Characterization of the Murine High $K_m$ Glucose Transporter GLUT2 Gene and Its Transcriptional Regulation by Glucose in a Differentiated Insulin-secreting Cell Line*

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In pancreatic β-cells, the high $K_m$ glucose transporter GLUT2 catalyzes the first step in glucose-induced insulin secretion by glucose uptake. Expression of the transporter has been reported to be modulated by glucose either at the level of mRNA accumulation or at the level of protein synthesis. In this study, we used the differentiated insulinoma cell line INS-1 which expresses high levels of GLUT2 and show that the expression of GLUT2 is regulated by glucose at the transcriptional level. By run-on transcription assays we showed that glucose induced GLUT2 gene transcription 3-4-fold in INS-1 cells which was paralleled by a 1.7-2.3-fold increase in cytoplasmic GLUT2 mRNA levels. To determine whether glucose regulatory sequences were present in the promoter region of GLUT2, we cloned and characterized a 1.4-kilobase region of mouse genomic DNA located 5' of the translation initiation site. By RNase protection assays and primer extension, we determined that multiple transcription initiation sites were present at positions -55, -64, and -115 from the first coding ATG and which were identified in liver, intestine, kidney, and β-cells mRNAs. Plasmids were constructed with the mouse promoter region linked to the reporter gene chloramphenicol acetyltransferase (CAT), and transiently and stably transfected in the INS-1 cells. Glucose induced a concentration-dependent increase in CAT activity which reached a maximum of 3.6-fold at 20 mM glucose. Similar CAT constructs made of the human GLUT2 promoter region and the CAT gene displayed the same glucose-dependent increase in transcriptional activity when transfected into INS-1 cells. Comparison of the mouse and human promoter regions revealed sequence identity restricted to a few stretches of sequences which suggests that the glucose responsive element(s) may be conserved in these common sequences.

The GLUT2 glucose transporter isoform plays a major role in glucose-induced insulin secretion in the pancreatic β-cell by catalyzing the uptake of glucose into the cell (1). The regulated expression of this transporter has been studied in vitro by cell culture and in a number of animal models which have an imbalanced glucose homeostasis (2-12). In diabetes, the expression of this transporter is regulated in a tissue-specific manner. In particular, in every animal model of diabetes studied such as the diabetic Zucker fa/fa rat, the neonatal low-dose streptozocin-induced diabetic rat, the GK rat, the db/db mouse or at the onset of diabetes in the BB/W rat, there is a strong reduction in GLUT2 gene expression which is restricted to the pancreatic β-cells while its expression in liver, intestine, or kidney is unaltered or slightly increased (6-12). From these data one could conclude that hyperglycemia of diabetic animals is responsible for the observed decrease in expression of GLUT2 in β-cells. However, exposure of pancreatic islets to high glucose in vitro leads to an increase in GLUT2 mRNA and protein levels. In diabetes it is therefore not clear what causes GLUT2 down-regulation. Experiments in which development of hyperglycemia was prevented by ascorbic acid treatment in Zucker diabetic rats showed that even in the presence of normoglycemia, GLUT2 expression steadily decreased over time (8). Taken together, the above observations suggest that in vitro glucose may directly stimulate GLUT2 gene expression while in the diabetic state the hyperglycemia causes a decreased GLUT2 expression.

To better understand the glucose-regulated expression of GLUT2, we studied its controlled expression by glucose in a highly differentiated insulinoma cell line and initiated the characterization of the promoter region of the transporter gene. We cloned and characterized the upstream regulatory region of the mouse GLUT2 gene and defined common initiation start sites in murine tissues which express GLUT2, i.e. pancreatic β-cells, liver, intestine, and kidney. The human and mouse promoters were functionally tested using a chloramphenicol acetyltransferase (CAT) reporter system transiently or stably transfected into a highly differentiated β insulinoma cell line, INS-1 (13). By increasing the concentration of glucose in the culture medium, a concentration-dependent transcriptional activation of the human and mouse GLUT2 gene was shown. Similarly, the endogenous GLUT2 gene expression was assessed in INS-1 cells by nuclear run-on and Northern blot analysis. As observed with CAT reporter constructs driven by the murine and human promoter, GLUT2 expression is transcriptionally regulated by glucose. Therefore, the 5'-regulatory regions of the human and mouse GLUT2 genes contain carbohydrate-responsive element(s) responsible for the glucose-induced gene transcription of GLUT2 observed in INS-1 cells.

