Concentrations of insulin in the blood decrease by 69.6±10.0% of the baseline levels following insulin secretion, the so-called glucose toxicity theory (reviewed in references 1 and 2). Normalization of blood glucose levels of type II diabetic patients partially restores defective insulin release (3–7), and normalization of glucose levels in several hyperglycemic animal models leads to full restoration of normal insulin secretion (8–10). However, no biochemical mechanism has been identified for gluco-toxic effects on the pancreatic β cell (2). Our previous observations (11) suggested that one potential explanation could involve an alteration in insulin gene expression.

The HIT-T15 cell is a clonal pancreatic β cell line derived from SV-40 transfected Syrian hamster pancreatic islets (12). This cell line synthesizes and releases insulin in response to physiological secretagogues including glucose (13–18). However, the HIT cell loses its ability to secrete insulin with increasing passage (12, 14, 18). Recently, Robertson et al. have shown that the loss of insulin secretion is associated with decreased insulin content and insulin mRNA, and that this loss can be prevented by passaging HIT cells in low glucose (0.8 mM) rather than the high glucose (11.1 mM) concentrations usually used when culturing these cells (11). The studies reported here were designed to determine whether a seemingly paradoxical decrease in insulin gene transcription might be a mechanism through which chronically culturing HIT cells in high glucose concentrations decreases insulin content and insulin mRNA.

We sought to answer three questions: (a) Does serially passaging HIT cells in high glucose concentrations lead to decreased insulin gene transcription? (b) Does serially passaging HIT cells in a low glucose concentration preserve insulin gene transcription? (c) Is there evidence for altered activity of insulin gene transcription factors that might explain the observed changes in insulin gene transcription?
CAT to RSVCAT expression to control for variability in transfection efficiency observed between cells cultured in low and high glucose concentrations.

Nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear extracts were made from various HIT cell passages according to methods originally described by Dignam et al. (21) and modified by Abmayr and Workman (22). Oligodeoxynucleotides Ins(−230/−201), Ins(−249/−220), and Ins(−94/−65) were annealed and then labeled with [32P]dCTP by filling overhanging 5′-ends with the large fragment of DNA polymerase I. Binding reactions and electrophoresis were performed as described by Shih and Towle (23) except that 0.3 μg of poly(dl-dc) and poly(dA-dT) were used as nonspecific competitors in the binding reactions.

Results

Insulin gene promoter activity in HIT cells serially passaged in either 11.1 mM or 0.8 mM glucose. Insulin gene promoter activity in early and late passages of HIT cells chronically cultured in 11.1 mM or 0.8 mM glucose was assessed by transiently transfecting the cells with a CAT reporter gene controlled by the 5′-regulatory domain (−326 to +30) of the human insulin gene. HIT cells from passage 73 to 76 cultured in 11.1 mM glucose readily expressed CAT activity when transiently transfected with either INSCAT or RSVCAT (Table 1). In early passages of HIT cells the relative expression of INSCAT to RSVCAT was 1.02±0.02. The relative expression of INSCAT to RSVCAT was 0.29±0.02 for late passages of HIT cells cultured in 11.1 mM glucose. The decrease in relative CAT activity observed in the late passages of HIT cells cultured in 11.1 mM glucose appears to be specific for INSCAT expression, since RSVCAT expression was the same for both early and late passages of HIT cells (Table 1).

Compared with late passages of HIT cells chronically cultured in 11.1 mM glucose, cells chronically cultured in 0.8 mM glucose expressed 2.4-fold (P < 0.005, n = 5) more relative CAT activity when transfected with INSCAT (Table 1). In late passages of HIT cells serially passaged in 0.8 mM glucose, insulin promoter activity was shown to be 69.6±10.0% compared with early passages of cells (Table 1). When early and late passages of HIT cells chronically cultured in either 11.1 mM or 0.8 mM glucose were cotransfected with both INSCAT and a RSV-driven luciferase reporter construct, to control for transfection efficiency, qualitatively similar results to those described in Table 1 were observed (data not shown). These data suggest that late passages of HIT cells chronically cultured in 0.8 mM glucose have preserved insulin promoter activity, which may explain the preservation of both insulin mRNA and insulin content (11).

