Biochem. J. (1991) 278, 609–619 (Printed in Great Britain)

REVIEW ARTICLE
Regulation of gene expression by insulin

Richard M. O'BRIEN and Daryl K. GRANNER
Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, 707 Light Hall, Nashville, TN 37232-0615, U.S.A.

INTRODUCTION

Insulin initiates its action by binding to a specific cell-surface receptor that is ubiquitously distributed [1]. The insulin receptor possesses an intrinsic tyrosine kinase activity [1,2] which is stimulated by insulin binding [3]. Evidence suggests a crucial role for this enzyme in mediating insulin's action [3], although physiologically important substrates, apart from the receptor itself, have yet to be identified [4].

Insulin performs a central role in homeostasis by regulating the activity or amount of critical proteins. Insulin-induced changes in the activity of enzymes through the mechanism of (serine/threonine) phosphorylation/dephosphorylation have been analysed in detail [5]. Recently there has been much interest in the identification of the insulin-stimulated serine/threonine kinases [6–9] and phosphatases [9] which catalyse these modifications. However, the connection between the insulin receptor tyrosine kinase and insulin-activated (serine/threonine) kinases/phosphatases has yet to be identified for any specific effect of the hormone [10].

In recent years it has become apparent that insulin also exerts profound effects on various cellular processes by altering the amount of critical proteins (see [11] for review). This facet of insulin action may not be mutually exclusive with its effect on enzyme activity: common signalling pathways may mediate both effects. Insulin changes the rate of protein synthesis in two general ways. First, it affects the rate of protein synthesis in selected tissues (liver, adipose tissue, skeletal and cardiac muscle) at the level of mRNA translation (see [12] for review). Total protein increases in such tissues without a similar change in total mRNA. Secondly, it also has positive and negative effects on the expression of specific genes (Table 1). The regulation of specific gene expression by insulin is clearly a major action of this hormone; in addition to the more than 50 known specific examples, numerous insulin-regulated genes of unknown identity have been detected in adipose tissue, skeletal and cardiac muscle and liver by using two-dimensional polyacrylamide-gel electrophoresis [105].

The genes regulated by insulin encode proteins involved in a variety of biological phenomena. Several of these mRNAs direct the synthesis of enzymes that have a well-established metabolic connection to insulin, while others represent major secretory proteins/hormones, integral membrane proteins and oncogenes/transcription factors (Table 1). The production of ovalbumin and casein is involved in reproductive function in birds and mammals; thyroglobulin plays an integral role in thyroid function and δ-crystallin is a structural protein. Insulin therefore regulates genes that represent a spectrum of different functions in a number of tissues, including liver, adipose tissue, muscle, connective tissue, pancreas, oviduct, mammary gland, lens and thyroid.

In this review we initially discuss some general considerations that are important in the study of insulin-regulated gene expression. We then concentrate on those genes regulated at the transcriptional level and describe the various approaches that can be used to define insulin-response DNA elements/sequences (IREs/IRSs). Several such elements have recently been delineated. Finally, progress on the identification of the *trans*-acting factors associated with these elements is reviewed and models that might explain their mechanism of action are proposed.

GENERAL CONSIDERATIONS

As shown in Table 1, the list of insulin-regulated genes is extensive and rapidly growing—compare with the list in [106]. Several different approaches have been used to establish that insulin regulates the expression of a particular gene. Some of these are only indirect. Moreover, the response of a given gene to insulin may vary between different tissues, between tissues and derived cell lines, and even between different cell lines from the same tissue source. As the examples below describe, this area of insulin-related research is no less frustrating, complex and arcane than others.

Tissue-specific variations in the response of a given gene to insulin are well-documented. Thus, insulin stimulates glucokinase transcription in the liver [45,46]; however, in the pancreatic β cell glucokinase activity is regulated by glucose [107]. This difference is explained by the observation that alternative promoters are utilized in these tissues [108]. Insulin has a selective effect on the expression of the GLUT2 glucose transporter in the pancreatic β cell compared to liver, though in this case the reason for the difference is unknown [55]. Similarly, the molecular basis for insulin's repression of phosphoenolpyruvate carboxykinase (PEPCK) expression in liver and adipose tissue but not kidney is unknown [106].

In many cases a direct effect of insulin on gene expression has not yet been established. Thus, the conclusion that insulin affects aldolase B and ATP–citrate lyase gene expression is based on the indirect observation that re-feeding starved mice or rats a high-carbohydrate/low-fat diet, a treatment that raises plasma insulin levels, increases the level of the respective mRNA [15,47]. Suitable tissue culture systems (primary cell culture, cell lines or tissue explants) are not currently available to study the hormonal regulation of several genes, including amylase [82–84], protein-disulphide isomerase [28], the GLUT2 glucose transporter [55], brown adipose tissue uncoupling protein [35,36] and the growth hormone receptor [50,51]. Thus, the conclusion that insulin regulates the expression of these genes is based on studies of diabetic and/or insulin-injected animals. However, studies of insulin action, particularly in intact animals, are complicated by the reciprocal effects of the so-called 'counter-regulatory' hormones that are secreted in response to insulin-induced hypoglycaemia. Many of these hormones regulate the gene in question

Abbreviations used: IRE, insulin response element; IRS, insulin response sequence; PEPCK, phosphoenolpyruvate carboxykinase; HRE, hormone response element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; GRU, glucocorticoid response unit.

Table 1. Insulin-regulated genes

* Indicates that the effect of insulin has been shown to be at the transcriptional level by use of the 'run-on' assay. Arrows indicate whether the effect of insulin is stimulatory (↑) or inhibitory (↓). In some cases, depending on the tissue culture system or tissue under investigation, insulin may have either positive or negative effects on gene expression.

