Liver-specific Enhancer of the Glucokinase Gene*

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Glucokinase gene regions that are important for liver specific expression of the enzyme have been functionally identified using transient transfection of rat hepatocytes. Maximal luciferase activity was elicited by a reporter plasmid with 3.4 kilobase pairs of genomic DNA flanking the liver glucokinase promoter. Deletion of a gene fragment between −1000 and −600 with respect to the start of transcription resulted in a 60% decrease in luciferase activity. Further reduction, close to background level, occurred upon deletion of a 90-base pair sequence between −123 and −34. Reporter plasmids with the liver glucokinase promoter and any length of flanking sequence were minimally active in INS-1 insulinoma cells, and conversely reporters with the β-cell-specific promoter were ineffective in primary hepatocytes. In FTO-2B hepatoma cells, a differentiated line expressing many liver-specific traits but not the endogenous glucokinase gene, the promoter proximal region between −123 and −34 markedly stimulated the expression of transfected plasmids above background. However, addition of the flanking region up to −1000 inhibited luciferase expression. The gene fragment from −1003 to −707 was shown to be a bona fide, hepatocyte-specific enhancer by the following criteria: 1) it stimulated reporter expression by more than 10- and 5-fold when inserted directly upstream of the glucokinase TATA box or complete promoter, respectively, regardless of orientation; 2) it stimulated gene expression from the heterologous SV 40 promoter -4-fold; 3) it was also effective from a downstream position; and 4) in contrast to the enhancer effect in primary hepatocytes, the sequence acted as a silencer in FTO-2B cells and was neutral in INS-1 cells. Both the promoter proximal and the enhancer regions were marked by DNase I hypersensitive sites in the chromatin of primary hepatocytes but not hepatoma or insulinoma cells. Seven footprinted elements termed A through G were mapped in the enhancer by the in vitro DNase I protection assay. Elements A–C may bind liver enriched factors, because they were not protected by spleen nuclear extract. In hepatocyte transfection, the downstream half of the enhancer containing elements A–C was about half as effective as the complete enhancer in stimulating glucokinase promoter activity. Site-directed mutagenesis of element A virtually abrogated the activity of the half-enhancer, whereas mutation of element C had a more moderate effect. The sequence between −732 and −578 upstream of the liver start of transcription in the human glucokinase gene displays 79% sequence identity with the downstream half of the rat enhancer. The human gene fragment ligated to the minimal rat liver glucokinase promoter was shown to work as an enhancer in the hepatocyte transfection system.

The mammalian glucokinase gene is a dual promoter transcription unit. Transcription can initiate at either one of two widely separated start sites, which are used in a mutually exclusive fashion in different cell types (1). The upstream promoter, termed the β-cell promoter, is active primarily in β-cells of the pancreatic islets and in rare cells of the gastrointestinal tract, lung, thyroid, and central nervous system. Short term transfection and transgenic mice experiments have indicated that a promoter proximal region of 300 bp of DNA upstream of the start site is necessary and sufficient for directing glucokinase gene expression in the above cell types (2, 3). The downstream promoter, designated the liver promoter, is known to be active only in hepatocytes (4). The genomic elements governing the function of the liver promoter have not been identified.

Several genes referred to as “liver-specific” are transcribed exclusively or preferentially in the liver (5). However, the hepatic expression of the glucokinase gene incorporates a number of added features not generally found in the liver-specific genes taken as a group, suggesting that particularly complex regulatory mechanisms of gene transcription might operate (6). The glucokinase gene is not transcribed in the fetal or neonatal liver, mRNA and enzyme appearing only at weaning time (7). Glucokinase is also known to be lost in the process of liver carcinogenesis (8). Glucokinase expression is thus restricted to mature, fully differentiated hepatocytes. In addition, liver transcription of the gene is dependent on the hormonal status of the animal, as shown by its complete repression in the insulin-deprived diabetic animal or in primary hepatocytes cultured without insulin, and its induction upon insulin treatment or addition to the culture medium (9–11). In order to understand these properties, it is necessary to identify gene regions harboring regulatory cis-acting DNA elements and subsequently the trans-acting factors binding to such elements.

The purpose of this work was to identify DNA elements responsible for the cell type specificity of the liver glucokinase promoter. A systematic transfection study of the promoter and flanking DNA is presented. Transient expression of reporter gene plasmids was monitored in primary rat hepatocytes and hepatoma and insulinoma cells. The functional data from the transfection system were correlated with the mapping of cell type-specific DNase I hypersensitive sites in chromatin and the identification of cis-acting elements using the in vitro DNase I footprinting assay.

