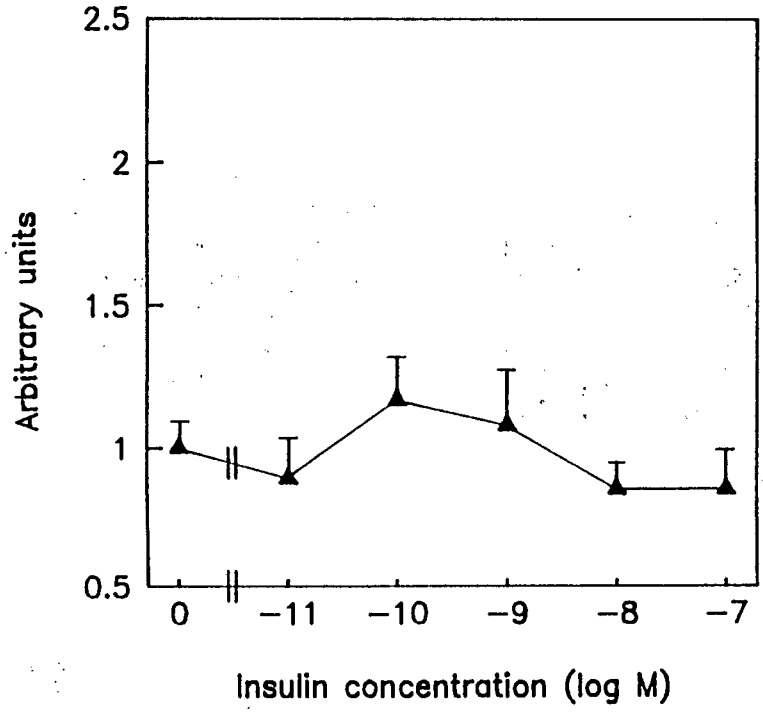

Figure 4.8 Effect of insulin added directly to isolated nuclei on the level of α-tubulin mRNA. Nuclei were incubated with insulin at concentrations ranging from 0,01 to 100 nM for 30 min and the total RNA isolated and probed. Each point is the mean result ± SD of three separate experiments.

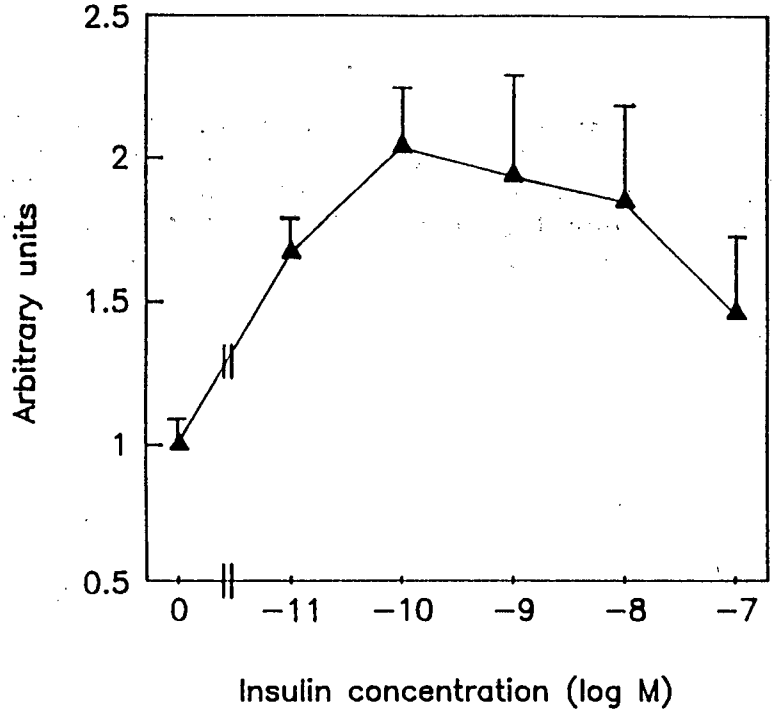

Figure 4.9 Effect of insulin added directly to isolated nuclei on the level of TAT mRNA. Nuclei were incubated with insulin at concentrations ranging from 0,01 to 100 nM for 30 min and the total RNA isolated and probed. Each point is the mean result ± SD of three separate experiments. There is a significant increase in the maximum level of TAT mRNA in insulin treated and untreated samples ($p < 0,005$). The difference in the maximum level of PK mRNA and the level of α-tubulin mRNA, a house-keeping gene, at corresponding insulin concentrations, is also significant ($p < 0,005$).

This result is depicted graphically in Figure 4.10. The same lack of response was also evident when transcription was continued for a further 30 min.

Insulin has been reported to decrease the constitutive rate of transcription of PEPCK in H4IIE cells. However, a more pronounced effect is observed when the cells are prestimulated with cAMP or dexamethasone prior to the addition of insulin (104). Whole cells were pretreated with dexamethasone or dibutyryl cAMP to stimulate the production of PEPCK mRNA prior to the isolation of the nuclei, and then subjected to insulin treatment in the *in vitro* system. No effect of insulin on the levels of PEPCK was noted.

4.3.4 EFFECT OF INSULIN ON THE LEVELS OF c-*fos* mRNA

The direct addition of insulin to isolated nuclei increased the amount of hybridizable mRNA for c-*fos* 2,6-fold after 30 min. This effect was concentration dependent being maximal at 1 nM and half maximal at 10-100 pM. This result is shown in Figure 4.11.

The amount of hybridizable mRNA for α-tubulin remained constant at all concentrations of insulin administration (Figure 4.8).

