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POU Domain Transcription Factor brain 4 Confers Pancreatic α-Cell-Specific Expression of the Proglucagon Gene through Interaction with a Novel Proximal Promoter G1 Element

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The proglucagon gene is expressed in a highly restricted tissue-specific manner in the α cells of the pancreatic islet, the hypothalamus, and the small and large intestines. Proglucagon is processed to glucagon and glucagon-like peptides GLP-1 and -2. Glucagon is expressed in α cells and regulates glucose homeostasis. GLP-1 is implicated in the control of insulin secretion, food intake, and satiety signaling, and GLP-2 is implicated in regulating small-bowel growth. Cell-specific expression of the proglucagon gene is mediated by proteins that interact with the proximal G1 promoter element which contains several AT-rich domains with binding sites for homeodomain transcription factors. In an attempt to identify major homeodomain proteins involved in pancreatic α-cell-specific proglucagon expression, we found that the POU domain transcription factor brain 4 is abundantly expressed in proglucagon-producing islet cell lines and rat pancreatic islets. In the latter, brain 4 and glucagon immunoreactivity colocalize in the outer mantle of islets. Electrophoretic mobility shift assays with specific antisera identify brain 4 as a major constituent of nuclear proteins of glucagon-producing cells that bind to the G1 element of the proglucagon gene proximal promoter. Transcriptional transactivation experiments reveal that brain 4 is a major regulator of proglucagon gene expression by its interaction with the G1 element. The finding that a neuronal transcription factor is involved in glucagon gene transcription may explain the presence of proglucagon in certain areas of the brain as well as in pancreatic α cells. Further, this finding supports the idea that the neuronal properties of endodermis-derived endocrine pancreatic cells may find their basis in regulation of gene expression by neuronal transcription factors.

The endocrine pancreas consists of isolated nests of hormone-producing cells, known as the islets of Langerhans, which are anatomically embedded in the exocrine pancreas. The hormones produced by the islets regulate nutrient homeostasis during feeding and fasting and include insulin (β cells), glucagon (α cells), and somatostatin (δ cells), all of which are synthesized in the form of prohormones. Proglucagon and prosomatostatin further undergo tissue-specific posttranslational processing to produce the bioactive hormones. The rat proglucagon gene is expressed in the α cells of the pancreatic islets, selected neurons of the hypothalamus and brain stem, and enteroendocrine cells of the small and large intestines (2, 6, 8). Transcription of the proglucagon gene results in the production of a single proglucagon mRNA that is identical in these tissues. Posttranslational processing of proglucagon is highly tissue specific, resulting in the formation of unique profiles of proglucagon-derived peptides in the pancreas, intestine, and brain (26, 31). Glucagon appears to be the most important biologically active proglucagon-derived peptide produced in pancreatic islets. Glucagon is secreted from the α cells of the endocrine pancreas during fasting and stimulates glycogenolysis and gluconeogenesis to maintain blood glucose levels in the absence of the intake of nutrients. In contrast to the pattern of posttranslational processing of proglucagon in the pancreas, the processing of proglucagon in enteroendocrine cells of the small and large intestines results in the alternative formation of the peptides oxyntomodulin, glucagon-like peptide (GLP)-1, and glicentin, as well as GLP-2 and two intervening peptides, IP-1 and IP-2 (26). Of these peptides derived from the intestine, GLP-1 is a potent insulinotropic hormone that stimulates insulin secretion and production (14). In the brain, glucagon gene expression is found in select ganglia of the brain stem and in the hypothalamus (6). Furthermore, GLP-1 is reported to be involved in the regulation of food intake and satiety signaling (47). GLP-2 appears to stimulate proliferation of the intestinal epithelium (7).

Experiments with islet cell lines have provided insights into both the signal transduction pathways and the *cis*-acting elements that mediate regulation of islet and α-cell-specific proglucagon gene transcription (5, 33). Two islet cell-specific enhancer-like elements (designated G2 and G3), a cyclic AMP response element, and an α-cell-specific promoter element (G1), have been identified within the first 300 bp of the rat proglucagon gene 5′-flanking sequence (16, 17, 33). Additional, more-detailed analyses of the G2 and G3 subdomains have identified specific DNA sequences that mediate inhibition of proglucagon gene transcription by HNF3β and insulin, respectively (34, 35). The G2 element includes a protein kinase C response element (9). Whereas G2 and G3 appear to display islet cell-specific enhancer-like properties (to enhance expression in α, β, and δ cells), the α-cell-specific promoter activity of the proglucagon gene has been mapped to the more-proximal G1 element. The G1 sequence interacts predominantly with proteins from islet α-cell nuclear extracts (27), suggesting that the highly restricted cellular specificity of the proglucagon promoter is mediated by the interaction of α-cell-specific proteins with subdomains of the proximal G1 promoter element (27).

Mutational analysis of the transcriptional and DNA-binding

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properties of the proglucagon gene G1 region (which extends from bp −100 to −52) localizes the determinants of islet cell-specific proglucagon gene transcription to a specific region containing three AT-rich motifs (27) characteristic of core elements that bind homeodomain-containing transcription factors. These observations suggest that the islet cell-specific expression of the proglucagon gene may be mediated by one or more homeobox-containing proteins expressed in the endocrine α cells of the pancreatic islets. Although the homeobox protein IDX-1 (STF-1/IPF-1/PDX-1) activates the insulin and somatostatin promoter and is important for pancreas formation, the lack of IDX-1 expression in the majority of glucagon-producing α cells (21, 25, 32) suggests that homeobox proteins other than IDX-1 must regulate the transcriptional control of the proglucagon promoters through the G1 element. Recently, the caudalrelated homeobox transcription factor cdx2/3 was shown to bind to AT-rich sites of the G1 element and to positively regulate transcriptional activity of the glucagon gene and was thus suggested to be an α-cell-specific regulator of the glucagon gene (15, 19).

Homeobox transcription factors that bind to promoters of hormone genes expressed in pancreatic islets have been identified by using several different experimental approaches. The far-box-linked AT-rich enhancer sequence of the insulin gene was used as a probe in expression cloning studies to isolate hamster far-box-linked AT-rich element-binding proteins, including the LIM domain protein lmx-1 and the caudalrelated protein cdx-2/3 (10). An alternative approach for the identification of islet homeobox sequences makes use of the high degree of conservation of amino acid sequences within homeobox proteins. This PCR-based strategy resulted in the isolation of several novel homeobox sequences from rat islet cDNA, including the IDX-1 gene product and sequences corresponding to members of the caudal gene family (25). A similar experimental approach was used to isolate homeobox DNA sequences from a hamster insulinoma cell cDNA library, including eight genes not previously known to be expressed in islet cells (38). Homeobox DNA binding regions are found in several classes of transcription factors subgrouped into Antennapedia (10, 21, 25, 29, 51), LIM (10), Extradenticle (32), PAX (paired box) (42, 43, 46), and POU (10).

An approach to identify important homeodomain-containing transcription factors with degenerate primers selecting for Antennapedia sequences in α cells has been unsuccessful (25). Therefore, we hypothesized that homeodomain transcription factors of groups other than Antennapedia may be involved in tissue-specific α-cell gene expression in pancreatic islets. To identify such candidate homeobox transcription factors we searched, by a degenerate reverse transcription (RT)-PCR approach, for POU domain transcription factors in islet cell lines.

