

The Proximal Islet-Specific Glucose-6-Phosphatase Catalytic Subunit-Related Protein Autoantigen Promoter Is Sufficient to Initiate but not Maintain Transgene Expression in Mouse Islets in Vivo

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We have previously reported the discovery of an islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) that is predominantly expressed in islet β -cells. IGRP has recently been identified as a major autoantigen in a mouse model of type 1 diabetes. The analysis of IGRP-chloramphenicol acetyltransferase (CAT) fusion gene expression in transiently transfected islet-derived hamster insulinoma tumor and β TC-3 cells revealed that the promoter region located between -306 and $+3$ confers high-level reporter gene expression. To determine whether this same promoter region is sufficient to confer islet β -cell-specific gene expression in vivo, it was ligated to a β -galactosidase reporter gene, and transgenic mice expressing the resulting fusion gene were generated. In two independent founder lines, this -306 to $+3$ promoter region was sufficient to drive β -galactosidase expression in newborn mouse islets, predominantly in β -cells, which was initiated during the expected time in development, around embryonic day 12.5. However, unlike the endogenous IGRP gene, β -galactosidase expression was also detected in the cerebellum. Moreover, β -galactosidase expression was almost completely absent in adult mouse islets, suggesting that *cis*-acting elements elsewhere in the IGRP gene are required for determining appropriate IGRP tissue-specific expression and for the maintenance of IGRP gene expression in adult mice. *Diabetes* 53:1754–1764, 2004

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CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; HNF, hepatocyte nuclear factor-6; IGRP, islet-specific G6Pase catalytic subunit-related protein; PAM, peptidyl-glycine alpha-amidating monooxygenase; PMSF, phenylmethylsulfonyl fluoride; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

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Islet-specific glucose-6-phosphatase (G6Pase) catalytic subunit-related protein (IGRP) is a putative enzyme that is specifically expressed in the islets of Langerhans (1–3), where it is localized predominantly in insulin-producing β -cells (4). IGRP was identified by screening a plasmid cDNA library prepared by subtraction of mouse insulinoma β TC-3 cDNA from mouse glucagonoma α TC-2 cDNA (1,5). As its name implies, IGRP is a homologue of the catalytic subunit of G6Pase, the final enzyme in the glycogenolytic and gluconeogenic metabolic pathways (6–9). G6Pase is located in the endoplasmic reticulum as a multicomponent system (6–9). The exact number of components in this system and their stoichiometry are unclear. However, the favored model includes a catalytic subunit and transporters for G6P, glucose, and phosphate (6–9).

Both the G6Pase catalytic subunit and IGRP are integral membrane proteins that are thought to span the endoplasmic reticulum membrane multiple times (1,3). The G6Pase catalytic subunit has its catalytic site oriented toward the lumen of the endoplasmic reticulum, so a G6P transporter is essential for delivering G6P from the cytosol to this active site (6–9). The spatial arrangement of the G6Pase system also serves to limit the function of the G6Pase catalytic subunit, which, in permeabilized microsomal membrane preparations, demonstrates relatively nonspecific phosphatase activity (6–9). Mouse IGRP is similar in size (355 amino acids [aa]; Mr [molecular weight] 40552) and sequence (~50% identity at the amino acid level) to the mouse G6Pase catalytic subunit (357 aa; Mr 40454) (1). However, we have been unable to identify a substrate for IGRP. Two observations suggest that IGRP does not catalyze G6P hydrolysis. First, most reports indicate that in isolated mouse islets and islet-derived cell lines, in which IGRP expression is high, G6Pase activity is low (6–9). Second, a similar low level of islet G6Pase activity is found in both rat and mouse islets (6–9), although IGRP is not expressed in the rat (3).

Whereas the role of IGRP in β -cell function remains unclear, IGRP has recently been identified as a major autoantigen in the NOD mouse model of type 1 diabetes (10). This form of diabetes is caused by an autoimmune reaction that leads to the destruction of the pancreatic

β -cell (11). Lieberman et al. (10) have shown that a substantial proportion (40%) of the CD8 T-cells infiltrating the islets in NOD mice appear to recognize a single peptide epitope in IGRP. Whether IGRP is an autoantigen in human type 1 diabetes is unknown; however, numerous similarities exist between disease etiology in NOD mice and human type 1 diabetic patients (12). Therefore, it is possible that studies on the IGRP promoter may be useful for the design of strategies aimed at slowing or averting β -cell destruction by preventing immune recognition through suppression of this β -cell antigen. The viability of this approach has already been suggested by studies in which reducing expression of another autoantigen, namely GAD, was shown to lower the incidence of diabetes (13). In addition, based on the precedent set principally through the characterization of the insulin promoter, studies on the IGRP promoter may lead to the identification of novel, islet-enriched transcription factors important for pancreatic development and/or function (14–16). For both of these reasons, we are interested in characterizing the IGRP promoter. In the islet-derived hamster insulinoma tumor (HIT) cell line, we have previously shown that the IGRP promoter region located between -306 and $+3$ is sufficient to confer high-level IGRP-CAT fusion gene expression (2). This article describes the generation and analysis of transgenic mice expressing an IGRP- β -galactosidase fusion gene containing this same promoter region. The results indicate that this IGRP promoter region is sufficient for driving β -galactosidase expression in developing and newborn mouse islets but is ineffective for maintaining that expression in adult animals. This suggests that distinct *cis*-acting elements in the IGRP gene are required for the initiation and maintenance of IGRP gene expression during islet development. As such, the IGRP gene provides a unique transcriptional model for the study of this process.

RESEARCH DESIGN AND METHODS

[α - 32 P]dATP ($>3,000$ Ci/mmol) and [3 H] acetic acid, sodium salt (>10 Ci/mmol), were obtained from Amersham and ICN, respectively.

Fusion gene plasmid construction. The construction of mouse IGRP-CAT fusion genes containing the promoter sequence from -911 to $+3$, -508 to $+3$, and -306 to $+3$ in the pCAT(An) expression vector (17) has been previously described (2). To construct an IGRP- β -galactosidase fusion gene for use in the generation of transgenic mice, the proximal IGRP promoter region, located between -306 to $+3$, was reisolated from the $-306/+3$ IGRP-CAT plasmid described above as a *Bam*HI-*Pst*I fragment. This fragment was then subcloned into the *Hin*DIII-*Pst*I digested pPD1.27 *LacZ* vector (18). After ligating the compatible *Pst*I ends, the Klenow fragment of *Escherichia coli* DNA polymerase I was used to fill in the noncompatible ends before blunt-ended ligation.

