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Glucose-Induced Transcription of the Insulin Gene Is Mediated by Factors Required for β-Cell-Type-Specific Expression

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The insulin gene is expressed exclusively in pancreatic islet β cells. The principal regulator of insulin gene transcription in the islet is the concentration of circulating glucose. Previous studies have demonstrated that transcription is regulated by the binding of *trans*-acting factors to specific *cis*-acting sequences within the 5′-flanking region of the insulin gene. To identify the *cis*-acting control elements within the rat insulin II gene that are responsible for regulating glucose-stimulated expression in the β cell, we analyzed the effect of glucose on the in vivo expression of a series of transfected 5′-flanking deletion mutant constructs. We demonstrate that glucose-induced transcription of the rat insulin II gene is mediated by sequences located between −126 and −91 bp relative to the transcription start site. This region contains two *cis*-acting elements that are essential for directing pancreatic β-cell-type-specific expression of the rat insulin II gene, the insulin control element (ICE; −100 to −91 bp) and RIPE3b1 (−115 to −107 bp). The gel mobility shift assay was used to determine whether the formation of the ICE- and RIPE3b1-specific factor–DNA element complexes were affected in glucose-treated β-cell extracts. We found that RIPE3b1 binding activity was selectively induced by about eightfold. In contrast, binding to other insulin *cis*-acting element sequences like the ICE and RIPE3a2 (−108 to −99 bp) were unaffected by these conditions. The RIPE3b1 binding complex was shown to be distinct from the glucose-inducible factor that binds to an element located between −227 to −206 bp of the human and rat insulin I genes (D. Melloul, Y. Ben-Neriah, and E. Cerasi, Proc. Natl. Acad. Sci. USA 90:3865–3869, 1993). We have also shown that mannose, a sugar that can be metabolized by the β cell, mimics the effects of glucose in the in vivo transfection assays and the in vitro RIPE3b1 binding assays. These results suggested that the RIPE3b1 transcription factor is a primary regulator of glucose-mediated transcription of the insulin gene. However, we found that mutations in either the ICE or the RIPE3b1 element reduced glucose-responsive expression from transfected 5′-flanking rat insulin II gene constructs. We therefore conclude that glucose-regulated transcription of the insulin gene is mediated by *cis*-acting elements required for β-cell-type-specific expression.

In higher eukaryotes the blood glucose level is controlled by extremely efficient mechanisms involving different hormones that function to modulate glucose uptake and production. Reduction in circulating blood glucose levels is primarily effected by secretion of the polypeptide hormone, insulin, from the β cells of the islets of Langerhans. The released insulin stimulates the uptake and metabolism of glucose in peripheral tissues as well as inhibiting glucose release from the liver. Insulin secretion and biosynthesis are regulated in β cells by glucose. The stimulatory effect of glucose is mediated by metabolism of the sugar in the β cell (1, 20). Studies have shown that insulin mRNA levels are elevated 4- to 10-fold in response to increased glucose concentration (4, 18). The sugar selectively increases the level of insulin mRNA by increasing both the stability of insulin mRNA (42) and the rate of transcription of the insulin gene (13).

Transcription of the insulin gene in β cells is dependent on *cis*-acting sequences in the 5′-flanking region of the gene (36). Cell-type-specific transcription is due to the recognition by specific *trans*-acting factors of the insulin enhancer region, which is located between nucleotides −340 and −91 relative to the transcription start site (10, 12, 44). Glucose-stimulated transcription is also directed by insulin enhancer elements (16, 19, 25). The *cis*-acting sequences of the rat insulin I gene that can mediate this effect are located between nucleotides −247 and −197 (16, 25). (Rats have two nonallelic insulin genes [termed rat I and rat II] which are almost equally transcribed in rat pancreas β cells and are not expressed in other cell types [7, 8].) Interestingly, this region of the rat insulin I gene can also direct pancreatic β-cell-type-specific expression from a heterologous promoter (17). Transcription from the −247 to −197 bp region is mediated by three *cis*-acting elements: Far (−241 to −233 bp; referred to here as the insulin control element [ICE]), Flat F (−222 to −218 bp), and Flat E (−213 to −209 bp) (17). Of these three elements, only the Flat E element is conserved within the corresponding region of the human and rat II insulin genes (37). As a result of the lack of sequence identity within this region of the rat insulin I and II genes, the homologous region of the rat insulin II gene (−237 to −185 bp) cannot direct β-cell-type-specific expression (17). Since there was clearly both sequence and functional divergence between the rat I and II insulin genes within the −247 to −197 bp region, we considered that this region of the rat insulin II gene might not be involved in glucose-responsive transcription.

In this report we show that glucose-stimulated expression of the rat insulin II gene is mediated by the sequences located between −126 and −91 bp. This region also contains several *cis*-acting elements important in directing β-cell-type-specific transcription of the rat insulin II gene (10, 33, 44). Glucose-stimulated expression was dependent upon two

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of these elements: RIPE3b1 (−115 to −107) and the ICE (−100 to −91 bp). RIPE3b1 factor binding activity was selectively induced in glucose-treated β cells. The ICE is the only conserved control element within the glucose-responsive region of the rat insulin I and II genes. Our results indicate that the same factors required for inducible transcription of the rat insulin II gene are also essential for regulating selective expression in the islet. Since RIPE3b1 and ICE sequences are conserved within the homologous region of the human (−135 to −102 bp) and rat insulin I (−138 to −103 bp) genes, we propose that these factors may also be important regulators in these genes.

MATERIALS AND METHODS

Tissue culture. The glucose-responsive HIT T15 β-cell line characterized by Zhang et al. (45) was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 15% (vol/vol) horse serum, 2.5% (vol/vol) fetal bovine serum, and 50 μg (each) of streptomycin and penicillin per ml. All experiments were performed with cells between passages 70 and 81. For the preparation of glucose-free medium, dialyzed horse serum (15% [vol/vol]) and fetal bovine serum (2.5% [vol/vol]) were added to DMEM without glucose (GIBCO/BRL). The initial glucose concentration of this medium was determined on a Beckman glucose analyzer and then supplemented to the desired concentration by addition of filter sterilized glucose.

