In most hepatoma cells, the high-\(K_m\) GLUT2/glucokinase proteins are replaced by the ubiquitous low-\(K_m\) GLUT1/hexokinase type I proteins. In the mhAT3F hepatoma cells, the stimulatory effect of glucose on gene expression and glycogen accumulation was not maximal at 5 mmol/liter glucose. This response to high glucose is observed in mhAT3F cells, where GLUT2 was expressed, but not glucokinase (assessed by Northern blotting and reverse transcription-polymerase chain reaction). A low-\(K_m\) hexokinase activity (19.6 ± 3.8 milliunits/mg of protein) was present, but a high-\(K_m\) (40 mmol/liter) hexokinase activity (13.9 ± 2.5 milliunits/mg) was also detected in mhAT3F cells. The high-\(K_m\) hexokinase activity was dependent on both ATP (or PP\(\_\)i) and glucose in the assay and was recovered in a 10–50-kDa fraction after filtration. A 30-kDa protein was detected using an anti-glucokinase antibody and localized by confocal microscopy at the same sites as glucokinase in hepatocytes. In FAO cells, the high-\(K_m\) hexokinase activity and 30-kDa protein were not found. We conclude that a high-\(K_m\) hexokinase activity is present in mhAT3F cells. This might explain why the effects of glucose on gene expression were not maximal at a glucose concentration of 5 mmol/liter. A 30-kDa protein identified using an anti-glucokinase antibody may be responsible for this activity present in mhAT3F cells.

Glucose regulates the expression of several genes, including liver pyruvate kinase (1), fatty-acid synthase (2), insulin (3), transforming growth factor-\(\alpha\) (4) glucose-6-phosphatase (5), and the facilitative glucose transporter type 2 (GLUT2) (6, 7). Glucose must be metabolized to stimulate the transcription of these different genes (8, 9). In the context of liver genes, the effect of glucose is potentiated by all stimuli able to favor glucose metabolism. For most genes, insulin, by increasing glucokinase gene expression, stimulates glucose phosphorylation and metabolism and potentiates the effect of glucose on liver pyruvate kinase, fatty-acid synthase, and glucose-6-phosphatase gene transcription (5, 10, 11). For the Glut2 gene, the stimulatory effect of glucose is not potentiated by insulin. Indeed, liver Glut2 gene expression is inhibited by insulin both in vivo (7, 12) and in cultured rat hepatocytes (7). Moreover, the presence of glucokinase does not seem to be required for glucose stimulation of Glut2 gene transcription since the Glut2 gene is stimulated in primary cultures of newborn rat hepatocytes that lack glucokinase (13).

In most hepatoma cells, the effect of glucose on gene expression is lost (14). This could be attributed to a partial dedifferentiation of hepatoma cells since the liver-specific proteins glucokinase and GLUT2 are replaced by the ubiquitous proteins hexokinase type I and GLUT1. By contrast, GLUT2 is still expressed in the mhAT3F cell line, whereas the phosphorylation of glucose is mediated by hexokinase type I and not by glucokinase (15, 16). Despite the absence of glucokinase, the maximal effect of glucose on gene expression is reached at glucose concentrations >10 mmol/liter (17). This was surprising because hexokinase type I is inhibited by glucose 6-phosphate, and its maximal activity is reached at low glucose concentrations (1–2 mmol/liter) (18). In addition, GLUT2 is not a rate-limiting step for liver glucose metabolism. This suggested that in mhAT3F cells, the phosphorylation of glucose was mediated by a high-\(K_m\) hexokinase. The aim of this work was to elucidate the mechanisms displayed in the mhAT3F cell line to stimulate gene transcription in response to glucose concentrations exceeding the \(K_m\) of hexokinase type I.

**MATERIALS AND METHODS**

*Primary Culture of Hepatocytes—*Female Wistar rats (200–220 g) housed at 24 °C with light from 07:00 to 19:00 h were used. Hepatocytes were isolated by the method of Berry and Friend (19) from rats in the post-absorptive period, i.e. at 09:00 h. The dissociation of the cells was performed in Hepes buffer (137 mmol/liter NaCl, 2.7 mmol/liter KCl, 0.7 mmol/liter Na\(_3\)HPO\(_4\), and 10 mmol/liter Hepes (pH 7.5) at 37 °C containing 0.1% collagenase (1.36 units/mg; Boehringer Mannheim, Meylan, France) and 5 mmol/liter CaCl\(_2\). For each hepatocyte preparation, the cell viability estimated by trypan blue exclusion was >90%.

