Stimulation of Glucose-6-phosphatase Gene Expression by Glucose and Fructose-2,6-bisphosphate

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Glucose-6-phosphatase, a key enzyme in the homeostatic regulation of blood glucose concentration, catalyzes the terminal step in gluconeogenesis and glycogenolysis. Glucose, the product of the glucose-6-phosphatase reaction, dramatically increases the level of glucose-6-phosphatase mRNA transcripts in primary hepatocytes (20-fold), and the maximum response is obtained at a glucose concentration as low as 11 mm. Glucose specifically increases glucose-6-phosphatase mRNA and L-type pyruvate kinase mRNA. In the rat hepatoma-derived cell line, Fao, glucose increases the glucose-6-phosphatase mRNA only modestly (3-fold). In the presence of high glucose concentrations, overexpression of glucokinase in Fao cells via recombinant adenovirus vectors increases lactate production to the level found in primary hepatocytes and increases glucose-6-phosphatase gene expression by 21-fold. Similar overexpression of hexokinase I in Fao cells with high levels of glucose does not increase lactate production nor does it change the response of glucose-6-phosphatase mRNA to glucose. Glucokinase overexpression in Fao cells blunts the previously reported inhibitory effect of insulin on glucose-6-phosphatase gene expression in these cells.

Raising the cellular concentration of fructose-2,6-bisphosphate, a potent effector of the direction of carbon flux through the gluconeogenic and glycolytic pathways, also stimulated glucose-6-phosphatase gene expression in Fao cells. Increasing the fructose-2,6-bisphosphate concentration over a 15-fold range (12 ± 1 to 187 ± 17 pmol/plate) via an adenoviral vector overexpression system, led to a 6-fold increase (0.32 ± 0.03 to 2.2 ± 0.33 arbitrary units of mRNA) in glucose-6-phosphatase gene expression with a concomitant increase in glycolysis and a decrease in gluconeogenesis. Also, the effects of fructose-2,6-bisphosphate concentrations on fructose-1,6-bisphosphatase gene expression were stimulatory, leading to a 5- to 6-fold increase in mRNA level over a 15-fold range in fructose-2,6-bisphosphate level. Liver pyruvate kinase and phosphoenolpyruvate carboxykinase mRNA were unchanged by the manipulation of fructose-2,6-bisphosphate level.

Overproduction of glucose by the liver is the major cause of fasting hyperglycemia in diabetes mellitus (1, 2). The terminal step in glucose production from the gluconeogenic and glycogenolytic pathways in the liver is catalyzed by glucose-6-phosphatase (Glu-6-Pase), a key enzyme in the homeostatic regulation of blood glucose concentration (3, 4). With the recent availability of cDNA clones for the Glu-6-Pase catalytic subunit (5, 6), it has become possible to study the regulation of gene expression for this enzyme in different hormonal and nutritional states. Increased Glu-6-Pase mRNA, protein, and activity have been found in several diabetic rat models, e.g., diabetic BB rats (7) and streptozotocin-induced diabetic rats (8, 9). Studies of hormonal regulation of the Glu-6-Pase catalytic subunit have shown that its mRNA levels are modulated similarly to that of other gluconeogenic enzymes, such as PEPCK and Fru-1,6-Pase (9, 10). Insulin decreases the activity and mRNA levels of the Glu-6-Pase catalytic subunit, whereas cAMP and glucocorticoids increase mRNA levels (10). The action of insulin is dominant over the action of glucocorticoids in Fao cells (10).

We have observed a stimulatory regulation of Glu-6-Pase mRNA levels by glucose. Glucose increases mRNA levels of the Glu-6-Pase catalytic subunit in rat hepatoma cells (9, 10). In an in vivo model, hyperglycemia per se stimulates hepatic Glu-6-Pase expression in diabetic rats (11). Also, the levels of Glu-6-Pase mRNA in liver from rat refed with a high carbohydrate diet are very high compared with PEPCK mRNA levels (9). Glucose stimulation of gene expression has previously been established for glycolytic enzymes, L-PK (12) and aldolase B (13), and for lipogenic enzymes, acetyl-CoA carboxylase and fatty-acid synthase (14) but not for gluconeogenic enzymes. In the regulation of glycolytic enzyme expression, the glucose effect on gene expression has been related to glucose metabolism. In one report, glucokinase expression has been linked to glucose stimulation of L-PK gene expression (15). Furthermore, 2-deoxyglucose, a glucose analog that is phosphorylated in cells, stimulates fatty-acid synthase and acetyl-CoA carboxylase gene expression, whereas 3-O-methylglucose, which is transported into the cell but not metabolized, has no effect (14).

In the present study we characterize the effect of glucose on the level of Glu-6-Pase message in primary hepatocytes, the system that has been used to demonstrate the effect of glucose on L-PK gene expression (12). Cultured cells have an advantage over in vivo models because they allow us to study specific changes in glucose concentration without inducing hormonal

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1 The abbreviations used are: Glu-6-Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; Fru-1,6-P 2ase, fructose-1,6-bisphosphatase; 6-PF-2/Fru-2,6-P 2ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fru-2,6-P 2ase, fructose-2,6-bisphosphatase; L-PK, liver type pyruvate kinase; GK, glucokinase; HKI, hexokinase I; 6-PF-1-kinase, 6-phosphofructo-1-kinase; Glu-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; FBS, fetal bovine serum; BSA, bovine serum albumin; kb, kilobase pair(s); pfu, plaque-forming unit(s).

