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Cell Specific Differences in DNase I Hypersensitivity between the Two Promoters of the Rat Glucokinase Gene

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Glucokinase (GK) gene transcription occurs in the liver and the ß cell of the endocrine pancreas where it is subject to different modes of regulation. This is accomplished largely through the use of two linked, cell-specific promoters separated by at least 12 kbp. We have used DNase I hypersensitivity to explore the chromatin structure surrounding the two promoters in cells that express either the liver or ß cell form of the GK gene, as well as cells that do not express GK. In RIN38 cells, a ß-cell-derived cell line, hypersensitive sites are detected over both the proximal and distal promoters. In liver, hypersensitive sites are present in the proximal promoter but not the distal promoter. Interestingly, in H4IIEC3 cells, a hepatoma cell line that has lost the ability to express GK, hypersensitive sites are also found in the proximal promoter but not the distal promoter.

Chromatin plays an important role in determining the transcriptional capability of a gene. In tissues where a gene is inactive, the chromatin is thought to be in a closed conformation, consisting of tightly packed nucleosomes. Transcription factors are unable to bind and the DNA is resistant to cleavage by DNase I (for review,1). On the other hand, when a gene is expressed, the chromatin appears to be in a more open conformation, thereby allowing access for transcription factor binding. This is reflected in the presence of hypersensitive sites (HS), which are discrete regions of the DNA that are extremely sensitive to DNase I. These hypersensitive sites correlate with protein binding within regulatory elements of a gene. There is also an increased general sensitivity to DNase I. For many genes, including ovalbumin (2), lysozyme (3), and apolipoprotein B (4), this increased sensitivity extends far beyond the region of DNA that is

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actually transcribed. Thus, the transcriptional functional unit can be viewed as a subset of a larger chromosomal structural domain.

We have examined the chromatin structure of the rat glucokinase gene. This gene was chosen because of its unusual structural organization. Distinct mRNAs are initiated by cell-specific promoters adjacent to cell-specific first exons, which are then processed to contain sequences from common exons 2-10 (see Figure 1). In the ß cell, the neuroendocrine transcriptional unit must necessarily contain the proximal promoter and thus both exons 1ß and 1L should be in the same open chromosomal domain. In contrast, the hepatic transcriptional unit does not contain exon 1ß. We were interested in determining whether the glucokinase chromosomal domain in liver contains both exons 1L and 1ß and, if so, whether we could locate sequences near exon 1ß that may play a role in hepatic GK gene transcription. Using indirect endlabelling for the identification of hypersensitive sites, as well as general DNase I sensitivity, we find that in RIN38 cells the regions upstream of each cell-specific first exon are open and are presumably in the same chromosomal domain. This is not the case in the liver. The sequences upstream of exon 1L are open and hypersensitive sites are detected; however, the neuroendocrine promoter appears to be in a closed conformation. Interestingly, in the hepatoma H4IIEC3 cells, where GK is no longer expressed, the conformation of the chromatin resembles that of the liver, indicating that an open chromatin does not necessarily generate effective transcription.

MATERIALS AND METHODS

Tissue Culture H4IIEC3 cells, a rat hepatoma cell line and XC cells, a rat fibrosarcoma cell line were maintained as monolayers in a 1:1 (v/v) mix of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 medium (Gibco BRL; Gaithersburg, MD) supplemented with 10% calf serum. RIN1046-38 (RIN38) cells (5), a rat ß cell line, were maintained as a monolayer in DMEM medium containing glucose at a final concentration of 25 mM and supplemented with 10% fetal bovine serum. All cells were grown at 37 °C in 95% air/5% CO_2.

Nuclei Isolation and DNase I Digestions Liver nuclei were isolated by the method of Gorski et al.(6). Nuclei were washed by resuspension in cold PBS (140 mM NaCl, 1.5 mM KH_2PO4, 8.4 mM Na_2HPO4, and 2.7 mM KCl) and then spun at 1500g, followed by two washes in buffer D (10 mM Tris (pH 7.4), 50 mM NaCl, 0.25 M sucrose, 0.5 mM spermidine, 0.15 mM spermine). Nuclei, suspended in buffer D, were adjusted to 25 A_{260} units(U)/ml. Nuclei from tissue culture cells were isolated as previously described (7).