Enzyme	Effect	Reference
Intracellular enzymes		
Pyruvate kinase*	↑	[13,14]
ATP citrate lyase	↑	[15]
Serine dehydratase*	↓	[16]
Fatty acid synthetase*	↑	[17]
Glutamine synthetase*	↑↓	[18–20]
Ornithine decarboxylase	↑	[21]
Tyrosine aminotransferase*	↑↓	[22–24]
Aspartate aminotransferase	↓	[25]
Fructose-1,6-bisphosphatase	↓	[26,27]
Protein disulphide isomerase*	↓	[28]
Carbamoyl-phosphatase synthetase I	↓	[29]
Phosphoenolpyruvate carboxykinase*	↓	[30,31]
Glucose-6-phosphate dehydrogenase	↑	[32]
Glycerol-3-phosphate dehydrogenase*	↑	[33,34]
Brown adipose tissue uncoupling protein	↑	[35,36]
Glyceraldehyde-3-phosphate dehydrogenase	↑	[37,38]
6-Phosphofructo-2-kinase/ fructose-2,6-bisphosphatase*	↑	[39–42]
Malic enzyme*	↑	[43,44]
Glucokinase*	↑	[45,46]
Aldolase B	↑	[47]
Integral membrane proteins		
Insulin receptor	↑↓	[48,49]
Growth hormone receptor	↑	[50,51]
Glucose transporter* (GLUT1)	↑	[52–54]
Glucose transporter (GLUT2)	↑	[55]
Glucose transporter (GLUT4)	↑	[56–58]
Proteins involved in reproduction		
Casein*	↑	[59–61]
Ovalbumin*	↑	[62]
Secreted proteins/hormones		
IGF 1	↑	[50,63,64]
IGF 2	↓↑	[65]
Prolactin*	↑	[66]
Glucagon*	↓	[67]
Growth hormone*	↓	[68]
Apolipoprotein B	↓	[69,70]
Lipoprotein lipase	↑	[71–73]
Hepatitis B surface antigen	↓	[74]
Pulmonary surfactant apolipoprotein A	↓	[75]
IGF binding protein 1	↓	[76–78]
IGF binding protein 2	↓	[79]
α-2$_u$ globulin*	↑	[80,81]
α-Amylase	↑	[82–84]
Albumin*	↓↑	[85,86]
Adipsin*	↓↑	[33,87–89]
Transcription factors		
c-*fos**	↑	[90–92]
egr-1	↑	[93]
c-*jun*, Jun B, Jun D	↑	[93]
c-*myc*	↑	[91]
Miscellaneous		
Gene 33*	↑	[94–96]
Thyroglobulin*	↑	[97]
Proline-rich protein	↑	[98]
Quiescence-specific gene	↓	[99]
δ-Crystallin*	↑	[100,101]
hsp 70	↑	[102]
S14*	↑	[103]
c-Ha-*ras*	↑	[104]

[40], so it is difficult to sort out cause–effect relationships. This observation may explain the disparate results obtained with insulin between animals and tissue culture with regards expression of the c-*fos* [16,90], fatty acid synthetase [17,179], malic enzyme [43,44] and albumin [86,109] genes. Studies in which hypoglycaemia is prevented, by use of a 'glucose clamp', obviate some of these problems, and can also be used to analyse whether glucose metabolism is required for an insulin response [76,110]. In the case of the glucose transporter gene family (for review, see [111,112]), changes in circulating glucose levels cannot explain the disparate results obtained for the regulation of GLUT1 and GLUT4 by insulin *in vivo* [56–58] and *in vitro* [52–54,111].

Clearly, tissue culture cell lines and primary cell culture are the systems of choice since they allow one to control the cellular environment exactly. It is possible, using these systems, to ask questions about the involvement of other hormones, or other factors such as glucose or amino acids. Thus, in a number of instances, the effect of insulin on gene expression is permissive, i.e., the effect of insulin is augmented by, or only seen in the presence of, other factors. For example, glucose is required for insulin's effect on pyruvate kinase [113,114] and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [41]. With respect to the ovalbumin [62] and casein [59,60] genes, oestrogen and prolactin/hydrocortisone, respectively, are the permissive factors.

Tissue culture has its own intrinsic complications. Thus, the effect of insulin on the expression of the genes for tyrosine aminotransferase [22–24], glutamine synthetase [18,19], albumin [85,86], lipoprotein lipase [71–73] and the insulin receptor [48,49] varies with the cell line or primary cell culture studied, even though these cells originated from the same tissue. Moreover, in some cases such as adipsin [33,52] and growth hormone [66,68], the effect of insulin varies within clones of the same cell line.

Physiological significance

As Table 1 clearly shows, the list of genes regulated by insulin is impressive and complex. However, the physiological significance of the regulation of many of these genes is unclear, mainly through lack of hard information. The half-life of the protein encoded by a given gene will determine whether the protein level oscillates significantly on a daily basis as insulin rises and falls after each meal. In cases where the protein has a long half-life insulin may normally only play a tonic, permissive role such that large variations in protein levels are only seen in extreme conditions such as starvation or diabetes.

The regulation of the genes of hepatic glucose metabolism are the best characterized with respect to physiological considerations (for review see [40]). The expression of the genes encoding the six key enzymes of glycolysis/gluconeogenesis are tightly and coordinately regulated [40] and it is interesting to speculate that this is because glucose is almost the only fuel source used by the brain. As explained above, studies of the effect of insulin on the expression of these genes in intact animals are complicated by the effects of 'counter-regulatory' hormones such as glucagon. This may be less of a problem in cases where the effect of insulin is dominant over glucagon (e.g. PEPCK; see [31]); however, in other genes (e.g. pyruvate kinase) the effect of glucagon is dominant [113]. Moreover, measurements of enzyme activity are

complicated in cases (e.g. pyruvate kinase) where insulin also has an effect on enzyme activity *per se*.

INSULIN CAN REGULATE GENE EXPRESSION AT SEVERAL STEPS

The steady-state level of a given mRNA represents the balance of its synthesis, nuclear processing and degradation, transport from nucleus to cytoplasm, and cytoplasmic degradation (see [12] for review). In addition to all these events, regulation of the translational efficiency of a given mRNA can determine the steady-state level of a protein [69,70]. Insulin probably regulates all of these steps in mammalian gene expression, but most attention has been given to its role in influencing the transcription of specific genes. Those genes regulated by insulin at the transcriptional level (only those measured directly, by using a 'run-on' transcription assay, are included in this list) are highlighted in Table 1.

In several cases insulin has both transcriptional and post-transcriptional effects, namely tyrosine aminotransferase [23,24], glycerol-3-phosphate dehydrogenase [33,34], prolactin [66], glucagon [67], albumin [86], malic enzyme [44], δ-crystallin [100], pyruvate kinase [114] and perhaps gene 33 [95,96]. In most cases the step at which post-transcriptional regulation occurs is unknown, though insulin has been shown to stabilize glycerol-3-phosphate dehydrogenase and pyruvate kinase mRNAs [33,34,114].

Although it is clear that insulin can regulate gene expression at several levels, the mechanism of this action of insulin remains an enigma [5]. In fact, several different mechanisms of insulin action probably exist. This may explain why the effect of insulin on the transcription of the albumin [86,109], casein [60] and pancreatic amylase [82] genes is slow in onset, whereas insulin has a rapid effect on transcription of the PEPCK [31], glucokinase [45], gene 33 [95] and c-*fos* [92] genes.