1 The abbreviations used are: bp, base pair(s); kpb, kilobase pair(s); PCR, polymerase chain reaction.

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EXPERIMENTAL PROCEDURES

Plasmid Constructions—Two rat genomic libraries in the EMBL 3 and Charon 4A vectors respectively (Clontech) were the source of DNA for the entire glucokinase gene including regions flanking the two promoter ends of the gene. Four clones were isolated from each library, and another was the generous gift of Dr. Christopher B. Newgard, The University of Texas Southwestern Medical Center at Dallas. Restriction fragments were subcloned in the pBluescript or pUC vectors, and subfragments were used for the construction of reporter plasmids and as probes. An approximately 400-bp BamHI-HindIII (blunted) genomic fragment containing the liver promoter was isolated and ligated to the BamHI and HindII (blunted) sites of the chloramphenicol acetyltransferase reporter plasmid pBLCAT3 (12) to generate pGK-400-CAT. This plasmid was digested with BamHI, and a 1-kbp HindIII genomic fragment was inserted in the sense orientation to obtain pGK-1400-CAT. Fragments Xhol (blunted)-Xhol from pGK-1400-CAT and pGK-400-CAT were subsequently transferred to the luciferase reporter plasmid pGL2 Basic (Promega) cut with Smal and Xhol, yielding plasmids pGK-1400-Luc and pGK-400-Luc. Plasmid pGK-1100-Luc was constructed by cloning a 1100-bp BstXI (blunted)-Xhol fragment from pGK-1400-Luc in the pGL2 vector cut with Smal and Xhol. Similarly, a 1000-bp NotI (blunted)-Xhol fragment from pGK-1400-Luc and a 700 bp fragment AflII (blunted)-Xhol fragment from pGK-1400-Luc were ligated into the pGL2 Basic vector digested with Smal plus Xhol to yield plasmid pGK-1000-Luc and pGK-700-Luc, respectively. Plasmids pGK-230-Luc and pGK-200-Luc were generated from pGK-400-Luc using XhoI digestion. Plasmid pGK-120-Luc was constructed by inserting a 132-bp DNA fragment synthesized by PCR and digested with BglII and KpnI in the corresponding sites of pGL2 Basic. The 5’ end points of the glucokinase gene sequence were nucleotides −1447 in pGK-1400-Luc, −1017 in pGK-1100-Luc, −915 in pGK-1000-Luc, −586 in pGK-700-Luc, −310 in pGK-400-Luc, −147 in pGK-230-Luc, −123 in pGK-200-Luc, and −34 in pGK-120-Luc, position 1 being the nucleotide preceding the liver start of transcription. The 3’ end point in all plasmids was position +79. An additional set of three deletion plasmids was constructed using another firefly luciferase vector called pGK-Luc (13). Plasmid pGK-230-Luc was obtained by subcloning a 3.4-kbp fragment of the glucokinase gene extending from a genomic HindIII site at approximately −3300 to the HindIII site at +79 upstream of the luciferase gene. Except for the vector backbone, plasmids pGK-1400-Luc and pGK-400-Luc were otherwise similar to pGK-1400-Luc and pGK-400-Luc.

The glucokinase enhancer genomic sequence from nucleotides −1003 to −707 with Smal sites added at both ends was synthesized by PCR, digested with Smal, and ligated in both orientations into the Smal site upstream of the glucokinase promoter in pGK-120-Luc to generate p-enhancer-GK-Luc and p-(r)-enhancer-GK-Luc. The fragment was similarly inserted upstream of the simian virus 40 early promoter in plasmid pGL2 Promoter (Promega) to generate p-enhancer-SV-Luc and p-(r)-enhancer-SV-Luc. The enhancer fragment was also subcloned in the Smal site of the vector pBluescript KS I, providing pB-enhancer. A BamHI-SallI fragment from pB-enhancer was inserted in the cognate sites of pGK-120-Luc and p-SV-Luc downstream of the luciferase gene to construct pGK-120-Luc-enhancer and p-SV-Luc-enhancer, respectively. The glucokinase promoter from −123 to +79 was ligated as a HindIII-Xhol fragment in pB-enhancer; the complete enhancer-promoter fragment was excised by digestion with Sall plus Xhol and was transferred to pGL2 Basic, yielding p-enhancer-GK-200-Luc.

The 3’ half of the enhancer was obtained from within the above plasmid as a BstEII-Smal fragment. After blunting the BstEII end, the approximately 150-bp fragment was subcloned in the Smal site of pGK-120 to obtain plasmids CBA-GK-120-Luc. A plasmid with the homologous human sequence was constructed with a DNA fragment generated by PCR, followed by Smal digestion. The primers were 5’-TCTCCCCGGA-TGAATCTACTGCTGCCATG-3’ and 5’-TCTCCCCGGA-TGCACTCACCC-3’, and the template was blood genomic DNA from a nodiabetic subject.

The 3’ part of the rat enhancer from nucleotides −812 to −708 with added Xhol and BstEII sites was synthesized by PCR and inserted in a Bluescript KS + plasmid in which the glucokinase promoter sequence from −1447 to −1017 had been subcloned into the Smal and Xhol sites. The SacI-Xhol fragment from the above plasmid was transferred to the pGL2 Basic to generate p-CBA-GK-200-Luc. Plasmids with block mutations in elements A and C were constructed in similar fashion using mutagenic primers. The DNA fragment with block mutation in the internal element B was synthesized with a single mutagenic primer by a two-step PCR procedure, the product from the first reaction serving as “megaprimer” in the second reaction, essentially as described (14, 15). For more details on the base changes on the DNA fragment sequence all DNA inserts generated by PCR or exonucle-ase III digestion was verified by dideoxy sequencing using the Sequenase polymerase. Molecular cloning methods were performed according to Sambrook et al. (16).

