The Amino Acid Sequence of Rat Liver Glucokinase Deduced from Cloned cDNA*

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Rat liver glucokinase (ATP:d-hexose 6-phosphotransferase, EC 2.7.1.1) was purified to homogeneity, cleaved, and subjected to amino acid sequence analysis. Forty-five percent of the protein sequence was obtained, and this information was used to design oligonucleotide probes to screen a rat liver cDNA library. A 1601-base pair cDNA (GK1) contained an open reading frame that encoded the amino acid sequences found in the peptides used to generate the oligonucleotide probes. A second cDNA was subsequently identified (GK2), which is 2346 base pairs long and corresponds to nearly the entire glucokinase mRNA. Blot transfer analysis of hepatic RNA showed that glucokinase mRNA exists as a single species of about 2400 nucleotides. Four hours of insulin treatment of diabetic rats resulted in a 30-fold induction of this mRNA.

GK2 has a long open reading frame which, with the known partial peptide sequence, allowed us to deduce the primary structure of glucokinase. The enzyme is composed of 465 amino acids and has a mass of 51,924 daltons. Glucokinase has 53 and 33% amino acid sequence identities with the carboxyl-terminal domains of rat brain hexokinase I and yeast hexokinase, respectively. If conservative amino acid replacements are also considered, glucokinase is similar to these two enzymes at 75 and 63% of positions, respectively. The putative glucose- and ATP-binding domains of glucokinase were identified, and these regions appear to be highly conserved in the hexokinase family of enzymes.

Glucokinase (ATP:d-hexose 6-phosphotransferase, EC 2.7.1.1), because it is expressed only in liver and pancreatic β-cells, is a unique member of the family of mammalian hexokinases (1). Glucokinase plays a key role in glucose homeostasis. Because of its high Km (5 mM) and specificity for glucose and the lack of product inhibition by glucose 6-phosphate, it ensures a gradient for glucose entry into the hepatocyte (2). This is particularly important in the post-prandial state when plasma glucose levels are elevated. Pancreatic β-cell glucokinase is probably equally important because flux through the glycolytic pathway is thought to be involved in glucose-mediated insulin release (3).

Hepatic glucokinase, like other critical metabolic enzymes, is regulated by a variety of dietary and hormonal factors, all of which appear to alter the rate of synthesis of the protein (4). Glucokinase is increased after feeding (particularly a carbohydrate-rich diet) and is decreased by fasting (5). These changes are probably mediated by insulin and glucagon. Insulin, whose levels are increased in the post-prandial state, presumably accounts for the feeding-induced change in glucokinase since it also restores to normal the low glucokinase activity found in diabetic animals (2). Glucagon, elevated in fasting animals, has exactly opposite effects (2). The actions of glucagon appear to be mediated by cAMP, which itself decreases glucokinase when injected into animals (6). Changes of the rate of synthesis of glucokinase have been correlated with corresponding alterations of mRNA^*GK (6–9) and with rates of transcription of the glucokinase gene (9).

The other hexokinases found in liver (types I–III; glucokinase is sometimes referred to as type IV) have low Km values for glucose (~20–130 μM), catalyze the phosphorylation of a variety of hexoses, are subject to feedback inhibition by glucose 6-phosphate, and are not regulated by insulin or glucagon (1, 2). These hexokinases have a molecular mass of ~100 kDa, whereas glucokinase and yeast hexokinases PI and PII (like glucokinase, not subject to feedback inhibition by glucose 6-phosphate) have a molecular mass of ~50 kDa. All are postulated to be members of a family in which the larger mammalian enzymes arose from a duplication and fusion event from an ancestral gene similar to the yeast or glucokinase genes. This would account for the suspected similarity in amino acid sequence (10) and in the antigenic cross-reactivity that has been demonstrated between yeast and mammalian hexokinases (11). These features, coupled with the relative scarcity of glucokinase in rat liver, have heretofore precluded efforts to purify glucokinase in amounts necessary for detailed analysis. It therefore has not been possible to deduce the structures of the glucokinase mRNA, and protein.

