Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3865–3869, May 1993
Biochemistry

Glucose modulates the binding of an islet-specific factor to a conserved sequence within the rat I and the human insulin promoters

(pancreatic islets/insulin gene expression/glucose regulation)

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Communicated by Paul E. Lacy, December 15, 1992

ABSTRACT **In cultured rat and human pancreatic islets, glucose stimulated transcription of the rat insulin I gene through the minienhancer (FF) located between residues −196 and −247. The glucose-sensitive element was delineated to the region −193 to −227. The minienhancer bound islet nuclear proteins to form three major complexes (C1–C3). A 22-bp subfragment, spanning the sequence −206 to −227, was sufficient to retain all binding activities of the entire FF. The homologous sequence of the human insulin promoter interacted with rat islet nuclear extracts to form a single complex, corresponding to the C1 complex of the rat insulin I sequence. C1 was present only in insulin-producing cells; it was the major complex detected in isolated human islets with both rat and human insulin sequences. Furthermore, the DNA binding activity of the C1 factor(s) was selectively modulated by extracellular glucose in a dose-dependent manner; a 4.5-fold increase in binding intensity was detected when rat islets were incubated for 1–3 h in the presence of 20 vs. 1–2 mM glucose. We therefore suggest that the factor(s) involved in the C1 complex corresponds to the glucose-sensitive factor and, consequently, may play a determining role in glucose-regulated expression of the insulin gene.**

Insulin gene expression is restricted to pancreatic beta cells. It has been shown by transfection of cultured cells (1, 2) and by gene transfer in mouse embryos (3) that the regulation occurs at the level of transcription and is controlled by cis-acting elements in the 5′ flanking region of the gene (4–10). These elements bind multiple beta-cell proteins (9–18), providing the basis for identifying and cloning putative insulin gene transcription factors (19–22).

Insulin production is regulated by glucose (23–25). The sugar acts both by increasing mRNA stability and by stimulating the transcription rate of the insulin gene (26–30). Recently, the expression of a chimeric gene containing the rat insulin I minienhancer was reported to be stimulated by glucose in transfected rat fetal islets (31). Fetal beta cells fail to recognize nutrients as inducers of the normal stimulus-secretion coupling (32, 33). It is therefore important to establish that in normal adult pancreatic islets, where glucose is the main physiologic stimulator, insulin synthesis is similarly activated through the interaction of trans-acting transcription factors with a cis-acting regulatory element of the insulin enhancer.

In this study, we describe an insulin-specific DNA binding protein whose binding activity is sensitive to extracellular glucose, providing evidence of a glucose-sensitive islet nuclear protein that may play a crucial role in glucose-regulated expression of the insulin gene.

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MATERIALS AND METHODS

Islet Isolation and Transfection. Male adult Sprague–Dawley or Sabra rats (Hebrew University) were used. Pancreatic islets were isolated by a modification of the method of Lacy and Kostianovsky (34); 200–300 rat islets and ≈100 human islets were plated on extracellular matrix as described by Kaiser *et al.* (35). Fifteen micrograms of chloramphenicol acetyltransferase (CAT) plasmid and 5 μg of β-galactosidase plasmid were transfected using the cationic lipid DOTAP as recommended by the manufacturer (Boehringer Mannheim) for 12 h; the medium was then replaced with fresh RPMI 1640 medium containing 10% fetal calf serum (FCS) and 2 or 20 mM glucose for another 48 h. The cells were then harvested; 100-μg protein extracts were used for overnight CAT assay (36) and 10-μg extracts were used for the β-galactosidase assay (37). CAT activity was expressed after normalization to β-galactosidase values.

Cell Cultures. Rat insulinoma cells (RIN) were grown in RPMI 1640 medium with 10% FCS; hamster insulinoma cells (HIT) and mouse beta tumor cell line (βTC1) were grown in Dulbecco's modified Eagle's medium (DMEM) with 15% horse serum and 2.5% FCS; and L8, Ltk⁻, and Rat-1 cells were grown in DMEM with 10% FCS. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to the medium.