MATERIALS AND METHODS

Cell Culture—The transplantaible x-ray-induced rat insulinoma INS-1 cell line was kindly provided by Asfari et al. (13). INS-1 cells were grown in RPMI 1640, 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM 4-morpholinepropanesulfonic acid. diHCO3

1 The abbreviations used are: CAT, chloramphenicol acetyltransferase; MOPS, 4-morpholinepropanesulfonic acid; bp, base pairs; kb, kilobase pair(s).

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was added to the various desired glucose concentrations. Passages (Life Technologies) was used for the basal RPMI media in which glucose CAT assay experiments were incubated overnight in RPMI containing transcription run-on and RNA expression studies. INS-1 cells for the additional glucose was added (10 and 20 mM final concentration) for the no glucose then, at time 0, glucose was added to the final concentrations described by Marie et al. (14) with the exception that 5% (w/w) Nonidet Tris, pH 8.3,40% glycerol, 5 mM MgCl2, 0.1 mM EDTA, flash frozen and water, re-precipitated, and washed. Counts were normalized for the containing 10 mM Tris, pH 8.0, 15-cm dish were used for each nuclear isolation. Nuclei were isolated according to the method of Vannice et al. (16). In brief, the nuclei were digested with 5 volumes of water, 100 μg of mRNA, and made 0.4% in SDS. An equal volume of 100 mM NaOAc, pH 5.0, 20 μM EDTA was added after which the RNA was extracted with an equal volume of phenol/H2O saturated and precipitated with a final concentration of 2 mM NAOAc and 2.5 volumes of ethanol. Pellets were resuspended in water, re-precipitated, and washed. Counts were normalized for the transcripts and subsequently hybridized to filter-bound GLUT2, β-ac- tin, and pGEM cDNA plasmids for 3 days at 42 °C. Zeta-probe membranes were used as filters (Bio-Rad) and the hybridization solution was 5 × SSPE (1 × SSPE, 0.15 mM NaCl, 10 mM NaOAc, 1 mM EDTA) 50% formamide, pH 6.5, 0.05% SDS, 1 μM EDTA, 2 × SSC, (5 × for prehybridization), and 200 μg/ml yeast tRNA. Filters were washed in 2 × SSC, 0.1% SDS at room temperature followed by two washes for 20 min in 0.2 × SSC, 0.1% SDS at 55 °C and then exposed to Hyperfilm-MP (Amer sham) at −70 °C for 7–10 days. Films were quantified using a Molecular Dynamics scanner (Sunnyvale, CA).

RESULTS

Identification of the Mouse Upstream Regulatory Region of the GLUT2 Gene—By screening a mouse genomic library with a GLUT2 cDNA probe, we isolated 4 overlapping bacteriophage clones which together contained most of the GLUT2 gene. The overall gene structure and exon-intron boundaries are similar to the recently published human GLUT2 gene (21). The 18-kb insert of clone 4 contains the first three exons of the gene and approximately 1.4 kb of the putative promoter region of GLUT2 (Fig. 1A). The nucleic acid sequence of the mouse upstream regulatory region is shown in Fig. 1B. By computer analysis, several potential consensus sequences for various transcription factors (22–28) are located within the 1.3-kb promoter region. The six elements include a cyclic AMP responsive element (CRE = ACGTCA 6/6), an AP-1, AP-4, and a CCAAT-box-binding transcriptional factor (CTF-NF1 10/14 TgatgTGAACCA). The role of these sequences in the control of GLUT2 gene expression needs to be established. A potential TATA-like motif and CAT box are located at −34 and −99 bp, respectively, of the major initiation start site and may be involved in mediating the basal transcriptional activity of the GLUT2 gene.

Nucleic acid comparison of the human and mouse 5′-regulatory regions of GLUT2 gene shows approximately 50% sequence identity between the human (−398 to +222) and the murine (−579 to −25) promoters which suggests that these conserved regions are of functional importance (Fig. 1C).

