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HEPATIC GLUCONEOGENESIS/ GLYCOLYSIS: Regulation and Structure/Function Relationships of Substrate Cycle Enzymes

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INTRODUCTION

Hormonal regulation of metabolic processes such as hepatic gluconeogenesis, glycolysis, and glycogen metabolism has been an active area of research for decades. Many important advances in our understanding of enzyme regulation by allosteric mechanisms, covalent modification, and changes in gene expression have resulted from this research. These advances include (*a*) the discovery of cAMP (184); (*b*) the discovery of a consensus sequence in the 5'-flanking region of the phosphoenolpyruvate carboxykinase (PEPCK) gene for regulation by cAMP (205), which led to the identification of similar sequences in other cAMP-regulated genes [see (192) for review]; (*c*) the pioneering studies on PEPCK gene expression that demonstrated specific regulation of gene transcription by insulin [see (71) for review]; (*d*) the phosphorylation of liver (L)-type pyruvate kinase (PK) by cAMP-dependent protein kinase [see (50) for review]; and (*e*) the discovery of Fru-2,6-P_2, an important regulator of glycolysis in many cell types [see (80, 155) for review]. The latter two discoveries have pinpointed the short-term action of hormones and cAMP on liver glycolysis/gluconeogenesis at a number of key regulatory enzymes located at two substrate cycles in the pathway: the first between Fru-6-P and Fru-1,6-P_2, which includes the enzymes 6-phosphofructo-1-kinase (6PF-1-K) and fructose-1,6-bisphosphatase (Fru-1,6-P_2ase); and the second between pyruvate and PEP, which includes PK as the most important regulatory step (79, 164, 165).

Hormonal regulation can be divided into short-term and long-term effects. The former, which occur in seconds to minutes, involve alterations in enzyme activity brought about by changes in allosteric effectors and/or phosphorylation state of enzymes while the latter involve changes in gene expression, a process that usually takes hours to days. The long-term effects of hormones not only involve enzymes of the Fru-6-P/Fru-1,6-P_2 and PEP/pyruvate substrate cycles but also the opposing enzymes of the glucose/G-6-P cycle.

In recent years a number of reviews have described the regulation of gluconeogenesis/glycolysis in great detail (23, 61, 79, 84, 97, 164, 165). In this review we restrict the discussion to important regulatory substrate cycle enzymes in the hepatic glycolytic/gluconeogenic pathway (PK, 6-PF-2-K/Fru-2,6-P_2ase, 6-PF-1-K, Fru-1,6-P_2ase, PEPCK, and GK) and we sum-

marize information, obtained during the last five years on enzyme regulation and structure/function relationships, that has allowed new insights into the nature, control, and evolution of these enzymes. For convenience, this review is divided into sections on short-term and long-term control. Each section begins with a summary of hormonal and/or nutritional regulation followed by a discussion of how our current knowledge of the structure/function relationships of the substrate cycle enzymes, analysis of their gene structure and regulation, and in some cases the use of molecular genetics have provided a basis for understanding their regulatory properties as well as their role in pathway control.

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GENERAL MECHANISMS OF SHORT-TERM HORMONAL REGULATION OF HEPATIC METABOLISM

Hormonal regulation of metabolic processes in liver occurs by two general mechanisms. The first mechanism includes those hormones (glucagon, β-adrenergic agonists) that interact with a plasma membrane receptor that is coupled to adenylyl cyclase activation, thereby resulting in an elevation of the level of intracellular cAMP. Elevation of cAMP levels leads to activation of cAMP-dependent protein kinases that then catalyze the phosphorylation of protein substrates and result in a physiologic response such as the stimulation of gluconeogenesis and inhibition of glycolysis (23, 61, 79, 84, 97, 164, 165). The second mechanism involves those hormones that act via changes in intracellular Ca^{2+} levels, by a mechanism involving the generation of two intracellular messengers: *myo*-inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (51). The change in intracellular Ca^{2+} level, in combination with calmodulin and/or other effectors, leads to activation of several Ca^{2+}-linked protein kinases including the Ca^{2+}- and calmodulin-dependent protein kinase. In general, with regard to gluconeogenesis and glycolysis, both cAMP- and Ca^{2+}-dependent phosphorylations lead to similar changes in pathway flux.

Insulin, on the other hand, opposes the action of cAMP-linked hormones, probably by its ability to activate cAMP phosphodiesterase, and results in lower cAMP levels (21). Precisely how this effect on phosphodiesterase is brought about is not known. The mechanism of insulin's action to oppose Ca^{2+}-linked hormones is also unknown at present. Insulin's effects may thus represent an additional general mechanism for regulation of metabolic processes in liver.

In liver the short-term control of glycolysis and gluconeogenesis poses a number of interesting regulatory problems. The fact that enzymes specific for gluconeogenesis, PEPCK, Fru-1,6-P_2ase, and G-6-Pase, are opposed in the cell by glycolytic enzymes PK, 6PF-1-K, and GK, respectively, raises the

possibility of cycling between the substrates and products of the enzymes. The simultaneous operation of opposing reactions makes possible a sensitive control system in which both the rate and direction of flux can be regulated by small changes in the concentration of effectors of one or more of the enzymes involved in the cycle and/or by covalent modification of these enzymes.

SHORT-TERM HORMONAL REGULATION IN THE GLYCOLYTIC/GLUCONEOGENIC PATHWAY

Regulation at the Pyruvate/Phosphoenolpyruvate Substrate Cycle

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EARLY EVIDENCE FOR REGULATION AT THE PYRUVATE KINASE STEP Some PEP is recycled to pyruvate during gluconeogenesis in perfused liver and isolated hepatocytes (59, 60, 193), and flux through PK is affected by nutritional state and hormones (193–195). Glucagon and cAMP strongly inhibit this flux, whereas epinephrine is only marginally effective (161, 176, 194). Insulin relieved the inhibition of PK flux caused by submaximal concentrations of glucagon (161, 176).

COVALENT MODIFICATION OF L-TYPE PYRUVATE KINASE These studies implicated PK in the regulation of gluconeogenesis. Liver-type PK is an allosteric enzyme that exhibits sigmoidal kinetics with regard to its substrate PEP. It is allosterically activated by Fru-1,6-P_2 and inhibited by alanine and ATP. With physiologic concentrations of alanine, ATP, and PEP, in vitro studies predicted that the enzyme would be completely inhibited unless it were activated by Fru-1,6-P_2 (56, 227).

Purified PK from rat liver can be phosphorylated by [γ-^{32}P]ATP in a reaction catalyzed by cAMP-dependent protein kinase (50, 123). Phosphorylation increases the phosphoenolpyruvate concentration required to reach 50% of the maximum activity, whereas no change in activity was observed in the presence of high concentrations of substrate or Fru-1,6-P_2 (123, 160, 189, 190). The phosphorylated enzyme is more readily inhibited by alanine and ATP than is the nonphosphorylated enzyme, although it is less readily activated by Fru-1,6-P_2 than is the nonphosphorylated enzyme (41, 50, 123).

The allosteric effectors of PK not only affect activity but also modulate phosphorylation of the enzyme by cAMP-dependent protein kinase (43, 53, 173). Both Fru-1,6-P_2 and PEP inhibit the rate of phosphorylation while alanine relieves the inhibition by physiologic concentrations of either Fru-1,6-P_2 or PEP. Thus, PK is a better substrate for cAMP-dependent protein kinase when the enzyme is inhibited than when it is activated.

Phosphorylation of hepatic PK is also catalyzed by Ca^{2+}-calmodulin-dependent protein kinase, and this phosphorylation results in inhibition of activity (202). This inhibition, like that seen with cAMP-dependent phosphorylation, is characterized by decreased affinity for PEP. The Ca^{2+}-calmodulin-dependent protein kinase catalyzes phosphorylation of two sites: the first at the same seryl residue phosphorylated by cAMP-dependent protein kinase and the second on a unique threonyl residue five residues C-terminal to the cAMP site (202, 207). Phosphorylation of only the threonyl residue also results in decreased affinity for PEP and inhibition of enzyme activity (202).

The effects of cAMP-dependent phosphorylation on purified PK suggested that hormones that raise cAMP levels should have similar effects on the enzyme. The addition of glucagon to isolated hepatocytes or to perfused liver, or administration in vivo, leads to increases in phosphate content of PK and, concomitantly, to inhibition of enzyme activity and flux (8, 20, 52, 57, 65, 90, 122, 189, 190, 210). In addition, the ability of submaximal concentrations of glucagon to phosphorylate the enzyme is modulated by elevated intracellular levels of Fru-1,6-P_2 or alanine (20, 57). Ca^{2+}-calmodulin-dependent phosphorylation accounts for the small effect of α-adrenergic agonists on PK activity and flux observed in isolated hepatocytes (14, 65, 210). Insulin also suppresses the effect of α-adrenergic agonists on PK activity by a cAMP-independent mechanism (20).

These studies showed that hormones stimulate gluconeogenesis, at least in part, by inhibiting PK. Figure 1 shows the regulation of this enzyme both by cAMP-dependent protein kinase–catalyzed phosphorylation and by allosteric effectors. The importance of Fru-1,6-P_2 in the regulation of PK is strengthened by the observation that this effector can control flux through PK in hepatocytes and that glucagon decreases its concentration (9, 20, 75, 161, 176, 241). The decrease in Fru-1,6-P_2 inactivates the enzyme directly and makes PK a better substrate for phosphorylation by cAMP-dependent protein kinase.

Regulation at the Fru-6-P/Fru-1,6-P_2 Substrate Cycle

EARLY EVIDENCE FOR HORMONAL REGULATION Fru-1,6-P_2 levels are controlled by the activities of the gluconeogenic enzyme Fru-1,6-P_2ase and the opposing glycolytic enzyme 6PF-1-K. Both enzymes are operative in vivo, in perfused liver, and in hepatocytes [see (23, 61, 79, 84, 97, 164, 165) for review], and flux through these enzymes is modulated by hormones and dietary status (18, 19, 98, 105, 230, 231). For example, both starvation and the addition of glucagon decreased flux through 6PF-1-K while increasing the rate of gluconeogenesis. These results suggested that the activity of either or both of these enzymes must be regulated by hormones. Subsequently, addition of glucagon to isolated hepatocytes was shown to inhibit 6PF-1-K activity

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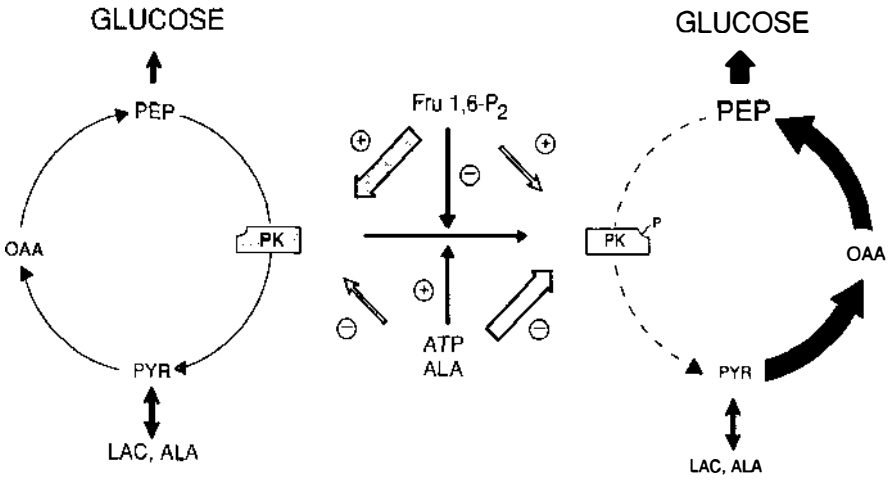

Figure 1 Regulation of hepatic PK by cAMP-dependent protein kinase–catalyzed phosphorylation and allosteric effectors. When cAMP levels are elevated (e.g. high glucagon-insulin ratio in plasma), PK is in its phosphoform and inhibited *(open box)*, recycling of PEP to pyruvate is inhibited *(dashed line)*, and gluconeogenesis is favored. Fru-1,6-P_2 inhibits, whereas alanine (ALA) and ATP enhance cAMP-dependent protein kinase–catalyzed phosphorylation of PK. Fru-1,6-P_2 activation of the enzyme is decreased by phosphorylation. ATP and ALA are more effective inhibitors of the phosphoform than of the nonphosphorylated form. LAC, lactate; OAA, oxaloacetate. Adapted from Ref. 7 with permission.

that was measured in crude extracts (13, 95, 177). Both enzymes were also found to be substrates for cAMP-dependent protein kinase in vitro and in hepatocytes, and initially this was thought to be the mechanism whereby their activities were altered (13, 24, 38, 39, 95, 163, 166, 188, 197). Meaningful changes in 6PF-1-K and Fru-1,6-P_2ase activities, however, were difficult to discern after the in vitro phosphorylation of 6PF-1-K and Fru-1,6-P_2ase by cAMP-dependent protein kinase [see (64) for review]. Furthermore, inhibition of 6PF-1-K disappeared after partial purification of the enzyme and subsequently was shown to be due to changes in a low molecular weight effector (25). The discovery of this effector, Fru-2,6-P_2, provided an alternative explanation for the regulation of both 6PF-1-K and Fru-1,6-P_2ase (22, 80, 155, 165).