Preparation of Nuclear Extracts. Nuclear extracts of islet, RIN, βTC1, L8, Ltk⁻, and Rat-1 cells were prepared (38) with the addition of protease inhibitors (aprotinin, 20 μg/ml; leupeptin, 10 μg/ml; Boehringer Mannheim). Nuclear extracts of HIT cells were prepared according to Dignam *et al.* (39). Protein concentrations were determined by the Bradford method (40).

Gel Electrophoretic Mobility-Shift Assay (41). Synthetic double-stranded oligodeoxynucleotides were end-labeled with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. DNA binding mixtures containing 10% (vol/vol) glycerol, 20 mM Hepes (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 μg of poly dI·dC, and 2–3 μg of nuclear protein extract were incubated for 10 min on ice. Approximately 0.2 ng of the probe was added for an additional 30 min on ice. The binding reaction was analyzed on a native 6% polyacrylamide gel with high ionic strength buffer (5). The oligodeoxynucleotides of the rat insulin I gene used were derived from the following sequence:

$^{-247}$CTTCATCAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTAAGTA^{-193}

Abbreviations: CAT, chloramphenicol acetyltransferase; GSE, glucose-sensitive element; TK, thymidine kinase.

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The R1 fragment spans the region from −196 to −247 (FF); R2 spans −193 to −227 (glucose-sensitive element; GSE), and R3 spans −206 to −227. The human insulin sequence corresponding to −206 to −227 is shown in Fig. 5. Simian virus 40 AP1 binding sequence was CATCTCAATTAGTCAGCAACCA.

RESULTS

The 5′ Flanking Sequence −196 to −247 of the Rat Insulin I Gene Confers Glucose Responsiveness in Transiently Transfected Rat and Human Islets. Sequences around −200 to −250 in the enhancer element of the rat I insulin gene enhanced the activity of the minimal thymidine kinase (TK) promoter in the insulinoma lines HIT (5, 10) and RIN (D.M., unpublished results) but not in baby hamster kidney (BHK) cells and Rat-1 fibroblasts. We cotransfected isolated adult rat and human islets with the reference plasmid RSV-βGal and the following test plasmids: TK–CAT, containing the herpes simplex virus TK promoter fused to the CAT gene, or FF–TK–CAT containing 5 copies of the rat insulin I FF minienhancer element (−196 to −247) multimerized and linked 5′ to the TK–CAT chimeric gene. As shown in Fig. 1, incubation at high glucose concentration of islet cells transfected with the chimeric gene containing insulin sequences induced 8- to 9-fold and ≈3-fold stimulation of CAT activity in rat and human cells, respectively.

Interaction of Rat Islet Nuclear Factors with the Rat Insulin I Minienhancer. Binding of islet nuclear extracts was assessed by gel electrophoresis mobility-shift assay; three major complexes (C1–C3) and two minor ones (C4–C5) were detected (Fig. 2, lane 1). To probe the specificity of binding, competition experiments were carried out with 180-nt-long DNA fragments containing block mutations throughout the enhancer (4). The wild-type competitor fragment (S-31), carrying mutations outside the minienhancer region from −322 to −330, as well as the fragments S-21 and S-22, carrying mutations from −223 to −232 and from −233 to −241, respectively, competed entirely for the binding of nuclear factors to FF (lanes 2, 5, and 6). S-19, containing mutations from −201 to −210, competed only partially for the signals, while mutations in −211 to −222 (S-20) abolished the competition for binding of nuclear factors to FF (lanes 3 and 4).

Identification of Sequences Within the Rat Insulin I Minienhancer That Bind the Islet Nuclear Factors. To delineate the DNA sequences responsible for the binding pattern, double-stranded oligodeoxynucleotides were synthesized spanning the sequences −193 to −227 (R2) and −206 to −227 (R3). These shorter sequences produced similar complexes with islet nuclear extracts as the complete FF (R1; Fig. 3*A*). The complexes obtained with the R1 probe (Fig. 3*B*, lane 1) were fully competed for by the shorter segments R2 and R3 (lanes 2 and 3). By using the shortest probe (R3), the retarded bands (lane 5) were efficiently displaced by the longer fragments R1 and R2 (lanes 6 and 7).