Surprisingly, we have found that the neuronal POU domain factor brain 4 is specifically present in high abundance in pancreatic α cells and is a strong positive regulator of glucagon gene expression by interaction with the α-cell-specific G1 element.

MATERIALS AND METHODS

Preparation of rat pancreatic islets. Rat pancreatic islets were isolated according to the method described by Lacey and Kosianovsky from adult male and female Sprague-Dawley rats weighing between 150 and 300 g (18).

Immunofluorescence cyto- and histochemistry and antisera. Rat pancreas and colon tissue were extracted, embedded, and sectioned as previously described (25). αTC-1 cells were plated in culture chambers prior to staining (Nunc, Inc., Naperville, Ill.). After fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and several rinses in PBS, samples were permeabilized with 100% methanol for 10 min at −20°C and then blocked with 3 to 5% normal donkey serum for 10 min at room temperature. The samples were then incubated with preimmune serum or antibodies raised against brain 4, cdx-2, glucagon, GLP-1, and IDX-1 (see below) at a dilution of 1:500 at 4°C overnight. Primary antisera were then rinsed off with PBS, and slides were again blocked with 3% normal donkey serum for 10 min at room temperature before being localized with donkey anti-rabbit indocarbocyanine (Cy3) dichlorotriazinylamino fluorescein (DTAF; Jackson ImmunoResearch Laboratories, West Grove, Pa.) for 30 min at room temperature. The slides were then rinsed with PBS and coverslipped with fluorescence mounting medium (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Images were obtained with a Nikon Epifluorescence microscope equipped with an Optronics TEC-470 charge-coupled device camera (Optronics Engineering, Goleta, Calif.) interfaced with a PowerMac 7100 installed with IP Lab spectrum analysis software (Signal Analytics Corp., Vienna, Va.).

Care was taken to analyze separate staining on adjacent (serial) sections of the tissue for identifying colocalization of GLP-1 with brain 4 or cdx-2 immunoreactivity.

Antisera raised in rabbits against brain 4 (4R2 and 4R3) (39), cdx-2 (45), and glucagon–GLP-1 were generously provided by M. G. Rosenfeld (University of California at San Diego), P. Traber (University of Pennsylvania, Philadelphia, Pa.), and G. W. Aponte (University of California at Berkeley), respectively. Antibody 523 raised against IDX-1 and CREB antiserum have been previously described (25). Normal donkey serum and fluorescently labeled antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pa.).

Expression and transcriptional reporter plasmids. Expression plasmids were all driven by a cytomegalovirus (CMV) promoter. pCMV-brain-4, pCEP-oct-3, and pRC-cdx-2 were generously provided by M. Mathis (Southwestern University, Dallas, Tex.), L. Dailey (Rockefeller University, New York, N.Y.), and P. Traber (University of Pennsylvania, Philadelphia, Pa.), respectively. Rat proglucagon gene 5′ flanking sequences from bp −60, −93, −168, and −361 to +58 inserted adjacent to the firefly luciferase reporter gene have previously been described (15, 34). Mutations in the G1 element were generated by the QuickChange mutagenesis kit (Stratagene, La Jolla, Calif.) according to the instructions of the manufacturer. The mutated construct was checked by sequencing. Adequate vectors without cDNA or promoter inserts, respectively, were used as controls. Transient transfection experiments were done in duplicate on at least three different occasions. The results of these assays are shown as means ± standard errors of the means (SE) for all results generated.

Cell culture and transfection. BHK-21 cells were obtained from the American Type Culture Collection. αTC-1 and -2 and βTC-1, -3, and -6 cells were generously provided by S. Efrat (Diabetes Center, Albert Einstein College of Medicine, New York, N.Y.). INR1-G9 cells and Glu-Tag cells were generously provided by S. Uchida (University of Tokyo, Tokyo, Japan) and D. J. Drucker (University of Toronto, Toronto, Canada), respectively. H35 mouse hepatoma cells were a gift from M. Alexander-Bridges (Harvard Medical School, Boston, Mass.). RIN 1027 B2 cells were derived in our laboratory (36). All cell lines, except INR1-G9 cells, were grown in Dulbecco's modified Eagle's medium (4.5 g of glucose per liter; Life Technologies, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, N.Y.) and 100 U of penicillin and 100 μg of streptomycin per ml. INR1-G9 cells were grown in RPMI medium (4.5 g of glucose per liter; Life Technologies) with identical additives. Cells were kept at 37°C in humidified 5% CO_2–95% air in 10-cm culture dishes (Life Technologies).

BHK-21 cells were transfected by the calcium phosphate precipitation method with a glycerol shock 4 h after transfection. αTC-1 cells were transfected by the Lipofectamine method with 8 μl of Lipofectamine reagent per 6-cm dish according to the instructions supplied by the manufacturer (Gibco BRL). The cells were harvested for analysis 48 h after transfection. The luciferase activities were analyzed, and values were normalized to the protein concentration in each extract (4). The luciferase activity of the specific reporter plasmid was normalized relative to the background luciferase activity obtained following transfection of the promoterless luciferase plasmid in the same experiment. Experiments were done in duplicate on at least three occasions. Means ± SE are of these assays are given.

RT-PCR. RNA from cultured cells and adult rat tissues (including isolated islets) was extracted with Trizol (Gibco BRL). The quality of the RNA was assessed by electrophoresis in a 5% formaldehyde agarose gel. RT was performed with oligo$(dT)_{18}$ as a primer and RNase H-reverse transcriptase according to instructions of the manufacturer (Superscript II; Life Technologies). Negative controls for the RT procedure and control for DNA contamination were performed by omitting the reverse transcriptase in parallel samples. Primers for PCR were degenerate oligonucleotides complementary to amino acids RIK/TLG or TICRFE (forward primer [fw]) and RVWFCN (reverse primer [rv]) of conserved regions of POU-specific and homeodomain sequences, respectively. PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 40°C for 30 s, and 72°C for 1 min and, finally, 72°C for 5 min. Viability of the RT product was controlled by separate PCR with primers specific for adenine-phosphoribosyltransferase of mice (fw, 5′-CACAGCGGCA AGATCGAC-3′; rv, 5′-CAAACATGG TTC CTCCTG-3′) or rats (fw, TCCGAATCTG AGTTG CAGC-3′; rv, CTGCACAC ATGGTT CCTC-3′), respectively. Cycle conditions were 95°C for 5 min; 30 to 35 cycles of 95, 55, and 72°C for 1 min each; and 72°C for 10 min as a final extension. The PCR products represented a mixed population of POU domain sequences which were cloned into pBluescript (KS^-)

TABLE 1. Results of degenerate RT-PCR amplification and cloning of expressed genes of pancreatic endocrine cell lines αTC-1 and βTC-1

Cell type[a]	No. of clones[b]	% Contribution[c]	GenBank match[d]
αTC-1	19	90	brain 4
	2	10	No match
βTC-1	9	50	brain 4
	4	22	oct-6
	2	11	oct-1
	2	11	mPOU
	1	5	No match

[a] Degenerate PCR primers (see Materials and Methods) for POU-specific and homeodomain sequences were used to amplify cDNA from αTC-1 and βTC-1 cell lines. Approximately 10% of αTC-1 cells stain immunopositive with insulin antiserum, while approximately 30% of βTC-1 cells immunostain positively for glucagon and GLP-1 (unpublished data). PCR products were cloned into pBSKS(−) (Stratagene), miniprepped, and sequenced.