ROLE OF FRU-2,6-P_2 Fru-2,6-P_2 is a potent allosteric activator of 6PF-1-K and a competitive inhibitor of Fru-1,6-P_2ase (22, 79, 80, 155, 162, 164, 165). The importance of the effects of Fru-2,6-P_2 on these two enzymes vis-a-vis regulation of hepatic gluconeogenesis is underscored by the observation that the level of this effector is subject to both nutritional and hormonal regulation (22, 140, 155, 156, 168, 226). The conclusion from these studies was that

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when rates of gluconeogenesis are high (starvation and diabetes), the level of Fru-2,6-P_2 is low, and when they are low (refeeding and insulin administration), the level of Fru-2,6-P_2 is high (22, 168, 226).

DISCOVERY, CHARACTERIZATION, AND REGULATION OF 6-PHOSPHOFRUCTO-2-KINASE (6PF-2-K)/FRU-2,6-P_2ASE Because the level of this effector varies with hormonal or nutritional status, the enzymes responsible for the synthesis and/or degradation of Fru-2,6-P_2 must be regulated. The synthesis of Fru-2,6-P_2 involves a 6PF-2-K reaction (Fru-6-P + ATP → Fru-2,6-P_2 + ADP), whereas its degradation is catalyzed by a specific Fru-2,6-P_2ase reaction (Fru-2,6-P_2 → Fru-6-P + P_i). A unique bifunctional enzyme, 6PF-2-K/Fru-2,6-P_2ase, catalyzes both of these reactions, and phosphorylation by cAMP-dependent protein kinase results in inhibition of kinase and activation of bisphosphatase, whereas dephosphorylation results in opposite changes in the two activities (22, 42, 45, 138, 174).

These changes in activity provided an explanation for the rapid modulation of Fru-2,6-P_2 levels that was seen when β-adrenergic agonists, glucagon, and insulin were added to isolated hepatocytes (22, 66, 156, 165). The increase in Fru-2,6-P_2 levels that occurs when α-adrenergic agonists are added to hepatocytes from fed rats is due to enhanced glycogenolysis and the provision of more Fru-6-P (85), and not to changes in phosphorylation state of the enzyme (22, 66).

The only protein kinase known to catalyze phosphorylation of hepatic 6PF-2-K/Fru-2,6-P_2ase is cAMP-dependent protein kinase; dephosphorylation of the enzyme is catalyzed primarily by protein phosphatase 2A (151). Recently, evidence for insulin stimulation of dephosphorylation of 6PF-2-K/Fru-2,6-P_2ase and PK has been obtained under conditions where cAMP levels did not change (3). The investigators postulated that insulin promotes the activity of one or more phosphatases, but these phosphatases were not identified or characterized. That insulin addition slightly increased phosphatase 2A activity in isolated hepatocytes has also been reported (54), but the physiologic relevance of this effect remains to be established.

Coordinated Regulation at the Fru-6-P/Fru-1,6-P_2 and Pyruvate/Phosphoenolpyruvate Substrate Cycles

The discovery of Fru-2,6-P_2 and the enzyme responsible for its synthesis and degradation, 6PF-2-K/Fru-2,6-P_2ase, provided the last piece in the puzzle of how hormones that act through cAMP regulate glycolysis/gluconeogenesis on a minute-to-minute basis in mammalian liver. Table 1 and Figure 2 summarize the acute effects of these hormones on PK, 6PF-2-K/Fru-2,6-P_2ase, gluconeogenesis, cAMP levels, and Fru-2,6-P_2 levels. Glucagon or a β-adrenergic agonist binds to its plasma membrane receptor and activates

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Table 1 Summary of hormonal regulation of gluconeogenesis, L-type PK, 6PF-2-K/Fru-2,6-P_2ase, and Fru-2,6-P_2 levels[a]

Hormone	Gluconeogenesis	Fru-2,6-P_2	Pyruvate kinase	6PF-2-K/FRU-2,6-P_2ase	Mechanism
Glucagon	Stimulates	↓	Inhibits	Inhibits/activates	↑ cAMP / ↑ cAMP-protein kinase
Insulin	Inhibits	↑	Activates	Activates/inhibits	↓ cAMP / ↓ cAMP-protein kinase and ↑ protein p'ase
Catecholamines					
β-agonist	Stimulates	↓	Inhibits	Inhibits/activates	↑ cAMP / ↑ cAMP-protein kinase
α-agonist	Stimulates	↑	Inhibits	— —	↑ Ca^{2+} / ↑ Ca^{2+}/CAM-protein kinase
Vasopressin	Stimulates	↑	Inhibits	— —	↑ Ca^{2+} / ↑ Ca^{2+}/CAM-protein kinase
Angiotensin	Stimulates	↑	Inhibits	— —	↑ Ca^{2+} / ↑ Ca^{2+}/CAM-protein kinase

[a] (—) indicates no effect on the phosphorylation state of 6PF-2-K/Fru-2,6-P_2ase. Fru-2,6-P_2 levels increase upon addition of α-agonists, vasopressin, and angiotensin because glycogen breakdown is enhanced by a Ca^{2+}-linked mechanism leading to elevated glycolysis and ipso facto elevated Fru-6-P.

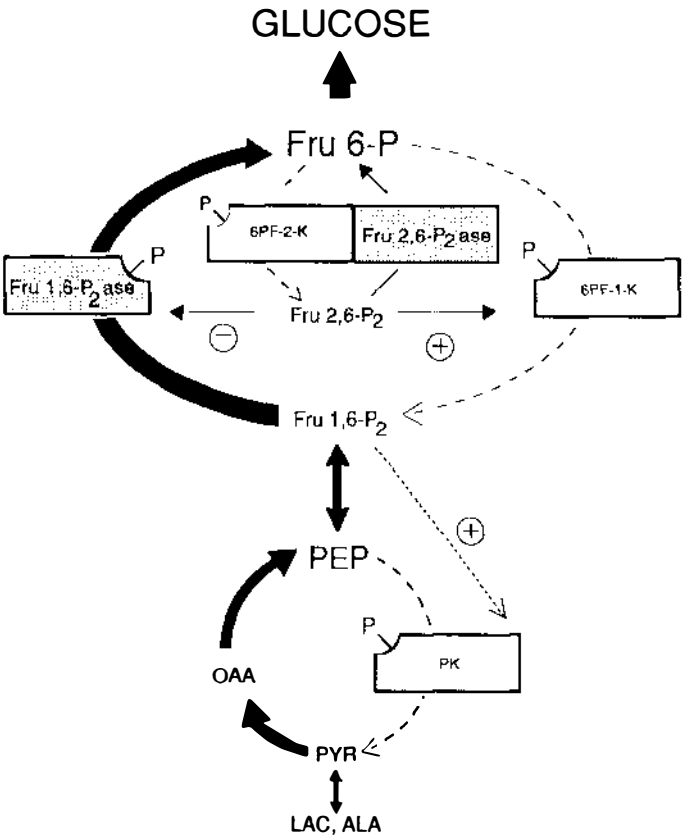

Figure 2 Sites of short-term regulation of hepatic gluconeogenesis by cAMP. Elevation of liver cAMP levels leads to phosphorylation of PK, 6PF-1-K, Fru-1,6-P_2ase, and 6PF-2-K/Fru-2,6-P_2ase, which are indicated by -P attached to appropriate enzyme box. *Shaded boxes*, enhanced enzyme activity; *open boxes*, enzyme inhibition. *Solid lines*, increased flux; *dashed lines*, diminished flux. ALA, alanine; LAC, lactate. 6PF-1-K and Fru-1,6-P_2ase activities are regulated by changes in the Fru-2,6-P_2 level, which is decreased as a result of 6-PF-2-K/Fru-2,6-P_2ase phosphorylation. Adapted from Ref. 7 with permission.

adenylyl cyclase, which results in increased intracellular cAMP levels and lower Fru-2,6-P_2 levels. Fru-1,6-P_2 is reduced owing to inhibition of 6PF-1-K and activation of Fru-1,6-P_2ase. The decrease in Fru-1,6-P_2 inactivates PK, both allosterically and by making the enzyme a better substrate for phosphorylation by cAMP-dependent protein kinase. These actions reduce PK activity and favor gluconeogenesis. Thus, the Fru-6-P/Fru-1,6-P_2 and PEP/pyruvate substrate cycles are linked together by the actions of Fru-2,6-P_2.

Stimulation of gluconeogenesis by α-adrenergic agonists is mediated not by

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cAMP but rather by a rise in intracellular Ca^{2+} and a subsequent increase in Ca^{2+}-calmodulin-dependent protein kinase activity (14, 65, 66, 210). This enzyme catalyzes phosphorylation and inactivation of PK (202, 207). However, Ca^{2+}-calmodulin-dependent protein kinase does not catalyze phosphorylation of 6PF-2-K/Fru-2,6-P_2ase, 6PF-1-K, or Fru-1,6-P_2ase, which may account for the smaller stimulation of gluconeogenesis by α-adrenergic agonists than by glucagon or β-adrenergic agonists.

Insulin counteracts the stimulation of gluconeogenesis by glucagon and β-adrenergic agonists by suppressing the rise in cAMP brought about by these hormones (157). This effect may be mediated by activation of a cAMP phosphodiesterase (21). The hormone's action to oppose glucagon and/or β-adrenergic agents under physiologic conditions can be totally explained by its ability to lower cAMP. Thus, insulin reduces the extent of phosphorylation of PK and 6PF-2-K/Fru-2,6-P_2ase, which results in a reversal of the effects described above and a decrease in the rate of gluconeogenesis. Insulin can also oppose the stimulation of gluconeogenesis by α-adrenergic agonists at the level of PK (20, 52). The mechanism of this cAMP-independent effect of insulin is unknown but may involve activation of protein phosphatase 2A (3, 21, 54).

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Structure/Function Relationships of Substrate Cycle Enzymes

L-TYPE PYRUVATE KINASE L-type PK plays a central role in glycolysis/gluconeogenesis by regulating carbon flux through the PEP/pyruvate substrate cycle (79, 84, 97, 115, 164, 165). Rat liver PK consists of four identical subunits with a subunit molecular weight of 57 kDa (87, 115). Mammals have four isoforms of PK: L, L', M1, and M2 (87, 131). The M forms are expressed in muscle and other cell types (89). The L and L' isoforms are expressed mainly in liver and red blood cells, respectively, and are encoded by the same gene while M1 and M2 are encoded by a different gene (96, 144, 152, 212). The L and L' mRNAs are identical except for the first exons, and two different promoters regulate expression of the two mRNAs in a tissue-specific fashion (26, 145). The L and L' isozymes are both phosphorylated by the cyclic AMP-dependent protein kinase (123, 130). The phosphorylation site is located near the N-terminus of the enzyme subunit, and the amino acid sequence is identical in the two isoforms (145). The L' isozyme consists of 574 amino acids, which is 31 residues longer than the L type and 44 residues longer than the M1 and M2 isozymes. The M forms do not contain cAMP-dependent phosphorylation sites and are regulated only by allosteric effectors. Both phosphorylation and allosteric effectors are important in the acute regulation of L-type PK (50, 165). Although the amino acid sequence of the L-type PK is known (124, 145), the residues involved in substrate and/or allosteric effector binding to this form are not known because no X-ray crystal structure is yet available.

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Since the cAMP-dependent phosphorylation site and the Ca^{2+}-calmodulin phosphorylation site are located just five residues apart at the N-terminus of the enzyme, it is not surprising that both phosphorylation events result in similar changes in kinetic properties of the enzyme (Table 2). Phosphorylation appears to modify the ease with which the enzyme undergoes conformational changes in response to its effectors. The end result is to shift the equilibrium from the active to the inactive form of the enzyme. Increasing concentrations of PEP and Fru-1,6-P_2 cause a conformational change in the enzyme by binding preferentially to the active form of the enzyme. Phosphorylation of the enzyme subunits may impede this conformational change and shift the equilibrium between active and inactive forms of the enzyme subunit, which results in decreased enzyme activity. High concentrations of PEP and Fru-1,6-P_2 can overcome this effect of phosphorylation. L-type pyruvate kinase appears to be an excellent model system for studying the structural basis for phosphorylation-induced activity changes. However, such studies require elucidation of the X-ray crystallographic structure of the liver isozyme. Only in the case of phosphorylase, whose crystal structure is known, has it been possible to study phosphorylation-induced changes in enzyme conformation (209).

6-PHOSPHOFRUCTO-1-KINASE Rat liver 6PF-1-K consists of four identical subunits with a molecular weight of 82 kDa (10). The liver enzyme exhibits homotropic cooperativity with regard to its substrate Fru-6-P (10, 132, 133, 163, 185, 186, 213). Allosteric activators of the enzyme include AMP, ADP, and cAMP, while ATP and citrate are allosteric inhibitors (10, 132, 185, 186). The ATP inhibition of the enzyme decreases markedly as the pH increases from 6.5 to 8.0 while citrate potentiates the inhibitory effect of ATP (150, 224).

