Review

Hormonal regulation of gluconeogenic gene transcription in the liver

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Glucose homeostasis in mammals is achieved by the actions of counterregulatory hormones, namely insulin, glucagon and glucocorticoids. Glucose levels in the circulation are regulated by the liver, the metabolic centre which produces glucose when it is scarce in the blood. This process is catalysed by two rate-limiting enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) whose gene expression is regulated by hormones. Hormone response units (HRUs) present in the two genes integrate signals from various signalling pathways triggered by hormones. How such domains are arranged in the regulatory region of these two genes, how this complex regulation is accomplished and the latest advancements in the field are discussed in this review.

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1. Introduction

Life in its several manifestations depends on the coordination between various processes that subserve many functions. In higher organisms, hormones that are secreted by a specific cell type in an organ enter the circulation and regulate the functions of cells of other tissues. This regulation can be by post-translational modification (as in glycogen phosphorylase) or at the level of transcription. In the case of the latter, the hormone activates a specific transcription factor, which binds to its cognate hormone response element, leading to either inhibition or activation of the target genes. The coordinated action of hormones on several tissues can be appreciated by analysing the maintenance of plasma glucose levels. The concentration of glucose is maintained within a narrow range (irrespective of fasting or feeding), by a delicate balance between intestinal absorption, glucose production by the liver (gluconeogenesis) and utilization of glucose by the peripheral tissues. In diabetes type II, these processes are deregulated. Understanding the nuances of these regulatory events enables us to interfere with the onset and progress of the disease.

The gluconeogenesis pathway is catalysed by several enzymes; the first and last ones being phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), respectively. *PEPCK* gene expression is increased not only in diabetes but also in hyperthyroidism and in various nutritional states such as high protein diets and fasting (Hanson and Reshef 1997). The *G6Pas*e gene is mutated in glycogen storage disease type 1a and type1b (van Schaftingen and Gerin 2002).

We attempt to give an overall view of how transcription of the *PEPCK* and *G6Pase* genes is altered by hormones in response to external and internal cues. A review of the G6Pase catalytic subunit gene family has recently been published, which addresses the biological function of the encoded

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Abbreviations used: AF, accessory factor element; C/EBP, CAAT- enhancer binding protein; cAMP, cyclic AMP; CBP, CREB-binding protein; CEBPβ, CAAT-enhancer binding protein β; COUP-TF, chicken ovalbumin upstream transcription factor; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; dAF, distant accessory factor element; G6Pase, glucose-6-phosphatase; GRE, glucocorticoid response element; GR-G, glucocorticoid receptor – glucocorticoid; HNF-1, hepatic nuclear factor 1; HNF-3 β, hepatic nuclear factor 3β; HNF-4α, hepatic nuclear factor 4α; HRU, hormone response unit; NF-1, nuclear factor 1; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor –γ coactivator-1α; PPARα, peroxisome proliferator activated receptor α; RARE, retinoic acid response element; RAR–RXR, retinoic acid receptor–retinoid receptor; SRE, SREBP response element; SREBP, sterol response element-binding protein; TORC2, transducer of regulated CREB activity 2; TRE, thyroid response element; TR-T3, thyroid receptor- thyroid hormone; TSS, transcription start site

proteins, their transcriptional regulation and their disease association (Hutton and O'Brien 2009). For an exhaustive coverage of the early literature on physiology and gene expression of *PEPCK*, readers are referred to Chakravarty *et al.* (2005). Hanson's group recently published reviews on the biological role of PEPCK (Yang *et al.* 2009a), on the role of chromatin structure and on *PEPCK-C* gene transcription in brief (Yang *et al*. 2009b). This review gives a distilled concentrate from previous studies and focuses in detail on the regulation of these two genes as a part of the pathway of gluconeogenesis. Finally, we give future directions that could take this field forward.

PEPCK (EC 4.11.32) catalyses the committed, first step in the gluconeogenesis pathway: the conversion of oxaloacetate to phosphoenolpyruvate. The activity of PEPCK is controlled only at the level of transcription as there are no known allosteric modifiers (Hanson and Patel 1994). Thus, this gene is a good model for understanding hormonal regulation at the level of transcription. In addition to participating in the gluconeogenesis pathway, G6Pase enzyme (EC 3.1.3.9) plays an important role in releasing free glucose from glucose-6-phosphate, the ultimate product of glycogen breakdown (van Schaftingen and Gerin 2002). Hormones that are secreted during fasting such as glucagon and glucocorticoids enhance the transcription of these two genes while insulin dominantly represses basal as well as hormone-induced expression. Transcription of the *PEPCK* gene is also enhanced by retinoic acid and thyroid hormone.

