Requirement of glucose metabolism for regulation of glucose transporter type 2 (GLUT2) gene expression in liver

Franck RENCUREL*, Gérard WAEBER†, Bénédicte ANTOINE‡, Francis ROCCHICCIOLÌ§, Paulette MAULARD*, Jean GIRARD* and Armelle LETURQUE*!

*Centre de Recherche sur l’Endocrinologie Moléculaire et le Développement, CNRS, 9, rue Jules Hetzel, 92190 Meudon, France. †Centre Hospitalier Universitaire Vaudois, Département de Médecine Interne B, CHUV, 1011 Lausanne, Switzerland. ‡Institut Cochin de Génétique Moléculaire, INSERM U129, 24, rue du Fbg St Jacques, 75014 Paris, France, and §Hôpital Saint-Vincent-de-Paul, INSERM U342, 82, avenue Denfert-Rochereau, 75614 Paris cedex 14, France

INTRODUCTION

Liver plays a crucial role in glucose homeostasis. In the postprandial state, glucose is taken up by the liver and is incorporated into glycogen and fatty acids or oxidized into CO₂. In the fasting state, liver provides glucose into the bloodstream via glycogenolysis and gluconeogenesis. Thus the transport of glucose across the liver plasma membrane is a bidirectional process. Liver glucose transport requires the presence of a high-Kₘ protein to allow a rapid equilibration of the intracellular glucose level with the extracellular glucose level within the physiological range of plasma glucose concentrations.

GLUT2 is a facilitative glucose transporter predominantly expressed in liver, pancreatic β cells and to a lesser extent in kidney, intestine and restricted area of the brain [1,2]. Several studies have shown that liver GLUT2 expression is regulated under different metabolic states. Liver GLUT2 expression is decreased during starvation and is restored to normal levels by refeeding fasted rats with a high-carbohydrate diet [3]. Liver GLUT2 mRNA concentrations are increased in streptozotocin-diabetic rats and are restored to control levels when hyperglycaemia is corrected by phlorizin [4], vanadate [5] or insulin administration [6]. GLUT2 mRNA levels are increased by high glucose concentration and in a dose-dependent manner in primary culture of adult rat hepatocytes [7,8]. These data strongly suggest that liver GLUT2 expression is regulated by glucose level both in vivo and in vitro.

In contrast, a decreased expression of pancreatic β cell GLUT2 has been reported in rat models of type II diabetes such as diabetic Zucker rat [9] and Goto–Kakizaki (GK) rats [10]. The decrease was correlated with the degree of hyperglycaemia. However, the inhibitory effect of glucose on pancreatic β cell GLUT2 expression has not been confirmed in vitro [11,12]. Moreover, when islets from diabetic or normal mice were cross-transplanted under the kidney capsule, it was shown that the loss of β cell GLUT2 was the consequence of the diabetic milieu [13] rather than of hyperglycaemia [14].

Glucose has been reported to regulate the expression of several other genes such as those for liver pyruvate kinase (L-PK) [15], fatty acid synthase (FAS) [16], insulin [17] and transforming growth factor α [18]. For L-PK and white adipose tissue FAS, the stimulatory effect of glucose requires its metabolism to glucose 6-phosphate and is potentiated by insulin [19,20]. In contrast, the effect of glucose on transforming growth factor α gene transcription involved the glucosamine pathway and not glycolysis [18]. Moreover, the stimulatory effect of glucose on liver GLUT2 expression was counteracted by insulin in vivo [6,8] and in cultured rat hepatocytes [8], which makes the regulation of this gene very unusual.

The aim of the present work was to investigate the possible intracellular mediators of glucose effects on GLUT2 expression by using a primary culture of rat hepatocytes and a well differentiated hepatocyte-like cell line: mhAT3F [21].

METHODS

Isolation of hepatocytes

Female Wistar rats (200–220 g) housed at 24 °C with light from 07:00h to 19:00h were used. Hepatocytes were isolated by the
Primary culture of hepatocytes

Hepatocytes were suspended in medium 199 containing Earle’s salts, 2.2 mg/l NaHCO₃, 2.2 mg/l NaHPO₄, 12H₂O, 10 mM Hepes, pH 7.5, at 37 °C) containing 0.1% collagenase (0.49 unit/mg; Boehringer Mannheim) and 5 mM CaCl₂. For each hepatocyte preparation the cell viability estimated by Trypan Blue exclusion was greater than 90%.

