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Transcriptional regulation of the human insulin gene is dependent on the homeodomain protein STF1/IPF1 acting through the CT boxes

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ABSTRACT **Insulin gene transcription is a unique feature of the pancreatic β cells and is increased in response to glucose. The recent cloning of insulin promoter factor 1 (IPF1) and somatostatin transcription factor 1 (STF1) unexpectedly revealed that these are mouse and rat homologues of the same protein mediating transactivation through binding of CT box-like elements in rat insulin 1 and somatostatin promoter/enhancer regions, respectively. By using oligonucleotides representing each of the three CT boxes of the human insulin (HI) gene enhancer and nuclear extracts from the mouse islet tumor cell lines βTC3 and αTC1, we have identified a β-cell-specific binding activity as reported for IPF1, which has maximal affinity toward the CT2 box. However, in pluripotent, HI-transfected rat islet tumor cells, NHI-6F, this binding activity is present prior to induction of (human) insulin gene transcription. Its migration is identical to that of *in vitro* translated STF1 in electrophoretic mobility-shift assays; it is specifically recognized by anti-STF1 antibodies and has an apparent molecular mass of 46 kDa. Mutation of the CT2 box decreases transcriptional activity of a HI reporter plasmid by ≈65% in βTC3 cells and blocks the glucose response in isolated newborn rat islet cells. Furthermore, cotransfection with STF1 cDNA into the glucagon-producing αTC1 cells increases the activity of the HI enhancer 4- to 5-fold, suggesting that STF1/IPF1 can confer on αTC1 cells the ability to transcribe the HI gene. We conclude that STF1/IPF1 is a necessary but not sufficient key regulator of insulin gene activity, possibly also involved in glucose-regulated transcription.**

The islets of Langerhans consist of four highly differentiated cell types which produce, store, and secrete their respective hormones. Insulin gene transcription is a unique feature of the β cell and has been found to increase in response to glucose (1, 2) by mechanisms that are not clearly understood.

Early gene transfection analysis demonstrated the presence of transcriptional control elements within the 5′ flanking region of the rat insulin I gene (3). Although not fully conserved in sequence among rodents and man, the 5′ flanking region is functionally conserved, allowing β-cell-specific transcription of the human insulin (HI) gene in transgenic mice (4–6) and in pluripotent rat islet tumors (7). The control of tissue-specific expression involves the interaction of specific DNA-binding factors with the 5′ flanking region of the gene. Two types of sequence motifs, the E and the CT boxes, have been shown to be important for transcriptional regulation of the HI gene (8). The E box (positions −110 to −104) binds IEF1 which is composed of helix–loop–helix proteins such as Pan1 or Pan2 [the human homologues are E47 and E12, respectively (9)] interacting with an unidentified protein of ≈25 kDa (10, 11). In contrast to the rat insulin I enhancer, the HI enhancer does not contain a second E box, but the corresponding sequence (−237 to −232) binds the helix–loop–helix protein USF (12). A negative regulatory element has been identified that decreases the transcriptional activity in both insulin-producing and non-insulin-producing cells (13, 14). Three CT boxes (CTAATG) are present in the HI enhancer at −81 to −76 (CT1), −214 to −209 (CT2), and −317 to −312 (CT3) binding the activity IUF1 (13, 14). The sequence from −227 to −206, containing CT2, was reported to bind to a factor that is more abundant in glucose-stimulated islets than in control islets (15). Several CT box-binding proteins that belong to the homeodomain family of transcription/differentiation factors have been cloned from islet-derived cell lines, including Isl-1, cdx3, and lmx1 (16, 17). A somatostatin gene transactivating factor, STF1, was cloned by use of a cDNA library from a somatostatin-expressing rat cell line, MSL-G2-Tu-6 (18, 19). STF1 contains a homeodomain and binds a CT box within the somatostatin enhancer. Sequences similar to the CT boxes are present in the rat insulin I enhancer and bind the β-cell-specific factor IPF1 (20). IPF1 has been cloned from a mouse βTC1 λgt11 cDNA library and found to be a homeodomain-containing transactivator of the insulin gene (21). Sequence comparison between IPF1 and STF1 revealed only 15 aa differences out of the 284 aa, indicating that IPF1 is the mouse homologue of STF1. IPF1 appears before insulin during ontogeny of the mouse pancreas, where it eventually is restricted to the β cells of the adult (21), whereas STF1 mRNA is also detected in rat duodenum (18).