A three-step PCR strategy (19) was used to create a site-directed mutant of the $-156/-152$ DeltaEF1 motif in the IGRP promoter. This construct was generated within the context of the -306 to $+3$ IGRP promoter fragment. Briefly, two complementary PCR primers were designed to mutate nucleotides within this motif. The sequence of the sense strand oligonucleotide was as follows: 5'-(-171)GGTCAGCATATACTGacaagCTTTCCACCCATT A-3' (mutated nucleotides in lowercase). With the -306 IGRP-CAT plasmid as the template, this sense strand oligonucleotide was used in conjunction with a 3' PCR primer to generate the 3' half of the IGRP promoter, whereas the complementary antisense strand oligonucleotide was used in conjunction with a 5' PCR primer to generate the 5' half of the IGRP promoter. The 3' primer (5'-CCGCTCGAGATCCAGATCCTC-3'; *Xho*I cloning site underlined) and 5' PCR primer (5'-CGGGATCCAAGCTCTAGCCAAGC-3'; *Bam*HI cloning site underlined) were designed to conserve the junction between the IGRP promoter and pCAT(An) 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 both primer and template in a

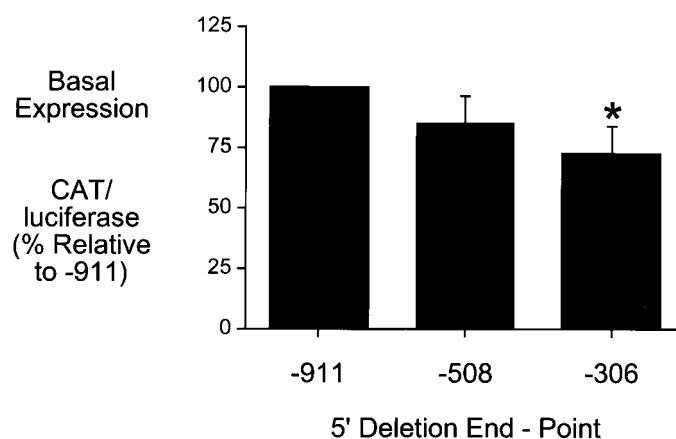


FIG. 1. The -306 to $+3$ IGRP promoter region confers high basal reporter gene expression in β TC-3 cells. β TC-3 cells were transiently cotransfected, as described in RESEARCH DESIGN AND METHODS, with an expression vector encoding firefly luciferase (0.5 μ g) and a series of IGRP-CAT fusion genes (2 μ g) with 5' deletion end points between -911 and -306 . After transfection, cells were incubated for 18–20 h in serum-containing medium. The cells were then harvested, and both CAT and luciferase activity were assayed as described in RESEARCH DESIGN AND METHODS. Results are presented as the ratio of CAT to luciferase activity and represent the mean of three experiments \pm SE, each using an independent preparation of each 5' truncated fusion gene plasmid. * $P < 0.05$ vs. -911 IGRP-CAT.

second PCR step to generate small amounts of the full-length mutated IGRP promoter fragment. Finally, the 5' and 3' PCR primers were then used to amplify this fragment, which was then completely sequenced to ensure the absence of polymerase errors. All plasmid constructs were purified by centrifugation through cesium chloride gradients (20).

Cell culture and transient transfection. Mouse pancreatic islet β -cell-derived β TC-3 cells were passaged as subconfluent cultures in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 2.5% (vol/vol) fetal bovine serum, and 15% (vol/vol) horse serum. β TC-3 cells were cotransfected with 0.5 μ g of an expression vector encoding SV40-firefly luciferase (Promega) and 2 μ g of the indicated CAT plasmids using the lipofectamine reagent (GibcoBRL) as previously described (21).

CAT and luciferase assays. Transfected β TC-3 cells were harvested by trypsin digestion and then homogenized in passive lysis buffer (Promega). After two cycles of freezing/thawing, firefly luciferase activity was assayed as described previously (22). The remaining β TC-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, results are expressed as the ratio of CAT to firefly luciferase activity.

Animal care. The animal housing and surgical facilities used for the mice in these studies met American Association for the Accreditation of Laboratory Animal Care standards. All animal protocols were approved by the Vanderbilt University Medical Center Animal Care Committee. Mice were housed in polystyrene cages with a 14-h light, 10-h dark cycle, with food (Purina Mouse Chow 5001 or, for pregnant and nursing mice, Purina Mouse Chow 5015; Ralston-Purina, St. Louis, MO) and water provided ad libitum.

Generation, identification, and breeding of transgenic mice. The IGRP- β -galactosidase fusion gene described above, containing the IGRP promoter region between -306 to $+3$, was isolated from the pPD1.27 *LacZ* vector by digestion with *Not*I and *Ase*I. The fusion gene was separated from the vector by electrophoresis on low-melting-point agarose and purified using GELase (Epicenter, Madison, WI) followed by phenol chloroform extraction. Transgenic mice were generated by the joint Vanderbilt Cancer Center/Diabetes Research and Training Center Transgenic Animal/Embryonic Stem Cell Core Facility. Transgenic mice were produced by microinjection of 3–10 pl of a 3 ng/ μ l solution of the IGRP- β -galactosidase fusion gene into the pronuclei of one-cell embryos from B6D2F1 females. Embryos were then injected into pseudopregnant ICR females. Transgenic founders were bred with B6D2F1 mice (The Jackson Laboratory, Bar Harbor, ME).

Mouse genotyping. Genomic DNA (10 μ g), isolated from tail clips of 3-week-old pups, was digested with either *Eco*RI and *Eco*RV or *Hpa*I, and DNA fragments were then separated by electrophoresis on 0.8% agarose gels containing ethidium bromide. The presence of the transgene was detected by

