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Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans

(hexokinase/insulinoma cells)

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ABSTRACT **Glucokinase, a key regulatory enzyme of glucose metabolism in mammals, provides an interesting model of tissue-specific gene expression. The single-copy gene is expressed principally in liver, where it gives rise to a 2.4-kilobase mRNA. The islets of Langerhans of the pancreas also contain glucokinase. Using a cDNA complementary to rat liver glucokinase mRNA, we show that normal pancreatic islets and tumoral islet cells contain a glucokinase mRNA species ≈400 nucleotides longer than hepatic mRNA. Hybridization with synthetic oligonucleotides and primer-extension analysis show that the liver and islet glucokinase mRNAs differ in the 5′ region. Glucokinase mRNA is absent from the livers of fasted rats and is strongly induced within hours by an oral glucose load. In contrast, islet glucokinase mRNA is expressed at a constant level during the fasting–refeeding cycle. The level of glucokinase protein in islets measured by immunoblotting is unaffected by fasting and refeeding, whereas a 3-fold increase in the amount of enzyme occurs in liver during the transition from fasting to refeeding. From these data, we conclude (*i*) that alternative splicing and/or the use of distinct tissue-specific promoters generate structurally distinct mRNA species in liver and islets of Langerhans and (*ii*) that tissue-specific transcription mechanisms result in inducible expression of the glucokinase gene in liver but not in islets during the fasting–refeeding transition.**

Glucokinase is one of the isoenzymes of the mammalian hexokinase group (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1). It has attracted considerable interest because of its distinctive structural and catalytic properties, as well as its typical tissue distribution (1, 2). Glucokinase activity is found only in liver and in the islets of Langerhans of the pancreas. Glucokinases of liver and pancreatic islets display similar chromatographic and electrophoretic behavior, suggesting that the enzyme from both tissues is the product of a single or two closely related genes (3, 4). In liver, glucokinase is considered to play a key regulatory role in glucose uptake and release (5). In the islets of Langerhans, it has been ascribed the role of glucose sensor in the regulation of insulin secretion (6). Further interest in this enzyme arises from its multihormonal regulation in liver (7, 8). In particular, it has been shown (9) that insulin rapidly stimulates transcription of the glucokinase gene in the liver of the diabetic rat. Virtually nothing is known, however, about a possible influence of nutritional or hormonal factors on glucokinase gene expression in pancreatic islets. Moreover, the mechanisms underlying the restricted tissue distribution of glucokinase have not been investigated at the molecular level. The cloning of cDNA for hepatic glucokinase (10) has allowed us to address these issues directly. The findings reported herein provide insight into the tissue-specific expression and regulation of the gene encoding this important enzyme of carbohydrate metabolism.

MATERIALS AND METHODS

Animals and Cells. Male Wistar rats (160–220 g) were used. For experiments assessing the tissue distribution of glucokinase mRNA, animals were allowed free access to a 10% (wt/vol) glucose solution in addition to water and food pellets for 3 days prior to an experiment, since liver glucokinase activity is known to be elevated under glucose-supplemented conditions (2). Two types of fasting/refeeding experiments were conducted. (*i*) Animals were allowed to feed ad lib on rat chow containing 58% (wt/wt) sucrose with free access to water, but no glucose solution for 3 days prior to the experiments. They were then deprived of food for 72 hr and refed by administration of 1 g of glucose through a gastric catheter, after which the animals had free access to food pellets and a 20% glucose solution for 6–18 hr. (*ii*) Animals, prefed as in experiment *i*, were deprived of food for 48 hr and allowed to refeed ad lib on a 58% sucrose chow for 4 hr without glucose gavage or supplementary glucose in their water. Tissues for isolation of RNA or assay of glucokinase were excised immediately after decapitation, washed with chilled isotonic phosphate-buffered saline, and either processed immediately or frozen in liquid N_2. Two insulinoma cell lines were also studied: (*i*) a clonal line derived in the Institut de Biochimie Clinique from RIN-m5F cells (11, 12) and (*ii*) the RIN 1027 line described by Philippe *et al.* (13). Culture conditions have been described (14). Pancreatic islets were isolated by a modification (15) of the well-established collagenase digestion procedure. For the assay of glucokinase by immunoblotting, the cytosol fraction from freshly isolated islets was prepared (3) and stored frozen at −75°C until used.