Nuclear DNase I digestions were carried out at 37 °C with the addition of 10 mM $MgCl_2$, 1 mM $CaCl_2$, and DNase I (Worthington; Freehold, NJ) at the concentrations indicated in the figure legends. At various time points after addition of DNase I, aliquots of nuclei were removed and added to a tube on ice containing EDTA at a final concentration of 50 mM. DNA was purified from the nuclei by standard methods (8).

Restriction Digest and Southern Blotting Twenty µg of genomic DNA was digested with the restriction enzymes indicated in the figure legends. After electrophoresis through a 0.8% Tris-acetate/EDTA (TAE; 1 x = 40 mM Tris-acetate and 1 mM EDTA) agarose gel, the DNA in the gel was depurinated, denatured, and transferred to Zeta Probe (Biorad; Hercules, CA) membrane and probed with genomic GK fragments (see Figure 1) according to manufacturer's suggestions.

RESULTS

Hypersensitive Analysis of the Distal Promoter

DNase I hypersensitivity assays were used to examine the chromatin structure in the

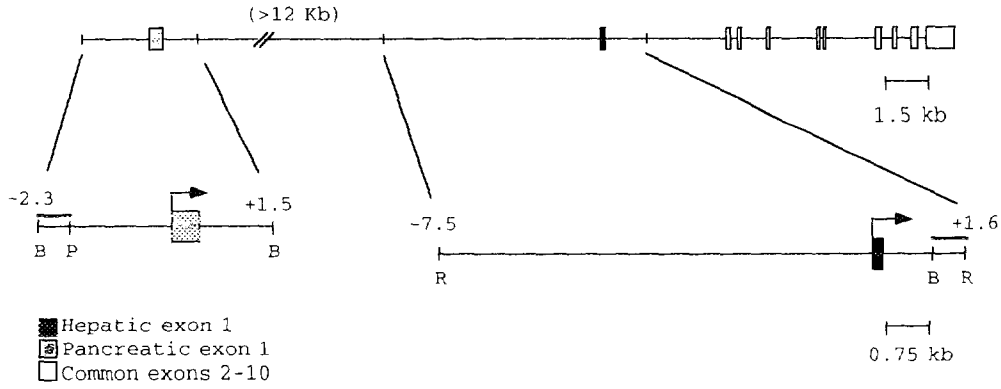


Figure 1. Structure of the Rat Glucokinase Gene. A schematic representation of the rat glucokinase gene is illustrated with each exon depicted as a box. Alternate first exons are used for cell specific gene expression. The genomic fragments containing exons 1L and 1ß, which were analyzed for DNase I hypersensitivity, are also illustrated. The restriction enzymes used to generate these genomic fragments and to isolate the genomic fragments used as probes are indicated (B= BamH I,P= Pst I, and R= EcoR I). The relative location of each genomic fragment with respect to either the neuroendocrine or hepatic transcription start site (denoted by an arrow) is also shown.

sequences surrounding exon 1ß in RIN38 cells. One broad hypersensitive site is visible in this region, HssA, and is located approximately at -0.1 kbp (see Figure 2, RIN38). This site is not seen in XC cells, a rat fibrosarcoma cell line that does not express GK. In addition, the overall chromatin in the XC cells is resistant to cleavage by DNase I relative to that in the RIN38 cells (Figure 2, XC). Finally, the organization of the GK neuroendocrine-specific promoter region was examined in liver cells and compared to that observed in the RIN38 cells. This region is resistant to DNase I and also shows an absence of hypersensitivity (Figure 2, liver).