During islet development, pluripotent islet stem cells develop into one of the four cell types by passing through a transient phase where several of the hormone genes are coexpressed (22, 23). From an x-ray-induced rat islet tumor the pluripotent MSL cells were isolated (24), which can be induced to represent different stages of islet cell differentiation (7, 25, 26). A stably transfected subclone, NHI-6F, carries a silent HI gene when grown *in vitro*, where it displays an α-cell-like phenotype expressing mainly glucagon and no insulin (NHI-Glu). When passaged *in vivo* the HI gene is coactivated with the rat insulin I and II genes, giving rise to insulinomas (7). Reintroduction to *in vitro* culture gives rise to NHI-Ins cells, which maintain insulin production during early passages (25). Cell lines representing more mature islet cells are the glucagon-producing αTC1 and insulin-producing βTC3 lines obtained from tumors of transgenic mice harboring the glucagon or the rat insulin II enhancer region, respectively, linked to the simian virus 40 tumor-antigen gene (27, 28).

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Abbreviations: CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus promoter; EMSA, electrophoretic mobility-shift assay; HI, human insulin.

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Here we analyze the distribution of CT-box-binding factors in nuclear extracts from the above cell lines. We provide evidence that STF1/IPF1 is a key β-cell-specific regulator of HI gene transcription in differentiated β cells. It is expressed in NHI-6F cells prior to insulin gene activation (i.e., in cells expressing glucagon), which may suggest an additional important function as a general hormone gene activator during early islet ontogeny.

MATERIALS AND METHODS

Cell Lines and Nuclear Extract Preparation. NHI-Glu and NHI-Ins [previously NHI-6F and NHI-6F-28, respectively (7, 25)] were grown in RPMI 1640 medium (GIBCO) with 10% fetal bovine serum (Sera-Lab, Crawley Down, Sussex, U.K.), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO/BRL). αTC1 (27) and βTC3 (28) were grown in Dulbecco's modified Eagle's medium (GIBCO/BRL) with the same supplements. Nuclear extracts were prepared as described (29).

Plasmids and Transfections. The wild-type construct contains 339 bp of the HI enhancer linked to the chloramphenicol acetyltransferase (CAT) gene (30), whereas pCT2m is mutated in the CT2 box (CTAATG → CTCCTG) by site-directed mutagenesis.

Transfection of αTC1 and βTC3 cells was done by the calcium phosphate coprecipitation method (31), using 5 μg of a firefly luciferase expression plasmid as internal control and 15 μg of reporter plasmid with or without 1 μg of cytomegalovirus promoter (CMV)–STF1 expression plasmid (18). Luciferase activities were determined (assay system from Promega), and CAT activities (31) were normalized to luciferase activities.

Islets were isolated from newborn rats (32) and transfected with 20 μg of reporter plasmid by electroporation (33) with the modification that electroporation was carried out in 0.5 ml of phosphate-buffered saline with 1-mF capacitance. Cells were then cultured in RPMI 1640 containing 2 or 20 mM glucose and with the same supplements as above. CAT assays were performed (31) after normalization to the protein concentration as determined by Bradford assay (Bio-Rad).

Oligonucleotides, Electrophoretic Mobility-Shift Assay (EMSA), and UV Crosslinking. Oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer, labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and annealed. Sequences (5′ to 3′) were as follows: CT1, AGGCCC**TAATG**GGCCAGGCGGCAGGGGTTG; CT2, CCCCTGGTTAAGACTC**TAATG**ACCCGCTGG; CT3, CAGCTCTCCTGGTC**TAATG**TGGAAAGTGGC; CT2m, GATCTGGTTAAGACTC**TCCTG**ACCCGCTGG. EMSA was performed as described (10) except that electrophoresis was performed in 0.5× TBE (1× is 130 mM Tris/89 mM boric acid/2 mM EDTA).

For UV crosslinking an EMSA reaction scaled up 5 times was run and the gel was exposed to UV light (320 nm) for 30 min and autoradiographed overnight. The retarded bands were excised and transferred to 1× sample buffer (2.5% SDS/2.5 mM Tris·HCl, pH 6.8/10% glycerol/0.025% bromophenol blue/100 mM dithiothreitol) and incubated at room temperature for 30 min. The gel pieces were loaded on top of a 1.5-mm-thick SDS/7.5% polyacrylamide gel with a 4% stacking gel. The gel was run at 4°C in 25 mM Tris/0.192 M glycine/0.1% SDS.