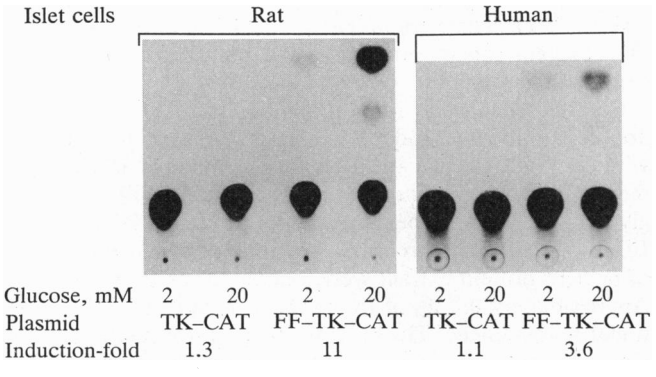

FIG. 1. Effect of glucose on CAT activity of transiently transfected adult rat and human islet monolayer cultures. TK–CAT is a plasmid containing the TK promoter fused to the CAT gene; FF–TK–CAT contains five copies of the minienhancer of the rat insulin I sequence −196 to −247 linked to the TK–CAT chimeric gene. Fifteen micrograms of each CAT plasmid was cotransfected with 5 μg of the RSV-βGal plasmid by the Lipofectin method. After transfection, the cells were cultured in medium supplemented with 2 or 20 mM glucose for 48 h. The relative -fold response to 20 mM glucose compared to 2 mM is the mean from three (rat) and two (human) independent experiments. One representative experiment each is shown.

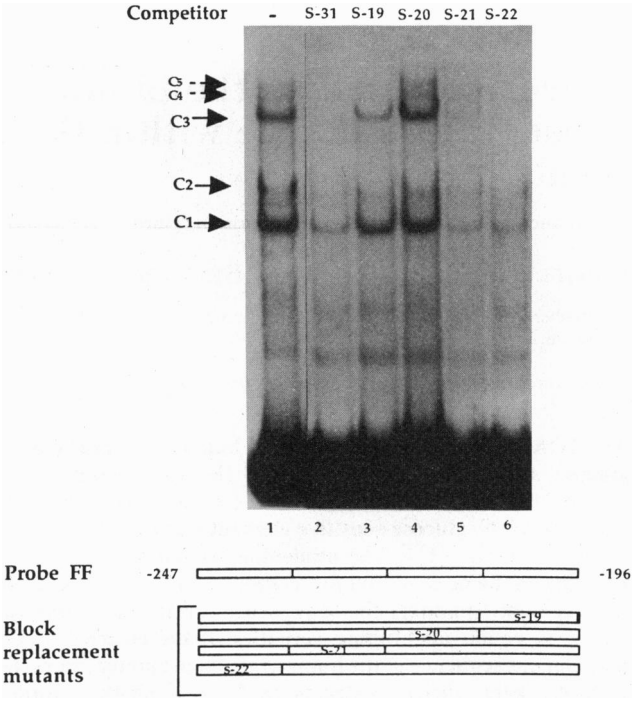

FIG. 2. Competition analysis of rat insulin I minienhancer (FF; −196 to −247) in rat islet nuclear extracts. DNA binding specificity was tested by a mobility-shift assay with a ^{32}P-labeled FF probe (lane 1). Competition was performed with a 50-fold molar excess of fragments carrying block mutations: S-19, from −201 to −210; S-20, from −211 to −222; S-21, from −223 to −232; S-22, from −233 to −241. S-31 contains the intact FF and a distant upstream block mutation (−322 to −330) and was used as a wild-type competitor. The complexes formed (C1–C5) are indicated by arrows.