Immunoblotting and Enzyme Assay of Glucokinase. The method for quantification of glucokinase by immunoblotting has been described (9). Autoradiograms of the blots were scanned with a densitometer to measure the area of the peak corresponding to the glucokinase band. Glucokinase levels were expressed in area units per mg of cytosol protein loaded on the gel. Glucokinase activity in the cytosol fraction of liver homogenates was measured by a spectrophotometric assay (10). Protein concentration in cytosol was measured by the Bio-Rad protein assay.

Isolation of RNA and Northern Blotting. Total RNA was extracted by the method of Chomczynski and Sacchi (16).

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Solid tissues were homogenized with a Polytron homogenizer and cells were subjected to several passes through a 21-gauge needle. Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography.

Gel electrophoresis of RNA in agarose gels and electrophoretic transfer to nylon membranes was performed as described (as in ref. 9 for Figs. 1*A* and 4*A* and as in ref. 17 for Figs. 1*B* and 4*B*). cDNA probes for hybridization were labeled by the random-priming procedure (ref. 18 for Figs. 1*A* and 4*A*) or nick-translation (Figs. 1*B* and 4*B*), and oligonucleotides were labeled using polynucleotide kinase. Hybridization with cDNAs was carried out at 42°C for 20 hr in a solution containing 50% (vol/vol) formamide, 25 mM Tris·HCl (pH 7.5), 0.8 M NaCl, 1% SDS, 10× Denhardt's solution, 10% (wt/vol) dextran sulfate, denatured salmon sperm DNA (125 μg/ml), and labeled probe at 0.5 ng/ml. (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) Blots were washed sequentially in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at room temperature, 1× SSC containing 1% SDS at 68°C, and 0.1× SSC at room temperature. Blotting with oligonucleotides was done in a solution containing 20 mM sodium phosphate (pH 7.0), 5× SSC, 10× Denhardt's solution, 7% SDS, salmon sperm DNA (200 μg/ml), and the probe at 2–3 ng/ml. Hybridization was for 16 hr at a temperature 10°C below the calculated melting temperature for each oligonucleotide (19). Blots were washed successively in 3× SSC at room temperature, 3× SSC containing 7% SDS at a temperature 8°C below the melting temperature, 1× SSC containing 1% SDS at the same temperature, and finally 1× SSC at room temperature.

Southern Blotting of Genomic DNA. High molecular weight DNA from rat liver was isolated and digested with restriction endonucleases by standard protocols (20). After gel electrophoresis in 0.7% agarose gels and alkali denaturation, DNA was transferred to nylon filters and hybridized to cDNA probes under the conditions used for RNA.

Synthesis of Oligonucleotides. Oligodeoxyribonucleotides were synthesized using automated DNA synthesizers (Gene Assembler, Pharmacia or Applied Biosystems model 381A) and purified by gel electrophoresis in urea/polyacrylamide gels.

Primer-Extension and DNA Sequencing. Poly(A)-containing RNA from liver or RIN 1027 cells was used as template for extension of a 5′-end-labeled oligonucleotide by reverse transcriptase, using a cDNA synthesis kit and protocols for the first-strand synthesis supplied by Boehringer Mannheim. Extension products and labeled size markers were resolved in parallel in a 5% polyacrylamide/urea sequencing gel.

