 Human Molecular Genetics, 2000, Vol. 9, No. 12 **1821–1827**

A mouse model of galactose-induced cataracts

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Received 22 March 2000; Revised and Accepted 31 May 2000

Galactokinase (GK; EC 2.7.1.6) is the first enzyme in the metabolism of galactose. In humans, GK deficiency results in congenital cataracts due to an accumulation of galactitol within the lens. In an attempt to make a galactosemic animal model, we cloned the mouse GK gene (*Glk1*) and disrupted it by gene targeting. As expected, galactose was very poorly metabolized in GK-deficient mice. In addition, both galactose and galactitol accumulated in tissues of GK-deficient mice. Surprisingly, the GK-deficient animals did not form cataracts even when fed a high galactose diet. However, the introduction of a human aldose reductase transgene into a GK-deficient background resulted in cataract formation within the first postnatal day. This mouse represents the first mouse model for congenital galactosemic cataract.

INTRODUCTION

GK is involved in the first step of the metabolism of galactose: the conversion of galactose to galactose-1-phosphate. In humans, homozygous GK deficiency results in galactosemia, galactosuria and cataracts (1); heterozygous individuals are susceptible to presenile cataracts (2). Cataracts form in GK-deficient individuals because ingested galactose cannot be metabolized and is therefore available for conversion to galactitol through the action of aldose reductase. Osmotically active galactitol accumulates within lens fiber cells causing swelling, cell lysis and eventually cataracts (3). Infantile cataracts can be prevented in GK-deficient individuals by eliminating galactose from the diet (reviewed in ref. 4).

The human galactokinase gene (*GK1*) and cDNA have been isolated and characterized (5). The *GK1* gene contains 8 exons and 7 introns as well as regions that encode the *GK* signature sequence and two ATP binding sites. Mutations in >20 GK-deficient patients have been mapped to various positions throughout the *GK1* gene (5–8), providing insight into the regions of the protein that are critical for function. For example, several human *GK1* mutations that render the protein inactive occur at or near the *GK* signature sequence. We isolated the mouse galactokinase cDNA (*Glk1*) and determined that its sequence encodes a protein which is 88% identical to the predicted amino acid sequence of the human GK protein (9). In both human and mouse, GK activity has been found in multiple tissues (9, and our unpublished data).

Animal models for galactosemia have been utilized to better understand this disorder. The majority of animal studies involve high galactose diets which overload the galactose metabolic pathway (10). In addition, a mouse deficient in the second enzyme of galactose metabolism [galactose-1-phosphate uridyl transferase (GalT)], has been generated and studied (11). However, whereas GalT-deficient patients display broad-range tissue damage (3), GalT-deficient mice appear normal except for elevated levels of galactose metabolites in tissues (11,12).

To broaden our understanding of galactosemia and galactosemic cataracts, we cloned the mouse genomic *Glk1* gene and disrupted it through homologous recombination. We find that GK-deficient mice are similar to GK-deficient patients in that they are unable to metabolize galactose. These mice also accumulate galactose and galactitol in their tissues. Interestingly, disrupting the mouse *Glk1* gene also causes early onset cataracts but only on a background of transgenic mice that over-express the human aldose reductase (*hAR*) gene. This study underscores the cooperative relationship between galactose metabolism and alcohol sugar production in the development of galactosemic cataracts.

RESULTS

Isolation and disruption of mouse *Glk1* gene

We previously reported the isolation and characterization of the mouse *Glk1* cDNA (9). To isolate the mouse *Glk1* genomic clone, a mouse genomic lambda library was screened using labeled, full-length mouse *Glk1* cDNA as a probe. One of the clones isolated was 15 kb. Restriction analysis of this clone revealed four *Xba*I fragments of 8, 4, 2 and 1.5 kb, respectively. To delineate the *Glk1* gene, all four fragments were subcloned into pBluescript, sequenced and compared with the mouse *Glk1* cDNA. The genomic mouse *Glk1* gene is ~4.6 kb, which is significantly smaller than the human *GK1* gene (>7 kb). Despite the size difference, the structure of the mouse and human galactokinase genes is very similar. Both genes contain