Determination of intracellular 2-deoxyglucose 6-phosphate concentration

After the attachment period, the culture medium was removed and hepatocytes were cultured for 1, 15 and 22 h in the presence of 5% fetal calf serum, 10 mM lactate, 1 mM pyruvate and 2-deoxyglucose (30 mM) (Sigma, St Louis, MO, U.S.A.) with a trace amount of 2-deoxy-[1-³H]glucose (0.74 MBq per dish). Control cultures were performed in the presence of 5% fetal calf serum, 10 mM lactate, 1 mM pyruvate and a trace amount of 2-deoxy-[α-³H]glucose (0.74 MBq per dish).

At the end of the culture period, the medium was removed, 1 ml of ice-cold 0.2 M NaOH was immediately added and the culture dish was frozen in liquid nitrogen. After scraping and neutralizing with 0.2 M HCl, 1 ml of 6% (v/v) HClO₃ was added to one aliquot fraction, which was then centrifuged. The radioactivity in the supernatant represented free 2-deoxyglucose and the cell-associated radioactive phosphorylated substrate concentration was obtained by subtraction and expressed as nmol per mg of protein.

Gas chromatography–mass spectrometry

Hepatocytes were broken by freeze–thawing. After homogenization, centrifugation and elution (AG 1-X2, 200–400 mesh; Bio-Rad) with hydrochloric acid, the supernatant was evaporated to dryness under a stream of dry nitrogen, and 100 µl of a (1:1) mixture of pyridine (Fluka, Buchs, Switzerland) and bis-(trimethylsilyl) trifluoroacetamide (Sigma) was added to the residue and heated at 60 °C for one hour. After cooling, 4 µl of the mixture was injected into the gas chromatographic–mass spectrometric system. Known amounts of 2-deoxyglucose 6-phosphate sodium salt (Sigma) were processed under the same conditions.

Use of inhibitors of protein and RNA synthesis

Puromycin (Sigma), an inhibitor of protein translation, was used at a final concentration of 0.25 mM. Hepatocytes were cultured overnight in the absence of glucose and were preincubated with puromycin for 1 h before adding the glucose to the culture medium.

An inhibitor of glucokinase, N-acetylglucosamine (Sigma), was used at a final concentration of 50 mM and was added to the fresh culture medium immediately after cell attachment. The cells were then cultured in the presence of different concentrations of glucose.

Actinomycin D and α-amanitin (Sigma), two inhibitors of transcription, were used at a final concentration of 5 µg/ml. Hepatocytes were cultured overnight in the presence of 20 mM glucose and the inhibitors were added to fresh culture medium. The culture was continued in the presence or absence of glucose for 24 h.

All these drugs had no effect on 18 S ribosomal RNA or on albumin mRNA levels (results not shown). This suggested that the drugs were not toxic for the cells under the experimental conditions used.

Quantification of GLUT2 mRNA concentration

Total RNA was isolated by the method of Chomczynski and Sacchi [23]. Northern blot and hybridization were performed as described previously [24]. The GLUT2 cDNA probe was kindly supplied by Dr. B. Thorens [1].

Transfection by lipofection of GLUT2/chloramphenicol-acetyltransferase (CAT) plasmid in cultured mHA3F

The mHA3F cells are a hepatocyte-like cell line derived from the tumorous liver of transgenic mice expressing the SV40 early
genes under the control of the liver-specific antithrombin III promoter [25]. This cell line was used for several reasons. First, glucose stimulated L-PK gene transcription in this cell line [26]. Secondly, the transfectability by lipofection of the cells was 50-fold higher than that of hepatocytes in primary culture [26]. Finally, the cells expressed GLUT2.