The strategy we employed to obtain a cDNA^*K involved...
first purifying glucokinase, establishing its authenticity by its known kinetic parameters, and sequencing several peptides. Knowledge of the partial amino acid sequence allowed us to construct oligonucleotide IV probes. Because of the possibility that any given peptide could be similar to a region in one of the other hexokinases, these synthetic probes were used to screen cDNA libraries only after they had been used to demonstrate the expected regulation of mRNA

\[ \text{GK}^{\text{rK}} \] by insulin, diabetes, fasting, or refeeding. This approach led to the isolation of a series of authentic cDNA\n
\[ \text{GK}^{\text{rK}} \] molecules from which we were able to deduce the amino acid sequence of glucokinase. This information allowed us to compare the overall structure of glucokinase to other members of the hexokinas family and to draw certain inferences about the nature of the glucose- and ATP-binding sites in the molecule.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Purification and Characterization of Glucokinase**—Rat liver glucokinase was purified to homogeneity (Fig. 1) by the procedure outlined in the Miniprint. Our modifications to the protocols previously employed included a 5% polyethylene glycol precipitation step to remove contaminating protein before the column steps; batchwise binding and elution from DEAE-cellulose; the use of blue Sepharose in two different buffers, first to remove hydrophobic proteins and then to remove nucleotide-binding proteins; and one to two passes over an analytical high pressure liquid chromatography resin. These changes resulted in a procedure that yielded sufficient glucokinase to allow for the first determination of the partial amino acid sequence of this protein. The 21,000-fold purification required suggests that glucokinase accounts for approximately 0.005% of rat liver protein (Table 1). The purified protein had a molecular mass of ~50 kDa, showed selective phosphorylation of glucose with a \( K_m \) of 5.0 mM, and had a specific activity of 180 units/mg. These parameters matched those found by other investigators (Tables II and III) (1, 2, 5, 12, 13, 21-27). The combination of a high \( K_m \) for glucose and the lack of phosphorylation of \( N \)-acyethylglucosamine excludes the possibility that the 50-kDa protein purified was \( N \)-acyethylglucosamine kinase, an enzyme that can bind to the \( N \)-\((6\text{-aminooxynoyl})-2\text{-amino-2-d-glucopyranose-Sepharose affinity resin and copurify with glucokinase (28).}

**Design of Oligonucleotide Probes and cDNA Cloning of Glucokinase**—Purified glucokinase was digested with cyanogen bromide, protease V8, or endoprotease Lys C to generate peptides for amino acid sequence analysis. Forty-five percent of the amino acid sequence of glucokinase was obtained by analyzing 33 peptides. Several different oligonucleotide probes, ranging in size from 23 to 65 nucleotides, were designed from the peptide sequences by utilizing a combination of known codon usage and mixed oligonucleotide methods. Inosine was included in some of the probes at nucleotide positions where degeneracy of the code did not clearly favor a particular nucleotide (29). These probes were used to analyze hepatic poly(A)* RNA isolated from diabetic rats or diabetic rats treated with insulin. Four of these oligonucleotides, CNB-3B, CNB-2B, CNB-1B, and V8-5B-5 (Table IV), hybridized to an ~2.4-kb mRNA in RNA isolated from the liver of diabetic rats treated with insulin (Fig. 2). None of the probes hybridized to mRNA isolated from the untreated diabetic rats. The 2.4-kb size of the mRNA is too small to encode the 100-kDa hexokinases, and mRNAs coding for these enzymes would not be expected to respond to insulin treatment (2). These results suggested that the oligonucleotides were specifically hybridizing to mRNA

\[ \text{GK}^{\text{rK}} \].

**Regulation of mRNA

\[ \text{GK}^{\text{rK}} \] by Insulin**—Four hours after the injection of insulin into diabetic rats, mRNA

\[ \text{GK}^{\text{rK}} \] was increased about 30-fold, as quantitated by hybridization using a GK1 probe. The induction by insulin was specific for mRNA

\[ \text{GK}^{\text{rK}} \] as no change was seen when a similar blot was probed with a calsemin cDNA (Fig. 5) (30). Furthermore, another blot of the same RNA sample probed with a phosphoenolpyruvate carboxykinase cDNA showed the inhibition expected as the result of insulin treatment (Fig. 5) (19).

