Differential Regulation of Islet-specific Glucose-6-phosphatase Catalytic Subunit-related Protein Gene Transcription by Pax-6 and Pdx-1*

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Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) is selectively expressed in islet β cells and is a major autoantigen in a mouse model of type I diabetes. The analysis of IGRP-chloramphenicol acetyltransferase (CAT) fusion gene expression through transient transfection of islet-derived βTC-3 cells revealed that a promoter region, located between −273 and −254, is essential for high IGRP-CAT fusion gene expression. The sequence of this promoter region does not match that for any known islet-enriched transcription factor. However, data derived from gel retardation assays, a modified ligation-mediated polymerase chain reaction in situ footprinting technique and a SDS-polyacrylamide separation/renaturation procedure led to the hypothesis that this protein might be Pax-6, a conclusion that was confirmed by gel supershift assays. Additional experiments revealed a second non-consensus Pax-6 binding site in the −306/−274 IGRP promoter region. Pax-6 binding to these elements is unusual in that it appears to require both its homeo and paired domains. Interestingly, loss of Pax-6 binding to the −273/−246 element is compensated by Pax-6 binding to the −306/−274 element and vice versa. Gel retardation assays revealed that another islet-enriched transcription factor, namely Pdx-1, binds four non-consensus elements in the IGRP promoter. However, mutation of these elements has little effect on IGRP fusion gene expression. Although chromatin immunoprecipitation assays show that both Pax-6 and Pdx-1 bind to the IGRP promoter within intact cells, in contrast to the critical role of these factors in β cell-specific insulin gene expression, IGRP gene transcription appears to require Pax-6 but not Pdx-1.

Islet-specific glucose-6-phosphatase catalytic-subunit-related protein (IGRP)§ is a putative enzyme specifically expressed in the insulin producing β cells of the Islets of Langleyrs (1–4). As its name implies, this protein is a homologue of the catalytic subunit of glucose-6-phosphatase (G6Pase), an enzyme that catalyzes the conversion of glucose 6-phosphate to glucose and orthophosphate (5–8). This activity is restricted to a limited number of tissues, principally the liver and kidney (5–8). In the liver, G6Pase is positioned at the junction of the metabolic pathways that mediate glycolysis and gluconeogenesis and thereby catalyzes the final step in hepatic glucose production. The role of G6Pase has also been studied in the context of other tissues, notably the pancreatic β cell (5–8). Interest in this cell type stems from the hypothesis that elevated G6Pase activity in certain settings, such as type II diabetes, could lead to the uncoupling of glucose-stimulated insulin secretion by reversing a required step in this process, the phosphorylation of glucose. Reports have varied, however, as to the level of and even existence of G6Pase activity/expression in islets (5–8). Interestingly, in studies that have been able to demonstrate islet G6Pase activity, different kinetic characteristics from those of the hepatic enzyme were observed, suggesting the presence of a unique islet G6Pase (1). The cloning of IGRP served to identify a candidate for this hypothetical enzyme (1, 2). Although initial studies failed to demonstrate any glucose 6-phosphate hydrolysis by IGRP (1), an independent study recently suggested that IGRP may possess low G6Pase activity (9).

Although the role of IGRP in β cell function remains unclear, a recent report identified IGRP as an autoantigen in a mouse model of Type I diabetes (10). This form of diabetes is caused by an autoimmune reaction that leads to the destruction of the pancreatic β cell (11–13). Current evidence suggests that autoreactive CD8-positive T cells play a key role in disease pathogenesis (14). In the NOD (non-obese diabetic) mouse, which spontaneously develops a disease similar to type I diabetes in humans, a substantial portion (40%) of the CD8 cells infiltrating the islet are IGRP-reactive (10). These results suggest that IGRP is a major autoantigen in this murine model. Whether these observations directly translate to humans is unknown, however, numerous similarities between disease etiology in NOD mice and Type I diabetes have been noted (15, 16).

Our work has focused on identifying the transcription factors that control IGRP gene expression (2, 3, 17, 18). Originally, this work was undertaken in an attempt to identify novel, islet-enriched transcription factors important for pancreatic development and/or function. We believe this approach is reasonable.

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The abbreviations used are: IGRP, islet-specific G6Pase catalytic subunit-related protein; G6Pase, glucose-6-phosphatase; CAT, chloramphenicol acetyltransferase; LMPCR, ligation-mediated polymerase chain reaction; GAD, glutamic acid decarboxylase; DMS, dimethyl sulfoxide; BSA, bovine serum albumin; NOD, non-obese diabetic; DTT, dithiothreitol; WT, wild type; PVDF, polyvinylidene fluoride; ChIP, chromatin immunoprecipitation.
given that similar work, focused on other islet-specific genes, has led to the identification of such proteins (19–21). With data now implicating IGRP as an autoimmuneype in Type I diabetes, the possibility exists that this work could also be useful for the design of strategies aimed at preventing immune recognition through suppression of β cell antigens. The viability of this approach has already been investigated using antisense RNA directed against glutamic acid decarboxylase (GAD), another protein identified as a type I diabetes autoantigen. In transgenic NOD mice expressing this antisense RNA, it was noted that suppression of GAD protein levels was associated with lower incidence of diabetes (22). Although controversial (23), the results of this study suggest that the manipulation of other autoantigens, such as IGRP, should be investigated as a potential means to slow or prevent β cell destruction and type I diabetes.

Previous studies investigating the molecular mechanisms that determine the islet-specific expression of the IGRP gene established the boundaries of a functional promoter (2). This sequence, which consists of 306 bp upstream of the transcription start site, is sufficient to drive expression of reporter genes specifically in β cell-derived cell lines in vitro and pancreatic islets in vivo (24). Further, the results of a 5′ deletion analysis, coupled with in situ footprinting, indicated that multiple cis-acting elements within this 306-bp region contribute to IGRP promoter activity (17). A recent publication examining the importance of two conserved E-Box motifs extended these findings by demonstrating that the transcription factors NeuroD/PDX2 and upstream stimulatory factor contribute to IGRP promoter activity (18). In this report we show that IGRP pro- moter activity in insulinoma cell lines is highly dependent on the binding of the transcription factor Pax-6 to two non-consensus binding sites. Interestingly, despite interacting directly with the IGRP promoter in situ, our data do not support a similar critical role for the transcription factor Pdx-1.

**EXPERIMENTAL PROCEDURES**

**Materials**

[α-32P]dATP (>3000 Ci mmol⁻¹) and [3H]acetic acid, sodium salt (>10 Ci mmol⁻¹) were obtained from Amersham Biosciences and ICN, respectively. Specific antisera to Pdx-1 and Pax-6 were obtained from Dr. Christopher Wright (Vanderbilt (25)) and Covance Laboratories, respectively. Rabbit IgG (sc-2027) was obtained from Santa Cruz Biotechnology, Inc.