Transcriptional Start Sites Are Common in GLUT2 Expressing Tissues—The initiation start sites were localized by RNase protection and primer extension assays. For the RNase protection assay, an antisense RNA probe corresponding to nucleotides −329 to +70 of the mouse promoter sequence was hybridized in solution with RNAs extracted from different tissues and purchased from Amersham. The products of the RNase protection or primer extension assays were separated on a 6% polyacrylamide/urea sequencing gel. A sequencing reaction primed with the same oligonucleotide as used for the primer extension was run as a sizing marker.

Transcriptional Reporter Constructs and Transfection Studies—Promoterless (DOTAP, Boehringer). The nucleic acid of either construct were usually co-transfected into 1–2 × 106 cells, with a pSV, galactosidase reporter gene (pSV,GAL) as internal control. Twenty four hours after transfection, glucose concentrations of the medium were changed (0, 5, 10, and 20 mM) and the cells incubated for an additional 24 h. The cells were harvested and then ruptured by three freeze-thaw cycles, as described by Pothier et al. (20). After removal of cellular debris, the extracts were held for 10 min at 65 °C to destroy endogenous deactetylating activity. Protein concentrations were determined using the BCA protein assay (Pierce). CAT assays were carried out using 100–150 μg of cell extracts and the acetylated chloramphenicol was separated on a thin layer chromatography plate. The results were normalized by the value of galactosidase activity measured from the co-transfection of pSV,GAL and/or adjusted to the protein concentration of the cell extracts. The CAT-enzyme-linked immunosorbent assay system (Boehringer) was also used in some studies to quantitate the CAT protein of the fusion mouse promoter CAT constructs was determined by primer extension of transfected INS-1 cell RNA using a complementary oligonucleotide to the CAT gene (5'- TTACGATGGCATGTGGG-3').
FIG. 1. Nucleotide sequence of the mouse GLUT2 promoter. A, diagram and partial mapping of the 18-kb insert of bacteriophage 4 obtained by screening a mouse genomic library with a GLUT2 cDNA probe. The 18-kb insert contains the first three exons of the gene and approximately 1.4 kb of sequence upstream the first coding ATG. B, sequence of the mouse promoter of GLUT2. A major initiation site was localized at -55 bp from the first coding ATG which determined the +1 bp coding system (arrow and T in bold character). Two minor start sites were also localized at -64 and -115 from the ATG encoding the first methionine. Potential TATAAA and CAAT boxes are present at -54 and -99 bp, respectively, of the major transcriptional start. Several potential consensus sites for various transacthing factors have been determined by computer analysis and shown with arrows (see results for description of the putative responsive elements). C, nucleic acid comparison of the human and mouse 5'-regulatory region of GLUT2 gene. Approximately 50% sequence identity was found between the human (-398 to +222) and the mouse (-579 to -25) promoters which may suggest that these conserved regions are of functional importance ( indicates identical sequences and A indicates gap in the sequence comparison).

a positive control was made from a sense RNA synthesized in vitro. Fig. 2A shows a typical RNase protection assay, where the 399-base probe protects 3 major transcripts after RNase A and T1 digestion, labeled 1, 2, and 3 in the figure. These transcripts define start sites localized at -115, -64, and -55 bases from the first coding ATG. The protected transcripts are present in the mouse islet, liver, intestine, and kidney RNAs but not in mouse heart, whole pancreas (the endocrine pancreas representing a
from a sense RNA synthesized to correspond to the expected amount of GLUT2 mRNA expression in RNA probe that contained the -329 to +70 bp region of the promoter. No mouse tissues RNAs hybridized in solution with a 399-base antisense defined by primer extension, and is considered as the major initiation sites of the mouse GLUT2 gene. A, Number GLUT2, the probe protected 3 major transcripts defined as tRNA, heart, and whole pancreas RNAs. A positive control was made protected transcripts were found in the negative controls which include these tissues: higher intensity is seen in islet and liver RNAs and lower intensity is found in the kidney and the intestine RNAs.