DNA constructs. The construction of the 5′-flanking rat insulin II gene wild type (WT) and −238 ICE mutant was described previously (29). Each plasmid is named according to the 5′ end point of the rat insulin II gene sequences. The −238 ICE mutant firefly luciferase (LUC) plasmid (−238 ICEm LUC) was constructed by deleting ICE sequences from −99 to −91 bp and substituting these with nonrelated sequences (29, 44). The pSV −90 WT LUC plasmid was made by subcloning the *Hin*dIII-*Bgl*II-digested simian virus 40 (SV40) enhancer-rat insulin promoter (−90 to +8 bp) sequences from pSV-90 CAT (43) just upstream from the LUC reporter gene in pSVOARPL2L (6). The pSV −126 WT LUC plasmid was prepared by replacing the insulin II promoter sequences in pSV −90 WT LUC with a −126 to +8 bp fragment of the rat insulin II gene prepared by PCR (30). Mutations in the RIPE3b1 and RIPE3a2 sites were created in the −238 WT LUC plasmid by using the in vitro oligonucleotide mutagenesis kit from Amersham. The RIPE3b1 mutant oligonucleotide (−120 CTGCAGCTCGAGCCCCTC TG −101) contains a two-nucleotide substitution (CG for TC) within the RIPE3b1 binding site, while the RIPE3a2 mutant oligonucleotide (−112 TCAGCCCCTCGATCCA TCTGCTG −90) contains a three-nucleotide substitution (GAT for TGG) within the RIPE3a2 binding site. These mutations eliminate RIPE3b1- and RIPE3a2-mediated activity from transfected rat insulin II enhancer-driven constructs in vivo and binding in vitro in the gel shift assay (33). The sequences of all the plasmids were confirmed by DNA sequencing.

DNA transfection and in vivo transcription assays. Approximately 18 h before transfection, 2.5×10^6 HIT T15 cells were plated onto 60-mm^2 plates. Transfection of plasmid DNA was performed with Lipofectin (Bethesda Research Laboratories) by modifying the manufacturer's suggested protocol. Eight micrograms of expression plasmid along with 2 μg of the SV40 chloramphenicol acetyltransferase (CAT) plasmid (pSV2CAT) per plate was transfected. The Lipofectin-plasmid DNA complex was prepared in DMEM lacking glucose and serum; Lipofectin (20 μg) was used at twice the concentration of plasmid DNA. The cells were washed two times with phosphate-buffered saline (PBS) and then incubated for 4 h with Lipofectin-DNA complex. The cells were then grown in fresh DMEM-serum plus glucose for 48 h. The cells were harvested, and CAT and LUC enzymatic assays were performed as described by De Wet et al. (11) and Nordeen et al. (26), respectively. The LUC activity is defined in arbitrary relative light units (RLU). The background LUC activity from mock-transfected cells was approximately 150 RLU. The LUC activity from the insulin expression plasmids was normalized to the CAT activity from pSV2CAT. Each experiment was repeated several times with at least two different plasmid preparations.

Extract preparation. HIT T15 cells were plated at a density of 5×10^6 cells per 175-cm^2 flask (Falcon) and grown for approximately 4 days. The medium was then removed, the cells were washed twice with PBS, and then either low (0.2 or 0.5 mM)- or high (11 or 20 mM)-glucose-containing medium was added. The cells were harvested after 24 h, and extracts were prepared either from isolated nuclei (32) or from whole cells (43). The same gel shift profiles were obtained from extracts prepared by these procedures. The gel shift results presented are representative of experiments performed with several independently prepared HIT T15 cell extracts.

Electrophoretic mobility shift assays. Double-stranded oligonucleotides to the ICE (5′-TCTGGCCATCTGCTGATC CT-3′), RIPE3a2 (5′-CTGCAGCTTCAGCCCCTCTGGCC ATC-3′), RIPE3b1 (5′-TGGAAACTGCAGCTTCAGCCCC TCT-3′), adenovirus type 5 major late transcription factor (MLTF) (5′-TAGGTGTAGGCCACGTGACCGGGTGTTC-3′), and Sp1 (5′-GATCGCCCCGCCCC-3′) factor binding sites were end labeled with [α-^{32}P]dATP (6,000 Ci/mmol) and the Klenow fragment of *Escherichia coli* DNA polymerase I and used as probe. The binding reactions with the RIPE3a2 and RIPE3b1 element probes were performed as described by Shieh and Tsai (33). The competition experiments were performed with double-stranded oligonucleotides corresponding to a RIPE3b1 mutant binding site (5′-TGGAAAC TGCAGCTCGAGCCCCTCT-3′) (33), a human −227 to −206 bp C1 binding site (5′-CCTGGTTAAGACTCTAA TGACC-3′) (25), and rat insulin II −214 to −192 bp (5′-CCTCTTAAGACTCTAATTACCCT-3′), which is homologous to the human C1 binding site. ICE and MLTF factor binding were detected under the conditions described by Whelan et al. (43). Sp1 binding reactions were performed as described by Sartorelli et al. (31), except that 1 mM dithiothreitol, 0.1 μg of poly(dI-dC) per μl, and 0.5 μg of bovine serum albumin per μl were also added to the binding buffer. After incubation of the binding mixtures at 4°C for 20 min, they were loaded onto a 6% nondenaturing polyacrylamide gel (acrylamide-bisacrylamide, 29:1) and were run in TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA [pH 8.5]). The gel was dried and subjected to autoradiography after electrophoresis. The gels were scanned on a Molecular Dynamics PhosphorImager.