12854 This paper is available on line at http://www-jbc.stanford.edu/jbc/
changes. We determined the specificity of glucose stimulation of Glu-6-Pase, L-PK, and several messages of glycolytic and gluconeogenic enzymes such as PEPCk, Fru-1,6-Pase, 6-PF-2-K/Fru-2,6-Pase, and GK. Since glucose metabolism is necessary for a glucose effect on gene expression, we examined the relationship between the response of Glu-6-Pase mRNA levels to glucose, to the levels of GK activity, and to glycolytic flux in primary hepatocytes and in Fao hepatoma cells. To correlate changes in Glu-6-Pase mRNA levels with changes in glucose metabolism, we manipulated the glycolytic flux in Fao cells by overexpressing GK via an adenovirus-mediated technique and measured the effect of glucose on Glu-6-Pase gene expression.

Here we also report the effect of cellular Fru-2,6-P2 concentration on the regulation of Glu-6-Pase gene expression in Fao cells. The cellular Fru-2,6-P2 concentration was manipulated via an adenovirus vector system (16) to alter glycolytic and gluconeogenic flux. The purpose of this alteration was to be able to correlate changes in glycolysis to changes in Glu-6-Pase gene expression as seen with high glucose. The effect of Fru-2,6-P2 concentration on the gene expression of other enzymes of the carbohydrate metabolism, namely Fru-1,6-Pase, glucose-6-phosphatase, L-PK, and several messages of glycolytic and gluconeogenic enzyme transcripts in primary hepatocytes. RNA was extracted from primary hepatocytes incubated 4, 24, or 48 h with 5.5 (L) or 27.5 mM (H) glucose. 20 µg of RNA were used for quantification by Northern blot analysis. The same blot was hybridized with six different specific probes made from cDNA templates identified in the figure. A 1.5-kb genomic EcoRI fragment of chicken brain PEPCK, a 1.2-kb RI fragment of Fru-1,6-Pase (23); 3) 0.9-kb RI fragment of Glu-6-Pase catalytic subunit (8); 4) 3.0-kb HindIII NotI fragment of GK (24); 4) a 0.4-kb Xml/HindIII fragment of L-PK (25); and 5) a 1.4-kb EcoRI fragment of rat liver 6-PF-2-K/Fru-2,6-Pase cDNA. A 1.5-kb genomic EcoRI/BamHI fragment containing the 5′-exon of the rat liver PEPCK gene was labeled to probe PEPCK mRNA (10), and a 2-kb PstI fragment of chicken brain β-actin cDNA (16) also was labeled and used to correct for variation in RNA loading and transfer efficiency. Northern blots were hybridized with the labeled probes and washed as described previously (10). Radioactive signals were quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed with IP-Lab Analyst Software (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis of Overexpressed Wild-type Rat Liver GK in Fao Cells—GK protein was extracted in homogenizing buffer (50 mM Tris/HC1, pH 7.4, 300 mM sucrose, 100 mM KCI, 1 mM EDTA, and 1 mM dithiotreitol) and concentrated by precipitation with 65% saturated (NH4)2SO4. The precipitate was dissolved in homogenizing buffer containing 0.5 mM phenylmethylsulfonfyl fluoride. Soluble protein (100 µg) was electrophoresed and electrophoretically transferred to a nitrocellulose membrane (Immobilon-P, Millipore, Bedford, MA) for Western blot analysis as described previously (16). The membrane was blotted with a 1:250 dilution of rabbit polyclonal antibody raised against human pancreatic GK (Lampire Biological Laboratories, Pipersville, PA) and then with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. Protein was visualized with a color reagent (Sigma). Protein levels were quantitated using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed with IP-Lab Analyst Software (Molecular Dynamics, Sunnyvale, CA).

Enzymatic Activity and Metabolite Concentration Determination Assays—Crude extracts of Fao cells and primary hepatocytes were prepared as described above. The activities were analyzed in 12,000 × g supernatants. Hexokinase maximal activity was measured at 0.5 mM glucose. GK activity was calculated as the difference between the glucose phosphorylation capacities at 100 mM and 0.5 mM glucose (26). Glu-6-Pase activity was determined in Fao cell homogenates treated with 0.1% Triton X-100 at 20 mM Glu-6-P concentration (10). Glucose concentration and lactate production in the incubation medium were measured using standard enzymatic methods (27). Fru-2,6-P2 was extracted at 100 °C in 0.1 M NaOH and measured using the 6-phosphofructo-1-kinase assay (28). Lactate and pyruvate concentrations were measured spectrophotometrically using standard enzymatic methods (27) in neutralized protein-free extracts as described previously by Arguda et al. (29).