**Fusion Gene Plasmid Construction**

The construction of a rat insulin II-chromaffin cell acetyltransferase (CAT) fusion gene, containing promoter sequence from –238 to +2 has been previously described (26) as it has a site-directed mutation of the A3 Pdx-1 binding site generated in the context of the –238 to +2 promoter region (27). The construction of mouse IGRP-CAT fusion genes, containing promoter sequence from –306 to +3, –273 to +3, and –254 to +3, has been previously described (2, 17).

The Pax-6 binding site located between –273 and –246 in the IGRP promoter was mutated by site-directed mutagenesis within the context of the –273 to +3 promoter fragment. Two constructs, designated –273 MUT 1 and –273 MUT 2 (Fig. 1), were generated using PCR and the following oligonucleotides as the 5′ primers: 5′-GGGATCCAAAGCT- (–273)TGAAGGGAATGATGTTAAAAATGCAAG-3′ (MUT 1) and 5′-GGGATCCAAAGCT–273 TGAAGGGAATGATGTTAAAAATGCAAG-3′ (MUT 2). BamHI cloning sites are underlined, and the mutated sequences are in lowercase letters. The 5′ end of the resulting IGRP promoter fragment was the same as that in the wild-type –306 IGRP-CAT fusion plasmid. The PCR products from each reaction pair were then combined and used themselves as primer and template in a second PCR reaction to generate a small amount of the full-length, mutated IGRP promoter fragment. Finally, the 5′ and 3′ PCR primers were then used to amplify this fragment.

Similarly, the Pax-6 binding site located between –306 and –274 in the IGRP promoter was targeted by site-directed mutagenesis within the context of the –306 to +3 promoter fragment. The construct, designated –306 Pax MUT B (Fig. 10), was generated, with the wild-type –306 IGRP-CAT plasmid as the template, using PCR with the same′ 5′ PCR primer as described above and the following oligonucleotide as the 5′ primer: 5′-GGGATCCAAAGCT–306 CTAGCCAAAGC-3′ (MUT 1). BamHI cloning site underlined) was designed to conserve the junction between the IGRP promoter and CAT vector to be the same as that in the wild-type –306 IGRP-CAT fusion gene plasmid. The PCR products from each reaction pair were then combined and used themselves as primer and template in a second PCR reaction to generate a small amount of the full-length, mutated IGRP promoter fragment. Finally, the 5′ and 3′ PCR primers were then used to amplify this fragment.

**Cell Culture and Transient Transfection**

Mouse pancreatic islet β cell-derived βTC-3 cells, α cell-derived αTC-6 cells, mouse adrenal chromaffin-derived Y1 cells, human cervix-derived HeLa cells, and rat liver-derived H4IIE cells were passaged as subconfluent cultures in Dulbecco’s modified Eagle’s medium. Cell cultures were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin and with either 2.5% (v/v) fetal bovine serum, 15% (v/v) horse serum (βTC-3 and αTC-6 cells), and 10% (v/v) newborn bovine serum and HeLa cells. Catania cell-derived baby bovine serum and 2.5% (v/v) fetal bovine serum (H4IIE cells). βTC-3 cells were co-transfected with 0.5 µg of an expression vector encoding SV40-firefly luciferase (Promega) and 2 µg of the indicated CAT plasmids using the LipofectAMINE reagent (Invitrogen) as previously described (17).

**CAT and Luciferase Assays**

Transfected βTC-3 cells were harvested by trypsin digestion and then solubilized in passive lysis buffer (Promega). After two cycles of freeze/thawing, firefly luciferase activity was assayed as described previously (30). The remaining βTC-3 lysate was heated for 10 min at 65 °C, and cellular debris was removed by centrifugation. CAT assays were then performed on the supernatant as previously described (2). To correct for variations in transfection efficiency, the results are expressed as the ratio of CAT/firefly luciferase activity. In addition, three independent preparations of each IGRP-CAT plasmid construct were analyzed to obtain the data shown in each figure.

**Gel Retardation Assay**

**Labeled Probes—Sense and antisense oligonucleotides representing various IGRP promoter sequences, as indicated in the figure legends, were synthesized with BamHI-compatible ends and subsequently gel-purified, annealed, and labeled with [32P]dCTP using the Klenow fragment of Escherichia coli DNA polymerase I to a specific activity of ~2.5 Ci/pmol (29).

**High Salt Nuclear Extract Preparation—High salt βTC-3 nuclear extract was prepared as described previously (31, 32), except that nuclei
were lysed by resuspension in a buffer containing 800 mM NaCl, 20 mM Hepes, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM diethiothreitol (DTT), 25% glycerol, and 1 mM phenylmethylsulfonyl fluoride. After incubation for 30 min at 4°C to ensure complete lysis, samples were centrifuged at 100,000 rpm in a Beckman TLA 100.3 rotor for 40 min at 4°C. The supernatant was dialyzed against buffer containing 100 mM KCl, 20 mM Hepes, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 20% glycerol, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration of the nuclear extracts was determined using the Bio-Rad assay and was typically ~1 μg μl⁻¹.

**Low Salt Nuclear Extract Preparation**—Low salt JTC-3, aTc-C, Y1, HeLa, and H4IE nuclear extracts were prepared as previously described (31, 32), except that the nuclear pellet was extracted with 20 mM HEPES, pH 7.9, 0.4 M ammonium sulfate, and the supernatant was used directly in gel retardation assays. The protein concentration of the nuclear extracts was determined using the Bio-Rad assay and was typically ~1 μg μl⁻¹.

**Binding Assays**—14 fmol of radiolabeled probe (~50,000 cpm) was incubated with 1.5–3 μg of the indicated nuclear extract in a final 20-μl reaction volume. For experiments using high salt nuclear extract, these reactions contained 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol (v/v), 1 mM DTT, 1 μM poly(dI-dC)-poly(dI-dC), and 100 mM KCl. In experiments using low salt nuclear extract the final concentrations of EDTA, EGTA, and glycerol were 0.1 mM, 1 mM, and 12.5%, respectively. In addition, the binding reaction contained 0.375 mM spermidine, 0.075 mM spermine, and 100 mM NaCl instead of 100 mM KCl. After incubation at room temperature for 20 min, samples were loaded onto a 6% polyacrylamide gel containing 1× TGE (25 mM Tris base, 190 mM glycine, 1 mM EDTA) and 2.5% (v/v) glycerol. Samples were electrophoresed for 1.5 h at 150 V in 1× TGE buffer before the gel was dried and exposed to Kodak X-B film with intensifying screens.