RESULTS

Identification of 5′-flanking sequences in the rat insulin II gene that regulate glucose-induced expression. Previous studies have indicated that the insulin enhancer region, which is located between −340 and −91 bp, mediates glucose-induced transcription (16, 19, 25). There is over 75% identity between the mammalian insulin genes within the sequences

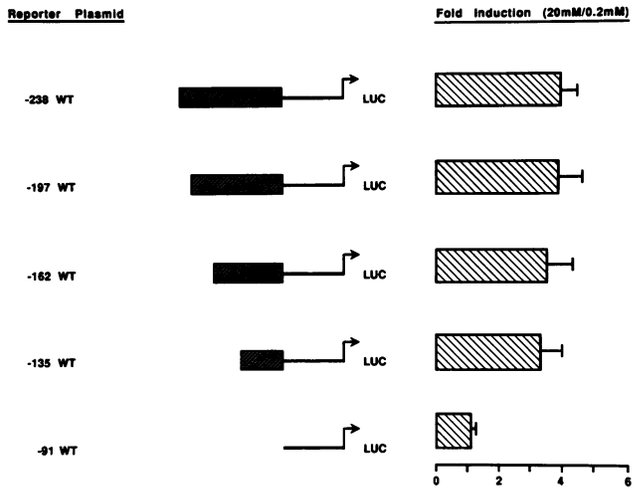

FIG. 1. Glucose-stimulated expression and structure of the 5'-flanking rat insulin II deletion mutants. Fragments of the 5'-flanking region of the rat insulin II gene were fused to the LUC gene in pSVOARPL2L (6). The hatched areas indicate enhancer sequences. All clones have a rat insulin II sequence with a 3' end at +2. Each mutant is named according to the 5' end point of deletion. LUC activity from each reporter plasmid was normalized to the CAT activity from the cotransfected pSV2CAT expression plasmid. The normalized activity from −238 WT LUC (3.1×10^5 RLU) is approximately 30-fold greater than that of −91 WT LUC (44). Fold induction is expressed as the ratio of the normalized LUC activity at 20 mM divided by the normalized LUC activity at 0.2 mM glucose. Results are presented as the mean ± standard error of the mean from four experiments.

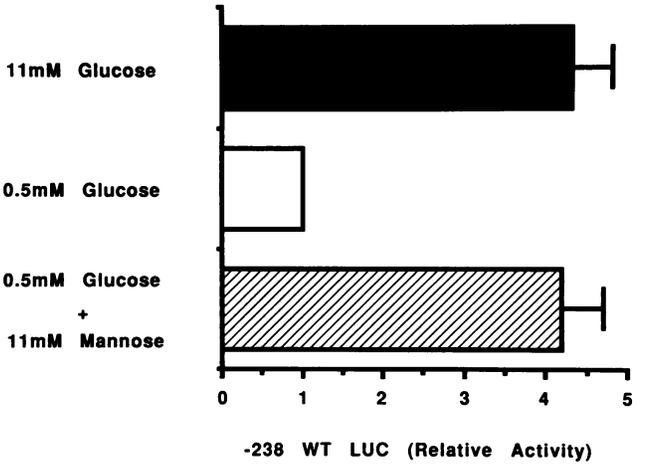

FIG. 2. Mannose induces insulin reporter gene expression. −238 WT LUC was transfected into HIT T15 cells grown in 0.5 or 11 mM glucose or 0.5 mM glucose plus 11 mM mannose. The results are presented relative to the 0.5 mM glucose point as the mean ± standard error of mean from five experiments. The average activity of −238 WT LUC in 0.5 mM glucose was 6.8×10^4 RLU.

located from −240 to −91 bp, whereas there is very little similarity upstream of −240 (37, 39). As a result, we believed that the *cis*-acting regulatory sequences within the rat insulin II enhancer region that mediated glucose-responsive transcription would be found between −240 and −91 bp. To identify the glucose response elements within the rat insulin II enhancer, a series of insulin expression plasmids containing rat insulin II gene sequences spanning the region from −238 to +2 bp linked to the LUC reporter gene were assayed for glucose-induced transcription. The insulin expression constructs were introduced into the hamster β-cell line HIT T15. After transfection, the cells were grown at a glucose concentration that results in basal (0.2 or 0.5 mM) or stimulated (11 or 20 mM) transcription from the insulin gene. (Insulin expression is induced in HIT T15 cells with a half-maximal response at 1.7 mM glucose [45].) Cell extracts were prepared 48 h after transfection and assayed for reporter gene activity. The LUC activity from each insulin reporter construct was normalized to the expression from the cotransfected pSV2CAT reporter plasmid.

The activities of all the 5'-flanking deletion expression constructs containing insulin enhancer sequences, including the −238 WT LUC, −197 WT LUC, −162 WT LUC, and −135 WT LUC, were induced approximately fourfold in response to glucose (Fig. 1). However, there was no significant change in expression from the −91 WT LUC plasmid under these conditions. Similarly, the expression mediated by the ubiquitously expressed Rous sarcoma virus and SV40 enhancers was unaffected by glucose concentrations that stimulated the activity of the insulin enhancer-driven constructs (data not shown).

Insulin mRNA levels increased approximately threefold in HIT T15 cells grown under basal (0.2 or 0.5 mM) versus stimulating (11 or 20 mM) glucose concentrations (data not shown). These results indicated that the rise in rat insulin II enhancer-mediated transcription parallels the rise in total insulin mRNA. In addition, since glucose-induced transcription was approximately fourfold above the basal level from all the rat insulin II constructs, except the −91 WT LUC insulin promoter expression plasmid, these results indicated that the sequences between −135 and −91 bp mediate glucose-responsive expression of the rat insulin II gene.