4.4 CONCLUSIONS

Insulin has a direct effect at the nucleus, in that it can regulate transcription when added to isolated hepatoma nuclei. This effect is observed when monitoring increases in total transcription in the nuclei, as well as increases in the levels of PK, TAT and c-*fos* mRNAs, three genes known to be regulated by insulin at the level of transcription in

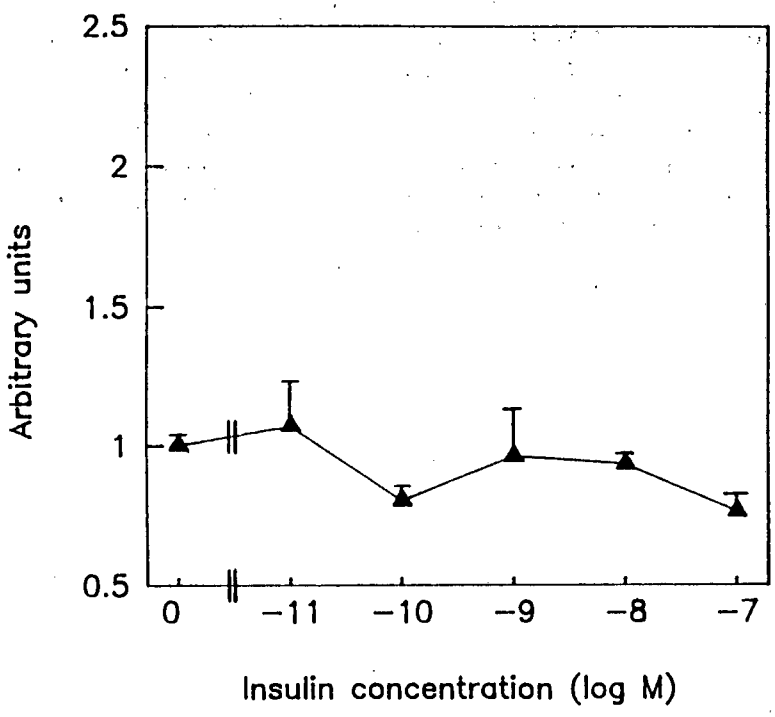

Figure 4.10 Effect of insulin added directly to isolated nuclei on the level of PEPCK mRNA. Nuclei were incubated with insulin at concentrations ranging from 0,01 to 100 nM for 30 min and the total RNA isolated and probed. Each point is the mean result ± SD of three separate experiments. There is no significant differenci in the levels of PEPCK mRNA in insulin treated and untreated samples ($p < 0,1$).

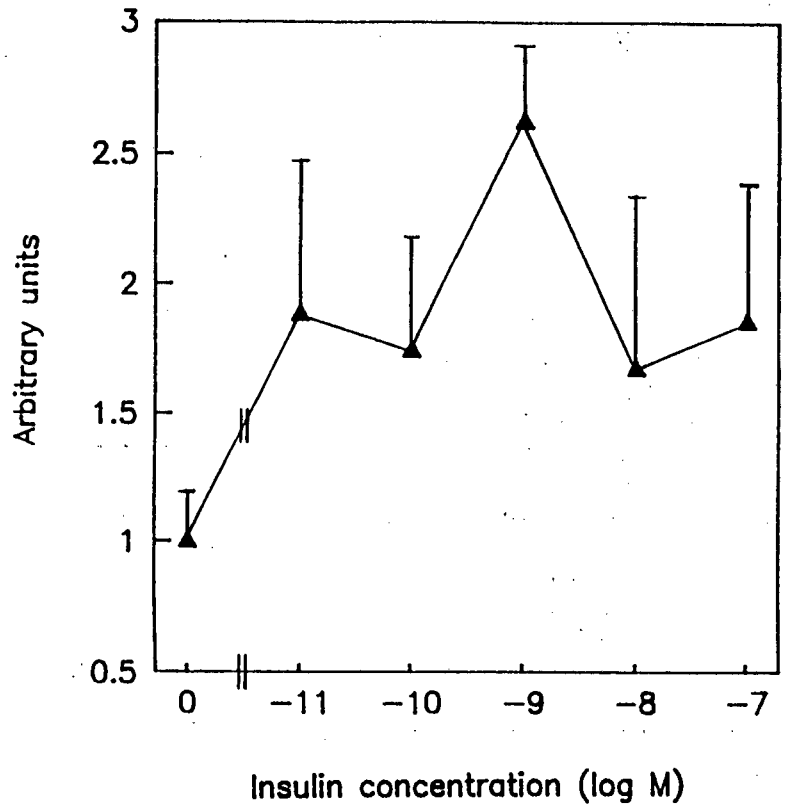

Figure 4.11 Effect of insulin added directly to isolated nuclei on the level of c-*fos* mRNA. Nuclei were incubated with insulin at concentrations ranging from 0,01 to 100 nM for 30 min and the total RNA isolated and probed. Each point is the mean result ± SD of three separate experiments. There is a significant increase in the maximum level of c-*fos* mRNA in insulin treated and untreated samples ($p < 0,005$). The difference in the maximum level of c-*fos* mRNA and the level of α-tubulin mRNA, a house-keeping gene, at corresponding insulin concentrations, is also significant ($p < 0,005$).

whole cells. The stimulation observed corresponds to the stimulation evident when insulin is administered to whole H4IIE hepatoma cells.

Insulin had no effect on the levels of PEPCK mRNA in isolated nuclei.

4.5 DISCUSSION

The mechanism whereby insulin regulates the expression of many genes in its target cells has always been a topic of much speculation and in particular, the means whereby signals elicited by insulin are transmitted from the plasma membrane to the nucleus to exert these effects.

Many reports have occurred showing the internalization of insulin and the subsequent localization of a proportion of that insulin to the nucleus in liver tissue (72-74,79). The binding of insulin to isolated liver nuclei has also been observed (81-83). These reports have not linked the nuclear association/ binding of insulin in a specific target tissue of the hormone directly to an effect on transcription.

The results of this study demonstrate that insulin can be its own mediator in regulating specific effects of gene expression. This brings added significance to the binding studies of insulin to isolated nuclei, as well as to studies relating to the function of internalized insulin.