RESULTS

Distribution of Glucokinase mRNA in Tissues. Earlier biochemical and immunochemical studies have identified glucokinase only in liver and islets of Langerhans of the pancreas, suggesting that the gene encoding this enzyme is expressed in a highly tissue-specific manner (3). We have now analyzed the tissue distribution of glucokinase mRNA by Northern blot hybridization using a cloned rat liver cDNA. The hybridization probe for the initial blotting experiments was a 1.8-kilobase (kb) partial glucokinase cDNA probe cloned from a rat liver cDNA library (10). This probe hybridizes to a 2.4-kb mRNA from liver, as described previously and further shown in Figs. 1 and 4. As may be seen, this mRNA is entirely liver-specific, even though RNA was isolated from glucose-supplemented animals in an effort to maximize glucokinase expression (2). On the other hand, RIN cells (as well as normal pancreatic islets, see below) display a glucokinase-related mRNA species of 2.8 kb, 400 nucleotides longer than the hepatic mRNA. RIN cells also contain a larger transcript of ≈4.4 kb that is more prevalent in RIN 1027 cells (Fig. 1*B*) than in RIN-m5F cells (Fig. 1*A*).

Glucokinase mRNAs of Liver and Insulinoma Cells Have Different 5′ Ends. To investigate the structural difference between the glucokinase mRNAs expressed in liver and islet cells, we have analyzed RNA from liver, normal islets, and RIN cells by Northern blotting with a number of liver cDNA subprobes and synthetic oligodeoxyribonucleotides. Consistent with extensive sequence homology between liver and islet mRNAs, these probes hybridized with both types of mRNA, with one exception. An oligonucleotide (oligo-1), 3′-GGTCTGTCAGGAGTGGACGTTG-5′, complementary to liver mRNA between nucleotides 31 and 52 of the sequence published by Andreone *et al.* (21) and mapping in the 5′ untranslated region of the mRNA, was shown to hybridize selectively to rat liver poly(A)-containing RNA and not to mRNA from RIN cells (Fig. 2*A*). In contrast, the 2.4-kb and 2.8-kb mRNAs typical of liver and islet cells, respectively, hybridized to a second oligonucleotide (oligo-2), 3′-GACCGTCTCAAGGTCGACGTCCTCCTTCTGGACTTCTT-5′, complementary to the published liver mRNA sequence between nucleotides 171 and 208—i.e., 57–94 nucleotides downstream of the putative initiator AUG. To map the position of the common probe, oligo-2, with respect to the transcription start site(s) of the glucokinase mRNAs in the two tissues, a primer-extension experiment was performed. As may be seen in Fig. 2*B*, a primary-extension product of ≈230 base pairs (bp) was generated from liver mRNA, as compared to a major product of 622 bp in RIN 1027 mRNA. A number of smaller, faint bands are seen in the liver primer-extension experiment that likely represent incompletely extended products, since only the 230-bp band is consistent with the known size of the liver glucokinase mRNA. A second less-prominent band of 900 bp was also obtained in the RIN extension reaction. These data predict that the liver glucokinase mRNA extends approximately 20 nucleotides on the 5′ side of the published cDNA