**Fractionation of Cell Proteins by SDS-PAGE, Elution, and Renaturation**—Nuclear extract proteins were boiled for 5 min in SDS-PAGE sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA, 100 mM b-mercaptoethanol) and resolved on 15% SDS-polyacrylamide gels using standard methods (29). For molecular weight standardization, samples containing high molecular weight markers (Rainbow Molecular Weight markers, Amersham Biosciences) were run in lanes adjacent to those containing experimental samples. Proteins were electrophoretically transferred from SDS-PAGE gel lanes to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) using the Bio-Rad Trans-Blot SD blotting apparatus according to the manufacturer’s instructions. Proteins were eluted from ~2-mm slices of PVDF membrane and renatured overnight in 100 μl of buffer containing 25 mM Hepes, pH 7.9, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X-100 (Surfact-Amp X-100, Pierce), and 0.1 mg/ml bovine serum albumin. Eluates were carried out in microcentrifuge tubes at 4°C with vigorous shaking. Samples were then flash-frozen on dry ice and later thawed for analysis in gel retardation experiments.

**Genomic DNA Isolation, in Situ and in Vitro Methylation, and Pipperidine Cleavage**—To analyze protein binding to the IGRP promoter, promoter methylation by DMS in vitro and in situ was compared, with differences interpreted as being indicative of trans-acting factor binding in situ. Maxam and Gilbert genomic DNA and strand cleavage by piperidine and heating was based on the Maxam and Gilbert method of DNA sequencing (33). The relative abundance of piperidine-cleaved DNA fragments, and hence the level of DNA methylation, was then assayed using the ligation-mediated polymerase chain reaction (LMPCR) footprinting technique (34).

**Hindlization and purification of βTC-3 genomic DNA and its subsequent isolation and purification were also performed exactly as previously described (17). Similarly, methylation of βTC-3 genomic DNA in situ and its subsequent isolation and purification were also performed exactly as previously described (17).** DNA was isolated from single 55-mm² cell culture dishes with a typical yield of ~200 μg per dish. After purification, DNA that had been methylated in situ or in vitro was treated using two separate methods for base displacement and strand scission as described by Maxam and Gilbert (33). In method A, DNA was resuspended in 180 μl of water and incubated with 20 μl of piperidine at 90 °C for 30 min, as previously reported (17). This method yields DNA products predominately cleaved at methylated guanine (G) residues with only a small level of cleavage at methylated adenine (A) residues. Alternatively, in method B, DNA was resuspended in 180 μl of phosphate-buffered water (10 mM sodium phosphate, pH 7) and heated at 90 °C for 15 min. 20 μl of piperidine was then added, and the incubation was continued at 90 °C for 30 min. This method yields DNA products cleaved at both methylated guanine (G) and methylated adenine (A) residues, with methylated guanine (G) residue cleavage predominating (33). In both methods, samples were then lyophilized to remove the piperidine. The DNA was resuspended in 100 μl of water and lyophilized another two times. The DNA was then resuspended in 100 μl of water, centrifuged briefly to pellet any debris, and ethanol-precipitated. Finally, the DNA was resuspended in 10 μl Tris-HCl (pH 8.0) and 1 μl EDTA to a final concentration of 1 μg μl⁻¹.

**Ligation-mediated PCR**—The ligation-mediated PCR (LMPCR) technique was used to analyze protein DNA interactions over the sense strand of the −273 to −246 region of the IGRP promoter in intact βTC-3 cells by comparing the level of G and A residue methylation over this region in situ and in vitro (34). LMPCR was performed exactly as previously described (17). Briefly, three nested oligonucleotides ( Primer Set A (17)) were used for each step of the LMPCR method, namely the first strand synthesis reaction, the amplification reaction, and the labeling reaction (17). The reaction products were separated by electrophoresis on 6% polyacrylamide/urea/TBE gels (29) and visualized by autoradiography.

**Chromatin Immunoprecipitation Assays**—Chromatin immunoprecipitation (ChiP) assays were performed exactly as previously described (18). Fragmented chromatin was immunoprecipitated using either Pax-6 antisera (Covance), Pdx-1 anti-serum (a generous gift from Dr. Chris Wright (25)), or rabbit IgG (sc-2027, Santa Cruz Biotechnology). Immunoprecipitated DNA was purified as previously described (18) and dissolved in nuclease free water (100 μl), and a sample (10 μl) was then used in a PCR reaction. PCR primers were designed to amplify the IGRP promoter (5′ – 414) GTTCCCTGGAACAGCACGAGATCG (~383) 3′ and 5′ – 119) CTCATCTGCTGTAGATTTAATGGGTTGAAAGAG (~154) 3′) and PEPCK promoter (5′ – 434) GAGTGACACCTCACAGCTG~GTGTTTG (~406) 3′ and 5′ – 98) GCCAGCGCTTGTGATCATG (~120) 3′). The IGRP and PEPCK promoters were amplified using the Qiagen Master Mix and the following reaction conditions: 95°C denaturation, 55°C annealing, and 72°C extension for 28 cycles. 6 μl of a 1:120 dilution of [α-32P]dATP (~3000 Ci mmol⁻¹) was included in the PCR reaction. The products were visualized by electrophoresis on 5% polyacrylamide gels containing 1× TGE (25 mM Tris base, 190 mM glycine, 1 mM EDTA). Samples were electrophoresed for 1.5 h at 150 V in 1× TGE buffer before the gel was dried and exposed to Kodak X-B film with intensifying screens. A Packard Instant Imager was used to count [32P] associated with the amplified promoter fragments.

**RESULTS**—The −273−254 IGRP Promoter Region Is Required for Maximal IGRP Fusion Gene Expression—In a previous study it was shown that the progressive 5′ deletion of IGRP promoter sequence, starting at −306, leads to a stepwise drop in promoter activity as measured by the analysis of IGRP-CAT fusion gene expression (17). The most dramatic reduction in activity occurred when the sequence located between −273 and −254 was removed, an observation that pointed to this element as being particularly important for maintaining transcription of the IGRP gene (17). This region is highly conserved between the mouse, rat, and human IGRP promoters (3). Promoter-scanning software (35) identified a number of binding sites for previously characterized transcription factors in the −273−254 promoter region, including binding sites for STAT (signal transducer and activator of transcription) and NFAT (nuclear factor activated in T cells). These candidates, however, were
Two mutations within the −273 to −254 IGRP promoter region reduce basal fusion gene expression. βTC-3 cells were transiently co-transfected, as described under “Experimental Procedures,” using a LipofectAMINE solution containing various IGRP-CAT fusion genes (2 μg) and an expression vector encoding firefly luciferase (0.5 μg). The IGRP-CAT fusion genes incorporated either the wild-type promoter sequence, located between −273 and −3 (−273 WT) or between −254 and +3 (−254 WT), or contained site-directed mutations of two promoter regions that are conserved between the mouse, rat, and human IGRP promoters (3). These mutations, designated −273 WT 1 and −273 WT 2, were generated within the context of the −273 to +3 promoter fragment. Following transfection, βTC-3 cells were incubated for 18–20 h in serum-containing medium. Cells were then harvested, and CAT and firefly luciferase activity were assayed as previously described (2, 30). Results are presented as the ratio of CAT:luciferase activity expressed as a percentage relative to the value obtained with the −273 WT fusion gene and represent the mean of three experiments ± S.E., each using an independent preparation of all fusion gene plasmids.