[b] Absolute numbers of picked clones.

[c] Relative contributions of the picked clones to the sequences.

[d] Sequences were matched with the BLAST function of the National Center for Biotechnology Information database. mPOU, mouse pou-homeodomain protein.

(Stratagene, La Jolla, Calif.) and used to transform JM109 *Escherichia coli*. Individual bacterial colonies were picked, and then miniprep plasmid DNAs were prepared for sequencing by the double-stranded dideoxy chain termination method (Sequenase kit; U.S. Biochemicals, Cleveland, Ohio). Sequences were compared with GenBank entries by using the BLAST Network Service through the National Center for Biotechnology Information.

PCR was additionally performed with an fw specific to both mouse and rat brain 4 (fw, 5′-ACGGATCCGCCACAGCTGCCTCGAAT-3′) and an rv annealing to a homeodomain sequence (5′-ATTACAGAACCAGAC ACG-3′) to confirm the presence of brain 4 RNA expression in pancreatic islet cell lines and adult rat pancreatic islets. The product was cloned into a TA cloning vector (Invitrogen, La Jolla, Calif.) for subsequent sequencing.

Nuclear protein extraction and EMSA. Nuclear proteins from cell lines αTC-1 and BHK-21 fibroblasts transfected with CMV–brain-4, CMV–cdx-2, and CMV–oct-3 were prepared as previously described (40). The synthetic oligonucleotides listed in Table 1 corresponding to glucagon promoter G1 sequences, the far-box-linked AT-rich region of the rat insulin I promoter (INS FLAT), and the sucrose-isomaltase footprint 1 (SIF1) (45) were annealed, end labeled with ^{32}P with T4 polynucleotide kinase, and purified over a Sephadex G-50 spin column (Boehringer-Mannheim, Nutley, N.J.). Electrophoretic mobility shift assays (EMSA) were performed by incubating approximately 5×10^4 cpm of end-labeled DNA probe (0.02 to 0.03 ng of DNA) with nuclear protein (0.75 to 7.5 μg) in a binding buffer (10 mM Tris-HCl [pH 8.0], 40 mM KCl, 6% glycerol, 1 mM dithiothreitol, 0.05% nonidet P-40) for 20 to 30 min at room temperature. For antibody interference experiments, nuclear proteins were premixed with diluted preimmune or specific antiserum in the binding buffer at room temperature for 20 min prior to adding the labeled DNA probe. Different dilutions of the immune sera had been tested prior to conducting the EMSA to rule out any nonspecific effects. For competition experiments, nuclear extracts were premixed with unlabeled double-stranded DNA oligonucleotides in binding buffer at room temperature for 20 min before addition of the radioactively labeled DNA probe for a further 20-min incubation. The reaction mixture was then analyzed by electrophoresis on a 5% nondenaturing polyacrylamide gel. Following electrophoresis, the gel was exposed to X-ray film for 16 to 72 h.

Western blot (immunoblot) analysis. Portions (7.5 μg) of nuclear extracts from various cell lines and 100 μg of rat islet whole-cell extracts (in radioimmunoprecipitation assay [RIPA] buffer) were size fractionated on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) gel and electrophoretically transferred onto a nitrocellulose filter. The immunoreactivities of brain 4 (antiserum 4R3) and CREB (25) were detected with the ECL Western analysis system (Amersham Life Sciences, Buckinghamshire, United Kingdom) with peroxidase-linked anti-rabbit immunoglobulin as the second antibody.

RESULTS

Expression of brain 4 in islet cells. RT-PCR of mRNA prepared from αTC-1 and βTC-1 cell lines with degenerate amplimers to conserved POU-specific homeodomain proteins revealed the expression of several known POU domain transcription factors (oct-1, oct-2, oct-6, and brain 4). Notably, we found that 90% of the clones derived from αTC-1 cells contained brain 4-specific sequences, whereas brain 4 was found in 50% of the clones derived from βTC-1 cells. Moreover, no other class III POU domain transcription factor (brain 1, brain 2, or oct-6) was amplified from the pancreatic α-cell line (Table 2).

To confirm further that brain 4 is present in endocrine pancreas-derived cell lines and in rat islets, RT-PCR was performed with specific primers for brain 4. Sequencing of the PCR products confirmed the presence of brain 4 in rat and mouse islet cell lines and also in adult rat islets. The validity of the RT and PCR was ascertained by amplification of mouse and rat adenosine phosphoribosyltransferase from the synthesized cDNAs. Amplification of genomic DNA was excluded by including samples that were not reverse transcribed prior to PCR which gave no amplified product (data not shown).

To ascertain whether the brain 4 mRNA transcripts that were detected are translated into protein, we prepared nuclear extracts from cell lines and protein extracts from adult rat islets for Western immunoblot analysis with antiserum to brain-4. Nuclear extracts prepared from BHK-21 cells transfected with a brain 4 expression vector were used as positive controls. brain 4 protein was detected in abundance in glucagon-producing islet cell lines αTC-1, αTC-2, and INR1-G9 and the intestinal cell line Glu-Tag, whereas insulin-producing tumor cell lines (βTC-3 and -6) and the somatostatin-producing cell line RIN B2 revealed only small amounts of detectable brain 4 immunoreactivity (Fig. 1). The finding that small amounts of brain 4 are present in these insulin- and somatostatin-producing islet cell lines is not surprising, because some overlap in the α- and β-cell phenotype between these tumor cell lines has been observed previously (37). Nonislet control cell lines (BHK-21, H35 hepatoma cells, and NIH 3T3 cells) showed no detectable brain 4 immunoreactivity. Most importantly, protein extracts prepared from adult rat islets revealed the presence of brain 4 immunoreactive protein (Fig. 1).

Adult rat pancreatic α cells express abundant brain 4 protein. Immunofluorescence staining reveals brain 4 immunoreactivity in abundance in αTC-1 nuclei. However, in contrast to

TABLE 2. Oligonucleotides used for EMSA

Oligonucleotide	Sequence
−93/−60[a]	5′ CCCATTATTTACAGATGAGAAATTTATATGTCAGCGTAA 3′
	GGGTAATAAATGTCTACTCTTTAAATATACAGTCGCATT
−93/−60m5′[b]	5′ CCCATCCCGCCCCAGTGAGAAATTTATATGTCAGCGTAA 3′
	GGGTAGGGCGGGGTCACTCTTTAAATATACAGTCGCATT
−93/−60m3′[c]	5′ CCCATTATTTACAGATGACAGCCCGCTATGTCAGCGTAA 3′
	GGGTAATAAATGTCTACTGTCGGGCGATACAGTCGCATT
−94/−74[d]	5′ CCCCATTATTTACAGATGAGAAA 3′
	GGGGTAATAAATGTCTACTCTTT
INS FLAT	5′ TTGTTAATAATCTAATTACCCTAG 3′
	AACAATTATTAGATTAATGGGATC
SIF1	5′ GTGCAATAAAACTTTATGAGTA 3′
	CTCGTTATTTTGAAATACTCAT

[a] Double-stranded oligonucleotide spanning from bp −93 to −60 with the wild-type sequence.