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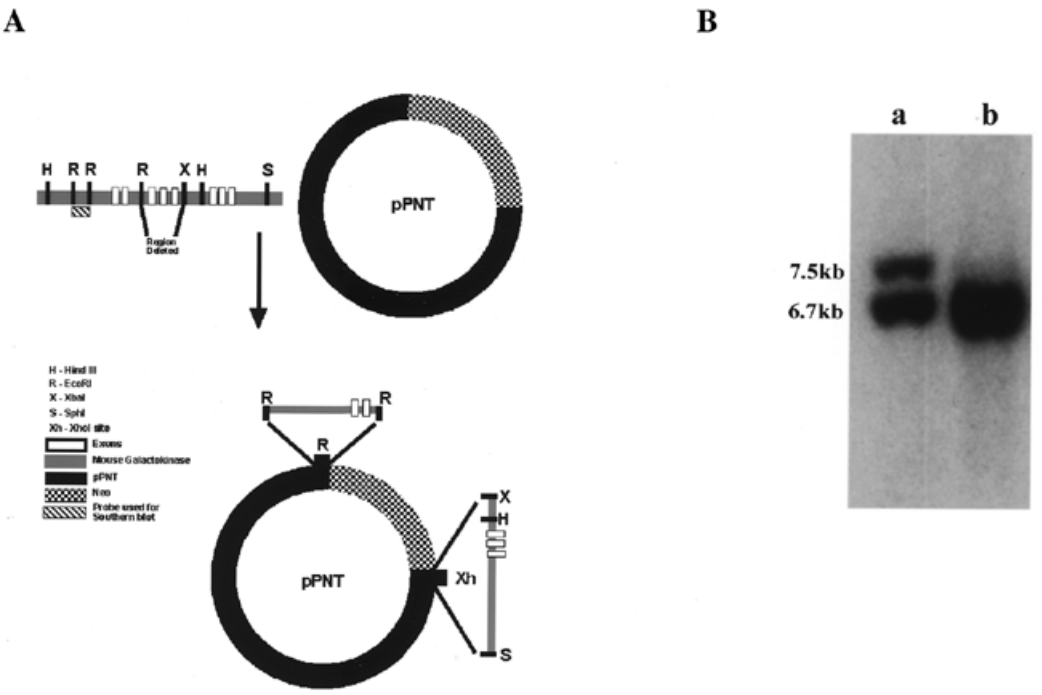

Figure 1. Construction of the *Glk1* knock-out vector and verification of its integration at the *Glk1* locus. (**A**) The configuration of genomic *Glk1* containing all eight exons is shown along with the empty pPNT cloning vector. The knock-out construct was created by inserting the *neo* gene between exons 2 and 6 and deleting exons 3, 4 and 5. (**B**) Southern blot of *Hin*dIII-cut DNA from two G418-resistant ES clones probed with a 900 bp *Eco*RI fragment from the 5′ end of *Glk1*. This probe hybridized to a 6.7 kb fragment when integration did not occur (b) and an additional 7.5 kb band when gene replacement was successful (a).

8 exons and 7 introns and the corresponding exons are identical in size. In addition, both genes contain regions encoding the *GK* signature sequence as well as two ATP-binding sites.

In order to construct a mouse model for galactosemic cataracts, the mouse *Glk1* gene was targeted for disruption by homologous recombination as described in Materials and Methods. TC1 embryonic stem (ES) cells (13) were transfected with a *Glk1* gene-targeting vector (Fig. 1A) containing the *neo* gene and G418-resistant clones were selected and analyzed by Southern blotting. An ES clone which gave the expected restriction pattern (Fig. 1B) was injected into blastocysts which were then implanted into pseudo-pregnant females. Germ-line chimeric mice that resulted from these injections were used to establish the *Glk1* null allele. To follow the disruption of *Glk1*, mice were screened by Southern blotting using *Hin*dIII-cut DNA and a 900 bp *Eco*RI probe from the 5′ end of *Glk1*.