To define the boundaries of the putative novel element present in the −273/−254 promoter region, the functional effect of introducing two block mutations in the −273/−254 region of the IGRP promoter on fusion gene expression was investigated (Fig. 1). These mutations, designated −273 MUT 1 and −273 MUT 2, were generated within the context of the −273 to +3 promoter region, and the level of reporter gene expression directed by fusion genes containing these mutations was then analyzed by transient transfection of βTC-3 cells (Fig. 1). For comparison, the CAT expression directed by the wild-type −273 IGRP-CAT and −254 IGRP-CAT fusion genes was also determined. The results of this analysis reveal that both block mutations lead to a large reduction in IGRP promoter activity (Fig. 1). Importantly, the level of basal CAT expression directed by these mutants is equivalent to that seen when the −273/−254 region was completely deleted, as is the case with the −254 5′ deletion end-point construct (Fig. 1). This result suggests that sequences encompassed by both block mutations are required for the activity conferred by the −273/−254 promoter region. Almost identical results were obtained when these fusion genes were analyzed by transient transfection of hamster insulinoma tumor (HIT) cells (data not shown).

A modified LMPCR in Situ Footprinting Method Reveals Broad Protein Binding over the Endogenous −273/−246 IGRP Promoter Region in βTC-3 Cells—To further define the boundaries of the putative novel element present in the −273/−254 promoter sequence, protein binding to this IGRP promoter region was analyzed using in situ footprinting. IGRP promoter methylation by dimethyl sulfate (DMS) in vitro and in βTC-3 cells in situ was compared, with differences interpreted as being indicative of trans-acting factor binding in situ. DMS methylates DNA on adenine (A) and guanine (G) residues and freely permeates cell membranes (36). The binding of a trans-acting factor to a gene promoter can result in either an increase or decrease in the level of methylation (34, 36). A decrease in the level of methylation is interpreted to occur as a result of steric hindrance, whereas an increase is interpreted to occur if the trans-acting factor causes a change in DNA conformation leading to greater reactivity of a base with DMS (36, 37).

In vitro and in situ methylated genomic DNA was cleaved either using piperidine under conditions that yielded DNA products predominately cleaved at guanine (G) residues with only weak cleavage of adenine (A) residues (33), as previously described (17). Alternatively, methylated genomic DNA was cleaved using piperidine under conditions that yielded DNA products cleaved at both G and A residues (33). The relative abundance of these piperidine-cleaved DNA fragments, and hence the level of DNA methylation, was then assessed using the LMPCR footprinting technique (17, 34). Differences in the frequency of methylation at a given G residue in situ result in the over or under representation of that piperidine-cleaved fragment in the subsequent PCR amplification in comparison to in vitro methylated DNA (Fig. 2) (17, 34).

Fig. 2 shows the in situ footprinting analysis of trans-acting factor binding to the sense strand of the −277/−245 IGRP promoter region. The positions of specific G and A residues in the IGRP promoter sequence are indicated, as are increases or decreases in the level of IGRP promoter methylation comparing in vitro (lane 1) and in situ (lanes 2–4) methylated βTC-3 genomic DNA. Only changes that were consistent between duplicate experiments are indicated. When methylated genomic DNA was cleaved using piperidine under conditions that yielded DNA products predominately cleaved at G residues, with only weak cleavage of A residues, hypomethylation of the guanine residues at −269, −268, and −257 and hypermethylation of the guanine residues at −267 and −262 was detected (Fig. 2; compare lanes 1 and 4), as previously described (17). In contrast, when methylated genomic DNA was cleaved using piperidine under conditions that yielded DNA products cleaved at both G and A residues, an additional hypermethylation of the adenine residues at −255 was detected (Fig. 2; compare lanes 1 and 2). None of these differences in methylation were influenced by the presence or absence of serum in the culture medium (Fig. 2; compare lanes 2 and 3). The results of this in situ footprinting analysis suggest either that multiple factors bind the −273/−254 IGRP promoter re-
tion or that a single factor makes broad contacts across this entire promoter region.

**Specific Protein Binding to the ~273/~254 IGRP Promoter Region in Vitro**—The fusion gene analysis (Fig. 1) indicated that one or more transcription factors required for expression of the IGRP gene interacts with the ~273/~254 region of the IGRP promoter and that two block mutations in this region (Fig. 1) disrupt binding of this factor or factors. To identify the factor(s) binding this element, a long oligonucleotide that includes sequence 3 of the ~254 5' deletion end point was used as the labeled probe in a gel retardation assay. When this labeled oligonucleotide, representing the wild-type (WT) IGRP promoter sequence from ~273 to ~246, was incubated with nuclear extract prepared from βTC-3 cells, four protein-DNA complexes were detected (Fig. 3A). Competition experiments, in which a 100-fold molar excess of unlabeled DNA was included with the labeled probe, were used to correlate protein binding with basal IGRP gene expression. The wild type (WT) ~273/~246 oligonucleotide competed effectively for the formation of three of these protein-DNA complexes, designated 1, 2, and 3 (Fig. 3A), indicating that complex 4 must represent a nonspecific protein-DNA interaction. By contrast, oligonucleotides, designated ~273/~246 MUT 1 and ~273/~246 MUT 2, that contain mutations identical to those described in the ~273 MUT 1 and MUT 2 constructs, respectively (Fig. 1), failed to compete with the labeled probe for formation of two complexes, designated 3 and 4 (Fig. 3A). This indicates that complex 3 (Fig. 3A; see arrow) represents a specific protein-DNA interaction and that its formation correlates with basal gene expression conferred by the ~273/~254 IGRP promoter region. In contrast, complexes 1 and 2 must either represent nonspecific protein-DNA interactions or protein binding to sequences outside the regions spanning the MUT 1 and MUT 2 mutations. A comparison of protein binding to the ~273/~246 WT oligonucleotide using nuclear extracts isolated from other cell lines suggests that the factor in complex 3 is restricted in its expression (Fig. 3B). Thus, of the cell lines analyzed, complex 3 was only detected using βTC-3 and αTC-6 nuclear extracts (Fig. 3B).