RESULTS

Glucose Response of Glu-6-Pase mRNA in Primary Hepatocytes—Incubating primary hepatocytes in a high glucose concentration (27.5 mM) increased the level of Glu-6-Pase mRNA 3-fold at 4 h, 9-fold at 24 h, and 20-fold at 48 h. However, in the presence of 5.5 mM glucose, Glu-6-Pase mRNA levels decreased from 4.2 ± 0.2 to 1 ± 0.1 (arbitrary units) during the first 24 h of incubation and remained constant for 48 h (Fig. 1). The stimulatory effect of glucose was seen despite the presence of 1 µM insulin in the incubation medium. The maximal stimulation of Glu-6-Pase mRNA was seen at 11 mM glucose, the lowest concentrations as low as 11 mM, and maximal stimulation was maintained at 27.5 mM glucose (Fig. 2). Although the medium was changed every 24 h, there was some lactate accumulation but without a significant change in medium pH. Since lactate production reached a maximum of 6 mM in 24 h (data not shown),

![Fig. 1](http://www.jbc.org/content/12855/1/25/178586)
we examined the effect of different concentrations of lactate on Glu-6-Pase mRNA in primary hepatocytes. A maximum 4-fold increase in Glu-6-Pase mRNA was obtained after 48 h incubation with 10 mM lactate (data not shown).

We compared the response of Glu-6-Pase mRNA in primary hepatocytes to that of L-PK, a well-characterized gene whose expression is stimulated by glucose (12). As shown in Fig. 1, the increase in L-PK mRNA response to glucose in primary hepatocytes was smaller than that seen with Glu-6-Pase mRNA. L-PK mRNA levels increased by 10 ± 0.7-fold at 24 h and 12 ± 2.3-fold at 48 h. The maximal response of L-PK mRNA to glucose was attained at 24 h, whereas for Glu-6-Pase mRNA the response doubled between 24 and 48 h.

To ascertain that the effects of glucose on Glu-6-Pase and L-PK mRNA were specific and not simply due to the cells being in a well-fed state, the expression of other gluconeogenic and glycolytic enzymes were measured and compared with values in low (5.5 mM) and high (27.5 mM) glucose as described above. The levels of Fru-1,6-Pase and 6-PF-2-K/Fru-2,6-Pase mRNA were increased slightly after 48 h incubation with high glucose, 3 ± 0.5-fold (Fig. 1) and 2-fold (data not shown), respectively. The mRNA level of PEPCK was unaffected by glucose at 48 h but became undetectable by 24 h, regardless of glucose concentration (Fig. 1). GK mRNA was unaffected by glucose treatment (Fig. 1). These results demonstrate that glucose specifically stimulates Glu-6-Pase and L-PK gene expression.

Effect of Glucose on Glu-6-Pase mRNA, GK Activity, and Glucose Metabolism in Fao Cells—As shown previously (9, 10), glucose also increased the Glu-6-Pase mRNA level in gluconeogenic rat Fao hepatoma cells; however, this increase was modest compared with that found in primary hepatocytes. In Fao cells, Glu-6-Pase levels at 27.5 mM glucose were increased 1.5-fold at 24 h and 3-fold at 48 h over Glu-6-Pase levels at 5.5 mM glucose (data not shown). To understand the difference in amplitude of the Glu-6-Pase mRNA response to glucose in primary hepatocytes versus Fao cells, we explored the differences of these models with respect to glucose metabolism. As previously reported (16) and shown in Table I, Fao cells exhibited a very low rate of glycolysis from glucose. The rate of lactate production from glucose was much lower in Fao cells than in primary hepatocytes (Table I, 0.27 ± 0.04 versus 23 ± 1 nmol/min/plate). The rate of lactate production in Fao cells was only slightly affected by glucose concentration, i.e. a 1.6-fold increase when glucose concentration was raised from 5.5 to 27.5 mM (Table I). In primary hepatocytes the rate of lactate production was increased 8-fold when the cells were incubated with 27.5 mM glucose compared with 5.5 mM (Table I) and at 27.5 mM glucose, 80% of the consumed glucose was metabolized to lactate (data not shown).

The low glycolytic rate with glucose and the modest effect of glucose concentration on this rate in Fao cells led us to compare the level of GK activity in this cell line with primary hepatocytes. As shown in Table II, the level of GK activity in Fao cells was one-fifth that of primary hepatocytes, whereas HK activity was 11-fold higher in Fao cells than in primary hepatocytes. In primary hepatocytes, the level of GK activity was constant during the 48-h experiment and was not dependent on glucose concentration (data not shown).

Metabolic Effects of Overexpression of Rat Liver GK and Relationship to Glu-6-Pase Gene Expression in Fao Cells—The difference in the amount of GK activity between Fao cells and primary hepatocytes could explain differences in glycolytic flux and in the response of Glu-6-Pase mRNA to glucose. To test this hypothesis, we overexpressed rat liver GK in Fao cells using recombinant adenoviral vectors (20). The amounts of GK protein, as determined by Western blot analysis, and GK activity (Table II) were quantified 72 h after transduction of the Fao cells with AdCMV-GKL or AdWT. The amount of protein increased 24-fold and the level of activity increased 13-fold in Fao cells transduced with AdCMV-GKL as compared with the endogenous level of GK in untransduced Fao cells or in cells transduced with AdWT (Table II). Lactate production was increased 130-fold in cells transduced with AdCMV-GKL, reaching a level comparable to that seen in primary hepatocytes (Table I). During this time, the glucose consumption was only increased 4-fold in cells overexpressing GK compared with cells transduced with AdWT (30 ± 3 versus 7 ± 1 nmol/min per plate, n = 3). Hence, the proportion of consumed glucose metabolized to lactate (Lac/2*Glc) was dramatically changed by the overexpression of GK. This proportion was only 4% in untransduced Fao cells or in AdWT transduced cells and approximately 63% when GK was overexpressed. Transduction of Fao cells with AdWT did not affect GK activity or lactate production (Tables I and II). In the cells in which GK was overexpressed, the endogenous low K_m hexokinase activity was decreased by 40% (Table II).