Characterization of GK-deficient mice

GK expression. To verify the disruption of the mouse *Glk1* gene, liver extracts were assayed for GK activity. The GK activity in wild-type mouse liver was 1.6 nmol galactose-1-phosphate/h/μg protein, whereas essentially no GK activity (0.007 nmol/h/μg) could be detected in the GK-deficient mouse. $Glk1^{-}/Glk1^{+}$ heterozygotes displayed intermediate GK activity of 0.9 nmol/h/μg. These results are consistent with the loss of functional GK activity due to gene disruption.

The disruption of *Glk1* expression was further confirmed by performing *in situ* hybridization on wild-type and GK-deficient mice using a wild-type *Glk1*-specific probe. In wild-type mice, *Glk1* transcript was detectable as early as embryonic day 10.5 (data not shown) and displayed a tissue-specific expression pattern throughout embryogenesis (Fig. 2A). Expression was found in tissues involved in galactose metabolism (liver, intestine and kidney) as well as in tissues in which the function of GK is not known (thymus and lung). As expected, no *Glk1*-specific signal was detected in the tissues of age-matched homozygous GK-deficient mice (Fig. 2B).

Oxidation of galactose. Patients deficient in GK are unable to metabolize galactose. This inability has been measured *in vivo* by injecting galactosemic patients with trace amounts of [^{14}C]-1-galactose and monitoring the relatively small levels of $^{14}CO_2$ produced (14). This experiment was performed in our laboratory with wild-type and GK-deficient mice to evaluate galactose utilization. Mice were injected with [^{14}C]-1-galactose, placed in sealed chambers and the amount of exhaled $^{14}CO_2$ was monitored (Fig. 3). In wild-type mice, the oxidation of [^{14}C]-1-galactose could be detected within 10 min of injection, with the rate of oxidation reaching a peak 90 min post-injection. In contrast, GK-deficient mice oxidized very little [^{14}C]-1-galactose. These results indicate that these mice, similar to GK-deficient patients, are unable to metabolize galactose.

