Glucokinase and Cytosolic Phospho*enol*pyruvate Carboxykinase (GTP) in the Human Liver

Regulation of Gene Expression in Cultured Hepatocytes

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Abstract

Glucokinase and phospho*enol*pyruvate carboxykinase are key enzymes of glucose metabolism in the rat liver. The former is considered to be instrumental in regulating glucose hepatic release/uptake according to the glycaemia level, and cytosolic phospho*enol*pyruvate carboxykinase is a major flux-generating enzyme for gluconeogenesis. The level of expression of both enzymes and the regulation of their mRNAs in the human liver cell were investigated. Surgical biopsies of liver from patients undergoing partial hepatectomies and parenchymal hepatocytes derived from the biopsies were used to assay glucokinase, hexokinase and phospho*enol*pyruvate carboxykinase activities. Hepatocytes were placed in culture and the actions of insulin, glucagon and cAMP on glucokinase and phospho*enol*pyruvate carboxykinase mRNAs were studied. The main results are: (*a*) glucokinase accounts for 95% of the glucose phosphorylation activity of human hepatocytes, although this fact is masked in assays of total liver tissue; (*b*) glucokinase activity is set at a lower level in human hepatocytes than in rat hepatocytes, and vice-versa for the gluconeogenic enzyme phospho*enol*pyruvate carboxykinase; and (*c*) as previously shown in rat liver, glucokinase and phospho*enol*pyruvate carboxykinase mRNAs are regulated in a reciprocal fashion in human hepatocytes, insulin inducing the first enzyme and repressing the latter, whereas glucagon has opposite effects. These data have interesting implications with respect to metabolic regulation and intracellular hormone signaling in the human liver. (*J. Clin. Invest.* 1995. 95:1966–1973.) Key words: glucokinase • liver • hepatocyte • insulin • phospho*enol*pyruvate carboxykinase

Introduction

The mammalian enzyme glucokinase, a member of the hexokinase family, is one of the regulatory enzymes of carbohydrate metabolism and is considered to play a crucial role in blood glucose homeostasis. Although the ATP-dependent conversion of glucose into glucose 6-phosphate is a ubiquitous metabolic step common to all cells of the body, the vast majority of cellular types in the organism catalyze this reaction via hexokinases type I to III and do not express glucokinase (hexokinase IV) (1). In the rat, glucokinase is limited in its tissue distribution to a very few cell types, which are known or presumed to occupy a central position in the regulation of blood glucose: (*a*) parenchymal cells of the liver (2); (*b*) β cells of the pancreatic islets of Langerhans (2); and (*c*) a small number of glucokinase expressing cells recently identified in the central nervous system and the gastrointestinal tract (3). The particular kinetic property of glucokinase with respect to glucose (half-saturation around 6 mM), together with abundant expression of a facilitative glucose transporter in the plasma membrane of the hepatocyte and the endocrine β cell, allow these cells to adjust their rate of glucose phosphorylation in function of the prevailing plasma glucose concentration. A variable rate of glucose phosphorylation by glucokinase in response to fluctuations of the glycaemia appears to be instrumental for the ability to regulate the net hepatic uptake/output of glucose, and the pancreatic rate of insulin secretion, two processes which in turn are major determinants of the blood glucose level (4). The recent identification of mutations of the human glucokinase gene as a probable cause for a subtype of non–insulin-dependent diabetes mellitus places further emphasis on this enzyme as a key regulator of the circulating glucose concentration (5–7).

Much of our knowledge about glucokinase comes from studies in the rat. Little is known, however, with respect to levels of activity, tissue distribution and possible regulation of glucokinase in human tissues. The presence of glucokinase mRNA in human liver and islets of Langerhans has been demonstrated by Northern blotting as well as reverse transcription/polymerase chain reaction (8). Only limited and sometimes conflicting data on glucokinase enzyme activity in the above tissues are available. Glucokinase activity has been detected in samples of microdissected human islets, at 50% of the level measured in rat islets with the same microassay procedure (9, 10). Published data on glucokinase activity in biopsies of human liver are widely divergent, from undetectable to levels as high as in rat liver (11, 12).

One purpose of this study was to establish a data base on the normal level of glucokinase enzyme activity in the human liver, an important issue in view of the inconsistencies mentioned above. A second objective was to determine whether the expression of the hepatic glucokinase gene obeys the same multihormonal regulation in man as in the rat. In rat liver cells, the glucokinase gene is known to be strictly dependent for its transcription on the presence of insulin, and inversely to be totally repressed under the action of glucagon (13). The gene is tran-

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Table I. List of Patients

Patient ID no.	Sex	Age	Weight	Plasma glucose	Tumor type
		yrs	*kg*	*mM*	
3	M	48	75	5.6	Metastasis*
4	F	54	58	5.5	Hydatid cyst
5	F	45	57	5.5	Hydatid cyst
6	F	27	48	5.5	Adenoma‡
7	M	6	20	5.2	Sarcoma§
8	M	44	67	5.5	Hemangioma
9	F	53	57	5.3	Hemangioma
10	F	33	73	4.7	Adenoma‡
11	F	52	55	5.4	Hemangioma

* Metastasis of colorectal adenocarcinoma. ‡ Hepatocellular adenoma. § Embryonic rhabdomyosarcoma of bile duct.

scriptionally inactive in fasting or diabetes (14, 15). Glucokinase activity is strongly depressed or absent under these conditions, and is restored after insulin treatment. We have used primary cultures of hepatocytes derived from surgical liver biopsies to examine whether these aspects of hormone action have been conserved in the human species. The availability of human hepatocytes in culture also provided the opportunity to investigate the multihormonal regulation of the gene for another key enzyme of carbohydrate metabolism, the cytosolic form of the gluconeogenic enzyme phospho*enol*pyruvate carboxykinase.