2. Basic concepts

Hormones such as insulin and glucagon that bind to plasma membrane-bound receptors convey the message into the nucleus by initiating a relay involving several proteins. Insulin receptor triggers several downstream steps, one of them being activation of PI3-kinase which, in turn, activates Akt to modulate the function of transcription factor. Glucagon receptor acts via G protein: stimulatory (Gs protein) to stimulate adenylyl cyclase, and results in an increase of cAMP level. This leads to activation of protein kinase A (PKA) and phosphorylation of cAMP-response element-binding protein (CREB), which then binds to its response elements (REs) in the promoter of the genes that are regulated at the level of transcription by glucagon. Hormones such as glucocorticoids, retinoic acid and thyroid hormone diffuse through the plasma membrane and bind to their respective cytosolic receptors. The hormone-bound receptor translocates into the nucleus and occupies the specific RE. The REs are specific DNA sequences which are docking sites for cognate transcription factors. They may be single consensus sequences or palindromes or direct repeats in which the half sites are separated by a few bases. CREB binds to its cognate sequence as a monomer while the ligand-bound glucocorticoid receptors (GRs) bind as dimers (homodimers or heterodimers with retinoid x receptor). Binding to the half-sites can be cooperative. Functional REs have been shown to impart hormone sensitivity to a heterologous (when inserted upstream) gene. Transcription factors that are activated by a stimulus recruit (a) co-activators to hire chromatin remodellers and histone acetyl transferases (HAT) to increase the accessibility of promoter DNA to proteins, or (b) co-repressors to engage histone deacetylases (HDAC) which have the opposite effect.

3. PEPCK (GTP)

Transcription of the cytosolic form (and not the mitochondrial form) of the *PEPCK* gene in the liver is subject to regulation by hormones as mentioned in the Introduction. Figure 1 illustrates the arrangement of various REs in the promoter region.

3.1 *Glucocorticoid response unit (GRU)*

The GRU of the *PEPCK* gene consists of two non-consensus, glucocorticoid response elements (GREs) GRE1 and GRE2; three accessory factor elements AF1, AF2 and AF3; and a cAMP response element (CRE). Initially, a set of serial 5' deletion mutants of the promoter, upstream of the transcription start site (TSS), was used to define the regulatory elements for the response to glucocorticoids in H4IIE rat hepatoma cells (Imai *et al.* 1990). DNase I footprinting analysis identified the exact sequence(s) that interact with the GR. The discrepancy between the boundary of the functional GRU domain and the GREs led to the discovery of accessory elements (Imai *et al.* 1990) and the factors that bound those sequences. The specific interactions of accessory factors were later confirmed using electrophoretic mobility gel shift assay, the GAL4 system, and the chromatin immunoprecipitation (ChIP) assay (Scott *et al.* 1996). Employing stopped flow fluorescence anisotropy measurements of protein–DNA interactions in nuclear extracts, Granner and co-workers demonstrated that GR binds to the GRE1 and GRE2 elements poorly as compared to a palindromic or consensus GRE. Accessory factors that bind to AF1 or AF3 create a high-affinity binding environment for GR to bind to GRE1 and GRE2 (Stafford *et al.* 2001). One function of the bound accessory factors (C/EBPβ) appears to be recruitment of co-activators (CBP) to the promoter (Duong *et al.* 2002). When the positions of AF1 and AF2 in the promoter are swapped, the response to glucocorticoids is reduced, indicating that the exact positions of these two elements are necessary for the full response (Wang *et al.* 1999).

Experiments in transgenic mice (a), transient transfections in HepG2 hepatoma cells (b) and the discovery

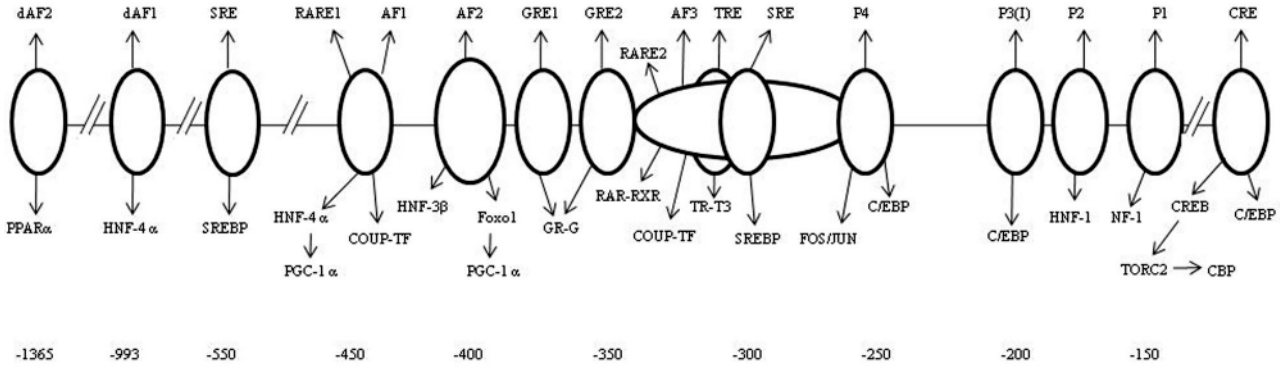

Figure 1. *PEPCK* gene promoter: response elements and the binding proteins. dAF2, distant accessory factor element 2; dAF1, distant accessory factor element 1; SRE, sterol response element; RARE1, retinoic acid response element 1; AF1, accessory factor element 1; AF2, accessory factor element 2; GRE1, glucocorticoid response element 1; GRE2, glucocorticoid response element 2; RARE2, retinoic acid response element 2; TRE, thyroid response element; AF3, accessory factor element 3; CRE, cyclic AMP response element; PPARα, peroxisome proliferator activated receptor α; HNF-4α, hepatic nuclear factor 4α; SREBP, sterol response element-binding protein; HNF-4α, hepatic nuclear factor-4α; HNF-3 β , hepatic nuclear factor 3β; COUP-TF, chicken ovalbumin upstream transcription factor; GR–G, glucocorticoid receptor–glucocorticoid; RAR–RXR, retinoic acid receptor–retinoid receptor; TR–T3, thyroid receptor–thyroid hormone; C/EBP, CAAT enhancer-binding protein; HNF-1, hepatic nuclear factor 1; NF-1, nuclear factor 1; CREB, cyclic AMP response element-binding protein; CEBPβ, CAAT enhancer-binding protein β; PGC-1α, peroxisome proliferator-activated receptor – γ coactivator-1α; TORC2, transducer of regulated CREB activity 2; CBP, CREB-binding protein

