Molecular Cloning of Glucokinase cDNA
DEVELOPMENTAL AND DIETARY REGULATION OF GLUCOKINASE mRNA IN RAT LIVER*

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A rat liver cDNA library enriched for glucokinase sequences was constructed using the phage expression vector λ gt11 and screened with an antiseraum to glucokinase. A positive phage clone termed λ-GK223 was isolated by several rounds of plaque purification. When introduced in the high frequency lysogenization strain Y1089, the phage was shown to encode a fusion protein containing epitopes specific to rat liver glucokinase. The 1800-base pair cDNA insert of λ-GK223 was subcloned in a pUC plasmid, and a resulting recombinant termed pUC-GK1 was used for hybrid selection of mRNA. The selected mRNA directed the synthesis in a cell-free translation system of a protein identified as glucokinase by electrophoresis and immunoprecipitation. The cloned cDNA was then used as a probe to measure the amount of glucokinase mRNA in rat liver during postnatal development. Glucokinase mRNA, 2.4 kilobases in length, was first detectable at day 14 after birth and increased 40-fold in amount from this age to day 31, in parallel with the emergence of glucokinase enzyme activity. In the adult rat, glucokinase mRNA was low during fasting and increased more than 50-fold above the fasting level within 6 h of an oral glucose load. However, maximal accumulation of glucokinase mRNA was short-lived and the mRNA level returned toward basal values by 18 h of refeeding. These data point to rapid and massive effects on the expression of the glucokinase gene at the transcriptional or post-transcriptional levels during ontogenic development and dietary changes in the adult animal.

Glucokinase, one of the isoenzymes of the mammalian hexokinase group (ATP:p-hexose 6-phosphotransferases, EC 2.7.1.1), exhibits interesting properties with respect to tissue-specific expression and hormonal regulation (1). Glucokinase is localized in the cytosolic compartment of the cell and consists of a single polypeptide chain with apparent Mr of 56,000 and pl of 5.6 (2, 3). In the rat, this enzyme has been identified only in liver, which is its main site of expression, and in the islet of Langerhans of the pancreas (3). Glucokinase is considered to play a key regulatory role in glucose metabolism in these two tissues (4, 5). Both the liver cell and the beta cell of the islet of Langerhans appear to be freely permeable to glucose, so that the intracellular concentration of glucose in these cells constantly mirrors the extracellular concentration (6, 7). Furthermore, the apparent Km of glucokinase for glucose is about 6 mM, a value corresponding to the physiological concentration of glucose in plasma (8). Therefore, the hepatocyte and the beta cell of the islet are able to respond to fluctuations of the blood glucose level by parallel changes in the rate of glucose phosphorylation, owing simply to substrate concentration effects on the rate of the glucokinase reaction.

Glucokinase activity appears in the liver of the rat during the third week of postnatal development, concomitantly with the shift in food intake from maternal milk to laboratory chow (9, 10). In the adult rat, glucokinase activity in the liver is high in carbohydrate-fed animals and low during fasting or in diabetes (11, 12). The latter observation suggests a role for insulin in the regulation of this enzyme, a suggestion supported by experiments with cultured hepatocytes (13). Changes in assayable glucokinase activity reflect changes in enzyme amount, which appear to result mainly from a stimulation or repression of the rate of synthesis of the protein (14-16). More recently, glucokinase mRNA assayed by translation in cell-free systems was found to increase during the fasting-refeeding transition or in diabetic rats injected with insulin (17, 18). Besides insulin, glucose itself or its metabolites may be involved as positive effectors for the induction of glucokinase (1, 16). Moreover, the glucocorticoids and thyroid hormones have been incriminated as permissive factors (19, 20). Other data showed that the administration of dibutyryl cAMP to well-fed animals caused a rapid fall in translatable glucokinase mRNA (18).