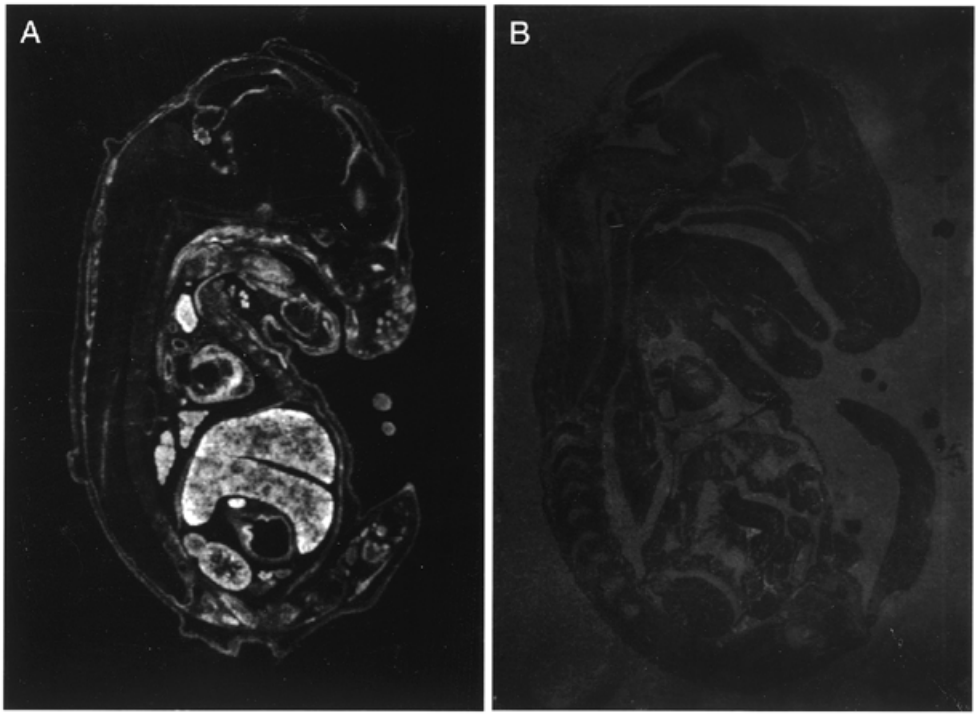

Figure 2. *In situ* hybridization in embryonic day 15.5 wild-type (**A**) and GK-deficient (**B**) mice using antisense *Glk1*-specific probe. Sections stained with sense or RNAse A-treated probes were negative.

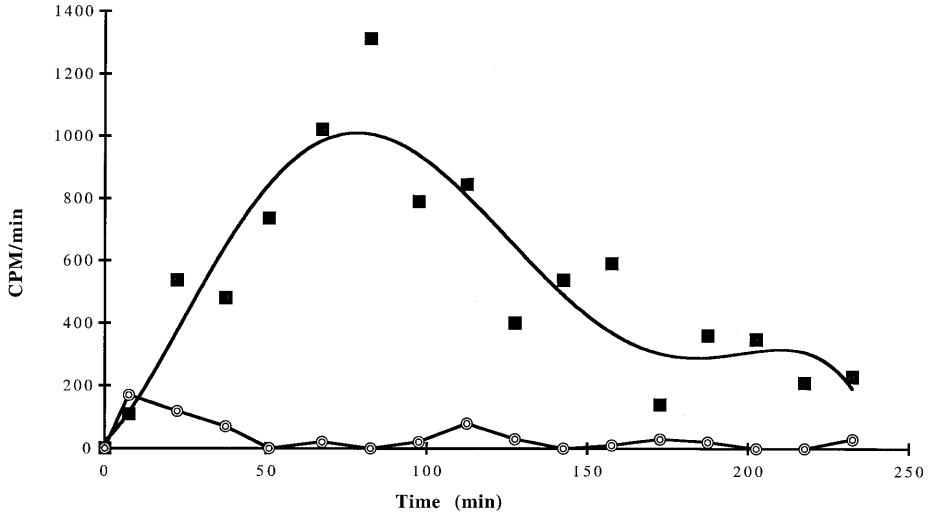

Figure 3. Rate of oxidation of [^{14}C]galactose in wild-type (closed squares) and GK-deficient (open circles) mice. Mice were injected with [^{14}C]galactose and exhaled air was collected and monitored for $^{14}CO_2$. Data are the average of two mice from each genotype.

Accumulation of galactose and galactitol. When GK-deficient patients ingest galactose, both galactitol and galactose accumulate in the urine (14–16). We next examined whether galactose metabolites accumulate in the tissues of GK-deficient mice. Wild-type and mutant mice were placed on high galactose or high glucose diets for 4 or 8 weeks, and tissues from these mice were assayed for galactose and galactitol (Table 1). In mice fed a 40% glucose diet, a modest but detectable accumulation of galactose and galactitol was seen in the liver, brain and kidney of homozygous GK-deficient mice, but not in wild-type mice. In contrast, mice fed a 40% galactose diet showed a marked elevation in both galactose and galactitol in liver, brain and kidney. The higher galactose metabolite levels were seen in both wild-type and GK-deficient mice. Because this higher

Table 1. Galactose and galactitol levels in tissues from wild-type and GK-deficient mice