of hypersensitive sites in the rat *PEPCK* gene promoter (c) suggested that the originally described GRU (–467 to +63) extends upstream of the –540 starting from transcription start site. Further, Reshef and group (Cassuto *et al.* 2005) showed in HepG2 cells and transgenic mice the presence of distal accessory sites, dAF1 (–993) and dAF2 (–1365).

3.2 *Retinoic acid response unit (RARU)*

The accessory elements AF1 and AF3 serve as sequences that bind to the retinoic acid receptor (RAR). RAR interacting with AF1 and a coregulator supposed to be retinoid receptor, RXR binding to an adjacent site C, enhance the expression of PEPCK gene (Hall *et al.*1992). AF3 supports the function of the RAR–RXR heterodimer (Sugiyama *et al.* 1998). The DNA-binding elements of GRU and RARU are overlapping and suggest the existence of functional cooperativity. In fact, the combination of dexamethasone (an agonist at the glucocorticoid receptor) and retinoic acid has a synergistic effect on endogenous *PEPCK* gene expression in both primary hepatocytes and H4IIE hepatoma cells. This synergism is abolished by selective reduction of a coactivator, p300, by siRNA (Wang *et al.* 2004).

3.3 *Cyclic AMP response unit (CRU)*

The CRU is composed of one CRE that is weak, three CAAT enhancer-binding protein (C/EBPα) sites and one AP-1 site. Initial studies by Hanson's group identified promoter elements necessary for cAMP effect: DNase I footprinting studies followed by introduction of block mutations (10–15 base pairs of random sequence in a region of the promoter between –490 and +73) identified multiple elements of the CRU (Liu *et al.* 1991). Of these, the CRE region is important for both basal (when C/EBPα binds to CRE) and cAMP-induced (when CREB binds to CRE) transcription (Roesler 2000). CREB, which is phosphorylated at Ser 133 by PKA, binds to CRE and, in turn, recruits CREB-binding protein, CBP (Chrivia *et al.* 1993), a HAT. Interestingly, both C/EBPα and CREB bind to this element with equal affinity. In fact, C/EBPα can substitute for CREB to mediate cAMP responsiveness, and the constitutive transactivation and hormone-inducible responsiveness of C/EBPα is exerted by different mechanisms (Roesler *et al.* 1998). In db/db diabetic mice, when siRNA to C/EBPα is introduced, hepatic glucose production is diminished. In these mice, CREB levels are found to be low and the effect of lowered CREB with C/EBPα siRNA treatment brings down the glucose production as both these transcription factors are required for gluconeogenic gene expression in response to cAMP (Qiao *et al.* 2006).

3.4 *Thyroid hormone response unit (TRU)*

In the presence of thyroid hormone, the thyroid hormone receptor (TR) binds to its RE as a heterodimer with RXR. In addition, another site, P3(I) binds the accessory factor

C/EBPα. Initial studies focused on the role of C/EBPα on the effects of T3 and cAMP because it is highly expressed in the liver. Park *et al.* (1995) showed that C/EBPβ in concert with TR can mediate T3 induction of *PEPCK* transcription. The P3(I) site is involved in induction of the *PEPCK* gene by both T3 and cAMP. Interestingly, CBP, which is needed for cAMP effect via its action as a coactivator for CREB, also enhances transcription of PEPCK by thyroid hormone. CBP can interact with steroid receptor coactivator (SRC-1), which is recruited by the TR in the presence of hormone (Jurado *et al.* 2002).

3.5 *Insulin response unit (IRU)*

Several gene regulatory proteins that mediate the action of insulin have been identified but the insulin response unit (IRU) has not been clearly demarcated. The sequence of the AF2 site of GRU confers insulin inhibition to a heterologous thymidine kinase promoter (O'Brien *et al.* 1990). AF2 is considered to be the IRE in the *PEPCK* gene. Insulin triggers the dissociation of the transcription initiation complex formed on the promoter in response to glucocorticoids (Hall *et al.* 2007).

Foxo1 is another transcription factor that is critical for gluconeogenesis. Insulin inhibits C/EBPα-dependent transcription (which is enhanced by Foxo1) in ChIP (chromatin immunoprecipitation) experiments (Sekine *et al.* 2007). However, mutating the IRE of *PEPCK* did not lead to corresponding alterations in insulin response in H4IIE rat hepatoma cells (Yeagley *et al.* 2001). Other mediators of the function of insulin have been reported. Insulin increases the protein levels of liver-enriched inhibitory protein (LIP), a natural inhibitory form of C/EBPβ which displaces its activating counterpart liver-enriched activating protein (LAP) *in vivo*. This further disrupts the association of CBP and Pol II on the promoter, resulting in the inhibition of transcription (Duong *et al.* 2002). Involvement of sterol regulatory element-binding proteins (SREBPs) in the mediation of insulin effect has also been reported. Mutating the binding sites for these proteins SREBP response element (SRE) increases the basal as well as PKA-stimulated transcription of the PEPCK promoter (Chakravarty *et al.* 2004).