Fru-2,6-P_2 activation Fru-2,6-P_2 is a potent allosteric activator of rat liver 6PF-1-K (22, 79, 164, 165). In the absence of any effectors, the enzyme exhibits a low affinity and a high degree of positive cooperativity toward its substrate, Fru-6-P. Fru-2,6-P_2 increases the affinity of the enzyme for Fru-6-P but has no effect on maximum activity of the enzyme (22, 79, 164, 225, 232). The K_a for Fru-2,6-P_2 is about 0.05 μM, consequently, this sugar bisphosphate is 50–100 times more effective than Fru-1,6-P_2 (79, 164, 165, 170, 208) and 2500 times more effective than glucose-1,6-bisphosphate (80). Fru-2,6-P_2 also overcomes inhibition by high concentrations of ATP (80, 225, 226), potentiates the activation by AMP (79, 164, 165, 174, 232), and acts synergistically with AMP to relieve ATP inhibition (225). Fru-2,6-P_2 also reportedly protects 6PF-1-K against inactivation by heat, low pH, or 6PF-1-K phosphatase (208, 225). Muscle 6PF-1-K bound 1 mol of Fru-2,6-P_2 per enzyme subunit, and the binding exhibited negative cooperativity, which

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Table 2 Effect of phosphorylation on the kinetic properties of L-type pyruvate kinase[a]

Kinetic parameters	L-type unphosphorylated	Phospho-L-type (cAMP)	Phospho-L-type (Ca^{2+},CAM)	Phospho-L-type (cAMP+Ca^{2+},CAM)
$S_{0.5}$ for Fru-6-P	0.6 mM	1.2 mM	1.0 mM	1.48 mM
Hill coefficent	2.0	2.7–2.9	2.5	3.0–3.1
Activation by Fru-1,6-P_2 (Ka)	0.02 μM	0.3 μM	0.09 μM	0.45 μM
Inhibition by ATP ($K_i$app)	[redacted] mM	0.4 mM	0.6 mM	0.2 mM
Inhibition by alanine ($K_i$app)	[redacted] mM	0.3 mM	0.4 mM	0.1 mM

[a] L-type pyruvate kinase was unphosphorylated or phosphorylated by cAMP-dependent protein kinase or by the Ca^{2+}, CAM-dependent protein kinase. Phosphorylation of only the threonine residue by the Ca^{2+}, CAM-dependent protein kinase was achieved after removal of phosphate from the cAMP-dependent site by reversal of the cAMP-dependent protein kinase reaction as described previously (33, 34).

suggests that Fru-2,6-P_2 binds to the enzyme at the same allosteric site as does Fru-1,6-P_2 [(103) and see (101) for review].

Role of covalent modification The role of phosphorylation in regulating the activity of 6PF-1-K remains uncertain, but the consensus now is that phosphorylation probably is not a physiologically relevant form of regulation in liver (22, 79, 158, 164, 165). Cyclic AMP-dependent protein kinase catalyzes phosphorylation of liver 6PF-1-K at a single C-terminal site (163), but compared to PK the enzyme is a very poor substrate for phosphorylation [see (158) for review]. Both a high- and a low-phosphate-containing form of rat liver 6PF-1-K have been isolated, and they exhibit different kinetic properties (62, 197). However, the changes observed in the regulatory properties of the enzyme were small. Phosphorylation may regulate hepatic 6PF-1-K activity under certain conditions in vitro. However, in the case of glucagon- or insulin-induced alterations in liver enzyme activity that were measured in crude extracts, partial purification of the enzyme results in complete disappearance of the hormone effect (25, 156). This finding suggests that changes in Fru-2,6-P_2 concentration are the most important regulating factor (22, 25, 156, 158).

Evolution of phosphofructokinases Recent analysis of the structure of bacterial and mammalian 6PF-1-Ks, pyrophosphate-dependent 6PF-1-K, and the 6PF-2-K domain of 6PF-2-K/Fru-2,6-P_2ase suggests that all these proteins are related (Figure 3). Bacterial 6PF-1-Ks have subunit molecular weights of about 36 kDa (78). Mammalian 6PF-1-K is a homotetramer with a subunit molecular weight of 82 kDa, a size about twice that of the bacterial enzymes (114). Since the N-terminal and C-terminal halves of the mammalian enzymes are homologous to each other and with the bacterial enzyme, it has been postulated that mammalian 6PF-1-K arose as a result of gene duplication of a progenitor gene (182). Analysis of the N-terminal and C-terminal sequences suggests that mutations of certain active site residues in the C-terminal half of the mammalian enzyme have resulted in allosteric binding sites, e.g. for Fru-2,6-P_2 (182). This would explain the lack of effect of Fru-2,6-P_2 on the bacterial 6PF-1-K (80). The same scenario applies to yeast 6PF-1-K, a heterodimer in which each subunit apparently arose from gene duplication and tandem ligation (77). Recent evidence also suggests that the α- and β-subunits of pyrophosphate-dependent 6PF-1-K are related and may have evolved from a common ancestral gene (12). Isolation of full-length cDNAs and elucidation of the amino acid sequence of the α- and β-subunits revealed that the subunits have 40% identity. A comparison of their sequences with that of the ATP-dependent 6PF-1-K from *Escherichia coli* showed little sequence homology except between regions involved in the binding of Fru-6-P and Fru-1,6-P_2 and

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possibly between regions binding pyrophosphate and the β- and α-phosphates of ATP/ADP. A comparison of the derived structures of the α- and β-subunits of pyrophosphate-dependent enzyme with the known secondary structure of the *E. coli* 6PF-1-K also suggested that the overall structures of these enzymes were analogous. The α- and β-subunits of the plant enzyme and the bacterial 6PF-1-K consist of alternating α- and β-structures that are thought to form a nucleotide-binding domain in the ATP-dependent enzyme (12). The sequence comparison also revealed that active site residues were conserved in the β-subunit, which was postulated to be the catalytic subunit of the pyrophosphate-dependent enzyme. A similar nucleotide binding fold has been found in all ATP-dependent 6PF-1-Ks as well as in the 6PF-2-K domain of 6PF-2-K/Fru-2,6-P_2ase (5), suggesting that the structure of the phosphofructokinase active site in all these enzymes has been conserved. Apparently, the mammalian and yeast 6PF-1-Ks arose as a result of gene duplication and fusion, the pyrophosphate-dependent 6PF-1-K by gene duplication without fusion, and the 6PF-2-K/Fru-2,6-P_2ase by gene fusion of a phosphotransferase with a phosphohydrolase domain (Figure 3).

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FRUCTOSE-1,6-BISPHOSPHATASE Mammalian liver Fru-1,6-P_2ase is a homotetramer with a subunit molecular weight of 38–41 kDa, depending on the species (83). Fru-1,6-P_2ase displays hyperbolic kinetics with regard to its substrate, Fru-1,6-P_2 (179, 199, 220). However, the enzyme is generally considered to be allosteric because of the noncompetitive nature of its inhibition by AMP.

Fructose-2,6-P_2 inhibition Since many of the effectors of 6PF-1-K affect the activity of Fru-1,6-P_2ase in a reciprocal manner, it is not surprising that Fru-2,6-P_2 was found to be a potent competitive inhibitor of the latter enzyme (169, 229). The inhibition by low concentrations of Fru-2,6-P_2 displays hyperbolic kinetics with respect to substrate, indicative of competitive inhibition at the active site (169). Higher concentrations of Fru-2,6-P_2 result in a sigmoidal response to increasing substrate concentrations (167, 229), which suggests that Fru-2,6-P_2 may also interact with a site other than the catalytic site. However, studies on the binding of Fru-2,6-P_2 to Fru-1,6-P_2ase revealed that only 1 mol Fru-2,6-P_2 bound per mol enzyme subunit (135). Binding exhibited negative cooperativity and was competitive with methyl α- and β-D-fructofuranoside-1,6-P_2, competitive substrate analogs of Fru-1,6-P_2. Taken together, these results indicate that Fru-2,6-P_2 binds to the catalytic site, and this conclusion has been confirmed by others using various kinetic approaches (63, 70, 128, 180).

In contrast to most of these findings, Francois et al (58) have argued that Fru-2,6-P_2 does not interact at all with the active site but instead binds to a separate allosteric site. The major points in favor of this view are (*a*) the

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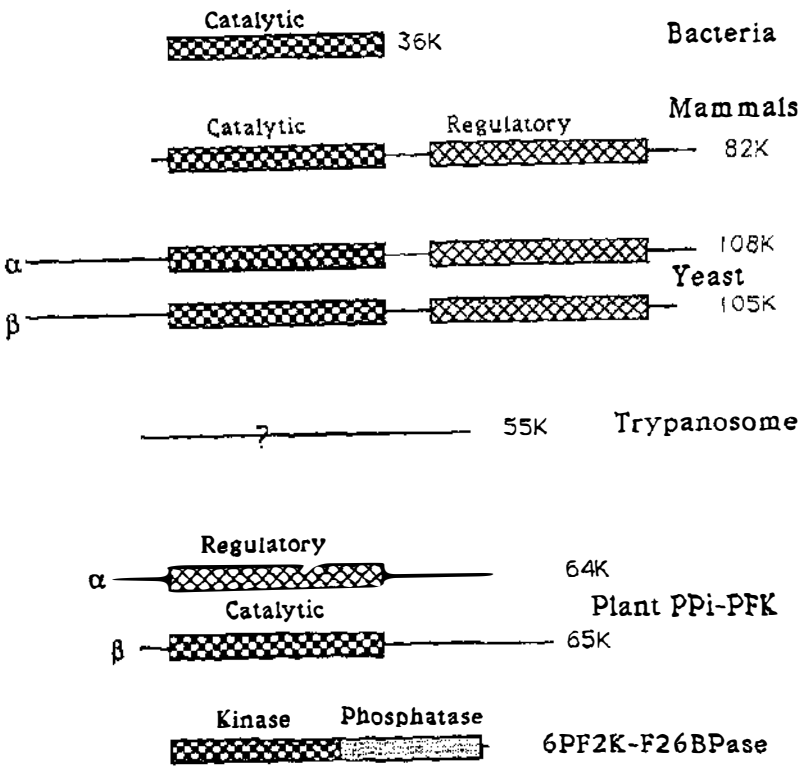

Figure 3 Evolution of phosphofructokinases. Phosphofructokinases from bacteria, yeast, plants, and mammals have analogous structures that have resulted from gene duplication of a *bacterial catalytic unit* (▩). In mammals the gene duplication was followed by tandem ligation and mutation of the gene-duplicated region, which led to the creation of effector sites for Fru-2,6-P_2 and ATP. The coding sequence arising from gene duplication is illustrated by (▩). The yeast enzyme is a heterodimer, whose two subunits also arose from gene duplication and tandem ligation. In this case, however, some controversy exists as to the identity of the catalytic unit. For trypanosomes and other protozoa no structural information is yet available. The plant pyrophosphate-dependent phosphofructokinase is a heterodimer, both subunits of which have analogous structures to the bacterial enzyme. Based on conservation of residues for ATP and Fru 6-P binding, the β-subunit is postulated to be the catalytic unit. 6PF-2-K/Fru-2,6-P_2ase arose as a result of gene duplication of a mutase/phosphatase and phosphotransferase catalytic units. The 6PF-2-K structure is also analogous to the bacterial 6PF-1-K (5).

sigmoidal substrate concentration curve in the presence of high concentration of Fru-2,6-P_2, (*b*) potentiation of AMP inhibition by Fru-2,6-P_2, and (*c*) the similar responses of Fru-2,6-P_2 and AMP inhibition to temperature.

The mechanism whereby Fru-2,6-P_2 potentiates the inhibition of Fru-1,6-P_2ase by AMP has been intensively studied (64, 70, 135). Binding studies

demonstrated that this effect was due to the ability of Fru-2,6-P_2 to enhance the affinity of the enzyme for AMP (135), presumably by bringing about a conformational change that facilitates AMP binding. Compatible with this hypothesis is the finding that both Fru-2,6-P_2 and AMP induce UV-difference spectra with saturable absorbance maxima at the same wavelengths (135). Studies using NMR and EPR show that the catalytic and AMP sites are in close proximity to one another, and this may explain why similar conformational changes are brought about by Fru-2,6-P_2 and by AMP (7, 64). In addition, ^1H and ^{31}P NMR have shown that the distances between the phospho group of Fru-6-P and enzyme-bound Mn^{2+} and between the 6-phospho groups of Fru-2,6-P_2 or α-methyl-D-fructofuranoside-1,6-P_2 and enzyme-bound Mn^{2+} were the same (64). The presence of Fru-2,6-P_2 caused the proton resonances of AMP to narrow, indicating that Fru-2,6-P_2 affects the exchange between AMP and the enzyme. Liu et al (120) used NMR techniques to measure distances from the structural metal site and from a spin-labeled cysteinyl residue to the active and Fru-2,6-P_2 binding site and found them to be equally distant. These findings are consistent with Fru-2,6-P_2 affecting the interaction of AMP with Fru-1,6-P_2ase by interacting with the active site [see (119) for review].

Final resolution of the question of where Fru-2,6-P_2 binds on the Fru-1,6-P_2ase subunit came with the elucidation of the X-ray crystallographic structure of the pig kidney Fru-1,6-P_2ase (99, 100). Ke et al (100) concluded from X-ray diffractions studies that Fru-2,6-P_2 binds at a single site, the active site, of the pig kidney cortex Fru-1,6-P_2ase. Further analysis of the X-ray crystallographic structure of liganded and unliganded forms of Fru-1,6-P_2ase should reveal the mechanism whereby Fru-2,6-P_2 affects AMP binding.

Role of covalent modification The first suggestion that rat liver Fru-1,6-P_2ase activity may be regulated by a phosphorylation mechanism came from the observations that injection of glucagon or cAMP into rats increased the activity of the enzyme (15, 137, 218, 219). Consistent with this idea was the observation that $^{32}P_i$ could be incorporated into the rat liver enzyme in vivo (188) and the demonstration of hormone-stimulated $^{32}P_i$-incorporation into the enzyme in isolated hepatocytes (24). In addition, Riou et al (188) reported that in vitro phosphorylation of the enzyme by the cAMP-dependent protein kinase resulted in a small increase in the V_{max}. This finding has been confirmed (39), and investigators have also reported that phosphorylation decreased the K_m for Fru-1,6-P_2 and decreased inhibition of the enzyme by both AMP and Fru-2,6-P_2 (37, 38). Despite these observations, the role of phosphorylation in the hormonal regulation of Fru-1,6-P_2ase is dubious, at best. Even though glucagon stimulated $^{32}P_i$ incorporation into the enzyme in isolated hepatocytes (24), no glucagon-induced activity changes have been

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observed in hepatocytes (S. J. Pilkis et al, unpublished results). Furthermore, the concentration of glucagon needed for half-maximal stimulation of $^{32}P_i$-incorporation was much higher than that needed for half-maximal stimulation of gluconeogenesis (24). These results suggest that Fru-1,6-P_2ase activity, like that of 6PF-1-K, is regulated primarily by hormone-induced changes in the level of Fru-2,6-P_2 (22, 79, 80, 164, 165).

