

MafB

An Activator of the Glucagon Gene Expressed in Developing Islet α - and β -Cells

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The large Maf family of basic leucine-zipper-containing transcription factors are known regulators of key developmental and functional processes in various cell types, including pancreatic islets. Here, we demonstrate that within the adult pancreas, MafB is only expressed in islet α -cells and contributes to cell type-specific expression of the glucagon gene through activation of a conserved control element found between nucleotides -77 to -51. MafB was also shown to be expressed in developing α - and β -cells as well as in proliferating hormone-negative cells during pancreatogenesis. In addition, MafB expression is maintained in the insulin⁺ and glucagon⁺ cells remaining in mice lacking either the Pax4 or Pax6 developmental regulators, implicating a potentially early role for MafB in gene regulation during islet cell development. These results indicate that MafB is not only important to islet α -cell function but may also be involved in regulating genes required in both endocrine α - and β -cell differentiation. *Diabetes* 55:297-304, 2006

The pancreatic islets of Langerhans are composed of α -, β -, δ -, and pancreatic polypeptide cells, which independently produce the hormones glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. Collectively, these hormones regulate both fuel and energy metabolism, with insulin and glucagon key to controlling glucose homeostasis (1). Thus, glucagon secreted from α -cells stimulates the mobilization of glucose through gluconeogenesis and glycogenolysis to prevent hypoglycemia, whereas β -cell-secreted insulin promotes glucose storage. Physiological glucose levels are maintained through the counter-regulatory actions of glucagon and insulin in peripheral tissues, with defects in α -

and β -cell function playing a significant part in the ability of diabetic patients to maintain glycemic control.

The identification and characterization of the transcription factors regulating insulin and glucagon expression have not only revealed their significance in islet function but also during pancreatogenesis. The pancreas develops from dorsal and ventral epithelial bud evaginations from the foregut, with glucagon-producing cells first appearing at mouse embryonic day (E) 9.5 in the dorsal bud (2-4). Insulin-producing cells emerge at E10.5 (3), whereas somatostatin and pancreatic polypeptide⁺ cells are not detected until E15.5 and E18.5, respectively (5). Insulin- and glucagon-producing cells appear in waves during development, with the functional α - and β -cells that will populate the islet produced starting at \sim E13.5 (5). This latter phase is termed the "secondary transition," and these cells continue to proliferate but are only organized into islet structures and become glucose-responsive shortly after birth (6).

The transcription factors associated with controlling cell-specific expression of the insulin and glucagon genes are principal regulators of islet cell formation, including Pdx1 (7-10), Pax6 (11,12), Pax4 (13,14), and NeuroD1 (15,16). Pdx1 is necessary for the growth of the endocrine and exocrine compartments, with pancreatic agenesis observed in *pdx1* mutant mice (17,18). In contrast, the loss of *pax6* (12,19,20), *pax4* (21,22), and *neuroD1* (23) only affects islet cell development. Heterozygous mutations in *Pdx1* (24), *Pax6* (25), and *NeuroD1* (26) also contribute to the formation of diabetes in humans, establishing a key conserved role for these factors in islet cell activity.

MafA is another essential activator of the insulin gene (27-31). Specific roles for MafA in processes important to islet cell formation and function have been suggested upon observing that MafA is first produced in insulin-producing cells of the secondary transition and was also capable of independently inducing insulin expression in non- β -cells (27,32). Its significance to β -cell function has recently been confirmed in MafA-deficient mice, which are glucose intolerant and develop diabetes (33). Interestingly, no developmental islet cell phenotype was reported in MafA^{-/-} animals, a surprise considering its embryonic expression pattern and that all other closely related members of the large Maf transcription factor family are associated with processes necessary for cell formation, including the brain (i.e., MafB [34,35]), cartilage (c-Maf [36]), and immune system (c-Maf [37]).

In addition to MafA, MafB is capable of activating

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CMV, cytomegalovirus; MOI, multiplicity of infection; PEPCK, phosphoenolpyruvate carboxykinase.