Overexpression of GK in Fao cells (Fig. 3) in the presence of 27.5 mM glucose increased levels of Glu-6-Pase mRNA by 21-fold (lane 2), compared with levels of Glu-6-Pase mRNA in the presence of 27.5 mM glucose in untransduced Fao cells (lane 1) or in AdWT transduced cells (lane 2). Overexpression of GK in the absence of glucose (lane 4) did not increase Glu-6-Pase mRNA levels. As with Glu-6-Pase mRNA, L-PK mRNA levels were increased when GK was overexpressed in Fao cells (Fig. 3; lane 3 versus lanes 2 and 1). Glucose stimulation of L-PK mRNA levels was smaller than that for Glu-6-Pase mRNA.

| Cell type | Treatment | 5.5 mM Glc | 27.5 mM Glc |
|-----------|-----------|------------|-------------|
| Primary hepatocytes | Untreated | 3 ± 0.3 | 23 ± 1 |
| Fao | Untreated | 0.17 ± 0.02 | 0.27 ± 0.04 |
| AdWT | ND | 0.15 ± 0.04 |
| AdCMV-GKL | ND | 19 ± 1.8 |
Regulation of Glucose-6-phosphatase Gene Expression

Table II

| Cell type          | Treatment   | Glucokinase Overexpression | Hexokinase Protein |
|--------------------|-------------|----------------------------|--------------------|
|                     |             | μmol/min/mg                | μmol/min/mg        |
| Primary hepatocytes | Untreated   | 14 ± 0.5                   | 1.4 ± 0.1          |
| FAO                 | Untreated   | 3.7 ± 0.8                  | 15 ± 1.1           |
| AdWT                | AdWT        | 13 ± 0.3                   | 16 ± 0.8           |
| AdCMV-GKL           | AdCMV-GKL   | 24 ± 0.9                   | 9 ± 0.7            |

9-fold for L-PK compared with 21-fold for Glu-6-Pase. These changes in mRNA level were reflected in an increase in Glu-6-Pase catalytic activity of the Fao cells (from 14 ± 2 to 31 ± 3 nmol/mg protein). Overexpression of GK, in the presence of high glucose, increased only Glu-6-Pase and L-PK mRNA levels. Fru-1,6-Pase and PEPCK mRNAs were unchanged by GK overexpression (data not shown). Since overexpression of GK in the presence of a high concentration of glucose led to lactate accumulation in the medium, approximately 6 mM in 24 h, we examined the effect of high lactate concentrations on Glu-6-Pase mRNA. We found that a 48-h incubation with 5 or 10 mM lactate, renewed every 24 h, increased the Glu-6-Pase mRNA level by 4-fold and the L-PK mRNA level by 2-fold (data not shown).

To determine whether the endogenous low $K_m$ hexokinase present in Fao cells was a limiting step for glycolysis, we overexpressed rat liver hexokinase I (HKI) in these cells. A 3-fold overexpression of HKI did not significantly increase lactate production at 27.5 mM glucose in Fao cells (data not shown) and did not affect the levels of Glu-6-Pase or L-PK mRNA relative to untreated cells (Fig. 3; lane 7 versus lane 1), whereas a 2-fold overexpression of GK was sufficient to raise the lactate production by 50-fold and the Glu-6-Pase mRNA level by 7-fold (data not shown). Thus, only overexpression of GK increased lactate production and was able to increase the response of Glu-6-Pase and L-PK mRNAs to glucose.

Glucokinase stimulation of Glu-6-Pase mRNA was seen in the presence of a high insulin concentration in primary hepatocytes (see above), whereas the same insulin concentration completely inhibited Glu-6-Pase mRNA in Fao cells, even in the presence of high glucose concentrations (9, 10). To test whether the discordant effects of insulin on Fao cells and primary hepatocytes were due to differences in GK expression in these cell types, we compared the insulin effect between control Fao cells and those overexpressing GK. As shown previously (10), the inhibitory effect of 24 h exposure to insulin was 50% at 1 nM (Fig. 3, lane 1 compared with lane 6) and 100% at 1 μM. When GK was overexpressed (Fig. 3, lane 3 and lane 5), the Glu-6-Pase mRNA level was only decreased 10% by 1 nM insulin (lane 5 compared with lane 3) and 30% by 1 μM insulin (data not shown). Thus, overexpression of GK in the presence of high glucose concentrations partially prevented the inhibitory effect of insulin on Glu-6-Pase message levels in Fao cells. The insulin effect on L-PK gene expression in untransduced Fao or in AdWT transduced cells (lanes 1 and 2) was not quantifiable due to low L-PK mRNA levels. When GK was overexpressed in the presence of 27.5 mM glucose, 1 nM insulin decreased L-PK mRNA levels by 20% (lane 5 compared with lane 3) and 1 μM insulin decreased L-PK mRNA by 50% (data not shown).