THE *cis/trans* MODEL OF TRANSCRIPTIONAL CONTROL

The *cis/trans* model of transcriptional control is used as a working hypothesis in studies of the regulation of gene transcription by insulin. The fidelity and frequency of initiation of transcription of eukaryotic genes is mediated by the interaction of *cis*-acting DNA elements with a variety of *trans*-acting factors. In eukaryotic cells a *cis*-acting element regulates contiguous DNA and does not code for a protein. A *trans*-acting factor is expressed by a gene not associated with the DNA sequence being regulated. In this *cis/trans* model two general types of DNA elements are involved. One class, located near the transcription initiation site, generally acts in an orientation- and position-dependent manner to regulate basal transcription and assure the accuracy of initiation. Another class of *cis*-acting elements, and their associated *trans*-acting factors, is often located farther from the initiation site and acts in an orientation- and position-independent manner to promote (enhance) or inhibit (silence) transcription. These elements can function in the context of their cognate promoter, or when attached to a heterologous promoter.

Most hormone response elements (HREs) fall into the enhancer/silencer class of *cis*-acting elements since these DNA sequences, and their associated *trans*-acting factors, can function through heterologous promoters and in a relatively position- and orientation-independent manner [115]. However, in some cases HREs also function as basal promoter elements [116]. Various combinations of positive and negative *cis*-acting DNA elements are also involved in tissue-specific and growth- or differentiation-related control of transcription.

USE OF FUSION GENES TO IDENTIFY INSULIN RESPONSE SEQUENCE(S)

The technique of fusion gene analysis [117] is used to identify HREs. A region of the promoter of the gene of interest is ligated to a reporter gene, such as that encoding the bacterial enzyme chloramphenicol acetyltransferase (CAT), which is not normally expressed in eukaryotic cells. The presence of an HRE is proven if the DNA segment confers hormone responsiveness upon the otherwise unresponsive reporter gene. Additional fusion genes containing progressively shorter regions of promoter DNA are used to localize the HRE. It is generally assumed that an effect on a reporter gene reflects an increase of transcript initiation caused by the *cis*-acting DNA element. This is not necessarily the case, as elements in the 5′ flanking region of genes also regulate transcript elongation [118], which is a very different mechanism of control. Therefore, the fusion gene analysis should follow, and not supplant, the 'run-on' transcription assay. When analysing fusion gene expression it is important to establish that normal physiology is reproduced, and it follows that the hormonal regulation of the fusion gene should mimic that of the endogenous gene. In addition, the effect of various concentrations of hormones on expression of the fusion gene and the endogenous gene should be comparable if both are regulated by similar mechanisms. If so, one can infer that the same regulatory mechanisms are operative in each case.

Four approaches have been used to analyse the effect of insulin on fusion gene constructs. These approaches, which have been described in detail elsewhere [117], are illustrated with specific examples in each case.

TRANSIENT TRANSFECTION

This technique requires the availability of a tissue culture system in which the gene of interest is expressed and regulated by insulin. The fusion gene construct, in the form of supercoiled plasmid DNA, is introduced into these cells by a variety of techniques, most commonly as a calcium phosphate co-precipitate, or alternatively, by lipofection or electroporation. Following transfection, cells are incubated in the presence or absence of insulin and after an appropriate period of time, between 4 and 60 h depending on the cell line under study, the cells are harvested and the expression of the reporter mRNA or protein is analysed. The supercoiled plasmid DNA is only expressed transiently in the cell nucleus prior to inactivation/degradation; hence the name of the technique. Transient transfection experiments have been used to analyze the effect of insulin on several genes, including c-*fos* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

c-*fos*

c-*fos* is the cellular homologue of the transforming gene of FBJ murine osteosarcoma virus [119]. Insulin stimulates c-*fos* gene transcription in H4IIE cells [92] and the rapid accumulation of c-*fos* mRNA in 3T3-L1 fibroblasts, adipocytes [90] and Chinese hamster ovary (CHO) fibroblasts [120]. In contrast, earlier studies showed no effect of insulin on c-*fos* mRNA levels in primary cultures of rat hepatocytes [121], PC12 rat pheochromocytoma cells [122] and murine fibroblasts [123]. The reason for these differences is unclear but may in part reflect a requirement for additional tissue-specific transcription factors whose expression varies between cells.

Phorbol esters and insulin induce a similar increase in c-*fos* mRNA levels in 3T3-L1 adipocytes [90] and H4IIE hepatoma

cells [91] though insulin acts through a protein kinase C-independent pathway [90,91]. The response of the c-*fos* gene to insulin is modified by protein synthesis inhibitors such as cycloheximide. In 3T3-L1 adipocytes, cycloheximide has little effect on c-*fos* mRNA levels but superinduces the insulin response [90]. In contrast, in H4IIE cells cycloheximide dramatically increases c-*fos* mRNA levels and insulin has no additional effect [92]. Interestingly, in both cell lines the dose–response for insulin-stimulated c-*fos* mRNA accumulation correlates closely with receptor occupancy [90,92]. This is in contrast to the effect of insulin on PEPCK mRNA levels, where the maximal insulin effect is seen at less than 2% receptor occupancy [124]. This observation adds support to the currently popular idea of multiple pathways of insulin action.

Transient transfection experiments using c-*fos*/CAT fusion genes have shown that the c-*fos* promoter sequence from −356 to +109 is sufficient to mediate the insulin response [120]. The CHO cell line used in these experiments overexpresses the human insulin receptor. In contrast, a CHO cell line expressing a kinase-defective human insulin receptor failed to induce CAT activity in response to insulin [120].

A sequence between −320 and −299 in the c-*fos* promoter mediates the response of the c-*fos* gene to serum [125]. Stumpo *et al.* [120], showed that mutation of four base pairs in this serum response element (SRE), known to abolish the response to serum [126], also nullifies the effect of insulin on the expression of the c-*fos*/CAT construct. To confirm that this region contains an IRS it will be necessary to demonstrate that it transfers the insulin response to a heterologous promoter. c-*fos* belongs to the 'immediate-early' class of genes that are thought to play a central role in the transition from quiescence to cell proliferation. The expression of other members of this class, including *egr*-1, c-*jun*, Jun B, Jun D and gene 33, is also stimulated by insulin [93]. The promoters of all of these genes, except gene 33, contain functional SREs so this may explain how insulin stimulates their expression [93].