***In Vitro* Translation of STF1 and Antibodies Against STF1.** CMV–Pan1 (17) (generously provided by M. German, University of San Francisco) and CMV–STF1 (18) expression plasmids were transcribed (Riboprobe; Promega) and translated (rabbit reticulocyte lysate system; Promega) *in vitro*, and 5-μl samples of the reaction mixtures were used for EMSA. Anti-STF1 serum was raised against aa 196–214 (18).

RESULTS

Identification of a β-Cell-Specific CT-Binding Activity. EMSA using CT1, CT2, and CT3 oligonucleotides and nuclear extracts from αTC1 and βTC3 confirmed the presence of a β-cell-specific binding complex previously termed IUF1 (14) (Fig. 1, arrow; data not shown for the CT3 box). The distribution of IUF1 thus parallels that of the homeodomain protein IPF1 (20, 21). Interestingly, the same CT-binding complex (IUF1) was formed with nuclear extracts from the pluripotent HI-transfected rat islet tumor line NHI-6F before (NHI-Glu, Fig. 1) as well as after (NHI-Ins, Fig. 1) the induction of insulin gene transcription. The slight variation in mobility between the mouse and rat complexes (Fig. 1, βTC3 versus NHI-Glu or NHI-Ins) most likely reflects species differences of the CT-box-binding protein. Its absence in αTC1 cells (Fig. 1) is in good agreement with the reported lack of expression of IPF1 in αTC1 (20, 21). However, the low expression levels detected on longer exposures (Fig. 2, lanes 1 and 3) may reflect the presence of a low frequency of insulin-producing cells in αTC1 (34). This has been confirmed by immunocytochemistry showing that STF1/IPF1 immunoreactivity is colocalized with insulin to this small subpopulation in αTC1 (P.S., unpublished observations). The sequence specificity of complex formation was confirmed in competition experiments using the mutated CT2m oligonucleotide (Fig. 2), indicating that the TAAT motif often found in homeodomain recognition sites is essential. Similar results were obtained with the CT1 and CT3 oligonucleotides and their mutated versions (data not shown). In cross-competition experiments, using the CT1 (data not shown) or CT2 (Fig. 3) probe and a 200-fold molar excess of CT1, CT2, or CT3 oligonucleotide competitor, the highest relative affinity was toward the CT2 box for nuclear extract from βTC3, NHI-Glu, or NHI-Ins.

IUF1 Is Identical to STF1/IPF1. UV crosslinking experiments were performed to identify the molecular weight of the CT box-binding protein. EMSA reactions were scaled up 5-fold with nuclear extracts from βTC3 and NHI-Ins and CT1 and CT2 probes. After exposure to UV light the retarded bands were excised and analyzed by SDS/PAGE (Fig. 4). A band at ≈65 kDa appeared for either of the probes and nuclear extracts. Subtraction of the molecular mass of the

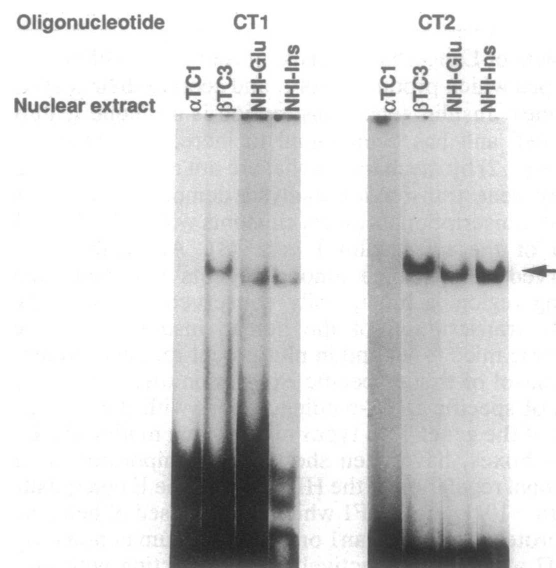

FIG. 1. Identification of a β-cell-specific CT box-binding complex by EMSA analysis using the CT1 or CT2 oligonucleotides. The complex formed is β-cell specific when mouse αTC1 and βTC3 cells are compared but is present in both NHI-Glu and -Ins phenotypes (arrow).