Manipulation of Fru-2,6-bisphosphate Levels—In studies using an adenoviral vector overexpression system, we have previously shown that Fru-2,6-P$_2$ levels are increased only when a double mutant form (Ad-PP2KMut) of the 6-PK-2-K/Fru-2,6-P$_2$ase is overexpressed (16). Mutation of Ser-32 to Ala in the kinase domain prevents cAMP-dependent phosphorylation of the enzyme (32), and mutation of the bisphosphatase active site His-258 to Ala abolishes bisphosphatase activity (33, 34). Therefore, the double mutant possesses only kinase activity which cannot be down-regulated by cAMP-dependent phosphorylation. Fig. 4 shows that Fru-2,6-P$_2$ level is dependent on the level of the multiplicity of infection with Ad-PP2KMut. The curve is sigmoidal between 0 and 10 pfu/cell and becomes linear above 10 pfu/cell. The activity of 6-PF2-K (kinase) increased in parallel with the Fru-2,6-P$_2$ level (data not shown). Also, as found previously (16), overexpression of the wild-type form of 6-PK-2-K/Fru-2,6-P$_2$ase (Fig. 4) decreased the level of Fru-2,6-P$_2$ by 70% relative to the control (Table III).

Effect of Fru-2,6-bisphosphate on Glycolysis and Glu-6-Pase Gene Expression—To demonstrate that changes in Fru-2,6-P$_2$ level affect glucose metabolism, we measured glycolysis from glucose in Fao cells for different levels of Fru-2,6-P$_2$, obtained with the transduction of different amounts of Ad-PP2KMut. Lactate plus pyruvate production in Fao cells was directly proportional to the level of Fru-2,6-P$_2$ (Fig. 5). During the overexpression of the mutant bifunctional enzyme, the increase in Fru-2,6-P$_2$ level and lactate plus pyruvate production was not limited by glucose concentration in the medium. These parameters showed no change when glucose concentration was increased from 11 to 30 mM. The mRNA level of overexpressed wild-type (lanes 3–7) and double mutant (lanes 8–12) forms of bifunctional enzyme is shown in Fig. 6. A uniform presence of these two mRNAs is seen when compared with the nontransduced (lane 1) or AdWT (lane 2) -transformed Fao cells.

Fig. 6 and Table III also show that transduction of Fao cells with Ad-PP2KMut increased Glu-6-Pase mRNA, whereas
transduction with Ad-PF2KWT decreased Glu-6-Pase mRNA levels. This effect was related to the change in Fru-2,6-P₂ concentration measured in the same experiment as shown in Table III. Compared with AdWT (empty vector), the AdPF2KWT-transduced cells showed a 70% decrease in Fru-2,6-P₂, which decreased Glu-6-Pase mRNA by 50% (0.5 arbitrary units). The AdPF2KMut-transduced cells showed a 3-fold increase in Fru-2,6-P₂, which increased Glu-6-Pase mRNA 3-fold. The Fru-2,6-P₂ effect on Glu-6-Pase mRNA is maximal between 140 ± 20 and 187 ± 17 pmol/plate and not at maximal Fru-2,6-P₂ levels. Fig. 7 shows the quantitation of the relative changes where a 15-fold range in Fru-2,6-P₂ level (10 ± 1 compared with 191 ± 18 pmol/plate) led to an overall 6-fold (2.2 ± 0.33 versus 0.32 ± 0.1 arbitrary units) change in Glu-6-Pase mRNA level. These changes in mRNA were reflected in an increase in Glu-6-Pase catalytic activity in these Fao cells (from 14 ± 2 to 27 ± 3 nmol/mg protein).

**Effect of Fru-2,6-bisphosphate on the Gene Expression of Other Gluconeogenic and Glycolytic Enzymes—** By measuring mRNA levels with other specific cDNA probes, the effect of Fru-2,6-P₂ concentration on the gene expression of other gluconeogenic and glycolytic was also determined. High Fru-2,6-P₂ concentration increased Fru-1,6-P₂ase mRNA, and low Fru-2,6-P₂ concentration decreased Fru-1,6-P₂ase mRNA (Fig. 6).

![Fig. 4. Titration of Fru-2,6-P₂ levels in Fao cells transduced with Ad-PF2KMut. 48 h after transduction of 55-cm² plate of Fao cells with Ad-PF2KMut, Fru-2,6-P₂ was extracted and measured as described under "Experimental Procedures." Different amounts of AdWT (□) or Ad-PF2KMut (●) were used at plaque-forming unit per cell (pfu/cell) values between 3 and 40. The pfu/cell value equal to 0 represents the value of Fru-2,6-P₂ in control Fao cells. Results are the means ± S.E. of 4 experiments.](http://www.jbc.org/)

The amplitude of change in Fru-1,6-P₂ase mRNA was in the same range as that of Glu-6-Pase mRNA, namely a 5–6-fold change for a 15-fold change in Fru-2,6-P₂ level (Table III), L-PK and PEPCK mRNA levels were unchanged by variations in Fru-2,6-P₂ level (Fig. 6).