Hepatocytes were suspended in medium 199 containing Earle’s salts and 2.2 mg/liter NaHCO\(_3\) (Life Technologies, Inc., Cergy Pontoise, France) with penicillin (10 units/ml), streptomycin (100 mg/ml), and kanamycin (50 mg/ml) and supplemented with fetal calf serum (5%, v/v; Jacques Boy, Reims, France). Hepatocytes were plated on 100-mm plastic dishes (8–10 × 10\(^5\) cells/dish). For attachment, cells were cultured for 4 h in medium 199 supplemented with 5% (v/v) fetal calf serum, 1 mmol/liter insulin (Actrapid, Novo, Copenhagen, Denmark), 100 mmol/liter dexamethasone (Soludecadron, Merck & Co., Riom, France), 2.2 mmol/liter glutamine (Life Technologies, Inc.), and 0.05% (v/v) serum albumin. The medium was then replaced by fresh medium 199 containing only 5% (v/v) fetal calf serum. We used 5% fetal calf serum in the culture medium since, in preliminary experiments, we showed that fetal calf serum allowed the maintenance, for at least 48 h, of the Glut2 mRNA concentration at a level comparable to the one observed in liver before cell dissociation (data not shown).

When the cultures were performed in the absence of glucose, 10 mmol/liter lactate and 1 mmol/liter pyruvate were added as oxidative substrates. Nevertheless, when the cells were cultured in the absence of glucose in the medium, hepatocytes produced glucose from lactate, pyruvate, and amino acids present in the culture medium, and the glucose concentration in the culture medium was 1.2 mmol/liter after 24 h of culture.

*Hepatoma Cell Culture—*The mhAT3F hepatocyte cell line was...
rived from the tumorous livers of transgenic mouse expressing simian virus 40 early genes under the control of the liver-specific antithrombin III promoter (15). Cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 Glutamax medium (Life Technologies, Inc.) supplemented with 100 mmol/liter insulin, 1 mmol/liter dexamethasone, 1 mmol/liter sodium pyruvate (Sigma, St Quentin Fallavier, France), and 10% (v/v) fetal calf serum. To study the glycogen content, cells were cultured overnight without glucose to deplete the glycogen stores, and then glucose was added in medium as required.

**Cellular Extracts**—The medium was removed after 24 h of culture for the hepatocytes or after confluency for the mhAT3F cells; the cells were then washed two times in ice-cold 0.9% NaCl. Cells were scrapped into 1 ml of homogenization buffer containing 0.15 mol/liter KCl, 10 mmol/liter Tris, 1 mmol/liter EDTA, 0.5 mmol/liter NADP+, and 2 mmol/liter β-mercaptoethanol (pH 8) at 4 °C. The cell suspension was homogenized for 2 min in a Potter homogenizer surrounded by ice. The cell homogenate was centrifuged at 1000 × g for 1 h at 4 °C. Western blotting was performed with 100 μg of protein from the supernatants.

The cellular extracts prepared for hexokinase activity determination followed the same protocol, except that the buffer contained 100 mmol/liter KCl, 25 mmol/liter Hepes, 7.5 mmol/liter MgCl₂, and 4 mmol/liter dithiothreitol (pH 7.4). Homogenates were vortexed and centrifuged at 10000 × g for 1 h at 4 °C.

**Hexokinase Activity**—Hexokinase activity was measured in 100 μl of cellular extracts prepared as described above, in the scraping buffer supplemented with 1 mmol/liter NAD, 4 mmol/liter ATP, and 100 or 0.5 mmol/liter glucose at pH 7.4. The reaction was started by the addition of 5 micromolar of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim), and the activity was followed at 340 nm for 15–20 min at 30 °C. One unit of glucose-6-phosphate dehydrogenase activity represents the production of 1 μmol of NADH/min at 30 °C. Enzyme activity is expressed as milliunits/mg of protein. The activity of high-Kₘ hexokinase was calculated as the difference between activities calculated at 100 and 0.5 mmol/liter glucose (20). The Kₘ value for glucose was determined by measuring the hexokinase activities as a function of added glucose (12.5, 25, 50, and 100 mmol/liter), and the results were drawn according to a Lineweaver-Burk double reciprocal plot. The phosphoryl donor was determined at pH 7.4 by measuring the activity of high-Kₘ hexokinase in the presence of 4 mmol/liter ATP, ADP, GTP, or PP₃.