Results of experiments in which insulin retained full biological activity when coupled to a large agarose complex unable to enter the cell, have been questioned, as the insulin-agarose complex is unstable in the presence of biological fluids and the released insulin is sufficient to account for all the activity detected (179). Thus, the

results reported now, are not contradictory to previous findings.

It has been reported that in rat liver, the effect of insulin on the increased expression of PK, as measured 18 h after injection of insulin, requires ongoing protein synthesis (99). The fact that insulin increased the transcription of PK in isolated nuclei within 30 min, may indicate that protein synthesis is required only for the ongoing effects of insulin and not for the immediate effects observed after 30 min of transcription *in vitro*.

The fact that no effect of insulin on PEPCK levels could be observed indicates that insulin exerts its positive and negative effects on transcription by different means. This result was unexpected, as PEPCK is the only gene of those employed in this investigation that is known to have insulin responsive sequences in the 5'end (122,125).

Although a definite effect of insulin on transcription *in vitro* was observed, the standard errors of the mean are large in comparison to those observed in whole cells. Several factors were found to play a role. These include the condition and degree of confluency of the cells prior to isolation of the nuclei, the speed of isolation of the nuclei and the freshness of the prepared solutions of insulin and nucleotides.

ATP and other NTPs inhibit the binding of insulin to its receptor on the plasma membrane (180). In order to try to maximize any binding to the nuclei, and so maximize possible responses mediated through binding, the nuclei were preincubated with insulin before the addition of the NTPs. This had no effect on the levels of incorporation monitored.

The fact that the response of the nuclei to insulin is reduced at high concentrations of the hormone is difficult to explain. In whole cells, this effect was attributed to a down-regulation of insulin receptors. Insulin purified by HPLC (see 5.1) gave the same effect, however, the presence of RNases or other interfering contaminants in the HPLC-purified insulin preparation cannot be excluded.

It is interesting to speculate on the mechanism by which insulin, interacting directly with the nucleus, can affect transcription. Several possibilities exist.

If insulin elicits its effects on transcription following binding to a nuclear membrane receptor, two main alternative pathways then arise. The first is that insulin does not enter the nucleus, but mediates its effects from the nuclear membrane. The second is that binding to the receptor facilitates the transport of insulin into the nucleus, where it affects gene expression. Insulin or an insulin-induced effector molecule could promote the effects on transcription through altered initiation, elongation (181) or termination (182). The exact locality of insulin in the nucleus is not clear. Some groups have found insulin mainly on the nuclear membrane (82), whereas others have reported its association with the nuclear matrix (79).

An important mechanism for the control of gene expression in eukaryotic cells is the regulation of transcription initiation. For genes transcribed by RNA polymerase II, accurate and efficient transcriptional initiation is dependent on multiple *cis*-acting DNA sequences, which constitute a transcriptional promoter (181). To date, four such *cis*-acting DNA sequences required for the regulatory control by insulin of the expression of specific genes have been identified in whole cells, through experiments with hybrid genes (145,140,33,125).

The *cis*-acting DNA elements recognize and bind one or more sequence-specific DNA binding proteins, which activate (or repress) transcriptional initiation (183). Omission of one or several of these factors may lead to reduction or even complete loss of transcriptional activity. Modulating the amount, activity or cellular localization of these factors may constitute an important mechanism for the control of the initiation of transcription (181,183,184). Insulin or an insulin-induced effector molecule could be regulating gene expression by acting as such a transcription factor, or alternatively, by controlling the amount, activity or subcellular location of specific transcription factors. If insulin binds to the transcription initiation complex in some way, it could stabilize this complex.

No direct binding of insulin to DNA has been observed (71). This would suggest that insulin acts via protein-protein rather than protein-DNA interactions. However, a monoclonal antibody, specific for the DNA-binding domain of the glucocorticoid receptor, also binds the B chain of insulin. The sequence of this epitope of the B chain is homologous to many sequences in DNA binding regions of steroid receptors (185).

Insulin or an insulin-induced molecule may also alter the local configuration of a gene, as such changes may determine the accessibility of the gene to the various cellular factors required for transcription. Local configuration changes can be affected through alterations in the methylation pattern of the gene (186).

These results open up many possibilities for further investigation as to the detailed mechanism of the stimulation of gene expression by insulin.

CHAPTER FIVE

THE BIOLOGICAL ACTIVITY OF INSULIN-ANALOGUES AT THE LEVEL OF TRANSCRIPTION

The analogues were synthesized by colleagues in this laboratory, to be used in independent studies relating to the intracellular location of insulin and to binding studies of insulin to nuclei or nuclear components. However, the interpretation of such studies is limited without the knowledge that the labelled molecule retains full biological activity. The following experiments were therefore conducted in order to ascertain the biological activity of the derivatives, and specifically, their effect on the accumulation of mRNAs for PK, TAT and PEPCK in whole H4IIE hepatoma cells. Insulin is known to regulate these mRNAs at the level of transcription (102,112,118).

The analogues studied were as follows:
2-(*p*-azidosalicylamido)-1,3'-dithiopropionate-insulin (ASD-insulin);
$N^{\alpha,B1}$-Biotinylinsulin (biotinylinsulin);
A14-[^{127}I]monoiodoinsulin (A14-[^{127}I]insulin).

5.1 SYNTHESIS OF INSULIN-ANALOGUES AND PREPARATION OF NATURAL INSULIN

Insulin was labelled with SASD (Sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate), the

heterobifunctional, photoreactive cross-linking reagent that is also iodinatable on the phenol ring. ASD-insulin was synthesized by G.P. Sabbatini according to the method of Knutson (187). The product was subjected to reversed phase HPLC (high pressure liquid chromatography) on a μ-Bondapak C-18 Radial Pak column to separate out the derivatized insulin from uncoupled insulin and SASD. All procedures involving SASD were performed under stringent red light conditions to avoid photoactivation of the azido moiety.