−238 WT LUC activity is induced by mannose. The stimulatory effect of glucose on insulin secretion and synthesis is mediated by metabolism of the sugar in the β cell (1, 20). Other nutrients which can be metabolized, like mannose, glyceraldehyde, and *N*-acetylglucosamine, can substitute for glucose and induce insulin expression (1). To test whether mannose will also induce rat insulin II enhancer expression, −238 WT LUC was transfected into HIT T15 cells and grown in medium containing 11 mM mannose and 0.5 mM glucose (Fig. 2). As previously illustrated in Fig. 1, −238 WT LUC activity is stimulated approximately fourfold in cells grown in 11 versus 0.5 mM glucose. We found that 11 mM mannose stimulated −238 WT LUC activity to a similar extent as 11 mM glucose. These results indicate that sugars which can be metabolized in the β cell, like mannose, also activate reporter gene expression regulated by the 5'-flanking enhancer sequences of the insulin gene.

Rat insulin II sequences from −126 to −91 bp impart glucose-responsive expression to the heterologous SV40 enhancer. The results from Fig. 1 indicated that there are glucose-responsive *cis*-acting elements in the rat insulin II gene located between −135 to −91 bp. This region spans three elements that are important in directing β-cell-type-specific expression of the rat insulin II enhancer: RIPE3b1 (−115 to −107), RIPE3a2 (−108 to −99 bp), and the ICE (−100 to −91) (33). Previously we had demonstrated that the ICE alone could direct cell-specific transcription from the ubiquitously expressed SV40 enhancer (44). To examine further whether the sequences between −135 and −91 bp were capable of directing glucose-inducible transcription,

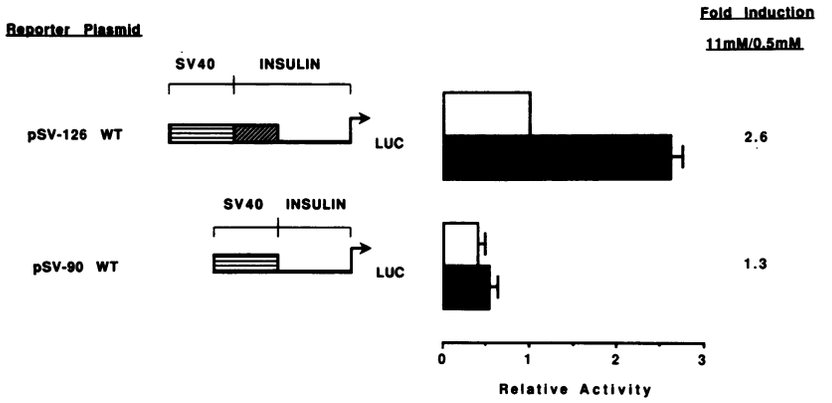

FIG. 3. Rat insulin II gene −126 to −91 sequences activate expression in a glucose-responsive manner when linked to a heterologous enhancer. The lined box represents the SV40 enhancer region, and the shaded box refers to rat insulin II enhancer sequences in the pSV-126 WT LUC and pSV-90 WT LUC constructs. The insulin LUC plasmids were cotransfected with pSV2CAT. The cells were then grown in either 11 mM (filled box) or 0.5 mM (open box) glucose. The results are presented relative to those of pSV-126 WT LUC grown in 0.5 mM glucose as the mean ± standard error of the mean from three experiments. The average activity of pSV-126 WT LUC in 0.5 mM glucose was 6.3 × 10^4 RLU. Fold induction is expressed as the ratio of the relative activity of each construct at 11 and 0.5 mM glucose.

the effects of 11 and 0.5 mM glucose on SV40 enhancer-mediated activity were compared in insulin-LUC reporter constructs containing rat insulin II sequences from −126 to +2 bp (pSV −126 WT LUC) or −90 to +2 bp (pSV −90 WT LUC). Expression from pSV −126 WT LUC was stimulated by glucose approximately threefold (Fig. 3). In contrast, glucose had little or no effect on pSV −90 WT LUC activity. These results indicated that SV40 enhancer-mediated transcription was stimulated in glucose-treated cells by *trans*-acting factors that interact and regulate expression from the −126 to −91 bp region of the rat insulin II gene. In addition, since the level of glucose-induced expression from pSV-126 WT LUC was comparable to that of −238 WT LUC (Fig. 1), these results also indicate that the rat insulin II sequences from −238 to −127 are not essential for glucose-responsive expression in HIT T15 cells.

RIPE3b1 binding activity is induced in glucose-treated cells. The gel mobility shift assay was used to determine whether glucose treatment of HIT T15 cells affected the levels of the cellular factors that interact with insulin *cis* elements from −126 to −91 bp. Extracts were prepared from HIT T15 cells grown for 24 h in the presence of either 0.2 or 20 mM glucose. Binding reactions were conducted with an equal amount of extract protein and ^{32}P-labeled oligodeoxynucleotide probe corresponding to RIPE3b1, RIPE3a2, or ICE sequences. Control reactions were also conducted to analyze the binding activities of two ubiquitously expressed cellular transcription factors in these extracts: the adenovirus 5 MLTF and the Sp1 transcription factor. The results from a typical gel shift experiment conducted with the insulin and general transcription factor probes are shown in Fig. 4a and b, respectively. There were no significant differences in ICE and RIPE3a2 binding activities in extracts prepared from either 20 or 0.2 mM glucose-treated cells (Fig. 4a). Similarly, there were also no changes in MLTF or Sp1 element binding (Fig. 4b). In contrast, the level of RIPE3b1 binding increased by eightfold in extracts prepared from 20 mM glucose-treated cells compared with that at 0.2 mM. We have also observed that the addition of 20 mM glucose directly to HIT T15 cells grown in 0.2 mM glucose selectively induces RIPE3b1 binding activity (data not shown).