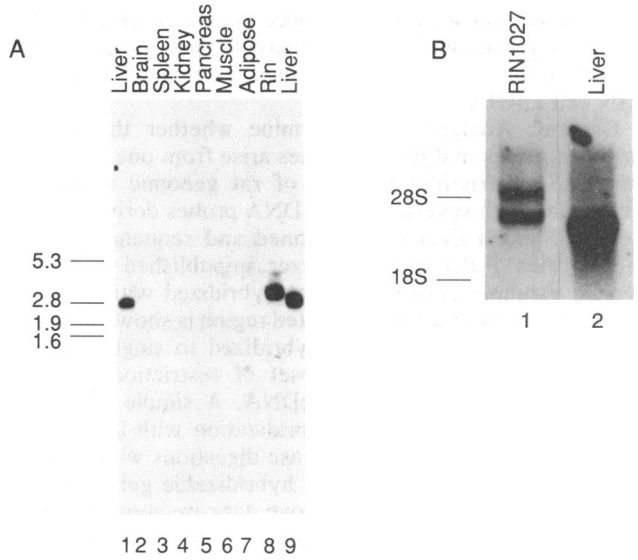

FIG. 1. Tissue-specific expression of the glucokinase gene. (*A*) Northern blot hybridization of poly(A)-containing RNA from rat tissues with the 1.8-kb cDNA insert of plasmid pUC-GK1 (10). The following amounts of RNA were loaded on gels. Lanes: 1, 5 μg; 2–7, 15 μg; 8 and 9, 10 μg. Lanes: adipose, white adipose tissue from the epididymal fat pad; muscle, skeletal muscle from the hindlimb; RIN, RIN-m5F rat insulinoma cells (12). The size of RNA markers is given in kilobases. (B) Hybridization to poly(A)-containing RNA was performed with the same probe used in *A*. The following amounts of RNA were loaded on gels. Lanes: 1, 10 μg; 2, 5 μg. RIN1027, RIN 1027 rat insulinoma cells (13).

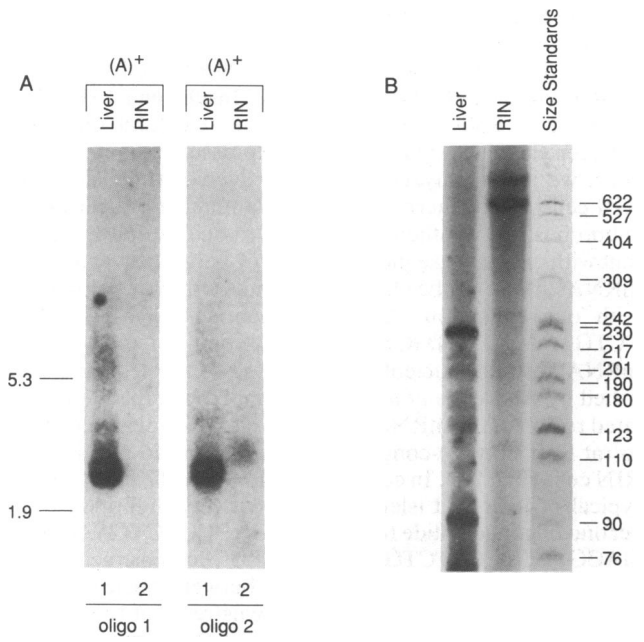

FIG. 2. (*A*) Northern blot hybridization of glucokinase mRNA from liver and RIN (RIN-m5F) cells with synthetic oligonucleotides. Duplicate blots with poly(A)-containing RNA from liver of a 6-hr glucose-refed rat (lanes 1, 2 μg) and RIN cells (lanes 2, 18 μg). Oligonucleotide probes 1 and 2 are described in the text. (*B*) Mapping of 5′ end of glucokinase mRNA by primer-extension analysis. The primer was oligo-2. Poly(A)-containing RNA from the liver of a fasted–refed rat (5 μg) or rat insulinoma cells (15 μg, RIN 1027) was used as template. To generate size standards, pBR322 DNA was cut with *Msp* I and end-labeled with the Klenow fragment of DNA polymerase I; sizes of the labeled fragments are indicated in base pairs.

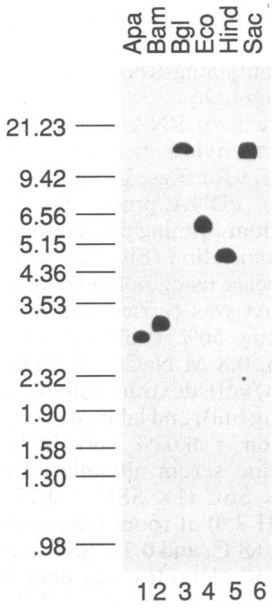

FIG. 3. Southern blot hybridization of rat DNA digested with restriction endonucleases *Apa* I (lane Apa), *Bam*HI (lane Bam), *Bgl* II (lane Bgl), *Eco* RI (lane Eco), *Hin*dIII (lane Hind), and *Sac* I (lane Sac). Hybridization was performed with a 303-bp 3′-end cDNA subprobe obtained by *Ava* I digestion of the near-full-length rat liver glucokinase cDNA plasmid pB-GK2. The size of markers is given in kilobase pairs.

sequence (21). Furthermore, the difference in size between the liver and the major RIN cell-extension products is 392 bp, in close agreement with the difference in size observed for the main glucokinase mRNAs in the two cell types, suggesting that the size difference is due to a unique, extended 5′ end in the RIN cell mRNA.