**Hexokinase Activity in Cellular Extracts Containing Proteins with a Molecular Mass between 50 and 10 kDa**—Cells were washed twice in ice-cold PBS and frozen in liquid nitrogen. Cells were thawed and scrapped in homogenization buffer at room temperature. Cells were then washed two times in ice-cold 0.9% NaCl. Cells were then added into 1 ml of homogenization buffer containing 0.15 mol/liter KCl, 10 mmol/liter Tris, 1 mmol/liter EDTA, 0.5 mmol/liter NADP+, and 2 mmol/liter β-mercaptoethanol (pH 8) at 4 °C. The cell suspension was homogenized for 2 min in a Potter homogenizer surrounded by ice. The cell homogenate was centrifuged at 1000 × g for 1 h at 4 °C. Western blotting was performed with 100 μg of protein from the supernatants.

**Western Blots**—Ten μg of protein extracts prepared as described above was separated by Western blotting using SDS-PAGE (14% acrylamide). After an overnight incubation in 5% (v/v) albumin and Tris-buffered saline (TBS), the blot was washed for 20 min in TBS and 0.1% (v/v) Triton X-100. A polyclonal antibody raised against a recombinant glutathione S-transferase-glucokinase fusion protein produced in sheep was a kind gift from Dr. M. A. Magnuson (21). The blot was incubated for 90 min at room temperature with the antibody diluted 1:1000 (v/v) in TBS, 0.05% (v/v) Triton X-100, 0.01% (v/v) Tween 20, and 3% (v/v) albumin. Then the blot was washed four times in TBS and 0.1% Tween and incubated for 60 min with the secondary antibody (an anti-sheep antibody) coupled to horseradish peroxidase (Pierce). This secondary antibody was diluted 1:20000 (v/v) in TBS, 0.1% Tween, and 5% (v/v) nonfat dry milk. The final blot was washed in TBS containing 0.1% (v/v) Tween 20. The peroxidase activity was revealed with the Amersham Pharmacia Biotech detection system as described by the manufacturer. Blots were exposed for 5 min (Hyperfilm, Amersham Pharmacia Biotech, Les Ulis, France). This antibody can detect mouse and rat glucokinases as well.

The anti-hexokinase type III antibody was a kind gift from Dr. J. Wilson (Michigan State University, East Lansing, MI). It was raised against a purified enzyme from rat Novikoff hepatoma (22). Ten μg of total protein extracts prepared as described above for hexokinase activities was separated by SDS-PAGE (10% acrylamide) and transferred to a membrane. After an overnight incubation with 5% (w/v) albumin and TBS, the blot was washed for 20 min in TBS and 0.1% (v/v) Triton X-100. The blot was incubated for 3 h at room temperature with the antibody against hexokinase type III diluted 1:200 in PBS, 0.1 mmol/liter EDTA, 0.5% (v/v) Triton X-100, and 1% (w/v) albumin. The washing and the detection procedures were identical to those described above, except that an anti-rat antibody was used as the secondary antibody.

**RT-PCR**—Poly(A)+ RNAs extracted from liver and mhAT3F and FAO hepatoma cells were reverse-transcribed and amplified with appropriate controls (23). Using 0.5 μg of mRNA as template, primer extension was performed with 3 units of *Thermus thermophilus* DNA polymerase (Promega) from 15 pmol of the 5′-primers in the N terminus and catalytic site of glucokinase and in hexokinase type I. The reaction proceeded for 10 min at 25 °C, for 2 min at 57 °C, and for 20 min at 70 °C and then was stopped by 5 min at 99 °C. The amplifications were performed with 2.5 μl of reverse-transcribed RNA and with 15 pmol of the 5′-primers of the domains to amplify. For each set of primers, controls without RNA and without T. *Thermophilus* DNA polymerase were performed.

Specific primer sequences were in the N terminus of liver glucoki- nase (5′-GAGCCCGATGTGGACTTCTG and 5′-TGTTCTTACGCCCTCACGTG), yielding an amplified fragment of 285 bp. Another set of primers selected in the catalytic region of glucokinase (5′-GATCA-GATGAAACACAGAAA and 5′-AGAGGGGCTTGGATGGGAT) gave a fragment of 190 bp (GenBank™ accession number M52807). The amplified products were separated in a 3% agarose gel containing 5 μl of the 5′-primers of the domains to amplify. For each set of primers, controls without RNA and without T. *Thermophilus* DNA polymerase were performed.