Two lines of investigation have to be pursued in order to elucidate the molecular mechanisms responsible for the tissue-specific expression of the glucokinase gene and its multihormonal regulation. One line is the design of a cell culture system for delineating the role of individual effectors in the regulation of the enzyme. The second is the isolation of nucleic acid probes for studies of the structure and regulated expression of the glucokinase gene. As a contribution in this direction, we now describe the molecular cloning of a cDNA encoding glucokinase. We also present experiments in which the cloned cDNA was used to measure the abundance of glucokinase mRNA in the livers of rats at various stages of postnatal development and in adults during the fasting-refeeding transition.

EXPERIMENTAL PROCEDURES

Animals—Male Wistar rats were used in all experiments. Newborn animals were left with the mother until day 22 after birth. Thereafter, the animals were fed a standard laboratory chow containing 57% carbohydrate, 10% lipid, and 33% protein in terms of energy supply. Rats weighting 140 to 220 g were used for the fasting-refeeding experiments. The rats were deprived of food for 27 h. They were next refed by gavage with 6 ml/rat of a 20% (w/v) D-glucose solution.

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6032
Liver Glucokinase mRNA Assayed with Cloned cDNA Probe

Lever was homogenized in Teflon-glass homogenizers in a buffer containing 40 mM Tris/HCl, pH 7.0, 0.15 M KCl, 5 mM EDTA, 4 mM MgCl₂, and 2 mM dithiothreitol. Homogenates were centrifuged at 60,000 × g for 75 min and the clear supernatant (cytosol) was assayed for enzyme activity immediately or after freezing and storage at −75°C. The spectrophotometric assay was performed at 30°C essentially as described (21), except that the coupling enzyme glucose-6-phosphate dehydrogenase was from Leuconostoc mesenteroides and that NAD rather than NADP was used as coenzyme. A unit of the free polyribosomes in the supernatant, as described by Ramsey in addition to water and allowed to eat food pellets placed in cages were placed in a dark room for the period of refeeding. The glucokinase reaction was incubated with a sheep antiserum to rat liver glucokinase under conditions described in detail previously (3). The only modification was the use in the third step of the procedure of goat antibodies to rabbit immunoglobulins conjugated to alkaline phosphatase instead of peroxidase. The first round of screening was done after 30 min. Positive plaques were picked and phages were plated at a density of 150/8.5-cm dish. Positive plaques were subjected to one more round of screening after plating phages at a density of 60/8.5-cm dish. This process led to the isolation of a putative glucokinase cDNA clone termed pUC-GK1.

Liver Glucokinase mRNA Assayed

The antiserum to glucokinase was a generous gift of Dr. Hans J. Seitz of the University of Hamburg School of Medicine and has been termed pUC-GK1, was grown in host strain JM83 for large-scale production of fusion protein containing putative glucokinase epitopes. DNA. A resulting recombinant plasmid containing the 1800-bp insert, described by Gubler and Hoffman (28), except that Escherichia coli DNA ligase was not included in the reaction. This procedure yielded 3.5 µg of double-stranded cDNA from 10 µg of RNA. Double-stranded cDNA was next made blunt-ended by treatment with T4 DNA polymerase, methylated with EcoRI methylase as described by Parnes et al. (34). Hybridization and elution of mRNA was performed exactly as described, with an input of 5 µg of size-selected poly(A)-containing RNA.

Construction of cDNA Library in Phage λ gt11—A selected size fraction of poly(A)-containing RNA from free polyribosomes was used as template for synthesis of cDNA. Synthesis of the second DNA strand was accomplished with RNase H and DNA polymerase I, essentially as described by Hubler and Hoffman (28), except that.

Hybrid Selection of mRNA—Three µg of plasmid pUC-GK1 DNA was denatured and bound to a 3 × 4-mm filter of nitrocellulose as described by Parnes et al. (34). Hybridization and elution of mRNA was performed exactly as described, with an input of 5 µg of size-selected poly(A)-containing RNA.