[b] Double-stranded oligonucleotide spanning from bp −93 to −60 with mutations in the 5′ AT-rich site of the G1 element of the glucagon gene promoter (underlined sites are mutated regions).

[c] Double-stranded oligonucleotide spanning from bp −93 to −60 with mutations in the 3′ AT-rich site of the G1 element of the glucagon gene promoter (underlined sites are mutated regions).

[d] Wild-type oligonucleotide spanning from bp −94 to −74 carrying only the 5′ AT-rich site and missing the 3′ AT-rich site of the G1 element of the glucagon gene promoter.

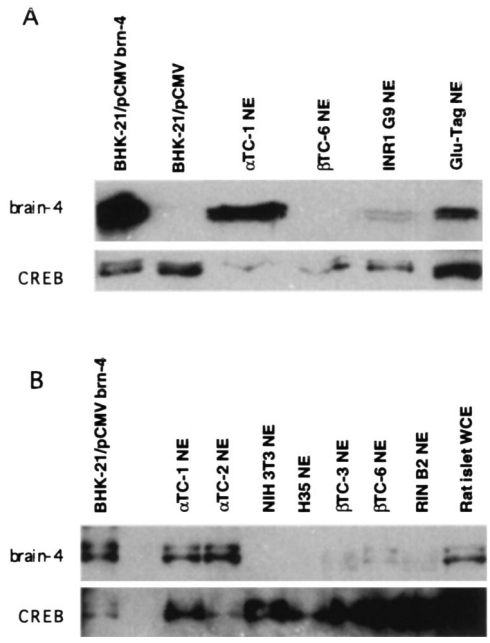

FIG. 1. Expression of brain 4 protein in proglucagon-producing cells determined by Western immunoblot analyses. Twenty micrograms of nuclear extracts (NE) from BHK-21 cells transfected with brain 4 and from BHK-21 cells transfected with empty expression vector, three proglucagon-producing cell lines (αTC-1 [mouse], INR1-G9 [hamster], and Glu-Tag [mouse]), and an insulin-producing cell line (βTC-6) (A) and 20 μg of nuclear extracts from BHK-21 cells with a transfected brain 4 expression vector, two proglucagon-producing pancreatic cell lines (αTC-1 and -2), NIH 3T3 fibroblasts, H35 mouse hepatoma cells, the somatostatin-producing rat cell line RIN B2, insulin-producing cell lines βTC-3 and -6, and 100 μg of adult rat islet whole-cell extract (WCE) (B) were size fractionated on an SDS–12% polyacrylamide gel and transferred electrophoretically to a nylon membrane. Immunoreactive brain 4 protein was detected with a rabbit antibody directed against the N-terminal portion of brain 4 and was visualized by using a peroxidase-linked anti-rabbit immunoglobulin and the ECL Western analysis system. Western immunoblotting of CREB immunoreactivity was performed to additionally control protein loading. Wild-type BHK-21 cells lack brain 4 immunoreactivity, while transfected BHK cells readily demonstrate nuclear brain 4 immunoreactivity. Glucagon-producing cell lines show abundant brain 4 immunoreactivity. Low-level immunoreactivity is seen in βTC-3 and -6 cells, as well as in RIN B2 cells. Adult rat islets also reveal brain 4 immunoreactivity.

the findings in adult rat tissue (see below), αTC-1 nuclei also stained strongly for cdx-2 (data not shown).

Immunofluorescence histochemistry was used to locate the expression of brain 4 in adult rat islet tissue (Fig. 2). Expression of brain 4 immunoreactivity in adult rat pancreas localizes to nuclei of glucagon-producing cells located in the mantle of the rat islet. Because cdx-2 has been reported previously to transactivate the glucagon gene, we attempted to detect cdx-2 immunoreactivity by immunohistochemistry of rat pancreas and gut tissues. cdx-2 immunoreactivity was detected at various intensities in a few scattered cells throughout adult rat islets but did not show a clear localization to the glucagon-producing cells in the islet periphery. As a control, cdx-2 was found in abundance in gut epithelial cells (Fig. 2). brain 4 immunoreactivity was not detected in adult rat gut epithelial cells (14a). Taken together, these data indicate that the expression of brain 4 occurs predominantly in glucagon-producing cell lines and in vivo in the adult rat is largely restricted to glucagon-producing α cells of the pancreatic islets.

brain 4 activates the proglucagon promoter in BHK-21 fibroblasts and αTC-1 pancreatic endocrine cells. The detection of brain 4 in glucagon-producing islet α cells suggested that this transcription factor may be a candidate regulator of proglucagon gene transcription. To examine this hypothesis, we transfected BHK-21 fibroblasts (which do not contain detectable brain 4 mRNA) with several different proglucagon promoter-luciferase fusion genes (Fig. 3). As expected, the basal levels of transcriptional activity of the proglucagon-luciferase reporter plasmids were low in BHK-21 cells; cotransfection of proglucagon-luciferase and rat brain 4 expression plasmids resulted in a 15- to 80-fold activation of the proglucagon promoter (Fig. 3A). The brain 4-dependent activation of the proglucagon promoter was observed in serially deleted fusion genes containing the deletion of bp −1031 to −93 of the rat proglucagon gene 5′-flanking sequences. Deletion of proglucagon gene sequences from bp −1031 to −361 revealed no change in brain 4 activation of the proglucagon gene. Further reduction of promoter segments (bp −361 to −93) led to enhanced capability of brain 4 to transactivate the respective proglucagon reporter constructs. Deletion from bp −93 to −60 led to a dramatic reduction in proglucagon gene expression, indicating that the promoter sequences from bp −93 to −60 of the rat proglucagon promoter which contain the G1 element also contain target sequences for brain 4 binding and transactivation (Fig. 3A). To assess the promoter specificity for activation by brain 4, we transfected brain 4 with a luciferase plasmid containing the proximal 410 bp of the insulin pro-