Methods

Tissue procurement and isolation of human hepatocytes. The protocol for these studies was approved by the Institutional ethics committee of the Department of Surgery and informed consent was obtained from the patients. Liver biopsies were taken from patients undergoing segmental hepatectomies for liver tumors. Basic physical data, fasting plasma glucose levels and types of liver tumors for the patients in this study are given in Table I. At the start of the intervention, a wedge of macroscopically normal tissue (15–30 grams) located within the part of the liver to be hepatectomized, was dissected while maintaining vascular connections intact and excised as biopsy. A small fragment was cut from the biopsy, immersed in ice-cold phosphate-buffered saline and immediately carried to the laboratory for assays of enzymes in fresh tissue. The major part of the biopsy was subjected in the operating room to perfusion for the isolation of hepatocytes, using a technique derived from that described by Seglen (16) for rat hepatocytes, except that the collagenase solution was not recirculated. Three to five catheters were introduced into ramifications of the portal vein visible on the biopsy section within minutes after the resection. Perfusion was initiated immediately after insertion of the first catheter with a calcium-free medium at a flow rate of 10 ml/min per catheter. After 25 min, the perfusion medium was shifted to a collagenase solution and perfusion continued for another 25 min at a flow-rate of 6 ml/min per catheter. The exact composition of the perfusion media has been described previously (17). After collagenase perfusion, the liver piece was incised with several cuts and liver cells were dissociated by brief manual shaking in a dish containing 200 ml of ice-cold RPMI 1640 culture medium supplemented with penicillin and streptomycin and 10% newborn bovine serum (hereafter termed wash medium). The cell suspension was filtered through surgical gauze and rapidly brought to the cell culture laboratory.

Hepatocyte culture. Cells from the original suspension were pelleted by centrifugation at 40 *g* and 4°C for 5 min and washed twice by resuspensions in 200 ml of wash medium and centrifugations. The washed cells were resuspended in RPMI 1640 medium supplemented with antibiotics, 10% fetal bovine serum and 10^{-8} M insulin (seeding medium). In 9 separate experiments, the total yield of cells was comprised between 70 and 450 $\times$ 10^6 cells, 70–92% of the cells excluding Trypan blue. Higher than 90% of the cells presented a morphology of hepatocytes. Hepatocytes were seeded at 4.5 $\times$ 10^6 viable cells per 10 cm dish (Falcon Primaria; Falcon Labware, Cockeysville, MD) in 12 ml of seeding medium. Cells were allowed to attach to the dishes for 4–7 h in seeding medium, which was then exchanged for fresh medium containing the same antibiotic and insulin concentrations but only 5% fetal bovine serum (recovery medium). After 22 h in this medium, the cells were withdrawn from insulin by three washes of 10 ml/dish of acid phosphate-buffered saline (pH 6), followed by two washes in standard phosphate-buffered saline (pH 7.4). Both buffers were supplemented with 5% newborn bovine serum. The cells were then fed RPMI 1640 medium with antibiotics, 5% fetal bovine serum and 10^{-8} M dexamethasone. Hormonal effectors were supplied to the cells 16 h after the last medium change and cells were harvested for RNA isolation at defined time intervals. Rat hepatocytes were isolated as described previously (13, 17) and cultured under the same protocol as human hepatocytes.

Enzyme assays. All manipulations before assays were performed at 4°C. Solid liver or freshly isolated hepatocytes that had been washed in phosphate-buffered saline were homogenized using Teflon glass Potter homogenizers, in three volumes of a buffer containing 20 mM Tris/HCl pH 7.5, 0.25 M sucrose, 80 mM KCl, 5 mM EDTA, 4 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.125 mM phenylmethlsulfonylfluoride, 2.5 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin. The homogenates were centrifuged at 17,000 *g* for 15 min. The resulting postmitochondrial supernatants were centrifuged at 180,000 *g* for 60 min in order to obtain cytosols. Hexokinase and glucokinase activities were measured by a spectrophotometric assay. The total reaction volume was 0.75 ml and the sample volume for human cytosols was generally 50 μl. The assay was conducted at 30°C for 15 min, in presence of 45 mM Tris/HCl pH 7.5, 110 mM KCl, 8 mM $MgCl_2$, 0.5 mM NADP, 0.9 U/ml glucose 6-phosphate dehydrogenase from yeast, 0.5 or 100 mM glucose and 0 or 5 mM ATP. The ATP-dependent rate of NADPH formation at 0.5 mM glucose was taken as hexokinase activity. Glucokinase activity was calculated as the ATP-dependent rate of NADPH formation at 100 mM glucose minus the rate at 0.5 mM. The coupling enzyme glucose 6-phosphate dehydrogenase from yeast and the coenzyme NADP were used rather than the more usual glucose 6-phosphate dehydrogenase from *leuconostoc mesenteroides* and NAD because the latter were found to give unacceptable background slope with human liver samples. A stoichiometry of 2 moles of NADPH formed per mole glucose phosphorylated was used in the calculations. Phospho*enol*pyruvate carboxykinase activity was measured in cytosol samples by a radiometric CO_2 fixation assay exactly as described (18). Units of enzyme activities are μmoles of products formed per min. Protein concentrations were measured using a commercial Bradford assay.