The mhAT3F cells were seeded in 60 mm culture dishes containing DMEM/F12 supplemented with 100 nM insulin, 1 mM dexamethasone, 1 mM tri-iodothyronine, 30 nM selenium (Na2SeO3) and 5% (v/v) fetal calf serum. When cells were at 60% confluence, they were maintained for 6 h in Dulbecco’s modified Eagle’s medium/nutrient mix F12 in the absence of glucose and insulin. Cationic liposomes (30 µg) (DOTAP, Boehringer Mannheim) were incubated for 15 min with 8 µg of the −338/+49 bp proximal region of the GLUT2 promoter linked to a CAT reporter gene (−338 CAT construct), with the promoterless plasmid (pCAT-Basic, Promega) or with a plasmid producing a strong expression of CAT (pCAT-Rous Sarcoma Virus promoter) in 100 µl of HEPES-buffered saline (20 mM Hepes, 150 mM NaCl, pH 7.4). The liposome-DNA complexes were added to mhAT3F cells cultured in a medium deprived of fetal calf serum. After an overnight culture, the medium containing liposomes was replaced by fresh medium supplemented with only 5% (v/v) fetal calf serum with or without glucose. Cells were harvested 36 h after removal of the liposomes. The chloramphenicol-acetyltransferase activity was assayed on 40 µg of total protein by TLC [27] and measured by liquid scintillation. The CAT activity was calculated as the ratio of the acetylated forms to the addition of acetylated plus non-acetylated forms of chloramphenicol per µg of cell proteins. The controls were expressed in m-units/µg of protein by comparison with a CAT enzyme (Sigma) used as standard in the assay. The effect of glucose was then expressed in fold stimulation over the CAT activity measured in cells cultured in the absence of glucose.

Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was performed with Student’s t-test for unpaired data.

RESULTS

Glucose concentration does not affect GLUT2 mRNA stability in primary culture of hepatocytes

The stimulatory effect of glucose on GLUT2 mRNA level observed after overnight culture in the presence of 20 mM glucose (Figure 1) could be partly due to the stabilization of GLUT2 mRNA. To test this hypothesis, one of two transcriptional inhibitors, actinomycin-D or α-amanitin (5 µg/ml each), was added after overnight culture in the presence of 20 mM glucose and the culture was continued in the presence or absence of glucose. The half-life of GLUT2 mRNA was similar in the absence and in the presence of glucose (about 10 h). This suggested that the stimulatory effect of glucose on GLUT2 mRNA did not involve a stabilization of GLUT2 mRNA (Figure 1).

The stimulatory effect of glucose on GLUT2 expression depends on continuing protein synthesis in primary culture of hepatocytes

The stimulatory effect of glucose on GLUT2 mRNA concentration could depend on continuing protein synthesis. To test this hypothesis, an inhibitor of protein synthesis, puromycin, was used. Hepatocytes were cultured overnight without glucose to decrease GLUT2 mRNA to a very low level. After this glucose-deprivation period, 20 mM glucose was added to the medium and the culture was continued in the absence (control) or in the presence of puromycin (0.25 mM). The accumulation of GLUT2 mRNA in response to glucose was completely abolished by puromycin (Figure 2). This suggested that continuing protein synthesis was required to mediate the glucose effect on GLUT2 expression.

The stimulatory effect of glucose on GLUT2 gene expression occurs at the transcriptional step

Endogenous GLUT2 gene expression was increased by glucose in a liver GLUT2-expressing cell line mhAT3F (Figure 3), in a similar manner to that found in cultured rat hepatocytes [8]. To investigate whether the effect of glucose on GLUT2 gene expression was transcriptionally regulated, mhAT3F cells were transfected by lipofection with a −338/+49 bp proximal region of the GLUT2 promoter linked to the reporter gene CAT (Figure 3). A 3-fold stimulation of CAT activity was observed in the presence of 17 mM glucose compared with the CAT activity measured in mhAT3F cells cultured in the absence of glucose.

Figure 1 Effect of glucose on GLUT2 mRNA half-life in primary culture of adult rat hepatocytes