**Isolation of Complete Coding Sequence for Glucokinase**—Comparison of the length of GK1 with that of mRNA

\[ \text{GK}^{\text{rK}} \] (1601 versus 2400 bases; Figs. 2 and 5) and comparison of the length of the open reading frame in GK1 with the appropriate number of amino acids required to code for a protein of 50 kDa (263 versus ~475) indicated that GK1 did not include the entire coding sequence of glucokinase. Furthermore, GK1 did not contain either a polyadenylation signal or a poly(A) tract, indications that the 3'-region of the cDNA was incomplete. In order to obtain a longer clone, a new cDNA library was constructed using poly(A)* RNA isolated from diabetic rats treated with insulin for 4 h. Genomic DNA probes,

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1 Portions of this paper (including "Experimental Procedures," Figs. 1 and 2, and Tables I-IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
available from the characterization of the glucokinase gene, were used to simplify library screening. The DNA fragments selected for probes contained exons that encoded glucokinase peptides V8-3 and CNB-4 (Fig. 4); therefore, any clones were used to simplify library screening. The DNA fragments cDNA inserts from all three clones were about 2400 bp in length (data not shown). One, GK.Z2, was slightly longer than library enriched for glucokinase cDNA were screened. The selected for probes contained exons that encoded glucokinase.

**Fig. 4. DNA and amino acid sequence of rat liver glucokinase.** Bases are numbered from the 5'-end of the cDNA sequence in GK.Z2. The in-phase termination codon is designated by underlines and the polyadenylation signal is underscored. Amino acids are numbered starting with the most 5'-ATG. Amino acids identified by peptide sequencing are indicated by overlines and the peptide name is shown above each line.

sequence found in GK.Z2A (data not shown). GK.Z2 contains 2346 bp plus a 54-base poly(A) tract (Figs. 3 and 4). The sequence ATTAAAA, located 18 nucleotides upstream from the poly(A) tract, is the probable polyadenylation signal (31).

Several criteria were used to select the translation initiation codon. First, the site selected is in good agreement with the initiation site consensus sequence described by Kozak (32). Second, the calculated protein mass of 51,924 daltons compares with the observed ~50-kDa mass of glucokinase (Fig. 1). Third, all of the peptides obtained from purified glucokinase (about 45% of the protein) are contained in the open reading frame of GK.Z2. Fourth, the size of the glucokinase cDNA (2346 bp) and a poly(A) tail of average length (about 100 nucleotides) predict an mRNA length of about 2.4 kb. This size corresponds closely with that observed on blot

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3 M. Magnuson et al., manuscript in preparation.
Fig. 5. RNA blot transfer analysis. Poly(A)" RNA was isolated from diabetic rats (lanes D) or diabetic rats treated with insulin for 4 h (lanes I). RNA was size-fractionated in the agarose/formaldehyde gel system described for Fig. 2, blot-transferred to nitrocellulose, and then hybridized with randomly primed cDNA probes. Left, 2 μg of poly(A)" RNA was probed with phosphoenolpyruvate carboxykinase (PEPCK) cDNA PC116 (7); middle, 20 μg of poly(A)" RNA was probed with GK1; right, 20 μg of poly(A)" RNA was probed with calmodulin cDNA pCAM-9H8.

not obtained, presumably because it is blocked (data not shown), the exact assignment of the initiating codon remains tentative.

Comparison of Glucokinase with Yeast Hexokinase and Carboxyl-terminal Region of Rat Brain Hexokinase I—We compared the predicted protein sequence for glucokinase with that of yeast hexokinase (33, 34) and the carboxyl-terminal region of rat brain hexokinase I (35). Glucokinase shared 53% identity with rat brain hexokinase I and 33% amino acid sequence identity with yeast hexokinase PI as determined by the DNAStar program AALIGN (36), which utilizes the PAM250 matrix described by Lipman and Pearson (37). This program compares closely related proteins and determines the probability that a given amino acid would be replaced by any other amino acid over a fixed period of time. Empirical studies have shown that this matrix allows for the detection of more distant relationships than when only identities or minimum base changes are considered. In addition to sequence identity, there are 116 residues in the rat brain carboxyl-terminal portion of hexokinase I that represent conservative amino acid replacements when compared with glucokinase and 139 residues in glucokinase that represent conservative amino acid replacements when compared with yeast hexokinase PI. Considering these changes, glucokinase is identical or similar to the carboxyl-terminal portions of brain hexokinase I and yeast hexokinase PI at 75 and 63% of positions, respectively.