Two specific and related sequences, −233 to −241 (IEB2 motif/Far box) and −104 to −112 (IEB1 motif/Nir box), were shown to be essential for expression of the rat insulin I gene in HIT cells (4). In our experiments, however, mutations in the IEB2 motif (S-22; Fig. 2, lane 6) did not prevent competition for binding of nuclear complexes to the R1 probe, nor did elimination of IEB2 in R2 and R3 prevent competition for any band (Fig. 3*B*, lanes 2 and 3). Furthermore, when the IEB2 sequence was used as competitor, it failed to displace any of the complexes detected with R1 (lane 4). Finally, while insulinoma cell extracts indeed contained a nuclear protein that bound the IEB2 motif (Fig. 3*C*, lanes 2 and 3), extracts from normal rat islets failed to demonstrate detectable binding activity (lane 1). In accord with the above, omission of IEB2 in the GSE sequence did not alter significantly glucose responsiveness in transfected islet cells (Fig. 4).

The 5′ Flanking Sequence −193 to −227 Serves as GSE in Rat and Human Islets. Since the shorter rat insulin I sequence R2 bound the nuclear proteins retained by the minienhancer FF, we verified its ability to confer glucose responsiveness in transfected islets. A GSE–TK–CAT hybrid gene, containing the R2 subfragment multimerized and linked upstream of TK–CAT, was constructed. As shown in Fig. 4, incubation of islets at 20 mM glucose induced 6- to 7-fold and 2.5-fold

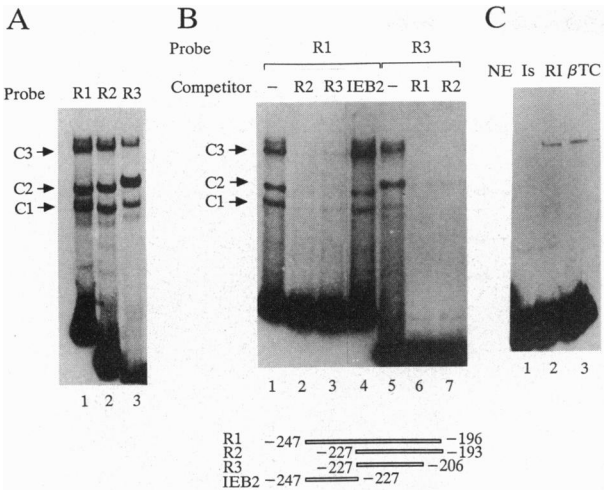

FIG. 3. Binding of islet nuclear factors to sequences in the minienhancer element of the rat insulin I gene. (*A*) Binding of islet nuclear extracts to rat insulin I sequences corresponding to the following probes: R1, −196 to −247 (FF); R2, −193 to −227 (GSE); R3, −206 to −227. (*B*) Competition analysis of the minienhancer FF sequence in islet nuclear extracts. Fifty-fold excess of unlabeled R1, R2, R3, or IEB2 sequence competitors was simultaneously added with R1 or R3 probes to the binding reaction mixtures as indicated. (*C*) Binding to the IEB2 motif. Binding of proteins was tested by gel mobility-shift assay with a ^{32}P-labeled IEB2 probe using nuclear extracts as follows: lane 1, adult rat islets; lane 2, RIN; lane 3, βTC1 cells. NE, nuclear extracts.

increases in the CAT signals in rat and human islets, respectively, when transfected with the plasmid containing insulin sequences; this is similar to the stimulation obtained with the full minienhancer (Fig. 1).

Binding of Rat Islet Nuclear Factors to the Sequence −206 to −227 of the Human Insulin Gene. The 5′ flanking region of mammalian insulin genes is highly homologous (42). Consequently, the human insulin sequence between −206 and −227 was synthesized and used as a probe to detect islet nuclear factors. Fig. 5 shows that a single complex was detected that comigrated with the C1 complex (lane 4). In cross-competition experiments, the human probe displaced only the C1 band, with the other complexes obtained with the rat sequence remaining essentially unaltered (lane 3). Both the human and the rat probe displaced efficiently the complex formed with the human sequence (lanes 5 and 6).