Quantification of specific mRNAs. Total RNA was extracted from cultured hepatocytes as previously described (13). Glucokinase mRNA, a low abundance message, was quantified using a reverse transcription/polymerase chain reaction procedure essentially as described by Chelly et al. (19). Reverse transcription was done with an input of 4 μg total RNA and random hexamers as primers, using a commercial system (Superscript Preamplification System, Life Technologies). After reverse transcription, template RNA was digested with RNAse H and the cDNA products were subjected to PCR using two pairs of sequence specific primers, one pair for glucokinase and one for ribosomal protein S26 used as internal control. Primers for amplification of glucokinase cDNA were: 5′-TGGACCAAGGGCTTCAAGGC-3′ (sense strand, positions 781–800 of cDNA sequence in reference 20) and 5′-CATGTAGCAGGCATTGCAGCC-3′ (antisense strand, positions 967–987). These oligonucleotide sequences are localized in exons 5 and 7, respectively, of the glucokinase gene and are identical in the human and rat genes (20, 21). The primers for amplification of S26 cDNA were: 5′-TCG-

TGCCAAAAAGGGCCG-3′ (sense strand, positions 50–67 of cDNA sequence in reference 22) and 5′-GCTCCTTACATGGGCTTT-GGTGG-3′ (antisense strand, positions 376–354). The structure of the S26 gene being unknown, the primers were selected near the extremities of the translated region of the mRNA to maximize the chance of straddling an exon boundary. The nucleotide sequence in the region of the sense primer is identical in the human and rat (22, 23), whereas the antisense primer has two base mismatches vis-a-vis the rat sequence near the 5′ end of the primer. The PCR program involved an initial denaturation step at 94°C for 4 min, followed by cycles of denaturation at 92°C, annealing at 58°C and elongation at 72°C each for 30 s. Samples were generally withdrawn after 18, 21, 24, 27, 30, and 33 cycles. An elongation step of 2 min at 72°C was allowed to take place at the end of each of the above cycles. The PCR reaction was performed with 10 μM of glucokinase primers and 5 μM S26 primers. Reaction products were resolved on 8% polyacrylamide gels and electrophoretically transferred to nylon membranes (GeneScreen; DuPont-NEN, Boston, MA). Membranes were hybridized with ^{32}P-labeled oligonucleotides with sequence 5′-CTCAGGAGCAGAAGGGAAC-3′ (sense strand, positions 801–819 of cDNA sequence in reference 20) for detection of the glucokinase product and 5′-GTCAGGAATCGATCTCGTG-3′ (sense strand, positions 273–291 of cDNA sequence in reference 22) for detection of the S26 products. Hybridization and washing of the membranes were performed as described previously (24). The blots were autoradiographed on preflashed x-ray film with one intensifying screen for visualization of the amplification products. Control experiments demonstrated that amplification products were absent after reactions omitting reverse transcriptase. Quantification of the 207-bp glucokinase product and of the 327-bp S26 product was accomplished by phosphorimaging of the blots. The amount of glucokinase product was normalized for the amount of S26 product in the same gel lane. At least two values within the exponential range of amplification were scored for each RNA sample. Finally, data for each experiment were expressed as ratios to the values in the control RNA sample from cells cultured in the absence of hormonal effector (relative mRNA level).

The analysis of mRNA by Northern blotting was performed by standard techniques (25). Hybridization was performed in Hybsol as described by Yang et al. (26).

Materials. The cDNA probe for human S26 ribosomal protein was produced from plasmid HSS26 kindly provided by Philippe Fort (Institut de Genetique Moleculaire, CNRS, Montpellier, France) (22). The cDNA probe for cytosolic phospho*enol*pyruvate carboxykinase was a 1.3-kbp SphI–SacI fragment derived from a full-length rat cDNA (27) provided by Richard Hanson (Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH). The fragment is comprised within the translated region of the mRNA and presents 85% identity with the human sequence (28); hybridization and stringency washes of Northern blots with human RNA were done at 57°C.

Results

Enzyme activities in human liver. The assay of hexokinase and glucokinase enzyme activities in samples of human liver obtained during hepatic surgery revealed striking differences with similar measurements in extracts of rat liver. The first noticeable difference was the markedly lower glucokinase activity in human liver, approximately one twentieth of the activity in liver cytosols from 24 h–fasted rats (Table II, column 1). We were concerned that drugs used for induction and maintenance of general anaesthesia might directly or indirectly interfere with the glucokinase assay in the human samples. However, this possibility appears unlikely, since control experiments have shown that: (*a*) hepatic glucokinase activity was not decreased in rats maintained for 2 h under isoflurane anaesthesia and receiving high doses of the opioid analgesic fentanyl and the neuromuscular blocking agent pancuronium (two drugs used routinely in surgical patients); and (*b*) mixing experiments with rat and human liver cytosols revealed 100% recovery of glucokinase activity, even at high ratios of human/rat cytosols in the mixtures (data not shown).