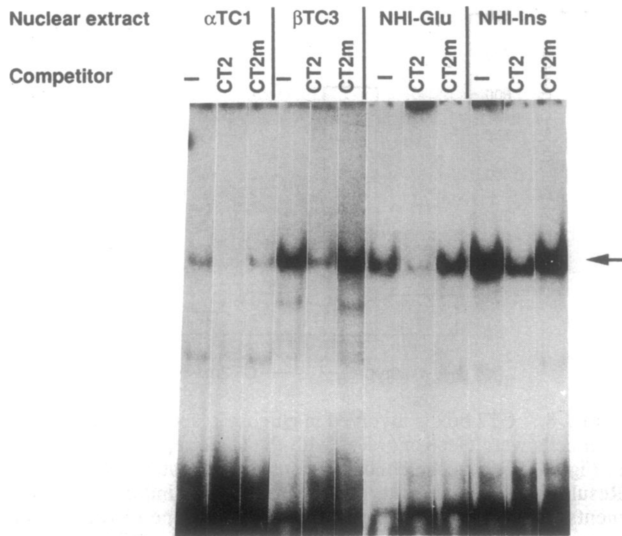

FIG. 2. Sequence specificity of the β-cell-specific complex. EMSA analysis used CT2 probe and 200-fold molar excess of either homologous (CT2) or mutated CT2 (CT2m) competitor. –, No competitor. Arrow points to the β-cell-specific complex.

probe (19 kDa) indicates that the protein is ≈46 kDa. STF1/IPF1 has a predicted molecular mass of 31 kDa (18, 21) but STF1 migrates on SDS/PAGE with an apparent size of 49 kDa (18) and could therefore be a candidate for binding the CT boxes within the HI enhancer. This was tested in EMSAs using CT1 probe and nuclear extract from βTC3, βTC3 plus reticulocyte lysate, and *in vitro* translated STF1 as well as Pan1 [to exclude the possibility that the 67-kDa Pan protein (17) would bind the CT boxes] (Fig. 5). Both *in vitro* translated STF1 (arrow) and βTC3 nuclear extract produced a band of identical mobility. The upper band observed with *in vitro* translated STF1 corresponded to a nonspecific component derived from the reticulocyte lysate. The high mobility band arose from degradation of STF1 (ref. 35; J.L., unpublished work). Further, the CT box-binding protein and *in vitro* translated STF1 reacted identically with an antibody raised against STF1 (Fig. 6). Anti-STF1 serum (18) inhibited binding to the CT box when nuclear extracts from the rat cell lines

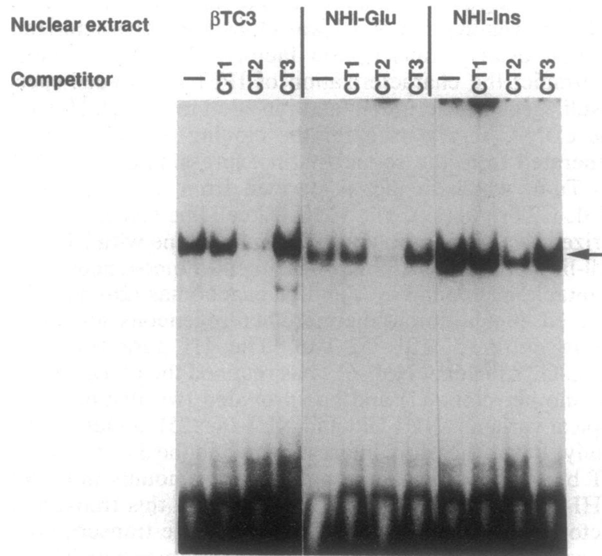

FIG. 3. High-affinity binding to the CT2 box. EMSA analysis used the CT2 probe and a 200-fold excess of CT1, CT2, or CT3 oligonucleotide as competitor. Arrow indicates position of the β-cell specific complex.