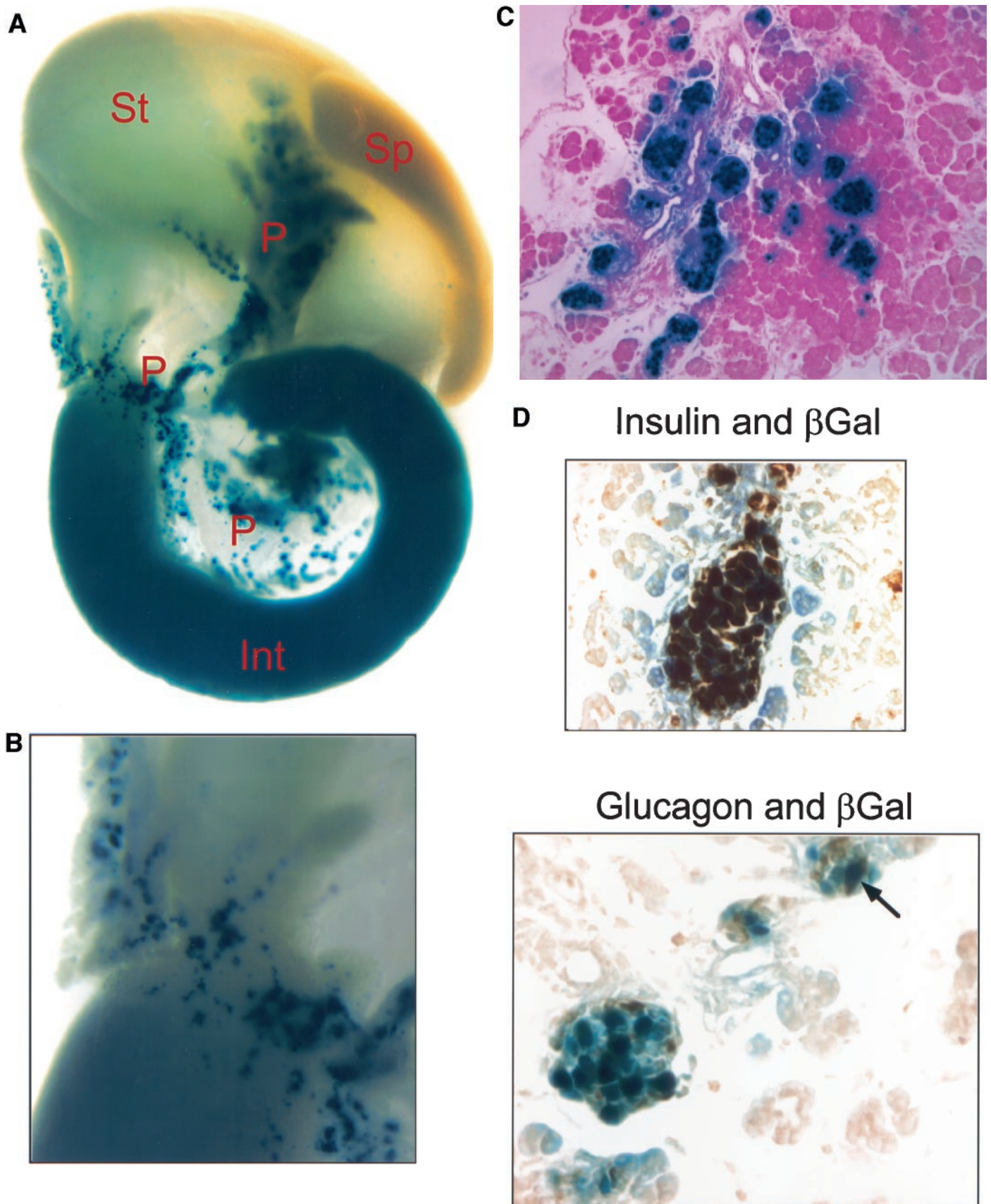


FIG. 2. The -306 to $+3$ IGRP promoter region is sufficient for directing transgene expression in newborn mouse islets in vivo. **A** and **B**: Whole-mount X-gal staining of neonatal (postnatal day 1) $-306/+3$ IGRP- β -galactosidase transgenic mouse organs. P, pancreas; St, stomach; Int, intestine; Sp, spleen. The punctate pattern of pancreatic staining suggests islet-specific expression. The blue staining in the intestinal lumen is caused by β -galactosidase activity in endogenous gut flora. Representative pictures are shown. Panel **A** is a $25\times$ magnification, whereas panel **B** is a $50\times$ magnification. **C**: X-gal staining of a neonatal (postnatal day 1) $-306/+3$ IGRP- β -galactosidase transgenic mouse pancreas section. The

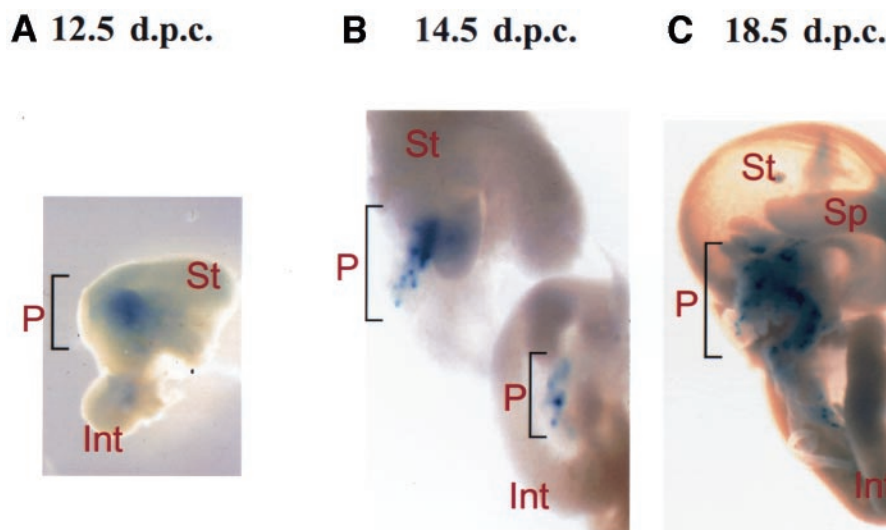


FIG. 3. The -306 to $+3$ IGRP promoter region is sufficient for conferring transgene expression around embryonic day 12 in mouse pancreas in vivo. Whole-mount X-gal staining of embryonic $-306/+3$ IGRP- β -galactosidase transgenic mouse organs during development. **A:** 12.5 days p.c. **B:** 14.5 days p.c. **C:** 18.5 days p.c. P, pancreas; St, stomach; Int, intestine; Sp, spleen. Representative pictures are shown.

DNA hybridization analysis using Zeta-probe membranes (Bio Rad). Both alkaline DNA transfer and hybridization were performed using the standard protocol according to the manufacturer's instructions. DNA probes of 1,893 base pairs (bp) and 625 bp, representing fragments of the LacZ reporter gene, were generated by digestion of the pPD1.27 LacZ vector with either *EcoRI* and *EcoRV* or *HpaI*, respectively. These probes were labeled by random oligonucleotide priming with [α - 32 P]dATP using the Stratagene Prime-It II random primer labeling kit according to the manufacturer's instructions.

Detection of β -galactosidase expression. The morning in which a vaginal plug was detected was considered 0.5 days post coitus. Digestive tissues and brains of newborn and adult mice as well as embryos at 12.5, 14.5, and 18.5 days p.c. were fixed upon dissection in 4% (wt/vol) paraformaldehyde in PBS for 40 min to 1 h at 4°C. Tissues were rinsed in PBS for 15 min at 4°C and permeabilized for 30 min at 4°C and then for 30 min at room temperature in 2 mmol/l MgCl₂, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 137 mmol/l NaCl, 2.7 mmol/l KCl, 4.3 mmol/l Na₂HPO₄, and 1.4 mmol/l KH₂PO₄, pH 7.4. Staining was performed overnight at 4°C by incubation of the permeabilized tissues in 2 mmol/l MgCl₂, 5 mmol/l K₃Fe(CN)₆, 5 mmol/l K₄Fe(CN)₆, 20 mmol/l Tris, 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), 137 mmol/l NaCl, 2.7 mmol/l KCl, 4.3 mmol/l Na₂HPO₄, and 1.4 mmol/l KH₂PO₄, pH 7.4. The samples were then rinsed in PBS and postfixed in 4% (wt/vol) paraformaldehyde (in PBS) for 1 h at 4°C. Tissues were then rinsed in PBS before storage in 70% ethanol at 4°C.

Immunohistochemical staining. Digestive tissues from transgenic mice were stained, as described above, to detect β -galactosidase expression. Tissues were then dehydrated through an ethanol series as previously described (23) and incubated with 100% isopropanol for 1 h to prevent leaching of the X-gal precipitate before embedding in paraffin. After dewaxing and rehydration (23), five to seven micron sections were incubated overnight at 4°C in PBS containing 5% (vol/vol) normal donkey serum and the following primary antibodies: guinea pig anti-insulin (Linco) and rabbit anti-glucagon (Linco), both at 1:1,000 dilutions. Sections were washed in PBS containing 0.1% Triton X-100, and primary antibody binding was then detected using secondary horseradish peroxidase-conjugated anti-rabbit (Vectastain ABC Kit; Vector Laboratories) or anti-guinea pig antibodies (Histomouse Kit Zymed Laboratories) according to the manufacturer's instructions. Sections were then counterstained using eosin (Surgipath Medical Industries). Images were recorded on an Olympus BX41 microscope and Optronics digital camera using the Magnafire program. Images were processed in Adobe Photoshop.