1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; GSTF, glucose-sensitive transcription factor.
Percent conversion represents the mean of duplicate transfections for each HIT cell passage. *P < 0.001, significance of relative INSCAT to RSVCAT expression between early and late passages of HIT cells serially passaged in 11.1 mM glucose. **P < 0.005, significance of relative INSCAT to RSVCAT expression between late passages of HIT cells serially passaged in either 11.1 mM or 0.8 mM glucose.

Alteration in insulin gene 5'-regulatory domain binding proteins in HIT cells serially cultured in 11.1 mM or 0.8 mM glucose. To determine whether decreased insulin promoter activity might be associated with altered activity of transcription factors that interact with insulin gene regulatory sequences, nuclear extracts were prepared from early passages (74 and 80) and late passages (119 and 133) of HIT cells cultured either in 11.1 mM or 0.8 mM glucose. EMSAs were then performed with two overlapping sequences, -249/-220 and -230/-201, of the human insulin promoter. The human insulin promoter sequence -249/-220 contains a strong positive regulatory element (24), while sequences -230/-201 have been shown to bind the β cell specific transcription factor, IUF-1 (25).

With an oligodeoxynucleotide probe containing sequences -230/-201 of the human insulin gene, at least 5 DNA-protein complexes were detected when using nuclear extracts from early passages of cells (Fig. 1 A, lane 2). Of these, one prominent DNA-protein complex was absent in nuclear extracts from late passages of HIT cells cultured in 11.1 mM glucose (Fig. 1 A, lane 3). In contrast, nuclear extracts made from later passages of cells serially passaged in 0.8 mM glucose retained the ability to form this DNA-protein complex (Fig. 1 A, lane 4). The specificity of the DNA-protein complexes formed with the different extracts was determined by use of an excess of unlabeled DNA from -230/-201 to compete for binding (Fig. 1 A, lanes 5–7). This slow mobility complex, present in early passages and late passages of cells cultured in 0.8 mM glucose and absent in late passages of cells cultured in 11.1 mM glucose, is designated as the glucose-sensitive transcription factor (GSTF). To validate that the loss of a specific DNA binding protein in nuclear extracts from HIT cells serially passaged in 0.8 mM glucose was not due to an aberration of the nuclear extraction, we tested the various preparations for activity of the ubiquitous MLTF nuclear factor. All the HIT cell nuclear extractions shifted an MLTF DNA probe to the same extent (data not shown), suggesting that all the nuclear preparations were active. Furthermore, several other nuclear factors binding to the -230/-201 fragment were also unchanged in the different preparations (Fig. 1 A).

The oligodeoxynucleotide probe containing the sequences -249/-220 of the human insulin gene was used to search for changes in other DNA binding proteins from HIT cells passaged either in 11.1 mM or 0.8 mM glucose. Nuclear extracts from early and late passages of HIT cells serially passaged in 11.1 or 0.8 mM glucose shifted this oligodeoxynucleotide to the same extent (Fig. 1 B). These data demonstrate that the differences in INSCAT expression in the early and late passages of HIT cells serially passaged in either 11.1 mM or 0.8 mM glucose.

Table 1. Expression of INSCAT and RSVCAT in Early Passages and Late Passages of HIT Cells Serially Passaged in Either 11.1 mM or 0.8 mM Glucose

<table>
<thead>
<tr>
<th>Experiment</th>
<th>P70 11.1 mM glucose</th>
<th>P130 11.1 mM glucose</th>
<th>P130 0.8 mM glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INSCAT</td>
<td>RSVCAT</td>
<td>INSCAT</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>4.0</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>1.8</td>
<td>5.6</td>
<td>1.04</td>
</tr>
<tr>
<td>3</td>
<td>4.9</td>
<td>4.6</td>
<td>1.05</td>
</tr>
<tr>
<td>4</td>
<td>4.4</td>
<td>4.5</td>
<td>0.96</td>
</tr>
<tr>
<td>5</td>
<td>5.1±0.2</td>
<td>5.0±0.2</td>
<td>1.02±0.02</td>
</tr>
</tbody>
</table>