Hepatocytes were cultured for 18 h in the presence of 20 mM glucose. Then inhibitors of transcription (α-amanitin or actinomycin D, each at a concentration of 5 µg/ml) were added to the culture medium and the culture was continued for 4–24 h in the absence or presence of 20 mM glucose. (A) Representative Northern blot. (B) The 100% GLUT2 mRNA value was obtained after 18 h of culture in the presence of 20 mM glucose. The GLUT2 mRNA values were normalized by hybridization with 18 S rRNA probe to correct for RNA loading. The values are means ± S.E.M. for four different experiments; no statistical difference was observed between results from the absence or the presence of glucose.
After overnight culture in the absence of glucose (G0), puromycin (0.25 mM) and glucose (20 mM) were added and the culture was continued for 24 h. **(A)** Representative Northern blot. **(B)** Results expressed in fold stimulation over basal level measured at t0 (time of addition of glucose to the medium). The GLUT2 mRNA values were normalized by hybridization with 18S rRNA probe to correct for RNA loading. The results are means ± S.E.M. for three different experiments; after 8, 10 and 24 h of puromycin treatment the GLUT2 mRNA levels were statistically different (P < 0.05) from control values obtained in the absence of puromycin. (Figure 3). This suggested that glucose responsiveness is transcriptionally regulated and is likely to be present within the proximal region of the GLUT2 promoter. This effect is specific for GLUT2 because the CAT activities measured from the promoterless plasmid-CAT, and the positive control with the strong promoter Rous Sarcoma Virus construct RSV-CAT, were not regulated by glucose.

**The metabolism of glucose is required for transcriptional control of GLUT2 gene expression**

Effects of glucose analogues on GLUT2 expression in cultured rat hepatocytes

The effect of glucose on GLUT2 expression could be dependent upon the glucose molecule itself or on one of its intracellular metabolites. It was not due to hyperosmolarity because 20 mM t-glucose, which is unable to enter into the liver cell, did not stimulate GLUT2 expression (Table 1). In addition, 3-O-methylglucose (20 mM), a glucose analogue that enters hepatocytes via the glucose transporter GLUT2 but is not phosphorylated, did not stimulate GLUT2 expression (Table 1). When hepatocytes were cultured in the presence of 30 mM 2-deoxyglucose no accumulation of GLUT2 mRNA was observed. 2-Deoxyglucose has long been considered to enter the cells via the same glucose transporter as glucose, to be phosphorylated but no longer metabolized and accumulated under the form of 2-deoxyglucose 6-phosphate. The very high 2-deoxyglucose concentration used in this study (30 mM) aimed at producing an intracellular accumulation of 2-deoxyglucose 6-phosphate. There was a rapid increase in phosphorylated products after addition of 2-deoxyglucose to the culture medium and their level remained elevated over 22 h (Table 2). As it has been reported previously [28] that 2-deoxyglucose 6-phosphate was not the major product of 2-deoxyglucose metabolism in liver, we have checked, by means of gas chromatography–mass spectrometry, whether the phosphorylated products accumulated in response to 2-deoxyglucose exposure were indeed 2-deoxyglucose 6-phosphate. The quantity of 2-deoxyglucose 6-phosphate in hepatocyte extract was calculated by measuring the ratio of the areas of the two ions to the areas of the authentic 2-deoxyglucose 6-phosphate in a given amount (100 nmol). We found that 66% of the phosphorylated products were 2-deoxyglucose 6-phosphate, i.e.
Stimulation of glucose transporter expression by glucose metabolism

Table 1  Effect of D-glucose, L-glucose, 3-0-methylglucose (3-0-MG), 2-deoxyglucose (2-DOG), DHA and sorbitol on GLUT2 mRNA concentration in cultured hepatocytes from adult rats

After the attachment period, hepatocytes were cultured with glucose analogues and DHA or sorbitol for 24 h. When the cells were cultured in the absence of glucose and in presence of glucose analogues that are not metabolized, a mixture of lactate and pyruvate (10 and 1 mM respectively) was added to the culture medium. Results are expressed in arbitrary units as means ± S.E.M. for three different experiments. *P < 0.05, **P < 0.01, significantly different from control values obtained when hepatocytes were cultured in the absence of glucose.

<table>
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<th>10</th>
<th>20</th>
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<td>1</td>
<td>2.1 ± 0.4*</td>
<td>5.5 ± 1.1**</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.2</td>
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<tr>
<td>L-Glucose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.1 ± 0.2</td>
<td>–</td>
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<tr>
<td>3-0-MG</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.1 ± 0.2</td>
<td>–</td>
</tr>
<tr>
<td>2-DOG</td>
<td>–</td>
<td>2.9 ± 0.2**</td>
<td>3.9 ± 0.1**</td>
<td>–</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>DHA</td>
<td>–</td>
<td>2.9 ± 0.2**</td>
<td>3.9 ± 0.1**</td>
<td>–</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>–</td>
<td>4.4 ± 1.4**</td>
<td>5.3 ± 1.8**</td>
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Table 2  Intracellular phosphorylated product concentrations in hepatocytes cultured for 1, 15 or 22 h in the presence of lactate and pyruvate (10 and 1 mM respectively) and 30 mM 2-deoxyglucose

The results are expressed as nmol of phosphorylated products per mg of protein. Results are means ± S.E.M. for four different experiments. *Statistical significance P < 0.001 when compared with control values obtained when no glucose was added to the culture medium.