Diet	*Glk1* genotype	Tissue	Galactose (μmol/g wet wt) (mean ± SE)	Galactitol (μmol/g wet wt) (mean ± SE)
40% glucose	Wild-type (n = 12)	Brain	ND[2]	ND
		Kidney	ND	ND
		Liver	ND	ND
	GK-deficient (n = 12)	Brain	0.08 ± 0.03	0.03 ± 0.01
		Kidney	0.12 ± 0.02	0.14 ± 0.01
		Liver	0.17 ± 0.03	0.05 ± 0.01
40% galactose	Wild-type (n = 10)	Brain	2.1 ± 0.5	2.6 ± 0.2
		Kidney	6.4 ± 1.0	1.8 ± 0.1
		Liver	1.7 ± 0.5	0.20 ± 0.04
	GK-deficient (n = 7)	Brain	2.6 ± 0.2	3.0 ± 0.3
		Kidney	12.6 ± 2.0	3.7 ± 0.2
		Liver	2.3 ± 0.4	0.51 ± 0.05

ND, not detected.

accumulation occurred in galactose-fed but not glucose-fed mice, galactose and galactitol are most certainly by-products of exogenous galactose.

In galactose-fed animals, the levels of galactitol (Table 1) in liver and kidney were consistently higher in GK-deficient mice compared with wild-type mice (P = 0.005). However, the difference in brain galactitol levels between galactose-fed GK-deficient and wild-type mice was not statistically significant (P = 0.12). The reason for the similar galactitol accumulation between wild-type and mutant brain is probably the low expression of aldose reductase in mouse brain (17). In addition, not all tissues accumulated galactitol to the same extent; for example, in both wild-type and mutant mice, liver had the lowest amount of galactitol.

Cataracts in GK-deficient hAR transgenic mice. When we examined the eyes of GK-deficient mice, no evidence of cataract formation was found, even after 6 months on a 40% galactose diet. Since aldose reductase is present in very low amounts in the mouse lens (18), high levels of galactitol cannot be made. Perhaps without increased lens galactitol, GK-deficient mice may have difficulty producing cataracts. To examine this possibility, we took advantage of mice expressing the human aldose reductase gene under the control of the murine αA–crystallin promoter (19). Previous studies showed that *hAR*$^+$/*hAR*$^-$ heterozygous transgenic mice overexpress the *hAR* gene and this expression is limited to lens epithelial cells. Furthermore, *hAR*$^+$/*hAR*$^-$ mice are prone to cataracts when fed a high galactose diet, unlike their non-transgenic littermates.

Wild-type and GK-deficient mice were crossed with *hAR*$^+$/*hAR*$^-$ mice and progeny were monitored for the formation of cataracts (Table 2). As mentioned above, GK-deficient mice fed a 40% galactose or 40% glucose diet did not display cataracts. Transgenic *hAR*$^+$/*hAR*$^-$ mice with wild-type *Glk1* develop cataracts after being fed a high galactose diet (19). These previously reported results were confirmed in our studies. In contrast, GK-deficient *hAR*$^+$/*hAR*$^-$ mice developed cataracts in the absence of a high galactose diet (Table 2). In GK-deficient *hAR*$^+$/*hAR*$^-$ mice, evidence of cataract formation was detected within the first postnatal day (P0), even when the mothers were maintained on a 40% glucose diet (Fig. 4). At this age, cataracts were observed microscopically as vacuoles at the periphery of the lens.

The most likely cause of cataracts in the GK-deficient *hAR*$^+$/*hAR*$^-$ mouse was an accumulation of lens galactitol. To test this, we assayed the galactose and galactitol level in mouse lens by mass spectroscopy (Table 2). For these experiments, lenses from several age-matched mice were pooled and assayed. Lenses from 2-week-old GK-deficient mice without the *hAR* transgene contained small but detectable levels of both galactose and galactitol. The addition of the *hAR* transgene onto the GK-deficient background had little effect on the galactose levels but resulted in a large increase in galactitol (from 6.12 to 154 μmol/g dry wt). Interestingly, even lenses from P0 GK-deficient *hAR*$^+$/*hAR*$^-$ mice had markedly high galactitol levels (65 μmol/g dry wt).