Cellular Distribution of the DNA–Protein Complex Common to the Rat and Human Insulin Genes. To ascertain the islet specificity of the C1 DNA–protein complex, nuclear extracts

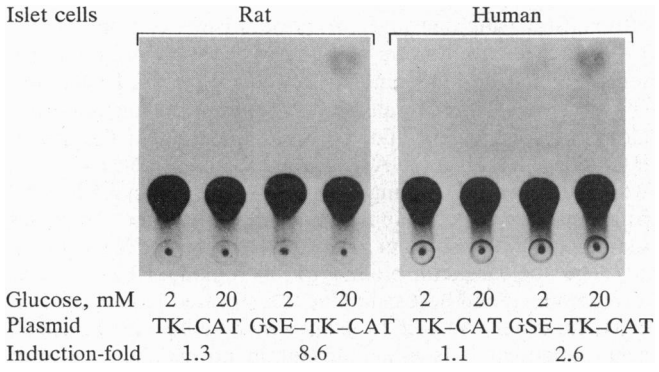

FIG. 4. Effect of glucose on CAT activity of rat and human islet monolayers transiently transfected with a hybrid gene carrying the sequence from −193 to −227 of the rat insulin I gene (R2). GSE–TK–CAT contains three copies of the sequence −193 to −227 linked to the TK–CAT chimeric gene. For details, see Fig. 1.

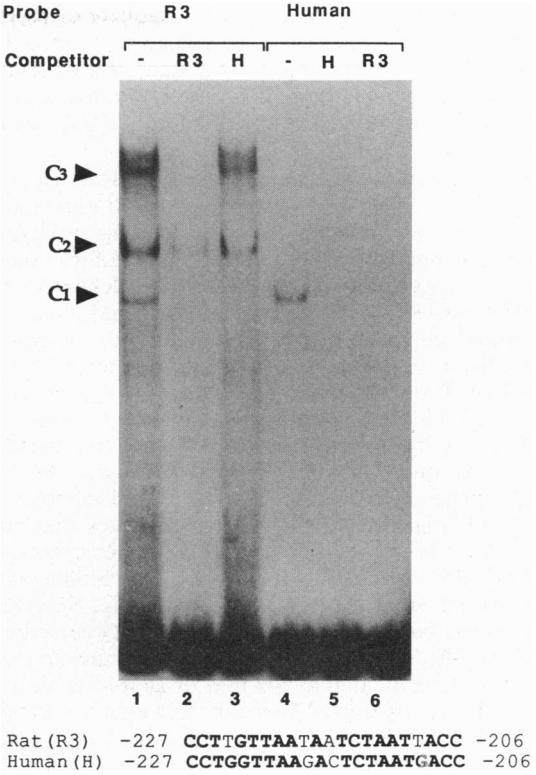

FIG. 5. Binding of rat islet nuclear proteins to rat I and human insulin DNA sequences. Electrophoretic mobility-shift assay was performed with rat islet nuclear extracts using the rat R3 and the human insulin probes (see respective sequences). Competition was carried out with 50-fold excess of unlabeled double-stranded oligonucleotides of the rat sequence (R3) (lanes 2 and 6) or the human sequence (H) (lanes 3 and 5).

from a variety of tissues and cell lines were tested. Several DNA–protein complexes were observed by using the rat probe; however, C1 was present only in insulin-producing cells (Fig. 6, lanes 1–4). Similarly, the homologous human probe bound selectively to extracts from insulin-producing cells to form the C1 complex (lanes 8–11). No binding activity was ever detected in any other insulin nonproducing tissue or cell line tested (lanes 12 and 13; data not shown).