$N^{\alpha,B1}$-Biotinylinsulin was synthesized by P.J. Smith. N^{α}-Boc-Gly^{A1},N^{6}-Boc-lys^{B29} insulin (di-Boc-insulin) was prepared according to the method of Grant and von Holt (188). The di-Boc-insulin was biotinylated using *N*-Hydroxy-succinimidobiotin, and the product purified by reversed phase HPLC on a μ-Bondapak C-18 Radial Pak column.

A14-[^{127}I]monoiodoinsulin was synthesized by P.J. Smith according to the lactoperoxidase method of Linde et al. (189), and purified by reversed phase HPLC on a μ-Bondapak C-18 Radial Pak column according to the method of Benzi et al. (190). ^{127}I will be replaced by ^{125}I in binding studies.

Natural insulin was also purified by reversed phase HPLC on a μ-Bondapak C-18 Radial Pak column using a 0-100% acetonitrile gradient in 0,05% trifluoroacetic acid. Thus, any changes in the activity of the insulin-analogues could not be ascribed to the effects of solvents or procedures used in processing by HPLC.

5.2 THE REGULATION OF mRNA LEVELS BY INSULIN AND ITS ANALOGUES

The protocol used in the determination of the effects of insulin and its analogues on gene expression is the same as

that described in 2.2 and detailed in (8.4.1.1). H4IIE hepatoma cells were changed from growth in normal medium (8.2.1.1) to growth in serum-free medium. Fresh serum-free medium was added 20 h later with either natural insulin, ASD-insulin, biotinylinsulin or A14-[^{127}I]insulin at a concentration of 10 nM, for 3 h. Control cultures were re-fed fresh serum-free medium. Total RNA was isolated and the levels of specific mRNAs quantitated using dot blot hybridization to the labelled cDNAs for PK, TAT and PEPCK.

5.2.1 EFFECTS ON PK mRNA LEVELS

As has been shown in 2.2.1, natural insulin causes a marked increase in the levels of PK mRNA in H4IIE rat hepatoma cells. The levels of PK mRNA were 2,4-fold greater in insulin treated cultures than in non-treated cultures following 3 h of insulin treatment at 10 nM.

The three insulin-analogues were all able to increase the levels of PK mRNA in hepatoma cells, however, the relative biological potency varied. Biotinylinsulin displayed biological activity most close to that of natural insulin, the increase being 2,2-fold above that of the control. ASD-insulin and A14-[^{127}I]insulin caused increases of 1,93- and 1,65-fold respectively.

The result of this experiment is depicted graphically in Figure 5.1.

5.2.2 EFFECTS ON TAT mRNA LEVELS

The addition of insulin to H4IIE hepatoma cells maintained in serum-free medium resulted in a 1,67-fold increase in the levels of TAT mRNA following 3 h of insulin treatment at 10

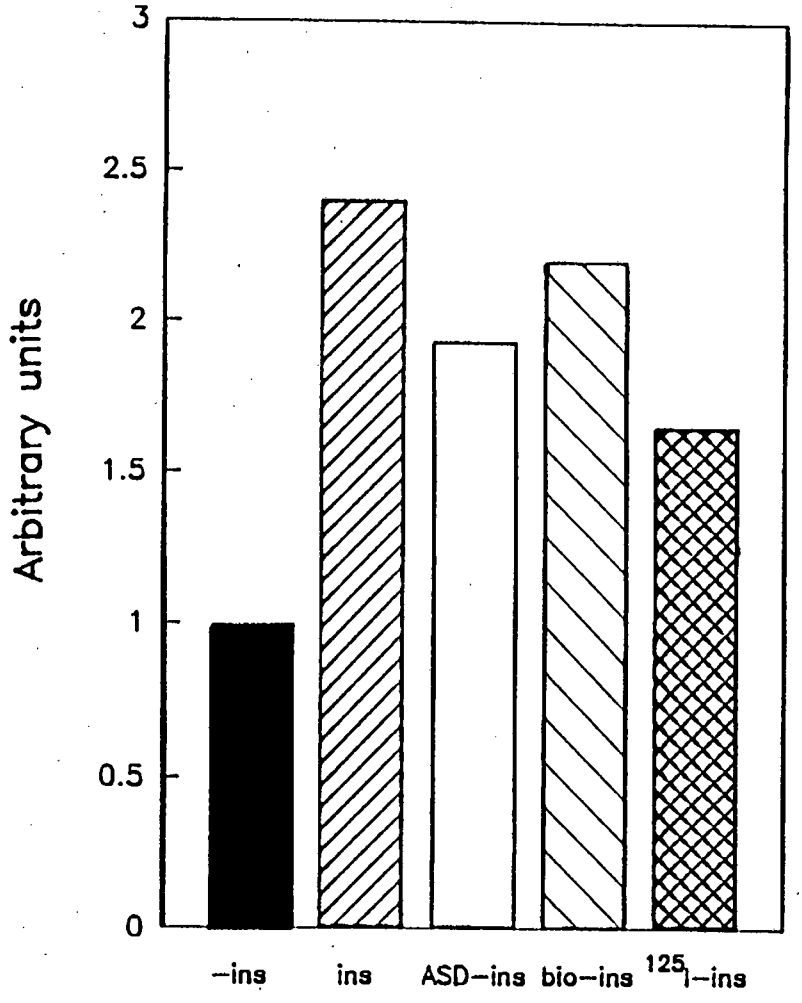

Figure 5.1 Stimulation of PK mRNA production by natural insulin (ins), ASD-insulin (ASD-ins), biotinylinsulin (bio-ins) or A14-[^{127}I]insulin ([^{127}I]-ins). H4IIE hepatoma cells were incubated with insulin or its analogue at 10 nM for 3 h and the total RNA isolated and probed. The average SEM for two separate experiments was 0,2.

nM insulin. This result has been described in 2.2.2.