Cell Culture and Transfection—For isolation of hepatocytes, the livers of adult male Wistar rats fasted for 48 h before the experiments were perfused with collagenase as specified previously (17). Cells were dispersed in 80 ml of RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% newborn calf serum. The cells were centrifuged at 23 × g for 2 min and washed twice in 100 ml of the same medium and once in 50 ml of a solution of termorebuttal electrofusor containing 20 mM Hepes, pH 7.15, 137 mM NaCl, 5 mM KCl, 0.7 mM NaHCO3, and 0.5 mM CaCl2 and MgCl2 added at the rat plasma concentrations. The final cell pellet was resuspended in ice-cold electrofusor buffer supplemented with 300 µg/ml sonicated salmon testes DNA to provide a cell density of 15 × 106 viable cells/ml and 0.8-ml samples of cell suspension were pipetted into electrofusor cuvettes with an electrode gap of 4 mm. Plasmids were added in amounts of 20 µg of pGL2 Basic or equimolar amounts of GK luc plasmids. For co-transfection, 3 µg of pCMV β-galactosidase (pCMV β, Clontech) were added, and the cuvettes were placed in ice for 5 min. The cells were carefully resuspended by manual shaking and electrofused with a GenePulsar apparatus (Bio-Rad) at a capacitance of 960 µF and voltage of 250 V. The cuvettes were returned to ice for 10 min. Cells from the cuvettes were then transfected to obtain containing 4.3 ml of RPMI 1640 plus antibiotics and 10% fetal calf serum and samples of 0.510 ml of cell suspension (1.5 × 106 cells) were pipetted into wells of 6-well culture dishes (Falcon Primaria) containing 3 ml of the above medium. After 3 h of culture in a CO2 incubator, the medium was replaced by a medium containing 5% fetal calf serum and 10–100 µM dexamethasone. Culture was continued for 20 h before cell harvest for measurements of luciferase and β-galactosidase enzyme activities.

Rat hepatoma cells of the FTO-2B line (18) were cultured in Dulbecco’s modified Eagle’s medium/Nutrient Mix Ham’s F-12 medium supplemented with antibiotics and 10% fetal calf serum in T75 culture flasks (Falcon). When cells reached 50–60% confluence, they were fed fresh medium and harvested by trypsinization 20 h later. The cells were washed once in culture medium containing 10% newborn calf serum and once in 50 ml of ice-cold electrofusor buffer with centrifugations at 90 × g for 5 min. The cell pellet was resuspended in electrofusor buffer containing 300 µg/µl salmon testes DNA. The cells were electrofused as described above, except that voltage was 280 V. The electroporated cells were seeded in 6-well dishes at a density of 3 × 106 cells/ml and allowed to attach for 3 h. The culture medium was then replaced by fresh medium, and culture continued for 20 h prior to harvest for reporter enzyme assays.

Rat insulinoma cells of the INS-1 line (19) were cultured as described (20). One day after being fed fresh medium, cells at 50–60% confluence were harvested from T75 flasks by trypsinization, washed twice in culture medium containing 10% newborn calf serum, and suspended in culture medium at a density of 0.5 × 106 cells/ml. Samples of 3 ml of cell suspension were transferred to tubes containing calcium phosphate/DNA precipitates formed with 10 µg of plasmid essentially as described by Kingston et al. (21). The tube contents were mixed by inversion and plated into 6-well culture dishes. Extracts of nontransfected INS-1 cells were added to have a high background in the β-galactosidase assay, making the co-transfection of pCMV β impracticable as a test of transfection efficiency. In each experiment, duplicate transfection mixtures were formed with each individual plasmid and plated in duplicate wells. The cells were cultured for 5 h in presence of the precipitate, washed twice with serum-free medium, fed 3 ml of medium with 10% fetal calf serum, and cultured for 20 h prior to harvest for assay of luciferase activity. Separate cell extracts were obtained from duplicate wells to monitor the reproducibility of the transfection efficiency. Replicates generally varied by less than 10%. For reporter enzyme assays, cell monolayers were washed twice with 2.5 ml of phosphate-buffered saline and scraped in 0.16 ml of a lysis buffer containing 25 mM Tris/phosphate, pH 7.8, 2 mM dithiothreitol, 1% Nonidet P-40, and 1% Triton X-100. Extracts were allowed to stand at room temperature for 15 min and centrifuged in a microfuge for 5 s, and the protein concentration of the supernatant was assayed using the Bio-Rad reagent. The extracts were then adjusted to a fixed protein concentration and stored in ice until assayed for enzyme activities. The luciferase assay was performed by adding to 30 µl of cell extract, 150 µl of a reagent....
containing 20 mM Tricine, pH 7.5, 4 mM MgCl₂, 0.1 mM EDTA, 33 mM dithiothreitol, 530 μM ATP, 270 μM Caa, and 300 μM firefly luciferin (22). The sample was immediately mixed by inversion and introduced in a liquid scintillation counter (Beckman LS 8100 or LS 6500), and light emission was measured in the single photon mode for 1 min. Cell extracts were used undiluted or diluted with lysis buffer supplemented with 0.1% bovine serum albumin to provide an input of 20–60 μg of protein/30 μl. The background recorded with extracts from nontransfected HepG2 cells was subtracted from measurements with transfected samples. The same extracts were used for the measurement of β-galactosidase activity by a chemiluminescent assay (23) using commercially available reagents (Galacto-Light, Tropix). The assay was performed using 0.15–1 μg of protein from primary hepatocytes and 0.1 μg of protein from FTO-2B cells. The incubation time was comprised between 40 and 120 min. Light emission was measured as above and a blank with nontransfected extracts was subtracted from gross counts.