The in vitro phosphorylation of the rat liver enzyme by the cyclic AMP-dependent protein kinase has been well characterized (188). Four moles of phosphate are incorporated per mole of enzyme or 1 mol of phosphate per mole of subunit. Fru-1,6-P_2ase is not as good a substrate for the cyclic AMP-dependent protein kinase as PK or 6PF-2-K/Fru-2,6-P_2ase because the latter two enzymes contain two and three arginine residues, respectively, on the N-terminal side of the phosphorylated serine whereas Fru-1,6-P_2ase contains only one [see (158) for review]. The sequence around the phosphorylated serine in rat liver Fru-1,6-P_2ase is Ser-Arg-Pro-Ser(P)-Leu-Pro-Leu-Pro (166). Rittenhouse et al (191) identified a second cAMP-dependent phosphorylation site, Arg-Ala-Arg-Glu-Ser(P)-Pro, at the C-terminal region of the subunit, but no effect of phosphorylation on the activity of the enzyme was detected.

Both phosphorylation sites of rat liver Fru-1,6-P_2ase are located near the C-terminus of the enzyme (83, 86, 191). Hosey & Marcus (83) have noted that, among Fru-1,6-P_2ases from livers of a number of mammalian species, only the rat enzyme contained a phosphorylation site within the C-terminal region. Limited proteolysis was not responsible for the absence of the phosphorylation site on the rabbit liver enzyme, since immunoprecipitation of in vitro translational products yielded a rat liver enzyme that was larger than the rabbit liver form (40). The finding of a C-terminal phosphorylation site only in the rat liver enzyme is consistent with lack of a universal role of hormonal modulation of phosphorylation in the regulation of mammalian liver Fru-1,6-P_2ase activity.

Fru-2,6-P_2 inhibition of Fru-1,6-P_2ase is the most primitive regulatory system Analysis of the effect of Fru-2,6-P_2 on 6PF-1-K and Fru-1,6-P_2ase throughout evolution provides some basis for speculation about the time that Fru-2,6-P_2 appeared during evolution. Fru-2,6-P_2 is found in trypanosomes, euglenoids, plants, fungi, and animals, but not in bacteria [see (233) for review]. All 6PF-1-Ks from mammalian sources and from fungi that have been tested so far are stimulated by this sugar bisphosphate (233). However, bacterial 6PF-1-Ks are unaffected by Fru-2,6-P_2 (80). In contrast, bacterial Fru-1,6-P_2ases are inhibited by Fru-2,6-P_2 (128), and Van Schaftingen and coworkers (233) have speculated that this was the original function of Fru-2,6-P_2. The inhibition of bacterial Fru-1,6-P_2ases by Fru-2,6-P_2 is also

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consistent with Fru-2,6-P_2 binding to the Fru-1,6-P_2ase active site, which has been conserved throughout evolution [see (101, 119) for review]. In contrast, Fru-2,6-P_2 activation of 6PF-1-K required the construction of an allosteric site for the effector by means of gene duplication, tandem ligation, and mutation. Thus, in higher organisms the role of Fru-2,6-P_2 was enlarged to include stimulation of glycolysis at the 6PF-1-K level as well as feedback inhibition of gluconeogenesis at the level of Fru-1,6-P_2ase.

6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BISPHOSPHATASE A key finding for understanding the molecular basis for the two reactions catalyzed by 6PF-2-K/Fru-2,6-P_2ase and for elucidation of the structural basis for the enzyme's bifunctionality was the demonstration that the enzyme could catalyze exchange reactions between ATP/ADP and Fru-6-P/Fru-2,6-P_2 (175). This demonstration suggested that one or both reactions proceeded via a two-step transfer mechanism involving a phosphoenzyme intermediate. Such an intermediate was detected when the enzyme was incubated with [2-^{32}P]Fru-2,6-P_2 but not with [γ-^{32}P]ATP, and the labeled amino acid was identified as a histidine (178). The phosphoenzyme was subsequently shown to be an integral part of the normal reaction pathway of the bisphosphatase (211). Location of the active-site residues for the two activities in the enzyme subunit was not known, but evidence from various protein modification studies suggested that the two reactions were catalyzed at discrete active sites [see (158) for review].

The primary sequence of the protein was determined by direct protein sequencing of the purified rat liver enzyme and by cloning methods involving antibody screening of a rat liver cDNA expression library in λgt11 (28, 34, 121) (Figure 4). The enzyme is a dimer with identical subunits that contain 470 residues. The N-terminal residue is an acetylated serine. The cAMP-dependent phosphorylation site is located at Ser-32, and the sequence adjacent to this residue, which is typical of those found in many substrates of the cAMP-dependent protein kinase, contains several basic residues separated by a single residue N-terminal to the phosphorylated serine. In the case of the bifunctional enzyme, the three arginyl residues render the protein an excellent substrate for cAMP-dependent protein kinase (68a, 138, 158). Two adenine nucleotide-binding-site signature sequences are present at residues 48–55 (GLPARGKT) and 274–281 (GLSARGKQ), of which the former represents part of the ATP-binding site of 6PF-2-K. Three cysteinyl residues, which are critical for Fru-6-P binding in the kinase reaction, are located at residues 160, 183, and 198 (47). Because a bisphosphatase active-site tryptic peptide had already been sequenced (172), it was possible to identify the bisphosphatase active-site phosphoacceptor histidine as residue 258.

Identification of the two catalytic domains was aided by the discovery that a high degree of sequence conservation existed between the Fru-2,6-P_2ase

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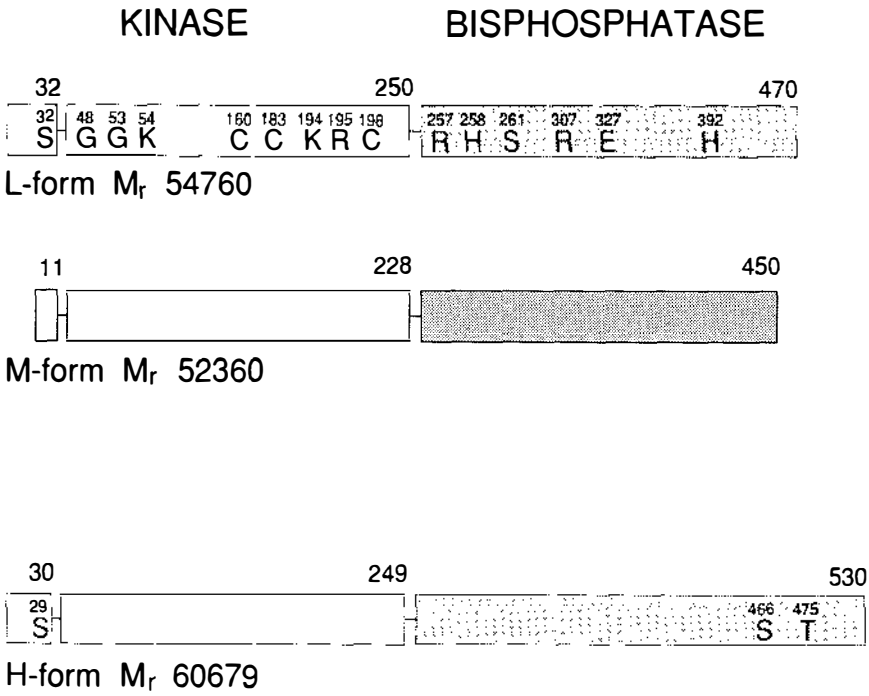

Figure 4 Representation of the different isoforms of 6PF-2-K/Fru-2,6-P_2ase. There are at least three isoforms of 6PF-2-K/Fru-2,6-P_2ase in mammalian tissues: a liver form, a muscle form, and a heart form. The liver and muscle forms are generated by alternative splicing of a single gene while the heart form is encoded by a different gene. (*a*) The liver form contains 470 amino acids, which can be divided into a regulatory region (residues 1–32), the kinase domain (residues 33–250), and the bisphosphatase domain (residues 251–470). Residue 32 is a cAMP-dependent phosphorylation site. In the kinase domain, residues 48–55 (GX_4GKT) represent part of the adenine nucleotide-binding site. Cysteinyl residues at 160, 183, and 198 are important for Fru-6-P binding in the kinase domain as are Lys-194 and Arg-195. The active-site phosphoacceptor histidine for the bisphosphatase is at residue 258. Other residues in the bisphosphatase (Arg-257, Ser-261, Glu-327, His-392) are implicated as active-site residues by homology with phosphoglycerate mutase. (*b*) The muscle form is generated by alternative splicing from the same gene as the liver form except that the first exon is 20 amino acids shorter and does not contain a cAMP-dependent phosphorylation site. The kinase and bisphosphatase coding regions and corresponding nucleotide sequence are identical to the liver form. (*c*) The heart form contains 530 amino acids. The phosphorylation sites for cAMP-dependent protein kinase and protein kinase C are located in a C-terminal extension. Another potential cAMP-dependent site is serine-29. The active site regions of the kinase and bisphosphatase domains are conserved when compared to the liver and muscle forms.

active site and the active site of the phosphoglycerate mutase family of enzymes, which also catalyze their reactions via a phosphohistidine intermediate (121, 172, 196). Weak sequence similarities were also shown to span the entire mutase sequence when it was compared with the C-terminal se-

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quence of the bifunctional enzyme. Indeed, Fru-2,6-P_2ase is similar enough to the mutases that it can be phosphorylated by 1,3-bisphosphoglycerate at the same histidyl residue that is phosphorylated by Fru-2,6-P_2 (214). These results provided strong support for the hypothesis that the C-terminal domain corresponds to Fru-2,6-P_2ase. They also suggested that the kinase activity resides in the N-terminal half of the subunit.

Structural similarities with other proteins were also sought for the N-terminal region of the bifunctional enzyme based on the presence of a nucleotide-binding fold signature sequence at residues 48–55 (5). An N-terminal domain from the bacterial 6PF-1-K family of structures showed a convincing similarity. Alignment of spatially equivalent bacterial 6PF-1-K residues to the nucleotide-binding fold sequence in the N-terminal half of the bifunctional enzyme showed that invariant or closely conserved residues played important structural or functional roles in 6PF-2-K (5). For example, 6PF-2-K residues 148–162 (EHGY[6AA]SICND) matched the *Bacillus stearothermophilus* 6PF-2-K residues 114–129 (EHGF[7AA]TIDND). The latter motif is highly conserved in all 6PF-1-K sequences, and these residues form part of the active-site cavity of 6PF-1-K (4, 5, 44). The sequence and structural similarity between 6PF-2-K and the 6PF-1-K family extended to amino acid 201 in rat liver 6PF-2-K, but the N-terminal 40 amino acids did not appear to form an integral part of this domain. Thus, the similarity to 6PF-1-K provided further indirect evidence that the N-terminal half of the bifunctional enzyme contained the 6PF-2-K.

Direct proof for the location of the two domains was obtained when each domain was separately expressed in *E. coli* with recombinant DNA techniques (216, 217). When the Fru-2,6-P_2ase domain (residues 251–470) was expressed, its kinetic properties were essentially identical to those of the bisphosphatase domain in the bifunctional enzyme structure. The only exception was that the expressed bisphosphatase domain eluted as a monomer from gel-filtration columns, whereas the bifunctional enzyme behaved as a dimer. These results demonstrated that the C-terminal domain represents the bisphosphatase domain and that the kinase domain contains regions of the subunit responsible for dimerization. Active kinase domain (residues 1–250) was also expressed in *E. coli,* but its affinity for Fru-6-P and ATP was greatly reduced compared with that of the kinase domain in the bifunctional structure (216). This result confirmed the designation of the N-terminal half of the subunit as the 6PF-2-K domain but suggested structural and/or active-site interactions between the two domains of the enzyme. Consistent with this idea, cAMP-dependent phosphorylation at Ser-32 affects both activities in a reciprocal manner (22, 138). Indeed, when Ser-32 is changed to Asp by site-directed mutagenesis, the same reciprocal changes in kinase and bisphosphatase activities are observed, suggesting that the introduction of negative charge in the N-terminal region plays a large role (107, 108). This N-terminal tail may

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wrap around the bifunctional molecule and influence subunit association or contact the linked bisphosphatase domain. On the basis of these results, the bifunctional enzyme appears to be a result of a gene fusion event (4, 5, 214, 216, 217).