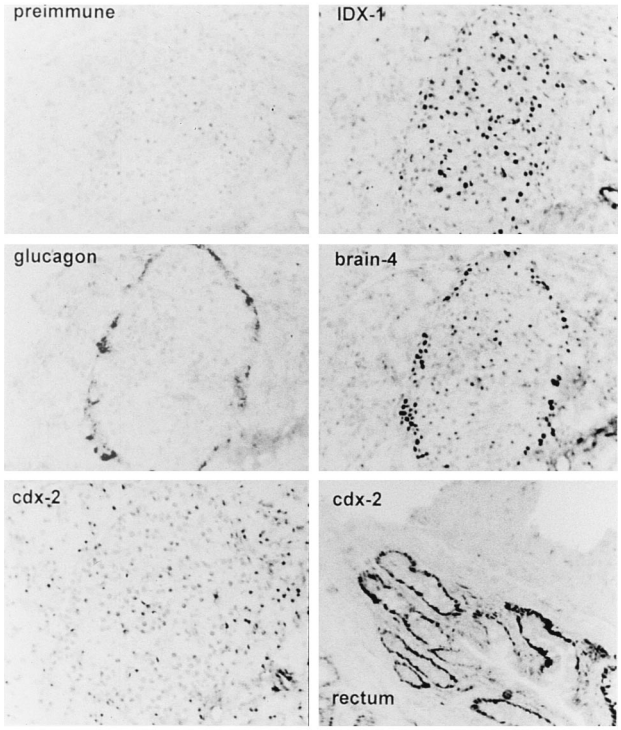

FIG. 2. Immunohistochemistry of adult rat tissues. Serial sections (5 μm) of frozen pancreas from an adult male Wistar rat were fixed in 4% paraformaldehyde in PBS, permeabilized with 100% methanol, and blocked with normal donkey serum. Sections were then incubated with preimmune antiserum and brain 4, cdx-2, and glucagon–GLP-1 antisera overnight at 4°C. Binding of primary antibody was detected with secondary antibodies with donkey anti-rabbit Cy3 DTAF. Images were obtained with an epifluorescence microscope equipped with a CCD camera, interfaced to IP Lab spectrum software. cdx-2 staining of rat rectal tissue (treated simultaneously and identically as the pancreatic tissue) was the positive control for cdx-2. The nuclear immunoreactivity of brain 4 colocalized in the cells staining for glucagon in the periphery of the rat pancreatic islet. Few cells with variable nuclear cdx-2 immunoreactivities were scattered throughout the islet.

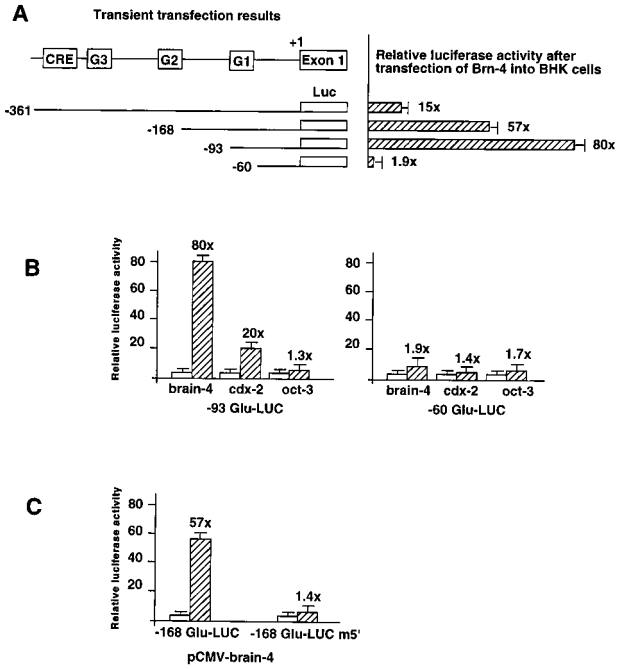

FIG. 3. brain 4 (Brn-4) activates the proglucagon promoter in BHK-21 fibroblasts. (A) A series of 5′ deletion constructs of the proglucagon-luciferase fusion genes encompassing the control elements cyclic AMP response element (CRE), G3, G2, and G1 (5 μg each) were cotransfected into BHK-21 cells with 1.5 μg of expression vector pCMV–brain-4. Luciferase (Luc) activity was measured 48 h after transfection. (B) Relative luciferase activity in response to cotransfection with expression vectors for brain 4, cdx-2, and oct-3 and the −93 Glu-LUC reporter containing the G1 element and luciferase activity from the same experiment performed with the gene for the −60 Glu-LUC reporter lacking the control element. (C) Relative luciferase activity in response to cotransfection with 1.5 μg of expression vector for brain 4 and either wild-type −168 Glu-LUC or −168 Glu-LUC with a mutated 5′ AT-rich site of the G1 promoter element (Glu-LUC m5′) corresponding to the mutation −93/−60m5′ in Table 2. Open bars, control cotransfections with empty expression vector. The data are means ± SE normalized to activity following transfection of an empty CMV expression vector in the same experiment and normalized to the protein content of the cell extract.

moter. In contrast to brain 4-dependent activation of the proglucagon promoter sequences, brain 4 did not activate the insulin promoter (data not shown).

To determine whether additional POU-domain transcription factors were also capable of activating the bp −93 proglucagon promoter, we cotransfected BHK-21 fibroblasts with the 3 bp −93 Glu-LUC plasmid and CMV–Oct-3. In contrast to brain 4, no activation of the proglucagon promoter was detected following cotransfection with the POU domain factor oct-3 (Fig. 3B). In support of our findings, pit-1, another POU domain transcription factor present in pituitary endocrine cells, has been shown previously to be incapable of transactivating the glucagon gene in BHK-21 fibroblasts (15).

To compare the effects of cdx-2 and brain 4 on proglucagon gene expression, we cotransfected BHK-21 cells with the bp −93 proglucagon promoter-luciferase reporter construct together with CMV-driven expression vectors of cdx-2 and brain 4. brain 4 consistently showed a four- to fivefold stronger transactivating capability compared to that of cdx-2 (Fig. 3B).

To determine whether brain 4 also transactivates the glucagon gene in pancreatic endocrine cells, αTC-1 cells were cotransfected with the CMV-driven brain 4 expression plasmid and reporter constructs with unaltered and mutated brain 4 binding sites. As expected, the promoter-luciferase construct −168 Glu-LUC was activated in the glucagon producing αTC-1 cells. Overexpression of brain 4 enhanced expression of the luciferase reporter. Mutation of the brain 4 binding site leads to a greatly reduced expression of the luciferase reporter which cannot be stimulated by overexpressing brain 4 (Fig. 4). Taken together, these results indicate that brain 4 is capable of stimulating expression of the glucagon gene in pancreatic endocrine cells.

brain-4 activation of the proglucagon promoter correlates with binding to proximal G1 promoter sequences from bp −93 to −60. To identify the sequences within G1 that are important for brain 4 activation of the glucagon gene promoter, we prepared synthetic oligonucleotides that spanned the bp −93 to −60 region of the glucagon promoter that includes the G1 element and used these sequences as probes in EMSA experiments. Several specific DNA-protein complexes were generated with these probes and αTC-1 nuclear extracts. To determine whether brain 4 is one of the proteins present in the complexes, we carried out DNA-binding (EMSA) studies in the presence of either preimmune serum or brain 4-specific antiserum (Fig. 5A). Since cdx-2 has been shown to bind to the G1 element of the glucagon promoter, we also conducted EMSA with cdx-2 antiserum in order to assess potential differences in the binding sites for brain 4 and cdx-2.