DISCUSSION

Many attempts have been made to develop an animal model for galactosemic cataracts. As early as 1935, Mitchell and Dodge (20) were able to produce cataracts in rats by feeding them a diet high in galactose. Using similar diet regimens, many publications have shown the ease of producing cataracts in other animals (reviewed in ref. 10). However, galactose loading has been unsuccessful in producing cataracts in wild-type mice. Lee *et al*. (19) produced a transgenic mouse expressing the *hAR* gene under the control of the α-crystallin promoter. When these mice were fed a normal diet they remained cataract-free but when they were fed a high galactose

Table 2. Lens galactose metabolite levels and cataract formation in glucose-fed mice

Glk1 genotype	hAR	Age[a] (days)	No. of pooled lenses	Galactose (μmol/g dry wt)	Galactitol (μmol/g dry wt)	No. of cataractous mice/no. of mice examined
Wild-type	–	P0	4	ND	ND	0/2
Wild-type	–	P17	4	ND	ND	0/2
Wild-type	+	P0	4	ND	ND	0/2
Wild-type	+	P17	12	ND	ND	0/6
GK-deficient	–	P0	4	ND	ND	0/2
GK-deficient	–	P14	12	0.65	7.05	0/6
GK-deficient	–	P15	8	3.40	6.12	0/4
GK-deficient	+	P0	4	8.59	65.73	2/2[b]
GK-deficient	+	P14	10	2.59	155.49	5/5
GK-deficient	+	P15	8	2.18	152.03	4/4

ND, not detected.
[a]Mothers maintained on 40% glucose diet throughout gestation and suckling. All mice in Table 2 were suckling.
[b]Lens vacuoles detected on microscopic examination.

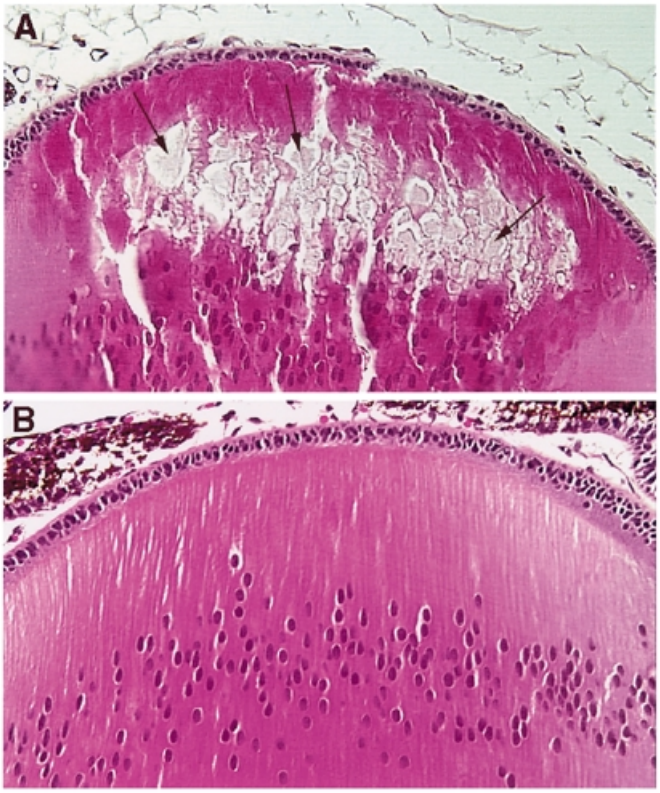

Figure 4. Lens structure of P0 wild-type and GK-deficient *hAR*$^+$/*hAR*$^-$ mice. (**A**) Eye section from *Glk*$^-$/*Glk*$^-$ mouse (P0) containing the *hAR* gene. Cortical vacuoles with irregular size are readily seen (arrows). (**B**) Representative section from wild-type mouse at P0. H&E stained.

diet they quickly developed cataracts (in some animals, cataracts began forming 1 day after instituting the high galactose regimen). The GK-deficient *hAR*$^+$/*hAR*$^-$ mouse reported here is the first galactosemic animal model that develops cataracts without a large exogenous galactose load.