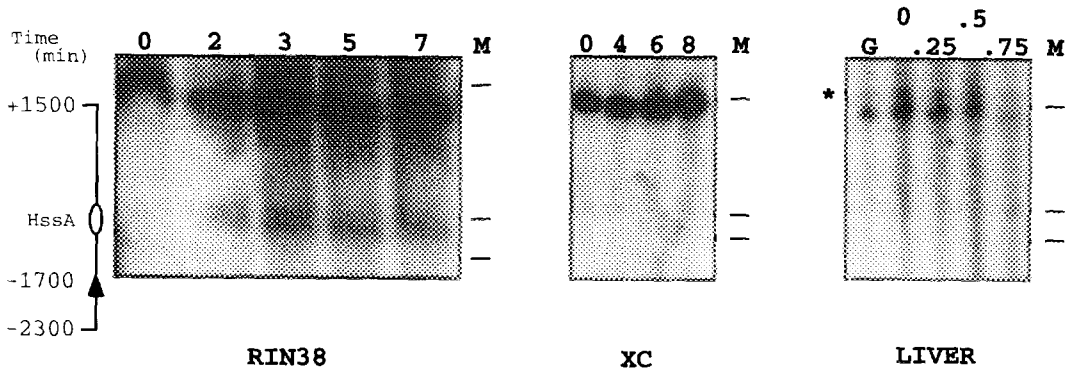


Figure 2. DNase I Hypersensitivity Analysis Over the Distal Promoter Isolated nuclei from RIN38 cells, XC cells and rat liver were treated with 10 u/ml, 20 u/ml and 15 u/ml of DNase I for the times indicated along the top of each panel, respectively. The DNA was digested with BamHI prior to indirect endlabelling. A fragment upstream of the neuroendocrine transcription start site (see Figure 1) was used to probe the blot. A schematic map of the area examined, the relative location of the probe (vertical arrow) and the position of the HS sites (ovals) are indicated at the far left of the figure. *G* represents genomic DNA. * is an artefact of the BamHI digest. *M* represents size markers: 4.4 kbp, 2.3 kbp, and 2.0 kbp.

Hypersensitive Analysis over the Proximal Promoter

In liver, we observe the rapid production of four hypersensitive sites, located at -0.1 kbp (Hss1), -0.7 kbp (Hss2), -1.9 kbp (Hss2b), and -5.0 kbp (Hss3), (Figure 3, Liver). These results differ slightly from earlier studies in that hypersensitive site 2b was not described previously (9). Using a different indirect endlabelling approach, Bossard et al. (9) also detected an additional hypersensitive site at -6.0 kbp (Hss4). The remaining sites were detected in both studies. Nuclei from XC cells were also treated with DNase I for detection of hypersensitive sites (Figure 3 XC). As was the situation for these cells over the pancreatic promoter, no hypersensitive sites were detected and the chromatin was resistant to cleavage by DNase I. Thus, it appears to be in a closed conformation. When this same region was probed in RIN38 nuclei, where the proximal promoter is inactive, Hss3 was present but all of the more proximal sites were absent (Figure 3, RIN38). Additionally, no hypersensitive sites unique to the RIN38 cells were found in this region. Although only one hypersensitive site was detected over the broad region upstream of the hepatic start site, this region was highly sensitive to overall DNase I digestion, showing rates of digestion comparable to that seen for the same region in the expressing liver cells and much greater than that for the nonexpressing XC cells. This suggests that the sequences upstream of exon 1L are in an overall open configuration, though the lack of some of the hypersensitive sites may reflect an absence of liver specific transcription factors.