The bp −93 to −60 portion of the G1 element of the proglucagon promoter contains two AT-rich sequence motifs that are putative binding sites for homeodomain-containing DNA-binding proteins. To identify the binding site of brain 4 within this element, oligonucleotides harboring mutations of either the 5′ or the 3′ localized AT-rich sites were used for EMSA combined with interference assays with specific antisera. The results of the experiments indicate that brain 4 present in αTC-1 nuclear extracts preferentially binds to the 5′ AT-rich site within bp −93 to −60. The brain 4 DNA-containing complexes migrate as a doublet; these were designated complexes A and B. Complex A was completely disrupted with brain 4 antiserum, whereas the intensity of complex B was attenuated, indicating that some brain 4 is also associated in complex B. When both brain 4 and cdx-2 antisera were added to the incubation, complex B was further attenuated but not completely abolished (14a), indicating that cdx-2 is capable of binding the 5′ AT-rich site of the G1 element in vitro but that an additional, still unknown protein(s) also appears to participate in this complex. The presence of cdx-2 in complex B is also

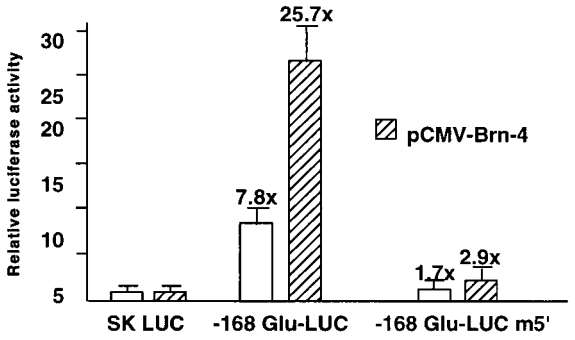

FIG. 4. brain 4 (Brn-4) activates the proglucagon promoter in αTC-1 pancreatic endocrine cells. Relative luciferase activity in response to cotransfection with 1.5 μg of expression vector for brain 4 and 5 μg of either wild-type −168 Glu-LUC or −168 Glu-LUC with a mutated 5′ AT-rich site at the G1 promoter element (Glu-LUC m5′) corresponding to the mutation −93/−60m5′ in Table 2. Open bars, control cotransfections with empty expression vector. Data are means ± SE normalized to cotransfections with the luciferase vector without any promoter sequences (SK Luc) and to the protein content of the cell extract.

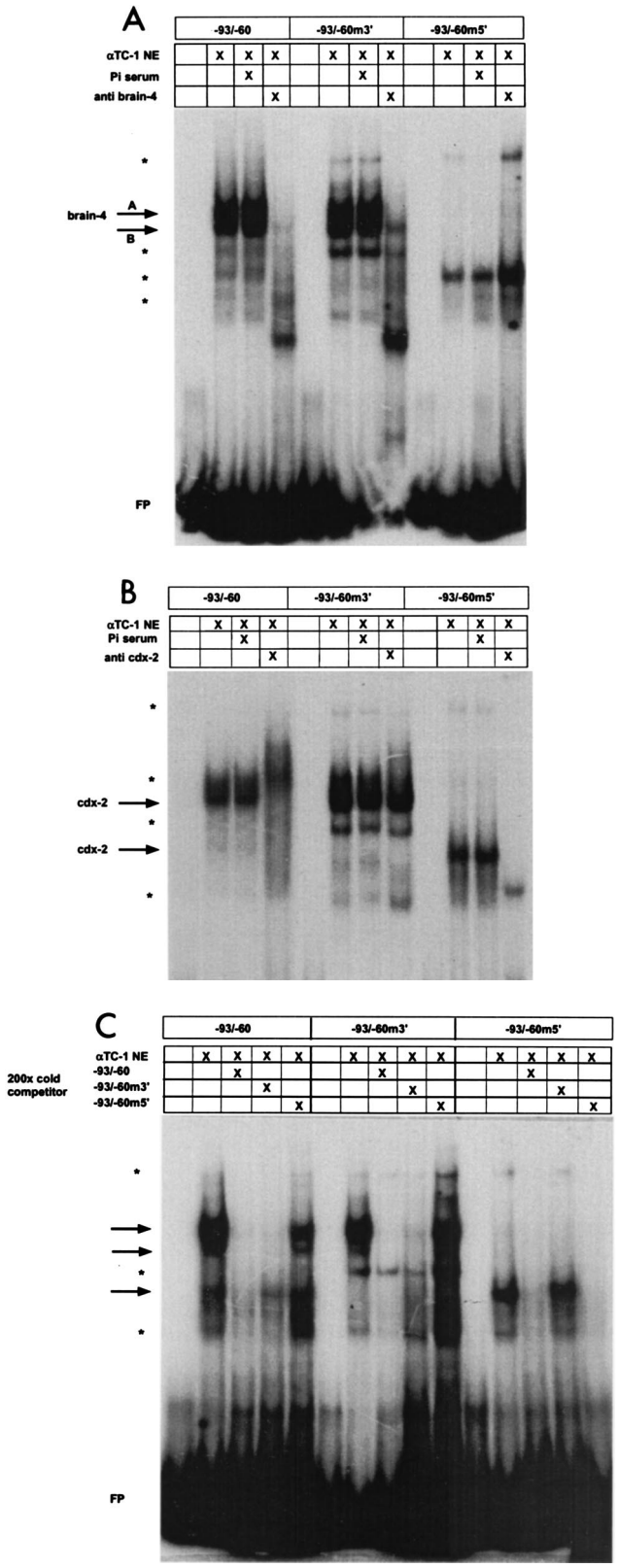

FIG. 5. (A) EMSA with islet αTC-1 nuclear extracts to identify G1 binding proteins. Nuclear extracts (10 μg each) were incubated with the G1 probes listed in Table 2 either alone or with 1 μl of preimmune serum (Pi) or 1 μl of brain 4 antibody (diluted 1:5), following which DNA-protein complexes were resolved by PAGE and autoradiography. brain 4 antiserum identifies the specific complexes by interference of added antibody (arrows). brain 4 preferentially binds to the 5′ AT-rich site of the oligonucleotide spanning from bp −93 to −60 bp (−93/−60) of the G1 element of the proglucagon promoter, as documented by abolished binding of brain 4 subsequent to mutation of the respective site (probe −93/−60m5′). (B) The experiment described for panel A used to detect cdx-2 binding. cdx-2 preferentially binds to the 3′ AT-rich site of the −93/−60 oligonucleotide spanning the G1 element of the proglucagon promoter. The major lower cdx-2 complex is missing in the 3′ mutated probe (−93/−60m3′). Also, there is an upper cdx-2 complex that comigrates with the lower of the two brain 4 complexes (complex B [A]). (C) EMSA with islet αTC-1 nuclear extracts to identify G1 binding proteins by using wild-type and mutated oligonucleotide probes; cross-competition of wild-type (−93/−60) and 3′ mutated (−93/−60m3′) and 5′ mutated (−93/−60m5′) AT-rich sites. Nuclear extracts (10 μg each) were incubated with the G1 probes listed in Table 2 either alone or in the presence of a 200-fold molar excess of unlabeled wild-type or mutated oligonucleotides, following which DNA-protein complexes were resolved by PAGE and autoradiography. Cross-competition of wild-type and 3′ mutated probes is possible by a 200-fold excess of wild-type and 3′ mutated probes but not of the 5′ mutated probe. The 5′ mutated probe is competed by a 200-fold excess of wild-type and 5′ mutated probes but not of the 3′ mutated probe. FP, migration position of unbound probe; NE, nuclear extracts; asterisks, nonspecific protein-DNA complexes.

indicated by the EMSA in Fig. 5B, lane 4. Mutation of the 3′ AT-rich site, which is preferentially bound by cdx-2, abolishes detectability of any cdx-2 bound to the 5′ AT-rich site. These results are in accord with the observations of Laser et al. that cdx-2 preferentially binds to the 3′ AT-rich site of the G1 promoter element of the glucagon gene and that binding of cdx-2 to the 5′ AT-rich site is possible when the 3′ AT-rich site is intact (19).