To determine the specificity of the protein-DNA complexes formed with the ICE, RIPE3a2, and RIPE3b1 probes, excess unlabeled WT and mutant oligodeoxynucleotides were included in the binding reaction mixtures. The results of such a competition analysis on the formation of the RIPE3b1 complex are shown in Fig. 5. The extract from 20 mM glucose-treated cells was incubated with labeled RIPE3b1 binding site probe either alone or with increasing amounts of unlabeled WT or mutant binding site oligodeoxynucleotide. The WT and mutant RIPE3b1 oligonucleotides contained rat insulin II sequences from −126 to −101 bp. The RIPE3b1 mutant contains a two-nucleotide substitution (CG for TC) at nucleotides −112 and −111 which eliminates RIPE3b1 binding in vitro in the gel shift assay (33). Although there are several protein-DNA complexes detected with the RIPE3b1 element probe in HIT T15 extracts, only one was affected by the presence of specific (i.e., WT) but not a nonspecific (i.e., mutant) RIPE3b1 oligonucleotide in the binding reaction mixtures. In addition, the RIPE3b1 element-specific complex was not detected in extracts prepared from non-insulin-producing cells (data not shown). Our analyses of the glucose-induced binding activity are consistent with it corresponding to the β-cell-specific RIPE3b1 binding complex described by Shieh and Tsai (33). The specificity of the RIPE3a2 and ICE activator complexes in Fig. 4a was also confirmed in similar studies (data not shown).

Melloul et al. (25) have recently shown that in islet β cells glucose induces the binding of a specific complex, termed C1, to oligodeoxynucleotide probes corresponding to −227 to −206 bp of the human and rat insulin I genes. The C1 factor appears to be interacting with the Flat E element in these DNAs. The sequences of this element are conserved within all mammalian insulin genes (37). Although the sequences required for Flat E element binding within the −227 to −206 bp probe (5′-CCTGGTTAAGACTCTAATGA CC-3′; underlined nucleotides eliminate binding to the Flat E element [3, 17]) are distinct from those required for RIPE3b1 binding (5′-TGGAAACTGCAGCTTCAGCCCCTCT-3′; underlined nucleotides span the RIPE3b1 binding site [33]), we analyzed in competition assays whether the human insulin −227 to −206 bp region and the corresponding −214 to −192 bp region of the rat insulin II gene influenced RIPE3b1 binding. An 80-fold excess of each of the C1 site competitors to the RIPE3b1 probe was used in these assays. Under these

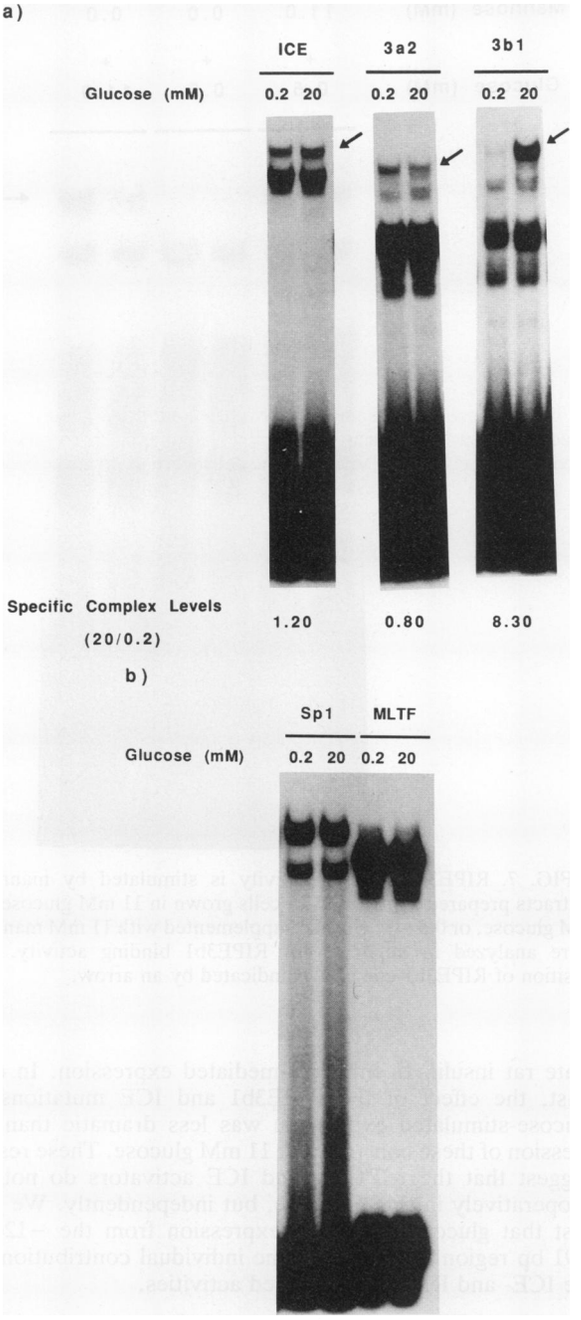

FIG. 4. RIPE3b1 binding activity is stimulated by glucose. Equal concentrations of HIT T15 protein extract prepared from cells grown in either 20 or 0.2 mM glucose were analyzed for ICE, RIPE3a2, RIPE3b1, Sp1, and MLTF binding. The relative levels (level at 20 mM/level at 0.2 mM) of the specific complexes were determined by densitometric scanning. (a) ICE, RIPE3a2, and RIPE3b1 binding activity. Arrows indicate the positions of specific complexes. (b) MLTF and Sp1 binding activity.