Genomic Analysis. To determine whether the distinct mRNA species in different tissues arise from one or several genes, Southern blot analysis of rat genomic DNA was performed with several partial cDNA probes derived from a near full-length liver cDNA cloned and sequenced in our laboratories (P.B.I. and D. Hayzer, unpublished results). A typical example depicting a blot hybridized with a 303-bp probe located in the 3′ untranslated region is shown in Fig. 3. As may be seen, this probe hybridized to single genomic fragments generated by a subset of restriction enzymes known not to cut within the cDNA. A simple restriction pattern was also seen after hybridization with a 413-bp 5′ cDNA probe, although in this case digestions with *Bam*HI, *Eco*RI, or *Hin*dIII yielded two hybridizable genomic fragments (data not shown). The above data are consistent with a single glucokinase gene per haploid genome in the rat.

Regulation of Expression of the Glucokinase Gene in Liver and Islets. We have shown (10) that glucokinase mRNA is massively induced in liver within a few hours of refeeding glucose to fasted rats. It was of interest to investigate the effect of glucose refeeding on glucokinase gene expression in the islets of Langerhans and to compare the effect of glucose gavage and ad lib refeeding. As may be seen in Fig. 4, pancreatic islets express a 2.8-kb glucokinase mRNA, as also found in insulinoma cells. Fig. 4*A* demonstrates that glucose refeeding does not alter the content of glucokinase mRNA in the islets relative to animals that have been fasted for 72 hr. Similarly, as shown in Fig. 4*B*, no difference in islet glucokinase mRNA levels are observed when comparing animals fasted for 48 hr with either ad lib fed or fasted and ad lib refed animals. For comparison, RNA samples from the livers of fasted, fed, and fasted–refed rats were analyzed in the same way. As expected from our previous work (10), glucokinase mRNA was undetectable in liver in the fasting state and was strongly induced 4–6 hr after either an oral glucose load or in response to ad lib refeeding on a 58% sucrose chow.

In the absence of change in mRNA level, the amount of glucokinase in islets might still be regulated during the fasting–refeeding cycle, through a translational mechanism or an effect on the turnover of the enzyme. We tested these possibilities by quantifying glucokinase protein in islets and livers of animals that were fasted for 72 hr and, in the case of refeeding, tube-fed with a glucose solution and given free access to food pellets 18 hr prior to removal of tissues. Glucokinase in liver and islets was quantified by immunoblotting using affinity-purified anti-glucokinase antibodies prepared as described (9), as shown in Fig. 5. These experiments were performed with six batches of islets pooled from five to eight animals for each batch and several livers. The amount of immunoreactive glucokinase in islet extracts was found to be unchanged after 18 hr of refeeding as compared to the fasted level, whereas there was a 3-fold increase in immunoreactive liver glucokinase protein. We have established that the increase in immunoreactive enzyme in liver upon refeeding correlates with a similar increase in glucokinase activity measured by enzyme assay. Thus, subsequent measurements of glucokinase in liver were by enzyme assay rather than immunoblotting, because the former procedure is technically simpler and better suited for large numbers of samples. In keeping with the previous findings, liver glucokinase activity was 2.6-fold greater in the fasted–refed animals compared to fasted animals (42.7 ± 3.3 milliunits/mg of protein in fasted–refed liver; 16.2 ± 1.1 milliunits/mg of protein in fasted liver when six pools of five to eight liver samples for each condition were analyzed). It should be pointed out that, whereas one previous study suggests that