What is the physiological significance of the induction of c-*fos* mRNA by insulin? The product of the c-*fos* gene encodes a transcription factor thought to be involved in cell proliferation and differentiation [127]. Insulin has growth factor activity [128] but also has many effects on fully-differentiated tissues [5]. In both cases, it has been speculated that the stimulation by insulin of c-*fos* gene expression may mediate insulin's effect on the expression of other genes [90]. An emerging paradigm in the regulation of gene expression is that *trans*-acting factors can communicate through protein–protein interactions, or by competing for binding to the same sequence. In this regard it is interesting that c-*fos* (and c-*jun*) have been shown to modulate the binding of the oestrogen, glucocorticoid, retinoic acid and vitamin D receptors to *cis*-acting elements in the ovalbumin, proliferin and osteocalcin gene promoters, respectively [129–132]. Hence, the stimulation of c-*fos* gene expression may also represent a mechanism by which insulin could indirectly regulate the ability of other effectors/hormones to affect gene expression. For example, this could explain the synergistic effect insulin and oestrogen have on ovalbumin gene expression [62].

Glyceraldehyde-3-phosphate dehydrogenase

Insulin induces the mRNA encoding the glycolytic enzyme GAPDH in the H4IIE hepatoma and 3T3 F442A adipocyte cell lines [37,38]. 'Run-on' assays have yet to show whether this effect occurs at the transcriptional level. Indeed, GAPDH expression is regulated post-transcriptionally in several rat tissues [133]. Moreover, GAPDH catalyses an equilibrium reaction in the liver so the significance of this regulation by insulin is unclear; however, in adipose tissue GAPDH is one of the least active glycolytic enzymes and has been postulated to catalyze a rate-limiting step [134]. Interestingly, GAPDH gene expression is elevated in adipose tissue from genetically obese Zucker rats [134], though this may not be a primary lesion.

Alexander *et al.* [38], using the transient transfection of GAPDH/CAT fusion genes, demonstrated that the stimulatory effect of insulin on human GAPDH gene expression is mediated through *cis*-acting sequences located between −488 and +21. Similar results were obtained with both the H4IIE hepatoma and 3T3 F442A adipocyte cell lines [38]. From the results of further transient transfection experiments Nasrin *et al.* [135] concluded that the GAPDH promoter contains two independent insulin responsive elements, designated IRE-A and IRE-B. The IRE-A sequence has a number of close similarities to sequences in the promoters of other insulin-regulated genes [135]. However, confirmation of IRE-A and B as insulin response elements will require a demonstration that these sequences confer the insulin response to a heterologous promoter.

By using the fusion gene and transient transfection approach, broad insulin responsive regions have been identified in a number of other genes. Thus, the sequences shown in parentheses appear to be sufficient to mediate insulin's effect on the IGF binding protein 1 (−529 and −107) [136], growth hormone (−497 and +2) [137] and δ-crystallin (−120 and −43) [101] genes. In the β-casein gene the effect of insulin is permissive but an insulin/hydrocortisone/prolactin responsive region has been localized to either 5.3 kb of 5′ or 1.6 kb of 3′ flanking sequence [61].

STABLE TRANSFECTION

In transient transfection experiments expression of the fusion gene is mainly directed from free, supercoiled plasmid DNA in the recipient cell nucleus. These plasmids, with a low efficiency (that varies with the cell line under study), may become integrated into the genome of the recipient cell. When cells are co-transfected with the reporter gene, and with a gene that encodes a selectable marker, such stable transfectants can be isolated, since cells that take up one gene have a high likelihood of taking up the other. Thus, the construction of stable transfectants allows for the analysis of fusion gene expression whilst integrated in the genome, which is particularly significant in cases where chromatin structure is important for hormonal regulation of gene expression.

Phosphoenolpyruvate carboxykinase

Stable transfectants have been used extensively in the study of insulin-regulated PEPCK gene expression. PEPCK catalyses the rate-limiting step in gluconeogenesis and is present at relatively high specific activity in liver, kidney and adipose tissue. Although all species studied contain immunologically distinct cytoplasmic and mitochondrial forms of PEPCK, only the cytoplasmic form responds to hormonal and dietary stimuli (for review, see [106]).

In rat liver, and in the H4IIE hepatoma cell line, glucocorticoids and cyclic AMP increase transcription of the PEPCK gene [31,138]. Insulin inhibits basal and cyclic AMP/glucocorticoid-stimulated transcription, and the effect of insulin is dominant [30,31]. These effects of glucocorticoids, cyclic AMP and insulin are exerted within minutes, can be equally rapidly reversed, and do not depend on on-going protein synthesis [31]. The inhibitory effect of insulin, which is independent of insulin-stimulated glucose uptake [139], is primarily on transcript initiation, though insulin also causes a 3-fold reduction in the rate of transcript elongation [140]. Cyclic AMP [141] and glucocorticoids [142] have secondary effects on PEPCK mRNA stability, but insulin has no notable effect in this regard. Recently, the vitamin A derivative retinoic acid has been shown to stimulate PEPCK gene transcription [143,144]. Studies are in progress to