Insights into structure-function relationships of the bisphosphatase domain have been obtained from secondary structural analyses, based on homology to yeast phosphoglycerate mutase, whose three-dimensional structure is known (242). The yeast enzyme does not bind nucleotides, but it assumes a nucleotide-binding fold-like structure favorable for binding phosphoglycerates and for spatial grouping of catalytic residues that are far apart in sequence. Thus, the phosphorylated His-8 is juxtaposed with His-179 in the yeast tertiary structure to form a "clapping-hands" structure. Alignment of the yeast enzyme with the bisphosphatase domain indicated that His-392 is the likely companion to phosphorylated His-258 in the bisphosphatase active site (4, 5, 44) (Figure 5). In support of this structural analysis, bisphosphatase activity and the ability to form a phosphoenzyme intermediate at His-258 were abolished when His-258 was mutated to alanine, confirming its designation as the phosphoacceptor histidine (215). Additional site-directed mutagenesis experiments revealed that mutation of His-392 to Ala also dramatically reduced bisphosphatase activity and decreased the rate of phosphoenzyme formation by 2000-fold (215). This result indicates that His-392 is an important catalytic residue; possibly, it acts as a proton donor to the leaving group, Fru 6-P. Another residue that is conserved in the mutase/bisphosphatase alignments is Glu-86 in the yeast PGM, whose corresponding residue in the bisphosphatase domain is Glu-327. Modelling studies indicate that this residue is located in the active site pocket in close proximity to His-392. Mutation of Glu-327 to either Ala or Gln produced mutants that had unaltered kinase activity, had very low bisphosphatase activity, and did not form a phosphoenzyme intermediate (117, 118). Mutation of Glu-327 to Asp produced a mutant that retained 50% of the wild-type activity. Thus, this residue is also important for bisphosphatase catalysis and it may influence the protonation state of the active-site histidyl residues.

The same experimental approach to structure-function relationships has been used for the kinase domain, where there is homology to *E. coli* 6PF-1-K, an enzyme whose crystal structure is known (204). The sequences of the two enzymes were aligned to determine which regions of 6PF-2-K correspond to the known secondary structure of 6PF-2-K. A predicted three-dimensional structure was built for the kinase, and in conjunction with site-directed mutagenesis, the function of 6PF-2-K was analyzed. Site-directed mutagenesis of residues in the nucleotide-binding-fold signature sequence (residues 48–55) results in almost complete loss of 6PF-2-K activity, as predicted by the model (107). The 6PF-2-K model also predicted that Arg-195, which corresponds to Arg-162 in the *E. coli* 6PF-1-K, would be important for

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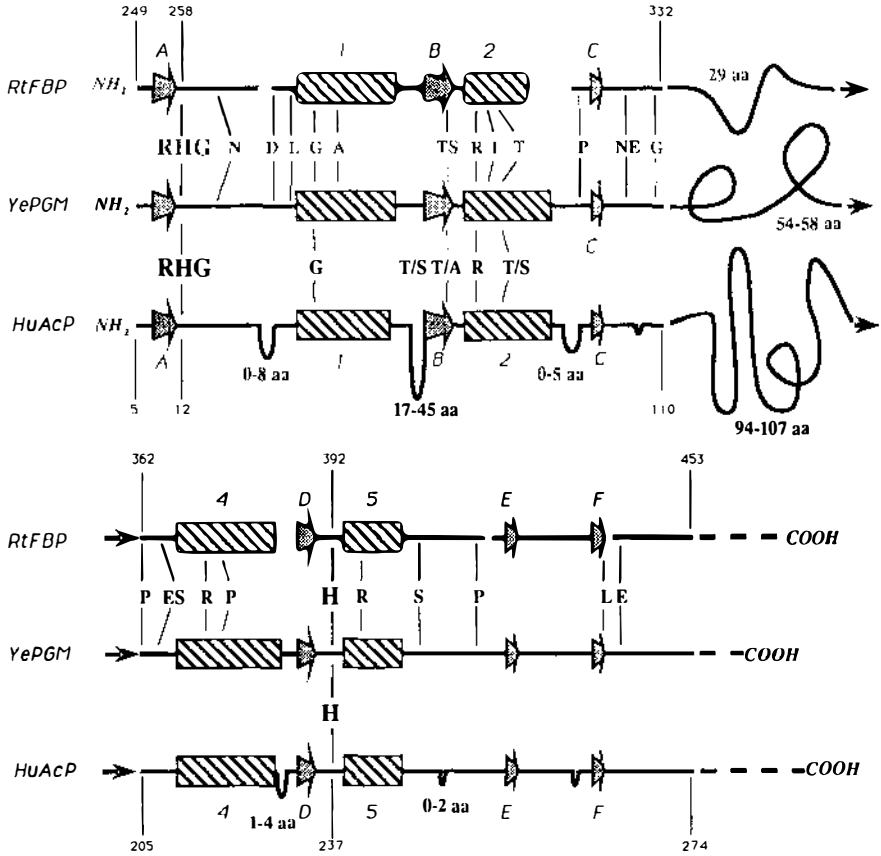

Figure 5 Alignment of Fru-2,6-P_2ase (RtFBP), yeast phosphoglycerate mutase (YePGM), and human prostatic acid phosphatase (HuAcP). *Stippled arrows*, β-strands labeled A–F; *hatched boxes*, helices labeled numerically. Conserved residues in all three structures are shown. Yeast phosphoglycerate mutase numbering system is followed. Different lengths of surface loops in each structure refer to varying lengths found in the particular family of enzymes (mutase or acid phosphatase). Adapted with permission from Ref. 7.

binding of Fru-6-P. When Arg-195 was changed to Ala the resulting mutant enzyme had 1/1000 the activity of the wild-type enzyme and the K_m for Fru-6-P was increased 500-fold (116). Thus, Arg-195 is important for Fru-6-P binding, a result that is consistent with the postulated analogous structure of 6PF-2-K and 6PF-1-K (Figure 3).

In addition to Fru-2,6-P_2ase and the phosphoglycerate mutase family of enzymes, several acid phosphatases also reportedly have an obligatory phosphoenzyme intermediate that involves the phosphorylation of a histidyl resi-

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due (228). This functional similarity with the catalytic mechanism of mutases and bisphosphatases, and the acid pH optimum of Fru-2,6-P_2ase, suggested a similarity with the acid phosphatases in either amino acid sequence or predicted secondary structure. Alignment of the five complete (and 1 partial) acid phosphatase sequences showed that the yeast *E. coli* and human enzyme were distantly related to each other (5). The regions surrounding two histidyl residues, typically spaced ~200–260 amino acids apart in the acid phosphatase alignment, showed a significant degree of similarity to active-site histidyl residues in the mutase/bisphosphatase alignment (Figure 5). The active-site His-8 of yeast phosphoglycerate mutase or His-258 of rat Fru-2,6-P_2ase is embedded in a conserved R-H-G-E/D motif, which is matched by an analogous N-terminal histidyl residue in a similar R-H-G-E/D motif in all the acid phosphatases. A merged alignment of the sequences of phosphoglycerate mutases, Fru-2,6-P_2ase, and acid phosphatases, in conjunction with a secondary structural analysis of acid phosphatase sequences, showed that predicted acid phosphatase secondary structural elements match those in yeast phosphoglycerate mutase, although there are striking differences in chain length between proposed active-site histidyl residues in the acid phosphatase and mutase/bisphosphatase sequences (Figure 5). Bazan and co-workers concluded that the acid phosphatase family of enzymes is distantly related to the mutase/bisphosphatase group and suggested that they share a common protein fold with yeast phosphoglycerate mutase (4, 5). A reasonable prediction is that this homology extends to similarities in the respective enzyme mechanisms; i.e. all members of this acid phosphatase family catalyze their reactions via a phosphohistidine intermediate, and the conserved N-terminal histidyl residue in the R-H-G-E/D motif is involved in phosphoenzyme formation.

The analysis by Bazan et al (5) strongly suggests that mutase, acid phosphatase, and bisphosphatase enzymes are evolutionarily related. Although these enzymes exhibit a divergence of amino acid sequences, preferred substrates, and biochemical pathways, there is conservation of a basic core structure and catalytic mechanism. The progenitor of mutase and bisphosphatase enzymes may have had an original affinity for small, negatively charged molecules in a binding site formed by the favorable nucleotide-binding fold-like arrangement of secondary structural elements (4, 5). The formation of a legitimate active site in this fold may have followed the acquisition of one or two reactive groups (e.g. the imidazole ring of histidine) in the binding crevice. The subsequent divergence of protein surface features, the gain of intruding loops, and changes in the peripheral residues of the active site have resulted in catalytic differences between mutases and bisphosphatases and in differences in the three activities (mutase, phosphatase, and synthase) of the enzymes of the mutase family (196). This evolutionary scenario is reminis-

cent of that suggested for the superfamily of binding/transport proteins, which includes sugar-binding proteins, aliphatic amino acid-binding proteins, and sulfate- and phosphate-ion-binding proteins (127). All of these proteins share a nucleotide-binding fold-like structure but with a different topology than that for phosphoglycerate mutase. As in the case of mutases, acid phosphatases, and bisphosphatases, binding/transport proteins have significantly diverged at the primary sequence level.

LONG-TERM HORMONAL CONTROL IN THE GLYCOLYTIC/GLUCONEOGENIC PATHWAY

Integrated Regulation of Hepatic Gluconeogenesis

Hepatic glucose production and utilization involve the movement of substrates through the glucose/G-6-P, Fru-6-P/Fru-1,6-P_2, and PEP/pyruvate substrate cycles. The direction and magnitude of this movement are controlled by enzymes whose activity is modulated by acute and long-term regulatory mechanisms (Figure 6). As mentioned earlier, the acute regulation of hepatic glucose metabolism occurs through hormone-mediated changes in enzymatic activity, principally the phosphorylation or dephosphorylation of two key enzymes, PK and 6-PF-2K/Fru-2,6-P_2ase, and the regulation of 6-PF-1K and Fru-1,6-P_2ase by Fru-2,6-P_2. Hormones also exert important long-term effects on glucose metabolism by changing the rate of enzyme synthesis. These long-term effects are mediated through alterations of the rate of mRNA synthesis and, in some cases, by changes in the rate of degradation of specific mRNAs.

Regulation of a complex pathway like gluconeogenesis involves a complex integration of minute-to-minute and long-term regulatory processes (73, 164). This integration is illustrated by the changes that occur in gluconeogenesis, Fru-2,6-P_2 levels, enzyme phosphorylation, and enzyme activities during brief (24 h) and long-term (72 h) starvation and in diabetes (Figures 6 and 7). In fed animals, the rate of gluconeogenesis is low because of a high rate of cycling at both the Fru-6-P/Fru-1,6-P_2 and pyruvate-phosphoenolpyruvate substrate cycles, which in turn is due to the high activity of 6PF-1-K and PK. The activity of PEPCK is also low. Glucagon or β-adrenergic agonists cause decreased recycling of PEP to pyruvate and Fru-6-P to Fru-1,6-P_2, which increases the rate of gluconeogenesis.

As the animal begins to fast, the rate of gluconeogenesis begins to rise, and insulin levels begin to fall. The decrease in insulin allows hormones such as glucagon and β-adrenergic agonists to stimulate adenylyl cyclase and increase cAMP levels. The increase in cAMP stimulates gluconeogenesis through its ability to inhibit 6PF-1-K, activate Fru-1,6-P_2ase, and inhibit PK.

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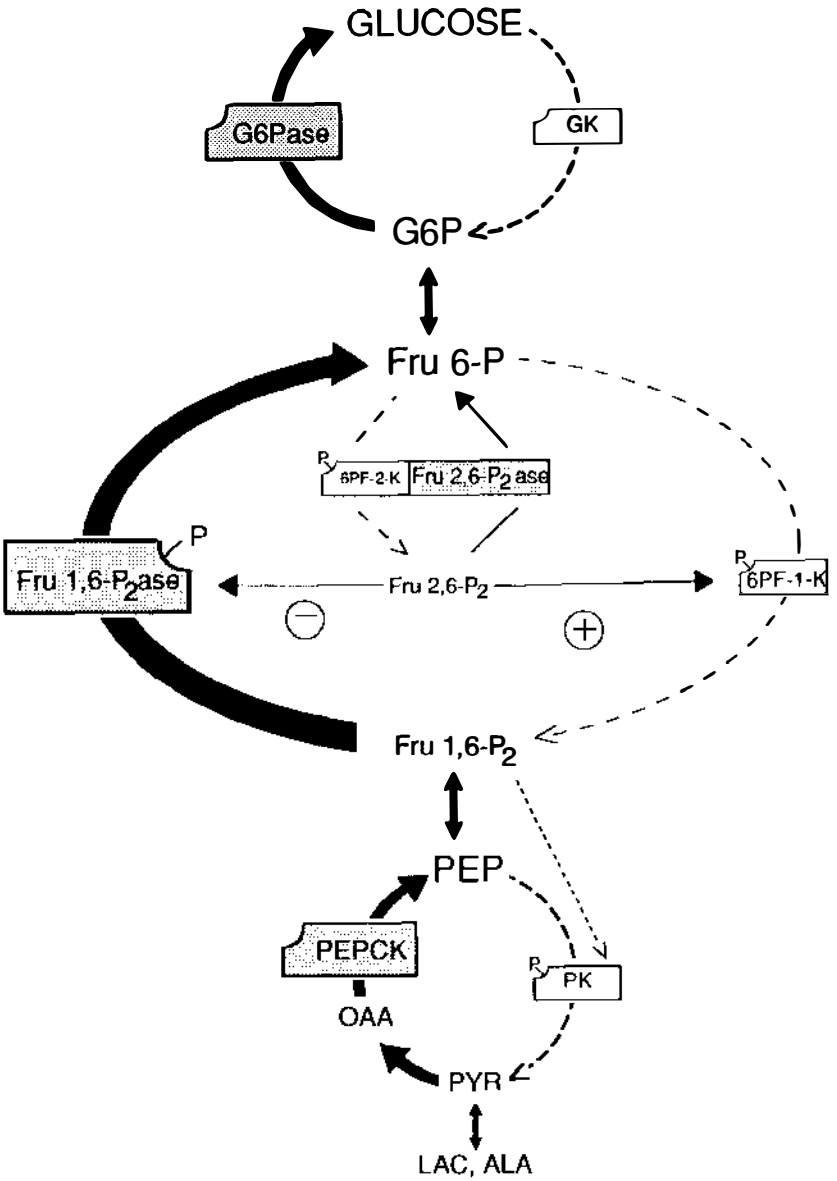

Figure 6 Integrated regulation of hepatic gluconeogenesis in long-term starvation and diabetes. Phosphorylated enzymes are indicated by –P attached to enzyme box. *Open boxes,* inhibition of activity; *shaded boxes,* stimulation. Size of box corresponds qualitatively to amount of enzyme present. *Solid lines,* increased flux; *dashed lines,* decreased flux. G6Pase, glucose-6-phosphatase; GK, glucokinase; G6P, glucose-6-phosphate; Fru-6-P, fructose-6-bisphosphate; 6PF-2-K, 6-phosphofructo-2-kinase; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase; Fru-1,6-P_2ase, fructose-1,6-bisphosphatase; 6PF-1-K, 6-phosphofructo-1-kinase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; ALA, alanine; PEP, phosphoenolpyruvate; OAA, oxaloacetate; PYR, pyruvate. Adapted with permission from Ref. 7.