To further characterize the protein(s) present in complex 3, nuclear extracts were subjected to a fractionation/renaturation protocol, a technique that allows for the determination of the molecular weight of transcription factors that retain their DNA binding activity in this assay. βTC-3 extract was fractionated by SDS-polyacrylamide gel electrophoresis, and proteins were transferred to a PVDF membrane. Eluates from slices of this membrane were then subjected to a gel retardation assay using the ~273/~246 WT oligonucleotide probe. Fig. 3C shows the results of the fractionation/renaturation procedure and includes a lane with crude nuclear extract (NE) so that the migration of fractionated binding activities can be compared with the complex of interest (see arrow). This gel retardation assay indicates that the eluate from slice D contains a binding activity that co-migrates with the complex of interest observed in crude extracts (Fig. 3C). From comparison with molecular weight standards it was concluded that proteins eluting from this slice were ~45 kDa in size. The reconstitution of this binding activity from a single membrane slice also suggested that the complex may contain a single protein species. Transcription factors that bind to DNA as heterodimers, in contrast, may be separated from their partners during fractionation depending on their respective molecular weights.

**Pax-6 Stimulates IGRP Fusion Gene Expression through the ~273/~246 Promoter Region**—Although computer analyses do not identify the ~273/~246 promoter region as a Pax-6 binding site, the ability to recognize a broad DNA element (consistent with the *in situ* footprinting analysis (Fig. 2)), the expression in both βTC-3 and αTC-6 nuclear extracts (Fig. 3B), and the molecular mass of ~45 kDa (Fig. 3C) are all characteristics of Pax-6 (38, 39). Therefore, a Pax-6 antiserum was tested for its
ability to alter formation of the specific −273/−246 complex in gel retardation assays. Fig. 4 demonstrates that preincubating βTC-3 nuclear extract with antibodies raised against this protein results in the disappearance of the specific complex and the appearance of a lower mobility, or supershifted complex. As a control, antibodies raised to various other transcription factors known to be enriched in pancreatic islets were tested for their ability to alter the abundance or migration of the −273/−246 complex in gel retardation assays, but they were without effect (data not shown). This result suggests that the −273/−246 complex contained the transcription factor Pax-6. Pax-6 is able to bind DNA as a monomer (38), an observation that is consistent with the ability to retain DNA binding activity after fractionation/renaturation (Fig. 3C).

To confirm this conclusion, competition experiments were performed using Pax-6 binding sites from promoters of other genes expressed in pancreatic islets (Fig. 5A). Pax-6 has been shown to interact with the C2 element of the rat insulin I promoter, and various lines of evidence indicate that this factor is required for the expression of the insulin gene (39). In addition to the regulation of β cell-specific genes such as insulin, Pax-6 is also expressed in the α cells of the pancreatic islet and has been shown to activate the glucagon promoter through two elements designated G1 and G3 (39, 40). Consistent with the results of the supershift analysis (Fig. 4), when unlabelled oligonucleotides representing these three Pax-6 sites were included in gel retardation reactions with the −273/−246-labeled probe, they competed effectively for the formation of the Pax-6 protein-DNA complex in a concentration-dependent manner. Representative autoradiographs are shown in Fig. 5B, whereas Fig. 5C shows quantified data from multiple experiments. These comparisons indicate that the C2, G1, and G3 elements all compete more effectively for Pax-6 binding than the IGRP element (Fig. 5B) and that Pax-6 binds to the G1 element with ~10-fold higher affinity than the IGRP element (Fig. 5C).

The results of these competition studies demonstrate that the affinity of Pax-6 for the IGRP −273/−246 region is relatively weak compared with other characterized binding sites. However, given that the promoter-scanning software did not identify this site even at low stringency, the question arises as to how this protein can bind to the IGRP element at all, albeit with low affinity. Pax-6 has two separate DNA binding domains, a paired-type domain and a homeodomain (41). Most studies, including the determination of the consensus Pax-6 binding site, have focused on the paired domain (38). The −273/−246 IGRP sequence bears a very weak resemblance to a consensus Pax-6 paired domain binding site (Fig. 5A), and thus is not recognized by the promoter-scanning software. It has been observed, however, that the homeodomain can bind to sites opposed to paired domain binding sites and can increase the affinity of Pax-6 for DNA in a cooperative manner (41).

Interestingly, there is an A/T-rich sequence in the −273/−246 region of the IGRP promoter, which is similar to the consensus TAAT recognized by many homeodomains (Fig. 5A). It is possible that, although the −273/−246 IGRP promoter region contains neither a strong Pax-6 paired domain binding site nor a strong homeodomain binding site, such that neither of these sequences by themselves would be predicted to bind the two respective Pax-6 DNA binding domains, these weak binding sites could cooperate to mediate an interaction. Consistent with this idea, a truncated −273/−250 IGRP oligonucleotide probe, in which the 3′ A/T-rich sequence has been removed, no longer binds Pax-6 (data not shown). Collectively, the results of these experiments suggest that, although Pax-6 binds to the −273/−246 region relatively weakly, this interaction is critical for maintaining maximum IGRP promoter activity.

**Pdx-1 Binds to Four Sites in the IGRP Promoter**—The studies described above were driven by the initial observation that deletion of the −273/−254 IGRP promoter region resulted in a large drop in IGRP promoter activity. As an alternative approach aimed at identifying transcription factors relevant to IGRP expression, candidates were sought that had been well studied in the context of β cell gene expression. The homeodomain transcription factor Pdx-1 fulfills this criterion based on the numerous reports investigating its role in the expression of β cell-specific or enriched proteins such as insulin, GLUT2, IAPP, and even Pdx-1 itself (42). The function of this transcription factor has been most closely examined in the context of studies investigating the cell type-specific activity of the insulin promoter. Although not sufficient by itself to mediate transcriptional activation, Pdx-1 is critical for insulin promoter activity and most likely functions in a higher order complex containing other islet-enriched factors (43).