In vitro. In all tissues expressing GLUT2, the probe protected 3 major transcripts defined as 1, 2, and 3. Number 3 is the strongest signal and corresponds to the start site defined by primer extension, and is considered as the major initiation site for RNA polymerase II. The intensity of the protected transcript corresponds to the expected amount of GLUT2 mRNA expression in these tissues: higher intensity is seen in islet and liver RNAs and lower intensity is found in the kidney and the intestine RNAs. B, the same RNA used to determine the start sites were hybridized with an actin antisense probe to show equivalent RNA loading in the various columns in the ribonuclease protection assay.

small percentage of the organ), or tRNA which were used as negative controls. An actin antisense RNA probe was hybridized to the same RNAs to quantitate the amount of RNA (Fig. 2B). The protected transcript mapped at -55 bases (number 3, Fig. 2A) is the major start site as confirmed by primer extension and several other RNase protection assays using a larger 1400-base antisense RNA probe (data not shown). Additional lower frequency start sites were also identified (transcripts 1 and 2 in Fig. 4A) in all tissues expressing GLUT2 which suggests the presence of multiple initiation start sites for the RNA polymerase II. The intensity of the protected transcripts of Fig. 2A corresponds to the abundance of GLUT2 mRNA expressed in these tissues. The highest level being found in pancreatic islets and liver, whereas, in kidney and intestine, it is lower.

By primer extension (using a complementary oligonucleotide to sequence +49 to +70 of the mouse promoter sequence) one major start site was identified (data not shown), which corresponds to the -55 bp transcript (transcript number 3, Fig. 2A), and was subsequently used to define the start site (bp +1 of the sequence shown in Fig. 1B).

Mouse and Human Promoters Are Functionally Active and Glucose-responsive When Transiently or Stably Transfected into a Differentiated β Insulinoma Cell Line—Regions of the mouse (−1311 to +49) and human (−1296 to +312) promoters were cloned into the eukaryotic expression vector pCAT Basic (Promega) and transiently transfected into a differentiated β insulinoma cell line, INS-1, which expresses a high level of GLUT2. Cells were then exposed for 24 h to a medium containing fetal calf serum and 0 or 20 mM glucose. As shown in Fig. 3, basal transcriptional activity, as measured by CAT assay, is higher for the mouse promoter in comparison to the promoterless vector pCAT Basic. Furthermore, the mouse promoter CAT fusion construct shows a glucose inducibility of 2–3-fold with 20 mM glucose in comparison to the basal transcriptional rate seen during incubation with 0 mM glucose. The CAT activity was normalized by protein content of the transfected cell extracts and/or by the activity of P-galactosidase expressed from a co-transfected pSV2 galactosidase reporter gene. Quan-

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**Fig. 2.** Determination of the transcriptional initiation start sites of the mouse GLUT2 gene. A, ribonuclease protection assay of mouse tissues RNAs hybridized in solution with a 399-base antisense RNA probe that contained the -329 to +70 bp region of the promoter. No protected transcripts were found in the negative controls which include tRNA, heart, and whole pancreas RNAs. A positive control was made from a sense RNA synthesized in vitro. In all tissues expressing GLUT2, the probe protected 3 major transcripts defined as 1, 2, and 3. Number 3 is the strongest signal and corresponds to the start site defined by primer extension, and is considered as the major initiation site for RNA polymerase II. The intensity of the protected transcript corresponds to the expected amount of GLUT2 mRNA expression in these tissues: higher intensity is seen in islet and liver RNAs and lower intensity is found in the kidney and the intestine RNAs. B, the same RNA used to determine the start sites were hybridized with an actin antisense probe to show equivalent RNA loading in the various columns in the ribonuclease protection assay.