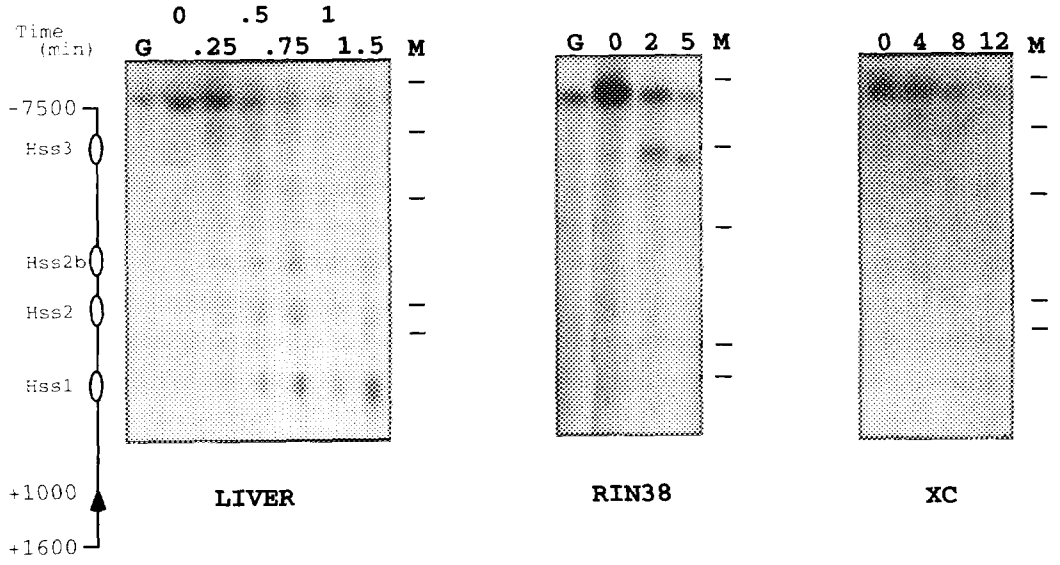


Figure 3. DNase I Hypersensitivity Analysis Over the Proximal Promoter Isolated nuclei from rat liver, RIN38 cells and XC cells were treated with 4 u/ml, 10 u/ml and 20 u/ml of DNase I for the times indicated along the top of each panel, respectively. The DNA was digested with EcoRI prior to indirect endlabelling. A fragment located downstream of the hepatic transcription start site (see Figure 1) was used to probe the filter. A schematic map of the area examined, the relative location of the probe (vertical arrow) and the position of the HS sites (ovals) are indicated at the far left of the figure. *M* represents size markers: 9.1 kbp, 6.6 kbp, 4.4 kbp, 2.3 kbp and 2.0 kbp. *G* represents genomic DNA.