Footprinting with the DNase I Protection Assay—Nuclear protein extracts were prepared from rat liver and spleen as described by Gorski et al. (24) and Maire et al. (25). Because transcription of the glucokinase gene is markedly stimulated by glucose refeeding after a fast, nuclei were isolated from animals that had been deprived of food for 40 h and given an oral glucose load 2 h before killing. Probes were labeled using [32P]labeled DNA were added to reaction mixes containing 20 mM Hapes, pH 7.7, 48 mM KCl, 4.6 mM MgCl₂, 0.08 mM EDTA, 0.8 mM dithiothreitol, 1 μg of poly(dI-dC) and 5–15 μg of nuclear protein in a total volume of 24 μl. The mixtures were allowed to stand in ice for 30 min after the addition of 0.1–0.3 unit DNase I depending on the nuclear protein input. Digestion with this enzyme was carried out at 4°C for 3 min and terminated by adding 80 μl of a solution containing 20 mM Tris/HCl, pH 7.5, 20 mM EDTA, 0.5% SDS, 6 μg of sonicated salmon testes DNA, and 100 μg/ml proteinase K. After a 45-min incubation at 50°C, DNA was phenol extracted and ethanol was precipitated. Products were analyzed on 6% acrylamide, 8 M urea sequencing gels. The protected elements were mapped by reference to the migration of Maxam-Gilbert sequencing products.

DNase I Hypersensitive Sites—Cultured rat hepatocytes, hepatoma FTO-2B cells, and insulinoma INS-1 cells were analyzed for DNase I hypersensitive sites in the glucokinase gene using the method of Stewart et al. (26). Cell monolayers in 10-cm culture dishes were washed at room temperature with PBS and overlayed with 4 ml of a solution containing 15 mM Tris/HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM β-mercaptoethanol, 0.3 mM sucrose, DNase I (Boehringer, Grade 1) at concentrations ranging from 50 to 400 units/ml as specified in the figure legends and Nonidet Nonidet P-40 at 0.3% for primary hepatocytes and 0.2% for the cell lines. Two dishes were used for each DNase I concentration. The DNase I solution was aspirated after 3.5-min incubation at room temperature, and digestion was terminated at 4 min by the addition of 3 ml of 50 mM Tris/HCl, pH 8, 20 mM EDTA, 1% SDS, and 0.2 μg/ml proteinase K. Digestion with this enzyme was done at 50°C for 5 h with intermittent agitation. The lysates from duplicate dishes were pooled and extracted with phenol-chloroform and the nucleic acids were precipitated with ethanol in presence of 2.5 M NH₄ acetate. The samples were digested with Rnase A, extracted once with phenol-chloroform and once with chloroform. After ethanol precipitation, DNA was digested with a restriction enzyme for 5 h, phenol extracted, and precipitated. The DNA was dissolved in TE buffer, its concentration was measured, and samples of 25 μg of DNA were electrophoresed in 0.8% agarose gels 20 cm in length. Southern transfer to nylon membranes by electroblotting and hybridization to short DNA probes for indirect end-labeling were performed by standard techniques. The size of DNA fragments was calibrated with HindIII and EcoRI-digested λ DNA M₁ markers. For accurate mapping of the hypersensitive sites with respect to internal restriction sites, samples of DNase I-digested DNA that had been cut with two endonucleases were run alongside the test samples. Outside of the gene regions described under “Results,” the following restriction fragments, with their position relative to the liver promoter, were analyzed and found to harbor no hypersensitive sites: HindIII from −14 to −7 kb, BamHI from +3 to +13 kb, and EcoRI from +12 to +17.5 kb.

RESULTS

Progressive Deletion of 5’ Flank of Liver Glucokinase Promoter—Hepatoma cell lines that have generally been used for transfection studies of genes transcribed in liver do not express the endogenous glucokinase gene. We therefore decided to use primary hepatocytes as target cells for transient transfection.

FIG. 1. Deletion analysis of liver glucokinase promoter and flanking region. Freshly isolated rat hepatocytes were transfected by electroporation and plated for primary culture. After 20–22 h in culture, cells were lysed, and fixed amounts of cellular protein were assayed for reporter enzyme activities as specified under “Experimental Procedures.” Luciferase activities were normalized for the β-galactosidase activity elicited by the co-transfected CMV-β plasmid and expressed relative to the activity of p-GK-1400-Luc taken as 100. Data are the means ± S.E. of three to five independent experiments using at least three separate plasmid preparations for each construct. The analysis reveals a promoter proximal (between the 5’ end points of p-GK-120-Luc and p-GK-200-Luc, −34 and −123) and a remote region (between the 5’ end points of p-GK-700-Luc and p-GK-1100-Luc, −586 and −1017) essential for high reporter gene activity.

studies. Preliminary experiments using the chloramphenicol acetyltransferase reporter gene showed that the glucokinase promoter and 5’ flanking DNA did not consistently elicit chloramphenicol acetyltransferase activity above background after transfection by either calcium phosphate-DNA co-precipitation or lipofection. In our hands, the only method allowing to quantify glucokinase promoter activity in a reliable manner has been the electroporation procedure described under “Experimental Procedures” combined with the use of firefly luciferase reporter plasmids. Luciferase activity after transfection of reporter plasmids driven by the liver glucokinase promoter and upstream sequence is shown in Fig. 1. Maximal luciferase activity was observed with a construct including 3.4 kb of flanking sequence and was maintained at similar levels in constructs comprising 1.4 and 1.1 kb of sequence upstream of the promoter. Deletion of the next 400 bp resulted in a 3-fold decrease in luciferase expression, delineating the region between −1017 (5’ end point in plasmid GK-1100-Luc) and −586 (5’ end point in plasmid GK-700-Luc) as a potential enhancer. Luciferase activity was little affected by further sequence deletion down to position −123 (plasmid GK-200-Luc). Deletion of the next 90 bp to position −34 (plasmid GK-120-Luc) was accompanied by a pronounced drop of expression, residual luciferase activity being only marginally above the background level noted with the promoterless luciferase vector (GL2 Basic plasmid). Thus, the 100-bp region directly upstream of the TATA box homology (TATTT from nucleotides −29 to −25) is essential for liver glucokinase promoter activity.