*P < 0.001, significance of relative INSCAT to RSVCAT expression between early and late passages of HIT cells serially passaged in 11.1 mM glucose. **P < 0.005, significance of relative INSCAT to RSVCAT expression between late passages of HIT cells serially passaged in either 11.1 mM or 0.8 mM glucose.

Figure 1. Analysis of protein binding to the insulin promoter sequences from -230 to -201 and -249 to -220 using nuclear extracts from an early passage and late passages of HIT cells serially passaged in either 11.1 mM or 0.8 mM glucose. (A) EMSA using 32P-labeled insulin promoter sequence -230 to -201. Lane 1, free probe; lanes 2, 5, 8, 11, 14, and 17, nuclear extract from control HIT cell extract; lanes 3, 6, 9, and 12, nuclear extract from HIT cell extract containing 11.1 mM glucose; lane 4, 7, 10, 13, and 16, nuclear extract from HIT cell extract containing 0.8 mM glucose. Competitor added, lanes 5–7, 100-fold molar excess of unlabeled wild type sequence -230 to -201; lanes 8–10, 10-fold molar excess of unlabeled mutated -230 to -201. Arrow indicates DNA-protein complex present in early passages of HIT cells and late passages of HIT cells cultured in 0.8 mM glucose, but not in late passages of HIT cells cultured in 11.1 mM glucose. (B) EMSA using 32P-labeled insulin promoter sequence -249 to -220. Lane 1, free probe; lanes 2–4, nuclear extracts from HIT cell passage 74 and passage 133 serially passaged in 11.1 mM glucose and 0.8 mM glucose, respectively. This figure represents results observed for two independent nuclear extractions from each HIT cell passage described. These data also represent a minimum of three independent experiments per condition (n = 3).
glucose are not due to changes in the binding of nuclear factors to this strong enhancer element.

The CT2 motif (CTAATG) located at -215/-210 of the human insulin promoter has previously been shown to be the binding site for a transcription factor, IUF-1 (25). To test whether this sequence might be a site for binding of GSTF, the sequence CTAATG was mutated to ACGGGT in the -230/-201 oligodeoxynucleotide. This mutated oligodeoxynucleotide no longer competed for the specific DNA-protein complex that was only present in the early passages cells and late passages of HIT cells serially passaged in 0.8 mM glucose (Fig. 1A, lanes 8–10). These data suggest that GSTF binds to the IUF-1 binding site.

Since GSTF appeared to bind the CT2 motif, the nuclear extracts from the various HIT cell passages were tested for binding to an oligodeoxynucleotide containing the sequences -94/-65 of the human insulin gene. This oligodeoxynucleotide probe contains the CT1 motif (CTAATG), located at -82/-77 of the human insulin promoter, which has been shown to be an additional binding site for IUF-1 (25). With the -94/-65 oligodeoxynucleotide probe, one prominent DNA-protein complex was observed with nuclear extracts from passage 74 cells (Fig. 2, lane 2). Nuclear extracts from passage 133 HIT cells chronically cultured in 11.1 mM glucose were unable to form this DNA-protein complex (Fig. 2, lane 3). In contrast, nuclear extracts from passage 133 HIT cells chronically cultured in 0.8 mM glucose retained the ability to form this DNA-protein complex (Fig. 2, lane 4). The specificity of the DNA-protein complex formed with nuclear extracts from HIT cell passage 74 was determined by using an excess of unlabeled -94/-65 deoxynucleotide probe to compete for binding (Fig. 2, lanes 5, 6).