RNA blot analysis. Total RNA was isolated from adult mouse frontal brain and cerebellum using TRI Reagent (Molecular Research Center) according to the manufacturer's instructions. Poly A⁺ RNA was then prepared using an

Oligotex mRNA Midi Kit (Qiagen) according to the manufacturer's instructions. Mouse pancreas Poly A⁺ RNA was purchased from BD Biosciences Clontech. Poly A⁺ RNA (5 μ g cerebellum and frontal brain, 2 μ g pancreas) was separated by electrophoresis in an agarose gel containing MOPS and formaldehyde and subsequently transferred to a nylon membrane (Zeta-probe; Bio-Rad) (20). Blots were hybridized for 16 h at 65°C in 0.5 mol/l sodium phosphate buffer, pH 7.2, 7% SDS, and 1 mmol/l EDTA, with a 32 P-radiolabelled randomly primed probe corresponding to the full-length mouse IGRP open reading frame. This probe was isolated from the pSVSPORT clone 2A5 (1) and labeled by random oligonucleotide priming with [α - 32 P]dATP using the Stratagene Prime-It II random primer labeling kit according to the manufacturer's instructions. After hybridization, blots were washed twice in 40 mmol/l sodium phosphate buffer, pH 7.2, with 5% SDS and 1 mmol/l EDTA at 65°C for 30 min, and then they were washed once in 40 mmol/l sodium phosphate buffer, pH 7.2, with 1% SDS and 1 mmol/l EDTA at 65°C for 30 min before visualization by autoradiography. Blots were then stripped by washing in 0.1 \times SSC, 0.1% SDS at 100°C and reprobed with a labeled mouse β -actin cDNA probe to confirm similar mRNA loading levels in each lane.

Gel retardation assays

Labeled probes. Sense and antisense oligonucleotides representing the IGRP promoter sequence between -170 and -141 were synthesized with *Bam*HI-compatible ends and subsequently gel purified, annealed, and labeled with [α - 32 P]dATP using the Klenow fragment of *E. coli* DNA polymerase I to a specific activity of ~ 2.5 μ Ci/pmol (20).

High-salt nuclear extract preparation. High-salt β TC-3 nuclear extract was prepared as described (24,25), except that nuclei were lysed by resuspension in a buffer containing 800 mmol/l NaCl, 20 mmol/l HEPES, pH 7.9, 0.75 mmol/l spermidine, 0.15 mmol/l spermine, 0.2 mmol/l EDTA, 2 mmol/l EGTA, 2 mmol/l dithiothreitol (DTT), 25% glycerol, and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF). 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 mmol/l KCl, 20 mmol/l HEPES, pH 7.9, 0.2 mmol/l EDTA, 0.2 mmol/l EGTA, 2 mmol/l DTT, 20% glycerol, and 1 mmol/l PMSF. The protein concentration of the nuclear extracts was determined using a Bio-Rad assay and was typically ~ 1 μ g/ μ l.

Binding assays. DeltaEF1 binding assays were based on the method of Sekido et al. (26). For the assay, ~ 14 fmol of radiolabeled probe ($\sim 50,000$ cpm) was incubated with 3 μ g of β TC-3 nuclear extract in a 20- μ l reaction containing 20 mmol/l HEPES, pH 7.9, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 15% glycerol (vol/vol), 2 mmol/l DTT, 1.5 μ g poly(dA-dT) \cdot poly(dA-dT), 50 mmol/l KCl, 0.1 mg/ml BSA, 2 mmol/l MgCl₂, and 0.1 mmol/l ZnSO₄. After incubation

section was counterstained with eosin. The staining pattern is consistent with that of neonatal mouse islet expression. No expression was detected in acinar cells or ducts. A representative picture is shown. Panel C is a 100 \times magnification. **D:** Immunohistochemical staining of a neonatal (postnatal day 1) $-306/+3$ IGRP- β -galactosidase transgenic mouse pancreas section with anti-insulin (top panel) and anti-glucagon (bottom panel) antibodies. Newborn transgenic mouse pancreas was stained to detect β -galactosidase activity before embedding and immunohistochemical staining as described in RESEARCH DESIGN AND METHODS. Occasional cells were detected that were both glucagon positive and expressed the transgene (see arrow). Representative pictures are shown. Panel D is a 400 \times magnification.

at room temperature for 20 min, samples were loaded onto a 6% polyacrylamide gel containing $0.25 \times$ TBE (90 mmol/l Tris base, 90 mmol/l boric acid, 2 mmol/l EDTA) and 2.5% (vol/vol) glycerol. Samples were electrophoresed for 1.5 h at 150 V in $0.25 \times$ TBE buffer before the gel was dried and exposed to Kodak XB film with intensifying screens.

Competition experiments and gel supershifts. For competition experiments, unlabeled competitor DNA was mixed with the radiolabeled oligomer at a 100-fold molar excess before addition of nuclear extract. For supershift experiments, a specific DeltaEF1 antisera (2 μ l), a generous gift from Dr. Michel Sanders (27), was preincubated with β TC-3 nuclear extract for 10 min on ice before the addition of the labeled wild-type IGRP promoter oligonucleotide probe. All subsequent steps were carried out as described above.

Statistical analysis. Transfection data were analyzed for differences from the control group values using an unpaired Student's *t* test. The level of significance was $P < 0.05$ (two-sided test).

RESULTS

The -306 to $+3$ IGRP promoter region directs high basal reporter gene expression in situ. We have previously demonstrated that the proximal IGRP promoter region located between -306 and $+3$, relative to the transcription start site, is sufficient to confer maximal basal IGRP-CAT fusion gene expression in HIT cells; a distal IGRP promoter region between -911 and -307 could be deleted without a reduction in fusion gene expression (2). Before generating transgenic mice expressing an IGRP- β -galactosidase fusion gene that contains this promoter region, we wanted to ensure that similar results could be obtained in another islet β -cell-derived cell line. Therefore, a series of truncated IGRP-CAT fusion genes were transiently transfected into β TC-3 cells, and the level of basal reporter gene expression directed by these fusion genes was analyzed (Fig. 1). Figure 1 shows that deletion of the IGRP promoter region between -911 and -307 resulted in a 25% decrease in basal fusion gene expression in β TC-3 cells, in contrast to the result previously seen in HIT cells (2). However, the -306 to $+3$ promoter region still conferred high basal fusion gene expression in β TC-3 cells, suggesting that it might contain all of the *cis*-acting elements required to confer islet-specific expression of the IGRP gene. Although this is a relatively short DNA sequence, Magnuson and colleagues (28,29) have previously shown that the proximal region of the upstream glucokinase promoter, located between -280 and $+14$, is sufficient to confer maximal basal expression of that gene in HIT cells and appropriate transgene expression in vivo. Interestingly, Magnuson and colleagues (28) have also shown that multiple *cis*-acting elements within this proximal region of the upstream glucokinase promoter are required for maximal fusion gene expression in HIT cells, as is the case with the IGRP promoter (21).