Cross-competition experiments with wild-type and mutated oligonucleotides confirm the specificities of the protein-DNA complexes matching the presumed binding sites of brain 4 and cdx-2 (Fig. 5C). The binding of brain 4 to the 5′ AT-rich site was further confirmed by EMSA with an oligonucleotide probe spanning bp −94 to −74 (Table 2) of the G1 element (Fig. 6A).

To determine specificities and exclude nonspecific effects of the antisera, EMSA for brain 4 were performed with element −94/−74 and EMSA for cdx-2 binding were performed with element SIF1, which has previously been shown to bind cdx-2 (45) (Table 2). The results exclude nonspecific effects and cross-reactivities of the antisera between brain 4 and cdx-2 (Fig. 6B).

Transactivation of proglucagon gene by brain 4 is abolished by mutating its binding site in the G1 element. To test the functional significance of the brain 4 binding site in the G1 element of the proglucagon gene, we inserted sequences containing the 5′ mutations described above (also Table 2) in the G1 element of a Glu-LUC reporter plasmid at bp −168. Transient transfection of BHK-21 and αTC-1 cells with the 5′ mutated AT-rich site abolished transactivation of the reporter construct by the cotransfected CMV-brain 4 expression plasmid. Thus, the 5′ AT-rich site is critical for transactivation of the proglucagon gene by brain 4 (Fig. 3C and 4).

DISCUSSION

The proximal promoter element G1 (bp −100 to −52) of the proglucagon gene is sufficient for cell-specific expression of the proglucagon gene in α cells (27, 33). Underscoring the importance of this element for proglucagon gene transcription is the fact that the rat and human G1 sequences are identical (27, 33). The data presented here provide identification of a specific transcription factor that mediates G1-dependent activation of proglucagon gene transcription in islet cell lines. Furthermore, the presence of brain 4 immunoreactivity in predominantly glucagon-producing cell lines and an intestinal

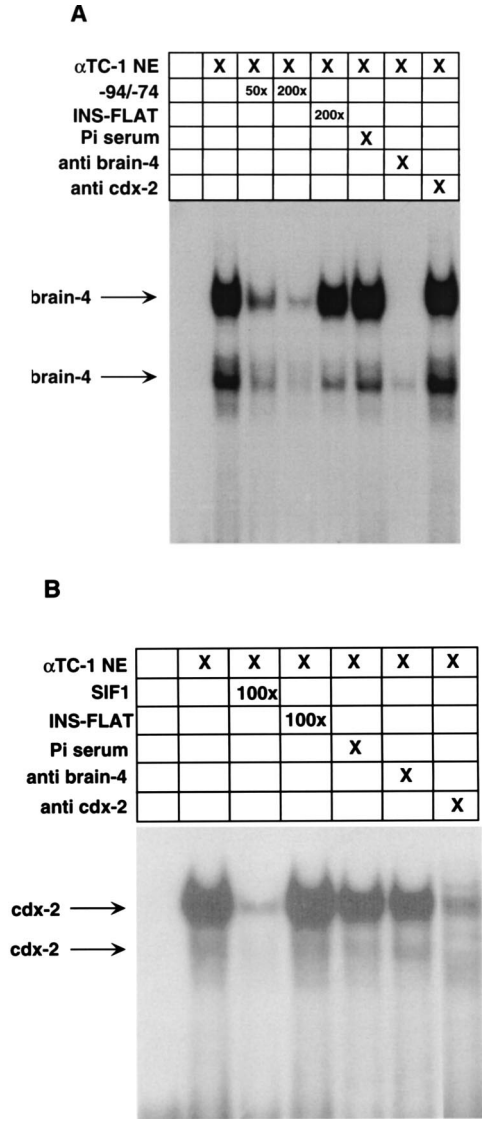

FIG. 6. EMSA with islet αTC-1 nuclear extracts (NE) and probes −94/−74 (spanning bp −94 to −74) missing the 3′ AT-rich site of the glucagon gene G1 promoter element (A) and probe SIF1 (B) (see Table 2). Nuclear extracts (10 μg each) were incubated with the respective probes either alone, in the presence of unlabeled specific and nonspecific (INS FLAT) DNA competitor, or in the presence of 1 μl of preimmune (Pi) serum or 1 μl of brain 4 (diluted 1:5) or cdx-2 (diluted 1:5) antibody, following which DNA-protein complexes were resolved by PAGE and autoradiography. Panel B shows the two expected cdx-2 complexes (46) and is a high-exposure scan of an EMSA autoradiography. Antibodies to brain 4 and cdx-2 disrupt DNA binding of the respective DNA-protein complexes and do not cross-react with each other.

cell line (Glu-Tag) and previous reports that brain 4 is expressed in the brain (13, 20, 24) are consistent with the known distribution of proglucagon-producing cell types. The present finding that a neuronal transcription factor is found in high abundance in pancreatic endocrine tissue is in accord with previous findings made with other transcription factors, i.e., Isl-1 (49), PAX4 and -6 (42, 43, 46) and Beta2/neuroD (28), and is consistent with the observation that pancreatic endocrine cells possess neuronal properties (1).

Based on the amino acid sequence similarities within POU domains, brain 4 is designated a class III POU factor (50). Other members of this class are brain 1, brain 2, and SCIP (Tst-1/oct-6). The mouse and rat brain 4 POU domain sequences were first isolated by screening genomic libraries for class III POU domain transcription factors (13, 20, 24) and by approaches using degenerate primers in RT-PCR screens (13, 20, 24). The transcripts of brain 4 were identified in neuronal tissues by in situ hybridization of rat embryos (13, 20, 24) and Northern analysis (13, 20, 24). Notably, brain 4 transcripts were not detected in pancreatic tissue by these techniques. This circumstance likely reflects the difficulty in detecting low-abundance RNA transcripts present in islet cells, which contribute to only a small percentage of total pancreatic RNA, as was also the case for cdx-2 (15).

By Western blot analysis we consistently detected a doublet of closely migrating but distinct immunoreactive brain 4 (Fig. 1) that was also present in BHK-21 cells transfected with a brain 4 expression plasmid. These two apparent brain 4 isoforms may arise as a consequence of at least two possibilities: first, the presence of an additional in-frame start site of brain 4 in reported sequences (13, 20, 24) and, second, alternate migration of postranslationally modified brain 4 protein which carries a putative N-glycosylation site at Asn-121. The possible differences in the functions of these two distinct forms of brain 4 remain to be elucidated.

Although the homeodomain is sufficient for the recognition of specific DNA sequences, the POU-specific domain increases the affinity of binding to the elements (50). Recently, an extensive analysis of the binding specificities of brain 4 was conducted by random oligonucleotide site selection (23) and yielded a brain 4 DNA-binding consensus sequence, CAATA TGCTAAT, with a fixed spacing requirement of two nucleotides between putative CAATAT and TAAT half-sites (23). However, reports on the binding of brain 4 to regulatory elements of the dopamine receptor gene (30) and the present study reveal a divergence of brain 4 DNA recognition sites from the reported consensus site (Table 3). It is important to note that the brain 4 binding site determined in the present study is, with the exception of one nucleotide, identical to the site published previously (30) (Table 3). Taken together, comparison of the data obtained from analysis of the proglucagon promoter with the results of previous studies suggests that brain 4 is capable of recognizing significantly diverse DNA target sequences. The functional relevance of the nucleotide differences in brain 4 target sequences for brain 4 binding remains undetermined. In this regard, a DNA-binding protein has recently been identified that interacts with the POU transcription factor oct-2 in B lymphocytes and determines cell-specific expression of immunoglobulin genes (11, 12, 41, 44). The presence of a similar factor in α cells which additionally would determine DNA binding and specific functional properties of brain-4 may be hypothesized.