**Fig. 3.** Glucose responsiveness of the mouse GLUT2 promoter. CAT activity was measured in the transiently transfected β insulinoma cell line (INS-1) exposed for 24 h to a medium without or with 20 mM glucose. The promoterless reporter CAT vector (pCAT basic) shows no glucose responsiveness, whereas 1311 bp of the mouse promoter CAT fusion construct shows a 2–3-fold increase in CAT activity with 20 mM.
GLUT2 Gene Expression

A, a typical CAT assay of transiently transfected INS-1 cells with the human (hΔ1296CAT) and the mouse (mΔ1311CAT) promoter CAT fusion constructs compared to the promoterless pCAT-basic vector (pCAT-basic). The human and mouse promoters were glucose-responsive in a dose-dependent manner from 0 to 20 mM of glucose for an incubation time of 24 h. The arrows designated the mono-, bi-, and triacetylated chloramphenicol forms were separated by thin layer chromatography. B, quantitative assessment of transcriptional glucose responsiveness of the human and mouse GLUT2 promoters. Six different transfection experiments were carried out and CAT activity or protein measured by counting 14C-acetylated chloramphenicol or by the CAT-enzyme-linked immunosorbent assay system. The CAT activity was normalized by protein content of cell extracts and/or by the co-transfection of a pSV2 galactosidase reporter gene. Glucose induced a dose-dependent transactivation of the mouse GLUT2 promoter: there was an increase in CAT activity of 1.4-, 2.5-, and 5.1-fold at glucose concentrations of 5, 10, and 20 mM, respectively, compared to baseline (glucose = 0 mM). Similarly, glucose induced a 1.3-, 1.8-, and 2.8-fold transactivation of the human GLUT2 promoter when exposed to 5, 10, and 20 mM (* = p < 0.05, ** = p < 0.01, and *** = p < 0.001). Data are shown as the mean ± S.E.

DISCUSSION

In this study, we have structurally characterized 1.4 kb of the mouse GLUT2 promoter and shown that this sequence shares...

FIG. 4. Dose-dependent transcriptional activation of the mouse and human promoters exposed to increasing concentrations of glucose. A, a typical CAT assay of transiently transfected INS-1 cells with the human (hΔ1296CAT) and the mouse (mΔ1311CAT) promoter CAT fusion constructs compared to the promoterless pCAT-basic vector (pCAT-basic). The human and mouse promoters were glucose-responsive in a dose-dependent manner from 0 to 20 mM of glucose for an incubation time of 24 h. The arrows designated the mono-, bi-, and triacetylated chloramphenicol forms were separated by thin layer chromatography. B, quantitative assessment of transcriptional glucose responsiveness of the human and mouse GLUT2 promoters. Six different transfection experiments were carried out and CAT activity or protein measured by counting 14C-acetylated chloramphenicol or by the CAT-enzyme-linked immunosorbent assay system. The CAT activity was normalized by protein content of cell extracts and/or by the co-transfection of a pSV2 galactosidase reporter gene. Glucose induced a dose-dependent transactivation of the mouse GLUT2 promoter: there was an increase in CAT activity of 1.4-, 2.5-, and 5.1-fold at glucose concentrations of 5, 10, and 20 mM, respectively, compared to baseline (glucose = 0 mM). Similarly, glucose induced a 1.3-, 1.8-, and 2.8-fold transactivation of the human GLUT2 promoter when exposed to 5, 10, and 20 mM (* = p < 0.05, ** = p < 0.01, and *** = p < 0.001). Data are shown as the mean ± S.E.
GLUT2 Gene Expression

Fig. 6. Effect of glucose on GLUT2 gene transcription rate in INS-1 cells. A, INS-1 cells were incubated in 2, 10, and 20 mM glucose for 4, 8, and 24 h. Nuclei were isolated and nascent transcripts were labeled and hybridized to the rat GLUT2 cDNA, pGEM, and β-actin cDNA. Glucose induced GLUT2 gene transcription in a concentration-dependent manner. B, quantitative assessment by laser densitometric scanning of GLUT2/actin transcription measured by nuclear run-on assay. A maximal 3.4- and 4.0-fold increase in GLUT2 gene transcription was observed with 10 and 20 mM glucose concentrations, respectively. Data were normalized to the β-actin densitometric value.