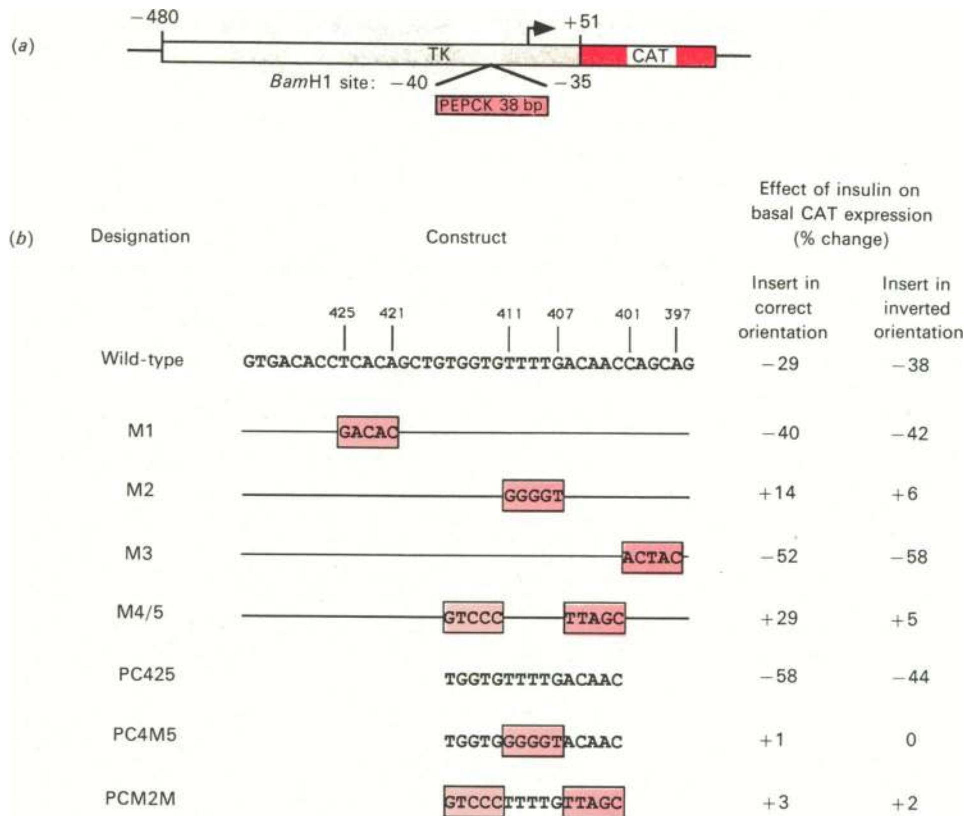

Fig. 1. Localization of the PEPCK IRS in the sequence −433 to −396

(*a*) Schematic representation of the TKC-VI vector which contains TK promoter sequence from −480 to +51 ligated to the chloramphenicol acetyltransferase (CAT) reporter gene. (*b*) Various mutants of the PEPCK sequence between −433 and −396 were synthesized with *Bam*HI (GATC) ends and cloned into the TKC-VI vector in both orientations. The effect of insulin on CAT expression was analysed by transient transfection [156]. Results are the ratio of CAT activity in insulin-treated versus control cells (expressed as percentage change) and represent the mean of six to fourteen separate transfections for each construct. Maximum error (±S.E.M.) was 7%. The boxed areas contain the mutant sequences. Reprinted with permission from *Science* (© AAAS 1990).

Gene	5′	Sequence	Match	Effect of insulin	References
PEPCK	−416	T G G T G T T T T G	10/10	I	[156, 157]
δ1-Crystallin	−1692	T G G T G T T C T G	9/10	S	[101, 158]
Gene 33	−954	T G G C G T T T T G	9/10	S	[95, 159]
Adipsin	−75	T G G T T T T C T G	8/10	I	[33, 160]
Glucokinase	−83	T G G T T C T T T G	8/10	S	[45]
PF-2-K/F-2,6-BP	−166	T G T G G T T T T G	8/10	S	[41, 161]
Malic enzyme	−692	T A T T G T T T T G	8/10	S	[44, 162]
α-Amylase	−165	G T T T A T T T T G	6/10	S	[163, 164]
IGFBP-1	−284	T G T C T T T T T G	7/10	I	[136, 165]
Aspartate aminotransferase	−1374	T G G T G T T T T G	10/10	I	M. Aggerbeck & R. Barouki, personal communication

Fig. 2. The 10-bp PEPCK IRS sequence (−416 to −407) is present in other insulin-regulated genes

All the sequences shown, except for aspartate aminotransferase, are from the coding strand of the respective gene promoter (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is abbreviated to PF-2-K/F-2,6-BP). Locations are expressed relative to the transcription start site. The effect of insulin on the expression of these genes is shown as (I) inhibition or (S) stimulation. In every case, apart from amylase and IGF binding protein-1 (IGFBP-1), the effect of insulin has been shown, using a 'run-on' assay, to be at the transcriptional level.

determine whether insulin is dominant over retinoic acid-induced PEPCK gene transcription just as it is over dexamethasone and cyclic AMP.

In contrast with the studies in rat liver and H4IIE cells, it should be noted that in primary hepatocytes [145,146] and regenerating liver [93] the effect of insulin is no longer dominant. Moreover, insulin has no effect on PEPCK gene expression in kidney [147]. The explanation for these differences is not known but may reflect the presence/absence of critical transcription factors.

In H4IIE cells, phorbol esters and diacylglycerol, presumably acting through protein kinase C, also inhibit PEPCK gene transcription [148]. Like insulin, the effects of these compounds are dominant over those of cyclic AMP and dexamethasone. Moreover, the effects of insulin and phorbol esters are additive and the inhibitory effect of insulin on PEPCK gene transcription is not mediated by protein kinase C [149], nor by a mechanism that involves changes in cyclic AMP concentration [150].

Although the basal promoter elements [116], cyclic AMP response element [116,151,152], retinoic acid response element [144] and glucocorticoid response unit [153] in the PEPCK gene have been mapped, the precise location of the insulin responsive sequence(s) (IRS) remained elusive for several years. Magnuson *et al.* [154] used transient transfection of H4IIE cells to analyse the regulation of a fusion gene in which the PEPCK promoter sequence between −2100 and +69 was ligated to the CAT reporter gene. Insulin prevented the increase of CAT activity usually noted after the addition of cyclic AMP or dexamethasone [154] and thus the regulation was qualitatively similar to that of the endogenous gene [31]. Since about 250 5′, 3′ and internal deletion mutants of the PEPCK promoter were available, localization of the IRS should have been a relatively simple task. For reasons that are unclear, transient transfection experiments with these constructs failed to give definitive and reproducible data with respect to the location of the IRS (M. A. Magnuson, P. G. Quinn & D. K. Granner, unpublished work).

Since the insulin response may require complex chromatin structure, the stable transfection approach was adopted. A series of stable transfectants was isolated by selection for expression of the co-transfected neomycin resistance (*neo*) gene [155]. Analysis of a stable transfectant expressing a fusion gene which contained PEPCK sequence between −2100 and +69 ligated to the CAT reporter gene showed that this transfectant demonstrated the same qualitative hormone responsiveness and concentration-dependence as seen in the endogenous gene [155]. Having demonstrated the validity of the stable transfection approach to locate an IRS, the region of the PEPCK promoter responsible for the inhibitory effect of insulin was further delineated. CAT activity was induced in a series of transfectants containing PEPCK/CAT 5′ deletion mutations by stimulation with a combination of cyclic AMP and dexamethasone to amplify the inhibitory effect of insulin. All the stable transfectants responded to insulin; however, the effect varied in magnitude [155]. Inhibition was almost complete (a mean value of 90%) for deletions with 5′ end points at −2100, −600, −467 and −437. When shorter fragments were used, e.g. −402, −306 or −271, this decreased to a mean value of 57%. Thus, the region of the PEPCK gene situated between −437 and +69, relative to the transcription start site, provides the full insulin effect and the reduction in the magnitude of the insulin effect between deletion endpoints −437 and −402 represents the loss of at least one IRS. One or more IRSs presumably resides within −402 and +69, and, since there is no apparent reduction in the insulin effect between −402 and −271, the other element(s) must be 3′ from the latter [155].