To prepare cDNA insert of pUC-GK1 DNA was isolated after electrophoresis in an 0.8% agarose gel by incision on a DEAE-membrane as described by Lizardi (35). Radioactive labeling of the cDNA was done with [α-32P]dCTP by the method of

The abbreviations used are: SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-D-thiogalactopyranoside; bp, base pair; kb, kilobase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Liver Glucokinase mRNA Assayed with Cloned cDNA Probe

Feinberg and Vogelstein (36, 37), using a commercial system under conditions described by the manufacturer. The specific activity of the probes was 1.7 to 2.0 × 10^6 dpm/μg DNA.

Electrophoresis and Blotting of RNA—Electrophoresis was done in 1% agarose gels after denaturation of the RNA samples with glyoxal and dimethyl sulfoxide, as described by McMaster and Carmichael (38). Prior to transfer of RNA, gels were stained with acridine orange and destained to localize M, markers. Subsequently, RNA bands were transferred electrophoretically to a nylon membrane. Dot blots of probes was 1.7 to 2.0 mRNA as starting material. Since cytosol proteins are synthesized in vitro, rabbit reticulocyte lysate, EcoRI-digested alkaline phosphatase-treated λ gt11, in vitro packaging extract, and E. coli strain Y1090 (r−) and Y1089 (r−) from Promega Biotech (Madison, WI) L-[35S]methionine (specific activity 1130 Ci/mmol) and GeneScreen nylon membrane from Du Pont-New England Nuclear; Triton X-405 from Fluka (Buchs, Switzerland); cDNA synthesis system, multiprime labeling system, and [α-32P]dCTP (specific activity > 3000 Ci/mmol) from Amersham Corp.; EcoRI methylase and EcoRI endonuclease from New England Biolabs; nitrocellulose and DEAE-membranes from Schleicher & Schuell; and glucose-6-phosphate dehydrogenase and IPTG from Boehringer Mannheim. The recombinant β-actin plasmid (pAL41) was cloned by Alonso et al. (39).

RESULTS

Construction and Screening of cDNA Library—Glucokinase is a scarce protein of liver. We estimated previously from immunoblotting data that this enzyme represents 0.1% of total soluble protein in the livers of glucose-fed rats (3). Assuming some relationship between the abundance of a specific protein and that of its mRNA, one would expect glucokinase mRNA to belong to the low abundance class of mRNAs. In order to increase the percentage of glucokinase cDNA in our library, we used RNA enriched in glucokinase mRNA as starting material. Since cytosol proteins are synthesized on free polyribosomes, poly(A)-containing RNA was isolated from free polyribosomes from livers of glucose-fed rats. This RNA was then size-fractionated in sucrose gradients and RNA from each fraction was translated in a rabbit reticulocyte lysate system. The total translation products encoded by the various size fractions and the corresponding glucokinase immunoprecipitates are shown in Fig. 1. Functional glucokinase mRNA was enriched in the RNA fraction with an average sedimentation coefficient of 21 S. From this experiment, we predicted that glucokinase mRNA should be about 2.4 kb in length (40).

Complementary DNA was synthesized from the 21 S RNA fraction and used for the construction of a cDNA library in the vector λ gt11, as described under “Experimental Procedures.” Sixty thousand recombinant phages from the original library, without prior amplification, were screened immunologically for the production of putative β-galactosidase-glucokinase fusion proteins. A positive clone was isolated by three successive rounds of screening and amplified by the plate lysate method for further analysis. This clone is henceforth designated λ-GK223. Upon digestion with EcoRI nuclease, the phage DNA was found to contain an insert of 1800 bp.

Characterization of Fusion Protein of λ-GK223—The high frequency lysogenization host strain Y1089 (r−) was infected with λ-GK223 and used as a source of fusion protein for immunochromatographic characterization. Protein extracts from IPTG-induced and noninduced cells were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with the antiserum to glucokinase. As shown in the Coomassie Blue-stained gel in Fig. 2A (lanes 4 and 5), cells infected with λ-GK223 displayed an IPTG-inducible protein with an apparent Mr of 155,000. The immunoblot (Fig. 2B) demonstrates that this inducible protein reacted specifically with the glucokinase antiserum. Since the β-galactosidase moiety in a recombinant λ gt11 phage accounts for 114,000 daltons of molecular mass, we concluded that the fusion protein of λ-GK223 contained a foreign polypeptide sequence accounting for 41,000 daltons. A parallel experiment with a negative recombinant phage from the cDNA library was performed. As may be seen in Fig. 2 (lanes 2 and 3), this phage encoded an IPTG-inducible fusion protein with an apparent Mr of 125,000. This protein did not stain in the glucokinase immunoblot.