The protein-DNA complex formed with the HIT cell passage 74 nuclear extract and the -94/-65 oligodeoxynucleotide was directly competed by the addition of excess unlabeled -230/-201 DNA (Fig. 2, lanes 9, 10). These data suggest that the transcription factor present in nuclear extracts from passage 74 HIT cells and passage 133 HIT cells cultured in 0.8 mM glucose, but absent in passage 133 HIT cells cultured in 11.1 mM glucose, which binds to -94/-65 probe, may be related to GSTF. To further investigate this possibility, the CT1 motif (−82/−77, CTAATG) contained within the −94/−65 oligodeoxynucleotide probe was mutated to GCACGC and tested for its ability to compete for nuclear extract binding to the wild type −94/−65 deoxynucleotide probe. This mutated oligodeoxynucleotide no longer competed for the specific DNA-protein complex present in passage 74 HIT cells (Fig. 2, lanes 7, 8). Overall these data suggest that GSTF binds to the CT motifs, which have been previously shown to bind IUF-1.

**Mutations of GSTF binding sites within 5′-regulatory domain of the human insulin gene lead to decreased expression of INS CAT reporter gene.** To test whether the loss of GSTF correlates to the decreased expression of INSCAT in late passages of HIT cells serially passaged in 11.1 mM glucose, site-directed mutations of the GSTF binding sites within the INSCAT vector were constructed and tested in the various HIT cell passages. Three mutant INSCAT vectors were constructed: mCT2CAT in which the CT2 motif was mutated to ACGGGT, mCT1CAT in which the CT1 motif was mutated to GCACGC, and mCT1CT2CAT in which the CT1 and CT2 motifs were mutated to GCACGC and ACACGC, respectively. The expression of the mCT1CT2CAT vector, in which both GSTF binding sites were mutated, is markedly decreased in early passages of HIT cells and in late passages of HIT cells cultured in either 11.1 mM or 0.8 mM glucose (Fig. 3). Mutation of the individual GSTF binding sites (mCT1CAT or mCT2CAT) also tended to decrease CAT expression in all HIT cell passages, but these effects were not always statistically significant. These data indicate that the GSTF binding sites present within the 5′-regulatory domain of the human insulin gene play a functional role in the regulation of insulin gene transcription and that the loss of GSTF binding leads to decreased insulin gene transcription.

**Discussion**

In experiments designed to examine potentially adverse effects of high glucose concentrations on insulin gene transcription, we found that the activity of the insulin gene promoter is decreased in HIT cells serially passaged in maximally stimulatory concentrations of glucose. This can be partially prevented by culturing HIT cells in a lower glucose concentration. The changes in the activity of the insulin promoter correlate with changes in insulin mRNA levels for the different HIT cell passages (11). The observation that chronic exposure of HIT cells to high glucose concentration leads to decreased insulin gene expression represents an apparent time-related paradox. Acute increases in glucose concentrations have been shown to increase insulin mRNA levels (26–30) by both stabilizing insulin message (30) and increasing the rate at which the insulin gene

**Figure 2.** Analysis of protein binding to insulin promoter sequences from -94 to -65 using nuclear extracts from an early and late passage of HIT cells serially passaged in either 11.1 mM or 0.8 mM glucose. Lane 1, free probe; lane 2, 5–10, nuclear extract from HIT cell passage 74; lane 3, nuclear extract from HIT cell passage 133 serially passaged in 11.1 mM glucose; lane 4, nuclear extract from HIT cell passage 133 serially passaged in 0.8 mM glucose. Competitor added, lanes 5–6, 50- and 100-fold excess of unlabeled wildtype -94/-65; lanes 7–8, 50- and 100-fold molar excess of unlabeled mutated -94/-65; lanes 9–10, 50- and 100-fold molar excess of unlabeled -230/-201.
is transcribed (26, 31, 32). The chronic effect of glucose on insulin gene transcription that we describe and which can be partially prevented by chronically culturing HIT cells in lower glucose concentrations, may represent a direct glucose toxic effect on a insulin-gene specific transcription factor(s).