The -306 to $+3$ IGRP promoter region is sufficient for directing transgene expression in newborn mouse islets in vivo. It is relatively unusual for *cis*-acting elements identified in transfection studies to only be important in that experimental system and be irrelevant to the actual elements required for the regulation of gene transcription in vivo; however, examples do exist (30,31). Moreover, as with all cell lines, islet β -cell-derived cell lines are not perfect models of β -cells in vivo. Therefore, to determine whether an identical region of the IGRP promoter was sufficient to confer islet cell-specific expression in vivo, we generated transgenic mice that express an IGRP- β -galactosidase fusion gene containing the IGRP

promoter sequence between -306 and $+3$. β -Galactosidase was used as the reporter gene for these studies because histochemical staining, using X-gal as the substrate, could be used to visualize the tissues in which the transgene was expressed. The β -galactosidase reporter contained a nuclear localization signal (18) to enhance the clarity of histochemical staining.

Nine founder animals were generated and used to establish lines. Of these nine founder animals, only two expressed the IGRP- β -galactosidase transgene. For all of the analyses described below, we have obtained identical results with both transgenic lines, indicating that the results are not an artifact of the site of transgene integration. In addition, no differences were observed in comparisons of IGRP- β -galactosidase fusion gene expression in male and female mice.

Figures 2A and B show that transgene expression was not detected in the stomach or the spleen of neonatal mice, but it was restricted to pancreatic tissue, with a punctate staining pattern consistent with that of islet-specific expression. The islet-specific nature of pancreatic expression was confirmed by examining β -galactosidase expression in pancreatic sections (Fig. 2C). Mouse islets are remodeled after birth (32,33), so the appearance of islets in newborn mice differs from that in adult animals (compare Figs. 2C and 4). Nevertheless, immunohistochemical labeling studies using antisera to insulin and glucagon reveal that this pancreatic β -galactosidase expression was predominantly found in islet β -cells (Fig. 2D). This is consistent with the expression profile of the endogenous IGRP gene, which is transcribed in most insulin-positive cells but in only a few glucagon-positive cells (4). Inspection of multiple slides revealed that β -galactosidase expression was detected in the majority of insulin-positive cells (data not shown). In addition, occasional cells were detected that both were glucagon positive and expressed the transgene (Fig. 2D, see arrow). These analyses also revealed that the occasional β -galactosidase staining seen in individual cells in pancreatic sections (Fig. 2C) represents expression in isolated insulin-positive cells that are seen in the neonatal, but not adult, mouse pancreas (data not shown).

An initial wave of insulin gene expression occurs between embryonic days 9.5 and 12.5 during pancreas development, the former corresponding to the time when the pancreatic buds are being formed (34,35). Although it is unclear what function, if any, these early insulin-producing cells perform, a second wave of insulin-producing cells is generated beginning around embryonic day 14.5 (36). Similarly, Hutton and colleagues (1) have demonstrated using RT-PCR that, in the mouse, endogenous IGRP gene expression can be detected at embryonic day 12, although prominent expression only becomes apparent around embryonic day 14. Figure 3 shows that IGRP- β -galactosidase fusion gene expression can also be detected around embryonic day 12, and that this expression is maintained at embryonic days 14.5 and 18.5. This embryonic expression of IGRP- β -galactosidase in the developing pancreas was much greater than the background X-gal staining detected in nontransgenic littermates that is caused by the presence of an endogenous acid β -galactosidase (37 and data not shown). These results suggest that the -306 to $+3$ IGRP

promoter region contains the *cis*-acting elements required for conferring the correct time of initiation of IGRP gene expression.

The -306 to +3 IGRP promoter region is insufficient for preventing transgene expression in brain in vivo.

Additional analyses showed that IGRP- β -galactosidase transgene expression was not detected in liver, skeletal muscle, heart, and lungs of newborn mice. However, of particular interest was the question as to whether the IGRP- β -galactosidase transgene was expressed in brain because islet and brain both express many of the same transcription factors. Thus, although IGRP mRNA was not detected in whole-brain tissue by RNA blot analysis using poly A⁺ RNA (3), it was possible that it might still be expressed in specific brain nuclei. Such expression could have been missed using a whole-brain mRNA preparation because of the dilution of IGRP mRNA in the sample. By analogy, expression of the islet-specific isoform of glucokinase was not detected when total-brain mRNA was analyzed by Northern blotting, but analysis of glucokinase- β -galactosidase transgene expression in mice revealed expression in the medial hypothalamus (29). This led to the discovery that endogenous glucokinase was expressed in this same location (29). Figure 4 demonstrates that the IGRP- β -galactosidase transgene was indeed expressed in the developing cerebellum of newborn mice (Fig. 4A) and in adult mice (Fig. 4B). However, RNA blotting experiments failed to detect IGRP mRNA in poly A⁺ RNA prepared specifically from the cerebellum of normal mice (Fig. 4C). Moreover, immunohistochemical labeling of adult mouse cerebellum using an IGRP antiserum also failed to detect IGRP (data not shown). These results suggest that the endogenous IGRP gene is not expressed in the cerebellum and that the -306 to +3 IGRP promoter region lacks *cis*-acting elements required to prevent IGRP gene expression in the brain. As described above, these results are in contrast to those obtained with the glucokinase gene, in which a short region of the upstream promoter, located between -280 and +14, directs appropriate transgene expression in vivo. However, these results with the IGRP promoter mimic similar analyses performed with the insulin gene, in which a 668-bp fragment of the rat insulin II promoter was found to drive inappropriate transgene expression in the brain (38).

The -306 to +3 IGRP promoter region is insufficient for maintaining transgene expression in adult mouse islets in vivo.

Although the -306 to +3 IGRP promoter region was sufficient for directing transgene expression in mouse islets in vivo (Fig. 2), with the correct time of initiation of transgene expression (Fig. 3), IGRP- β -galactosidase transgene expression, in both founder lines, was markedly reduced in adult mouse islets, although a mosaic pattern of expression remained (Figure 5). This is in contrast to the IGRP- β -galactosidase transgene expression in the cerebellum, which was still detected in adult mice (Fig. 4B). This also contrasts with the endogenous IGRP gene that is expressed in a majority of β -cells in adult mouse islets (3,4). This reduction in IGRP transgene expression occurred within 5 weeks after birth (data not shown). The occasional expression of the transgene in adult islet cells was not a marker of the cell's potential to divide because transgene expression was detected both in

cells that were positive and in those that were negative for the proliferation marker, phosphorylated H3 histone (data not shown). This loss of expression was not caused by silencing of the transgene in situ (39) because the progeny of these mice also express the transgene in islets at birth. Silencing would presumably affect the IGRP transgene locus in both islet and germ cells. This result suggests that the proximal IGRP promoter, located between -306 and +3, is sufficient to initiate IGRP- β -galactosidase transgene expression in islets. However, additional regulatory elements must be required for the maintenance of IGRP- β -galactosidase transgene expression in adult islets.

The transcriptional repressor DeltaEF1 binds to the -156 to -152 region of the IGRP promoter in vitro.