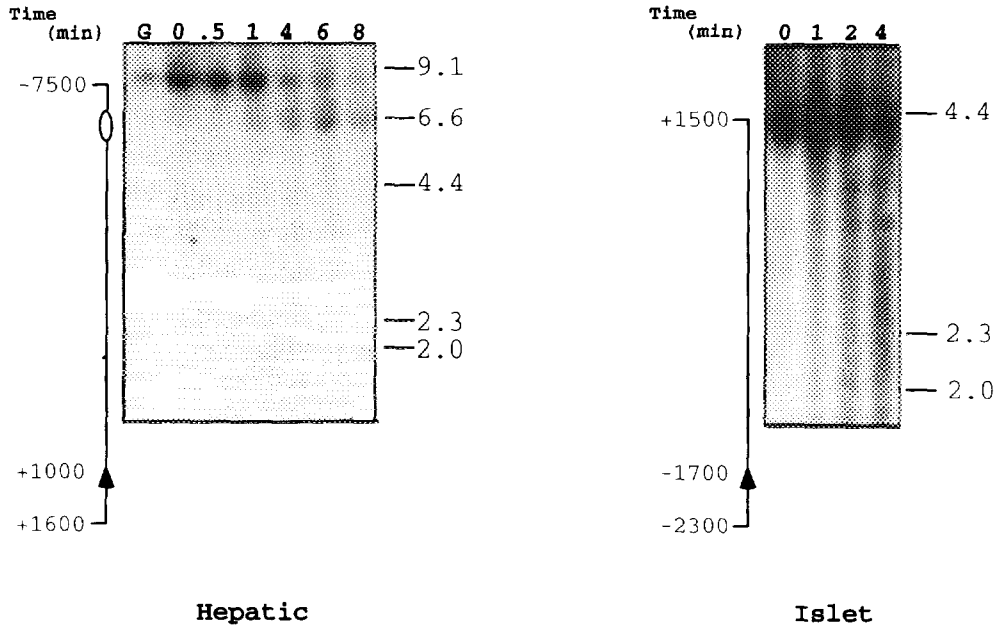


Figure 4. DNase I Hypersensitivity Analysis in H4 Cells *Hepatic:* Isolated nuclei were treated with 8 u/ml of Dnase I for the times indicated along the top of the panel. The DNA was digested with EcoRI prior to indirect endlabelling as described in the legend to Figure 3. *Islet:* Isolated nuclei were treated with 25 u/ml of DNase I for the times indicated along the top of the panel. The DNA was digested with BamHI prior to indirect endlabelling as described in the legend to Figure 2. A schematic map of the area examined, location of the probes (vertical arrows) and positions of the hypersensitive sites (ovals) are to the left of each panel. Size markers representing λ digested with HindIII are indicated on the right hand side of each panel.

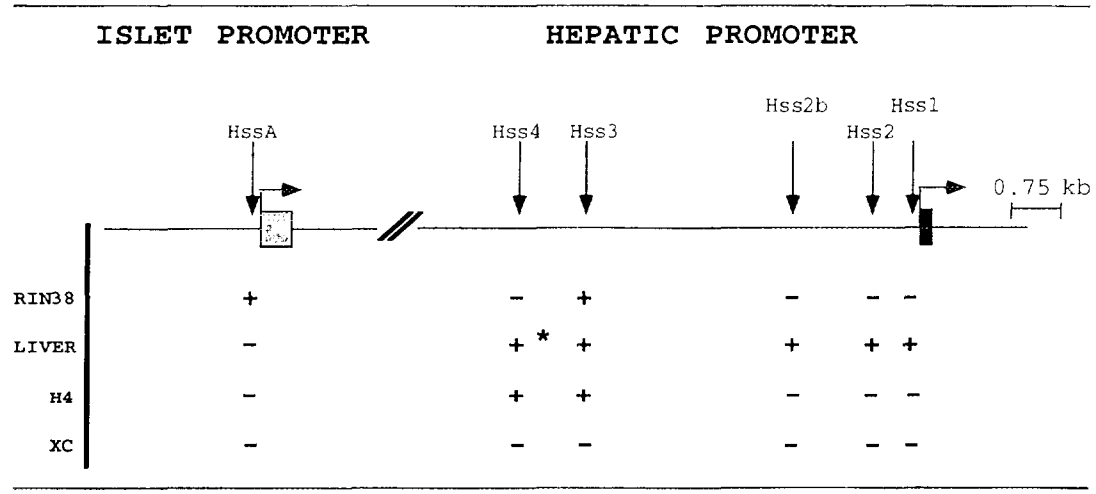
Hypersensitive Analysis in H4IIEC3 Cells

The activity of several liver-specific enzymes is significantly reduced or absent in a number of hepatomas (10). H4IIEC3 cells, a rat hepatoma cell line, does not express the GK gene, although it has retained the capacity to express other liver-specific genes such as phosphoenolpyruvate carboxykinase (PEPCK). We were interested in determining if the loss of expression of GK resulted in an alteration of the chromatin structure in these cells. Isolated nuclei from these cells were treated as described above and purified DNA was digested with EcoRI prior to indirect endlabelling to examine the region upstream of exon 1L. Two hypersensitive sites are visible (Figure 4, Hepatic). Hss3 is clearly visible in the H4IIEC3 cells; interestingly, Hss4 is also detectable. We also digested DNase I-treated, purified, DNA with BamHI to look at the neuroendocrine promoter (Figure 4, Islet). No hypersensitive sites were detected in this region nor does it appear to be particularly accessible to general DNase I activity.