The second difference pertained to the glucokinase/hexokinase ratio. In rat liver, glucokinase typically accounted for 90% of total hexokinase activity. In contrast, the ratio was only 35% in human liver (Table II, columns 1 and 2). The lower fractional activity of glucokinase raised the intriguing possibility that glucokinase might not be the predominant glucose phosphorylating enzyme in the human liver cell. A more trivial explanation would be that human liver tissue might contain a larger proportion of non-parenchymal cells (i.e., devoid of glucokinase activity) than rat liver. To distinguish between these possibilities, enzyme activities were assayed in isolated hepatocytes obtained after collagenase perfusion of human liver biopsies. The specific activity of glucokinase was enriched twofold in human hepatocytes as compared to fresh solid tissue, whereas the specific activity of low K_m hexokinase was reduced 10-fold (Table II, columns 1–4). The data show that glucokinase accounts for 95% of total hexokinase activity in the cytosol of human hepatocytes and is therefore the major glucose phosphorylating enzyme in the human parenchymal liver cell, as well as in the rat. However, the specific activity of glucokinase in hepatocytes from surgical patients was still sevenfold lower than in hepatocytes from 24 h–fasted rats.

In contrast to the relatively low level of glucokinase activity, the cytosolic extracts of the same hepatocytes displayed high activity of the gluconeogenic enzyme phospho*enol*pyruvate carboxykinase (GTP). The activity level of this enzyme was twofold higher in human than in rat samples (Table II, last column).

Insulin effect on glucokinase mRNA. Primary cultures of human hepatocytes were used to determine whether hepatic glucokinase is an insulin-inducible enzyme in the human liver, as has been previously shown for the rat enzyme both in the whole animal and in cultured hepatocytes. The level of glucokinase mRNA was assayed by an RT-PCR procedure with total cellular RNA as starting material. After reverse transcription, PCR amplification of glucokinase cDNA was performed with co-amplification in the same reaction tubes of the cDNA for a control mRNA serving as internal standard, thus allowing to normalize all data. For this purpose, the mRNA encoding protein S26 of the small ribosomal subunit was chosen (22), based on preliminary experiments showing that S26 mRNA quantified by Northern analysis was essentially invariant in human hepatocytes cultured under the present conditions (data no shown). The procedure was first applied for the measurement of glucokinase mRNA in hepatocytes stimulated for various periods of time with insulin. The autoradiograph of Southern blots of RT-PCR products is illustrated in Fig. 1 *A* and data obtained by Phosphorimager quantification of the results are plotted in Fig. 1 *B*. The addition of insulin to the cells was followed by a time-dependent rise in the relative level of glucokinase mRNA. The time-course of this process was characterized by a small but detectable increase at 2 h, a maximal induction around 10 h and a subsequent decline toward the baseline at 24 h after insulin. The effect of insulin was somewhat variable in distinct hepatocyte preparations, the mRNA increase ranging from 3- to 12-fold above the starting level at 6 to 8 h after hormone addition, with an average of 7-fold in nine separate experiments

Table II. Enzyme Activities in Liver Tissue and Freshly Isolated Hepatocytes from Humans and Rats

	Liver tissue		Isolated hepatocytes		
Species	Glucokinase	Hexokinase	Glucokinase	Hexokinase	Phospho*enol*pyruvate carboxykinase
Rattus norvegicus	20.6±0.77	2.0±0.39	22.6±2.80	0.5±0.17	91±4.9
Homo sapiens	1.6±0.49	2.7±0.27	3.4±0.33	0.2±0.04	206±57.3

Liver tissue from human subjects was obtained by surgical biopsy in patients undergoing partial hepatectomies. None of the patients was diabetic. The patients received their last meal at 6:00 PM on the day before the operation. Liver tissue from 24 h–fasted male Wistar rats was excised after killing the animals by decapitation. Human hepatocytes were isolated by collagenase perfusion of surgical biopsies. Rat hepatocytes were isolated by collagenase perfusion of the liver. Glucokinase, hexokinase and phospho*enol*pyruvate carboxykinase activities were assayed in the cytosolic fraction of homogenates as described in methods. Activities are expressed in mU/mg protein in cytosols. Data are means+SE of five independent determinations with human samples and of 4 determinations with rat samples. Data for both tissue and isolated hepatocytes were obtained from each patient and animal.

(Fig. 1 *B*). The effect was elicited at physiological insulin concentrations (half-maximal stimulation between 10^{-10} M and 4×10^{-10} M insulin, data not shown).