**Glycogen Content**—Glycogen content was measured in confluent cells on 60-mm dishes after 24 h of culture under different experimental conditions. Cells were washed twice with ice-cold 0.9% NaCl and scrapped into 0.2 mol/liter sodium acetate (pH 4.5). The cell homogenates were sonicated for 1 min (1 pulse/s). Cell homogenates (100 μl) were filtered for 1 h at 30000 g (Amicon) with a molecular mass cutoff at 10 kDa by centrifugation at 20000 × g for 1 h at 4 °C to concentrate the preliminary filtrate. The hexokinase assay was performed on 100 μl. To verify the molecular mass of the proteins in the sample, proteins were separated by 14% SDS-PAGE, and the blots were stained with Ponceau red.

**Glut2 mRNA Concentration**—Total RNA was isolated by the method of Chomczynski and Sacchi (24). Northern blotting and hybridization were performed with 20 μg of total RNA as described previously (7). The *Glut2* cDNA probe was kindly supplied by Dr. B. Thorens (25). The glucokinase cDNA probe was provided by Dr. M. A. Magnuson (26). The poly(A)+ RNAs were obtained using the mRNA purification kit from Amersham Pharmacia Biotech.

**Immunolocalization**—Hepatocytes and mhAT3F and FAO cells were grown on Permanox four-chamber slides (Lab-Tek, Nunc) overnight in the presence of 20 mmol/liter glucose as described above. The glucoki- nase was detected using the polyclonal antibody raised against a recombinant glutathione S-transferase-glucokinase fusion protein produced in sheep. Cells were washed three times in PBS and permeabilized for 4 min in PBS containing 0.1% (p/v) Triton X-100. Cells were incubated for 40 min in diluted antibody (1:2000 dilution in PBS and 0.2% gelatin) at room temperature, washed three times in PBS containing 0.5% (v/v) Tween 20, and incubated for 1 h with fluorescein isothiocyanate–conjugated rabbit anti-sheep IgG (1:128 dilution; Sigma). The slides were mounted in glycerol/PBS mounting medium (Cytifluor), and confocal laser scanning microscopy was performed using a Leica confocal imaging system (TCS-4D) and an immersion lens (63×, numerical aperture 1.4 plan Apochromat). Micrographs were printed directly from the computer on a dye sublimation printer (Colorace, Eastman Kodak Co.).

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1 The abbreviations used are: PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s).
Unusual High-$K_m$ Hexokinase

RESULTS

Glucose Stimulates Glut2 mRNA and Glycogen Accumulation in mhAT3F Cells in a Dose-dependent Manner—When mhAT3F cells were cultured for 24 h in the presence of increasing glucose concentrations (0, 5, and 17 mmol/liter), an accumulation of Glut2 mRNA was observed (Fig. 1). Furthermore, glycogen accumulated in mhAT3F cells with a dose-response curve similar to the one observed for Glut2 mRNA (Fig. 1).

When mhAT3F cells were cultured in the absence of glucose, the concentration of glycogen left in the medium after 24 h was 0.03 ± 0.01 mmol/liter. When mhAT3F cells were cultured in the presence of an initial concentration of glucose of 17 mmol/liter, the concentration of glucose left in the medium after 24 h was 8.65 ± 0.1 mmol/liter.

Hexokinase Activities—The maximal level of Glut2 mRNA in mhAT3F cells was reached at glucose concentrations higher than 5 mmol/liter, suggesting the presence of a high-$K_m$ hexokinase. To determine the type of hexokinase present in mhAT3F cells, we performed assays of hexokinase activity at two glucose concentrations: 0.5 and 100 mmol/liter. Two hepatoma cell types were used: mhAT3F, in which a stimulation of Glut2 mRNA was observed (Fig. 1); and FAO, in which no response to glucose was detected (Table I).

When mhAT3F cells were cultured in the absence of glucose, the enzyme was found to utilize PPi much more efficiently than it does ATP (Table I). Other nucleotides like GTP and ADP did not serve as substrates (Table I).