Insulin opposes the action of glucagon, which is via CREB, by triggering phosphorylation of CBP. Insulin signalling phosphorylates CBP at Ser 436, a site near the CREB-binding domain, which may interfere with the interaction of CBP with CREB (Zhou *et al.* 2004). These experiments in 'knock-in' mice containing the *CBP* gene with S436A mutation had higher levels of gluconeogenic mRNAs in the fed state.

The data on the dominant action of insulin at the transcription level suggest that there could be several factors mediating insulin response. For instance, AF2, a component of GRU, is required for disrupting the dexamethasone-induced transcription initiation complex by insulin (Hall *et al.* 2007). Supposedly, the same site may be involved in interference of the RAR response by insulin. Interestingly, one of the SREs corresponds to the thyroid response element; again suggesting that the mechanism of action of insulin lies in the displacement of TR binding. Thus, these overlapping structural elements support the distinct functions of hormones. Insulin directly blocks the action of Foxo1 by phosphorylation through the PI3-kinase pathway. Recent studies indicate that insulin opposes the action of glucagon at the level of another co-activator, TORC2 (described at length later), which has been shown to recruit CBP to cAMP-regulated genes (Ravnskjaer *et al.* 2007).

4. Glucose-6-phosphatase (G6Pase)

G6Pase is located in the endoplasmic reticulum and is a multicomponent system with a catalytic unit and transporters for glucose-6-phosphate and glucose (Hutton and O'Brien 2009). Unlike PEPCK, whose gene expression has been probed intensively since the 1980s, this aspect of G6Pase started to receive attention relatively recently. The catalytic unit of G6Pase is subject to hormonal regulation and the promoter of the catalytic unit of the *G6Pase* gene has been demarcated and dissected into various components. The list of REs and the proteins that bind to these REs are given in table 1. Only the important features that are unique to *G6Pase* gene expression as induced by hormones and are totally different from those of PEPCK are described in this section.

4.1 *Hormone response units (HRUs) of G6Pase*

The occupancy of three positive elements by GR and, in addition, binding of HNF-1, HNF-4, CRE-binding factors and FKHR (Foxo1a) are essential for the complete induction of G6Pase by glucocorticoids. The single negative GRE, nGRE4, and the associated negative accessory factor element (–260/–255) are involved in the inhibition of glucocorticoid response (Vander Kooi *et al.* 2005). nGRE4 can compete with the labelled consensus GRE probe for binding of the GR DNA-binding domain, but with a lower affinity. Although this appears to be the first gene reported to have both positive and negative GREs whose significance is still not clear, their existence may indicate the presence of a feedback loop which is operational at higher concentrations of the hormone to limit the biological response.

Interestingly, an HRU for insulin has been described in this gene; it is composed of Region A and Region B. Region A serves as an accessory element for the binding of HNF-1 (Vander Kooi *et al.* 2003). Region B contains

Table 1. Glucose-6-phosphatase–DNA-binding sites

Response element – Protein	Location	Reference
GRU:		
GRE1 (GR)	-197/-183	(Vander Kooi *et al.* 2005) (mouse)
GRE2 (GR)	-180/-166	(Vander Kooi *et al.* 2005) (mouse)
GRE3 (GR)	-156/-142	(Vander Kooi *et al.* 2005) (mouse)
nGRE4 (GR)	-239/-225	(Vander Kooi *et al.* 2005) (mouse)
FKHR B (Foxo1a &-3a)	-141/-135	(Vander Kooi *et al.* 2005) (mouse)
FKHR C (Foxo1a &-3a)	-110/-104	(Vander Kooi *et al.* 2005) (mouse)
HNF-3B- HNF-3β	-144/-133	(Vander Kooi *et al.* 2005) (mouse)
HNF-3C- HNF-3β	-117/-106	(Vander Kooi *et al.* 2005) (mouse)
HNF-4α	-76/-64	(Vander Kooi *et al.* 2005) (mouse)
HNF-1	-222/-210	(Streeper *et al.* 1998) (mouse)
CRU:		
CRE-1-CREB	-153/-160	(Thiel *et al.* 2005) (human)
CRE-2-CREB	-129/-136	(Thiel *et al.* 2005) (human)
HNF-6	-114/-99	(Streeper *et al.* 2001) (mouse)
C/EBP- α &β	-608/-603	(Gaultier-Stein *et al.* 2005) (rat)
HNF-4α	-694/-500 & -79/-67	(Gaultier-Stein *et al.* 2005) (rat)
IRU:		
Region A		
HNF-1	-231/-199	(vander Kooi *et al.* 2003) (mouse)
Region B		
IRE-1-Foxo1a	-188/-182	(vander Kooi *et al.* 2003) (mouse)
IRE-2-Foxo1a	-174/-168	(vander Kooi *et al.* 2003) (mouse)
IRE-3	-166/-160	(vander Kooi *et al.* 2003) (mouse)

three insulin response elements (IREs) designated IRE 1, -2 and -3. FKHR binds IRE1 with high affinity and IRE2 with low affinity but both are equally important for the insulin response. Interestingly, IRE3 cannot bind FKHR and confers an inhibitory effect of insulin on the expression of a heterologous fusion gene, suggesting the involvement of another transcription factor.