The comparative effect of the addition of an insulin-analogue at 10 nM, for 3 h, is shown in Figure 5.2.

The insulin-analogues caused an increase in the levels of TAT mRNA in a manner similar to that observed in 5.1.1 for PK mRNA. Biotinylinsulin was the most active analogue, causing a 1,88-fold increase in the levels of TAT mRNA above those in control cultures without insulin. ASD-insulin treatment resulted in a 1,42-fold increase and A14-[^{127}I]insulin in a 1,31-fold increase in the total hybridizable TAT mRNA.

5.2.3 EFFECTS ON PEPCK mRNA LEVELS

In contrast to the increased levels of PK mRNA and TAT mRNA following insulin treatment, insulin causes a decrease in the levels of PEPCK mRNA in H4IIE hepatoma cells. This is a well known effect of the hormone, as has been reported by a number of laboratories (113,115). These findings were repeated for the purposes of this thesis. PEPCK mRNA levels, in cultures treated with 10 nM insulin, decreased to 24% of those in untreated control cultures (2.2.3).

The insulin-analogues studied were also effective in reducing the levels of PEPCK mRNA. Biotinylinsulin decreased PEPCK mRNA levels to 19%, ASD-insulin to 26% and A14-[^{127}] insulin to 28% of the levels in untreated control cultures.

The inhibition of PEPCK mRNA synthesis by insulin and its analogues is shown graphically in Figure 5.3.

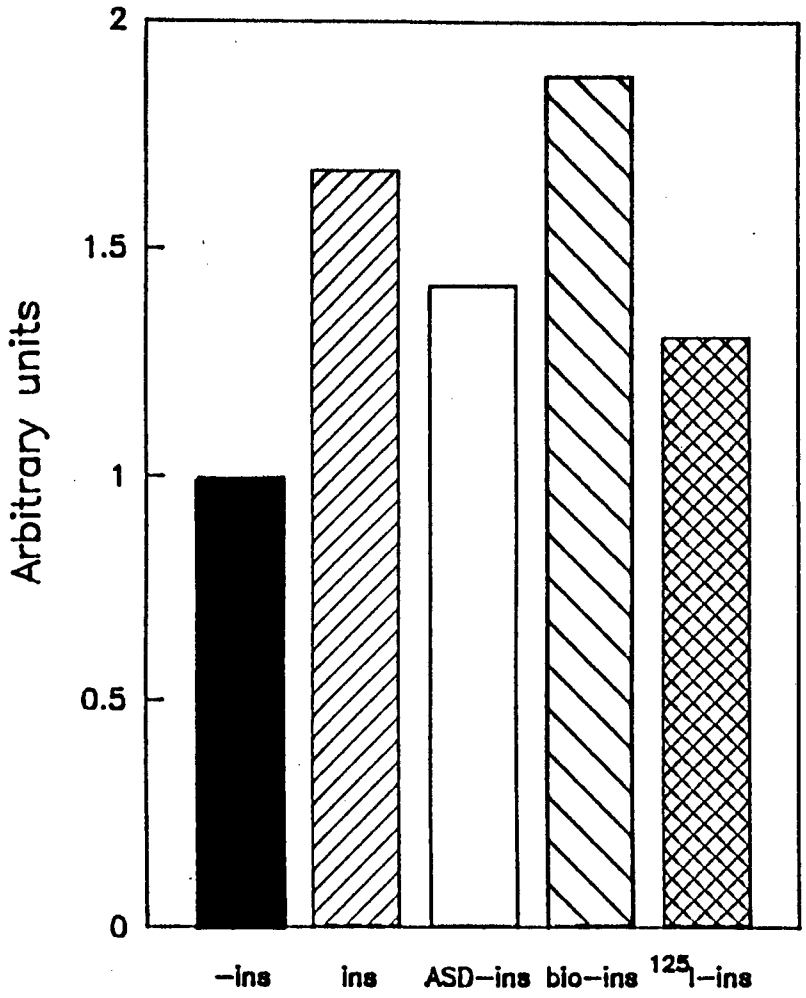

Figure 5.2 Stimulation of TAT mRNA production by natural insulin (ins), ASD-insulin (ASD-ins), biotinylinsulin (bio-ins) or A14-[^{127}I]insulin ([^{127}I]-ins). H4IIE hepatoma cells were incubated with insulin or its analogue at 10 nM for 3 h and the total RNA isolated and probed. The average SEM for two separate experiments was 0,2.