In order to test the cell type specificity of the effects described above, comparative transfection experiments were performed with primary rat hepatocytes, INS-1 insulinoma cells,
Investigated in both orientations and also in a 3-GK-120-Luc. For some of the constructs, the test fragment was complete promoter in GK-200-Luc and the minimal promoter in the homologous promoter, plasmids were constructed both with the constitutive SV40 196-bp early promoter. With respect to the homologous promoter or a heterologous promoter, namely the glucokinase promoter in GK-120-Luc, reporter expression in primary hepatocytes was enhanced 10-fold with the test fragment upstream of the luciferase coding region. The results of short term transfection in primary hepatocytes and FTO-2B hepatoma cells are summarized in Fig. 3. With the complete homologous promoter in GK-200-Luc, the upstream fragment stimulated luciferase activity 5-fold in hepatocytes, whereas a 2-fold decrease was noted in hepatoma cells. With the minimal glucokinase promoter, plasmid was active in both normal hepatocytes and hepatoma cells but not in insulinoma cells. The remote regulatory region has a positive effect in hepatocytes and a negative effect in hepatoma cells.

and FTO-2B hepatoma cells. Experiments in primary hepatocytes (Fig. 2A) demonstrated that the expected drop in luciferase activity when plasmid GK-400-Luc was compared with GK-1400-Luc and the sharp decrease in activity between GK-200-Luc and GK-120-Luc. Moreover, a plasmid with the β-cell glucokinase promoter, plasmid βGK-400-Luc, was unable to elicit luciferase expression in the hepatocytes. Conversely, this plasmid was active when transfected in INS-1 cells, whereas all the constructs driven by the liver promoter and flanking sequence displayed only marginal luciferase activity (Fig. 2B). In FTO-2B hepatoma cells, luciferase activity was background with the minimal liver promoter construct GK-120-Luc and maximal with plasmid GK-200-Luc. Inclusion of the genomic sequence containing the putative enhancer identified in primary hepatocytes did not further augment but on the contrary resulted in a significant 2-fold decrease in reporter gene expression (compare plasmid GK-400-Luc with GK 1400-Luc) (Fig. 2C). Thus, both the promoter proximal region and the upstream putative enhancer contribute to the cell type-dependent activity of the liver glucokinase promoter, but the upstream enhancer appears more specific in that its function is restricted to normal hepatocytes. The remainder of this work focuses on the function of the upstream regulatory region.

Enhancer in Primary Hepatocytes and Silencer in Hepatoma Cells—The ability of the upstream region to function as a enhancer was further tested by ligating a DNA fragment spanning positions -1003 to -707 upstream of the homologous glucokinase promoter or a heterologous promoter, namely the viral SV40 196-bp early promoter. With respect to the homologous promoter, plasmids were constructed both with the complete promoter in GK-200-Luc and the minimal promoter in GK-120-Luc. For some of the constructs, the test fragment was investigated in both orientations and also in a 3’ position downstream of the luciferase coding region. The results of short term transfection in primary hepatocytes and FTO-2B hepatoma cells are summarized in Fig. 3. With the complete homologous promoter in GK-200-Luc, the upstream fragment stimulated luciferase activity 5-fold in hepatocytes, whereas a 2-fold decrease was noted in hepatoma cells. With the minimal glucokinase promoter in GK-120-Luc, reporter expression in primary hepatocytes was enhanced 10-fold with the test fragment inserted in a 5’ position regardless of orientation and slightly more than 2-fold from a 3’ position. By contrast, no stimulation occurred in FTO-2B cells. When tested with the SV40 promoter in primary hepatocytes, the inserted fragment enhanced luciferase level more than 3-fold from a 5’ location and slightly less than 2-fold from a 3’ position. In FTO-2B cells, reporter enzyme activity was reduced by 50%, similar to the result with the homologous promoter. In summary, the upstream regulatory region functions as a typical enhancer in primary liver cells but negatively affects both homologous and heterologous promoter activities in hepatoma transformed cells. In INS-1 cells, the enhancer sequence neither stimulated or inhibited promoter activity, as tested with the SV40 promoter (data not shown).

DNase I Footprinting Analysis of Enhancer—The ability of nuclear proteins to interact with specific DNA sequence elements in the enhancer was investigated using the in vitro DNase I protection assay. The analysis was performed with nuclear extracts from rat liver or spleen and the enhancer fragment labeled with T4 polynucleotide kinase on either strand. With the probe labeled on the nonsense strand, three protected elements designated A, B, and C were visualized after electrophoresis of the digestion products obtained with liver nuclear protein (Fig. 4A). These elements were not protected by spleen nuclear extract, indicating that they could
represent binding sites for liver-enriched trans-acting factors. The borders of elements C were delineated more accurately following protection assay using a shorter probe, consisting of the downstream half of the enhancer labeled on the sense strand (Fig. 4B). In the 5' part of the enhancer, four protected elements G, F, E (Fig. 4C), and D (not shown) could be mapped after digestion in presence of hepatic nuclear protein. This region was also protected by spleen nuclear extract, but the protection was more diffuse, and distinct elements could not be separated. Whether ubiquitous or more specific factors bind DNA in this region should be elucidated in future investigations. The nucleotide sequence of the enhancer (27) and the localization of the footprints are schematically depicted in Fig. 4D. Several prominent sites of increased cleavage in the presence of liver nuclear extract both within and between the protected elements are also depicted (Fig. 4D, arrows). Elements G, C, B and A were strongly protected at a protein input of 5 μg of liver protein, whereas elements F, E, and D required 15 μg for similar protection. This suggests that the putative transacting factors are present at different concentrations in the nuclear extract or that their affinities for the respective binding sites vary.