The responsiveness to cAMP of the G6Pase promoter depends on cooperation between the proximal and distal regions, and involves HNF4α, C/EBP- and CREB-binding sites (Gaultier-Stein *et al.* 2005).

Interestingly, G6Pase gene transcription as well as stability of its mRNA is increased by glucose (Massillon 2001) and this effect is discussed in the section on TORC2.

5. PEPCK and G6Pase

5.1 *The co-activator PGC-1α*

The fact that β-adrenergic agonists induce the co-activator PGC-1α in brown fat through the cAMP system, and glucagon also acts via the same second messenger, suggest a possible role of PGC-1α in the molecular changes that take place in fasting. The time course of fasting coupled with measurement of mRNA for PEPCK, G6Pase and PGC-1α reveal the importance of PGC-1α in the induction of gluconeogenic genes. Dexamethasone and a cAMP analogue increase the levels of PGC-1α and the induction by cAMP is mediated by CREB (Herzig *et al.* 2001). Infusion of adenoviruses containing recombinant PGC-1α increased mRNA levels of both PEPCK and G6Pase in mice (Yoon *et al.* 2001). PGC-1α functions by coactivating both HNF-4α and the glucocorticoid receptor. Insulin acts via Akt/PKB and phosphorylates and inactivates PGC-1α (Li *et al.* 2007).

Transgenic mice expressing the synthetic acidic zipper DNA-binding domain of CREB (A-CREB) reduced the expression of PEPCK and G6Pase 4-fold. Also, PGC-1α promoter activity was induced 3–4 fold by a cAMP agonist that was blocked by A-CREB, indicating CREB activation of PGC-1α expression (Herzig *et al.* 2001). In the A-ZIP factors, the leucine zipper region mediating dimerization specificity is fused to an acidic extension with charge complementary to the basic residues that mediate site-specific DNA recognition. A-ZIP: bZIP prevent binding of the native factor.

Many studies used H4IIE hepatoma cells that do not possess PGC-1α. Despite this, the cells showed appropriate responses to dexamethasone, cAMP and insulin as in the liver. However, when PGC-1α was introduced by adenovirus infection, G6Pase and PEPCK were stimulated 10- and 5-fold, respectively. It is possible that some protein with a lower affinity, which exists in these cells, takes over the role of PGC-1α or that PGC-1α is an amplifier rather than an essential protein involved in regulation as observed in H4IIE

cells (Herzog *et al.* 2004). The profound effect of PGC-1α on G6Pase rather than on PEPCK could be due to the closeness of the HNF4α-binding site to the TATA box in *G6Pase* gene than are the AF1 and AF3 elements in the PEPCK promoter. It should be mentioned that dexamethasone and cAMP increase the transcription of PEPCK and G6Pase by 60- and 30-fold in hepatocytes, while it is increased only 8- and 6-fold in H4IIE cells. This report highlights the importance of experiments in live animals and primary cultures, as exact values obtained in the cell lines may reflect a change in the necessity of the tissue-specific function of proteins such as PEPCK and G6Pase.

SIRT1 (a sirtuin), an NAD-dependent deacetylase, is induced in fasting, and this enzyme deacetylates PGC-1α and increases its interaction with HNF-4α, resulting in the induction of gluconeogenic genes (Rodgers *et al.* 2005). Sirtuins augment longevity in *Caenorhabditis elegans* and also in mammalian models of calorie restriction, in which changes in glucose metabolism are the key events (Canto and Auwerx 2009). Since calorie restriction extends the lifespan and SIRT1 is the modulator of these pathways, therapeutic studies have been initiated for treating ageing-related diseases such as diabetes 2 (Tang and Chua 2010). Resveratrol, a small molecule, activates SIRT1 and increases hepatic glucose production. SIRT1 activators can modulate glucose homeostasis because gluconeogenesis is activated by both PGC-1α and Foxo1 (the effect on Foxo1 is given later). However, there are conflicting reports of the effect of SIRT1 activation on *PEPCK-C* gene transcription (Yang *et al.* 2009c).

5.2 *The co-activator TORC2 (transducer of regulated CREB)/CRTC2*

Montminy's group (Koo *et al.* 2005) observed that intraperitoneal injection of either glucagon or insulin leads to comparable CREB phosphorylation, suggesting that perhaps the CREB–CBP pathway does not discriminate between fasting and feeding signals in the liver. This, in turn, triggered a search for other CREB co-activators and the TORC2 family seemed appropriate as cAMP dephosphorylates and promotes nuclear entry of these proteins. Moreover, TORCs interact with the CREB DNA-binding domain. Since TORC2 activity is abundant in the liver, its phosphorylation in response to glucagon or fasting was examined and was found to relate well. In addition, ChIP experiments demonstrated the presence of TORC2 on G6Pase, PEPCK and PGC-1α promoters in liver tissue samples. Furthermore, knock-down of either PGC-1α or TORC2 with Ad-RNAi constructs suggest that PGC-1α acts downstream of TORC2 in the gluconeogenic pathway. In primary hepatocytes, upon glucagon addition, PEPCK expression reaches a peak in 2 h and returns to normal levels by 4 h, which is due to a feedback loop involving salt-inducible kinase (SIK1).

Insulin signalling activates SIK2, which phosphorylates and subjects TORC2 to ubiquitination and degradation (Dentin *et al.* 2007). TORC2 is subject to glycosylation (Dentin *et al.* 2008) at the same sites that are phosphorylated by insulin (Dentin *et al.* 2007). Phosphorylation causes exclusion of TORC2 from the nucleus.