**DISCUSSION**

Previous work has demonstrated that glucose increases expression of genes encoding glycolytic enzymes such as L-PK (12) and aldolase B (13), whereas it decreases expression of gluconeogenic enzymes such as PEPCK (32). In this study, we report that in primary hepatocytes, the gene expression of a gluconeogenic and glycogenolytic enzyme, Glu-6-Pase, is greatly increased by glucose, as is the gene expression of a glycolytic enzyme, L-PK. The glucose response is specific since Fru-1,6-P₂ase and 6-PF-2-K/Fru-2,6-P₂ase mRNA levels were only slightly changed by glucose, and PEPCK and GK mRNA levels were unchanged. It is paradoxical that glucose simultaneously increases both the mRNA levels of L-PK, the terminal enzyme of the glycolytic pathway, and the mRNA levels of Glu-6-Pase, the terminal enzyme of the gluconeogenic and glycogenolytic pathways. This counter-regulation may play a role in controlling glycogen storage in the liver by avoiding excessive storage during the postprandial period. Alternatively, glucose stimulation of Glu-6-Pase mRNA levels may have a limited physiological role in normal animals since postprandial hyperglycemia is usually modest and transient, whereas it takes at least 4 h for the Glu-6-Pase mRNA to respond to a high glucose concentration. However, glucose regulation may play an important deleterious role in diabetes where chronic hyperglycemia per se has been shown to increase the level of Glu-6-Pase mRNA and protein in diabetic rats (11).

Glucose stimulation of the L-PK gene has been shown to be dependent on GK expression in primary hepatocytes (15, 33). A response to glucose is seen when GK expression is maintained by insulin or when the primary hepatocytes are transfected with a GK expression vector (15). L-PK mRNA is also responsive to glucose in the INS-1 β-cell line where GK expression is high (34), whereas there is no response of L-PK mRNA to glucose in Fao, Faza, and FTO hepatoma cell lines where GK expression is very low or absent (35). An exception, however, is that L-PK transcripts respond to glucose in mAsT3F hepatocyte-like cells in which only low Kₘ hexokinases are expressed (36). As is generally the case for L-PK transcripts, the response of Glu-6-Pase to glucose is also dependent on GK expression. This is evidenced by an increased response of Glu-6-Pase

**TABLE III**

| Sample numbers | Type of adenovirus | Virus titration | Fru-2,6-P₂ | mRNA |
|----------------|-------------------|----------------|-----------|------|
| 1              | Control           |                |           |      |
| 2              | AdWT              | 20             | 49 ± 6    | 1.2  |
| 3              | Ad-PF2KWT         | 2              | ND        | 0.7  |
| 4              | Ad-PF2KWT         | 4              | ND        | 0.8  |
| 5              | Ad-PF2KWT         | 6              | ND        | 0.7  |
| 6              | Ad-PF2KWT         | 8              | ND        | 0.6  |
| 7              | Ad-PF2KWT         | 10             | 12 ± 1    | 0.5  |
| 8              | Ad-PF2KMut        | 5              | 106 ± 12  | 2.3  |
| 9              | Ad-PF2KMut        | 10             | 140 ± 18  | 2.7  |
| 10             | Ad-PF2KMut        | 12.5           | 140 ± 20  | 2.2  |
| 11             | Ad-PF2KMut        | 15             | 187 ± 17  | 2.8  |
| 12             | Ad-PF2KMut        | 25             | 205 ± 22  | 2.8  |

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mRNA to glucose from 3- to 21-fold in Fao cells via GK overexpression, presumably by increased glucose metabolism. Also, the glucose response of both L-PK and Glu-6-Pase transcripts in primary hepatocytes was correlated with an increase in glucose metabolism and in lactate production. These findings are consistent with the postulate that regulation of hepatic gene expression by carbohydrate is mediated by increases in glucose metabolism via glycolysis (33, 37).

HKI overexpression did not increase lactate production in Fao cells incubated at 27.5 mM glucose nor could it substitute for GK overexpression with respect to Glu-6-Pase message stimulation. In a previous study, overexpression of HKI in primary hepatocytes was shown to increase lactate production at low but not at high glucose levels, leading to the suggestion that overexpressed HKI becomes metabolically inactive at high glucose concentrations via an increase in the level of its allosteric inhibitor, Glu-6-P (38, 39). As we did not measure the impact of HKI overexpression on lactate production or Glu-6-Pase overexpression at low glucose levels, it is possible that effects would be observed under these conditions. Nevertheless, the results clearly indicate that the effects of the two hexoki-nase isofroms are not equivalent and that GK may be uniquely capable of enhancing glucose metabolism, since only GK overexpression was able to confer potent regulation of Glu-6-Pase expression by high glucose. This regulation is likely mediated through a metabolite generated by the increase in glucose metabolism.

We also observed that GK overexpression significantly decreased HKI activity. While we have no explanation for this, we can speculate that the decreased activity may be due to 1) transcriptional down-regulation of HKI expression by GK or 2) inhibition by high levels of Glu-6-P, either directly or by displacement of the HKI from its mitochondrial site, the latter event is known to decrease its activity (40).