Site-directed Mutagenesis of Cis-acting Elements in 3' Part of Enhancer—To ascertain the role of the putative cis-acting elements A–C identified above, block mutations were introduced by site-directed mutagenesis in these elements (Fig. 5A) and functionally tested in the hepatocyte transfection system. The DNA fragment from nucleotides –812 to –708 containing the wild type elements C, B, and A, when ligated upstream of the glucokinase proximal promoter region, conferred a slightly less than 2-fold enhancement in reporter gene activity (compare

![Diagram](image-url)
Liver-specific Enhancer of Glucokinase Gene

**A**

| RN | -1000 | TGTCCACCCACATGGAGGCTGCGACC | C | GTTCCACCCACATGGAGGCTGCGACC |
| RN | -892 | AGGTGCACTACGCTGTCACGCTGAC | G | AGGTGCACTACGCTGTCACGCTGAC |
| RN | -942 | GTGATGCTGACAGCTGTCATGCAC | E | GTGATGCTGACAGCTGTCATGCAC |
| RN | -852 | TGGGCTGACAGCTGTCATGCAC | D | TGGGCTGACAGCTGTCATGCAC |
| RN | -1083 | TGGGCTGACAGCTGTCATGCAC | C | TGGGCTGACAGCTGTCATGCAC |
| RN | -772 | AGGTGCACTACGCTGTCACGCTGAC | B | AGGTGCACTACGCTGTCACGCTGAC |
| RN | -712 | GGTGATGCTGACAGCTGTCATGCAC | A | GGTGATGCTGACAGCTGTCATGCAC |
| RN | -716 | GCCGACGAGCAGGAGGAC | | GCCGACGAGCAGGAGGAC |
| RN | -593 | AGTCAGCCGACGAGGAC | | AGTCAGCCGACGAGGAC |

**B**

![Luciferase Activity](image)

**Fig. 6. Enhancer of the human glucokinase gene.** A, alignment of the rat (RN) gene enhancer with the homologous region of the human (HS) gene using the FASTA program. Nucleotides are numbered from the nucleotide preceding the start of transcription (27, 28). Putative cis-acting elements mapped in Fig. 4 are highlighted as shaded rectangles in the rat sequence. The open frame encloses the part of the enhancer that was tested in the experiments illustrated in the lower panel. B, functional activity of the human half-enhancer. The gene fragment from human origin harboring elements C, B, and A was ligated in both orientations upstream of the minimal rat liver glucokinase promoter and tested in the primary rat hepatocyte transfection system. The plasmid with the rat half-enhancer in natural orientation was included as reference. The data are absolute values of luciferase activity expressed in arbitrary light units and are presented as the means ± S.E. of six distinct transfections.

plasmids **GK-200 luc** and **CBA-GK-200 luc**, (Fig. 5B). The block mutation in element A nearly abolished this enhancer effect. In contrast, a block mutation in element B did not reduce, and if anything tended to slightly increase, the luciferase activity of transfected cells. Mutation of element C resulted in an approximately 50% decrease in the enhancer activity. As expected from the results of individual mutations, the combination of mutations in elements A and C totally abrogated the enhancer effect. Finally, a DNA fragment with only element A intact, generated by joint mutagenesis of elements B and C, exhibited about half the activity of the wild type fragment.

**Enhancer of Human Gene—A** computer analysis of the human glucokinase gene revealed that the region between nucleotides −892 and −578 with respect to the hepatic start site of transcription (28) presented strong identity with the rat gene enhancer. The alignment of the two sequences generated with the FASTA program is shown in Fig. 6A. The level of nucleotide identity over the 300-bp region is 75%. The extent of similarity falls off markedly on both sides of the enhancer. Alignment of the adjacent upstream or downstream 100 bp yields figures of 63 and 56%, respectively. The human and rat DNA fragment harboring the putative cis-acting elements C, B, and A were cloned upstream of the rat liver glucokinase minimal promoter in plasmid GK-120-Luc and tested for their ability to activate luciferase expression in transfected primary rat hepatocytes (Fig. 6B). Insertion of the fragments in sense orientation resulted in 5- and 3-fold activation for the rat and human sequences, respectively. The activation was about two times stronger with the human DNA in reverse than in sense orientation. A similar orientation effect was consistently observed with the rat sequence (data not shown). These results demonstrate the conservation of a functional enhancer in the human gene.