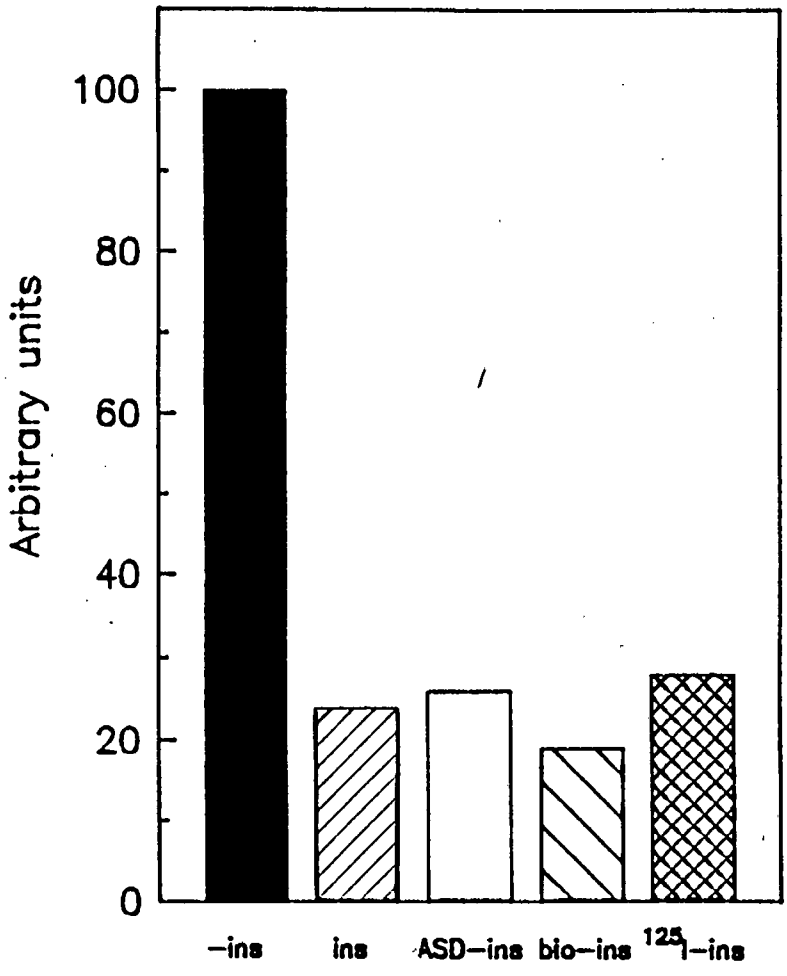

Figure 5.3 Inhibition of PEPCK mRNA production by natural insulin (ins), ASD-insulin (ASD-ins), biotinylinsulin (bio-ins) or A14-[^{127}I]insulin ([^{127}I]-ins). H4IIE hepatoma cells were incubated with insulin or its analogue at 10 nM for 3 h and the total RNA isolated and probed. The average SEM for two separate experiments was 0,2.

5.3 CONCLUSIONS

All the insulin-analogues studied are biologically active at the level of transcription. However, the relative biological potency appears to vary.

Biotinylinsulin has full biological activity at the level of transcription. Interestingly, in two instances, namely, the increase in TAT mRNA and the decrease in PEPCK mRNA, biotinylinsulin appeared more potent that natural insulin, resulting in mRNA levels marginally greater than those observed following treatment with natural insulin.

ASD-insulin was on average slightly less active than natural insulin, with values of comparative activity approximately equal to three quarters of that of natural insulin. Although there was considerable variation in the percentage difference from natural insulin, ASD-insulin in all three probes studied, showed less activity than natural insulin or biotinyl-insulin, but greater activity than A14-[^{127}I]insulin.

A14-[^{127}I]insulin had an average activity approximately equal to two thirds of that of natural insulin. This was, thus, the least transcriptionally active insulin-analogue.

In summary, the analogues of insulin studied, namely, biotinylinsulin, ASD-insulin and A14-[^{127}I]insulin, all induce transcriptional responses in H4IIE hepatoma cells that are of similar magnitude to those observed following treatment with natural insulin.

5.4 DISCUSSION

The interpretation of the test results can only be done within the framework of the limitations of the test used, i.e. the measurement of the levels of a specific mRNA at one insulin concentration and at one time point. Thus, the reported biological activities do not necessarily give absolute values, but rather, an indication that differences do exist, as for each mRNA studied a specific dose and time course is most likely.

Biotin can be attatched to a macromolecule under mild conditions with a high degree of efficiency and limited loss of biological activity. It has proved to be a useful tool in the labelling of many biologically significant molecules (191,192). Hofmann et al. (193), first synthesized biotinylinsulin and showed it to have biological activity 94% of that of natural insulin on lipid synthesis assays in isolated rat epididymal fat cells. Biotinylinsulin-avidin complex has been used in insulin receptor studies (194). The full biological activity of biotinylinsulin at the level of transcription, adds credence to this already useful insulin derivative.

ASD-insulin is a relatively new tool in insulin studies. It was previously synthesized by Knutson and used in the study of the dynamics of the plasma membrane insulin receptor (187).

Much controversy has surrounded the use of radioactive iodine as a tracer in insulin studies. Iodinated insulin with a high specific activity is readily prepared, and for this reason, has remained a popular marker despite reports that iodination may result in a loss of biological activity (195). A14-[^{127}I]insulin, prepared using the lactoperoxidase method, is the only iodinated insulin analogue that has been

shown to retain the activity of natural insulin in the stimulation of lipogenesis (189) and to exhibit maximal insulin binding (196). However, the activity of A14-[^{127}I]insulin at the level of transcription has not previously been determined. Although the results obtained indicate the reduced biological potency of A14-[^{127}I]insulin at the level of transcription, this effect may have possibly been due to lower concentrations of A14-[^{127}I]insulin at the plasma membrane of the cells. A14-[^{127}I]insulin is more hydrophobic than either biotinylinsulin or ASD-insulin and therefore, more of the A14-[^{127}I]insulin could have been lost due to non-specific binding of the insulin to various surfaces.

CHAPTER SIX

THE EFFECT ON mRNA LEVELS OF NERVE GROWTH FACTOR ADDED DIRECTLY TO ISOLATED PC12 NUCLEI

The previous studies reported in Chapter 4, detailed the stimulation by insulin, of the transcriptional activity of rat hepatoma nuclei. The present investigation extends these observations, by demonstrating that a second peptide hormone, namely, nerve growth factor, can induce similar effects in the nuclei of its target cells.

Nerve growth factor (NGF) is a peptide hormone that is essential for the development and survival of sensory and some sympathetic neurons. NGF influences many aspects of cellular metabolism including ion flux, neurotransmitter synthesis, protein phosphorylation, protein synthesis and RNA synthesis. (Reviewed in (197)).

The PC12 cell line, derived from a rat adrenal medulla pheochromocytoma, has proved extremely useful in the study of NGF action at the molecular level (198). Although NGF is not required for the survival of these cells, it can stimulate differentiation and neurite outgrowth. In the presence of NGF, PC12 cells take on many of the properties of the mature sensory nerve (198).