The effect on the first two enzymes is mediated by lower Fru-2,6-P_2 levels owing to phosphorylation of 6PF-2-K/Fru-2,6-P_2ase, whereas the effect on PK is mediated by enzyme phosphorylation and lower Fru-1,6-P_2 levels. These changes in hormones and the associated rise in cAMP also produce a two- to three-fold elevation of PEPCK activity (222). Because carbon flux through all the substrate-cycle enzymes is high in the livers of fed animals, cAMP-mediated changes in PK and 6PF-2-K/Fru-2,6-P_2ase phosphorylation state and induction of PEPCK mRNA all contribute to the increase in gluconeogenesis (21).

After 24 h of starvation, hepatic cAMP levels and the rate of gluconeogenesis are elevated, whereas the level of Fru-2,6-P_2 is reduced to 10% of that in livers of fed animals. Substrate cycling is diminished because of increased flux through Fru-1,6-P_2ase and PEPCK and diminished flux through 6PF-1-K and PK. Further stimulation of gluconeogenesis by glucagon or catecholamines is small because the activities of enzymes (PK, 6PF-2-K, and Fru-1,6-P_2ase) acutely responsive to these hormones in the livers of fed animals have already been altered by phosphorylation mechanisms. Only PK inhibition is known to occur in response to glucagon in the starved state (74).

In diabetes or long-term starvation, the rate of gluconeogenesis is further elevated because of increased substrate supply and, most importantly, by

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REGULATION OF HEPATIC GLUCOSE METABOLISM

	GENE EXPRESSION			ENZYME ACTIVITY	
Enzyme	Ins	cA	Gc	Ph	Allosteric
GK	$\uparrow^{t}$	$\downarrow^{t}$	$\not\Delta$	No	No
6-PF-1-K	$\uparrow^{(?)}$	$\downarrow$	?	No	Yes
PK	$\uparrow^{tgs}$	$\downarrow^{ts}$	P	Yes	Yes
6-PF-2-K/Fru-2,6-P_2ase	$\uparrow^{tg}$	$\downarrow$	$\uparrow^{g}$	Yes	Yes
PEPCK	$\downarrow^{t}$	$\uparrow^{ts}$	$\uparrow^{ts}$	No	No
Fru-1,6-P_2ase	$\downarrow$	$\uparrow$	?	No	Yes
G-6-Pase	?	?	?	No	?

Figure 7 Hormonal regulation of the genes involved in regulation of glucose synthesis and utilization; t, gene transcription measured; g, effect requires glucose; ↓, dominant effect; P, permissive requirement; Δ, no change; s, mRNA stability affected; ?, no information available. Yes or No as to whether enzyme is regulated by phosphorylation/dephosphorylation (Ph) or allosteric effectors. Ins, insulin; cA, cyclic AMP; Gc, glucocorticoids.

changes in concentration of various enzymes that are brought about by insulin and/or cAMP regulation of their gene expression (73, 164). Activities of the gluconeogenic enzymes Fru-1,6-P_2ase and G-6-Pase are increased while PEPCK is further increased. Conversely, the activities of glycolytic enzymes GK, 6PF-1-K, and PK are reduced, as is the amount of the bifunctional enzyme. The elevated level of cAMP and the absolute (diabetes) or relative (long-term starvation) lack of insulin promote the changes in enzyme amounts and mRNAs that are observed.

Acute effects of glucagon and catecholamines on gluconeogenic flux are not observed during long-term starvation or in diabetes because the enzymes responsive to these hormones are already phosphorylated. Restoration of acute hormone responsiveness by refeeding or insulin administration, respectively, takes many hours to achieve. The elevated levels of cAMP must first decrease and the level of Fru-2,6-P_2 increase. Full restoration requires that insulin inhibit expression of the PEPCK and Fru-1,6-P_2ase genes and induce mRNA for GK, PK, and 6-PF-2-K/Fru-2,6-P_2ase. Clearly the extent of carbon flux in the gluconeogenic pathway depends in a complex way on many factors whose importance varies depending on the nutritional and hormonal status of the animal.

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Control of Substrate Cycle Enzyme Gene Expression

With the exception of G-6-Pase, complete cDNA molecules have been obtained for all of the cytoplasmic enzymes involved in the substrate cycles of hepatic glucose metabolism. These have allowed measurement of the specific mRNA amounts and gene transcriptions in response to various hormone treatments (Figure 7), and also have permitted the isolation and characterization of the cognate genes (Figure 8). In some cases, the promoter regions have also been isolated, and studies of the interaction of specific *cis*-acting DNA elements with the *trans*-acting transcription factors that mediate hormone action have begun. In the following sections, we summarize briefly the mechanisms by which hormones control gene expression of the key regulatory enzymes in the glycolytic/gluconeogenic pathway.

L-TYPE PYRUVATE KINASE Chronic regulation of the liver form of PK (PK-L) mRNA by hormones and dietary factors is extremely complex. Hepatic PK activity and mRNA, decreased in starvation and diabetes, are restored to normal by a high carbohydrate diet and insulin administration (136, 181). Glucagon, acting via cAMP, inhibits transcription of the PK-L gene and also accelerates the degradation of PK-L mRNA (143, 234). Carbohydrates stimulate mRNA accumulation (143, 234). This stimulation appears to involve transcription and mRNA stabilization, and the permissive action of thyroid hormones and glucocorticoids may be required (234). The stimulatory effect

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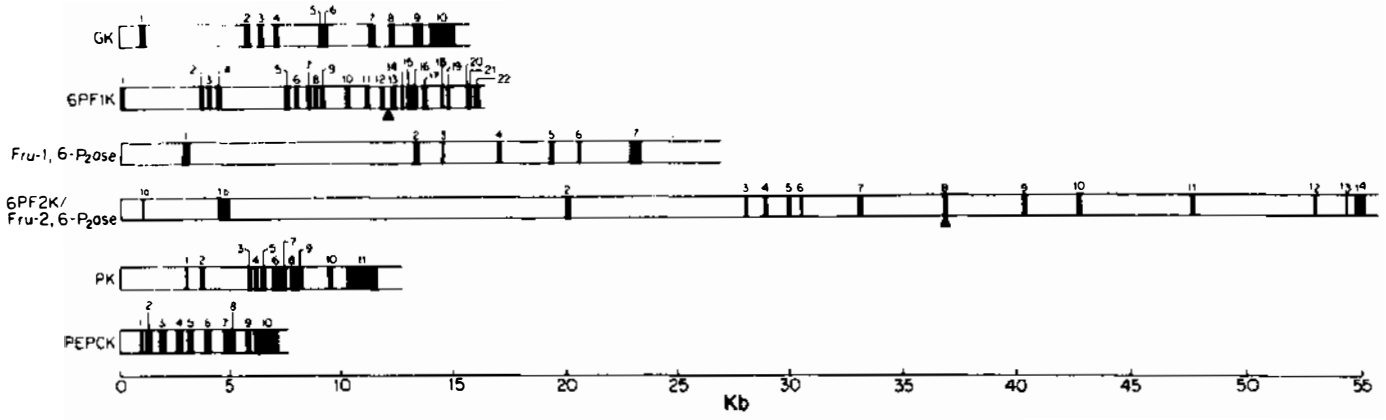

Figure 8 Intron/exon organization of hepatic genes of glucose metabolism. Exons are shown in black and are numbered. Introns are not shaded. Diagram was adapted from data on genomic structures for rat GK, 6PF1K (rabbit muscle), rat Fru-1,6-P_2ase, rat 6PF2K/Fru-2,6-P_2ase, rat pyruvate kinase (PK), and rat PEPCK genes. Arrow in exon 8 of 6PF-2-K/Fru-2,6-P_2ase gene denotes border of kinase and bisphosphatase domains. Arrow before exon 13 in 6PF1K gene denotes putative gene duplication position.

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of insulin is slow at first and requires ongoing protein synthesis; the induction of another gene product may be a prerequisite (143). PK-L gene expression is stimulated by the combination of glucose and insulin in primary cultures of adult rat hepatocytes, provided that thyroid hormone and glucocorticoids are present (35). Neither glucose nor insulin administered alone can stimulate PK-L gene expression. Glucagon inhibits the synthesis of PK-L mRNA both in cultured hepatocytes (35) and in vivo (234). Glucagon also increases the rate of degradation of PK mRNA, whereas glucose/insulin increase the stability of this mRNA (35). The DNA sequences necessary for glucose-stimulated expression of the L-type pyruvate kinase gene have been localized to sequences that are between –197 and –96 base pairs from the transcriptional start site (102).

Consensus CRE (cAMP regulatory element) sequences are present in the PK-L gene but are far upstream (–2.3 kb) and downstream (+5.8 kb) from the cap site (26). A sequence resembling a glucocorticoid-responsive element was also identified (26). Surprisingly, no studies have been conducted on localization of functional hormone response elements in the hepatic L-type PK promoter; instead, work has centered on DNA-binding proteins that confer tissue specificity.

DNase I hypersensitivity can be used to identify regions of DNA involved in the binding of regulatory proteins. Two DNase I hypersensitive sites are located upstream from the L promoter of the L-PK gene; detection of the proximal site (–24 to –40 bp with respect to the L cap site) is correlated with transcriptional activity, whereas the distal site (–3.2 to –3.0 kb) is present whether the gene is active or not (223). DNase footprinting and gel mobility shift assays have been used to identify *cis*-acting elements and to follow purification of specific nuclear proteins that bind to the *cis*-acting DNA elements. Four sites located in a region 183-bp upstream from the transcription start site were specifically protected by nuclear extracts from rat liver (236). Two of the four proteins are liver-specific; the other two are present in all tissues tested. The nucleotide sequences of the liver-specific, protein-binding elements are similar to those of hepatocyte nuclear factor 1 and liver factor A1, DNA-binding proteins that regulate transcription of other genes expressed in liver. The nucleotide sequences of the nontissue-specific, protein-binding elements are similar to those of nuclear factor 1 and a ubiquitous nuclear factor that binds to the major late promoter of adenovirus 2 and some other genes. These DNA-binding factors were present at similar concentrations in nuclear extracts of livers from both carbohydrate-fed and starved rats, thus indicating that the concentrations of these factors probably do not play a role in the nutritional regulation of transcription of the pyruvate kinase gene. Possibly, these protein factors may be regulated in some manner (e.g. phosphorylation/dephosphorylation or low molecular weight effectors), and this regulation may operate during changes in nutritional or hormonal states.

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Similar approaches have been used to determine which DNA elements are involved in the hepatocyte-specific expression of the L-type PK (243). Three positive regulatory regions located at –76 and –94 (PKL-I), –126 and –149 (PKL-II), and –150 and –170 (PKL-III) were found to be responsible for tissue-specific expression. Only PKL-I showed enhancer activity. Various combinations of these elements had synergistic effects suggesting that they functioned as a unit. PKL-I contained a sequence homologous to the sequence of the LF-B1 binding site; PKL-II contained a sequence similar to that of the LF-A1 binding site; and PKL-III included a sequence similar to that of the binding site of adenovirus major late transcription factor. LF-B1 bound to PKL-I, but *trans*-acting factors binding to PKL-II and PKL-III were different from LF-A1 and major late transcription factor. Conclusions from these experiments were not consistent with those of Vaulont et al (235, 236) except in the case of LF-B1. The DNA elements and their cognate binding proteins interact in a complex manner that will only be clarified by further experimentation.

A 186-bp fragment of the promoter/regulatory region of the rat L-type pyruvate kinase gene was ligated upstream of the reporter gene, bacterial chloramphenicol acetyltransferase (CAT), and transiently transfected into rat hepatocytes maintained in mass cultures, hepatoma cell lines, and mouse fibroblasts of the 3T6 and L cell lines (68). CAT activity was detected only in hepatocytes or liver-derived hepatoma cells, which suggests that this fragment of DNA contained *cis*-acting sequence elements that specified tissue-specific transcription of this gene in liver.

The responses of L-type pyruvate kinase to hormones in hepatocytes in culture are consonant with important roles those hormones play in the nutritional regulation of the genes' expression in intact animals. However, to definitively prove this point it will be necessary to employ intact animal models such as transgenic animals.