The construction of further IGRP- β -galactosidase transgenes containing additional IGRP promoter and/or intron sequences should eventually lead to the identification of the regulatory elements that are required for the maintenance of IGRP- β -galactosidase transgene expression in adult islets. However, a second topic that remains to be addressed is the elucidation of the mechanism involved in the reduction of -306 IGRP- β -galactosidase fusion gene expression that is seen in adult mice (Fig. 5). One possibility is that transgene expression is actively repressed in adult mice by a factor binding this IGRP promoter region. An analysis of putative *cis*-acting elements in this IGRP promoter region, using MatInspector sequence analysis software (40), identified a binding site for the transcriptional repressor DeltaEF1, also known as BZP and ZEB, between -156 and -152 (Fig. 6A) (41-44). We have previously shown, using the ligation-mediated PCR footprinting technique, that this binding site is adjacent to a guanine residue whose accessibility to methylation by dimethyl sulfate is altered by *trans*-acting factor binding within intact β TC-3 cells (21). This indicates that a transcription factor binds at or near the DeltaEF1 motif in the IGRP promoter in situ.

The gel retardation assay was used to investigate whether DeltaEF1 can bind to the IGRP promoter in vitro. When a labeled double-stranded oligonucleotide, designated IGRP WT, representing the wild-type IGRP promoter sequence from -170 to -141 (Fig. 6A), was incubated with nuclear extract prepared from β TC-3 cells, two protein-DNA complexes were detected (Fig. 6B). Competition experiments, in which a 100-fold molar excess of unlabeled DNA was included with the labeled probe, were used to investigate the specificity of this protein binding. The wild-type -170/-141 IGRP oligonucleotide competed effectively for the formation of both of these protein-DNA complexes (Fig. 6B). By contrast, a random oligonucleotide (designated RAN) and an oligonucleotide (designated IGRP MUT) that contains a mutation in the putative DeltaEF1 binding site (Fig. 6A) failed to compete with the labeled probe for formation of the upper complex (Fig. 6B, see arrow). This indicates that this upper complex represents a specific protein-DNA interaction, whereas the other complex detected in the assay represents a nonspecific protein-DNA interaction. To determine whether the specific protein-DNA complex contains DeltaEF1, the effect of preincubating β TC-3 nuclear extract with either preimmune serum or an antiserum specific for DeltaEF1 on the migration of this complex was investigated (Fig.

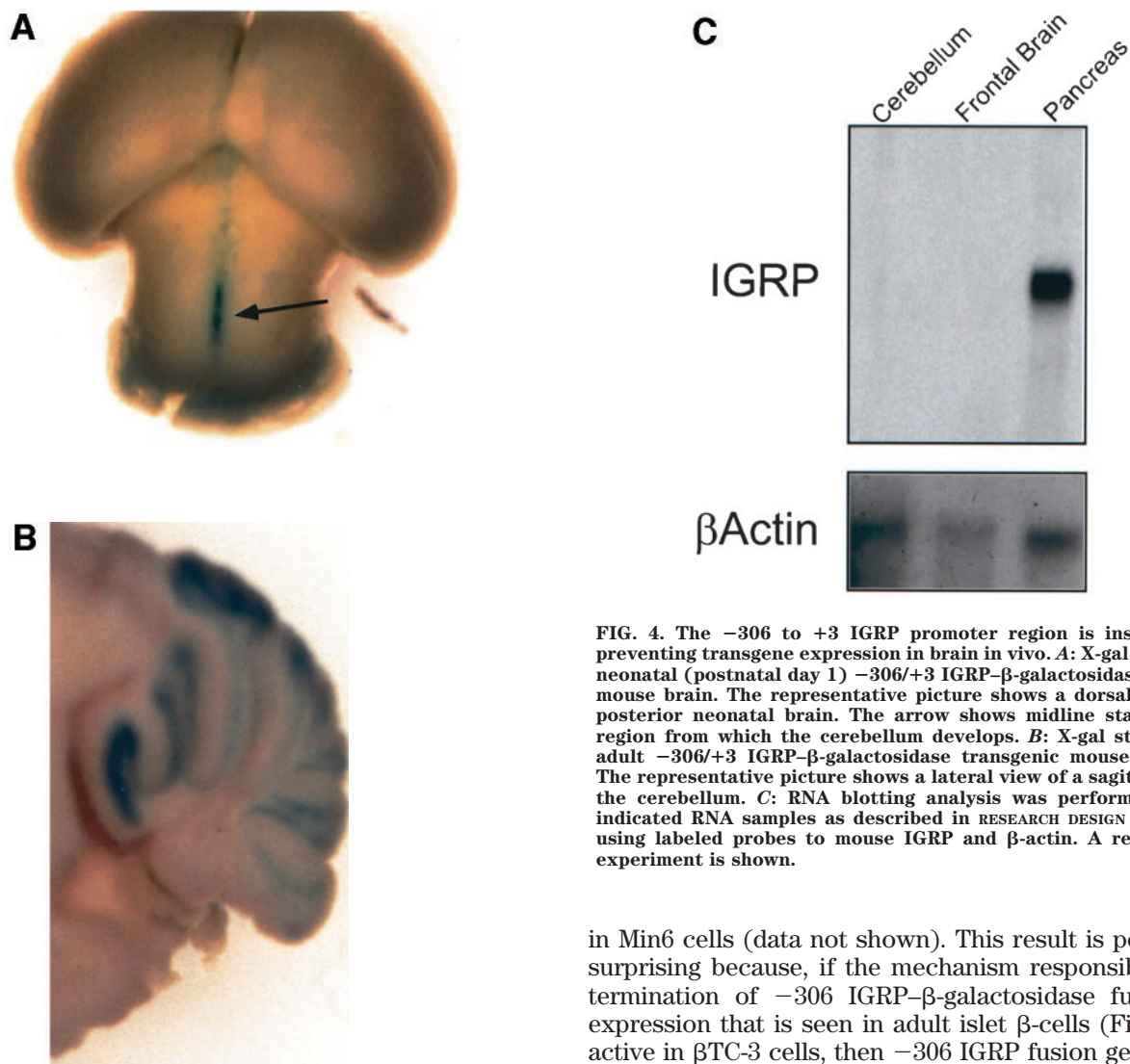


FIG. 4. The -306 to $+3$ IGRP promoter region is insufficient for preventing transgene expression in brain in vivo. **A:** X-gal staining of a neonatal (postnatal day 1) $-306/+3$ IGRP- β -galactosidase transgenic mouse brain. The representative picture shows a dorsal view of the posterior neonatal brain. The arrow shows midline staining in the region from which the cerebellum develops. **B:** X-gal staining of an adult $-306/+3$ IGRP- β -galactosidase transgenic mouse cerebellum. The representative picture shows a lateral view of a sagittal section of the cerebellum. **C:** RNA blotting analysis was performed with the indicated RNA samples as described in RESEARCH DESIGN AND METHODS using labeled probes to mouse IGRP and β -actin. A representative experiment is shown.