NGF alters the expression of several genes in PC12 cells. The genes induced by NGF include β-actin (199), c-*fos* (199,200), c-*myc* (199), ornithine decarboxylase (ODC)(201), Thy-1 (coding for a cell surface glycoprotein)(202), NF68 (coding for a neurofilament protein)(202), GAP-43 (203), NGF1-A (204,205), genes coding for sequences related to calcium-binding proteins (206), PC4 (an interferon-related

gene)(207), and several unknown genes, *a-2*, *d-4* and *d-5* (208). The induction of some of these genes is required for neurite formation to occur (199), while others are possible transcription factors (204). However, the precise role of these genes in NGF's mechanism of action is unclear.

The mechanism by which NGF regulates gene expression is not known. Results of studies of four genes regulated by NGF in PC12 cells have indicated that it is complex, involving several distinct mechanisms (208). Two adjacent promoter elements, one of which is the serum response element (SRE), have been identified in the c-*fos* gene, and can mediate NGF induction of c-*fos*. The two sequences confer inducibility independently of each other, suggesting that different pathways are involved in their activation (209).

The role of second messengers in mediating the effects of NGF have been studied. cAMP can mimic many of the effects of NGF, including neurite outgrowth (210). However, cAMP cannot be the mediator of all of the responses to the hormone. For example, clonal variants of PC12 cells with defects in cAMP-dependent protein kinase activity, did not induce ODC in response to adenosine or cAMP analogues, but were completely responsive to NGF (211). Diacylglycerol was also investigated as a possible mediator of NGF effects, as NGF is known to activate protein kinase C (212). However, phorbol 12-myristate 13-acetate (PMA), a phorbol ester which also activates protein kinase C, was unable to mimic the effects of NGF on neurite outgrowth (213).

An alternative possibility is that internalized NGF or the NGF-receptor complex, could mediate the effects on gene expression. Intact NGF, bound to high-affinity receptors, has been found on the nuclear membrane, and neurite outgrowth is dependent on NGF reaching the nucleus (197). NGF binding sites have also been identified on the chromatin

of PC12 cells (214), but the significance of these binding sites is not known. These observations suggest the direct involvement of NGF in mediating effects on gene expression.

The following preliminary studies were conducted in order to further investigate the general applicability of the proposed mechanism for peptide hormone action directly at the level of the nucleus.

6.1 EFFECT OF NGF ON THE LEVELS OF mRNAs FOR c-*fos*, ODC AND α-TUBULIN IN PC12 CELLS

NGF has been shown to induce a rapid and transient increase in c-*fos* gene expression. The effect is maximal after 30 min and returns to basal levels after 1 h of exposure of PC12 cells to NGF at 50 ng/ml (199,200). The reported induction of ODC by NGF in PC12 cells is a delayed response, being maximal after 4 to 6 h of treatment at 100 ng/ml (201). The levels of α-Tubulin mRNA are not significantly affected by NGF and were thus used as an internal control (199).

PC12 cells were cultured routinely as described in 8.2.1.1. Mouse 2.5S NGF was added to the cells at a concentration of 50 ng/ml, in fresh Dulbecco's Modification of Eagle's Medium (DMEM), containing 1% horse serum. RNA was isolated from the cells immediately, and after 30 min and 6 h of NGF treatment (8.3.2). The effects of NGF on the levels of the mRNAs coding for c-*fos*, ODC and α-tubulin were determined using a dot blot hybridization assay (8.4.2.1) with cDNA probes labelled by nick-translation (8.3.3.2).

The results of the experiment are reported in Table 6.1. The values are the mean result of duplicate samples from a single experiment.

Two flasks, one with and one without NGF were kept in the incubator for 48 h to assess the ability of the added NGF to stimulate neurite outgrowth.

Table 6.1 The effect of NGF treatment of PC12 cells on the levels of mRNA coding for c-*fos*, ODC and α-tubulin. Values represent arbitrary units taken from densitometer readings of the dot blots.

cDNA probe	Time 0 h	0,5 h	6 h
c-*fos*	1,0 (± 0,5)	105 (± 17)	2,0 (± 1)
ODC	1,0 (± 0,3)	1,3 (± 1)	15 (± 5)
α-tubulin	1,0 (± 0,2)	1,0 (± 0,1)	1,1 (± 0,2)

The response of the PC12 cells maintained in culture in this laboratory, to added NGF, has been determined visually by monitoring neurite outgrowth. The response at the level of transcription has now also been confirmed. NGF caused a 105-fold increase in the level of c-*fos* mRNA isolated from these cells. This response is in keeping with published values of greater than 100-fold induction (199). The maximal effect of NGF on ODC mRNA levels has been reported to be a 20- to 40-fold induction at 100 ng/ml. At 50 ng/ml the expected levels are reduced by approximately 3% (201). The levels obtained in this experiment of a 15-fold induction are thus lower than expected, but are still a very positive indication of the induction of ODC. The levels of α-tubulin mRNA remained constant at all three time intervals measured.

6.2. ISOLATION OF PC12 NUCLEI

Nuclei were isolated from logarithmically growing PC12 cultures that had reached approximately 80% confluency, according to the melittin method described in 8.3.1.

Melittin was used at a final concentration of 1 mg/10^8 cells. The cells required 3-5 strokes of the tight-fitting hand-held glass Dounce homogenizer to disrupt the cell membrane. Nuclei were isolated in yields of 90-100% at greater than 99% purity (see 3.2.2).

6.3 EFFECT OF NGF ON THE LEVELS OF THE mRNAs FOR c-*fos*, ODC AND α-TUBULIN WHEN ADDED TO ISOLATED NUCLEI

The *in vitro* system developed for use with the H4IIE hepatoma nuclei was used for the PC12 nuclei without modification (8.4.1.3). The parameters of the concentrations of $MgCl_2$ and KCl were not altered as a good incorporation of labelled nucleotide was observed.