Insulin/cAMP interaction. Previous work from this laboratory has shown that glucagon or cell-permeant analogs of cAMP repress the transcription of the hepatic glucokinase gene in the rat, even in the presence of maximal stimulatory concentrations of insulin (13, 29). In the present experiments, the new RT-PCR assay was used to compare effects of cAMP analogs, singly or in combination with insulin, on glucokinase mRNA in rat and human hepatocytes cultured under identical conditions. In rat liver cells, results in agreement with published data were obtained (Fig. 2, *left panels*). Similar responses took place in human hepatocytes: (*a*) insulin alone caused a clear-cut increase of the glucokinase mRNA and (*b*) the inductive effect of insulin was abolished when cAMP was presented to the cells together with insulin (Fig. 2, *right panels*). Glucokinase mRNA was actually reduced by cAMP below the baseline level prevailing in control cells receiving no effectors. In four experiments with separate preparations of human liver cells (Fig. 3), the stimulatory effect of insulin amounted to a sevenfold increase in glucokinase mRNA. Addition to the cells of chlorphenylthio-

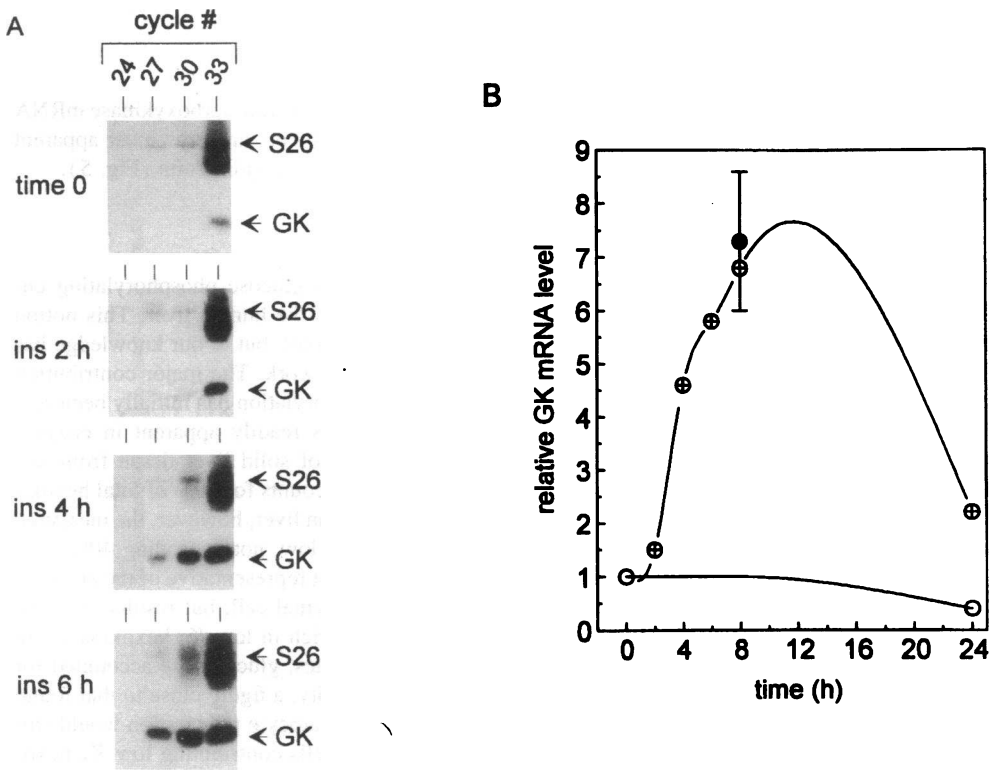

Figure 1. Induction of glucokinase mRNA by insulin in human hepatocytes. Hepatocytes were isolated from surgical biopsies of liver and cultured as detailed under Experimental Procedures for 2 d before addition of insulin (6.4×10^{-9} M) to the culture medium (time 0 on the abscissa). Total RNA was extracted from cells at several time points after addition of insulin and assayed for glucokinase mRNA by RT/PCR. Co-amplification of cDNA for invariant ribosomal protein S26 mRNA in the same reactions served for normalization purpose. The amplification products were separated on polyacrylamide gels, electroblotted to nylon membranes and hybridized to radiolabeled glucokinase or S26 oligonucleotide probes. (*A*) Autoradiograph of blots showing the 207-bp glucokinase (*GK*) PCR product and the 327-bp S26 product obtained from RNA samples taken at the indicated times following insulin addition. (*B*) Diagram showing the quantification of glucokinase mRNA in the samples shown in *A* as well as additional samples taken at later points of the time course experiment. Quantification was done with a Phosphorimager. (⊕) RNA from insulin stimulated cells; (○) RNA from control cells in absence of insulin. Also shown is the increase in glucokinase mRNA at 8 h after addition of insulin in nine independent experiments (•, mean±SE)