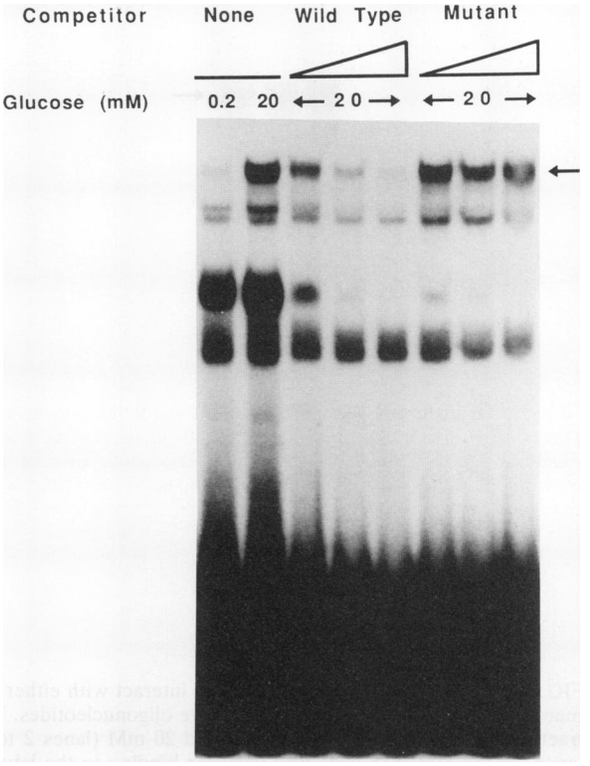

FIG. 5. The glucose-inducible binding activity corresponds to the RIPE3b1 complex. The molar ratio of cold WT and mutant RIPE3b1 (−126 to −105 bp) competitor to probe are 20, 40, and 80. The extract prepared from 20 mM glucose-treated HIT T15 cells was used in the analysis. The position of RIPE3b1 specific complex is indicated by an arrow.

conditions, the RIPE3b1 competitor completely eliminated RIPE3b1 complex formation (Fig. 5). However, neither of the C1 competitors had any effect on the formation of this complex (Fig. 6). These results suggest that glucose-stimulated transcription of the insulin gene is mediated by, at least, two distinct transcription factors, RIPE3b1 and C1.

RIPE3b1 binding activity is induced by mannose. In Fig. 2 it was demonstrated that 11 mM mannose also stimulated −238 WT LUC expression in transfected HIT T15 cells. To determine whether mannose exerts its effects by influencing RIPE3b1 element binding, HIT T15 cell extracts were prepared from cells grown in 0.5 mM glucose supplemented with 11 mM mannose. The RIPE3b1, RIPE3a2, and ICE binding activities in this extract were compared with those of the basal (0.5 mM) and stimulated (11 mM) glucose-treated HIT T15 extracts. The levels of RIPE3b1 binding in the extracts from mannose- and high-glucose-treated cells were similar (Fig. 7). However, mannose did not have any effect on either the ICE or the RIPE3a2 binding activities (data not shown). These experiments also indicate that RIPE3b1 binding activity is specifically and selectively induced under conditions that stimulate insulin gene transcription in vivo.

Both the ICE and the RIPE3b1 element are important for glucose-induced transcription of the rat insulin II gene. Since the binding activity of the transcription factors that regulate glucose-induced expression of the hepatic S14 and L-type pyruvate kinase genes is unchanged by glucose treatment (34, 38), we considered that the ICE and the RIPE3a2 element may also be involved in glucose-stimulated expression. In addition, the ICE is the only *cis*-acting element conserved within the glucose response regions of the rat insulin I (−247 to −197 bp) and II (−126 to −91 bp) genes. Thus, the contribution of the RIPE3b1 and RIPE3a2 elements and ICE towards glucose-stimulated expression in HIT T15 cells was assayed. Appropriate mutations within these elements in the −238 WT LUC expression plasmid

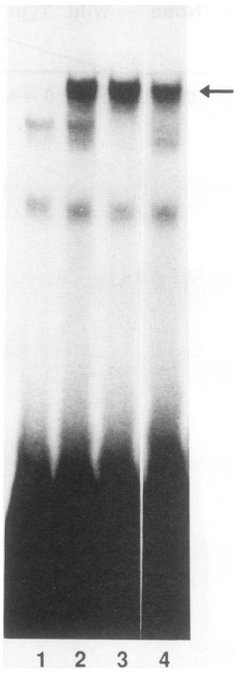

FIG. 6. The RIPE3b1 complex does not interact with either the human or rat insulin II C1 factor binding site oligonucleotides. The extract prepared from 0.2 mM (lane 1) and 20 mM (lanes 2 to 4) glucose-treated HIT T15 cells was used for binding to the labeled RIPE3b1 probe in the competition analysis with an 80-fold excess of the cold competitor. Lanes 1 and 2, no competitor; lane 3, human insulin −227 to −206 bp competitor; lane 4, rat insulin II −214 to −192 bp competitor. The position of RIPE3b1 specific complex is indicated by an arrow.

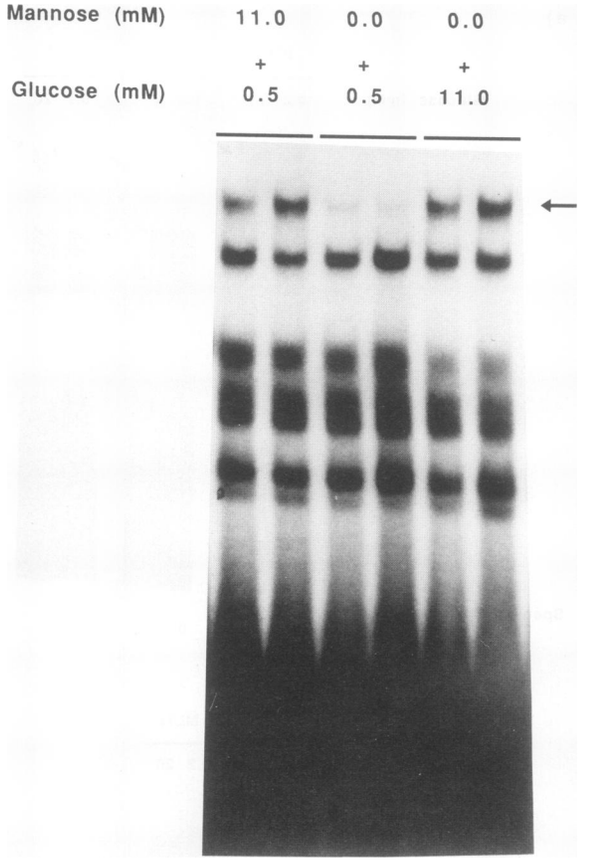

FIG. 7. RIPE3b1 binding activity is stimulated by mannose. Extracts prepared from HIT T15 cells grown in 11 mM glucose, 0.5 mM glucose, or 0.5 mM glucose supplemented with 11 mM mannose were analyzed in duplicate for RIPE3b1 binding activity. The position of RIPE3b1 complex is indicated by an arrow.

were made, and their effects were analyzed in vivo. The RIPE3b1 binding site mutant (termed −238 b1m LUC) was created by a two-nucleotide substitution (CG for TC) at nucleotides −112 and −111, and the RIPE3a2 mutant in −238 a2m LUC was made by changing the nucleotides at −102 to −100 (GAT for TGG), while ICE sequences from −99 to −91 bp were changed in −238 ICEm LUC.