In transgenic mice containing an intact rat L-type pyruvate kinase gene with 3.2 kb of 5'-flanking DNA, rat L-type pyruvate kinase mRNA was expressed specifically in liver (234). Furthermore, abundance of the rat mRNA was low in the livers of starved mice and greatly increased by refeeding. The refeeding-induced stimulation was blocked by administration of glucagon. Changes in the abundance of the rat mRNA paralleled changes in the levels of the endogenous mouse mRNA. Another line of mice contained a rat L-type pyruvate kinase gene that was identical except for the deletion of exons 2 through 9. This DNA construction contained exons 1, 10, and 11, plus the same 5' and 3' flanking DNA as the intact rat gene, and was regulated in the same manner. These results suggest that the *cis*-acting sequences involved in regulation of the L-type pyruvate kinase gene are localized in the 3.2 kb DNA fragment and that the genomic DNA between exons 1 and 10 or the

mRNA coded by exons 2 through 10 is not involved in regulation of mRNA abundance by diet, glucagon, or tissue type. However, the possibility that important sequence elements are located at the 3' end of the gene cannot be ruled out.

As pointed out recently by Goodridge (69), the transgenic mouse is an attractive physiologic approach to analysis of the nutritional and tissue-type regulation of promoter/regulatory regions of genes. However, functional identification and detailed characterization of hormone regulatory regions of the L-type pyruvate kinase gene have not been done in cell culture systems or with transgenic animals.

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PHOSPHOENOLPYRUVATE CARBOXYKINASE The PEPCK gene was isolated before any of the other genes discussed in this review and has been extensively studied with regard to hormonal control. It is a relatively short gene, 7 kb in length, and contains 10 exons and 11 introns (6) (Figure 8). It is subject to positive and negative regulation by nutritional factors and hormones (91, 240). In contrast to the glycolytic enzymes, hepatic PEPCK activity is markedly reduced in carbohydrate-fed animals and is increased in fasted animals (222). These changes are largely, if not entirely, due to coincident changes of plasma glucagon and insulin. The increased plasma insulin following a carbohydrate meal results in a decreased rate of PEPCK synthesis (1, 72), which is directly due to a decrease in PEPCK mRNA, which, in turn, is due to the fact that insulin rapidly inhibits transcription of the PEPCK gene (72, 200). This effect, studied most extensively in H4IIE hepatoma cells, occurs in minutes, is mediated through the insulin receptor, is promptly reversed upon removal of insulin from the culture medium, and does not require ongoing protein synthesis (148). The elevated plasma glucagon (or intracellular cAMP) characteristic of the fasting condition induces PEPCK synthesis, which reflects enhanced transcription of the PEPCK gene (72, 109, 200), although cAMP also stabilizes PEPCK mRNA against degradation (82). Glucocorticoid hormones increase PEPCK by stimulating transcription of the gene, and they also stabilize PEPCK mRNA (153). Both glucocorticoids and cAMP-induced stabilization of PEPCK mRNA are mediated by nucleotide domains contained within the 3' region of the mRNA (81, 153). The effects of glucocorticoids and cAMP on transcription are additive, and insulin exerts a dominant negative effect on transcription, whether these effectors are added singly or in combination (200).

The basal PEPCK gene promoter consists of three major elements: a CAAT box, a combined basal enhancer element and CRE, and a TATA box (6). The PEPCK gene CRE, which was used to formulate the first consensus sequence for a CRE, mediates most, but not all, of the effect cAMP has on PEPCK gene transcription (72, 200, 205).

Glucocorticoid hormones stimulate PEPCK gene transcription through a uniquely complex glucocorticoid response unit (GRU) (88). This GRU spans ~110 bp (from –465 to –350) and it consists of two accessory factor binding sites (AF1 and AF2) and two glucocorticoid receptor binding sites (GR 1 and GR2). All are required for a maximal response to glucocorticoids. The function of these accessory factor sites is not clear, but it is interesting that 5' deletion analysis places one insulin-responsive element within the sequence –416 to –402 (149). The location of an element that acts both as a part of the GRU and as an insulin-responsive sequence could account for the dominant negative effect insulin has on the glucocorticoid response. An explanation for the dominant effect of insulin on the cAMP response has not yet been forthcoming, however.

DNA elements responsible for the tissue-specific expression of cytoplasmic PEPCK have been studied in transgenic mice (134). The transgene contained 460 bp of the rat PEPCK gene ligated to the structural gene for bovine growth hormone (bGH). Lines of mice that contained significant bGH in their blood expressed the PEPCK/bGH gene only in liver and kidney. Tissue-specific expression also was obtained with chimeric DNAs that had only 355 bp of PEPCK flanking DNA. Developmental expression of the transgene in liver paralleled that of the endogenous PEPCK gene. Expression of the chimeric PEPCK/bGH gene also responded to dietary manipulations and cAMP in the same manner as the endogenous mouse PEPCK gene. Feeding a high carbohydrate diet decreased blood level of bGH to 5% of that in starved animals, whereas a high protein diet, free of carbohydrate, resulted in high levels of growth hormone in the blood. The response of the transgene to the high carbohydrate diet suggests the presence of an insulin regulatory element in the 460 bp upstream from the transcription start site of the PEPCK gene. This hypothesis is consistent with the recent localization of one insulin response element within the sequence –416 to –402. Administration of cAMP to the mice resulted in a twofold increase in blood bGH within 90 min, which is consistent with the presence of a cAMP regulatory element in this DNA fragment. The transgene also was expressed selectively in the periportal portion of the liver, a pattern characteristic of the endogenous PEPCK gene.

Modified retroviruses have provided another way to introduce the PEPCK/bGH gene into cells and animals (76). The stably integrated PEPCK/gGH gene was expressed in livers of animals infected in utero and in regenerated livers of rats infected immediately after partial hepatectomy. Feeding a high carbohydrate diet decreased, and administering glucocorticoid plus cAMP increased, the level of bGH in the blood.

6-PHOSPHOFRUCTO-1-KINASE Hepatic 6-PF-1K activity is reduced in fasting and diabetes and is restored to normal levels by refeeding and insulin

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administration, respectively (36). When fasted animals are refed a high carbohydrate diet, hepatic 6-PF-1K mRNA is increased (67). This increase is partially blocked by administration of dibutyryl cyclic AMP. The mRNA is also increased in livers of diabetic animals treated with insulin (67). These results suggest that 6-PF-1K gene expression, like that of PEPCK, GK, and 6-PF-2K/Fru-2,6-P_2ase, is under reciprocal control by insulin and cAMP. A brief report in which direct measurements of transcription were made tended to confirm this hypothesis (237).

Human tissues contain three 6-PF-1K isozymes, each encoded by a separate gene (238). The human muscle gene contains at least two alternative promoters and produces three different mRNAs (139). The mouse liver type 6-PF-1K gene has recently been isolated (237). However, its expression has not been studied in a cultured cell system.

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FRUCTOSE 1,6-BISPHOSPHATASE Fru-1,6-P_2ase, which catalyzes the hydrolysis of Fru-1,6-P_2 to Fru-6-P, is induced by diabetes and starvation, and the 10-fold increase of hepatic Fru-1,6-P_2ase mRNA in diabetic rats is reduced to control levels by insulin (48). In cultured hepatocytes, the addition of cAMP increases, and insulin decreases, Fru-1,6-P_2ase mRNA (46). The effects of insulin and cAMP on Fru-1,6-P_2ase gene expression are similar to their effects on PEPCK gene expression.

The availability of a cDNA clone for liver Fru-1,6-P_2ase has allowed its overexpression in *E. coli* with a T7 RNA polymerase-based expression system (49). This system should allow production of the liver enzyme in amounts necessary for crystallization, for structure/function studies on catalytic mechanism, and for the functional identification of the binding sites for Fru-2,6-P_2 and AMP.

The 7-exon Fru-1,6-P_2ase gene extends over approximately 23 kb (200) (Figure 8). The promoter contains a consensus CRE located at −169. When a promoter-reporter gene (CAT) construct containing the consensus CRE was transfected into kidney cells, CAT activity increased after addition of cAMP (46), which strongly suggests that cAMP acts, at least in part, by enhancing the transcription rate of the gene. Although the regulation of this gene has not yet been studied extensively, one can reasonably predict that it will closely resemble the regulation documented for the PEPCK gene.

6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BISPHOSPHATASE The amount of bifunctional enzyme protein is decreased during starvation and in diabetes and can be restored by refeeding a high-carbohydrate diet or by insulin administration, respectively (29, 31, 171). The increase in mRNA with refeeding or insulin administration correlated with the increase in the amount of enzyme protein. However, bifunctional enzyme mRNA was not

decreased in starvation or diabetes, which suggests that the decreased enzyme protein in those states is due to a posttranslation effect and/or enhanced protein degradation (29, 31, 171).

Adrenalectomy also reduced the amount of 6PF-2-K/Fru-2,6-P_2ase protein and its mRNA abundance (129, 171). Subsequent administration of glucocorticoids increased mRNA levels for 6PF-2-K/Fru-2,6-P_2ase by increasing the transcription rate of the gene. The addition of glucocorticoids to primary cultures of hepatocytes increased mRNA levels by ~100-fold (106, 110). Furthermore, only glucocorticoids prevent the loss of bifunctional enzyme mRNA that occurs when hepatocytes are placed into primary culture. These results suggest that glucocorticoids are the most important factor in maintaining expression of this gene in liver cells.

While in vitro studies and work with primary hepatocyte cultures suggested a multihormonal control of 6PF-2-K/Fru-2,6-P_2ase gene expression, elucidation of the molecular mechanisms involved has been facilitated by development of a cell culture system that expresses 6PF-2-K/Fru-2,6-P_2ase mRNA and responds to hormones. Studies with rat hepatoma (FTO-2B) cells showed that addition of insulin or dexamethasone increases bifunctional enzyme mRNA by 10- to 20-fold and that these effects were completely blocked by the addition of cAMP analogues (17). Insulin and dexamethasone increased gene transcription because their effects were blocked by actinomycin D and did not invoke alterations in mRNA stability. Both hormones also stimulated mRNA synthesis in transcription run-on assays (17). These effects required the presence of glucose, which suggests that glucose or a metabolite of glucose acts to modulate gene expression. Thus, a complex control of 6PF-2-K/Fru-2,6-P_2ase gene expression probably plays an important role in the long-term regulation of hepatic gluconeogenesis.

The genomic sequence of rat 6PF-2-K/Fru-2,6-P_2ase has recently been determined (32, 33, 110). The 6PF-2-K/Fru-2,6-P_2ase gene is 55 kb in length, contains 15 exons, and is at least twice as long as that of genes of other glycolytic/gluconeogenic enzymes that have been reported (Figure 8). The bifunctional enzyme gene encodes at least two isozymes in a tissue-specific manner by alternative splicing from two promoters (29, 32, 33, 110) (Figure 4). A muscle-specific transcript is initiated at an upstream promoter that is processed to mRNA by incorporating exon 1a and splicing out exon 1b (32). A liver-specific transcript is initiated at a promoter 5 kb downstream that incorporates exon 1b. Exons 2–14 are common to both messages. The first 32 N-terminal residues of the liver isoenzyme are replaced by 11 amino acids in the muscle isoenzyme, and the phosphorylation site residue Ser-32 of the liver is replaced by an alanine residue (32). Consistent with these differences, liver mRNA is 0.2 kb larger than muscle mRNA (29, 32, 110). Alternate exon use is advantageous, since 6PF-2-K/Fru-2,6-P_2ase regulation in muscle and liver

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would be expected to be different. Stimulation of glycogenolysis by β-adrenergic agents in muscle would be expected to result in enhanced glycolysis. In muscle, increased cAMP levels do not inactivate the bifunctional enzyme, since it does not have a cAMP phosphorylation site.

The mRNA of rat hepatoma cell line FTO-2B was also found to contain exon 1a of the muscle form but is the same size as liver mRNA and therefore may represent another alternative splicing event (17). Fao cells, another human hepatoma cell line, also express bifunctional enzyme mRNA, and this expression is also stimulated by dexamethasone and insulin and inhibited by cAMP (C. Espinet, A. J. Lange, S. J. Pilkis, unpublished results). The mRNA in Fao cells appears to contain exon 1b rather than 1a.

Another distinct bifunctional enzyme isozyme is found in heart tissue (44a, 104, 187, 198) (Figure 4). The heart isoform is encoded by a separate gene (112, 113). Its primary sequence contains both conserved kinase and bisphosphatase active site regions when compared to the liver and skeletal muscle isozymes (198). This isozyme, which contains 530 amino acids, contains a C-terminal extension that includes both a cAMP-dependent and protein kinase C phosphorylation site (198). Cyclic AMP-dependent phosphorylation at the C-terminal site has been reported to inhibit heart 6PF-2-K (104, 198). Interestingly, inspection of the N-terminal region also reveals another potential cAMP-dependent phosphorylation site (residues 26–29, –KKCS–), but there is no experimental evidence for phosphorylation at that site. For reasons which are unclear, the heart isozyme has little bisphosphatase activity (44a, 104, 187) even though it contains a bisphosphatase coding region with a high degree of homology with the liver enzyme. The regulation of the heart bisphosphatase activity by phosphorylation/dephosphorylation has not been reported. Additional work on the regulation of this interesting form and the relationship of this regulation to pathway flux is required. HTC cells also contain an isozyme of 6PF-2-K/Fru-2,6-P_2ase that has no bisphosphatase activity even though the coding sequence for the bisphosphatase domain is present (32).