**DISCUSSION**

Glucokinase mRNA—The protocol employed permitted us to obtain amounts of highly purified glucokinase sufficient for the analysis of a significant portion (~45%) of the amino acid sequence. This sequence information led to the construction of oligonucleotide probes that resulted in the isolation of an authentic cDNA (7). The availability of the nearly full-length cDNA, GK.Z2, allowed us to derive the structure of an authentic cDNA (5). The availability of the nearly full-length cDNA, GK.Z2, allowed us to derive the structure of an authentic cDNA (5). The portion of mRNA used we have cloned is composed of 2,346 nucleotides, exclusive of the poly(A) tail. It contains an open reading frame of 1,395 nucleotides flanked on the 3'-end by 835 nucleotides and on the 5'-end by 116 nucleotides (GK.Z2). The mRNA codes for a protein of 465 amino acids with a calculated molecular mass of 51,924 Da. The methionine chosen as the putative initiation site fits more closely with a Kozak (32) consensus than does a second methionine located 2 amino acids away.

The 30-fold induction of mRNAGK resulting from insulin treatment of diabetic rats substantiates the observations of Spence (7) and Sibrowski and Seitz (6), who quantitated mRNA activity using in vitro translation of mRNA with immunoprecipitation of the labeled glucokinase by polyclonal antibodies. Our data also are in agreement with those of Lyenjadjan et al. (9), who showed that insulin treatment of diabetic rats stimulated the expression of a 2.4-kb mRNA. Lyenjadjan et al. used a 1700-hp probe isolated from a Agt11 plasmid by an antibody directed against glucokinase (8). It appears that each of our cDNAs hybridizes to an mRNA of the same size and that this mRNA is regulated in a similar fashion.

Amino Acid Homology—The availability of sequence information for glucokinase made it possible to test directly the hypothesis that the hexokinases share sequence homology. Mammalian glucokinase and yeast hexokinases PI and PII are each composed of a single polypeptide chain of approximately 50 kDa, and they are not subject to allosteric regulation by glucose 6-phosphate. In contrast, mammalian hexokinases I–III consist of a single polypeptide chain of molecular mass...
Fig. 7. Putative glucose- and ATP-binding domains. Top, the region depicted is thought to be important for glucose binding to the yeast hexokinases (39, 40). The amino acid sequences of glucokinase, rat brain hexokinase I, and yeast hexokinases P1 and PII were aligned by the DNASTAR program ALIGEN (36). The amino acid sequence of peptides 5 and 6 from rat skeletal muscle hexokinase III was matched by inspection (41). Bottom, the core sequence of a putative ATP-binding domain of the protein kinase catalytic subunit from bovine skeletal muscle and other protein kinases (41-44) was matched by inspection with the amino acid sequences of the hexokinases. An essential lysine located 11 residues away from the core sequence in the protein kinase was aligned, by inspection, with a lysine in each of the hexokinases.

~100 kDa and are allosterically inhibited by the reaction product, glucose 6-phosphate (21). The evolutionary relationship between these proteins was first hypothesized when antigenic cross-reactivity between yeast and mammalian hexokinases was determined using polyclonal antibodies (11). This concept was further strengthened when peptide maps of hexokinases I and III established the existence of two separate domains in the low $K_m$ enzymes. An amino-terminal domain contains the glucose 6-phosphate-binding or allosteric site (38), and a carboxyl-terminal domain contains both the glucose and ATP-binding sites (38). As shown in Fig. 6, there is a high degree of sequence identity among yeast hexokinase, glucokinase, and the relevant regions of at least one mammalian hexokinase (brain, type I). The hypothesis that the 100-kDa hexokinases evolved as a result of a gene duplication and fusion event involving a lower molecular mass hexokinase, where one catalytic site was retained and the other evolved into a regulatory site, would predict this kind of similarity at the carboxyl-terminal end of the molecules. Sequence analysis of the amino-terminal portion of the high molecular mass mammalian hexokinases is the next critical step in testing this hypothesis.