The location of an IRS between −437 and −402 was confirmed by using a vector (TKC-VI) containing the heterologous herpes simplex thymidine kinase (TK) promoter ligated to CAT (Fig. 1*a*). Since this promoter has a high intrinsic activity, unlike the PEPCK promoter, introduction of putative IRS sequences in this promoter allows for an analysis of the effect of insulin on basal CAT activity, i.e., unlike the PEPCK/CAT studies there is no necessity to induce CAT expression to amplify the inhibitory effect of insulin. A double stranded oligomer containing PEPCK sequence between −433 and −396 conferred an insulin-responsive inhibition of CAT expression in transient transfection assays (Fig. 1*b*; [156]).

To delineate further the boundaries of this element, the sequences with the end points −425/−421, −411/−407 and −401/−397 were changed to produce mutations designated M1, M2 and M3, respectively. These were inserted into the TKCAT vector and the transient expression of CAT activity was analysed in H4IIE cells. The M2 mutation abolished the insulin effect, but plasmids containing both orientations of the M1 and M3 mutations still responded to insulin (Fig. 1*b*). Another mutation, designated M4/5, in which the 5 bp sequences between −416/−412 and −406/−402 were changed on either side of the wild type −411/−407 sequence, failed to give an insulin-dependent inhibition of CAT expression in either orientation (Fig. 1*b*).

A 15-bp core sequence which spans −416 and −402 (PC425) showed insulin-dependent, orientation-independent, inhibition of CAT expression. Mutations equivalent to M2 and M4/5 within PC425, designated PC4M5 and PCM2M, respectively, abolished the inhibitory effect of insulin, as did identical mutations in the full-length oligomer (Fig. 1*b*). The 15-bp sequence, −416 to −402, is therefore a functional IRS [156].

Subsequently, experiments have shown that the 10-bp sequence between −416 and −407 (TGGTGTTTTG) is sufficient to mediate the insulin-response [181]. A comparison of this sequence with other insulin-regulated genes demonstrates some potentially interesting similarities (Fig. 2). Of particular note is the observation that this sequence is present in genes whose transcription is stimulated by insulin. Thus, perhaps the same 10-bp sequence can mediate both positive and negative effects of insulin. If so, it will be important to define the parameters that determine whether the insulin response is positive or negative. Although the similarity is low, the 10-bp sequences shown from the amylase and IGF binding protein 1 genes (Fig. 2) are within regions implicated in mediating the effect of insulin on the expression of these genes [136,164]. These similarities, if significant, may indicate considerable flexibility in the interaction between the insulin response sequence and one or more *trans*-acting factors. This would not be unprecedented. The identical DNA sequence can bind several transcription factors [180] and a single transcription factor (e.g. C/EBP) can bind to several, apparently unrelated, DNA elements [166]. The identification of *cis*-acting elements requires the demonstration of functional competence; comparisons of sequence similarity *per se* are insufficient. For example, the δ-crystallin gene promoter contains a sequence that has a 9/10 match with the PEPCK IRS (Fig. 2) but this is outside the region defined as sufficient for mediating the insulin response [101].

TRANSGENIC ANIMALS

Fusion gene constructs can also be analysed following their introduction into the germ line of animals (for review see [167]). Such transgenic animals offer the opportunity to study the hormonal regulation of the fusion gene under more physiological conditions although, unlike cell culture, the results may be

complicated by the presence of other hormones. Transgenic animal technology has been used extensively in the study of insulin-regulated amylase gene expression.

Amylase

α-Amylase catalyses the digestion of dietary starch. Two classes of pancreatic-specific amylase genes, designated Amy-2.1 and Amy-2.2, are present in the mouse genome. Pancreatic amylase mRNA is reduced to $< 1\%$ of normal in diabetic rats [82] and mice [83,84] but can be restored to normal by insulin administration. The Amy-2.2 promoter is also active in stomach, though at much lower levels (0.05 %) than pancreas, but it is not regulated by diabetes/insulin in this tissue [164]. Similarly, the amount of parotid gland amylase mRNA is unaffected by either insulin or diabetes [82]. Since the Amy-2 genes account for 15–25 % of total pancreatic mRNA [168], the induction of amylase mRNA is one of the largest effects of insulin on a specific gene.

Since a suitable cell line is unavailable, the transgenic approach has been used to study insulin regulation of pancreatic amylase gene expression. Osborn *et al.* [84] first established two lines of transgenic mice, the first containing a single copy of the complete Amy-2.2 gene as well as 9 kb of 5′ flanking sequence and 5 kb of 3′ flanking sequence. The second line of transgenic mice contained multiple copies of a minigene containing the complete Amy-2.2 coding sequence, intron 1, plus 208 and 300 bp of 5′ and 3′ flanking sequence, respectively. The transgene was expressed exclusively in the pancreas in both lines and was regulated by diabetes/insulin in the same manner as the endogenous gene [84]. Thus, *cis*-acting sequences in the minigene are sufficient for mediating tissue-specific and insulin-responsive amylase expression. These studies were not designed to pin-point the location of these *cis*-acting elements. Subsequent studies, using an amylase/CAT fusion gene introduced into the germ line of mice, demonstrated that the sequence between −208 and +19 of the amylase promoter is responsible for mediating tissue-specific expression and the response to diabetes/insulin [163].

More recently, Keller *et al.* [164] analysed hybrid genes containing sequences from the amylase promoter ligated to the insulin-unresponsive elastase promoter, which itself was coupled to the CAT reporter gene. These heterologous constructs were introduced into the germ line of mice and the effect of diabetes on their expression was analysed. An elastase/CAT construct containing a 30 bp fragment of the amylase promoter (−167 to −138) was much less active in diabetic mice relative to control mice. Keller *et al.* [164] therefore concluded that this 30 bp fragment is an insulin response sequence.

This IRS in the Amy-2.2 promoter contains a sequence with a 6/10 match with the PEPCK IRS (Fig. 2). Whether this is a coincidence, or a key to insulin action, remains to be established. The promoters for Amy-2.2 and Amy-2.1 differ slightly over this region [84], which may also be significant, since the Amy-2.2 gene exhibits a greater response to insulin than does Amy-2.1 [83].

Finally, it should be noted that a 'run-on' assay has not been performed to prove that this regulation is accomplished at the transcriptional level, though the aforementioned studies would suggest this is highly likely. More importantly, the long insulin administration times used in these studies [84,163], as well as the complex metabolic changes induced by diabetes, raise the question as to whether a direct insulin effect is involved in this regulation, or whether a secondary factor such as changes in glucose levels is involved.