6B). Both the preimmune serum and DeltaEF1 antiserum reduced the formation of the nonspecific complex, whereas only the latter reduced the formation of the specific complex (Fig. 6B). Furthermore, the formation of this specific complex was dependent on the presence of zinc in the gel retardation assay (Fig. 6B), a requirement for optimal DeltaEF1 binding (26). These data suggest that the specific complex that forms with the $-170/-141$ IGRP WT oligonucleotide contains DeltaEF1. It has been previously shown that DeltaEF1 is present in the endocrine pancreas (42), but this is the first report of its binding to the promoter of an endocrine pancreas-specific gene.

To investigate the functional significance of these observations, the DeltaEF1 motif was mutated in the context of the -306 to $+3$ IGRP promoter region, and the level of reporter gene expression directed by a fusion gene containing this mutation was then analyzed by transient transfection of β TC-3 and Min6 cells. The mutation introduced was identical to that described in the IGRP MUT oligonucleotide (Fig. 6A), which disrupts DeltaEF1 binding in vitro (Fig. 6B). Figure 6C shows that in β TC-3 cells, mutation of the DeltaEF1 motif resulted in no significant change in promoter activity. A similar result was obtained

in Min6 cells (data not shown). This result is perhaps not surprising because, if the mechanism responsible for the termination of -306 IGRP- β -galactosidase fusion gene expression that is seen in adult islet β -cells (Fig. 5) were active in β TC-3 cells, then -306 IGRP fusion gene expression would not be detected in these cells. The fact that the -306 to $+3$ IGRP promoter is active in β TC-3, Min6, and HIT cells (21) suggests that these cell lines may be more characteristic of fetal/neonatal β -cells, rather than adult β -cells. Alternatively, it is possible that these cell lines are more characteristic of adult β -cells than fetal/neonatal β -cells, except that the mechanism responsible for directing the loss of IGRP transgene expression in vivo is not active in these cells in situ.

DISCUSSION

Transient transfection experiments show that the region of the IGRP promoter between -306 and $+3$ is sufficient to confer high basal fusion gene expression in HIT cells (2) and β TC-3 cells (Fig. 1). However, although the -306 to $+3$ IGRP promoter fragment is sufficient to drive highly islet-selective expression in newborn mice (Fig. 2 and data not shown) that initiates during the expected time in development (Fig. 3), it is insufficient to maintain expression in adult mouse islets (Fig. 5). This suggests that *cis*-acting elements elsewhere in the IGRP gene must be required for the maintenance of IGRP gene expression in adult mice. The results reveal that IGRP represents a unique transcriptional model for the study of islet development in that different *cis*-acting elements in the IGRP gene must be

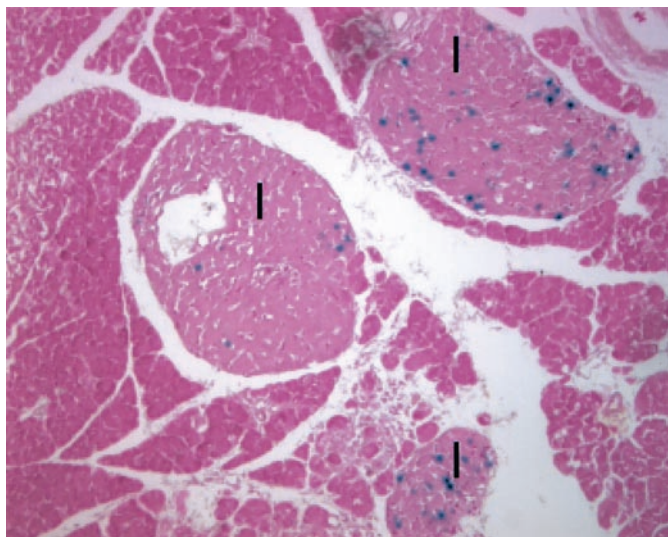


FIG. 5. The -306 to $+3$ IGRP promoter region is insufficient for maintaining transgene expression in adult mouse islets *in vivo*. X-gal staining of an adult (8 months old) $-306/+3$ IGRP- β -galactosidase transgenic mouse pancreas section. The section was counterstained with eosin. Only a few β -cells still express β -galactosidase in adult islets (*I*). A representative picture is shown. The picture is a $40\times$ magnification.

important for driving IGRP gene expression at different stages of development. Interestingly, Gannon et al. (45) were able to demonstrate a similar phenomenon in experiments that involved the ligation of fragments of the Pdx-1 promoter to a heterologous promoter. Thus, a transgene containing a *PstI*-*BstEII* fragment of the mouse Pdx-1 promoter, representing the promoter sequence from $-2,731$ to $-1,743$, ligated to a hsp68- β -galactosidase fusion gene, was expressed in all endocrine cell types starting at embryonic day 11.5, with expression maintained in adult islets (23,45). In contrast, a transgene containing an *XhoI*-*BglIII* fragment of the mouse Pdx-1 promoter, representing the promoter sequence from $-1,772$ to -727 , ligated to the hsp68- β -galactosidase fusion gene, was expressed specifically in islet β -cells starting at embryonic day 14.5, but expression was not maintained in adult islets (45). Whether these Pdx-1 promoter regions function at different stages of development in the context of the native Pdx-1 promoter remains to be determined.

The loss of IGRP- β -galactosidase transgene expression in adult islets suggests that the islet morphogenesis and acquisition of mature islet function that occurs after birth (32,33) results in a switch in the nature of the *cis*-acting elements and associated factors that are required for IGRP expression. This is consistent with the observations that gastrin (46), thyroid-releasing hormone (47), and peptidyl-glycine α -amidating monooxygenase (PAM) (48) gene expression in islets are markedly reduced during this transition, although transcription factors that account for these changes have not been identified. The scope of this remodeling is apparent from the results of a recent gene CHIP analysis that identified multiple genes whose expression are reduced during this transition (49).

The simplest model to explain the decrease in islet gastrin, thyroid-releasing hormone, and PAM gene expression at birth would be that a factor that is required to

initiate expression of these genes in embryonic islets is decreased in the neonatal-to-adult transition. Alternatively, a repressor, such as DeltaEF1 (Fig. 6), may be induced/activated during the neonatal-to-adult transition, which switches these genes off. The same two models can be invoked to explain how the truncated IGRP promoter is initially active in newborn mice and then becomes inactive in adult mice. However, our transgenic results reveal that the regulation of endogenous IGRP gene expression must be more complex. Thus, neither model accounts for the fact that the endogenous IGRP gene remains active in adult mice. The simplest possibility here is that an IGRP promoter region 5' of -306 contains a binding site(s) for a factor(s) required to maintain expression of the IGRP gene in adult mice. Studies on the IGRP promoter show that the islet-enriched transcription factors NeuroD/BETA2 (50), hepatocyte nuclear factor (HNF)-3 (HNF-3/FOXA) (21), and Pax-6 (C.C.M. and R.M.O., unpublished observations) bind the -306 to $+3$ promoter region and stimulate IGRP fusion gene transcription. However, the expression of these factors in islets does not decrease in the neonatal-to-newborn transition (51), suggesting that they are not involved in the loss of $-306/+3$ IGRP- β -galactosidase transgene expression in adult islets. Interestingly, all of these factors are also expressed in islet α -cells, even though endogenous IGRP gene expression in these cells is very low (4). This suggests that other factors must be involved in the selective induction of IGRP gene expression in islet β -cells or that IGRP gene expression is selectively regulated in islet α - or β -cells at a posttranscriptional level.