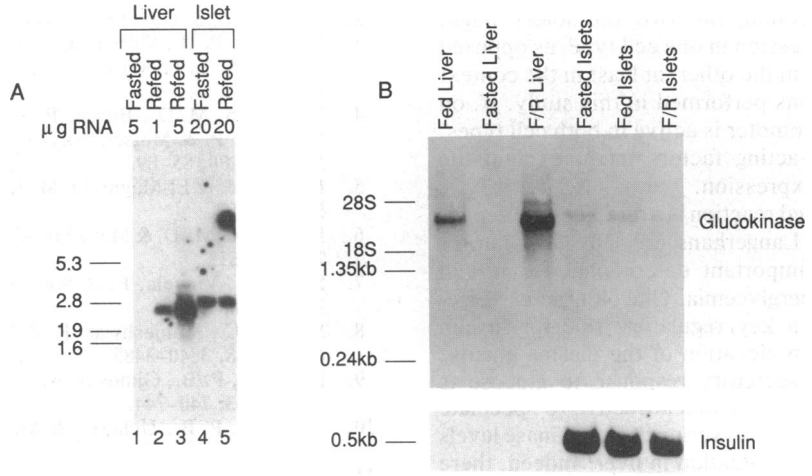

FIG. 4. Effect of fasting and refeeding on glucokinase mRNA in liver and pancreatic islets. (*A*) Effect of 72 hr of fasting and glucose refeeding. Total RNA in the indicated amounts was analyzed by Northern blotting with the cDNA of pUC-GK1 labeled by random priming as probe. Tissues from refed animals were removed 6 hr after the start of refeeding. (*B*) Effect of a 48-hr fast and a 4-hr ad lib refeeding on 58% sucrose chow. Poly(A)-containing RNA was probed with nick-translated pUC-GK1 (10). The following amounts of RNA were loaded. Lanes: 1–3, 5 μg of liver RNA; 4–6, 2 μg of islet RNA. In a separate experiment, the blot in *B* was probed with a nick-translated cDNA probe for rat insulin (22).

liver glucokinase activity peaks 18 hr after refeeding (8), another shows a further increase between 24 and 48 hr after a meal (23).

DISCUSSION

Of all tissues examined here, only liver and the islets of Langerhans contain glucokinase mRNA. This suggests that trans-acting factors necessary for the transcription of the glucokinase gene are present in proper combination and amounts only in liver and islets. Unexpectedly, we found that the gene is transcribed into two mature mRNA species, a 2.4-kb mRNA specific to liver and a 2.8-kb mRNA specific to islets. From the ability of the islet mRNA to hybridize with several DNA probes covering various regions of the liver mRNA, we conclude that the regions of sequence identity are distributed over most of the mRNA length. However, an oligonucleotide probe complementary to a region of the 5′ untranslated leader of the liver mRNA failed to hybridize to RIN cell mRNA, pointing to a sequence difference in the 5′ area of the two mRNAs. Hybridization of both mRNAs to a second oligonucleotide spanning the region of the liver cDNA sequence encoding amino acids 19–32 localizes the difference to a region upstream of the codon for amino acid 19. Primer-extension analysis with the common oligonucleotide further establishes that the RIN cell mRNA has a unique extended 5′ region. Tissue-specific differential splicing and/or the use of alternative promoters could explain this difference. Multiple promoters have been described for a number of eukaryotic genes (24–26). In a few cases, the choice among alternative promoters is specific to particular cell types or developmental stages and is accompanied by alternative splicing, so that transcripts initiated at the upstream promoter in one tissue subsequently undergo processing with removal of the leader sequence found in the mRNA transcribed from a downstream promoter in another tissue or at another stage of development (26–28). It is tempting to speculate that such a mechanism might apply to the glucokinase gene. Earlier studies have shown that glucokinase from islets or RIN cells has biochemical and immunological properties very similar to those of the liver enzyme, including electrophoretic mobility in one- and two-dimensional gel systems and peptide maps after limited proteolysis (3, 4, 14). This led to the suggestion of a common primary structure. It remains to be seen whether the distinct liver and islet cell mRNAs identified here are entirely identical in the protein coding region. To resolve these issues, we have prepared rat pancreatic islet cDNA libraries (J.L.M., C.Q., and C.B.N., unpublished data).

