Cell-specific Expression and Regulation of a Glucokinase Gene Locus Transgene*

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Transgenic mice containing one or more extra copies of the entire glucokinase (GK) gene locus were generated and characterized. The GK transgene, an 83-kilobase pair mouse genomic DNA fragment containing both promoter regions, was expressed and regulated in a cell-specific manner, and rescued GK null lethality when crossed into mice bearing a targeted mutation of the endogenous GK gene. Livers from the transgenic mice had elevated GK mRNA, protein, and activity levels, compared with controls, and the transgene was regulated in liver by dietary manipulations. The amount of GK immunoreactivity in hepatocyte nuclei, where GK binds to the GK regulatory protein, was also increased. Pancreatic islets displayed increased GK immunoreactivity and NAD(P)H responses to glucose, but only when isolated and cultured in 20 mM glucose, as a result of the hypoglycemic phenotype of these mice (Niswender, K. D., Shiota, M., Postic, C., Cherrington, A. D., and Magnuson, M. A. (1997) J. Biol. Chem. 272, 22604–22609). Together, these results indicate that the region of the gene from −55 to +28 kilobase pairs (relative to the liver GK transcription start site) contains all the regulatory sequences necessary for expression of both GK isoforms, thereby placing an upper limit on the size of the GK gene locus.

Glucokinase (GK)1 plays an essential role in maintaining euglycemia, both in humans and rodents (2–7). GK gene expression is cell type-specific and involves two alternate promoters that are differentially regulated (8–10). Expression of the islet GK isoform, which is determined by the upstream promoter, is a key determinant of the insulin secretory response to glucose (11, 12). The upstream promoter is also expressed in several other rare neural/neuroendocrine cell types in the brain and gut, including pituitary corticotropes, although the functional significance of GK in these locations remains to be determined (13). The downstream GK promoter determines expression of the hepatic GK isoform, which is thought to be a rate-determining step for hepatic glucose utilization (11, 14, 15).

Studies of the cis-regulatory elements involved in cell-specific expression of islet GK have shown that a 294-bp DNA fragment containing the upstream promoter is sufficient for reporter gene expression in a variety of neural/neuroendocrine cell types, including pancreatic β cells (13). Elements in the proximal promoter region have been identified that contribute to transcription in both insulinoma cells (16, 17) and AtT-20 cells, a pituitary corticotrope cell line (18). Distal elements may also be involved, although none have been reported (17). GK gene transcription in the islet appears to be largely constitutive, although glucose acts at a post-transcriptional level to regulate islet GK activity. Increased glucose leads to a 3–4-fold elevation in GK activity, islet glucose usage, and insulin secretion, without a parallel increase in islet GK mRNA (19–21).

The regulation of the downstream GK promoter, which is expressed only in hepatocytes, is both more complex and less well understood than that of the upstream promoter. Insulin increases hepatic GK gene transcription, whereas glucagon, acting via cAMP, decreases transcription (8, 22–24). Thyroid hormone and biotin also increase GK gene transcription in liver (25, 26). The cis-regulatory elements that determine hepatocyte-specific expression and regulation of the hepatic GK isoform are largely undefined as a DNA fragment able to confer both hepatocyte-specific and hormone-regulated expression to a reporter gene has not yet been identified. While Iynedjian et al. (27) have recently reported that a 1-kb fragment of the rat downstream promoter is transcriptionally active in primary hepatocytes, it is not regulated by insulin. Furthermore, in transgenic mice a 7.5-kb fragment of the rat downstream promoter DNA does not confer position-independent expression on a reporter gene (28).