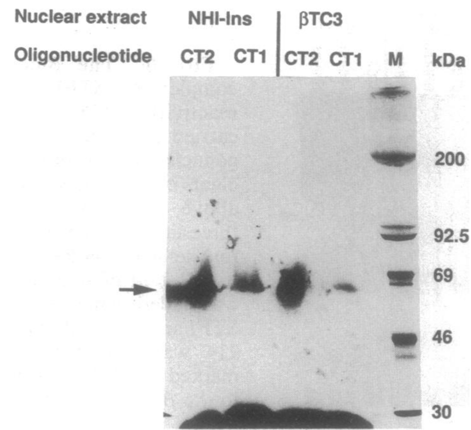

FIG. 4. Molecular size of the CT box-binding protein. A standard EMSA reaction was scaled up 5-fold, and the retarded bands were excised after UV exposure and subjected to SDS/PAGE. The nuclear extracts analyzed by a given oligonucleotide used as a probe are indicated above the lanes. Arrow indicates the migration of the β-cell-specific complex. Lane M, molecular size markers.

NHI-Ins and NHI-Glu were used (Fig. 6, lanes 7–9). The peptide sequence (rat) used for immunization has one amino acid substitution in the corresponding mouse IPF1 sequence, which may explain the inability of this antibody to recognize the CT box-binding protein from the mouse cell line βTC3.

Functional Importance of the CT2 Box and STF1 in Transcription from the HI Promoter. Since the CT2 box was shown to have the highest relative affinity for the STF1/IPF1-like protein, we determined the functional role of this element. The wild-type and pCT2m reporter plasmids were used in transient-transfection analysis. The wild-type HI enhancer was 10 times more active in βTC3 than in αTC1 (data not shown; ref. 14). Mutation of the CT2 box decreased the activity of the HI enhancer by ≈65% relative to the wild-type activity in βTC3 cells (Fig. 7), indicating that the CT2 box is important for the transcriptional activity of the HI gene. Since STF1 is absent from αTC1 cells, we tested whether STF1 was responsible for the 10-fold higher activity seen in the βTC3 cell line. αTC1 and βTC3 cells were cotransfected with the wild-type or pCT2m construct and an STF1 expression vector. STF1 increased the activity of the HI enhancer 4- to 5-fold in αTC1 (up to 60% of the wild-type activity seen in βTC3 cells), whereas only a slight increase was observed in βTC3 (Fig. 7). Thus, STF1 can confer on αTC1 cells the ability to transcribe the HI gene. Although the activity of the HI enhancer is decreased to 65% when the CT2 box is mutated, STF1 is still able to increase the activity of the mutated enhancer in αTC1 cells. This may suggest that the additional low-affinity CT boxes are functionally interacting with STF1.

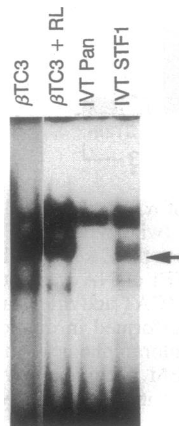

FIG. 5. *In vitro* translated STF1 binds the CT2 box. EMSA analysis with the CT2 probe was used to compare βTC3 nuclear extract, nuclear extract plus reticulocyte lysate (RL), and *in vitro* translated (IVT) STF1 and Pan1 as indicated above the lanes. Note that the migration of the β-cell-specific complex is identical to that of the STF1 complex (arrow).

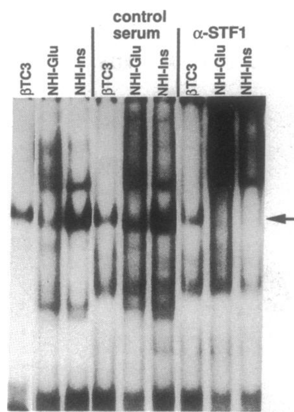

FIG. 6. The β-cell-specific complex has STF1-like immunoreactivity. EMSA analysis was carried out using the CT2 box oligonucleotide as probe. The nuclear extract used is indicated above each lane. For the first three lanes no serum was added; for the lanes marked "control serum," 1 μl of preimmune serum was added, whereas 1 μl of anti-STF1 serum raised against aa 196–214 was added for the lanes marked "α-STF1." Arrow points to the β-cell-specific complex.

CT2 Box Is Involved in the Glucose Response. To investigate the importance of the CT2 box in the transcriptional response of the HI enhancer to glucose, isolated, precultured newborn rat islet cells were transfected with the wild-type and pCT2m constructs and grown for 2 days in 2 or 20 mM glucose before CAT activity was assayed (Fig. 8). Relative to wild-type activity in 2 mM glucose, a 6-fold increase was observed with 20 mM glucose. Moreover, mutation of the CT2 box completely blocked the stimulatory effect of the high glucose, which strongly suggests that this element is involved in glucose-regulated transcription.