McGrane *et al.* [169] used a fusion gene containing the PEPCK promoter sequence between −460 and +73 ligated to the human growth hormone gene to produce a series of transgenic mice. From an investigation of the effects of diet on the hepatic expression of this construct, it was inferred that this fragment of the PEPCK promoter contained an IRS, an observation consistent with our results (see above). Transgenic mice have also been produced which express a pyruvate kinase/CAT fusion gene. The IRS in this gene is within a region of the promoter from −3000 to +37 (170).

TRANSCRIPTION *in vitro*

An ultimate goal of molecular endocrinology is to establish cell-free systems that can be used to analyse how hormones regulate transcription at the biochemical level. This approach uses the same fusion gene constructs described previously in an *in vitro* system with (ideally) purified components of the transcriptional machinery and purified *trans*-acting factors. Transcription *in vitro* is now applicable to studies of steroid hormone and cyclic AMP action, since the relevant *cis*-acting elements are known and cognate, purified *trans*-acting factors are available [171]. With regards to insulin, though *cis*-acting elements in some genes have been defined, the associated *trans*-acting factors have not been purified (as described below). Nevertheless, progress has still been made with this approach, particularly with respect to the gene 33 and albumin genes, by comparing the ability of nuclear extracts isolated from control or diabetic rats to promote transcription *in vitro*.

Albumin

The regulation *in vivo* of albumin gene expression by insulin is complex. Insulin increases the amount of albumin mRNA in diabetic rats [109]. This effect also occurs in primary cultures of chick embryo hepatocytes [172] and the hormone increases albumin gene transcription in primary cultures of rat hepatocytes [85]. However, insulin does not induce albumin mRNA in normal rats, and in cultured rat hepatoma H4IIE cells insulin decreases albumin mRNA. This latter effect apparently occurs at both transcriptional and post-transcriptional levels [86]. Though the explanation for these discrepancies is at present unclear, Wanke *et al.* [173] have shown regulation of albumin gene transcription *in vitro*. Hepatonuclear extracts obtained from diabetic rats supported transcription at 25 % of the level afforded by using extracts isolated from control rats [173]. Although the nature of the *trans*-acting factor(s) involved is unknown, this approach has been used to identify a *cis*-acting insulin response region between −650 and +22 [173].

Gene 33

The mRNA transcribed from gene 33 encodes a protein of about 55 kDa whose function is unknown [94], although it may be a member of the 'immediate-early' gene family [93]. In rat liver and H4IIE hepatoma cells, insulin stimulates gene 33 transcription [94–96]; however, gene 33 expression is several times less sensitive to insulin than is that of PEPCK [95]. This observation may be explained by the existence of multiple pathways of insulin action. The regulation of two genes, PEPCK and gene 33, by insulin in the same H4IIE cell, but in opposite directions, should allow for an analysis of this possibility. The structure of the gene has recently been elucidated and its basal promoter characterized [159,174].

Sato *et al.* [175] have assayed the ability of nuclear extracts from H4IIE cells to promote transcription *in vitro* from the gene 33 promoter. Nuclear extracts prepared from insulin-treated H4IIE cells promoted an enhanced rate of transcription relative to nuclear extracts isolated from control cells [175]. They concluded that a region of the gene 33 promoter from −1500 to +1 was sufficient to mediate insulin's effect on gene 33 transcription [175]. This region contains a sequence with a 9/10

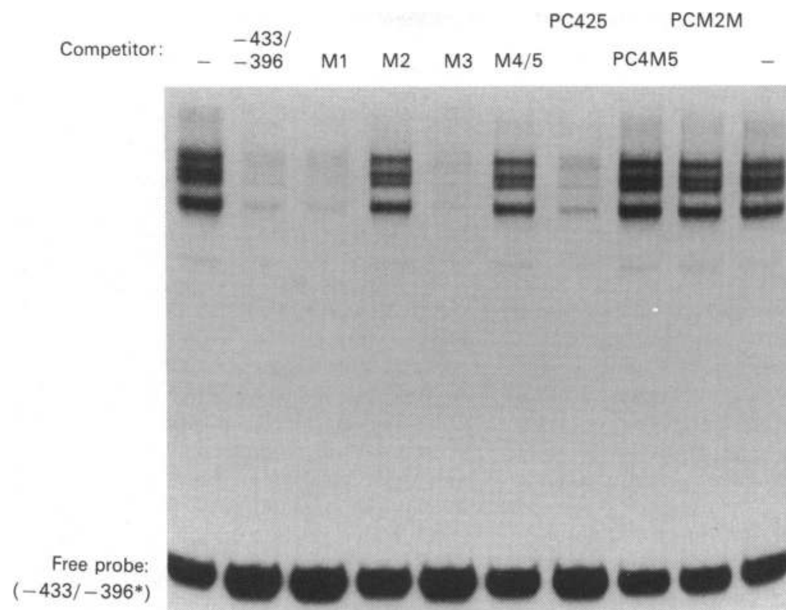

Fig. 3. Correlation of protein binding to the PEPCK IRS with insulin response

Gel retardation assays were performed as described [156]. The −433 to −396 PEPCK sequence was used as the labelled probe. Unlabelled competitor DNA fragments (a 100-fold molar excess) were added for competition analyses. Reprinted with permission from *Science* (© AAAS 1990).

match with the PEPCK IRS (Fig. 2) though the functional significance of this is not known.

IRS BINDING PROTEINS

The preceding discussion documents the identification of insulin response sequences/elements in a variety of genes. What is known about the *trans*-acting factors that presumably bind to these sequences? Three assays have been used to analyse protein–DNA binding, namely, DNAase 1 footprinting, methylation interference and gel retardation.

The PEPCK IRS is the site of a DNAase 1 footprint [153] and four major protein–DNA complexes indicative of specific interactions were detected when the PEPCK wild type −433/−396 sequence was used as the labelled probe in a gel retardation assay (Fig. 3; [156]). Sequences that conferred a response to insulin in transient transfection (Fig. 1), including −433/−396, M1, M3 and the 15-bp −416/−402 sequence (PC425), effectively competed for binding with the labelled probe. Sequences that did not confer the insulin response, i.e., the M2, M4/5, PC4M5 and PCM2M mutations, failed to compete (Fig. 3; [156]). Thus, the DNA–protein interactions correlate with the insulin response. A comparison of hepatonuclear extracts obtained from control, diabetic and insulin-treated diabetic rats revealed no change in the binding pattern [156].