We have previously reported the cloning and partial sequencing of an 83-kb DNA fragment of the mouse GK gene locus (28). These studies revealed that the two promoters in the mouse GK gene are separated by 35 kb, and that the gene contains at least eight liver-specific DNase I-hypersensitive sites that span over 20 kb of DNA (28). Given the complexity of the GK gene locus, both in terms of the widely separated promoter regions, and the multiple, widely dispersed hypersensitive sites, we sought to place an upper limit on its size by inserting the 83-kb fragment of cloned genomic DNA into the genome of mice. The studies presented here indicate that this DNA fragment, which spans from −55 to +28 kilobase pairs relative to the liver transcription start site, is expressed and regulated both in the liver and in the islet, strongly suggesting that it contains the entire mouse GK gene locus. In addition to placing an upper limit on the size of the GK gene locus, these studies have generated a novel animal model for determining

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1 The abbreviations used are: GK, glucokinase; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction.
FIG. 1. Detection and characterization of the GK gene locus transgene. A, diagram of the GK gene fragment used. The insert was excised from the P1 vector using NruI. This left short vector DNA sequences on the 5’ and 3’ ends that enabled detection of the transgene. B, PCR detection of the 5’ and 3’ ends of the transgene in two of three founders. C, Southern blot analysis using probes isolated from three different parts of the gene (locations are shown in A). The same three probes were used to determine the transgene integrity and calculate copy number. This analysis was performed using mice that were both homozygous (hom.) and heterozygous (het.) for the transgene, as well as non-transgenic controls (cont.).

the metabolic ramifications of increased GK gene copy number, as are described separately (1).

EXPERIMENTAL PROCEDURES

Transgene Preparation—the insert from P1–305, which contains all GK coding sequences and both promoter regions (28), was isolated using a Qiagen Maxi plasmid purification kit (Qiagen, Chatsworth, CA). Restriction of P1–305 DNA with NruI left 392 bp of vector sequence on the 5’ end and 202 bp of vector sequence on the 3’ end of the insert, thereby enabling the transgene to be distinguished from the endogenous mouse GK gene locus. The 83-kb genomic DNA fragment was isolated as a single fragment by pulsed-field gel electrophoresis and thereby enabling the transgene to be distinguished from the endogenous GK gene locus (7) were identified using a 268-bp PCR fragment from pmGK28.H2 using the primer pair 5’-AGACGGCCAATTTATTAGAGC or a 1.3-kb fragment from the 5’ end vector sequence (5’-CGAGGCGCCAT-TAGGCCCTAC and 5’-GGCTTCAGTGCTGCTCTCGT). Southern blot analysis was performed using standard procedures (30) with the 5’ end-specific PCR fragment used to routinely identify transgenic animals. For copy number analysis, probe 1, which lies near the 5’ end of the transgene, was a 2.3-kb SacI fragment from plasmid mGK28.H2.S1. Probe 2, which is located near the middle of the transgene, was a 1.3-kb SacI-BamHI fragment from plasmid mGK40.5B. Probe 3 was a 500-bp BamHI-EcoRI fragment from pgMK18 BS, which contains sequences near the 3’ end of the transgene. Mice bearing the targeted mutation of the endogenous GK gene (7) were identified using a 268-bp PCR fragment amplified from pgMK28.H2 using the primer pair 5’-AGCTTTG-GCAATAAGCAT and 5’-TGAATTCAACTCATAAGGT. The DNA was cut with BglII and analyzed by Southern blot analysis. A 2.5-kb band was specific for the inactivated GK allele, and a 9-kb band indicated a wild type GK allele, as described previously by Bailer et al. (7). An ~1-kb DNA fragment (generated by PCR using the primers 5’-CGCTCTTG-GAATGATGGGATTC and 5’-GGAACAGCGTACACACAGGCTAG) containing sequences from a neomycin resistance gene was used as an internal probe for the targeted GK locus. Quantitation of band intensity was performed by phosphorimager analysis (GS-250 Molecular Imager, o-Rad).

All mice were housed in specific pathogen-free barrier facilities, maintained on a 12-h light/dark cycle, and fed a standard rodent chow (Purina Mills, Inc., St. Louis, MO). Females from both transgenic lines were used for most analyses. The gene rescue experiment was conducted using animals from line 37 only. Non-transgenic offspring of similar genetic backgrounds from within the same mouse colony served as controls. Animals were killed at 0600, which corresponds to a fed state (31), except for those in Fig. 5, which were killed in the afternoon.