The relative activities of −238 WT LUC, −238 b1m LUC, −238 a2m LUC, and −238 ICEm LUC in HIT T15 cells grown in either 0.5 or 11 mM glucose are shown in Fig. 8. The ICE and RIPE3b1 mutations had two major effects on −238 WT LUC activity. First, the overall activity of both the RIPE3b1 and ICE mutant constructs was reduced relative to that of −238 WT LUC at either glucose concentration. The activity of the RIPE3b1 and ICE mutant −238 expression constructs was decreased by about 56 and 92%, respectively, in cells grown in 11 mM glucose. Second, both of these mutations reduced glucose-stimulated expression. We found that induced expression was 4.4-fold from −238 WT LUC while only 2.8- and 2.2-fold from −238 b1m LUC and −238 ICEm LUC, respectively. Thus, these mutations resulted in a 38 and 51% decrease, respectively, in glucose-stimulated expression from the RIPE3b1 and ICE mutant constructs. In contrast, the RIPE3a2 mutation had little effect on −238 WT LUC activity in cells grown in 11 mM glucose or on glucose-responsive expression.

The reduction in −238 WT LUC activity by the RIPE3b1 and ICE mutations at 11 mM glucose is consistent with the findings of Shieh and Tsai (33), who suggested that the RIPE3b1 and ICE activators interact synergistically to stimulate rat insulin II enhancer-mediated expression. In contrast, the effect of the RIPE3b1 and ICE mutations on glucose-stimulated expression was less dramatic than expression of these constructs at 11 mM glucose. These results suggest that the RIPE3b1 and ICE activators do not act cooperatively in this response, but independently. We suggest that glucose-stimulated expression from the −126 to −91 bp region is a result of the individual contributions of the ICE- and RIPE3b1-mediated activities.

DISCUSSION

The principal transcriptional regulatory signal for the insulin gene in differentiated β cells is the concentration of circulating glucose. The results described here demonstrate that the *cis*-acting sequences that reside between nucleotides −126 to −91 of the rat insulin II gene are involved in glucose-induced expression. We identified two elements within this region that are required for glucose-stimulated expression: RIPE3b1 (−115 to −107 bp) and the ICE (−100 to −91 bp). It was also shown that the DNA-binding activity of the RIPE3b1 activator was selectively induced in glucose-treated β cells. The ICE and the RIPE3b1 element are also essential regulators of β-cell-type-specific expression of the rat insulin II gene. Hence, our results indicate that the *cis*-acting elements that are essential for mediating glucose-

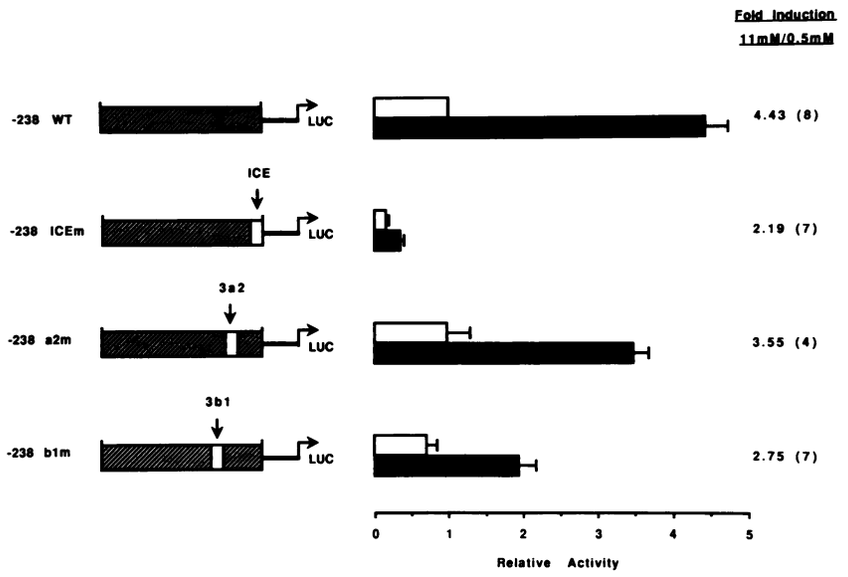

FIG. 8. The ICE and the RIPE3b1 element are important for glucose-mediated transcription of the rat insulin II gene in vivo. The structure of the LUC expression plasmids −238 WT LUC, −238 b1 mutant (−238 b1m LUC), −238 a2 mutant (−238 a2m LUC), and −238 ICE mutant (−238 ICEm LUC) are shown. The hatched box represents rat insulin II enhancer sequences, and the arrows indicate the site of the mutation. Plasmids were cotransfected with pSV2CAT. The transfected cells were grown in either 11 mM (filled box) or 0.5 mM (open box) glucose. The results are presented relative to the LUC activity of −238 WT at 0.5 mM glucose as the mean ± standard error of the mean. The average activity of −238 WT at 0.5 mM glucose was 7.6×10^4 RLU. The fold induction is calculated as the ratio of the relative activity of each construct at 11 mM glucose to that at 0.5 mM glucose. The values in parentheses correspond to the number of independent experiments performed.

stimulated transcription of the rat insulin II gene are also required for β-cell-type-specific expression.