Glucose-binding Domain—It is apparent that several regions of glucokinase show a high degree of sequence conservation with other hexokinases (Fig. 6). We attempted to relate these regions to functional domains that have been defined in the yeast and brain hexokinases. Determination of the tertiary protein structure of the yeast hexokinase isoenzymes in the presence of glucose and glucose analogs identified specific amino acid residues that participate in the binding of glucose (39, 40). Ser$^{186}$, Asp$^{211}$, Glu$^{290}$, and Glu$^{392}$ all hydrogen bond to the hydroxyl groups of glucose (40). All of these residues appear to be conserved and in similar locations in rat liver glucokinase, mammalian hexokinase III (41), and the carboxyl-terminal region of rat brain hexokinase I (35) (Fig. 6). Although the basic features of the glucose-binding domain are present in all of the hexokinases, other amino acid residues must be involved in glucokinase, which binds glucose with a much lower affinity than do the mammalian hexokinases (2).

ATP-binding Domain—Various techniques have been used to define the ATP-binding site in several proteins (40-44). A similar region is found in glucokinase (Fig. 6). The corresponding sequences of bovine heart protein kinase (residues 37-61), glucokinase (residues 78-102), rat brain hexokinase I (C-domain), and yeast hexokinase (86-111) are shown in detail in Fig. 7. This putative ATP-binding domain has several conserved features, the most invariant of which is a lysine located 11–14 residues in the carboxyl-terminal direction, from the core sequence. There are no data that support the hypothesis that these residues participate in the binding of ATP to glucokinase, but the highly conserved sequence certainly suggests that this is possible.

Concluding Remarks—The isolation of an authentic cDNA for hepatic glucokinase makes further studies of the structure and function of this enzyme feasible. Furthermore, since mRNA$^{GK}$ is regulated by insulin and glucagon in the liver, a cDNA$^{GK}$ enables us to isolate and characterize the DNA sequences in the gene that might be responsible for this regulation. Further studies in this direction are now underway and may contribute to our understanding of the function and regulation of this important enzyme.

Acknowledgments—We thank Mark Lively (Oncology Research Center, Bowman Gray Medical School) and Tom Lucas and Paul Matrisian (Howard Hughes Medical Institute, Vanderbilt University), who helped perform the amino acid sequence analysis. Warren Zimmer provided the chicken calmodulin cDNA (pCAM-9H8). Patrick Fran provided a critical analysis of the manuscript. The expert technical assistance of Steve Koch and Elisabeth Zimmerman is gratefully acknowledged, as is the help Deborah Caplenor provided in preparing the manuscript.

REFERENCES


**Supplemental Material**

To the Main Acid Sequence of Rat Liver Glucokinase cDNA

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**Methods**

**Experimental Procedures**

- **Purification of Glucokinase**: Male Sprague-Dawley rats (250–750 g; Harlan Brothers, Indianapolis, IN) were used. Glucokinase was extracted from rat liver against KCl, centrifuged, and then treated with 5% PEG to remove the precipitate. The glucokinase was then dialyzed against a solution of 50% ethanol and 50% water, pH 7.0. The glucokinase was then purified by gel filtration on a Sephadex G-25 column.

- **Mass Spectrometry**: The glucokinase was then purified by ion exchange chromatography on a MonoQ column.

- **Nuclear Magnetic Resonance (NMR)**: The purified glucokinase was then analyzed by NMR spectroscopy.

**Results**

- The glucokinase was then analyzed by NMR spectroscopy.

**Discussion**

- The results were then analyzed and discussed.

**Conclusion**

- The conclusions were then drawn.

**Acknowledgments**

- The acknowledgments were then given.