GK-deficient mice fed a 40% glucose diet accumulated detectable levels of galactose and galactitol within their tissues. One possible source of galactose in glucose-fed GK-deficient mice is the mother's milk which the pups ingested during the study period. We detected high lens galactitol levels in newborn GK-deficient *hAR*$^+$/*hAR*$^-$ mice, even though the mothers were maintained on a 40% glucose diet, which contains no bio-available galactose. Currently, we cannot be sure that the newborn pups drank enough mother's milk to produce the lens galactitol measured. It is possible that the accumulated galactose is derived not from mother's milk but from the metabolism of endogenous glycoproteins. Alternatively, the animals may utilize an alternative pathway to synthesize galactose from UDP-glucose (specifically, UDP-glucose→UDP-galactose→Gal-1-P→galactose). We are currently investigating all these possibilities as the source of galactitol.

Our data do support a role of galactitol as the causative agent in the formation of galactosemic cataracts. Because aldose reductase expression is low in the lens of GK-deficient mice, galactitol does not accumulate, even when these mice are fed a galactose-rich diet. When aldose reductase levels are increased by introducing the *hAR* transgene, galactitol levels increase substantially and cataracts form. Furthermore, it is likely that the variations in galactitol levels between different mouse tissues reflect differences in the expression of AR within the various tissues. Indeed, Gui *et al*. reported that aldose reductase activity is high in mouse kidney and very low or undetectable in mouse liver and lens (18). In agreement with

this, we consistently find higher levels of galactitol in mouse kidney than mouse liver. These data demonstrate that a block in galactose metabolism and the presence of aldose reductase activity are both key components for galactosemic cataract formation.

Due to the critical roles that aldose reductase plays in galactosemia and diabetes, aldose reductase and its inhibitors have often become the target for the study of both subjects. Due to the similarity between galactosemia and diabetes, the study of AR inhibitors in a galactosemic mouse model would not only help to identify potential therapeutic reagents but could also add insight into the role of sorbitol in diabetes.

MATERIALS AND METHODS

Library screening

To isolate a mouse genomic *Glk1* clone, a mouse genomic library in lambda phage FixII (Stratagene, La Jolla , CA) was screened with a ^{32}P-labeled mouse *Glk1* cDNA clone (9). Positive clones were re-screened using the same probe. One clone that consistently hybridized with the *Glk1* probe was further analyzed by restriction digests, subcloning and sequencing. The sequence of the mouse *Glk1* gene has been deposited into GenBank (GenBank accession no. AF246459).

Transgenic and knock-out mice

A *Glk1* gene-targeting vector was created by excising a 3.7 kb *Eco*RI fragment from the 5′ end of the mouse *Glk1* gene containing exons 1 and 2 and subcloning it into the *Eco*RI site of pPNT vector (21). A 5.5 kb *Xba*I–*Sph*I fragment containing the 3′ end of the mouse gene, including exons 6, 7 and 8, was blunt-ended and then subcloned into the blunt-ended *Xho*I site of the vector. The final construct, which lacked *Glk1* exons 3, 4 and 5 and carried the 1.8 kb *neo* gene cassette (Fig. 1A), was used to transfect TC1 ES cells which are from mouse strain 129S6/SvEv-Tac (13). G418-resistant ES cell clones were picked and further analyzed by Southern blot analysis to verify complete integration of the gene-targeting construct. To check integration of the 5′ end of the construct, DNA from ES cells was digested with *Hin*dIII, separated by agarose gel electrophoresis and blotted. The blots were probed with a 900 bp *Eco*RI fragment from the 5′ end of the mouse genomic clone. A 7.5 kb band on the Southern blot indicated the presence of a disrupted *Glk1* gene whereas the endogenous mouse *Glk1* gene gave a 6.7 kb band. Integration of the 3′ end was detected by probing a Southern blot containing *Sca*I-digested DNA with a 1.1 kb *Sph*I fragment from the 3′ end of the *Glk1* gene.