Although the -306 to $+3$ IGRP promoter region does not contain an HNF-6 binding site, this factor is a candidate to explain the decreased expression of at least some genes in islets in the neonatal-to-adult transition. HNF-6 is a dual cut domain and homeodomain-containing transcription factor that is broadly expressed in the developing endoderm, including the pancreas (52-54). HNF-6 is expressed early in development throughout the pancreas, but it becomes downregulated specifically in endocrine cells at e18.5, coinciding with islet morphogenesis (53,54). Interestingly, Gannon et al. (23) demonstrated the critical importance of this downregulation of HNF-6 expression for normal islet physiology by using an islet-specific enhancer from the Pdx-1 gene promoter to maintain HNF-6 expression in islets past the developmental time point at which it is normally downregulated. Mice in which HNF-6 continued to be expressed in islet endocrine cells after e18.5 had disrupted islet architecture and function, such that these mice were diabetic at weaning (23). These studies support the concept that the induction and downregulation of the expression of specific transcription factors are both necessary for normal islet morphogenesis and mature islet function.

In summary we show here that the -306 to $+3$ IGRP promoter region is sufficient to drive transgene expression in newborn mouse islets, but that it is insufficient to maintain this expression in adult mice. Future experiments will focus on the identification of the *cis*-acting elements and their associated factors that are required for the maintenance of IGRP gene expression in adult mice.

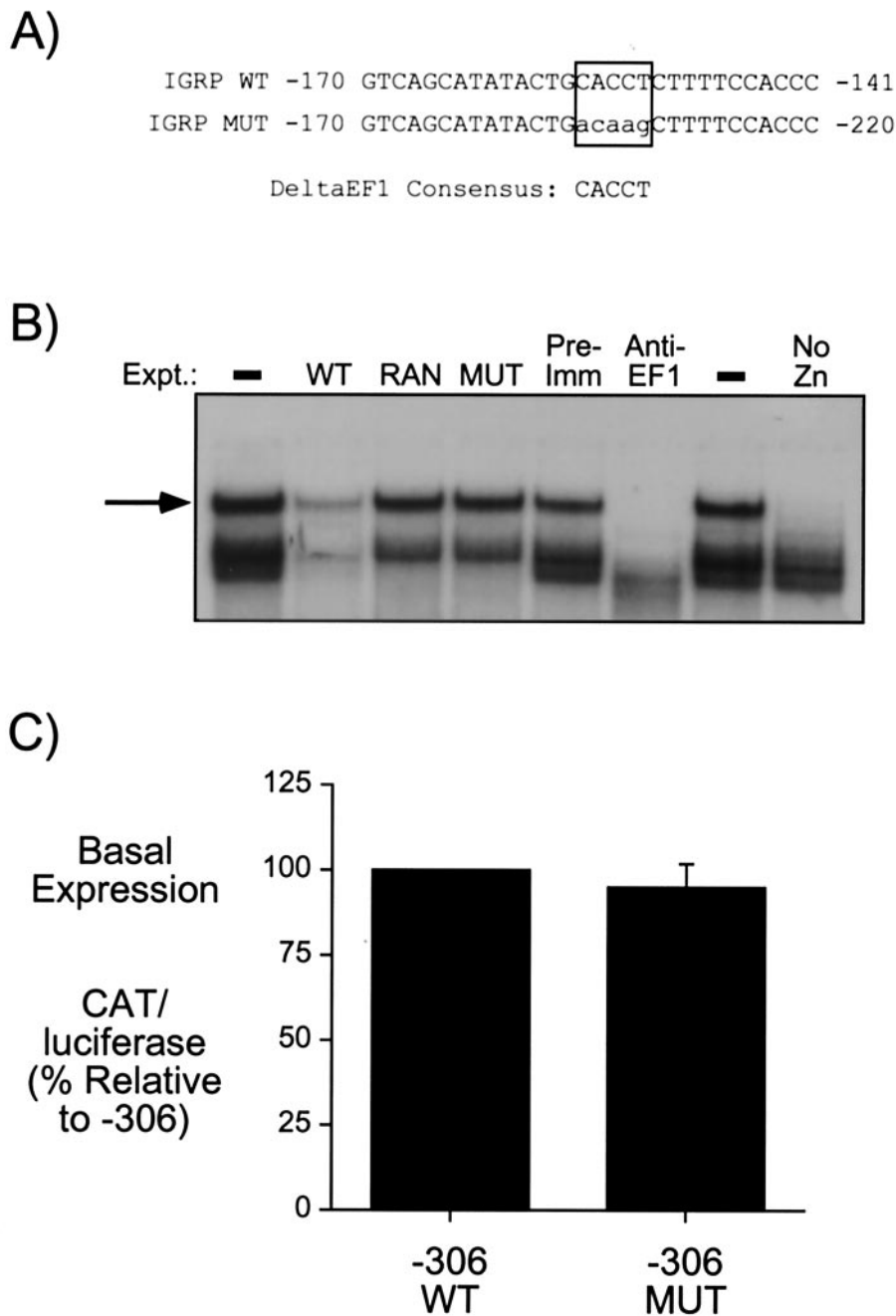


FIG. 6. The -156 to -152 region of the IGRP promoter binds DeltaEF1 in vitro. **A:** The sequence of the wild-type (WT) and mutant (MUT) oligonucleotides used in these studies. The DeltaEF1 binding motif is boxed, and the altered base pairs in the mutant IGRP sequence are shown in lower case letters. The consensus DeltaEF1 binding motif is taken from the report by Sekido et al. (26). **B:** A labeled oligonucleotide representing the wild-type $-170/-141$ IGRP sequence was incubated in the absence (-) or presence of a 100-fold molar excess of the unlabeled competitor DNAs shown, representing the wild-type (WT) and mutant (MUT) $-170/-141$ IGRP sequences (A) or a random (RAN) sequence. β TC-3 nuclear extract was then added, and protein binding was analyzed using the gel retardation assay in the presence of 0.1 mmol/l zinc sulfate, as described in RESEARCH DESIGN AND METHODS. Alternatively, preimmune (Pre-Imm) serum or DeltaEF1 antiserum (Anti-EF1) was included in the binding reaction or zinc sulfate was omitted. The representative autoradiograph only shows the retarded complexes and not the free probe, which was present in excess. The specific DeltaEF1 complex is indicated by the arrow. **C:** β TC-3 cells were transiently cotransfected, as described in RESEARCH DESIGN AND METHODS, with an expression vector encoding firefly luciferase (0.5 μ g) and either an IGRP-CAT fusion gene (2 μ g) containing the wild-type promoter sequence located between -306 and $+3$ (-306 WT) or an IGRP-CAT fusion gene containing a site-directed mutation of the DeltaEF1 motif (-306 MUT) generated within the context of the -306 to $+3$ promoter fragment. After transfection, cells were incubated for $18-20$ h in serum-containing medium. The cells were then harvested and both CAT and luciferase activity assayed as described RESEARCH DESIGN AND METHODS. Results are presented as the ratio of CAT to luciferase activity and represent the mean of three experiments \pm SE, each using an independent preparation of each fusion gene plasmid.

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