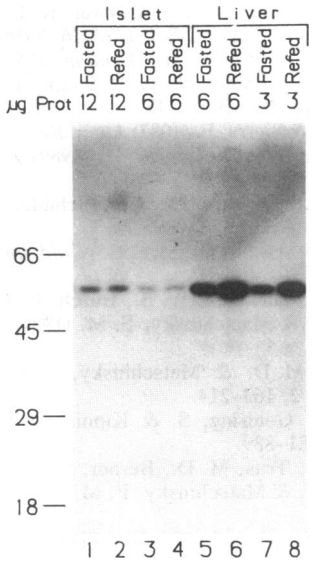

FIG. 5. Effect of fasting and carbohydrate refeeding on glucokinase enzyme in liver and pancreatic islets. Cytosol protein in the indicated amounts was resolved by SDS/polyacrylamide gel electrophoresis in a 11% polyacrylamide gel and transferred to nitrocellulose for immunoblotting. Fasting was for 72 hr. Tissues from refed animals were excised 18 hr after the start of refeeding. The molecular weights of size markers are indicated in kDa.

Glucokinase mRNA in islets is expressed at similar levels in fasting and after carbohydrate refeeding, whereas in liver it is absent in the fasting state and is dramatically induced within a few hours of glucose intake. Insulin, which is known to be secreted in response to an oral glucose load, appears to be the key transcriptional inducer of glucokinase in liver (9). Although the mechanism for gene activation in response to insulin is unknown, it evidently depends on the interaction of hormonally regulated transcription factors and particular cis-acting elements in the promoter regulatory region of the gene. The present data indicate that this mechanism operates in a tissue-specific manner. If transcription of the gene is initiated from different promoters in liver and islets, distinct

cis-acting elements controlling the two promoters might account for regulated expression in one cell type, as opposed to unregulated expression in the other, at least in the context of the dietary manipulations performed in this study. If, on the other hand, a single promoter is active in both cell types, distinct subsets of trans-acting factors might explain the different modes of gene expression.

The glucokinase-catalyzed reaction is a rate-limiting step for glycolysis in the islet of Langerhans (29, 30). The rate of glycolysis is in turn an important determinant for insulin release in response to hyperglycemia. Glucokinase is, therefore, considered to play a key regulatory role for insulin secretion in response to an elevation of the plasma glucose concentration. Since the secretory response to glucose is blunted in fasting animals (31), it was attractive to speculate that the defect might be due to a lowering of glucokinase levels in islets, by analogy with the situation in liver. Indeed, there is a report in the literature showing an ≈30% reduction in islet glucokinase activity, as measured by enzymatic assay, after 3 days of fasting. The activity was restored to normal after 24 hr of refeeding (32). From the present results, it appears that this effect would result from an alteration in the catalytic activity of the enzyme, without change in its content due to specific effects at the transcriptional, post-transcriptional, or translational levels.

Various lines of RIN cells derived from a radiation-induced insulinoma of the rat are in use in a number of laboratories. Given the time-consuming procedure necessary for isolation of pancreatic islets from laboratory animals, and their limited yield, insulinoma cells provide a useful model system for investigation of islet physiology. It should be realized, nevertheless, that such cells display heterogeneous phenotypes, as shown in particular by variable expression of the genes encoding pancreatic hormones (13). The cell lines investigated here contain levels of glucokinase mRNA and enzyme protein roughly comparable to those in normal rat islets. We have noted, however, that different clones of RIN cells contain far lower amounts of glucokinase mRNA (33) consistent with very low levels of glucokinase activity reported by another laboratory (34). In addition, the two lines examined herein contain markedly different levels of an alternative glucokinase transcript of 4.4 kb. This larger transcript may represent a splicing intermediate that is more long lived in some insulinoma lines compared to others or to normal islets. A less likely possibility, given the stringent washing and hybridization conditions employed, is that the larger transcript represents cross reactivity of the glucokinase probe with one of the hexokinase isozyme mRNAs. Resolution of the exact mechanism involved in generating tissue and cell-specific glucokinase transcripts will await further investigation.

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