In diabetes, circulating glucose levels are high, and the effect of glucose on gluconeogenic gene expression has been under investigation. As expected, *PEPCK* transcription is lower in the presence of glucose (Foufelle *et al*. 1994). Interestingly however, transcription of the *G6Pase* gene as well as stability of its mRNA is increased by glucose (Massillon 2001). This observation suggests that glucose-6-phosphate levels are prevented from reaching high levels so that the glycogen deposits can be maintained at reasonable levels, allowing the system to store energy in the form of fat in the adipocytes. Furthermore, glucose needs to be metabolized to stimulate the expression of *G6Pase*. Montminy's group observed O-glycosylation of TORC2 in the presence of glucose or glucosamine, and its translocation to the nucleus and the subsequent hike in G6Pase mRNA levels (Dentin *et al.* 2008). Since TORC2 is involved in the transcription of both *PEPCK* and *G6Pase*, how *PEPCK* expression is reduced and, in contrast, *G6Pase* transcription is increased is a puzzle that remains to be solved. The role of Foxo1 also needs to be explored in this context. Selective interactions between proteins that bind to the promoters of each gene might play an important role.

5.3 *Energy status of the cell – gluconeogenesis*

It is well established that ATP depletion and activation of AMP-activated kinase (AMPK) under energy stress blocks the gluconeogenic programme. Adenovirus-mediated gene transfer of a constitutively active form of AMPKα2 is sufficient to decrease blood glucose levels and reduce hepatic gluconeogenic gene expression (Foretz *et al.* 2005). In mice with hepatic deletion of AMPK isoform α2, regulation of gluconeogenesis by leptin and adiponectin, but not by insulin, was impaired (Andreelli *et al.* 2006). Phosphorylation and inactivation of TORC2 by AMPK has also been demonstrated, coordinating the energy levels of the cell with the actions of hormones (Koo *et al.* 2005).

5.4 *Foxo1, the transcription factor*

In mice, antisense oligonucleotides to Foxo1 reduce the amount of Foxo1 and mRNA levels of *PEPCK* and *G6Pase* in the liver (Samuel *et al.* 2006). Foxo1 interacts with PGC-1α and increases hepatic glucose production (HGP) (Puigserver *et al*. 2003). However, there are reports that

provide evidence against the involvement of Foxo1 in insulin response (Herzig *et al.* 2001). However, recent reports provide strong evidence in favour of Foxo1 as a stimulator of gluconeogenic gene expression. When hepatocytes from normal mice were transfected with PGC-1α, a coactivator for Foxo1, *G6Pase* and *PEPCK* expression levels increased 2500- and 80-fold, respectively. These were reduced (>95%) in hepatocytes from Foxo1-deficient mice (Matsumoto *et al.* 2007). This could indicate that Foxo1 is the major stimulatory factor for *G6Pase* but not for *PEPCK*, as discussed later.

Ubiquitination and acetylation of Foxo1 are well established (Brunet *et al.* 2004) and glycosylation of Foxo1 has been recently reported to increase in diabetic rats and stimulate gluconeogenic gene expression. This appears to be a direct control of transcription by nutrients (Housley *et al.* 2008). Addition of insulin leads to phosphorylation of Foxo1 and its export from the nucleus to the cytoplasm (Greer and Brunet 2005). Deacetylation by the NAD-dependent deacetylase SIRT1 and the resultant increase in the nuclear residence time of Foxo1 promotes Foxo1-dependent transcription. The effect was more prominent on *G6Pase* than on *PEPCK* (Frescas *et al.* 2005). In addition to inactivating Foxo1 by phosphorylation through Akt, insulin induces the expression of COP1, which increases the ubiquitination of Foxo1 and its degradation via the ubiquitin–proteasome pathway (Kato *et al.* 2008), and regulates the expression of gluconeogenic genes. It should be noted that COP1 also downregulates the CREB co-activator TORC2 (Dentin *et al.* 2007).

5.5 *Foxo1 and TORC2*

Sequential actions of TORC2 and Foxo1 in the regulation of initial and subsequent phases of gluconeogenic gene expression during fasting have been reported lately (Liu *et al.* 2008). During feeding, insulin activates SIK2, which phosphorylates and inactivates p300, the enzyme that acetylates TORC2. Glucagon signalling activates p300 and, as a result, TORC2 is acetylated. The acetyl group protects TORC2 from degradation and enhances expression of both the gluconeogenic genes. It is interesting that insulin alters the stability and activity of TORC2 by inhibiting p300 indirectly and also inhibits TORC2 directly by promoting SIK2-mediated phosphorylation (Dentin *et al.* 2007). TORC2 activity is further controlled by SIK1, a component of the feedback loop constituted by the CREB–TORC2 pathway itself (Koo *et al.* 2005). Foxo1 induced by glucocorticoids (Sekine *et al.* 2007) and that activated by glucagon via dephosphorylation accumulates in the nucleus. At the same time, PGC-1α is induced by glucagon (Yoon *et al.* 2001). Concomitantly, changes in glucose levels and/or pyruvate increase synthesis of the SIRT1 protein, which accumulates gradually (Rodgers *et al.* 2005). Thus, the situation with high NAD/NADH, active SIRT1, favours the deacetylation and activation of the accumulated Foxo1 and its co-activator PGC-1α for the takeover from TORC2 (which is inactivated by the same modification), during long-term fasting. As shown by this study, it is important to separate the acute effects of fasting on *PEPCK* gene transcription from the chronic effects at a molecular level. Understandably, a complete picture would emerge from further studies that examine the effect in hepatocytes of glucocorticoids and glucagon on the transcription of these two gluconeogenic genes in fasting at different time points.