To determine if Pdx-1 interacts with the IGRP promoter, potential binding sites were first identified. Based on previously studied Pdx-1 binding sites, it appears that this transcription factor recognizes a core TAAT motif, which as mentioned above, is recognized by many different homeodomain proteins (44). A search of the IGRP promoter identified three such motifs, all of which are conserved across human, mouse, and rat species. The flanking sequences surrounding these TAAT motifs do not match those of consensus Pdx-1 binding sites, so promoter-scanning software (35) did not predict that these motifs would bind Pdx-1. Nevertheless, the potential of these sites to interact with Pdx-1 was examined using oligonucleotide probes representing all three sites in gel retardation experiments. These probes were designated Site 1, Site 3, and Site 4 and are depicted in Table I along with another probe labeled Site 2, which is discussed below. When the labeled Site 1 oligonucleotide was incubated with nuclear extract prepared from βTC-3 cells a single, major protein-DNA complex was formed (Fig. 6A). As described above, the specificity of this protein-DNA interaction was investigated by including various cold competitors in the gel retardation assay. Fig. 6A shows that the wild-type (WT) unlabeled Site 1 oligonucleotide competed for formation of the major protein-DNA complex, whereas an unrelated (UNR) oligonucleotide was completely ineffective, demonstrating the specificity of this interaction. Further, an oligonucleotide containing a block mutation in the Site 1 TAAT motif (Table I) competed relatively weakly, sug-
FIG. 5. Relative affinity of Pax-6 binding sites in the insulin, glucagon, and IGRP promoters. A, Pax-6 binding sites in the insulin, glucagon, and IGRP promoters and the consensus Pax-6 binding site are shown. This consensus was taken from Ref. 38. Increases (G) or decreases (E) in DMS methylation between in situ versus in vitro methylated mouse βTC-3 cell DNA over the 273 to 244 IGRP promoter region are shown; this information was derived from Fig. 3. B, prior to the addition of high salt βTC-3 cell nuclear extract, the labeled oligonucleotide representing the wild-type 273/246 IGRP promoter sequence was incubated in the absence (−) or presence of the indicated molar excess of the unlabeled competitor (comp.) DNAs shown, representing the wild-type 270/246 IGRP sequence, the insulin C2 element, and the glucagon G1 and G3 elements. The IGRP 273/246 and 270/246 oligonucleotides bind Pax-6 with the same affinities (data not shown). Protein binding was then analyzed as described under "Experimental Procedures." In the representative autoradiographs shown only the retarded complex is visible and not the free probe, which was present in excess. C, the protein binding shown in panel C was quantified by using a Packard Instant Imager to count 32P associated with the retarded complex. The data represent the mean ± S.E. of three experiments.
mutated nucleotides are in lowercase letters.

The wild-type (WT) and mutated (MUT) TAAT-like motifs are boxed. All sequences are labeled relative to the transcription start site at +1, and mutated nucleotides are in lowercase letters.

| Site 1 WT  | Site 1 MUT |
|------------|------------|
| -306       | -306       |
| CTAAGGAGCTGGAATGCACTGCTGTGCACTTTA | -271 |

| Site 2 WT  | Site 2 MUT |
|------------|------------|
| -200       | -200       |
| AGAAATCCATGGTCCCGAAATGCCCC | -173 |

| Site 3 WT  | Site 3 MUT |
|------------|------------|
| -156       | -156       |
| CACCTCTTCTCCACGCTAAAGCTATAGGATGAG | -119 |

| Site 4 WT  | Site 4 MUT |
|------------|------------|
| -61        | -61        |
| CACCTGATGCTACAGTGCATCAAGG | -35 |

A) Site 1 probe

B) Site 3 probe

C) Site 4 probe

Fig. 6. Specific protein binding to the Site 1, Site 3, and Site 4 IGRP promoter regions. Labeled oligonucleotides representing the wild-type (WT) −306/−271 (Site 1), −156/−119 (Site 3), and −61/−35 (Site 4) IGRP promoter regions were incubated in the absence (−) or presence of a 100-fold molar excess of the unlabeled competitor (comp.) DNAs shown, representing the WT or mutated (MUT) Sites 1, 3, and 4 IGRP promoter regions, respectively (Table I), or an unrelated (UNR) DNA sequence. High salt βTC-3 nuclear extract was then added, and protein binding was analyzed using the gel retardation assay as described under “Experimental Procedures.” In the representative autoradiograph shown only the retarded complexes are visible and not the free probe, which was present in excess. The formation of a single complex, indicated by the arrow, is selectively reduced by competition with the WT and not the UNR Site 1 oligonucleotides, whereas the formation of two complexes, designated X and Y, is selectively reduced by competition with the WT and not the MUT Site 3 and Site 4 oligonucleotides.

In contrast to this result with the Site 1 probe, when the labeled Site 3 and Site 4 oligonucleotides were incubated with βTC-3 nuclear extract several protein-DNA complexes were formed (Fig. 6, B and C). The wild type Site 3 and Site 4 oligonucleotides competed effectively for the formation of all of these protein-DNA complexes, whereas oligonucleotides containing mutations in the Site 3 and Site 4 TAAT motifs (Table I) failed to compete with the labeled probe for formation of two complexes, designated X and Y (Fig. 6, B and C). This indicates these complexes represent specific protein-DNA interactions and that the other bands detected in the assay must either represent nonspecific protein-DNA interactions or protein binding to sequences outside the regions spanning the TAAT mutations.

To assess the presence of Pdx-1 in these specific protein-DNA complexes, βTC-3 nuclear extract was preincubated with antisera raised to the amino terminus of this transcription factor (25). As can be seen in Fig. 7 (lane 3), this had no appreciable effect on formation of the complex formed with the Site 1 probe. In contrast, preincubation with this antiserum resulted in the disappearance of the specific complex Y and the appearance of a lower mobility, or supershifted complex, with both the Site 3 and Site 4 probes.

Also included in these supershift experiments was an oligonucleotide probe designated Site 2, which does not contain a TAAT motif (Table I). This probe encompasses a region in the IGRP promoter from −197 to −173 that, when deleted, results in a loss in promoter activity (17). Originally, this probe was used in gel retardation assays to identify the transcription factors responsible for this activity. These experiments resulted in the identification of two binding activities that mapped to an A/T-rich region of the Site 2 probe and co-migrated with complexes X and Y formed using Site 3 and 4 probes (data not shown). Interestingly, despite lacking the core TAAT motif, Pdx-1 was observed to interact with this probe as evidenced by the Pdx-1 antibody-induced supershift of one of the specific complexes, complex Y, that forms with the Site 2 probe (Fig. 7, lane 3, Site 2 panel). The identity of the specific complex that forms with the Site 1 probe and complex X that forms with the Site 2, 3, and 4 probes was next investigated. Based on the results of the competition analysis described above, it was apparent that the formation of these complexes was dependent on a TAAT motif (Fig. 6), or in the case of Site 2, an A/T-rich sequence (data not shown). These observations suggested the potential binding of another homeodomain protein. The molecular weight of the potential homeodomain protein present in the specific complex that forms with the Site 1 probe was estimated by utilizing the fractionation renaturation protocol described earlier. After fractionation of the βTC-3 nuclear extract and renaturation, binding activity was detected using the eluate from a particular slice that co-migrates with the specific complex that forms with the Site 1 probe (data not shown). From comparison with molecular mass standards, it was apparent that proteins eluting from this slice were ~45 kDa in size (data not shown). Because Pax-6 is also present in this slice (Fig. 3C) and because Pax-6
contains a homeodomain, antibodies to this factor were tested for their ability to alter the formation of the specific complex that forms with the Site 1 probe. Preincubation of βTC-3 nuclear extracts with Pax-6 antiserum reduced the abundance of this complex and resulted in the appearance of a slower migrating complex (Fig. 7, lane 4, Site 1 panel), demonstrating the presence of Pax-6. Further, these antibodies also disrupted and/or supershifted complex X, which formed when the Site 2, 3, and 4 probes were used (Fig. 7, lane 4). These experiments thus identify the unknown homeodomain protein binding to Sites 1 through 4 as Pax-6.