<table>
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<tr>
<th>Length of culture (h)</th>
<th>No glucose added</th>
<th>30 mM 2-deoxyglucose</th>
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<tr>
<td>1</td>
<td>4.0 ± 0.3</td>
<td>57 ± 10*</td>
</tr>
<tr>
<td>15</td>
<td>0.050 ± 0.006</td>
<td>27 ± 7*</td>
</tr>
<tr>
<td>22</td>
<td>0.06 ± 0.03</td>
<td>30 ± 9†</td>
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20 nmol 2-deoxyglucose 6-phosphate per mg of protein. Thus the addition of 2-deoxyglucose to the culture medium results in a substantial intracellular accumulation of 2-deoxyglucose 6-phosphate in hepatocytes. None of the glucose analogues was able to mimic the effect of glucose on GLUT2 expression.

Effects of an inhibitor of glucose phosphorylation on GLUT2 expression in cultured rat hepatocytes

When hepatocytes from adult rat were cultured in the presence of 20 mM glucose and 50 mM N-acetylglucosamine the stimulatory effect of glucose on GLUT2 expression was inhibited (Figure 4). At this high concentration, N-acetylglucosamine inhibits both glucokinase and hexokinase activities [29]. This suggested that glucose must be phosphorylated to exert its stimulatory effect on GLUT2 expression.

To test whether the presence of glucokinase, the enzyme phosphorylating glucose in the liver under physiological conditions, was an absolute prerequisite for the stimulatory effect of glucose on GLUT2 expression, we used hepatocytes from 14-day-old rats and mhAT3F cells, which do not express glucokinase [26,30]. A 2–3-fold increase in GLUT2 mRNA concentration was observed in hepatocytes from 14-day-old rats cultured in the presence of glucose (Figure 5). This was 50% lower than the effect observed in hepatocytes from adult rat (Figure 5). The lower stimulation of GLUT2 expression in hepatocytes from 14-day-old newborn compared with adult rats might be explained by the absence of glucokinase in suckling rat hepatocytes and by the lower glucose phosphorylating capacity of hexokinase than glucokinase. In mhAT3F cells a stimulatory effect of glucose on GLUT2 mRNA accumulation was also observed (Figure 3) despite the absence of glucokinase [26] in this cell line. These data suggest that the presence of glucokinase is not a prerequisite for the effect of glucose on GLUT2 gene expression.
The aim of the present study was to investigate some mechanisms involved in the stimulatory effect of glucose on liver GLUT2 expression.

**DISCUSSION**

Glucose stimulates GLUT2 gene transcription without stabilization of its transcript

The accumulation of GLUT2 mRNA in response to glucose was due to an activation of transcription, as shown by transfection experiments in the mhAT3F cell line expressing liver GLUT2. Indeed, in transient transfection studies of a [-338/+49] bp proximal region of the GLUT2 promoter linked to a reporter gene CAT in the mhAT3F cell line, a 4-fold stimulation of the CAT activity is observed in the presence of 17 mM glucose. This suggests that the regulatory cis elements conferring glucose responsiveness must be present within this region of the GLUT2 promoter. A 5-fold stimulation of CAT activity in response to glucose has also been reported when [-1311] bp GLUT2 promoter/CAT constructs were transfected into a highly differentiated pancreatic β cell line (INS1 cells) [31]. This region does not possess the classical glucose responsive elements such as ChoRE, capable of binding the major late transcription factor or upstream stimulating factor described for other genes [32]. This suggests that unidentified DNA binding sequences are required for mediating the glucose effect on GLUT2 gene transcription. Glucose had no effect on the half-life of GLUT2 mRNA (about 10 h) in the presence of transcription inhibitors. Nevertheless the stimulatory effect of glucose on GLUT2 expression requires continuing protein synthesis. A putative transcription factor capable of binding to a ‘glucose-responsive element’ could be synthesized during glucose stimulation.