Several studies report higher activation of *G6Pase* than that of *PEPCK* mediated by Foxo1 and this could be due to the presence of only one response sequence for Foxo1 in the latter gene in comparison to three elements for this protein in the *G6Pase* promoter. It could be that the CREB–TORC2 pathway is more important for the stimulation of *PEPCK* gene expression, whereas the Foxo1 pathway might play a major role in the regulation of the *G6Pase* gene. If we look at the REs of Foxo1, CRE and GRE, there is an overlap between these three only in the *G6Pase* gene. Furthermore, overexpression of Foxo1 increases the expression of the catalytic subunit of *G6Pase* in response to cAMP and dexamethasone, and not that of *PEPCK* (Barthel *et al.* 2001). It is important to remember that G6Pase is the terminal step of gluconeogenesis and is important in releasing free glucose from glucose-6-phosphate liberated as a result of glycogen breakdown, and both these pathways operate during fasting with overlapping time frames. On the other hand, the logic behind the evolution of three REs for Foxo1 may lie in the fact that glucose taken in by cells is converted rapidly into glucose-6-phosphate for retention in the cell. Since G6Pase catalyses the conversion of glucose-6-phosphate to glucose, this gene may have to be more responsive to insulin and to inactivation of Foxo1 by insulin. In parallel, insulin also enhances glucose uptake by the hepatocytes, which is dependent on the concentration gradient of glucose between hepatocyte and blood, as facilitated by GLUT-2 in the plasma membrane; the steeper the gradient, the greater the transport. It is advantageous for the cell not to have a high intracellular concentration of glucose in order to facilitate more uptake. At the same time, glucose-6-phosphate that has accumulated until then can enter glycolysis, the pentose phosphate pathway or anabolic pathways that are triggered by insulin.

6. Development

The foetus receives nutrition via the placenta and thus does not require hepatic glucose production. Moreover, insulin, the dominant repressor of HGP, is high in the foetus. At the time of birth, this scenario needs to change, as the newborn does not receive a nutritional supply and has to maintain

euglycaemia. Therefore, glucagon and glucocorticoid levels increase with a reciprocal decline in insulin concentration, necessitating the action of PEPCK and G6Pase and expression of their genes. Villarroya and group (Yubero *et al.* 2004) report high levels of PGC1-*α* in foetal liver despite the lack of induction of the gluconeogenic programme. Hyperinsulinaemia and the absence of HNF-4*α*, which is a repressor of PGC-1*α* induction, partially explain the dissociation of this co-activator with the induction of the *PEPCK* and *G6Pase* genes. It should be remembered that PGC-1*α* induces gluconeogenic genes serving as co-activators of HNF-4*α*. At a certain level, HNF-4*α* may have a repressive effect on PGC-1*α* induction, again suggesting the presence of feedback loops.

C/EBP-*α* is a major regulator of liver differentiation and is abundant in the foetus, but does not affect the expression of *PEPCK*. Interestingly, Foxo1 is abundant in the neonate but scarce in embryonic day E14.5 and adult livers, and the level of this protein is increased by glucocorticoid treatment in E14.5 and primary hepatocytes. Foxo1 enhances C/EBP-*α* dependent *PEPCK* transcription and PGC1-*α* further augments this function (Sekine *et al.* 2007). Darlington and group have shown that C/EBP-*α* regulates *PEPCK* and *G6Pase* transcription early in the perinatal period as in C/EBP-*α*-null mice, at 2 h post partum, transcripts for cytosolic *PEPCK* were undetectable and the *G6Pase* gene was underexpressed by 70% relative to control mice. At 7 and 32 h post partum, mRNAs for these two genes matched those of the control mice, suggesting the importance of additional factors (Wang *et al.* 1995).

Since PEPCK and G6Pase are rate-limiting enzymes of the same metabolic pathway, one might expect that their genes would have a common induction programme. It has been shown that a certain level of HNF-6 in concert with HNF4*α*, both of which recruit PGC-1*α* during development, is required for the induction of G6Pase but not for PEPCK, which is considered as one of the markers of hepatic differentiation (Beaudry *et al.* 2006). This could be due to the distinct arrangement of components in the promoters of these two genes and the possible interactions among them. Physiologically, this may suggest the earlier onset of glycogenolysis than gluconeogenesis. This report highlights the importance of well-adjusted concentrations of transcription activators in regulating transcription during development as discussed later. The fact that HNF-6 is dispensable after birth (Beaudry *et al.* 2006) highlights the differences in stimuli that trigger the transcription of a particular gene between developing and differentiated cells

7. Perspectives

A chromatin environment is central to the regulation of transcription and studies have begun to investigate this aspect (Hall *et al.* 2007). Histones within the *PEPCK* gene promoter are highly acetylated even under unstimulated conditions, indicating an open chromatin configuration. The addition of insulin changes this pattern by deacetylating the histones associated with the promoter. Insulin treatment also results in the demethylation of H3-R17 (diMe) on the promoter, which corresponds to a change in the transcription rate. Thus, it appears that insulin action results in a compact/closed chromatin configuration at the *PEPCK* gene promoter (Hall *et al.* 2007).