Northern Blot Analysis—Total RNA was prepared by acid guanidinium thiocyanate extraction (32). RNA gels, blots, and hybridizations were performed as described previously (28). A 789-bp rat GK cDNA fragment from pgKZ1 (24) was used as the probe. The loading control probe was an ~700-bp HindIII-EcoRI cyclophilin DNA fragment (33). Densitometric analysis of autoradiograms was performed using NIH Image software (National Institutes of Health, Bethesda, MD) after digital scanning. GK values were corrected for loading differences with the cyclophilin value.

Western Blot Analysis—Western blot analyses were performed as described (34). To visualize GK antibody binding, the washed membranes were incubated in a 1:10,000 dilution of donkey anti-sheep horseradish peroxidase-conjugated antibody (Jackson Immunoresearch, West Grove, PA). Samples were run on 10% SDSPAGE gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked in 5% nonfat milk and washed with TBS-T (10 mM Tris-Cl pH 8.0, 0.1% Tween 20) for 1 h at room temperature. A solution consisting of 1.25 mM Luminol, 0.2 mM para-coumaric acid, and 0.009% H2O2 in 100 mM Tris (pH 8.5) was used to initiate the chemiluminescence reaction. The membranes were then dried-dried, wrapped in polyvinyl chloride film, and exposed to autoradiography film.

GK Activity Measurements—GK activities in crude liver extracts (as prepared for Western blotting) were estimated by determining the difference in glucose phosphorylation in the presence of 100 mM and 0.5 mM glucose. The final assay mixture contained 50 mM triethanolamine hydrochloride, 20 mM MgCl2, 100 mM KCl, 1 mM dithiothreitol, 0.1% bovine serum albumin, 10 mM ATP, 1 mM NADP, either 0.5 or 100 mM glucose, 4 μg/ml glucose-6-phosphate dehydrogenase (Boehringer Mannheim, and 100 μl of liver extract. Glucose phosphorylating activity was taken as the increase in NADPH fluorescence measured at 340 nm after 4 min at 32 °C.

Immunocytochemical Analysis—Tissues were processed for immunocytochemical analysis as described previously (34). Briefly, GK immunoreactivity was detected using an anti-GST-GK fusion protein antibody (34) with a CY3-conjugated donkey anti-sheep IgG secondary antibody (Jackson Immunoresearch, West Grove, PA). Samples were imaged using a Zeiss LSM410 confocal scanning laser microscope with the 543 nm line of a helium/neon laser, and digital images were quan-
Glucokinase Gene Locus Transgenic Mice

RESULTS AND DISCUSSION

Generation of GK Gene Locus Transgenic Mice, Transgene Integrity, and Copy Number—Pronuclear DNA microinjection experiments were performed to generate transgenic mice containing additional copies of the entire GK gene, including both promoter regions and all exons (28). Three founders were generated, two of which passed the transgene through the germ-line (animals 37 and 41). The founder that did not pass the transgene was presumed to be mosaic. Transgene inheritance patterns indicated that the exogenous GK gene sequences had integrated into the X chromosome in founder 37 and into an autosome in founder 41. Because of the large size of the transgene, both PCR and Southern blot experiments were performed to exclude fragmentation or rearrangement of the DNA upon integration into the genome. These analyses suggest that transgene integration occurred without any major rearrangements or deletions in both lines of mice (Fig. 1, A–C). Quantitation of Southern blot band intensities (Fig. 1C) indicated that line 37 had ~1 transgene copy per haploid genome, whereas line 41 had ~4 copies.

Expression and Regulation of the Transgene in Liver—To determine whether the downstream GK promoter in the transgene is expressed in liver, GK mRNA, GK activity, and GK immunoreactivity were assayed in transgenic and control mice. In fed animals from line 37, GK mRNA levels were elevated 1.8-fold in heterozygotes and 2.6-fold in homozygotes, compared with the controls (Fig. 2A, left). Similar expression level differences were evident in line 41; GK mRNA was 1.8- and 2.4-fold greater in the heterozygous and homozygous transgenic mice, respectively (Fig. 2D). Increased hepatic GK activity (Fig. 2, B and E) and increased GK protein (Fig. 2, C and F) were also observed in both lines of mice.