Given the relatively widespread expression of brain 4 in the central nervous system, it must be assumed that brain 4 is involved in the regulation of a variety of genes expressed there. In the central nervous system, brain 4 has been shown to regulate striatal D1a dopamine receptor gene transcription (30). The clear and strict localization of brain 4 expression in glucagon-producing α cells of the pancreas points toward a potentially important role for brain 4 in the determination of the α-cell phenotype. It is important to note that GLP-1, a product of the proglucagon gene derived by posttranslational processing, is expressed in nuclei of the hypothalamus and selected nuclei of the brain stem and is reported to regulate food intake behavior in rodents (47). Expression of brain 4 is detectable in hypothalamic nuclei, in agreement with the idea that it may be involved in hypothalamic transcriptional regulation of the proglucagon gene in addition to other functions.

Confirmation of the in vivo function of a gene requires

TABLE 3. Comparison of the DNA recognition sites of brain 4

Binding site[a]		Sequence	
Octamer motif	5′	ATGCAAAT	3′
		T	
oct-1 motif	5′	gAATATGCA	3′
Class III POU factors	5′	GCATnTAAT (n = 0, 2, or 3)	3′
brain 4 (by amplification)	5′	CAATATGCTAAT	3′
brain 4 (dopamine receptor gene promoter)	5′	AGCTCCCTGGGAATTATTAGGTTGGTGGGCT	3′
	5′	AGCTCTTGACGTGAGTAATGATGCCATT	3′
Glucagon G1	5′	CCCCATTATTTACAGATGAGAAA	3′

[a] DNA-binding consensus sequences of the octamer motif originally found to bind brain 4 (24), of the oct-1 motif (48), of the binding sites of other class III POU domain transcription factors (22), of brain 4 as determined by a site selection amplification method (23), of brain 4 binding sites on the dopamine receptor gene promoter (30), and of the brain 4 binding site determined in the present study (glucagon G1 at bp −94 to −74).

analysis of experimental animals in which the gene is nonfunctional (knockout) or of naturally occurring mutant strains of mice or human patients carrying the mutations. The knockout of the brain 4 gene in mice has not yet been reported. Mutations in or around the human brain 4 gene are associated with an X-linked mixed deafness (3). No overt metabolic disturbances in these patients have been reported. These metabolic disturbances may well be subtle and would require stimulation tests (i.e., hypoglycemia) which, to our knowledge, have never been performed with such patients. Glucagon responses to hypoglycemic episodes in individuals with mutations in the brain 4 gene may further reveal the role of brain 4 in metabolic control.

Previous reports have identified the homeodomain protein cdx-2/3 as a positive regulator for proglucagon gene expression in islet and intestinal cell lines (15, 19). In contrast to brain 4, cdx-2/3 can also be detected in rat exocrine cell lines (AR42J) which do not express glucagon, much in contrast with expression of brain 4 that is localized to endocrine pancreatic cell lines (14a). Immunocytological staining of islet-derived glucagon-producing αTC-1 cells revealed the presence of both brain 4 and cdx-2 in high abundance (data not shown). This observation is consistent with the idea that although these cells were originally derived from pancreatic endocrine cells, a certain overlap of these cells is found with proglucagon-producing intestinal cells (37). However, the intestinal Glu-Tag cell line also expresses brain 4 protein (Fig. 1). This observation may also be due to the fact that—as in αTC-1 cells—certain phenotypes of both intestinal and pancreatic endocrine tissue may overlap. STC-1, a different transformed endocrine gut cell line which expresses various pancreatic and intestinal hormones, including proglucagon (15, 19), does not express brain 4 protein (14a) but expresses cdx-2/3 (15, 19). This clearly indicates that results derived from transformed cell lines may not represent the actual in vivo situation and that such findings need to be confirmed for adult animals. It may thus be possible, according to the relative tissue distribution derived from immunohistochemistry of rat adult tissues (Fig. 4), that brain 4 is the predominant regulator of the proglucagon gene in the central nervous system and pancreatic α cells, whereas cdx-2/3 is the predominant tissue-specific factor binding to the G1 element in proglucagon-expressing cells of the intestine.

From the present EMSA results, it may be assumed that brain 4 predominantly interacts with the 5′ AT-rich site of the G1 element of the proglucagon promoter spanning bp −93 to −60, while cdx-2/3 predominantly binds to the 3′ AT-rich site. Complex A of the doublet formed in the EMSA is clearly disrupted by brain 4 antiserum (Fig. 5A, lane 4). The intensity of complex B is also attenuated by brain 4 antiserum. The signal in band B can further be reduced by the addition of cdx-2 antiserum (14a), indicating that cdx-2 may participate in complex B but that one or more additional proteins may also participate in the formation of complex B. A nonspecific effect of the brain 4 antiserum cross-reacting with cdx-2 can largely be excluded as a cause for the attenuation of complex B in the EMSA, since the same antiserum does not interfere with the cdx-2–sucrose–isomaltase promoter element complex (Fig. 6B). cdx-2 antiserum also recognizes cdx-2 in complex B in the wild-type −93/−60 G1 element but not in the 3′ mutated element. Thus, it appears that cdx-2 can bind to the 5′ AT-rich site of the G1 element as detectable in EMSA mainly in the presence of an intact 3′ AT-rich site. This is in accord with previous observations of Laser et al. that cdx-2 preferentially binds to the 3′ AT-rich site but can bind to the upstream AT-rich site when the downstream AT-rich site is intact (19). The integrity of complex A also seems to be somewhat altered by the cdx-2 antiserum. This is probably not due to a cross-reactivity of the antiserum to brain 4 (Fig. 6B) but suggests a possible interaction between brain 4 and cdx-2. Indeed, brain 4 and cdx-2 appear to interact indirectly in pancreatic endocrine cell nuclei (data not shown). As mentioned above, DNA-binding proteins similar to OBF-1, which directs the binding specificity of the POU transcription factor oct-2 in B lymphocytes, may be hypothesized to be involved in such interactions (11, 12, 41, 44).

The tissue distribution of cdx-2/3 reveals its presence not only in glucagon-producing α-cell lines but also in exocrine pancreas and insulin-producing pancreatic β-cell lines, as well as in the intestinal epithelium. However, given the fact that pancreatic endocrine cells are of endodermal origin but possess neuronal properties, a combination of endodermal and neuronal transcriptional regulation may be the basis of the different phenotypes found in these cells. In this regard, we have consistently observed additional distinct protein-DNA complexes during our EMSA studies, suggesting that further heretofore unidentified DNA-binding proteins are involved in the regulation of proglucagon gene expression via interactions with the G1 element. Identification of these additional proteins and the signals regulating the expression of brain 4 in α cells and additional target genes of brain 4 in α cells will likely further our understanding of the molecular control of cell-specific gene expression and phenotype determinations in the endocrine- and neuron-like cells of the pancreas.

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