In contrast, a comparison of nuclear extracts obtained from control versus insulin-treated 3T3-L1 adipocytes showed that protein binding to the GAPDH IRE-A is inducible [135]. The amount of this protein–DNA complex was also increased when hepatonuclear extracts from fed rats were compared with those from fasting rats. Whether this induction requires protein synthesis *de novo* has not been documented. Prager *et al.* [176] have reported insulin-inducible binding to the growth hormone promoter and in this case protein synthesis *de novo* is required. The functional significance of this result is unclear since these studies used CHO cell nuclear extracts, whereas transient transfection studies reported by this group were performed in other cell lines [68]. Neither the data reported for GAPDH nor that for growth hormone has been accompanied by a demonstration that these sequences, to which insulin-inducible proteins bind, function as IRSs when attached to a heterologous promoter. Moreover, it is not clear whether the time course of insulin-stimulated GAPDH or growth hormone gene expression correlates with (i.e. follows) the appearance of these proteins.

The amylase IRS overlaps the binding site for the pancreatic transcription factor PTF1 and Keller *et al.* [164] have suggested that insulin may mediate its effect on amylase expression via this protein (see below). Similarly, insulin may stimulate δ-crystallin gene expression by a mechanism involving the transcription

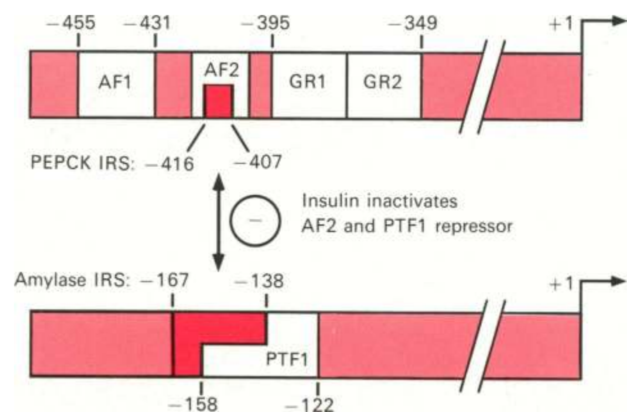

Fig. 4. Model of insulin-regulated PEPCK and amylase gene expression

DNA sequence is labelled relative to the transcription start site (+1). Details of the model are discussed in the text. The dark shaded regions represent the PEPCK and amylase insulin response sequences. In the PEPCK promoter the IRS (−416 to −407) coincides with AF2 (−420 to −402) whereas in the amylase promoter the IRS (−167 to −138) overlaps with the PTF1 binding site (−158 to −122).

factor SP1 [101]. Much work remains to be done before the causal relationships are established in any of these examples.

MODELS OF INSULIN-REGULATED GENE EXPRESSION

The study of insulin-regulated gene expression has advanced dramatically with the recent identification of several insulin response sequences. The purification and cloning of the associated *trans*-acting factors will permit an analysis of the protein–protein interactions through which these factors alter gene transcription. Various models of transcription repression have been proposed [177] and, in the case of the PEPCK and amylase genes, mechanisms through which these *trans*-acting factors could function can be hypothesized (Fig. 4).

How might insulin mediate its dominant inhibitory effect over glucocorticoid-stimulated PEPCK gene transcription? In the PEPCK gene a complex glucocorticoid response unit (GRU) mediates the stimulatory action of glucocorticoids [153]. This GRU consists of a tandem array (5′ to 3′) of two accessory factor binding sites (AF1, from −455 to −431; AF2, from −420 to −403) and two glucocorticoid receptor binding sites (GR1 and GR2, from −395 to −349). AF1 and AF2 do not function as glucocorticoid response elements themselves. However, transient transfection experiments show that when they are both mutated the promoter is no longer responsive to glucocorticoids. Thus, GR1 and GR2 are inert by themselves. Since the IRS (−416 to −407) coincides with AF2, it is ideally positioned to inhibit AF2 function. This could explain how insulin mediates its dominant negative effect on glucocorticoid-stimulated PEPCK gene transcription. Whether the same protein(s) mediate AF2 activity and the response to insulin is unknown, but mutations that disrupt the response to insulin concomitantly disable the response to glucocorticoids (J. Mitchell & D. Granner, unpublished work). Thus, the molecular physiology of gluconeogenesis with regards to glucocorticoids and insulin can be explained by this model.

The 30-bp amylase IRS (−167 to −138) includes part of the binding site for the pancreatic transcription factor PTF1. Keller *et al.* [164] have suggested that the effect of insulin may be explained by a mechanism involving interaction with PTF1 (Fig. 4). However, in gel retardation experiments a comparison of pancreatic nuclear extracts isolated from control or diabetic rats demonstrates that PTF1 binding is unchanged [164]. Since the promoters for elastase and chymotrypsin also bind PTF1, but are not regulated by insulin, these authors suggest that this interaction must involve an amylase specific *cis*-acting element and associated *trans*-acting factor, adjacent to or overlapping the PTF1 binding site, and hence be an indirect effect on PTF1. In their model, this *cis*-acting element would bind a repressor in the absence of insulin. Thus insulin may mediate its action on the expression of both the PEPCK and amylase genes by inactivating a protein: in one case (PEPCK) a positive factor (AF2) is disabled, in the other case (amylase) a negative repressor protein is inactivated.

SUMMARY AND FUTURE DIRECTIONS

The past 5 years have seen much progress in the field of insulin-regulated gene expression. The list of insulin-regulated genes has grown dramatically, though in many cases the physiological significance of this regulation remains to be established. There has also been much progress towards the identification of insulin response sequences. In some genes, such as PEPCK, GAPDH, c-*fos* and amylase the IRS has been mapped to a short DNA sequence (< 30 bp). In other genes only broad insulin response regions have been identified. The PEPCK and GAPDH IRSs have very different sequences so it will be interesting to see whether either of these IRSs will mediate an effect of insulin on the expression of other genes. If so, since insulin stimulates GAPDH expression but inhibits PEPCK expression, will the respective IRSs be confined to genes that are regulated in the same direction? Alternatively, both of these elements may be specific for the gene in which they were identified, and one or more different IRSs may regulate other genes. This would not be unprecedented. Phorbol esters, like insulin, have been shown to regulate the expression of more than 40 genes. At least four distinct phorbol ester response sequences are known [178].

There is also considerable interest in the proteins that bind to these insulin response sequences, so the isolation and cloning of these proteins is a major goal. Purification of these proteins will be required for an understanding of how they communicate (directly or indirectly) through protein–protein interactions with the transcription complex and hence mediate their effect on transcription. Moreover, the potential involvement of such proteins in the pathogenesis of type II diabetes will need to be investigated. Finally, since these proteins represent the final step in a signal cascade, it is hoped that it will be possible to elucidate the successive steps required for insulin action by working back from these proteins to the insulin receptor.

We thank D. Caplenor for preparing the manuscript. Our work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (AM35107) (D.K.G.) and an American Diabetes Association Mentor-Based Fellowship (R.M.O'B.).

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