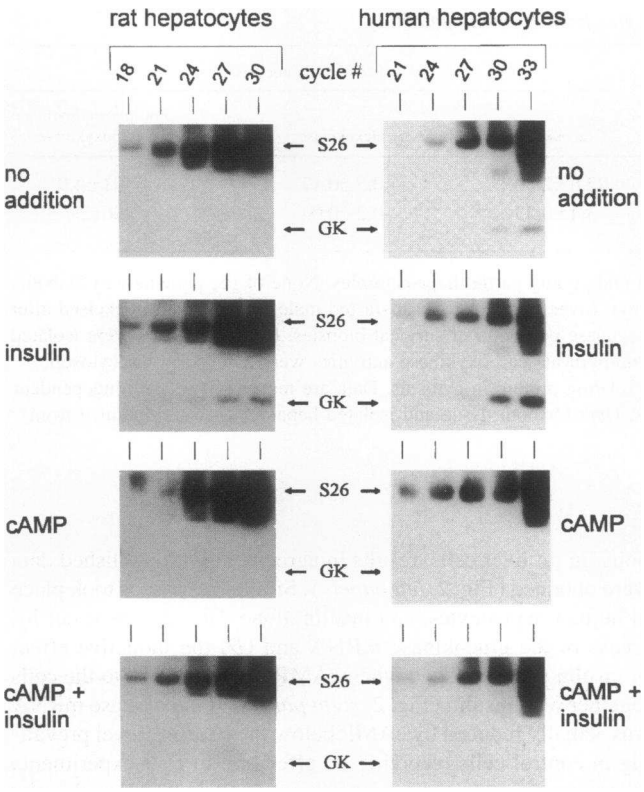

Figure 2. Negative effect of cAMP on glucokinase mRNA in rat and human hepatocytes. Rat and human hepatocytes were maintained in culture under identical conditions. On day 2 after isolation, insulin (6.4×10^{-9} M), chlorphenylthio-cAMP (100 μM) or both were added to the cells. Cells were harvested for RNA isolation 6 h later. Glucokinase (*GK*) mRNA level was estimated by RT/PCR using S26 mRNA as control. Autoradiographs of blots after electrophoretic separation of PCR products from typical experiments with rat (*left panels*) and human (*right panels*) are shown.

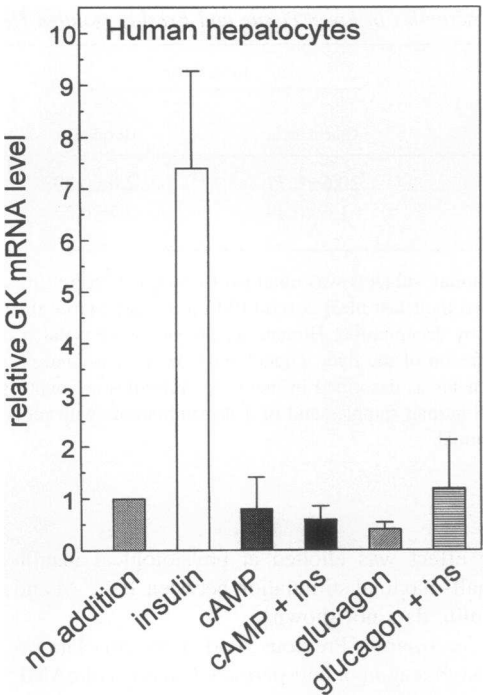

Figure 3. Effects of insulin, glucagon, and cAMP on glucokinase mRNA in human hepatocytes. Data obtained in four separate experiments with human hepatocytes are shown as means±SE. Relative glucokinase mRNA level was measured after RT/PCR as in Figs. 1 and 2. Bands on the blots were quantified using the Phosphorimager. In each experiment, data were expressed relative to the mRNA level measured in unstimulated cells, which was given the value of 1. Concentrations of effectors were: insulin (6.4×10^{-9} M), chlorphenylthio-cAMP (100 μM) and glucagon (10^{-9} M). All cells were harvested at 8 h after effector addition.

cAMP just before insulin completely prevented the induction. The physiological effector glucagon also strongly suppressed the effect of insulin (Fig. 3).

Hormonal effects on phosphoenolpyruvate carboxykinase mRNA in human hepatocytes. The cytosolic form of phospho*enol*pyruvate carboxykinase is transcriptionally regulated in rat liver (30, 31) and the regulation of this gene in rat hepatocytes (17) and hepatoma cell lines (32) is a classical model of multihormonal effects on gene expression. Whether the same type of regulation operates in the human liver is unknown. We have investigated the regulation of phospho*enol*pyruvate carboxykinase mRNA in parallel in rat and human hepatocytes. As expected from previous studies, glucagon and cAMP analogs elicited a strong increase in the amount of phospho*enol*pyruvate carboxykinase mRNA, and insulin reduced the mRNA level, in rat hepatocytes (Fig. 4, *left panel*). Hepatocytes from human subjects responded similarly. Phospho*enol*pyruvate carboxykinase mRNA markedly increased after stimulation of the cAMP signaling pathway by cAMP analogs or the physiologically relevant hormone glucagon (Fig. 4, *right panel*). A strong induction of the mRNA following stimulation of the cells by cAMP or glucagon occurred in four separate batches of human hepatocytes, confirming that these cells were consistently responsive to the activation of the cAMP system (Fig. 5). The negative effect of insulin on phospho*enol*pyruvate carboxykinase mRNA was also noted in all the experiments and was most apparent in cells supplied with both glucagon and insulin (Fig. 5).