Binding of Human Islet Factors to the Rat and Human Insulin Sequence −206 to −227. Using the rat R3 probe,

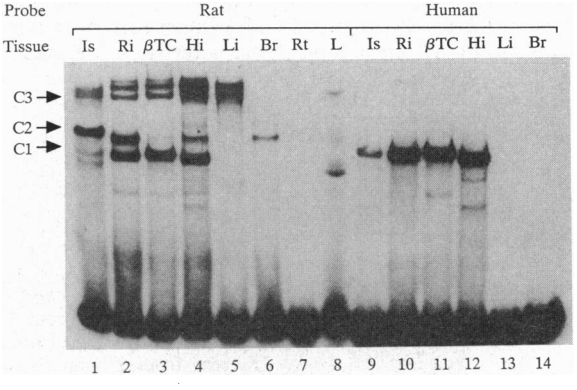

FIG. 6. Cellular distribution of nuclear proteins that bind to rat I and human sequences. Nuclear extracts were incubated with labeled rat and human sequences corresponding to the region between −206 and −227 as shown in Fig. 5. Is, rat islets; Ri, RIN (rat insulinoma line); βTC, mouse transformed beta-cell line; Hi, HIT (hamster insulinoma line); Li, liver; Br, rat brain; Rt, Rat-1 fibroblasts; L, Ltk$^-$ fibroblasts. (Occasionally, a faster band was observed when islet nuclear extracts were stored for longer time periods; see lane 1.)

several complexes were obtained with rat islet extracts; the affinity to C1 was lowest (Fig. 7, lane 1). In contrast, only two complexes were detected by R3 in human islet extracts, the predominant one comigrating with the C1 complex (lane 3). Again, the human sequence identified only the C1 complex (lanes 2 and 4).

The C1 Complex Is Glucose Sensitive. Rat islets were incubated for 3 h at various glucose concentrations; nuclear proteins were extracted and tested for binding to the rat (R3) and the human insulin genes. The C1 complex was sensitive to extracellular glucose concentrations: the density of the C1 band was 2- to 3-fold higher in islets at 11 mM glucose, and 4- to 6-fold augmented at 20 mM glucose, compared to extracts from islets at 2 mM glucose, whether the rat (Fig. 8*A*, lanes 1–3) or the human probe (lanes 4–6) was used. In contrast, the binding intensity of complexes C2 and C3 was not influenced by glucose (lanes 1–3). Extracts from rat islets incubated for only 1 h at 1, 5.5, and 20 mM glucose showed similar changes in binding activity of the C1 complex (data not shown). Fig. 8*A* (lanes 7–9) demonstrates that nuclear extracts from islets exposed to different glucose concentrations bound with equal intensity to the AP1 binding site (as well as to the SP1 site; data not shown). Fig. 8*B* gives the densitometric values of C1 bands formed between either the rat I (R3) or the human insulin sequence and nuclear extracts from rat islets incubated at 1–2 mM or 20 mM glucose for 1 or 3 h in 16 experiments. The mean -fold increase in binding intensity induced by high vs. low glucose was 4.4 ± 1.54 (mean ± SD; $P < 0.0001$).

DISCUSSION

The beta-cell-specific expression of insulin is controlled by multiple regulatory DNA sequences that bind trans-acting factors. These interactions determine the temporal expression of the gene and its inducibility by external stimuli (43). The tissue-specific regulatory element of the rat insulin I gene spanning the region −200 to −250 (refs. 5 and 10; D.M., unpublished data) was found to mediate glucose-stimulated expression of the CAT reporter gene in transiently transfected fetal islet cells (31). Here we present evidence that this effect is conserved in the adult rat and human islets, despite major differences in the physiological responses of beta cells at different stages of development. Furthermore, we limited the GSE to a fragment extending from residue −193 to −227.

Insulinoma cell lines have been used as a main source to study DNA–protein interactions in insulin promoters and, in some cases, to isolate the genes coding for trans-acting factors. Because transformed beta cells differ markedly from normal islet cells in many physiological characteristics, we made the effort of focusing our study on normal islets of Langerhans. Using the electrophoretic mobility-shift assay, multiple proteins binding the minienhancer of the rat insulin I promoter (FF) were detected. Competition experiments using mutations along the FF element allowed the delineation of the site of interaction to a 22-nt DNA fragment (residues −206 to −227), which retained the binding activities of the original FF. This fragment does not contain the IEB2 motif/Far box, which has a dominant role in insulin gene transcription in HIT cells (4, 10). The nuclear factor binding to this region, IEF2 (12), was also demonstrated here using the transformed RIN and βTC1 cells; however, no IEF2 signal was detected in rat islets, suggesting that it is either absent in normal beta cells or expressed at extremely low levels. The FF minienhancer contains the IEB2 motif and the proximal A+T-rich region or FLAT sequence (10). Here we show that the A+T-rich region (residues −193 to −227) is sufficient to confer glucose responsiveness in normal rat and human beta cells.