In the course of these binding analyses it was found that the ability to detect Pax-6 using some of the indicated probes depended on the salt concentration used for nuclear extraction. Thus, as an example, under high salt (800 mM NaCl) extraction conditions, both Pdx-1 and Pax-6 are observed to interact with the Site 2, 3, and 4 probes (Fig. 7, lanes 1–4). In contrast, the Pax-6 interaction is almost undetectable using proteins extracted from nuclei under low salt (200 mM NaCl) conditions (Fig. 7, lanes 5–8). Similarly, Pax-6 binding to the −270/−246 IGRP promoter sequence is reduced using βTC-3 nuclear extract prepared from nuclei under low salt nuclear conditions (Fig. 7). These observations most likely reflect differences in the extraction efficiency of Pax-6. We were able to take advantage of this to demonstrate Pdx-1 binding to the Site 1 probe by using βTC-3 nuclear extract prepared from nuclei under low salt nuclear conditions (Fig. 7, lane 7, Site 1 panel). The inability to detect Pdx-1 binding to this probe using high salt extracts may be explained by a combination of both larger amounts of Pax-6 protein in these extracts and different affinities of Pdx-1 and Pax-6 for the Site 1 probe. In summary, these gel retardation experiments demonstrate that both Pdx-1 and Pax-6 can bind to four and five different sites, respectively, in the IGRP promoter. Importantly, we do not detect Pdx-1 binding to the −270/−246 IGRP promoter sequence under any condition tested (Fig. 7, −270/−246 panel).

Pax-6 and Pdx-1 Bind to the IGRP Promoter in Situ—Although the gel retardation experiments were able detect binding of Pax-6 and Pdx-1 to oligonucleotides representing IGRP promoter sequences in vitro, they do not address whether these interactions occur inside intact cells. This caveat was addressed by using chromatin immunoprecipitation (ChIP) as-

FIG. 7. Pdx-1 and Pax-6 bind the Site 1, Site 2, Site 3, and Site 4 IGRP promoter regions. High or low salt βTC-3 cell nuclear extract was incubated in the absence (−) or presence of the indicated antisera for 10 min on ice prior to the addition of a labeled oligonucleotide probe representing the indicated IGRP promoter regions, and the incubation was continued for an additional 20 min at room temperature. Protein binding was then analyzed as described under “Experimental Procedures.” In the representative autoradiograph shown only the retarded complexes are visible and not the free probe, which was present in excess.
Pdx-1 and Pax-6 binding to the IGRP promoter were analyzed in situ using the chromatin immunoprecipitation (ChiP) assay. Chromatin from formaldehyde-treated βTC-3 cells was immunoprecipitated using anti-Pdx-1 or anti-Pax-6 antibodies or, as a control, using IgG. The presence of the IGRP promoter and the PEPCK promoter in the chromatin preparation prior to immunoprecipitation (1:100 input) and in the immunoprecipitates was then assayed using PCR as described under “Experimental Procedures.” Panel A shows a representative autoradiograph, whereas panel B shows the mean data ± S.E. of three experiments. A Packard Instant Imager was used to count 32P associated with formaldehyde-treated (data not shown).

Fig. 8. The IGRP promoter binds Pdx-1 and Pax-6 in situ. Pdx-1 and Pax-6 binding to the IGRP promoter were analyzed in situ using the chromatin immunoprecipitation (ChiP) assay. Chromatin from formaldehyde-treated βTC-3 cells was immunoprecipitated using anti-Pdx-1 or anti-Pax-6 antibodies or, as a control, using IgG. The presence of the IGRP promoter and the PEPCK promoter in the chromatin preparation prior to immunoprecipitation (1:100 input) and in the immunoprecipitates was then assayed using PCR as described under “Experimental Procedures.” Panel A shows a representative autoradiograph, whereas panel B shows the mean data ± S.E. of three experiments. A Packard Instant Imager was used to count 32P associated with formaldehyde-treated (data not shown).
Pax-6 binding to Sites 1 through 4 has a minimal effect on IGRP promoter activity. In contrast, it was demonstrated that disruption of Pax-6 binding to the $\text{-}273\text{/}246$ element leads to a large reduction in IGRP promoter activity (Fig. 1). Unlike the mutations in Sites 1 through 4, which were introduced into the full-length promoter extending from $\text{-}306$ to $\text{-}274$, the mutations in the $\text{-}273\text{/}246$ element were originally introduced into an IGRP-CAT fusion gene, designated $\text{-}273\text{/}246$-MUT, that is truncated at the 5' end. This truncated fusion gene therefore lacks the sequence that spans the $\text{-}306$ to $\text{-}274$ region, where Site 1 is located. This point became relevant when the effect of disrupting Pax-6 binding to the $\text{-}273\text{/}246$ element in the context of the full-length promoter was assessed. Thus, a fusion gene, designated $\text{-}306$-Pax MUT A, was generated that contains the same mutation as present in the $\text{-}273$ MUT 1 fusion gene (Fig. 1), except that the mutation was introduced in the context of the full-length $\text{-}306$ IGRP-CAT fusion gene. As shown in Fig. 10, following transient transfection of $\beta$TC-3 cells, the $\text{-}306$ Pax MUT A fusion gene directed a similar level of CAT expression as the wild-type $\text{-}306$ WT IGRP-CAT fusion gene, and represent the mean of three experiments ± S.E., each using an independent preparation of all fusion gene plasmids.

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salt βTC-3 nuclear extract Pax-6 binding to the Site 2, 3, and 4 elements is no longer detected (Fig. 7). This suggests that Pax-6 interacts with higher affinity to the Site 1 than to Site 2, 3, and 4 elements. As with the −273/−246 Pax-6 binding site, promoter-scanning software (35) does not predict Pax-6 binding to Site 1.