Discussion

Glucokinase is the preponderant glucose phosphorylating enzyme in the parenchymal cell of the human liver. This notion has been widely assumed in the past, but to our knowledge has not been established before this work. The major contribution of glucokinase to glucose phophorylation has initially been recognized in rat liver (33) and is readily apparent in enzyme assays performed with extracts of solid liver tissue from this species, in which glucokinase accounts for 90% of total hexokinase activity. In biopsies of human liver, however, the measured glucokinase/hexokinase ratio is low, not exceeding 40%. Our data show that this low ratio is not representative of the enzymic make up of the hepatic parenchymal cell, but results from the contribution of other cell-types rich in low K_m hexokinases. In freshly isolated human hepatocytes, glucokinase accounted for $> 95\%$ of total hexokinase activity, a figure close to that found in rat hepatocytes. Since any hepatocyte preparation would still contain some nonparenchymal cells contributing low K_m hexokinases, it is clear that glucokinase can be considered the sole functionally relevant hexokinase of the liver cell in the human species, as is the case in the rat. The predominance in the liver

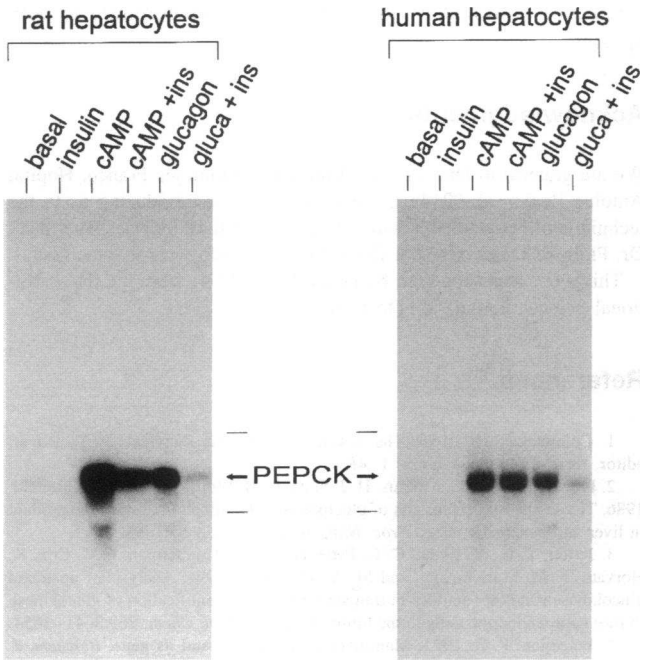

Figure 4. Phospho*enol*pyruvate carboxykinase mRNA is induced by cAMP and glucagon and de-induced by insulin in human and rat hepatocytes. The amount of mRNA for cytosolic phospho*enol*pyruvate carboxykinase mRNA was analyzed by Northern blot hybridization using a ^{32}P-labeled rat cDNA probe. Autoradiographs of typical blots are shown. Rat hepatocytes (*left panel*) and human hepatocytes (*right panel*) were cultured for 8 h with the effectors at concentrations as indicated in Fig. 3.

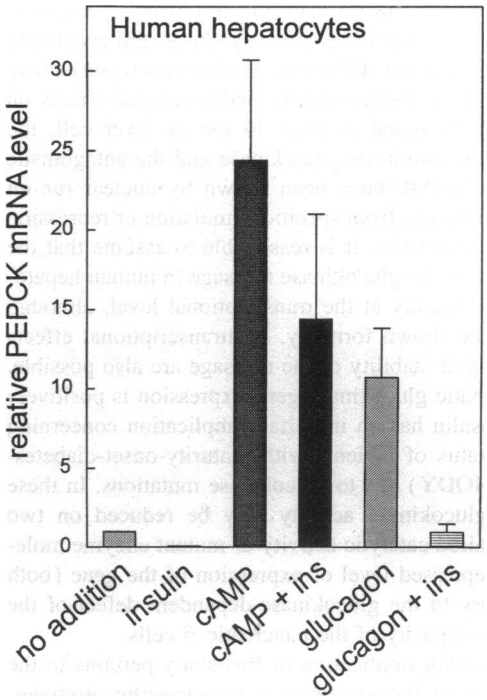

Figure 5. Effects of insulin, glucagon, and cAMP on phospho*enol*pyruvate carboxykinase mRNA in human hepatocytes. Hormonal effects on phospho*enol*pyruvate carboxykinase mRNA were measured in the same human hepatocytes analyzed in Fig. 3 for effects on glucokinase mRNA. The mRNA bands in Northern blots as shown in Fig. 4 were quantified using the Phosphorimager. Data were expressed relative to the value in cells incubated in absence of any effector which was taken as 1. Data are means±SE for $n = 4$.

cell of glucokinase, an enzyme with a uniquely low affinity for glucose (half-saturation around 6 mM glucose as compared with μM concentrations for the other mammalian hexokinases) is considered crucial for the ability of the liver to adapt its rate of glucose release or uptake according to the degree of glycaemia (34). In association with a nonlimiting glucose transport system at the plasma membrane level, glucokinase should be viewed as a glucose-sensitive device which allows the rate of glucose phosphorylation and further utilization to vary in response to physiological fluctuations of the plasma glucose concentration.