**Hepatocyte-specific DNase I Hypersensitive Sites Mark Promoter and Enhancer—Genomic sites where sequence-specific DNA binding proteins interact with DNA are often marked by hypersensitivity of the chromatin to DNase I digestion, presumably reflecting the displacement of nucleosomes (29, 30).** Hypersensitive sites were previously detected around the liver glucokinase promoter in nuclei from rat liver, but they were not mapped accurately (31, 32). We have now scanned the glucokinase locus for the presence of DNase I hypersensitive sites in primary rat hepatocytes, as well as FTO-2B and INS-1 cells. An improved method in which DNase I is made to act directly on cell monolayers permeabilized by detergent rather than on isolated nuclei was used. Our experiments covered the two promoters and the entire structural gene region. A total of seven hypersensitive sites were identified in a 32-kbp region extending from 14 kbp upstream of the liver start site of transcription to 2.8 kbp downstream of the polyadenylation site. In rat hepatocytes, five hypersensitive sites termed HS-1 to HS-5 were detected in a 9.2-kbp EcoRI fragment encompassing the liver promoter (Fig. 7, A and C). The three proximal site HS-1 to HS-3 were specific to primary hepatocytes, whereas the two distal sites HS-4 and HS-5 were present both in hepatocytes and hepatoma cells, but not in insulinoma cells. The latter cells displayed weak sites that did not correspond to any of the hepatic sites. The site HS-1 mapped to the promoter proximal region and HS-2 to the enhancer, corresponding to the two functionally important regions revealed by the transfection studies. However, our transfection experiments do not permit assigning a function to the third hepatocyte specific site HS-3, because plasmid GK-3400-Luc, which included that site, had an activity similar to GK-1400-Luc, which did not. It should also be mentioned that luciferase plasmids that included genomic DNA with the HS-4 and HS-5 sites were not more active than plasmid GK-1400-Luc in any of the three cell types tested (data not shown). Two additional hypersensitive sites designated HS+1 and HS+2 were identified in a HindIII fragment downstream of the liver promoter between exons 1L and 2A (Fig. 7, B and C). Site HS+1 was hepatocyte-specific, whereas HS+2 was found in the three cell types. A DNA fragment comprising the hepatocyte-specific HS+1 was inserted downstream of the luciferase gene in the GL-1400-Luc plasmid without effect on luciferase activity of transfected cells. A possible transcriptional regulatory role of HS+1 and HS+2 remains to be investigated with constructs mimicking the intronic position of these sites.

Two hypersensitive sites were detected in a 12.5-kbp EcoRI harboring the β-cell promoter. The first site, termed HSβ-1, maps to the promoter itself, known to comprise multiple transcriptional start sites (1). The second site HSβ-2 was localized 2.3 kbp downstream, within the first intron of the gene. Both sites were present in INS-1 insulinoma cells, which actively transcribe the gene, but not in primary hepatocytes nor hepa-
insulinoma cell line. This result suggests that elements within the proximal region contribute to confer cell type specificity to the transcription of this promoter.

A second region, spanning positions −1003 to −707 with respect to the transcription start site, augmented the expression of reporter plasmids exclusively in primary hepatocytes. This sequence exhibits the typical properties of an enhancer: 1) it acts from a remote site; 2) it is effective in both orientations; and 3) it is functional when placed either upstream or downstream of a promoter, although in our plasmid constructs the stimulatory effect was stronger from an upstream position. The enhancer was able to stimulate luciferase expression driven by both the glucokinase promoter and, to a lesser extent, by a heterologous promoter. Stimulation of reporter gene activity was 10–15-fold when the enhancer acted in conjunction with the minimal glucokinase promoter (TATA box) and more than 5-fold with constructs including the promoter proximal sequence with end point at −123. The enhancer effect on the heterologous SV40 promoter was approximately 4-fold.

A remarkable characteristic of the enhancer is its stringent cell type specificity, restricted to primary hepatocytes. The sequence was essentially neutral in insulinoma cells. More importantly, a negative effect was consistently noted in the FTO-2B hepatoma cells. These cells are considered a relatively well differentiated model of liver cells, because they retain the ability to express many hepatic traits. Nonetheless, expression of the glucokinase gene is extinguished in FTO-2B cells (20), as it is in most hepatomas. Whether the silencer effect shown in our experiments and the extinction phenomenon affecting the endogenous gene are related phenomena raises an intriguing question. One might hypothesize that one of the cis-elements of the enhancer binds a factor, of which a negatively acting variant form exists in the hepatoma cells. This explanation may be too simplistic, however, because the DNase I hypersensitive site HS-2 marking the enhancer in primary hepatocytes is lost in the FTO-2B cells. This suggests that the binding of sequence-specific factors to the enhancer might be altogether prevented in the chromatin of the FTO-2B cells.

No fewer than seven protected elements, termed elements A through G in the 3’ to 5’ direction, were detected using the *in vitro* DNase I footprinting assay. The 3’ half of the enhancer harbors three DNA elements, A–C, that were protected against DNase I digestion by nuclear protein from liver but not by protein from spleen nuclei. These data suggested that the footprints may represent cis-acting elements binding to liver-enriched trans-acting factors. A functional correlate to the DNase I protection assay was obtained by site-directed mutagenesis of the putative cis-acting elements and transient transfection assay of the resulting reporter plasmids. Large block mutations introduced in elements A and C resulted in a severe (element A) or more modest (element C) reduction in enhancer activity. We therefore propose that elements A and C bind factors that positively regulate transcription from the glucokinase promoter with a predominant role for element A. The preponderance of element A was further supported by showing that a DNA fragment with double mutations of elements B and C (in other words with only element A intact) was still able to stimulate luciferase expression. The function of element B is less apparent. However, disruption of this element slightly increased the activity of the transfected reporter plasmid, suggesting the involvement of a factor that might negatively modulate transcription.