To prove that the fusion protein of λ-GK223 does contain one or more authentic glucokinase epitope(s), antibodies from the crude antiserum to glucokinase were affinity purified with the phage protein immobilized on nitrocellulose and next reacted with a Western blot of total protein from rat liver. For affinity purification, a preparative blot of total protein from lysogenic cells was incubated with antiserum to glucokinase. A transversal strip of nitrocellulose was cut at the level of the fusion protein, and bound antibodies were eluted by treatment of the strip with acid. As shown in Fig. 3B (lanes 1 and 2), the epitope-selected antibodies reacted with a single hepatic protein comigrating with purified glucokinase. This was true irrespective of the protein load on the gel. On the contrary, a duplicate blot challenged with the crude antiserum displayed

![Figure 1](image-url)
Liver Glucokinase mRNA Assayed with Cloned cDNA Probe

Hybrid Selection of Glucokinase mRNA—To identify the cloned cDNA by hybrid-selected translation, the 1800-bp cDNA insert of clone λ-GK223 was subcloned in the EcoRI site of the pUC13 plasmid. A recombinant plasmid containing the 1800-bp insert and termed pUC-GK1 was used for hybrid selection of mRNA. The latter was performed with the 21 S RNA fraction of poly(A)-containing RNA from which the cDNA library was constructed. The hybrid-selected mRNA species as well as input RNA were translated in the rabbit reticulocyte lysate system, with results shown in Fig. 4. The hybrid-selected message directed the synthesis of a single polypeptide product, which comigrated in a SDS-polyacrylamide gel with pure rat liver glucokinase and bound to antibodies directed against this enzyme. Note that the few extraneous products seen in the lane for hybrid-selected mRNA (lane 3) are endogenous to the reticulocyte lysate, since they are also visible in the absence of added mRNA (lanes 1 and 4). The data presented above provide firm evidence for the identification of a glucokinase-specific cDNA.

Appearance of Glucokinase mRNA in Rat Liver during Ontogenic Development—Glucokinase belongs to a group of liver enzymes appearing in the third week of postnatal development in the rat, at the time of weaning (9, 10). We asked whether the emergence of glucokinase activity reflected the initial appearance of glucokinase mRNA during ontogeny. To this end, the cDNA insert of pUC-GK1 was used as a probe to estimate the amount of glucokinase mRNA in the livers of additional nonspecific bands, especially in the lane that was purposefully overloaded with liver protein (Fig. 3A, lane 2), as already noted in a previous publication (3).

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suckling and weaned rats. As shown in Fig. 5, the labeled cDNA hybridized to an mRNA molecule 2.4 kb in length, in good agreement with the size of glucokinase mRNA deduced from the translation experiment of Fig. 1. Glucokinase mRNA was undetectable in rats of 1 and 10 days of age, even after prolonged exposures of Northern blots. The message could be detected in 14-day-old animals and subsequently accumulated to high levels between days 21 and 31 after birth (Fig. 5). Quantitative data on glucokinase mRNA levels obtained by scanning autoradiograms of Northern blots with a densitometer are presented in Table 1, together with the glucokinase activity measured by enzyme assay in the liver of the same animals. Clearly, the rise of glucokinase activity occurs concomitantly with the accumulation of specific mRNA, suggesting that the appearance of enzyme activity reflects the turning on of specific gene transcription. In addition, these results provide further support for the identification of the cloned cDNA as a glucokinase gene sequence.