Unexpectedly, we detected a high-$K_m$ hexokinase activity in mhAT3F hepatoma cells (activity measured at 100 mmol/liter glucose minus activity at 0.5 mmol/liter glucose). The high-$K_m$ hexokinase activity measured in mhAT3F cells was similar to the one attributed to glucokinase in rat hepatocytes (14.4 ± 3 versus 15.6 ± 0.3 milliunits/mg of protein) (Fig. 2A).

This hexokinase showed a $K_m$ for glucose of ~40 mmol/liter (Fig. 2B). It catalyzed the phosphorylation of glucose using either PPi or ATP as phosphoryl donor (Table I). Other nucleotides like GTP and ADP did not serve as substrates (Table I). The enzyme was found to utilize PPi much more efficiently than it does ATP (Table I).

RT-PCR—To determine if glucokinase was expressed in mhAT3F cells at a level that was not detected by conventional Northern blotting (Fig. 3A), we performed a RT-PCR from poly(A)$^+$ RNA (Fig. 3B). We used isomorf-specific primers hybridizing with specific regions coding for the amino-terminal and catalytic domains of liver glucokinase and not present in other hexokinase mRNAs. We used mRNA from mhAT3F (upper panel), rat liver (middle panel), and FAO hepatoma (lower panel) cells, as presented in Fig. 3B. As shown in Fig. 3B, with RNA extracted from livers, we detected a 285-bp band corre-
Glucokinase

Glut2

**FIG. 3.** Detection of liver glucokinase transcripts by Northern blotting (A) and by RT-PCR (B) in mhAT3F cells. A, for Northern blotting, RNAs from fed rat liver and mhAT3F cells were hybridized with glucokinase (upper panel) and Glut2 (lower panel) cDNAs, respectively. B, poly(A)⁺ RNAs were extracted from fed rat liver (middle panel), mhAT3F cells (upper panel), and FAO cells (lower panel), cultured for 24 h in the presence of 17 mmol/liter glucose. Reverse transcriptions were performed as described under “Materials and Methods.” Reverse-transcribed RNA was amplified using primers specific for rat liver glucokinase, hexokinase type I, and *T. thermophilus* polymerase. The molecular size markers used were the kilobase ladder from Life Technologies, Inc. in lanes 1, 16, and 17 and the DNA VI from Boehringer Mannheim in lanes 8, 9, and 22. The controls without *T. thermophilus* polymerase with the appropriate primers are in lanes 5–7, 13–15, 20, and 21 (controls without RNA are not shown). Primers corresponding to a fragment of rat liver glucokinase mRNA used in lanes 4, 7, 12, and 15 yielded a fragment of 285 bp only in rat liver mRNA. Primers corresponding to a fragment of the catalytic domain of glucokinase used in lanes 2, 5, 10, 13, 18, and 20 yielded a fragment of 190 bp only in rat liver mRNA. Primers corresponding to hexokinase type I used in lanes 3, 6, 11, 14, 19, and 21 gave a fragment of 340 bp in mhAT3F and FAO mRNAs.

Responding to N-terminal domain and a 190-bp band to the catalytic domain of liver glucokinase. We did not detect these bands with RNA extracted from mhAT3F cells. The catalytic domain of glucokinase was not present in FAO mRNA. We did detect a 340-bp band specific for hexokinase type I with RNA extracted from mhAT3F and FAO cells. This demonstrated that glucokinase was not expressed in mhAT3F cells (Fig. 3).

**Hexokinase Proteins**—Using an anti-hexokinase type III antibody, we found a large signal located at ~100 kDa in lung extracts used as a positive control (Fig. 4). The hexokinase type III protein was not detected in mhAT3F cells and hepatocytes (Fig. 4).

Using an antibody against glucokinase, we observed an immunoreactive band at 50 kDa in extracts from rat hepatocytes (Fig. 4) and mouse liver (data not shown). We did not obtain a 50-kDa signal with extracts from mhAT3F and FAO cells, suggesting that glucokinase was not present in these cell lines (Fig. 4). Nonetheless, a band around 30 kDa was detected with the polyclonal anti-glucokinase antibody in cellular extracts from mhAT3F cells. This band was not found in rat hepatocytes. Such a band was not observed in extracts from FAO cells, suggesting that this protein was specific for the mhAT3F cell line.