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insulin enhancer–driven expression in non-islet cell transfection assays, even though MafB is primarily found in islet α -cells (28). The members of the large Maf transcription factor family all share a highly conserved NH₂-terminal activation domain and a COOH-terminal basic leucine-zipper DNA-binding domain. Here, we show that MafB is an important regulator of glucagon gene expression. Thus, MafB was found to bind to and activate at conserved nucleotides –77 to –51 of the α -cell–specific promoter control element (termed G1). MafB was also capable of specifically activating endogenous glucagon expression when overexpressed in a β -cell line. Furthermore, MafB was present in both insulin- and glucagon-producing cells during development, with expression only restricted to α -cells soon after birth. Our data imply that MafB may be involved in regulating genes integral to islet α - and β -cell formation, with a long-term role in glucagon transcription in islets.

RESEARCH DESIGN AND METHODS

Immunohistochemistry. Adult tissue and staged embryos were obtained from wild-type (Balb/c), Pax4^{–/–} (22), and Pax6^{sey^{NEU}/sey^{NEU}} (38) mice (sey/sey). The day of vaginal-plug discovery was designated stage E0.5. Immunofluorescence and confocal image analyses were performed on paraffin sections as described previously (28). The primary antibodies used were rabbit anti-MafB (1:5,000; Bethyl Laboratories BL658, Montgomery, TX), goat anti-Pdx1 (1:10,000), mouse anti-ki67 (1:500; BD Biosciences, San Jose, CA), guinea pig anti-Ngn3 (1:1,000), guinea pig anti-insulin (1:2,000; Linco Research, St. Charles, MO), guinea pig anti-glucagon (1:2,000; Linco Research), guinea pig anti-pancreatic polypeptide (1:2,000; Linco Research), and sheep antisomatostatin (1:2,000; American Research Products, Belmont, MA). Secondary antibodies were Cy3- or Cy5-conjugated donkey anti-guinea pig, anti-sheep, anti-goat, and anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA). Fluorescent images were captured with a Zeiss LSM 510 confocal microscope using an optical depth of 1 μ m. Nuclear counterstaining was performed using YoPro1 (Molecular Probes, Eugene, OR).

DNA constructs and transfection assays. The glucagon –476 luciferase reporter (–476 GLU) contains rat glucagon gene sequences from nucleotides –476 to +10. The glucagon G1 mutant was generated in –476 GLU using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA) with the rat G1 MUT^{–73}ATTTATATTGTCCTGGTAATATCTGCA^{–47} oligonucleotide; the mutated sequence is underlined. The construction of the cytomegalovirus (CMV)-driven MafB expression construct (CMV:MafB) in pcDNA3.1 has been described previously (28). An expression construct encoding amino acids 210–323 of MafB (CMV:MafB [210–323]) was prepared by PCR amplification (top primer, 5'-ATGCGCTTCTCTGATGACCAGCTGG-3'; bottom primer, 5'-TCACAGAAAGAACTCAGGAGAGG-3') and subcloned into pcDNA3.1 (Invitrogen). All mutated constructs were verified by sequencing.

Monolayer cultures of HeLa and α TC-6 were maintained as described previously (28,32). Transfections were performed using the lipofectamine reagent (Invitrogen) under the manufacturer's conditions. Extracts from cells were prepared 40 to 48 h after transfection and analyzed for glucagon-driven firefly luciferase and thymidine kinase–driven renilla luciferase (i.e., using pHRL-TK; Promega, Madison, WI) activity using the Dual-Luciferase Reporter Assay System (Promega).

Adenovirus infection and real-time PCR analysis. Mouse MafB cDNA (28) was used to generate recombinant adenovirus (Ad-MafB) as described previously (39). An adenovirus containing the coding sequences for GFP (Ad-GFP) was used as a control. Purified adenovirus was used to infect 2×10^6 β TC-3 and NIH3T3 cells at multiplicities of infection (MOI) ranging from 2 to 100 for 5 h. No change in cell viability was found between adenovirus and mock-infected cells, as was determined by trypan blue staining (Sigma). Total RNA prepared using the RNeasy kit (Qiagen, Valencia, CA), and nuclear extracts were collected from cells 24 h after infection. Isolated RNAs were also subjected to DNaseI treatment using the Versagene RNA DNase kit (Gentra Systems) to remove genomic DNA. TAQMAN RT-PCR reverse transcript reagents (Applied Biosystems) were used to generate cDNAs from 0.5 μ g DNase-treated RNA.