The exon/intron boundaries of the liver/skeletal muscle 6PF-2-K/Fru-2,6-P_2ase gene appear to conform to the enzyme domain alignments (5, 110). For example, the phosphorylation site domain of the liver enzyme and the major secondary structural domains of the kinase are almost entirely defined by these boundaries. An exception is the region of the gene that encodes the kinase-bisphosphatase junction that does not contain an intron separating the end of the kinase from the beginning of the bisphosphatase. If the bifunctional enzyme gene arose by fusion of precursors of these two genes, then there was either a fusion of exonic domains or the intervening intron was lost during the course of evolution.

Because expression of the 6PF-2-K/Fru-2,6-P_2ase gene has been shown to

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be regulated by insulin, glucocorticoids, and cAMP, the 5'-flanking region of the gene probably contains the respective hormone response elements. However, these elements have not yet been identified.

GLUCOKINASE Upon entering the hepatocyte, glucose is converted to G-6-P by the enzyme glucokinase (GK). GK, a member of the hexokinase (HK) family, is unusual in that its K_m for glucose is ~5 mM whereas that of HK I–III (GK is HK IV) varies between 20 and 120 μM [see (125) and (239) for review]. In the hepatocyte glucose is efficiently converted to G-6-P because GK, unlike HK I–III, is not subject to feedback product inhibition by G-6-P (239).

GK activity is not altered by covalent modification; thus, changes in activity are entirely due to changes in the amount of the protein. When insulin is low, gluconeogenesis is unrestrained, and glycolysis is inoperative, such as in starvation, the activity of glucokinase is diminished and mRNA levels decline (93, 141, 154, 203). Refeeding a diet high in carbohydrate increases GK activity and mRNA levels within hours (93, 141, 154, 203). In diabetic animals GK mRNA is very low and the GK gene is inactive (2, 93, 126, 206). Within 30–60 min after the injection of insulin into a diabetic rat or after its addition to primary cultures of hepatocytes, a 20–30-fold increase in GK gene transcription occurs (Figure 7) and GK mRNA increases accordingly (2, 93, 126, 206). Glucagon (or cAMP, its intracellular messenger) inhibits GK gene transcription and overrides the stimulatory effect of insulin (92, 126). These effects are not dependent on the presence of glucose in the medium (92).

Isolation of a specific GK cDNA, combined with extensive amino acid sequence information, led to the elucidation of the primary structure of the protein (2). A comparison of this structure with the sequences of yeast hexokinases and a partial sequence of mammalian HK I showed that the ATP-binding and glucose-binding domains are highly conserved between members of the HK gene family (2). A further analysis of mammalian HK I confirmed a long-standing hypothesis about the origin of the hexokinases. A primordial enzyme similar to the yeast HK (and GK) gave rise, by gene duplication with tandem ligation, to mammalian HK I and, presumably, to HK II and HK III (142, 201). An intriguing possibility has been suggested: namely, that the ATP-binding domain of heat shock protein has a three-dimensional structure which is identical to the corresponding ATP-binding domain of yeast hexokinase (55).

Using the GK cDNA, investigators have expressed rat liver GK in *E. coli* by means of a T7 RNA polymerase expression system (16). Analysis of the yeast HK crystal structure suggested that Asp-205 acted as a base catalyst in the hexokinase reaction. However, to date no functional data have confirmed the importance of this residue in the yeast HK or in any mammalian HK. The

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residue corresponding to yeast HK Asp-205 in GK is Asp-211. Site-directed mutagenesis has recently been used to mutate this residue to Ala. When this mutant was expressed in *E. coli* and then purified, it had only 1/500 the wild-type activity, but its K_m for glucose was little changed (111, 113). The conclusion was that Asp-211 acts as a base catalyst in the GK reaction. Additional site-directed mutagenesis studies with GK and other HKs should allow further analysis of structure/function relationships of this enzyme family. The ability to express high levels of liver GK in *E. coli* suggests that the crystallization of this enzyme family will also soon be possible.

The organization of the rat GK gene has recently been determined (126) (Figure 8). The GK gene in hepatic and pancreatic β-cells uses different first exons (1^H and 1^β are separated by ~12 kb), which means that different transcription initiation sites, promoters, and regulatory elements are functional in these cells, and alternative splicing results in different primary transcripts (125). Attempts to identify hormone response elements in the hepatic GK promoter have, to date, been unsuccessful, in part because of the lack of a tissue culture cell line in which the transgene gene is regulated.

GLUCOSE-6-PHOSPHATASE Glucose-6-phosphatase, a microsomal enzyme, has proved to be refractory to purification and cDNA cloning. Hepatic glucose-6-phosphatase activity is increased by starvation and in diabetic rats (146, 147). It will not be surprising if insulin reduces, and cAMP increases, glucose-6-phosphatase mRNA by exerting reciprocal effects on gene transcription just as occurs for PEPCK and Fru-1,6-P_2ase.

SUMMARY AND FUTURE DIRECTIONS FOR THE STUDY OF METABOLIC PATHWAY FLUX

Our understanding of the regulation of gluconeogenesis was initially based on analysis of metabolites of the pathway, but recent advances have come from the application of molecular genetics. Five years ago, little was known about the primary structure of the key regulatory enzymes. Since then, the primary sequence of liver GK, 6PF-2-K, Fru-1,6-P_2ase, PK, PEPCK, and 6PF-2-K/Fru-2,6-P_2ase has been derived from cDNA sequences and/or determined by direct protein sequencing. This information in turn has provided new insights into molecular mechanisms of catalysis and regulation by covalent modification of these proteins. Isolation of cDNA for these enzymes also has permitted the quantitation of specific mRNAs and analysis of hormonal control of their gene expression. The genes for these enzymes have been isolated and sequenced, their promoter regions are being identified and characterized, and several hormone regulatory elements have been delineated. The promoter regions for 6PF-2-K/Fru-2,6-P_2ase and Fru-1,6-P_2ase have also been identi-

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fied, and one area of active research in the future will be the elucidation of the mechanisms whereby hormones regulate the expression of these genes.

G-6-Pase represents another fertile area of future research; it is the only key regulatory enzyme in the pathway that has not been cloned. Much new information will be forthcoming after cDNAs are obtained for its catalytic unit and other putative associated regulatory subunits. For example, the catalytic subunit of mammalian G-6-Pase catalyzes its reaction via a phosphohistidine enzyme intermediate and has a molecular weight of 36,000 (30). Elucidation of its structure will probably reveal that it is a member of the Fru-2,6-P_2ase/phosphoglycerate mutase/acid phosphatase enzyme family. Which factors regulate expression of the G-6-Pase gene, and the underlying mechanism(s) of their effects, remain unresolved.

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Given our current state of knowledge about regulation of gene expression of hepatic glycolytic/gluconeogenic enzymes, a number of generalizations can be made. First, there is coordinate hormonal regulation of gene expression. The effects of hormones on gene expression are consistent with their physiologic actions. Insulin induces the mRNAs that encode glycolytic enzymes and represses the mRNAs that encode gluconeogenic enzymes; cAMP has opposite effects. Both can increase or decrease transcription. Whereas insulin and cAMP affect all of these mRNAs, glucocorticoids may have a more restricted action. They play an important role in increasing PEPCK and bifunctional enzyme mRNA and have a permissive action in the regulation of PK mRNA. Glucocorticoids have no effect on GK, and information is not available concerning their regulation of 6PF-1-K, Fru-1,6-P_2ase, or G-6-Pase.

The weight of evidence indicates that both transcriptional and posttranscriptional mechanisms are involved. Insulin and cAMP likely regulate the synthesis of all of these mRNAs. Relatively little is known about the regulation of mRNA stability, but it too is probably an important control mechanism. PEPCK mRNA is also stabilized by agents that increase the rate of transcription of the gene. Under appropriate metabolic signals, this dual control provides a long-term increase in PEPCK mRNA and protein. Studies with PK mRNA are less direct but suggest a similar dual mechanism. Whether multilevel regulation is restricted to these two mRNAs, or whether the stability of other mRNAs involved in glucose metabolism is also affected, needs to be determined.

Glucose also appears to be an important regulatory molecule for hepatic regulatory enzyme gene expression. The liver is responsible for converting excess dietary carbohydrate to triglycerides. Animals fed a high-carbohydrate and low-fat diet respond by inducing the synthesis of several key hepatic enzymes involved in glycolysis and fatty acid synthesis. Insulin, to have an effect on the PK and bifunctional enzyme genes, requires the presence of

glucose. Because these mRNAs encode enzymes that catalyze intermediate or distal reactions in the glycolytic pathway and the regulation of GK gene transcription by insulin is independent of glucose, possibly a glucose metabolite is the active agent and is generated as a consequence of insulin's stimulation of GK gene transcription. Increased catabolism of glucose could account for the fact that insulin is necessary but not sufficient for the induction of PK and the bifunctional enzyme. Even though several genes are controlled by a carbohydrate diet, only in the yeast enolase gene (27), the S_{14} gene (94), and perhaps the L-type pyruvate kinase gene (101) have glucose regulatory *cis*-acting DNA sequences been defined. In no instance have any *trans*-acting proteins been identified. Whether or not regulation of gene expression of gluconeogenic enzymes by insulin is dependent on glucose is uncertain, although this does not seem to be the case for PEPCK when cell culture systems are used.

It has been postulated that negative regulation is dominant for many of these genes (73,200). In this concept of dominance a negative agonist is effective in blocking even saturating concentrations of a positive agonist. Studies of the PEPCK gene showed that inhibition of transcription by insulin was dominant over the stimulatory effects of cAMP and glucocorticoids. Dominance is not a universal feature of insulin action, because its ability to stimulate transcription of glycolytic enzyme genes is overridden by the inhibitory effect of cAMP. This restraint of gluconeogenesis and glycolysis by the action of whichever hormone exerts the negative effect may be of central importance, but the molecular mechanisms for such dominance are unknown.

Analysis of the genomic organization of a number of genes reveals that alternate exon use allows for special types of regulation. Cell-specific regulation is accomplished, in part, by the fact that alternate first exons provide unique mRNAs for PK, GK, and the bifunctional enzyme in different tissues. Therefore, different promoter regions and control elements are employed, and an enzyme that catalyzes a specific reaction can serve a very different physiologic purpose in two different tissues (e.g. 6PF-2-K/Fru-2,6-P_2ase in the hepatocyte and the muscle cell). Alternate exon use appears to be a characteristic of the hepatic glycolytic enzyme genes (GK, 6PF-1-K, 6PF-2-K/Fru-2,6-P_2ase, PK) but not the gluconeogenic enzyme genes PEPCK and Fru-1,6-P_2ase.

We need to determine how these genes are regulated at the molecular level. Presumably, this regulation occurs via *trans*-acting protein factors that bind at the *cis*-acting DNA elements. The first step will be to isolate and identify these *trans*-acting factors and, ultimately, to clone them. The next step will be to determine how these *trans*-acting factors are regulated (e.g. covalent modification, allosteric effectors, changes in gene expression). Some formidable challenges exist. Little is known about how a hormone exerts positive

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and negative effects upon separate genes, and the fact that these effects can occur simultaneously within a cell is a complicating issue. The cis/trans model of gene regulation has been applied successfully to gluconeogenic enzyme gene promoters such as PEPCK and Fru-1,6-P_2ase but with little success to glycolytic enzyme genes such as PK, GK, and the bifunctional enzyme. Confounding this issue is the fact that many of these genes are not expressed or regulated in standard tissue culture cell lines. Additional complications can also occur. For example, in the case of the bifunctional enzyme 6PF-2-K/Fru-2,6-P_2ase, mRNA is expressed in hepatoma cells and is responsive to hormones, but the mRNA is spliced differently from that found in liver. Even when suitable cell lines are available, the fusion gene/transfection approach has not worked in some instances. If this continues to be true, especially as more of these promoters are analyzed, we must consider the possibility that the hormonal regulation of these glycolytic enzyme genes, which also employ alternate exon use, is somehow unique.

Despite major advances in knowledge of the structure and function of hepatic regulatory enzymes and the control of metabolism via phosphorylation mechanism, interest in metabolic regulation has fallen in recent years. This decline may have occurred because of a lack of new approaches for analyzing control of pathway flux, but the recent advances in DNA/RNA recombinant technology have opened new exciting avenues. For example, it is possible to transfect cell lines derived from the liver with genes for native and modified forms of key enzymes, express them, and determine their effects on metabolic flux. This approach will involve engineering chimeric genes with potent promoters so that increased amounts of these enzymes produced in transfected cells can be modulated. Such metabolic pathway engineering should allow investigators to sort out the complex interplay between short-term, acute regulation of enzyme activity via covalent modification and/or allosteric effectors and the long-term effects on gene expression. For example, this problem can be approached by transfection into appropriate cell lines of genes, linked to powerful, regulated promoters, for wild-type and modified forms of covalently regulated enzymes, such as 6PF-2-K/Fru-2,6-P_2ase, that lack phosphorylation sites. In addition, the use of transgenic mice will allow the analysis of promoter function as well as the expression of regulatory gene products under specific physiologic conditions. This approach has great potential for the physiologic analysis of metabolic pathways. Homologous recombination also is a potentially powerful analytical tool (11, 221). For example, homologous recombination can theoretically be used to inactivate genes for important regulatory proteins such as the key regulatory substrate cycle enzymes. The next step would be to express enzyme forms altered by site-directed mutagenesis. Thus, stable cell lines could be created with targeted mutations.

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The question of how the liver regulates the utilization and synthesis of glucose has been studied intensively by physiologists and biochemists for decades. Much has been learned, but many questions remain unanswered. However, the tools to approach these problems successfully are growing in number and incisiveness, and their use in the future should yield rich rewards.

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