As recently reported [33], a fragment of [338] bp of the promoter seemed to be sufficient to confer islet- and liver-cell-specific expression in transgenic mice. Therefore the [338] bp promoter region contains sufficient information to drive reporter gene expression in a liver transformed cell line and in the liver of transgenic mice. Within this region several cis elements and trans-acting factors have been identified that may play a role in this glucose responsiveness [34].

Glucose stimulates GLUT2 expression after being metabolized in the glycolytic pathway

Glucose must be metabolized to stimulate liver GLUT2 expression. Indeed, no effect was observed when glucose was replaced by analogues that do not enter the cell or that are not phosphorylated or that are accumulated as hexose 6-phosphate. When glucose phosphorylation is inhibited by N-acetylglucosamine, the effect of glucose on GLUT2 mRNA is completely abolished. Furthermore an effect of glucose is observed in cells devoid of glucokinase such as hepatocytes from 14-day-old rat or mhAT3F cells. This shows that the presence of a high capacity for glucose phosphorylation is not required for GLUT2 expression because the glucose phosphorylation rate is 20 times higher in hepatocytes from adult animals than in hepatocytes from newborns [29]. The regulation of GLUT2 gene expression by glucose is different from the regulation of L-PK, which required the presence of a high-β hexokinase (glucokinase) to activate the L-PK promoter maximally [35]. The absence of effect of 2-deoxyglucose on GLUT2 expression suggests that glucose 6-phosphate is not the metabolite involved in the stimulation of GLUT2 gene transcription in response to glucose. This is in contrast with previous studies on pieces of white adipose tissue in which 2-deoxyglucose was as efficient as glucose in inducing FAS mRNA accumulation and in which there was a good correlation between intracellular glucose 6-phosphate concentration and FAS mRNA levels [20]. In addition, fructose [7], sorbitol and DHA stimulate GLUT2 expression in cultured hepatocytes. Although these substrates are capable of increasing

Effects of dihydroxyacetone and sorbitol on GLUT2 expression in cultured rat hepatocytes

To determine whether other substrates that enter the glycolytic pathway at an earlier stage could exert a stimulatory effect on GLUT2 expression, dihydroxyacetone (DHA) and sorbitol were tested. DHA is phosphorylated by triose kinase to form dihydroxyacetone phosphate, as which it enters the glycolysis pathway. A 4-fold increase in GLUT2 mRNA was observed in hepatocytes cultured in the presence of 10 mM DHA (Table 1). Sorbitol is first converted into fructose by sorbitol dehydrogenase before entering into the glycolytic pathway at the level of fructose 6-phosphate. A 5-fold increase in GLUT2 mRNA concentration was observed in hepatocytes cultured in the presence of 20 mM sorbitol (Table 1). When 10 mM lactate and 1 mM pyruvate were provided as substrates for the tricarboxylic cycle, no increase in GLUT2 mRNA was observed.

Figure 5 Effect of glucose on GLUT2 mRNA level in cultured hepatocytes from 14-day-old newborn and adult rats

Glucose was added to the culture medium after the attachment period and the culture was continued for 24 h. (A) Representative Northern blot; Adh, cells harvested after attachment period; other abbreviations as in Figure 4. (B) Results expressed as fold increase of GLUT2 mRNA concentration by comparison with the level observed in hepatocytes cultured in the absence of glucose. GLUT2 mRNA values are normalized by hybridization with 18 S rRNA probe to correct for RNA loading. Means ± S.E.M. for three different experiments. **Significantly different (P < 0.01) from control values obtained without glucose in the culture medium.

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glucose 6-phosphate levels, they could also generate a metabolite in the lower part of the glycolytic pathway or in other pathways such as the polyol or pentose phosphate pathways, which could be important for stimulation of GLUT2 expression.

In conclusion, we have shown here that the stimulation by glucose of GLUT2 expression in cultured hepatocytes is due to an activation of gene transcription and is dependent upon an active glucose metabolism. The metabolite(s) responsible for this effect have not been identified but are located after glucose 6-phosphate formation.

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