To address the role of Pax-6 binding to Site 1 further, a mutation that targets the putative Pax-6 paired domain binding motif in Site 1 (Fig. 10A) was introduced in the context of the full-length wild-type −306 IGRP-CAT and −306 Pax MUT A fusion genes, resulting in the generation of constructs designated −306 Pax MUT B and −306 Pax MUT B, respectively (Fig. 10B). This paired domain binding motif mutation partially disrupts Pax-6 binding to Site 1 leaving Pdx-1 binding intact (data not shown). Consistent with the hypothesis that both Pax-6 binding elements in the IGRP promoter compensate for each other, this Site 1 mutation has no effect by itself as assessed by comparing the level of CAT expression directed by the wild-type −306 IGRP-CAT and −306 Pax MUT B fusion genes (Fig. 10). However, when coupled with the mutation of the −273/−254 Pax-6 binding motif, fusion gene expression was reduced by −50% (Fig. 10). Thus, loss of Pax-6 binding at either site individually is compensated for by Pax-6 interaction at the reciprocal site. This result explains why disruption of Pax-6 binding to the −273/−246 element has a significant impact on promoter activity in the context of a truncated IGRP promoter lacking Site 1, but not in the context of the full-length IGRP promoter. Site-directed mutagenesis of both Pax-6 paired domain binding motifs, as present in the −306 Pax MUT AB construct, reduces fusion gene expression to a lesser degree than deletion of both elements, as occurs in the −254 5′ deletion construct (compare Figs. 1 and 10). We hypothesize that this difference is explained by residual Pax-6 homeodomain binding to the TAAT-like motif in Site 1, consistent with the observation that mutation of the Pax-6 paired domain binding motif in Site 1 only partially disrupts Pax-6 binding (data not shown).

**DISCUSSION**

The results of this study provide insight into the molecular mechanisms controlling IGRP gene transcription. A role for Pax-6 was demonstrated based on data derived from *in vitro* and *in situ* binding studies, coupled with functional studies utilizing promoter fusion genes. Although Pax-6 is capable of interacting with at least five separate elements in the IGRP promoter *in vitro* (Fig. 7), only two of these, located in the −306/−273 (Site 1) and −273/−246 regions, appear critical for promoter activity (Figs. 1, 9, and 10). In fact, it was the marked loss in promoter activity observed when the −273/−246 IGRP promoter region was removed in the original 5′ deletion analysis (17) that directed our attention to this element and eventually led to the identification of Pax-6 as the factor binding this region. However, this reduction in promoter activity was not detected when this element is mutated using site-directed mutagenesis, because Pax-6 binding to Site 1 can compensate for the loss of Pax-6 binding to the −273/−246 element (Fig. 10B). A key result that led to the identification of Pax-6 as a candidate for the factor binding the −273/−246 element was the use of a modified *in situ* footprinting method, the results of which suggested that a single factor may make unusually broad contacts across this promoter region (Fig. 2). We have previously demonstrated the utility of this methodology by showing that multiple *trans*-acting factor binding sites identified in the IGRP promoter in βTC-3 cells by *in situ* footprinting correlate with regions of the IGRP promoter identified as being important for basal IGRP-CAT fusion gene expression in βTC-3 cells (17).

As an alternative method for identifying the factors controlling IGRP expression, we also took advantage of the accumulating literature regarding the transcription factors controlling the expression of other β cell-specific or -enriched proteins, such as insulin, to identify candidate proteins. Our efforts here were focused on the transcription factor Pdx-1, which was shown to interact with the IGRP promoter at four separate elements *in vitro* together with Pax-6 (Fig. 7). Both of these proteins bind to TAAT or TAAT-like motifs within these elements (Fig. 6 and data not shown), presumably through their respective homeodomains. It is interesting that this dependence of binding on the presence of the TAAT sequence is not absolute, as reflected in the observation that Pdx-1 and Pax-6 were able to interact with the element designated Site 2, which contains the sequence TAAA (Table 1). This observation suggests that the homeodomains in these proteins exhibit rather loose specificity *in vitro*. Because similar binding characteristics have been observed for other homeoproteins (44, 46, 47), the question arises: do these proteins achieve target specificity *in vitro*? One route through which this could occur is by cooperative binding with other transcription factors. With regard to Pdx-1, such a model is consistent with work showing that this factor can interact with the transcription factor Neu-roD/BETA2 to support cooperative binding to the A3 and E2 elements, respectively, in the rat insulin I promoter (48).

Based on data derived from site-directed mutagenesis (Fig. 9), it appears that Pdx-1 does not contribute significantly to IGRP promoter activity. Thus, it would seem that speculation about potential Pdx-1 interacting proteins with regard to IGRP promoter regulation is unwarranted. It is possible, however, that an interaction between Pdx-1 and another transcription factor could allow this protein to interact with the promoter in the absence of a binding site, thus masking the effects of site-directed mutagenesis. Consistent with this idea is the fact that Pdx-1 is associated with the IGRP promoter *in situ*, which suggests it plays a role in regulating IGRP expression (Fig. 8). Alternatively, the simpler explanation, that Pdx-1 is not critical for maximum IGRP expression, is consistent with studies on glucokinase gene expression. Thus, although Pdx-1 is able to interact with the glucokinase promoter *in vitro* (49), no alteration in glucokinase protein is observed in mice heterozygous for a Pdx-1 null allele or in animals whose β cells completely lack Pdx-1 (50, 51). Measurements of IGRP expression levels in the absence of Pdx-1 may definitively answer this question.

In addition to its role in regulating basal gene transcription, Pdx-1 also appears to contribute to the modulation of gene expression by glucose in β cells. Transcription of the insulin gene, for example, is induced when β cells are exposed to elevated glucose concentrations, and studies aimed at identifying the transcription factors responsible for this effect have shown that disruption of Pdx-1 binding to the insulin promoter impairs the glucose response (52). Consistent with the idea that Pdx-1 is a glucose-responsive factor, it has been demonstrated that this transcription factor translocates from the cytoplasm, or nuclear periphery, to the nucleus in response to elevated glucose levels (53, 54). In light of these data, it seems reasonable to hypothesize that, although we are not able to ascribe a major role for Pdx-1 in the maintenance of basal IGRP promoter activity, this transcription factor may play a role in the regulation of the IGRP gene by metabolites such as glucose. Similarly, in addition to its role in regulating basal gene transcription, Pax-6 also appears to contribute to the regulation of glucagon expression by insulin in α cells. Pax-6 binds the G3 element in the glucagon promoter (55), an element that is critical for the regulation of glucagon gene transcription by insulin (56). However, although this element is sufficient to confer insulin-regulated reporter gene expression in a hetero-
ogous context (56, 57), it is apparent that other promoter elements contribute to the regulation of glucagon gene transcription by insulin (57). Thus, although Pax-6 binds the promoters of several genes whose expressions are islet-enriched (58), it is possible that Pax-6 may only mediate an insulin response in specific promoter contexts. Future studies investigating the possible effects of glucose and insulin on IGRP promoter activity will address these issues.

Because IGRP is only one of a small group of proteins known to be highly enriched in pancreatic β cells, it offers a relatively unique opportunity to study the molecular basis for selective gene expression within this cell type. Our future goals include identifying the additional transcription factors required for the gene expression within this cell type. Our future goals include identifying the additional transcription factors required for the gene expression within this cell type. Our future goals include identifying the additional transcription factors required for the gene expression within this cell type. Our future goals include identifying the additional transcription factors required for the gene expression within this cell type.