Continuous efforts to explore the anatomical and physiological structure of the PEPCK and G6Pase promoters are revealing the importance of the components of one HRU in the complete effect caused by another hormone. For example, AF1 and AF3 elements, part of the GRU and RARU, are required for the complete effect of cAMP in the *PEPCK* gene. Chicken ovalbumin-upstream promoter-binding proteins (COUP-TF), CBP and SRC-1 participate in both the cAMP and glucocorticoid responses (Waltner-Law *et al.* 2003). The presence of RE overlaps in both the genes might be necessary for the additive and synergistic responses in the presence of combinations of hormones.

It appears that HRUs with multiple components are necessary for the fine control of graded physiological response to hormones, perhaps similar to many keys on the piano keyboard, which produce seamless sounds of music. Some keys are specific for certain notes and the sequence of notes is important for harmony. Only such a framework allows different permutations and combinations that are characteristic of living systems. The evolution of HRUs in mammalian genes has its origins in prokaryotes. Even the prototypic LAC operon contains two auxiliary operator sequences in addition to the main operator sequence. However, the same tetrameric repressor binds to the major operator and either of the accessory operators, thereby shutting down the expression of structural genes necessary for lactose metabolism. Binding at the auxiliary operators is necessary for complete effect. In contrast, various REs that exist in the *PEPCK* gene promoter bind different proteins: some, such as *α*- and *β*-isoforms of C/EBP and HNF-4*α*, impart tissue specificity to a response (liver-enriched in this case), while proteins such as GR, CREB, TORC2 and PGC-1*α* respond to external and internal stimuli. This provides for integration of response to signals that emanate as a result of cell functioning in a tissue-specific manner. The requirement of CRU for CREB or C/EBP*α* at CRE, and C/EBP*α*/C/EBP*β* at other distal (P1, P2, P4) sites (with AP-1 bound to P3) represents a built-in fail-safe mechanism. In addition, this arrangement offers various degrees of responsivity to hormone (Roesler 2000).

This system also points to the possibility of expression of different genes at gradually increasing concentrations of a hormone and contributes to temporal patterns in transcriptional networks – based on the nature of REs

(consensus or non-consensus as in the GRE of the *PEPCK* gene) and other components (accessory-binding sites AF1, AF2, AF3, as in the *PEPCK* gene) in those transcription units. Products of genes that are activated early might contribute further to the regulation of genes that are expressed later (cAMP induces PGC-1α through TORC2, and PGC-1α, in turn, activates the transcription of *PEPCK* and *G6Pase*) (Yoon *et al.* 2001). The presence of proteins that connect signals from several pathways enhances the efficiency of such a composite unit, one such protein being TORC2. TORC2 is glycosylated precisely at sites that are phosphorylated and thus integrates the effects of glucose on gluconeogenesis with insulin signalling (Dentin *et al.* 2008). O-GlcNAc-dependent activation of TORC2/CREB drives PGC-1α expression. PGC-1α, in turn, increases targeting of O-GlcNAc transferase to Foxos, further increasing gluconeogenesis (Housley *et al.* 2009). Importantly, the energy status-sensing kinase AMPK phosphorylates TORC2 and blocks hepatic glucose production – a key event in the convergence of energy-sensing and hormonal pathways (Koo *et al.* 2005). TORC2 is necessary for the recruitment of CBP to genes that are turned on by cAMP and not by stress signals, showing that it is at the level of the co-activator that specificity is achieved in response to two entirely different signals which result in the phosphorylation of CREB to an equal extent (Ravnskjaer *et al.*2007). Whether TORC2, like PGC-1α, serves as a coactivator to liver-specific factors such as HNF-4α remains to be seen. CBP also may be involved in the integration of signals. CBP supports transcription not only by CREB but also interacts with many transcription factors that regulate *PEPCK*, including thyroid receptor, glucocorticoid receptor, Jun and C/EBP (Leahy *et al.* 1999 and references therein). Even adenovirus early protein E1A interacts with CBP to completely inhibit cAMP induction of the gene in hepatoma cells.

Studies probing *PEPCK* and *G6Pase* promoters reveal further insights into the organization of the distal upstream region. Two distal accessory elements in *PEPCK* have been identified lately (Cassuto *et al.* 2005). How these sites control transcription in concert with the proximal sites and whether the mechanisms mimic those of enhancers, and to what extent protein–protein interactions and DNA bending contribute to this effect remain to be understood.

Regulation of transcription brought about by transcription factors is well established by now. However, various hormone-mediated modifications of transcription factors and cofactors such as Foxo1 and PGC-1α, respectively, and integration of signals from various pathways by TORC2 and CBP pose another question: are there other proteins that modulate transcription which have not yet been discovered or whose functions are yet to be discovered? In addition, how the constellation of proteins located at the 5' end of the gene transfers transcriptional activation signal to the RNA polymerase initiation complex remains to be elucidated. Integration of data from transcription factor databases with the data from genomewide location analysis (ChIP-on-chip) may provide information regarding active chromatin. Identification of the entire repertoire of proteins; paraphernalia required to regulate expression under different condition is required for further progress in this direction. This, in turn, would pave the way for understanding the synergistic effects of various hormones. At the same time, such studies would facilitate discovery of the order in which proteins are recruited to the promoter DNA in response to a hormone, and would lead to new therapeutic interventions.

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