**Hexokinase Activity in 10–50-kDa Proteins from mhAT3F Extracts**—To determine whether the 30-kDa protein recognized by the anti-glucokinase antibody in mhAT3F cells possessed a glucose phosphorylation activity, we measured the activity of hexokinase in fractions of protein selected by their molecular masses. To eliminate proteins with a molecular mass higher than 50 kDa, cellular extracts were filtered. We performed hexokinase assays on filtered extracts in the presence of 0.5 and 100 mmol/liter glucose (Fig. 5). A low-$K_m$ hexokinase activity was detected in the cellular extract; however, this activity was no longer found in the proteins under 50 kDa. A high-$K_m$ hexokinase activity, reminiscent of a glucokinase activity, was measured both in the cellular extract and in the 10–50-kDa mhAT3F proteins.

We verified that this activity could be attributed to a hexokinase by performing the assay under the following conditions: 1) in the absence of ATP and in the presence of 100 mmol/liter glucose (0.3 ± 0.02 milliunits/mg of protein) and 2) in the presence of ATP and in the absence of glucose (1.5 ± 0.7 milliunits/mg of protein). Under these conditions, we detected very low hexokinase activities, suggesting that the enzyme required both ATP and glucose as substrates. We did not measure any hexokinase activity in the presence of glucose-6-phosphate dehydrogenase alone, without cellular extracts, or in the presence of fructose (data not shown). Thus, the activity measured in the presence of 100 mmol/liter glucose was in accordance with the activity measured in crude extracts (14.6 ± 3.8 versus 13.9 ± 2.5 milliunits/mg of protein). This enzyme activity could explain why the transcriptional effect of glucose on Glut2 was not maximal at glucose concentrations lower than 5 mmol/liter.

**Immunolocalization of the Protein with an Antibody against Glucokinase**—The cellular localization of the 30-kDa protein in mhAT3F cells was determined by immunofluorescence using the antibody against glucokinase previously used for the Western blot experiments (Fig. 4). Images were analyzed by confocal laser scanning microscopy. As expected, glucokinase was mainly localized in the vicinity of the plasma membrane in cultured rat hepatocytes; glucokinase was also detected in the nucleus in other hepatocytes (Fig. 6). Glucokinase was not detected in FAO hepatoma cells (Fig. 6). On the contrary, the mhAT3F hepatoma cells did show a positive signal in the cytoplasm, near the plasma membrane, in some cells and in the nucleus in other cells (Fig. 6). This confirmed that the 30-kDa protein detected by the anti-glucokinase antibody was specific...
for mhAT3F cells. An identical localization was observed in hepatocytes and mhAT3F cells.

**DISCUSSION**

In the liver, glucose phosphorylation is the limiting step for glucose metabolism since the glucokinase activity is 100-fold lower than the glucose transport capacity mediated by GLUT2 (6, 27). In mhAT3F cells, which express GLUT2 but not glucokinase (16), Glut2 mRNA levels depended on glucose concentrations, as previously reported in hepatocytes (6, 7, 13). Thus, the presence of GLUT2 could be necessary for the stimulation of gene transcription in response to glucose. This is in agreement with a recent study demonstrating that pancreatic beta cells from Glut2 null mice had lost their capacity for stimulating insulin gene transcription in response to glucose (28). Nevertheless, the existence of a high-$K_m$ hexokinase in mhAT3F cells was hypothesized to explain the transcriptional response of GLUT2 to glucose concentrations higher than 5 mmol/liter and was tested.

An additional argument favoring the presence of the high-$K_m$ hexokinase in mhAT3F cells was the dose-dependent accumulation of glycogen in response to glucose. In hepatocytes that overexpressed hexokinase type I after adenovirus infection, glycogen synthesis was unrelated to glucose concentration in the culture medium (29). By contrast, in hepatocytes that overexpressed glucokinase after adenovirus infection, glycogen synthesis and lactate production were related to glucose concentrations in the medium (29). These differences could be attributed to the cellular localization of these two hexokinases. A number of studies have shown that liver glucokinase is translocated from a bound to a free compartment in response to glucose, fructose, and sorbitol (30–32). The translocation occurs in response to metabolic signals that cause dissociation of glucokinase from its regulatory protein (33). The synthesis of glycogen is very sensitive to a small increase in glucokinase activity and correlates more closely with the free glucokinase activity (34). This suggested that mhAT3F cell expressed a high-$K_m$ hexokinase that shared some common characteristics with liver glucokinase. Moreover, a $K_m$ for glucose of 40 mmol/
Unusual High-$K_m$ Hexokinase