Changes in lactate production were not always perfectly correlated with the response of Glu-6-Pase mRNA levels to glucose. Whereas the response of Glu-6-Pase mRNA to glucose was maximal for a glucose concentration of 11 mM, lactate production still increased linearly, up to concentrations of 27.5 mM glucose (data not shown). A saturation of the regulatory machinery could explain the absence of complete correlation between glycolysis and the glucose response. Also, insulin, which stimulates lactate production in Fao cells (data not shown), inhibited Glu-6-Pase gene expression. Dihydroxyacetone, another carbon source, was unable to increase the glucose response in Fao cells to a greater extent than that seen in Fao cells treated with 25 mM glucose, even though the lactate production was 10 times higher than with 25 mM glucose alone (data not shown). Hence, the response of Glu-6-Pase gene expression to glucose was well correlated with glucose metabolism, but this correlation does not extend to the end point of the glycolytic pathway, i.e. lactate production.

The signal mediating the glucose effect on Glu-6-Pase mRNA seems to be situated between Glu-6-P and Fru-1,6-P2 because glucose, but not dihydroxyacetone, is stimulatory. Dihydroxyacetone is phosphorylated by glycerokinase or α-triokinase to dihydroxyacetone phosphate (41), which is then converted either to glucose (30%) or lactate plus pyruvate (70%) in Fao cells (16). Hence, glucose and dihydroxyacetone do not use a common glycolytic pathway between Glu-6-P and dihydroxyacetone phosphate. The glycolytic metabolites Glu-6-P and Fru-6-P are branch points to the glyogen synthetic pathway, the pentose phosphate shunt, and fructose-2,6-bisphosphate formation (see below). Metabolites in these pathways are...
Regulation of Glucose-6-phosphatase Gene Expression

It has been also postulated that regulation of hepatic gene expression by glucose may be dependent on the non-oxidative branch of the pentose phosphate pathway via xylulose 5-phosphate (42). The role of the pentose phosphate shunt cannot be excluded since we did not measure the metabolite levels of this pathway. Because xylitol was able to stimulate the L-PK promoter via the glucose response element with no change in Glu-6-P concentration, Doiron et al. (42) excluded the role of the intermediates of the Embden-Meyerhof pathway in glucose response. It should be noted, however, that xylitol has been described as a stimulator of glycolysis in liver via a decrease in ATP concentration (43, 44). This may cast some doubt on the feasibility of eliminating glycolytic intermediates as possible regulatory metabolites.

Besides its role of maintaining GK expression and glucose metabolism in liver cells, the effect of insulin on L-PK gene expression has not been clearly established (15, 33). As previously shown by Meienhofer et al. (35) in MH1C1 cells, we found that long exposures to high concentrations of insulin decreased L-PK mRNA levels in Fao cells. It has been shown previously that insulin strongly inhibits Glu-6-Pase gene expression in Fao cells and primary hepatocytes (10). When glucose is metabolized via GK overexpression in Fao cells, the inhibitory effect of insulin is blunted and the stimulatory effect of glucose on Glu-6-Pase gene expression becomes dominant. A similar phenomenon was also observed in primary hepatocytes. When the cells were grown overnight in the absence of insulin, the addition of insulin strongly inhibited Glu-6-Pase gene expression (10), whereas when high concentrations of insulin were consistently maintained in the medium, as in this study, there was no apparent inhibitory insulin effect. The difference in insulin response between these two conditions is probably due to a difference in the level of GK activity and lactate production.

Manipulation of Fru-2,6-P2 concentration by adenovirus vector overexpression was used to change rates of glycolysis and examine the effect of this change on Glu-6-Pase gene expression in Fao cells. This system is well suited because it does not require hormone treatments that have confounding effects on gene expression (45), and the Fao cells are not dependent on hormones for attachment (46). Fru-2,6-P2 levels were manipulated to demonstrate the dependence of the stimulation of Glu-6-Pase gene expression on glycolysis without adding high levels of glucose. Since Fru-2,6-P2 is an allosteric activator of 6-PF-1-kinase and an inhibitor of Fru-1,6-P2ase (12–14), this manipulation of Fru-2,6-P2 levels increases glycolysis (high Fru-2,6-P2) and decreases gluconeogenesis (low Fru-2,6-P2) (16). High Fru-2,6-P2 levels increased gene expression of Glu-6-Pase and low levels decreased it.

Fru-2,6-P2 stimulation of gene expression of a gluconeogenic enzyme, such as Glu-6-Pase, is paradoxical because high Fru-2,6-P2 levels stimulate glycolytic flux and inhibit gluconeogenic flux (47, 48). However, a similar situation is seen in genetically obese (fa/fa) rats when compared with lean animals, where chronically elevated Fru-2,6-P2 is thought to be caused by hyperinsulinemia (47). In these animals, in vivo isotopic studies have shown increased glucose production in the presence of high levels of Fru-2,6-P2 (49). Stimulation of Glu-6-Pase gene expression by high Fru-2,6-P2 levels may therefore participate in the hyperglycemia found in insulin resistance syndrome in non-insulin-dependent diabetes mellitus (2).

The stimulatory effect of high Fru-2,6-P2 on Fru-1,6-P2ase gene expression is also paradoxical and counter-regulatory, because this enzyme is gluconeogenic and Fru-2,6-P2 is an inhibitor of Fru-1,6-P2ase (50). However, inhibition of Fru-1,6-P2ase activity is an acute effect of Fru-2,6-P2 and has been shown only in enzyme extracts (50). There is clearly a dichotomy between this acute regulation and the long term stimulatory regulation we have observed. Because the effects on Fru-1,6-P2ase and Glu-6-Pase were so similar, the 5'-flanking region sequences of the Fru-1,6-P2ase and Glu-6-Pase genes (to −800 bp) were compared to reveal possible common response elements. However, no regions of high homology were identified (9, 23).