DISCUSSION

The rat insulin I and II genes and the HI gene share substantial homology in the 5′ flanking region (36) and are probably regulated by similar trans-acting factors. CT boxes are thus shared among known insulin genes but are present in a variety of other gene promoter/enhancers, including those of the glucagon and somatostatin genes. The human enhancer harbors three CT boxes, whereas the nonallelic rodent insulin genes carry two each. A β-cell-specific activity, IUF1, binds the CT boxes in the HI enhancer (14), sequences resembling the P1 region in the rat insulin I enhancer. A mouse β-cell-specific homeodomain protein, IPF1, was cloned (21) and found to bind the P1 region and to increase the activity of the rat insulin I promoter when cotransfected into βTC cells. Simultaneously, an almost identical protein, STF1 (18) was

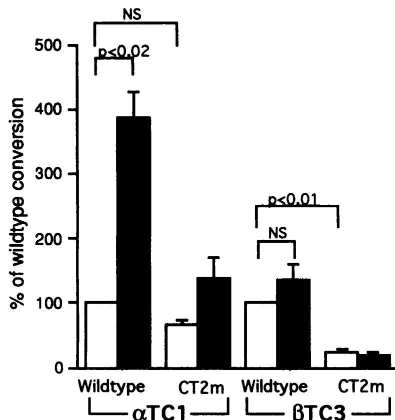

FIG. 7. Transient-transfection analysis of αTC1 and βTC3 cells carried out with 339 bp of the HI enhancer (wild type) or pCT2m, containing a mutated CT2 box (CT2m) linked to the CAT (open bars). Filled bars show the results when CMV–STF1 cDNA was cotransfected with the indicated constructs. Results (CAT activities) are the mean of three independent experiments (performed in duplicates) and are given as percentages of wild-type chloramphenicol conversion for each cell line. Error bars indicate SEM. Significance values (Student t test) are given relative to wild type in the respective cell line. NS, not significant.

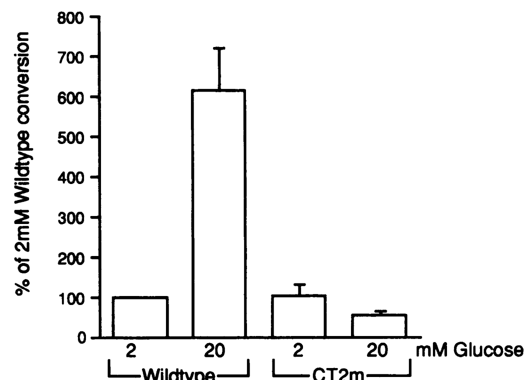

FIG. 8. CT2 box is involved in glucose-responsive transcription. Islet cells were electroporated with the same reporter constructs as in Fig. 7. The glucose concentrations are indicated below the bars. Results (CAT activities) are the mean of three independent experiments and are given as percentages of wild-type chloramphenicol conversion in 2 mM glucose. Error bars indicate SEM.

cloned from a rat somatostatinoma of more pluripotent character. STF1 binds a CT box within the somatostatin enhancer. STF1 is 1 aa shorter than IPF1 (283 vs. 284 aa) and varies in a total of 15 aa in the N- and C-terminal regions which flank the 100% conserved central homeodomain. In the present study we have investigated the importance of the CT boxes in the transcriptional regulation of the HI gene and shown that the major β-cell-specific CT box-binding protein as detected by EMSA analysis is immunologically indistinguishable from STF1.

Although it bound all three CT boxes, this factor showed the highest relative affinity toward the CT2 box. By UV crosslinking experiments followed by SDS/PAGE, the apparent molecular mass of the CT box binding protein was estimated to be ≈46 kDa. This is in good agreement with the reported migration of STF1 (49 kDa), although this factor has a predicted molecular mass of 31 kDa (18). Using *in vitro* translated STF1 in EMSAs, we have shown that STF1 indeed forms a complex with the CT boxes that comigrates with the β-cell-specific complex. Further, the use of anti-STF1 serum showed that the protein binding to the CT boxes has STF1-like immunoreactivity. These data strongly suggest that the IUF1 binding activity is equivalent to the STF1/IPF1 protein.