Four recombinant clones (nos 6, 66, 95 and 129) of 105 G418-resistant candidates gave the expected restriction pattern. Blastocysts from C57BL/6J egg donors were injected with clone no. 66 and then implanted into pseudo-pregnant female mice to establish the germline mutation. Mice were screened for gene replacement by Southern blotting as detailed above. The chimeras were mated to NIH Black Swiss outbred mice to check for germline transmission, then they were mated to 129/SvEv-Tac to keep them completely inbred. Homozygotes were then generated from F_1 crosses.

Mice carrying the human aldose reductase gene (*hAR*) under the control of the α-crystallin promoter (mouse line nos CAR222 and CAR648) were crossed with GK-deficient mice and offspring were genotyped for the *hAR* transgene by PCR using the primers described by Lee *et al*. (19). The presence of the *hAR* transgene was further confirmed by Southern blot hybridization using *hAR* as a probe. F_1 mice with the *hAR* transgene and one disrupted *Glk1* gene were mated to homozygous *Glk1* knock-out mice. Progeny heterozygous for the *hAR* transgene and homozygous GK-deficient were utilized for dietary studies. Transgenic mice and their non-transgenic littermates were fed chow containing 17.5% protein, 11% fat, 2.5% fiber and the remainder carbohydrate (PMI Feeds, Richmond, IN). In the 40% glucose and 40% galactose diets, the carbohydrate portion of the chow was replaced with glucose or galactose respectively.

Biochemical procedures

Oxidation of galactose was measured in whole animals as described (12). Briefly, mice were injected with 0.5 μCi [^{14}C]-1-galactose (sp. act. = 50 μCi/mmol; ARC, St Louis, MO) and placed in a small chamber through which room air was circulated. The effluent stream from the chamber was passed through a Kontes bubble collector containing benzetholium hydroxide to trap CO_2. Aliquots from the collector were removed and counted for radioactivity every 15 min. The rate of oxidation (c.p.m./min) was determined by dividing the amount of radioactivity in each aliquot by 15 min. The values were then plotted against the midpoint of each time point (e.g. 7.5 min for 15 min time point). Two animals of each genotype were used in the experiment and the data represent the average of these two data sets.

To measure GK activity, mouse liver was homogenized in 10 mM phosphate buffer, pH 7.4. Protein concentration was determined by the Bradford method and GK was assayed as described previously (2).

Levels of tissue galactose and galactitol were measured by gas chromatography/mass spectroscopy as described (12).

Histology

Eyes were dissected from P0 mice and fixed in 10% formalin for 3 h, prior to paraffin processing. Sections (6 μm thick) were hematoxylin and eosin (H&E) stained and analyzed for the presence of pathology.

In situ hybridization

Mouse embryos harvested at embryonic day 15.5 were fixed in 4% paraformaldehyde and processed for paraffin sectioning. Sections (6 μm thick) were cut sagittally, de-waxed and rehydrated through an ethanol gradient. *In situ* hybridization was carried out as described (22), with modifications. Specifically, fixed sections were treated with Proteinase K (10 μg/ml) for 30 min at 37°C, re-fixed in 4% paraformaldehyde for 20 min at 24°C and acetylated in 0.1 M TEA/0.25% acetic anhydride for 20 min at 24°C. Control samples were treated with RNase A (100 μg/ml) for 2 h at 37°C. Sections were pre-hybridized for 2 h at 60°C in 50% formamide, 2.5× Denhardt's 0.6 M NaCl, 10 mM Tris–HCl pH 8, 1 mM EDTA, 0.1% SDS, 10 mM DTT and 500 μg/ml *E.coli* tRNA. Hybridization was carried out with radiolabed RNA probes (sense or antisense) for 18 h at 60°C in the same buffer. Sections were then treated with

RNase A, washed, dehydrated, dried, coated with NTB-2 emulsion (Kodak, Rochester, NY) and exposed to film. Images of *in situ* autoradiograms were taken using a Toshiba 3 CCD camera mounted on a Nikon Microphot FXA scope.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health grants R01 EY09404 (D.S.) and P01 HD29847 (S.S.).

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