**References**

- A list of references was then provided.

**Tables**

- Several tables were then provided.

**Figures**

- Several figures were then provided.

**Appendix**

- An appendix was then provided.
Rat Liver Glucokinase cDNA

TABLE II
Glucose Phosphorylating Enzymes in Rat Liver

The molecular weight of glucokinase (GK) was determined by SDS-Polyacrylamide gel electrophoresis as shown in Figure 1. The specific activity was assayed according to Toker [12]. The specific activity was assayed according to Toker [12]. The specific activity was assayed according to Toker [12]. The specific activity was assayed according to Toker [12].

<table>
<thead>
<tr>
<th>Glucose Phosphorylating Enzymes</th>
<th>GPK (U/ mg)</th>
<th>B-Ag (U/ mg)</th>
<th>GPK/B-Ag</th>
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</thead>
<tbody>
<tr>
<td>Rat Liver (U/ mg)</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Sp. act. (U/mg)</td>
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<td>0.0</td>
<td>0.0</td>
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</table>

TABLE III
Substrate Specificity of Glucose Phosphorylating Enzymes in Rat Liver

The specificity of glucokinase (GPK), purified as described in the text, was determined for several substrates using SDS-Polyacrylamide gel electrophoresis. The values for the substrates 1-DP (1U) in glucose, and N-acetylglucosamine (N-AcGl) were taken from references 3, 5, 13, and 14. All values are expressed as a ratio equal to enzyme activity with substrate divided by enzyme activity with glucose.

<table>
<thead>
<tr>
<th>Substrate Specificity</th>
<th>GPK (U/ mg)</th>
<th>B-Ag (U/ mg)</th>
<th>GPK/B-Ag</th>
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<tbody>
<tr>
<td>Fructose</td>
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<td>1.1</td>
</tr>
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<td>Galactose</td>
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<td>0.5</td>
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<tr>
<td>N-acetylglucosamine</td>
<td>0.2</td>
<td>0.1</td>
<td>2.0</td>
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TABLE IV
Sequence of Synthetic Oligonucleotides

The oligonucleotides were designed from the various peptide sequences shown in Figure 4. The oligonucleotides were synthesized as shown in Table V. The hybridization to both RNA and DNA could be accomplished. Probe CM-38 (5 mer/8 species) was designed from peptide CM-1. Probe CM-28 (5 mer/8 species) was designed from peptide CM-1. Probe CM-19 (10 mer/8 species) was designed from peptide CM-1. Probe CM-18 (20 mer/8 species) was designed from peptide CM-1.

<table>
<thead>
<tr>
<th>Oligonucleotide Sequence</th>
<th>CM-18</th>
<th>CM-28</th>
<th>CM-38</th>
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<tr>
<td>CM-38</td>
<td>TGGAGCGGCCCAGAGCTGCTGGGGGTTGCGGGCAGTCATTGGATAGATCGCGATGCA</td>
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<td></td>
</tr>
</tbody>
</table>

Figure 1. SDS-Polyacrylamide gel electrophoresis of purified glucokinase. Panel A: Silver-stained SDS-Polyacrylamide gel containing 0.15 M DTT. Lane 1 contained 2 ug each of the following standards: phosphorylase B, 97.4 kD; bovine serum albumin, 66.0 kD; ovalbumin, 43.0 kD; carbonic anhydrase, 29.0 kD; cytochrome c, 12.4 kD, and lysozyme, 14.3 kD. Lane 2 contained 2 ug of purified glucokinase from preparation 1. Lane 3 contained 2 ug of purified glucokinase from preparation 2. Panel B: Coomassie blue stained SDS-Polyacrylamide gel of the same sample.

Figure 2. RNA blot transfer analysis. Poly(I)/RNA was isolated from the livers of diabetic rats and diabetic rats treated with insulin for 4 hours [7], as described in Materials and Methods. 4 ug of each RNA was run and fractionated in a 1% agarose gel containing 6.25 formaldehyde. The RNA was transferred to nitrocellulose and probed with oligonucleotides end-labeled with 32P. The mixed oligonucleotide probe sequences are shown in Table IV.