Fig. 5. Glucose effect on stably transfected INS-1 cells with human and mouse promoter CAT constructs. A, quantitative assessment of the mouse promoter CAT construct stably transfected into INS-1 cells. CAT activities were normalized to protein content. A maximal 1.9-fold increase in CAT activity was observed with 20 mM glucose concentration for 24 h. B, a maximal 3.6-fold increase in CAT activity was measured with 20 mM glucose concentration for 48 h using INS-1 cells stably transfected with the murine promoter CAT construct. C, quantitative assessment of the human promoter CAT construct stably transfected into INS-1 cells and exposed to 0, 5, 10, and 20 mM glucose for 24 or 48 h.
analogy to the distal L4 element of the pyruvate kinase gene (GTGCCC). Several AT-rich stretches are present in the murine and human promoters but these structurally defined sequences need to be functionally tested to see if they are able to confer glucose responsiveness on heterologous promoters.

We have demonstrated by nuclear run-on analysis that INS-1 cells exposed to medium with 10 and 20 mM glucose have a maximum 3.4- and 4-fold increase, respectively, in endogenous GLUT2 gene transcription. Furthermore, cytoplasmic RNA of the run-on analysis have shown a parallel induction of GLUT2 gene expression to a maximum 1.7- and 2.3-fold for the 10 and 20 mM glucose concentrations, respectively. Therefore the endogenous GLUT2 gene is transcriptionally regulated by glucose as is the case for the murine and human GLUT2 promoters. The modest differences between transcription rate and mRNA expression could possibly be explained by either a delay in the transfer of the transcripts from the nuclei to the cytoplasm and/or an alteration in GLUT2 mRNA stability induced by glucose. We have measured GLUT2 mRNA halflife in INS-1 cells in 11 mM glucose and determined that it was approximately 8 h. Further work will be necessary to demonstrate a possible role of glucose in altering GLUT2 mRNA stability.

Several reports have previously shown that GLUT2 gene expression is modulated by glucose in vivo and in vitro. In primary cultures of rat hepatocytes, Asano et al. (2) described a 3.2-fold increase in GLUT2 mRNA expression when these cells were incubated in 27.8 mM glucose for 20 h. Similar observations were recently reported by Postic et al. (3) who have studied GLUT2 mRNA expression in liver in vitro and in vivo: a maximal 4-fold increase in GLUT2 mRNA expression was found when hepatocytes were incubated in 20 mM glucose for 24 h. In the hamster beta-cell line HIT, Inagaki et al. (4) found a 40% increase in GLUT2 mRNA expression when these cells were incubated in 22.2 versus 11.1 mM glucose for 24 h. A 3-fold induction in GLUT2 expression was observed in primary rat islets in 11.1 versus 5.5 mM glucose concentration by Yasuda et al. (5). Chen et al. (6) described a 46% increase in GLUT2 mRNA expression in beta-cells of rats maintained hyperglycemic for 5 days. More recently, a maximal 10-fold increase in GLUT2 expression was described by Ferrer et al. (7) when rat islets were incubated in 11 mM glucose in comparison to 2 mM. In this study, a time course experiment of the effect of glucose on GLUT2 has shown a 2.5-fold induction of GLUT2 mRNA only after 8 h of culture in 16.7 mM glucose. Taken together, these observations clearly demonstrate that high glucose concentrations positively modulate GLUT2 gene expression in beta-cells and hepatocytes.

In diabetic animal models, a drastic beta-cell-specific reduction of GLUT2 expression has been reported for the NIDDM Zucker fa/fa, the neonatal low-dose STZ-induced diabetic rat, the Wistar Kyoto, GK rats, db mice, and the autoimmune diabetic BB rats (8–12). By cross-transplanting islets from db/db mice into control mice or vice versa, the decreased GLUT2 expression was shown to be reversible and induced by the diabetic environment of the animals (26). The pathogenic significance of the decrease in beta-cell GLUT2 is controversial: it has been claimed that this loss may be the primary cause of the specific glucose-induced secretory abnormality encountered in the diabetic state (1, 8, 11) although others consider this possibility unlikely (37). Whatever the pathogenic significance of the loss of GLUT2 expression, it is one of the earliest biochemical markers of the diabetic state. Since glucose regulates positively GLUT2 gene transcription in the normal state and since the carbohydrate-responsiveness of GLUT2 is selectively lost in the beta-cell of diabetic rodents, further work to identify the cis elements and trans-acting factors involved in the control of...
GLUT2 gene transcription is needed and this may lead to a better understanding of the pathogenic events involved in the early diabetic state.

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