It is intriguing that STF1 was cloned and characterized as a somatostatin transcription factor (18), which seems to contradict the characterization of IPF1 as a β-cell-specific insulin gene transcription factor in adult mice (21). However, the cDNA library used for the cloning of STF1 (18) was generated from the somatostatin expressing cell line MSL-G2-Tu-6, which in fact is derived from an insulinoma of MSL-G2 origin (19). The MSL-G2 cell line has been characterized as a pluripotent multihormonal clone with islet stem-cell-like properties from which highly homogeneous transplantable insulinomas (7) and glucagonomas (26) have been derived, in addition to the more heterogeneous somatostatinoma culture, MSL-G2-Tu6. The HI gene-transfected MSL-G2 subclone, NHI-6F, has retained the pluripotency of the mother clone (7) and has provided two distinct phenotypical variants, NHI-Glu and NHI-Ins (25), included in this study. It was therefore interesting to find the STF1/IPF1-like CT box-binding protein in comparable amounts in both the NHI-Ins and NHI-Glu cells showing that this transcription factor, which is necessary for insulin gene transcription, is present before induction of insulin gene expression. This is in agreement with the onset of IPF1 expression prior to insulin expression during mouse pancreatic development (21). We speculate that STF1/IPF1 in addition to participating in restricting insulin gene expression to the mature β cells, may

have another important function, as a general hormone gene transcription factor during early ontogeny, where several hormone genes are coexpressed (22, 23). If MSL-G2 cells and derived subclones in fact do represent this rather narrow window of islet development, it may not be surprising that a functional somatostatin transcription factor, STF1, has been isolated which in the adult islet may be restricted to function mainly in the insulin-producing β cells (IPF1).

Since STF1/IPF1 was found to have the highest relative affinity for the CT2 box, we introduced point mutations in the core sequence of this box to test its functional importance. The CT2-box mutation decreased the activity of the HI enhancer by 65% in the STF1/IPF1-positive βTC3 cells, whereas the effect in αTC1 cells was less pronounced. This may reflect the lack of CT box-binding protein in these cells, which may explain the low activity of the wild-type construct in αTC1 cells (only 10% of that observed in βTC3 cells). Indeed, cotransfection experiments with an STF1 expression vector produced a 4- to 5-fold increase of the wild-type activity in αTC1 cells, whereas only a slight increase was seen in βTC3 cells. When cotransfected with the mutated construct, STF1 had no effect in βTC3 cells but was still able to transactivate the insulin enhancer in αTC1 cells, although significantly less than with the wild-type construct. This may indicate that the lower-affinity CT1 and CT3 boxes become functional in the presence of STF1 in αTC1 cells. These data strongly suggest that STF1/IPF1 is necessary for insulin gene transcription and that its major site of action is through the CT2 box of the HI gene enhancer.

A glucose-induced increase in binding activity of transactivating factors to the region −227 to −206 of the HI gene enhancer has been reported (15). Since this region contains the CT2 box, we tested whether this element was involved in glucose regulation of transcription. In transiently transfected normal islet cells, we found a 6-fold increase in CAT activity of the wild-type construct in high vs. low glucose. This effect was blocked with the mutated construct, which strongly suggests that the CT2 box binds the glucose-responsive factor. This is in agreement with the findings of Olson *et al.* (37), where the inability of HIT cells to respond to glucose was associated with the inability to form DNA–protein complexes with CT motifs of the HI promoter.

In conclusion, we have identified a STF1/IPF1-like homeoprotein as a key regulator of HI gene transcription, exerting its effect mainly through the CT2 box. Further, this box is involved in glucose regulation of transcription. Thus, STF1/IPF1 is a candidate factor for modulating the transcriptional effect of high glucose levels. STF1/IPF1 has a β-cell-specific expression pattern in adult islets and highly differentiated islet tumor lines, such as αTC1 and βTC3, suggesting a role in restricting insulin gene expression to β cells. Moreover, forced expression of STF1 in αTC1 confers the ability to transactivate a HI reporter plasmid. However, IPF1 is detected before insulin during pancreas ontogeny (21), and STF1/IPF1 is expressed in pluripotent heterogeneous rat islet tumor cells prior to insulin gene activation. STF1/IPF1 is thus not exclusively linked to an insulin-producing phenotype and may therefore also function as a transcription factor for islet hormone genes in general during the transient multihormone phase observed during islet ontogeny.

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