Although the major hexokinase of the hepatocyte, glucokinase was present in human hepatocytes at an activity level only a fraction (16%) of that found in rat hepatocytes. The comparison here is between human subjects in the postabsorptive state and rats fasted for 24 h. This comparison is somewhat arbitrary, since glucokinase activity is adaptive to the nutritional status of the subject or animal. It should be noted that glucokinase activity is still above 10 mU/mg protein in the livers of rats after 3 d of fasting (P. B. Iynedjian, unpublished observations). The activity in the hepatocytes of human subjects in the postabsorptive condition (3.5 mU/mg protein) is therefore only ~ 30% of the activity remaining after severe food deprivation in the rat. The inherent medical condition of surgical patients might in principle contribute to a depression of hepatic glucokinase activity. However, our group of patients presented distinctly different pathologic conditions and the variation of glucokinase activity between patients was relatively small. For this reason, we do not believe that the pathologic factor had an important role to account for the lower enzyme activity in the human liver. Rather, our data suggest an interesting metabolic difference between the two species. The human liver appears not to be as well-equipped as the rat liver for the uptake of glucose and the immediate deposition of glycogen from circulating glucose by the so-called direct pathway. The high activity of cytosolic phospho*enol*pyruvate carboxykinase, on the other hand, argues for the preponderance of the gluconeogenic pathway in human hepatocytes and is consistent with a prevalent role for the indirect route of glycogen synthesis in the human liver (35).

Earlier work by other authors has suggested that glucokinase activity in the human liver was affected by the nutritional and hormonal condition of the subject. In surgical biopsies of liver, glucokinase could be detected by standard activity assay or following starch gel electrophoresis in the livers of well nourished patients, but was absent in poorly nourished patients (36). Glucokinase activity was depressed in needle biopsy specimens from healthy voluntary subjects after an 8-d period on a low-carbohydrate diet. Enzyme activity increased fourfold over the next 2 d on a high-carbohydrate diet (33). Two reports have indicated that glucokinase activity was reduced by about 50% in newly diagnosed insulin-dependent diabetics (37, 38). The present study shows that human hepatocytes in primary culture respond to the addition of insulin by induction of glucokinase mRNA. Glucagon acting via cAMP overcomes the action of insulin and exerts a dominant negative effect on the expression of glucokinase mRNA. A general assumption in this type of

study is that changes in mRNA leads to parallel and commensurate changes in the rate of the enzyme synthesis and eventually in enzyme protein amount. Therefore, the findings reported here provide a mechanism for the dietary and hormonal effects on glucokinase activity noted in vivo. In the rat liver cell, the inductive effect of insulin on glucokinase and the antagonistic role of glucagon/cAMP have been shown by nuclear run-on assay to result primarily from specific stimulation or repression of gene transcription (13). It is reasonable to assume that the hormonal effects on the glucokinase message in human hepatocytes are exerted mainly at the transcriptional level, although this remains to be shown formally. Posttranscriptional effects on the processing or stability of the message are also possible. The fact that hepatic glucokinase gene expression is positively controlled by insulin has an important implication concerning the metabolic status of patients with maturity-onset-diabetes-of-the-young (MODY) due to glucokinase mutations. In these subjects, liver glucokinase activity may be reduced on two counts: (*a*) impaired catalytic activity of mutant enzyme molecules and (*b*) depressed level of expression of the gene (both alleles) secondary to the glucokinase-dependent defect of the insulin secretory capacity of the pancreatic β cells.

Another interesting implication of this study pertains to the structure/function of the glucokinase liver-specific promoter. Regulatory DNA sequence elements that account for the cell-type specificity and hormone-responsiveness of gene transcription in the rat have yet to be defined. Our results suggest that homologous *cis*-acting elements might be found in the rat and human genes. A systematic comparison of the two promoters using the multiple experimental approaches available might provide insight into molecular mechanisms that have so far remained elusive.

The similarity between the human and rat species in the hormonal control of metabolically important liver genes extends to the cytosolic form phospho*enol*pyruvate carboxykinase. As in rat systems, mRNA for this flux-generating enzyme of gluconeogenesis accumulates massively in response to glucagon and cAMP in cultured human hepatocytes. Insulin causes a decline of the mRNA below the basal level and markedly reduces the glucagon-induced increase in mRNA. Interestingly, in both human and rat hepatocytes, insulin is far less effective in opposing the action of phosphodiesterase-resistant cAMP analogs than that of glucagon, suggesting that the negative effect of insulin might partly rely on a stimulation of the hormone-sensitive cyclic nucleotide phosphodiesterase. In the rat liver and rat hepatoma cells, the regulation of phospho*enol*pyruvate carboxykinase mRNA relies primarily on transcriptional effects of cAMP and insulin (31, 32, 39). In addition, the half-life of the mRNA is prolonged in hepatoma cells cultured in presence of cAMP (40). An array of *cis*-acting elements important for hormone responsiveness has been described in the promoter regulatory region of the rat gene (41, 42). The identity of *trans*-acting factors capable of binding to some of these DNA elements has been determined (43). In addition, recent studies in transgenic mice have suggested that the nutritional and hormonal regulation of the phospho*enol*pyruvate carboxykinase gene in the intact animal depend on the interplay between multiple elements, rather than on the functioning of any single element in particular (44). The similarity of hormonal effects on hepatic phospho*enol*pyruvate carboxykinase mRNA in rat and man supports the hypothesis that the transcription of the gene might be controlled by mechanisms that are common to both species.

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