A computer analysis of the enhancer sequence using the recently published Matinspector program (33) reveals that the complement of the sequence 5’-CTGACCACCTTGC-3’ in the middle of element C matches the consensus binding site (core

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**DISCUSSION**

Two regulatory gene regions important for the hepatic expression of glucokinase emerge from the present experiments. The first is a promoter proximal segment extending from positions −123 to −34 with respect to the start site of transcription that is the 90 bp of DNA immediately upstream of the TATA box homology at position −29. Whereas a luciferase reporter plasmid comprising only the TATA box is essentially inactive in all cell types tested, a construct with the additional −123 to −34 sequence elicits readily assayable luciferase activity when transfected in the primary hepatocyte system, amounting to 30% of the maximal activity seen with plasmids including 1.4 kbp or more of flanking sequence. The function of the promoter proximal sequence was also apparent in FTO-2B cells, a rat hepatoma cell line. However, it was minimal in the INS-1

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**FIG. 7.** Mapping DNase I hypersensitive sites around the liver glucokinase promoter. Controlled digestion of the chromatin with DNase I was performed directly on Nonidet P-40 permeabilized cell monolayers, by-passing the need for preliminary isolation of nuclei. The DNA was next isolated, digested with a restriction enzyme, resolved by agarose gel electrophoresis, and transferred to a Nylon membrane as specified under “Experimental Procedures.” A, autoradiograph showing a 9.5-kbp EcoRI fragment extending from 8 kbp upstream to 1.5 kbp downstream of the liver promoter. The probe used for indirect end-labeling is shown at the bottom of C as a thick line segment abutting the 3’ EcoRI site. The three cell types analyzed are indicated on top of the figure (Hepato, primary hepatocytes). Under the brackets, the left-hand lanes contain undigested chromatin, and the crescendo symbols denote concentrations of 100, 300, and 400 units/ml of DNase I. The side lanes contain DNA that was doubly digested with EcoRI plus HindIII (left) and EcoRI plus SacI (right) for accurate mapping of the hypersensitive sites. B, autoradiograph showing a 7.5-kbp HindIII fragment extending from 3.4 kbp upstream to 4.1 kbp downstream of the liver promoter. The probe is indicated at the bottom of C as the thick line segment abutting a BamHI site at the 3’ end of the fragment. Concentrations of DNase I were 50, 100, and 200 units/ml for hepatocytes and otherwise as in A. Lanes marked E and Bg contain DNA doubly digested with HindIII plus EcoRI and BgIII, respectively. C, schematic of hypersensitive sites HS-5 to HS-1 and HS+1 and HS+2. **Shaded rectangles** denote hepatocyte-specific sites, and **open rectangles** denote sites found in both primary hepatocytes and hepatoma cells. **Triangles** designate sites in insulinoma cells. The **middle line** represents genomic DNA with the transcription start site (curved arrow) and exons 1L and 2A (black rectangles). The **lower line** is a map of landmark restriction sites; E, EcoRI; H, HindIII; S, SacI; Bg, BgIII; B, BamHI.
underlined) for the trans-acting factor ROR alpha 2, a member of a new subfamily of orphan nuclear receptors (34). This family includes hepatocyte nuclear factor 4, a factor known to play an important role in liver-specific gene expression (35). The suggestion of binding of a factor of the same family to element C is of great interest. The positive identification of the factor binding to this and the other elements delineated here should be the subject of further investigations, in particular with the help of the electrophoretic mobility shift assay. In any event, the present results indicate that the downstream half of the enhancer may account for about 40% of the activity of the complete enhancer and may be crucial for its cell type specificity. The full function of this regulatory region probably requires a very complex interplay with additional elements in the upstream half of the enhancer.

The characterization of the liver enhancer of the rat gene led us to identify a region in the 5′ flank of the human liver glucokinase promoter, approximately 100 bp closer to the promoter than in the rodent gene, which displays 75% sequence identity with the rat enhancer. Sequence identity rises to 79% when the downstream half of the enhancers harboring the putative liver-specific cis-elements A–C is considered. By comparison, the 125 bp immediately 5′ to the transcription start sites in the two genes, namely the promoter proximal regions, exhibit only 66% identity. The degree of sequence conservation in itself argues for a critical function of the genomic segment. Indeed, our experiments demonstrate that the human sequence is a functional enhancer when ligated to the rat minimal promoter and transfected into primary hepatocytes. If, as our experiments suggest, the enhancer plays an important role for efficient transcription from the liver promoter, one can speculate that mutations of the enhancer, or of genes encoding proteins binding to the enhancer, could compromise hepatic glucokinase expression and lead to genetic disorders of liver glucose metabolism.

An essential aspect of glucokinase gene transcription in the liver is its induction by insulin and repression by cAMP. Hepatocyte transfection experiments were performed to test the hormone responsiveness of the reporter plasmids described here. None of them showed the expected regulation of luciferase, namely increased activity after culture in presence of insulin and reduced activity with cAMP analogs, in spite of a proper response of the glucokinase mRNA in the population of transfected cells. Several possibilities may account for the failure of transfected plasmids to reproduce correct hormone regulation: 1) hormone response elements may be located outside of the cloned gene regions; and 2) hormone action is disturbed in the particular hepatocytes that have taken up the reporter plasmid. In turn, this might be due to an impairment of the hormone signaling pathways in electroporated cells, or it may reflect the fact that regulation would necessitate that the gene be embedded in its chromatin environment for responses to hormones. Although we cannot rule it out, possibility number one does not appear very likely, because all the hepatocyte-specific DNase I hypersensitive sites that could be detected in a region spanning 35 kbp of DNA have been included in our reporter plasmids and failed to sustain the expected hormone responses. In addition, we found that a luciferase plasmid driven by the promoter of the rat phosphoenolpyruvate carboxykinase gene did not respond negatively to insulin, as should be expected from experiments in hepatoma cells or transgenic mice. Thus, we conclude that transient transfection of primary rat hepatocytes by electroporation may not be an adequate method for studying transcriptional responses to insulin or cAMP. On the other hand, the system has allowed us to define a promoter proximal region and an enhancer of the glucokinase gene that appear to play key roles for the hepatocyte-specific expression of this regulatory enzyme of glucose metabolism.

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