A glucose-responsive DNA element has been mapped to −247/−196 of the rat 1 insulin gene enhancer (31). Both ubiquitous transcription factors, Pan-1 and Pan-2 (33–36), and β cell specific factors (37–39) have been shown to bind to DNA sequences contained in this glucose-responsive region. A β cell specific factor (IUF-1) binds to the CT motifs (CTAATG) located at −215/−210 (CT2 motif) and −82/−77 (CT1 motif) in the human insulin gene enhancer (25). The CT motifs are highly conserved across all mammalian species, suggesting that these motifs may participate in the transcriptional regulation of the insulin gene. The CT2 motif of the human insulin gene is similar to sequences located in the FLAT element (−222/−208) of rat 1 insulin gene (40). Mutations of the FLAT element have been shown to decrease the rat 1 insulin gene promoter activity (41) and to alter the activity of a rat 1 insulin gene mini-enhancer linked to a heterologous promoter (40). Recently, German et al. has cloned two protein factors, lmx-1 and cdx-3, which bind to the FLAT element (42). Lmx-1, a β cell specific LIM-homeo domain protein, synergistically interacts with Pan-1 and increases the expression of the rat 1 insulin gene mini-enhancer in nonpancreatic beta cells (42). However, the relationship between IUF-1 and FLAT element binding proteins, lmx-1 and cdx-3, has yet to be determined.

Our data show that nuclear extracts made from late passages of HIT cells chronically cultured in a high glucose concentration fail to form a specific DNA-protein complex (GSTF) to sequences −230/−201 and −94/−65 of the human insulin gene and that GSTF requires the CT motifs contained within these sequences for binding. Our studies also demonstrate that mutations of the CT motifs decrease insulin pro-

cytomer activity in all passages of HIT cells. These data suggest that the CT motifs play a functional role in the regulation of the human insulin gene promoter activity. Our findings strongly suggest that the mechanism of action for the decrease in insulin gene transcription in cells cultured chronically in high glucose concentrations is the loss of GSTF binding to the CT motifs either because GSTF has been altered or is no longer present. The exact relationship between IUF-1 and GSTF has not been resolved, but they may be closely related to the FLAT binding proteins, Lmx-1 or cdx-3. The decrease in insulin gene transcription is not due to the loss of a transcription factor that binds to the strong enhancer element located between −249 and −220 of the human insulin gene because similar levels of DNA-protein complexes, assessed by EMSA, were formed when using nuclear extracts from HIT cells chronically cultured in high or low glucose concentrations.

The loss of insulin response to glucose in HIT cells serially passaged in high glucose concentrations and the ability to prevent this by passaging cells in a more physiological glucose concentration is reminiscent of the adverse effects of chronic hyperglycemia which occur in patients with type II diabetes mellitus and animal models fed excess glucose. Although it is generally thought that such glucose toxic effects occur at sites within the exocytotic pathway, our results suggest that these adverse effects occur at the level of the insulin gene and lead to a paradoxical decrease in the rate of transcription. Recently published data demonstrated that prolonged exposure of the human pancreatic islet to high glucose concentrations leads to impaired insulin secretion (43). This was attributed mainly to a decline in insulin content that was preventable by culturing the islets in low glucose concentrations (43). This conclusion is consistent with our suggestion that β cells are sensitive to direct glucose toxic effects on insulin gene transcription. We therefore propose a time-related distinction between the terms glucose desensitization and glucose toxicity: The former may be a more acute, reversible phenomenon at the level of insulin exocytosis, whereas the latter may be a more chronic, less reversible phenomenon at the level of insulin gene transcription.

To the extent that one can generalize from observations in HIT cells to the pathophysiology in type II diabetic patients, our data suggest that close regulation of glucose concentrations in type II diabetic patients might provide the previously unanticipated benefit of helping preserve insulin gene expression. A corollary of this logic is that prolonged hyperglycemia, despite treatment with oral hypoglycemic agents, may contribute to deterioration of insulin production and secretion and, thus, lead to so-called “secondary failure” of these drugs.

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