The stimulatory effect of Fru-2,6-P2 on Glu-6-Pase gene expression is seen when there is an increase in glycolysis. However, the correlation between an increase in glycolysis and an increase in Glu-6-Pase mRNA is not complete because the levels of Glu-6-Pase mRNA reach a maximum before saturation of glycolysis and Fru-2,6-P2. This lack of complete correlation favors a direct saturable effect of Fru-2,6-P2 on Glu-6-Pase gene expression or regulation of a saturable metabolite that builds up on a side pathway that is dependent on glycolytic flux.

High glucose and high Fru-2,6-P2 stimulate Glu-6-Pase gene expression. However, the Fru-2,6-P2 effect is less pronounced than the glucose effect; a 15-fold increase in Fru-2,6-P2 concentration increases Glu-6-Pase gene expression by 6-fold, whereas a 2-fold change in glucose concentration increases Glu-6-Pase gene expression by 20-fold, and both stimulate glycolysis 7–8-fold. However, even though both Fru-2,6-P2 in Fao cells and glucose in primary hepatocytes (at 27.5 mM glucose) increased glycolysis by 7–8-fold, the glycolytic flux was comparatively lower in Fao cells in the presence of high levels of Fru-2,6-P2 (191 ± 18 pmol/plate). The rates of glycolytic flux were 0.6 and 23 nmol/min/plate, respectively. In Fao cells overexpressing bifunctional enzyme, where GK message levels were found to very low, GK expression is thought to limit glycolysis and thereby limit the effect of Fru-2,6-P2 on Glu-6-Pase gene expression. This view was confirmed because the GK limitation of glycolysis was overcome by adenovirus-mediated co-overexpression of GK and mutant 6-PK 2-K/Fru-2,6-P2ase which doubled the stimulatory effect of Fru-2,6-P2 on Glu-6-Pase gene expression.

The effects of glucose and Fru-2,6-P2 on the expression of other genes of carbohydrate metabolism enzymes were also compared. Glucose stimulates L-PK mRNA levels, but Fru-2,6-P2 does not affect L-PK levels. Fru-2,6-P2 stimulates Fru-1,6-P2ase mRNA levels but glucose produces no effect. The differential effects of Fru-2,6-P2 and glucose on gene expression imply different mechanisms of action. Fru-2,6-P2 may act directly on gene expression or via a pathway metabolite different from that generated by glucose metabolism, even though both seem to be acting via glycolysis. The metabolic intermediate of glycolysis that increases with high Fru-2,6-P2 would be expected to be different than that which increases with the overexpression of GK or with high glucose concentrations. With high Fru-2,6-P2, which activates 6-PF-1-kinase and inhibits Fru-1,6-P2ase (50), Glu-6-P and Fru-6-P levels would be expected to be low, whereas with high glucose concentration or GK overexpression, all the intermediates between Glu-6-P and pyruvate would be expected to be high. The differential pattern of gene expression we observe may indicate that glucose acts on gene expression at the level of Glu-6-P or Fru-6-P. As mentioned previously, the stimulation of L-PK gene expression by glucose has been related to the non-oxidative branch of the pentose phosphate pathway through the intermediate xylulose 5-phosphate (42). This pathway would be expected to be stimulated with high glucose or GK overexpression but not by high Fru-2,6-P2 levels, making it unlikely that xylulose 5-phosphate
is responsible for the stimulation of Glu-6-Pase gene expression observed with high Fru-2,6-P₂.

The regulation of Glu-6-Pase gene expression can be divided into two types of regulation that are either metabolically logical or paradoxical. Previously, we have shown that insulin decreases Glu-6-Pase gene expression. Insulin in the postprandial state would be thought to limit glucose production by the liver. A decrease in Glu-6-Pase activity would be one mechanism by which glucose production could be lowered. The reported stimulatory effect of cAMP (10) on Glu-6-Pase gene expression, presumably acting via glucagon, can be similarly rationalized. Here we have shown that either high levels of glucose or Fru-2,6-P₂ paradoxically stimulate Glu-6-Pase gene expression. Clearly, gluconeogenic/glycolytic enzyme reciprocity may only seem paradoxical due to our limited knowledge of the functions of this enzyme. Other yet unknown functions may apply to Glu-6-Pase gene expression. Also, the present studies demonstrate that the catalytic subunit of Glu-6-Pase is highly regulated, which argues against regulation of the Glu-6-Pase system solely by substrate transport through the putative Glu-6-P translocase (52).

Work is in progress to determine whether or not the stimulatory effects of Fru-2,6-P₂ on Glu-6-Pase and Fru-1,6-P₂ase vice versa (51), does not apply to Glu-6-Pase gene expression. Also, the present studies demonstrate that the catalytic subunit of Glu-6-Pase is highly regulated, which argues against regulation of the Glu-6-Pase system solely by substrate transport through the putative Glu-6-P translocase (52).

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