NGF was added to the *in vitro* transcription system described in 8.4.1.4. Nuclei were incubated with or without NGF (50 ng/ml) for 20 or 40 min and the RNA isolated according to the method described in 8.3.2. The effects of NGF on the levels of the mRNAs coding for c-*fos*, ODC and α-tubulin were determined using a dot blot hybridization assay (8.4.2.1) with cDNA probes labelled by nick-translation (8.3.3.2).

6.3.1 EFFECT ON LEVELS OF c-*fos* mRNA

The effect of NGF on the levels of c-*fos* mRNA in the PC12 nuclei is shown in Figure 6.1.

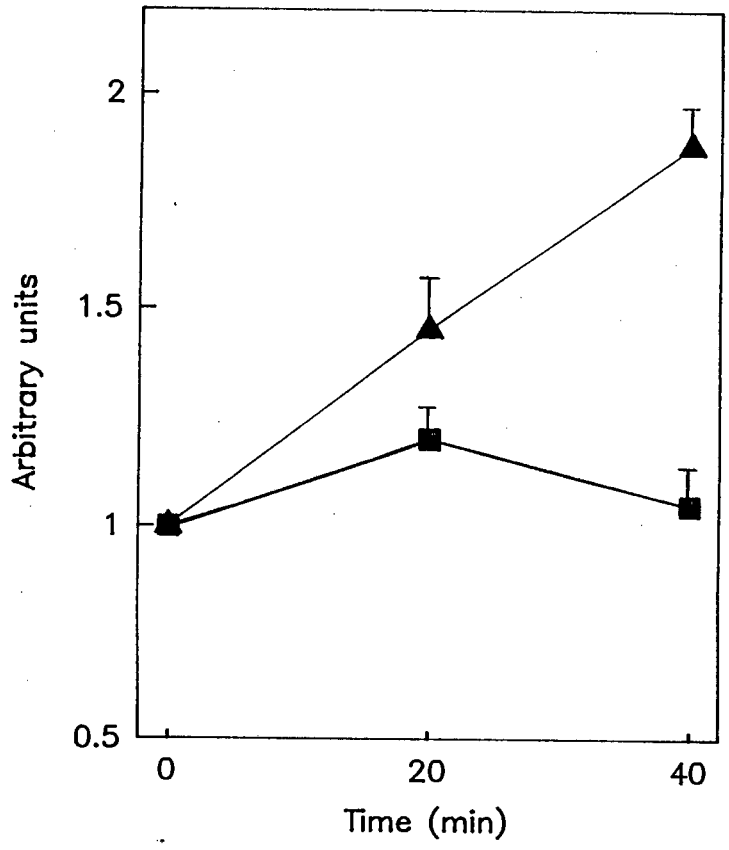

Figure 6.1 Effect of NGF added directly to isolated PC12 nuclei on c-*fos* mRNA levels. Nuclei were incubated for 20 or 40 min in an *in vitro* transcription assay with NGF at 50 ng/ml. Each value is the mean result of duplicate samples from a single experiment ± SEM.

The levels of c-*fos* mRNA increased in both the NGF treated and untreated incubations after 20 min. However, after 40 min of incubation, c-*fos* mRNA levels continued to increase in the NGF treated incubations, whereas no further increase was observed in untreated incubations. c-*fos* mRNA increased 1,88-fold after 40 min of NGF treatment at 50 ng/ml. Thus, NGF added directly to isolated PC12 nuclei has an effect on the levels of c-*fos* mRNA.

6.3.2 EFFECT ON LEVELS OF ODC mRNA

No effect of NGF on isolated nuclei was observed when monitoring the changes in the levels of ODC mRNA following exposure of the nuclei to NGF at 50 ng/ml. ODC mRNA was present in the nuclei but no changes in the levels occurred in the time course monitored.

6.3.3 EFFECT ON LEVELS OF α-TUBULIN mRNA

α-Tubulin mRNA levels also remained unchanged following NGF treatment or incubation in the transcription system for the 40 min time period monitored.

6.4 CONCLUSION

NGF can specifically increase the levels of c-*fos* and ODC mRNAs in PC12 cells maintained in culture. The levels of α-tubulin mRNA remain unchanged.

NGF can increase the levels of c-*fos* mRNA (a rapid response gene) when added directly to isolated nuclei whereas it does not alter the levels of ODC mRNA (a delayed response gene). As expected, the levels of α-tubulin mRNA remain constant.

6.5 DISCUSSION

The increased levels of c-*fos* mRNA in NGF treated and untreated incubations after 20 min are probably due to the run-off transcription of the c-*fos* gene which was induced prior to the isolation of the nuclei. The nuclei were isolated from cells that were maintained, according to routine culture procedures, in medium containing a total serum concentration of 15%. Thus, it is not unlikely that c-*fos* genes could be actively transcribing. However, the fact that this effect is not prolonged in incubations without NGF, but continues to increase in NGF treated incubations, indicates that NGF is playing a role in the isolated nucleus. Although firm conclusions cannot be drawn from a preliminary investigation, this observation does corroborate the findings of insulin's effects on hepatoma nuclei and thus, adds further to the proposed theory that peptide hormones can exert a direct effect on transcription at the level of the nucleus.

In a recent publication, Rudkin et al. demonstrated that the action of NGF on PC12 cells is cell cycle specific. Induction of c-*fos* by NGF was greater in serum-starved cells than in exponentially growing cultures (215). Further studies on the regulation of transcription by NGF should incorporate this knowledge. Serum-starvation would also be expected to result in a more significant increase in c-*fos* levels in isolated nuclei at early time intervals.

The lack of an NGF effect on ODC mRNA levels in isolated nuclei is not unexpected. Up to 66% of the increased levels of ODC mRNA observed in PC12 cells following NGF treatment, are known to require protein synthesis (201). Also, as the effect in whole cells is a long term one requiring 4-6 h for induction, a diminished response, if any, was expected.