Effect of Glucose Refeeding on Abundance of Glucokinase mRNA in Rat Liver—We next assessed the effect of glucose refeeding after a fast on the amount of glucokinase mRNA in the liver of adult rats. As shown by the autoradiogram of the RNA blot in Fig. 6A, glucokinase mRNA was undetectable in a fasting rat, appeared at 1 h after refeeding, and accumulated to much higher levels at 3 and 6 h. Interestingly, an additional minor band corresponding to a larger RNA of 3.6 kb was visible 3 h after glucose administration. As a control, a similar RNA blot was hybridized to a cloned β-actin cDNA probe. In contrast to the dramatic accumulation of glucokinase mRNA, the actin message exhibited only a small increase in amount 1 h after the glucose load and remained virtually stable thereafter (Fig. 6B).

Dot blot assays were done to follow the time course of change in glucokinase mRNA during glucose refeeding. The relative level of glucokinase mRNA in total liver RNA increased an average of 30-fold at 1 h after a glucose load, 60-fold at 3 h, and 85-fold at 6 h. Thereafter, in spite of the continued availability of dietary glucose, the amount of mRNA returned toward lower levels so that by 18 h of refeeding the mRNA was increased only about 10-fold above the fasting level (Fig. 7). From these experiments, we concluded retrospectively that the initial choice of a 15-h refeeding period before isolation of RNA as starting material for cDNA cloning was not the most judicious. For reference, β-actin mRNA was also quantified in these time course experiments. We noted a comparatively small, but reproducible 2- to 3-fold increase in the level of this message during a 6-h period after glucose administration (Fig. 7). It should be pointed out that the height of the bars in the figure does not reflect the abundance of one message relative to the other.

![Fig. 5. Appearance of glucokinase mRNA in liver during postnatal development.](image)

**Table 1**

Postnatal development of glucokinase mRNA and enzyme activity in rat liver

Glucokinase mRNA was quantified by densitometer scanning of autoradiograms similar to that shown in Fig. 5. The amount of mRNA was taken as the area under the peak corresponding to the mRNA and is expressed in arbitrary units. Glucokinase activity in liver cytosol from the same animals was assayed as described under "Experimental Procedures" and is expressed in milliunits/mg of cytosol protein. Data are the average of measurements for two animals at each age.

| Postnatal age (day) | Glucokinase mRNA amount (densitometer unit) | Glucokinase enzyme activity (milliunits/mg protein) |
|---------------------|---------------------------------------------|---------------------------------------------|
| 1                   | 0                                           | 0.0                                         |
| 10                  | 0                                           | 0.0                                         |
| 14                  | 5                                           | 0.8                                         |
| 22                  | 69                                          | 11.4                                        |
| 23                  | 132                                         | 15.7                                        |
| 31                  | 228                                         | 20.9                                        |
| 60                  | 215                                         | 29.8                                        |

![Fig. 6. Effect of glucose refeeding on glucokinase and β-actin mRNAs in liver.](image)
In this work we have isolated and characterized a cDNA molecule coding for glucokinase, a key regulatory enzyme of carbohydrate metabolism. The specific cDNA was initially isolated in a recombinant phage from a cDNA library from rat liver mRNA constructed with the expression vector λ gt11. The identity of the cloned cDNA was established by the following criteria. In the λ gt11 cloning vehicle, the cDNA was shown to code for a polypeptide moiety bearing epitopes specific to liver glucokinase. When subcloned in the pUC13 plasmid, the cDNA insert hybridized specifically with a mRNA from rat liver that directed the synthesis of a protein identified electrophoretically and immunologically as glucokinase. Furthermore, the cloned cDNA was found to hybridize to a mRNA that appeared in liver at the same time during ontogeny as glucokinase activity. Finally, the cognate mRNA was induced in rat liver after glucose refeeding, as would be anticipated for the glucokinase mRNA from earlier in vitro translation data (17, 18). Thus, all the available evidence shows that the cloned cDNA is a glucokinase gene sequence, although a formal proof will have to await primary structure information for both gene and gene product.