DISCUSSION

Table 1 summarizes the location of the DNase I hypersensitive sites detected over the regions upstream of exons 1L and 1ß in liver, RIN38, H4IIEC3, and XC cells. Several features

Table 1 Summary of HS sites in Glucokinase This table represents the hypersensitive sites detected around exons 1ß and 1L in liver, RIN38, H4IIEC3, and XC cells. Along the top is a schematic representation of the two promoters with the cell-specific first exons indicated by boxes and transcription start sites by horizontal arrows. Vertical arrows denote the positions of the HS sites with respect to each start site. The cell types are listed along the left hand side of the table. (+) beneath a HS site indicates its presence in a particular tissue, while (-) indicates its absence. * from Bossard et al. (9)



can be noted. First, in XC cells, where the glucokinase gene is transcriptionally silent, there are no hypersensitive sites in either the region upstream of the hepatic initiation site or upstream of the neuroendocrine initiation site. The chromatin is also resistant to general DNase I cleavage. Thus, we conclude that the GK chromosomal domain in XC cells is closed.

In RIN38 cells, transcription of GK occurs from the neuroendocrine-specific promoter. The chromatin in this region is open and accessible to DNase I digestion and one hypersensitive site is observed (HssA). HssA is located within a region already shown to be sufficient for directing islet-specific reporter gene expression in transgenic mice (11). In addition, all mice that harbor this construct exhibit position-independent transgene expression, indicating that this region is sufficient for maintaining an open chromatin structure.

The initiation site of hepatic glucokinase is contained within the neuroendocrine glucokinase transcriptional unit, as specified by the genomic organization of the gene (see Figure 1). Despite this, initiation from the hepatic promoter in RIN38 cells does not occur. This may possibly be due to the lack of liver-specific factors since the chromatin is in an open conformation as indicated by an overall sensitivity to DNase I and the detection of a hypersensitive site (HSS 3). This result may reflect the fact that RNA polymerase must traverse this region during transcription initiating from the neuroendocrine promoter in the ß cell.

Initiation of hepatic GK transcription occurs downstream of exon 1ß in liver cells; thus, this exon is not part of the hepatic GK transcriptional unit. It was possible, though, that sequences upstream of exon 1ß might be included within the hepatic chromosomal domain. However, no hypersensitive sites were detected in the region of the neuroendocrine promoter. Additionally, the

chromatin is resistant to cleavage by DNase I in this region. This argues that the sequences surrounding exon 1ß are in a closed conformation in liver cells.

In liver-derived H4IIEC3 cells, where the capability to transcribe GK has been lost, the chromatin surrounding exon 1ß is devoid of any hypersensitive sites and appears resistant to digestion by DNase I. Interestingly, in the region upstream of exon 1L, the chromatin is accessible to DNase I digestion and two hypersensitive sites are observed (Hss3 and Hss4). These sites are also seen in the liver. This is in contrast to the PEPCK gene where loss of PEPCK gene expression in a hepatoma (HTC cells) correlated with a lack of any hypersensitive sites and a general sensitivity to DNase I that was comparable to the XC cells, where the PEPCK gene is not expressed (12). Our results suggest that turning off a transcriptional unit does not necessarily lead to closing of its chromosomal domain.

In summary, we have analyzed the chromatin structure surrounding exons 1L and 1ß in the rat GK gene. The gene is organized such that there are two transcriptional units expressed selectively in different tissues, one of which is encompassed within the other. Two possible chromosomal domain models can be envisioned from this arrangement. In one scenario, there is a common chromosomal domain for all expressing tissues, as is seen in the human ß globin gene cluster (13). An alternative model would involve tissue-specific chromosomal domains. Our results support the latter model and is most clearly seen in the liver, where the distal neuroendocrine promoter is not used for transcriptional initiation and is located within a closed chromatin environment. In contrast, when transcription is initiated from the neuroendocrine promoter in RIN38 cells, the hepatic promoter is an unavoidable part of the transcription unit and it is, as expected, in an open conformation. Although we have identified liver-specific hypersensitive sites upstream of exon 1L, we know that these sites alone are not sufficient to open the liver chromatin. Constructs containing 7.5 kbp upstream of exon 1L were unable to confer liver-specific, position-independent expression in transgenic mice (14). We plan to attempt to identify the factors binding to the liver hypersensitive sites when additional cloning and transgenic experiments, which are currently underway, have identified the full extent of GK sequences upstream of exon 1L which are necesary for position-independent expression in the liver.

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