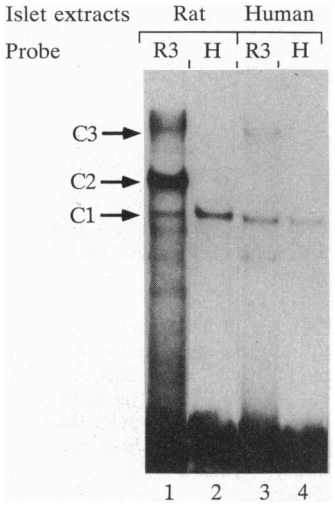

FIG. 7. Binding of nuclear factors from human islets to DNA sequences of rat and human insulin genes. Labeled sequences of rat I between −206 and −227 (R3) (lanes 1 and 3) and human insulin genes (H) (lanes 2 and 4) were incubated with nuclear extracts from isolated rat (lanes 1 and 2) and human islets (lanes 3 and 4).

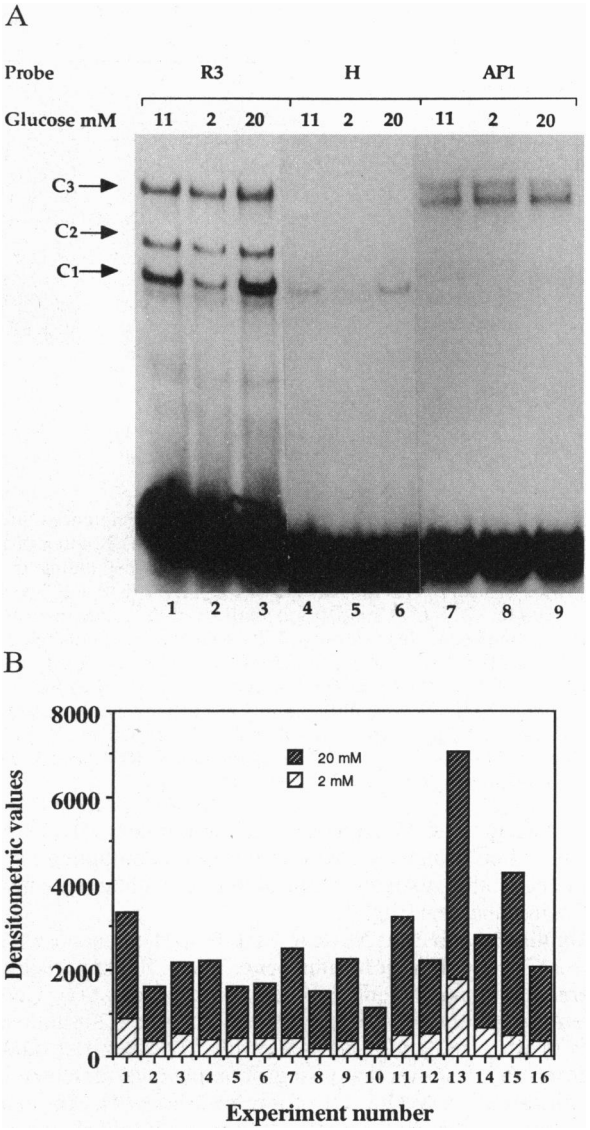

FIG. 8. Glucose sensitivity of the C1 complex. (*A*) Nuclear extracts from rat islets exposed to the indicated glucose concentrations for 3 h were incubated with the labeled sequences −206 to −227 of the rat I (R3) and the human (H) insulin genes as well as with the AP1 binding site. One representative example of 16 independent experiments is shown. (*B*) Intensity of the C1 complex formed with nuclear extracts from islets incubated at 1–2 mM and at 20 mM is given as individual densitometric values.