To assess the regulation of the transgene in liver, GK mRNA levels were determined in mice fasted for 34 h or fed diets that varied in carbohydrate content. In the 34-h fasted mice GK mRNA expression was decreased in both the transgenic and control mice to very low levels (Fig. 2A). In addition, hepatic GK mRNA was decreased by ~70% in mice fed a low carbohydrate diet, compared with those fed a high carbohydrate diet (data not shown). These results indicate that the GK transgene is expressed in liver, that there is an association between gene copy number and gene expression within a given line, and that the expression of the transgene in liver is affected by dietary manipulations known to alter expression of the endogenous GK gene (8, 24, 38–40). Identification of GK mRNA and GK immunoreactivity in the liver of a transgene-rescued GK null mouse (as shown below) also provides evidence that the downstream GK promoter in the transgene is transcriptionally active in hepatocytes.

The transmission pattern of the GK transgene in line 37 revealed that it had integrated into the X chromosome, as
Glucokinase Gene Locus Transgenic Mice

**Immunocytochemical analysis of GK subcellular localization in hepatocytes.** A, control liver from overnight fed mouse. A representative 1-mm² field is shown with a central vein near the center of the image. Faint GK immunoreactivity can be seen in a few nuclei as well as diffuse cytoplasmic GK immunoreactivity. Scale bar = 50 μM. B, corresponding field from a transgenic liver. C, quantitation of nuclear GK immunoreactivity (n = 2 mice/group, ~10 fields/animal; *, p < 10⁻⁵ compared with control). GKRP immunostaining patterns and numbers of positive nuclei were not different between transgenic and control mice (data not shown).

**Quantitative immunofluorescence analysis of islet GK content.** Immunoreactive GK content in pancreata from transgenic and control mice was determined as described under "Experimental Procedures." A, quantitation of islet GK immunoreactivity from line 37 mice (*, p < 10⁻⁵ compared with control). B, quantitation of islet GK immunoreactivity from line 41 mice (*, p < 10⁻⁵ compared with control). For both A and B, 2 mice/group and 8–15 islets/animal were analyzed. C, representative confocal micrograph of an islet from a line 37 mouse. D, representative confocal micrograph of a control islet.

**Altered Subcellular GK Localization in Hepatocytes—**Recent studies have revealed that GK translocates from the cytoplasm to nucleus in certain metabolic states (48, 49). Thus, to examine the subcellular location of GK in the livers of transgenic mice, we performed immunocytochemical studies. In livers from fed control mice, GK immunoreactivity displayed a peri-central zonation pattern typical of GK and other glycolytic enzymes (50) with only a few faintly immunopositive nuclei being observed (Fig. 3A). In contrast, the livers of fed transgenic mice showed both more pronounced nuclear GK immunostaining (Fig. 3B) and cytosolic GK immunoreactivity that was more intense than the controls (Fig. 3, compare A and B). Approximately 5-fold more nuclei stained positively for GK in both homozygous and heterozygous transgenic livers (Fig. 3C). In addition, the peri-central zone of both nuclear and cytoplasmic GK immunoreactivity was expanded in transgenic livers (data not shown). While the functional significance of GK localization in the nucleus of the hepatocyte remains to be determined, the enhanced nuclear localization of GK in transgenic hepatocytes appears to reflect nuclear sequestration of excess GK by binding to GKRP, since the latter has been found to be a nuclear protein (48, 51). Given that alterations in GK gene expression profoundly affect the plasma glucose concentration (1), increased binding of GK to GKRP in these mice may be a mechanism that protects the animals from the physiologic effects of excess hepatic GK catalytic activity.