The major species of glucokinase mRNA in rat liver is 2.4 kb in length. The apparent M, of glucokinase deduced from SDS-polyacrylamide gel electrophoresis is 56,000 (3), requiring a coding sequence 1.5 kb in length. Therefore, the glucokinase message comprises about 900 untranslated nucleotides. The cDNA insert of λ-GK223 and pUC-GK1 is 1,800 base pairs in length, that is 75% of full length. The foreign polypeptide moiety of the fusion protein of λ-GK223 accounts for 41,000 daltons in molecular mass, i.e. for 1,070 out of the 1,800 base pairs of the cDNA. Thus, the present glucokinase cDNA appears to contain most of the nontranslated nucleotides of the mRNA. It is likely that the cDNA copy lacks sequences located at the 5' end of the mRNA molecule, including a segment coding for the first hundred amino acids of glucokinase.

The glucokinase gene is not expressed at a significant level in liver during the first 10 days of extraterine life in the rat. The enzyme mRNA can first be detected around day 14 after birth and increases more than 40-fold from this age to day 31. The level of glucokinase in liver measured by enzyme assay follows a similar time course, with enzyme activity appearing at day 14 and increasing about 30-fold within the following 2 weeks. These data strongly suggest that the stimulus responsible for the developmental emergence of glucokinase acts by turning on the transcription of the gene. The nature of this stimulus has not been fully elucidated, but the event appears to be related to the dietary shift from maternal milk, which is rich in fat, to laboratory chow, which is rich in carbohydrate. The importance of diet composition was underlined in a recent study by Haney et al. (41), in which premature induction of glucokinase was elicited on the second day of neonatal life by administration of a carbohydrate-rich formula to newborn rats. These experiments further show that the competence of the liver to express the glucokinase gene is already established 2 weeks before the occurrence of the final triggering event during development. The respective roles of nutrients and hormones for de novo induction remain to be determined. At any rate, induction studies during the neonatal period should provide important new insight into the regulation of the expression of the glucokinase gene.

In the adult animal, glucose intake after a period of fasting causes a rapid and massive accumulation of glucokinase mRNA in the liver. The maximal rate of mRNA build-up was found to be relatively short-lived, not exceeding 6 h after the glucose load. Neither the magnitude nor the time course of increase in glucokinase mRNA were predicted from earlier estimates of glucokinase mRNA levels obtained by cell-free translation assay (17, 18). It is probable that translational assays underestimated changes in glucokinase mRNA because of problems with specificity in the immunoprecipitation of the glucokinase product. We do not know whether the dramatic increase in enzyme mRNA results from a transcriptional effect on the gene and/or from a stabilization of nuclear processing intermediates or of the mature RNA. In this context, the appearance of a 3.6-kb hybridizable RNA species at the time of most intense mRNA accumulation is interesting and suggests the possibility of build-up of a processing intermediate due to stimulated transcription. It is also possible that the longer RNA species is a second form of mature mRNA, resulting from alternative exon splicing or from the use of a more distal polyadenylation site in the gene.

The effect of refeeding on the mRNA for β-actin was unexpected. Although small in comparison with the increase in glucokinase mRNA, this effect points to a pleiotropic alteration in the expression of the genome in liver during the fasting-refeeding transition.

The findings reported here raise a number of questions regarding the signaling mechanism for the dietary effect of glucose on glucokinase mRNA. Is insulin action involved? Is

2 Data from translation assays consistent with the time course shown here were recently obtained in another laboratory (H. J. Seitz, personal communication).
Liver Glucokinase mRNA Assayed with Cloned cDNA Probe

there a direct effect of glucose or one of its metabolites in the hepatocyte? Is the induction actually the result of a derepression mechanism, as has recently been suggested in the case of the gene coding for L-type pyruvate kinase (42, 43)? The availability of a glucokinase cDNA probe now allows us to approach these issues directly.

A final point from this work deserves notice. It is known that glucokinase activity increases, at most, 3-fold within 24 h after refeeding glucose to fasting rats (11, 12). In this regard, the increase in enzyme mRNA occurring during the same time period appears entirely disproportionate. This observation is paradoxical, but not without precedent. Nutritional effects on the abundance of L-type pyruvate kinase mRNA are also known to outweigh changes in the amount of pyruvate kinase itself (42-44).

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