Cross-species comparisons show that the region between −206 and −227 of the insulin gene is highly conserved in mammals (42). Indeed, we found that the homologous human insulin sequence interacts with rat islet nuclear proteins to form a single complex, corresponding to C1, revealed with the rat sequence. This strongly suggests that a similar nuclear factor(s) interacts with a conserved element in both genes.

Scott *et al.* (18) reported that a similar human insulin sequence (residues −201 to −230) binds specific and ubiquitous proteins in insulinoma lines. A specific factor (IUF1) contained two components with different mobilities. Although our results show a single complex, this could be due to different experimental conditions, and one of the IUF1 complexes may indeed correspond to the C1 complex.

The rat islet factor(s) involved in formation of the C1 complex had the lowest binding affinity to the rat insulin I sequences compared to factors in the other complexes. Yet, this is the predominant complex detected in human islets using the rat probe, and the only one observed in rat and human islets with the human probe. It appears to be highly specific to insulin-producing cells, since it was present in rat and human islets and in insulinoma lines RIN, HIT, and βTC1, while it could not be detected in any of the insulin nonproducing cells tested, such as Rat-1 fibroblasts, rat L8 muscle cells, mouse Ltk^- cells, and rat brain and liver. Its similar interaction with rat I and human insulin sequences emphasizes its potential role in tissue-specific expression of the insulin gene.

The C2 complex appears to be a rat islet-specific factor since, while absent in insulin nonproducing cells, it was undetectable in human islets. A closely migrating complex was present in the insulinomas RIN and HIT, as well as in rat brain, and may correspond to the insulin gene-binding protein isl-1 isolated from a RIN cDNA library and that binds to the A+T-rich region of the minienhancer FF (22). isl-1 was also found in regions of the rat brain and was suggested to be important for motor neuron differentiation (44, 45). The other factors involved in complexes C3, C4, and C5 are not tissue specific since they were present in other cell types such as hepatocytes.

It could be questioned whether the glucose effect on insulin gene expression (30, 46) is evidenced at the level of the GSE-binding proteins. This is indeed the case: incubation of rat islets for 1–3 h at different glucose concentrations yielded nuclear factors with differential binding activities to the 22-bp fragment (−206 to −227) within the rat GSE and to the human homologous sequence. The C1 complex was quite sensitive to glucose, while neither the slower migrating C2–C5 complexes nor AP1 or SP1 binding proteins were affected. Thus, there was a selective dose-dependent modulation of the C1 binding protein(s) by extracellular glucose, an average 4.4-fold increase in the binding intensity being detected in islets cultured in high vs. low glucose. These changes correspond to the overall increase in insulin mRNA induced by glucose in different systems (27–30, 46) as well as to the increase in CAT activity in transfected islet cells (Fig. 1). We therefore suggest the term GSF (glucose-sensitive factor) for the C1 complex.

Glucose did not cause formation of a new DNA binding complex, rather, it induced a rapid change in the binding intensity of GSF. Various mechanisms have been described that affect the binding of preexisting trans-acting factors (for review, see ref. 47); these mechanisms could enhance GSF binding to GSE.

We are grateful to Mrs. L. Eylon for expert technical assistance and to Drs. N. Kaiser, R. Nesher, A. Rosenthal, U. Nir, and M. Walker for helpful advice. Human islets were graciously provided by Dr. P. E. Lacy, Dr. D. Sharp, and Ms. B. Olack (Washington University School of Medicine, St. Louis). Our sincere thanks to Dr. Yehudit Bergman for valuable suggestions and for critically reading the manuscript. This work was supported by generous grants from Mr. and Mrs. Jack A. Benaroya (Seattle) and Mr. and Mrs. Lewis Sandler (South Orange, NJ) and by the Koblitz–Matzkin Fund, the Wolfson Foundation, and the Grandis Endowment.

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