**GK Transgene Expression in Islets—**To assess expression of the upstream GK promoter in the transgene, we used quantitative methods to examine GK immunoreactivity in pancreatic islets. Given that small fragments of the upstream GK promoter have previously been shown to be expressed in both the islet and other rare neuroendocrine cell types (13, 52), we expected that a transgene with ~15 kb of additional 5′-flanking sequence for the upstream promoter would cause increased islet GK gene expression. Surprisingly, in freshly isolated pancreatic islets, the amount of immunoreactive GK in islets from both lines of transgenic mice was less than the controls: 66% and 9% compared with control (4). Representative islets from a line 37 mouse, compared with a control mouse, are shown in Fig. 4 (A and B). However, since glucose is known to regulate islet GK content (20, 53), and since these mice have a hypoglycemic phenotype, as is described in detail elsewhere (1), decreased islet GK content may reflect differences in the plasma glucose concentrations in transgenic mice.
after culturing islets under equivalent glucose conditions. After culturing control and transgenic islets from line 37 in 20 mM glucose, 1.5-fold more immunoreactive GK was present in the transgenic islets (Table 1), a difference that parallels the addition of the single extra GK gene in these mice (3 versus 2 gene copies). To further validate this result, differences in glucose metabolism were quantitatively assessed using two photon excitation microscopy to measure changes in NAD(P)H autofluorescence (37). The 1.5-fold increase in GK immunoreactivity observed after culturing the transgenic islets in 20 mM glucose was matched by a 1.4-fold increase in glucose-stimulated NAD(P)H autofluorescence. Finally, detection of GK immunoreactivity in β cells of the transgene-rescued mouse also provides clear evidence that the GK gene locus transgene is expressed in the islet (see below).

These results indicate that transgenic islets, when exposed to the same glucose concentrations as controls, express a proportionally greater amount of GK. Moreover, like the liver, the amount of GK immunoreactivity also appears to be GK gene copy number-dependent. While an extensive analysis of transgene expression in other neuroendocrine cell types known to express the islet GK isoform was not performed (13), GK mRNA levels were elevated to a similar extent (∼1.6-fold) in a sample of pooled transgenic pituitaries, suggesting that the GK transgene is probably expressed in all locations where the endogenous upstream GK promoter is transcriptionally active.

**Rescue of GK Null Lethality**—The effects of complete inactivation of the endogenous GK gene by gene targeting have been reported by two different groups (6, 7). While the time of death varies greatly in the two different studies, the availability of these mice provided an opportunity to directly test the expression of the GK transgene in the absence of endogenous GK gene expression. GK transgenic mice were first crossed with the heterozygous null GK mice generated and characterized by Bali et al. (7) to generate animals that were heterozygous for both the GK transgene and targeted endogenous GK alleles. The double heterozygous mice were then intercrossed, and 56 offspring were generated and characterized. Because the genomic Southern blot analysis was complicated by the presence of up to four GK genes in some mice (Fig. 5A), variable band intensities, and a random insertion of the targeting vector in the genome of the GK null mice (see extra band for the neomycin resistance gene probe in Fig. 5A), all candidate rescued mice were test-mated to a wild type mouse to verify transmission of one null GK allele to all offspring.

From these matings, a single female mouse was identified that passed one null GK allele to all offspring (n = 7, p < 0.01), as shown in Fig. 5B. This animal was killed for tissue analysis at ∼12 weeks of age and was euglycemic at that time (104 mg/dl). GK immunostaining in both liver and pancreatic tissue sections in the rescued mouse were indistinguishable from a control mouse (Fig. 5C). RNA blot analysis also indicated that there was a similar amount of hepatic GK mRNA expressed compared with the control (Fig. 5D). There was no evidence of transgene misexpression in a survey of several other tissues that do not express endogenous GK (Fig. 5D). More importantly, the transgene-rescued mouse lived well past the time when the GK null mice died (6, 7) and survived metabolic stresses such as weaning and pregnancy. While the results of this experiment need to be interpreted with caution, it suggests
that the GK gene locus transgene is able to fully compensate for the lack of endogenous GK gene expression in the GK null mice, thus providing additional evidence that the 83-kb DNA fragment tested in these mice defines the mouse GK gene locus.

**Conclusion**—The analyses performed here indicate that an 83-kb DNA fragment spanning from about −55 to +28 kilobase pairs relative to the liver transcription start site defines a functional mouse GK gene locus. By all criteria applied, expression of the GK gene locus transgene fully recapitulates the functional mouse GK gene. By all criteria applied, expression of the GK gene locus transgene fully recapitulates the functional mouse GK gene. By all criteria applied, expression of the GK gene locus transgene fully recapitulates the functional mouse GK gene. By all criteria applied, expression of the GK gene locus transgene fully recapitulates the functional mouse GK gene. By all criteria applied, expression of the GK gene locus transgene fully recapitulates the functional mouse GK gene.

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