Hepatocytes    

FAO    

Fig. 6. Immunolocalization of glucokinase in hepatocytes and glucokinase-like protein in FAO and mhAT3F cell lines. The 30-kDa protein in mhAT3F cells was localized by immunofluorescence using the anti-glucokinase antibody previously used for the Western blot experiments. In rat hepatocytes cultured in the presence of 20 mmol/liter glucose, glucokinase was mainly localized in the vicinity of the plasma membrane or in the nucleus of cells. The glucokinase-like protein was localized in the cytoplasm or in the nucleus of mhAT3F hepatoma cells. In FAO hepatoma cells, no glucokinase was detected. Images were obtained by confocal microscopy using a Leica confocal imaging system (TCS-4D) and an immersion lens (63×, numerical aperture 1.4 plan Apochromat). Micrographs were printed from the computer on the Colorease dye sublimation printer.

The high-$K_m$ hexokinase activity was found in mhAT3F cells, but not in FAO cells, which is another hepatoma cell line. Using Northern blotting and RT-PCR, we showed that liver glucokinase was not expressed in mhAT3F and FAO cells. Thus, glucokinase was not responsible for the high-$K_m$ hexokinase activity detected in mhAT3F cells. Two other enzymes could possibly be responsible for glucose phosphorylation. The presence of a high-$K_m$ glucose phosphorylation was reported previously in extrahepatic tissue (36). It was attributed to N-acetylglucosamine kinase, an enzyme that catalyzes the phosphorylation of glucose at high glucose concentrations ($K_m = 370$ mM) (36). This enzyme has a molecular mass of 55 kDa, close to the 50 kDa of glucokinase (36). In diabetic rats, the activity of liver glucokinase decreased, whereas the liver N-acetylglucosamine kinase activity remained steady (37). To rule out the possibility that the activity we measured in mhAT3F cells was N-acetylglucosamine kinase, filtration experiments were performed. After elimination of proteins with molecular masses higher than 50 kDa and lower than 10 kDa, we still detected a glucose phosphorylation activity that was thus not related to N-acetylglucosamine kinase. We also observed a lower glucose phosphorylating activity in cells cultured in the presence of 17 mM glucose than in cells cultured in absence of glucose. Thus, the high-$K_m$ hexokinase activity observed in mhAT3F cells cannot be attributed to N-acetylglucosamine kinase because the activity of this enzyme was not expected to be altered under these experimental conditions. The high-$K_m$ hexokinase activity observed in mhAT3F cells could also be attributed to the phosphotransferase activity of the glucose-6-phosphatase system (38). This seemed very unlikely because the glucose-6-phosphatase is located in the microsomes, whereas the high-$K_m$ activity was recovered in a soluble fraction after filtration. Furthermore, the enzyme activity was measured at pH 7.4, a pH at which the phosphotransferase was unable to use ATP as a phosphoryl donor (39).

The high-$K_m$ hexokinase activity was recovered in the proteins with molecular masses ranging from 50 to 10 kDa in mhAT3F extracts. In liver extracts, a major band at 50 kDa and a minor band at 30 kDa (considered as a degradation product of glucokinase) were detected with an anti-glucokinase antibody (40). This degradation product was not detected here in rat hepatocytes, but a 30-kDa band was present in mhAT3F cells. We were unable to amplify by PCR any N-terminal glucokinase sequences in mhAT3F cells, contrary to what was observed in rat liver. Thus, the presence of an N-terminal truncated glucokinase protein is unlikely. This band at 30 kDa cannot be attributed to the murine origin of mhAT3F cells since the antibody against rat glucokinase readily detects mouse liver glucokinase. Moreover, rat liver glucokinase cDNA can hybridize on Northern blotting with mouse glucokinase mRNA (data not shown). The ATP- and glucose-binding sites of glucokinase are localized within 272 amino acids (41), and these domains are likely to be epitopes that can be recognized by an antibody against purified recombinant glucokinase. Moreover, in mhAT3F cells, this protein was localized in the cytoplasm with accumulations in the vicinity of the plasma membrane in some cells and in the nucleus of other cells. These images were very similar to those observed in hepatocytes.

Taken together, these experiments suggest that a new protein whose function is to phosphorylate glucose at a $K_m$ for glucose of $\sim 40$ mmol/liter in an ATP-dependent manner is present in mhAT3F cells. This protein has a molecular mass of 30 kDa, shares epitopes with glucokinase, and displays a similar cellular localization in hepatocytes and mhAT3F cells.

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