The sequences between −247 and −197 bp can impart glucose-responsive expression to the rat insulin I gene (16, 25). The sequences homologous to this region were contained within the 5′-flanking rat insulin II deletion construct, −238 WT LUC, as there is a 12-bp deletion within the promoter region of the rat insulin II gene compared with rat insulin I (37). However, we found that there was no loss in glucose-stimulated expression in HIT T15 cells upon deletion of the −247 to −197 bp region from the rat insulin II gene enhancer (Fig. 1). Thus, the activities of the −238 WT LUC, −197 WT LUC, −162 WT LUC, and −135 WT LUC constructs were all induced approximately fourfold by glucose, whereas insulin promoter (i.e., −91 WT LUC) and Rous sarcoma virus and SV40 enhancer-mediated activities were unaffected. Furthermore, we found that the non-glucose-responsive SV40 enhancer cooperated effectively in mediating glucose-induced expression from −126 to −91 bp (Fig. 3). The inability of the upstream region of the rat insulin II gene to contribute to glucose-responsive expression may be due to the absence of two essential rat insulin I gene control elements, Flat F and the ICE, within this region of the gene (17). Alternatively, this discrepancy may be a consequence of our experiments being conducted with the HIT T15 cell line, whereas the studies with the rat I gene were performed with islets (16, 25).

Pancreatic β-cell-type-specific transcription can be recapitulated in cell culture with expression constructs driven by either the −126 to −91 sequences of the rat II gene (33) or the −247 to −197 sequences of the rat insulin I gene (17). Although it is generally accepted that the activity of these regulatory regions is the result of cooperative interactions between the transcription factors bound to their cognate recognition elements, glucose-inducible expression appears to result from the cumulative contributions of the individual glucose-responsive elements present in these regions. Thus, mutations within the RIPE3b1 element or ICE reduce rat insulin II enhancer-mediated activation by approximately 3- to 10-fold (Fig. 8) (10, 33), whereas mutations within the ICE and the RIPE3b1 element only reduced glucose-responsive expression by approximately 2-fold (Fig. 8). Melloul et al. (25) have also shown that eliminating the ICE sequences at −241 and −233 bp from the glucose response region of the rat insulin I gene (i.e., −247 to −197 bp) has only a modest effect on glucose-regulated expression in islets. Interestingly, they found that the −227 to −193 bp region alone can direct glucose-inducible expression from a heterologous promoter. They also found that the binding activity of a β-cell-specific-factor(s) to the −227 to −206 bp region, termed C1, was induced in glucose-treated cells. Interestingly, the C1 and RIPE3b1 binding factors do not appear to be closely related (Fig. 6). These results indicate that glucose-induced expression of the insulin gene is mediated, in part, by factors like the RIPE3b1 and C1 activators that are induced in response to stimuli. RIPE3b1 binding activity is rapidly induced in glucose-treated cells (32a), which provides further support for this factor being a major component of the insulin transcriptional apparatus involved in coordinating glucose-stimulated expression.

While the RIPE3b1 binding activity was induced in response to glucose in HIT T15 cells, ICE activator binding levels were not (Fig. 4a). The activator of ICE expression is a member of the basic helix-loop-helix (B-HLH) family. It appears to be composed of the ubiquitously expressed E2a gene products, E12 and/or E47 (or antigenically related proteins), and a factor that is uniquely distributed in pancreatic α and β cell types (9, 15, 27, 29, 33, 40). The heteromeric structure proposed for the ICE activator is analogous to the composition of the B-HLH regulators involved in tissue-

specific transcription of neuron- (5) and muscle-specific (28, 41) genes.

Increased activity of the ICE activator in the absence of any change in the level of the binding complex does not necessarily exclude this factor from playing a role in glucose-dependent stimulation, since activation may result from posttranslational modifications of a protein(s) in this complex. Posttranslational modifications are important in regulating transcription factor function (14, 22). Specifically, phosphorylation has been shown to augment *trans*-activation without affecting DNA-binding activity (22, 35). Alternatively, it is possible that ICE-mediated transcription is selectively stimulated in β cells as a result of the reduced expression of a repressor factor. c-*jun* may be a repressor of ICE-mediated activity in glucose-treated cells. This factor has been shown to inhibit B-HLH activation mediated by both the ICE activator (21) and the muscle-specific proteins MyoD and myogenin (2, 24). Importantly, repression of ICE activation by c-*jun* does not appear to result from direct binding to the ICE or by influencing ICE activator levels (21), a finding consistent with those of Li et al. (24) with the myogenic B-HLH activators. We hypothesize that c-*jun* regulates the activity of a coregulator of B-HLH function (21). Indirect evidence supporting a transcriptional regulatory role for c-*jun* in glucose-treated cells is provided by the observation that there is a concomitant decrease in c-*jun* mRNA levels in HIT T15 cells upon stimulation of insulin mRNA expression (23). Specifically, our findings indicate that ICE-mediated activation can be augmented by glucose in the absence of induced levels of the ICE activator binding complex.

We have demonstrated that the ICE and the RIPE3b1 element are involved in activation of rat insulin II gene transcription in glucose-treated β cells. The studies of Melloul et al. (25) indicate that Flat E element binding factors may also be involved in this pathway. The ICE, RIPE3b1, and Flat E enhancer elements are also essential for β-cell-type-specific expression of the insulin gene (3, 10, 17, 33, 44). Since the functional sequences necessary for the control mediated by these elements appear to be conserved within the transcription units of all mammalian insulin genes (37), the ICE, RIPE3b1, and Flat E may represent core *cis*-acting transcription control elements required for insulin gene expression. It is our objective to understand the roles that these individual elements and their transcription factors play in the formation of the functional insulin gene transcription complex and how their interplay determines cell-type-specific and -inducible transcriptional activation.

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