PCR mixes were assembled using SYBR Green Master Mix reagents (Applied Biosystems), reactions were performed, and the results were analyzed using the Applied Biosystems Prism 7000 sequence detection system and software. The following mouse cDNA primer sets were used in the amplification step: glucagon top (5'-CATTACCACGACTACAGCAA-3') and glucagon

bottom (5'-TCATCAACCACTGCACAAAATCT-3'); insulin I top (5'-GCAAGCA GGTCATTGTTTCAAC-3') and insulin I bottom (5'-AAGCCTGGTGGGTTTG G-3'); MafB top (5'-CAACAGCTACCCACTAGCCA-3'), insulin II top (5'-CCACC CAGGCTTTGTCAA-3'), insulin II bottom (5'-CCAGCTCCAGTTGTTCCAC-3'), and MafB bottom (5'-GGCGAGTTTCTCGCATTTGA-3'); 18S top (5'-AGTCC CTGCCCTTGTACACA-3') and 18S bottom (5'-GATCCGAGGGCCTCACTAAAC-3'); and γ -actin top (5'-GCACCCGGTGCTTCTGAC-3') and γ -actin bottom (5'-C CAGATGCATACAAGGAC-3').

Electrophoretic mobility shift assays. Binding analysis was performed with a radiolabeled, wild-type rat glucagon G1 (G1 wild type) site probe (–⁷⁷GAAATTTATATTGTCAGCGTAATATCT^{–51}). Binding reactions (25 μ l total volume) were conducted at 4°C for 30 min with 5 μ l in vitro–transcribed and –translated MafB produced from CMV:MafB and 400 fmol ³²P–radiolabeled probe in binding buffer containing 10 mmol/l Tris (pH 8), 100 mmol/l NaCl, 1 mmol/l EDTA, 2 mmol/l dithiothreitol, 10% glycerol, and 1 μ g poly(dI-dC). Binding analysis was also conducted with 10 μ g nuclear protein prepared from HeLa cells transfected with CMV:Maf (210–323). Competition assays were performed under the same conditions, except that a 50-fold molar excess of unlabeled wild-type or mutant G1 (–⁷⁷GAAATTTATATTGTCCTGGTAATA TCT^{–51}, mutated bases underlined) was added to the reaction along with the probe before the addition of protein. Anti-MafB BL658 (1 μ g) antibody was incubated with nuclear extract for 30 min on ice before adding the probe. MafB blocking peptide (1 μ g; Bethyl Laboratories) was incubated with anti-MafB for 30 min on ice before addition of MafB. G1 element binding complexes were resolved on a 5% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide, 29:1) and run in TGE buffer (50 mmol/l Tris, 380 mmol/l glycine, and 2 mmol/l EDTA, pH 8.5). Gels were dried, and binding complexes were visualized by autoradiography.

Chromatin immunoprecipitation assay. β TC-3 cells ($\sim 2 \times 10^7$) infected with Ad-GFP or Ad-MafB at an MOI of 100 or uninfected α TC-6 cells (~ 0.5 to 1.0×10^8) were formaldehyde cross-linked, and the sonicated protein-DNA complexes were isolated under conditions described previously (28). Sonicated chromatin was incubated for 1 h at 4°C with anti-MafB BL658 antiserum. Antibody-protein-DNA complexes were isolated by incubation with A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA). PCR was performed on one-tenth of the purified immunoprecipitated DNA using Ready-to-Go PCR beads (Amersham Pharmacia Biotech, Rockville, MD) and 15 pmol of each primer (glucagon, –³⁵³CCAATCAAGGGATAAGACCCTC and +⁷AAGCTCTG CCCTTCTGCACCAG; and phosphoenolpyruvate carboxykinase [PEPCK], –⁴³⁴GAGTGACACCTCAGCTGTGG and –⁹⁶GGCAGGCCCTTTGGATCATAG CC). The PCRs were performed under the following conditions: one cycle of 95°C for 2 min followed by 28 cycles for 30 s at 95, 61, and 72°C. The correctness of the PCR products was confirmed by sequencing. Amplified products were resolved on a 1.4% agarose gel in Tris-acetate EDTA buffer containing ethidium bromide.

Statistical analysis. The significance of the experimental findings was determined by Student's *t* test from multiple independent comparisons ($n > 3$). A *P* value of <0.05 was considered significant.

RESULTS

MafB is exclusively expressed in islet α -cells in the adult pancreas. To determine the distribution of MafB in mouse islets, immunohistochemical studies were performed with anti-MafB BL658, a recently generated peptide-specific antibody. MafB has been reported to be present in α - and β -cells, but the accuracy of this work was in doubt because of cross-reactivity of the antiserum with the β -cell–specific MafA protein (28). MafB was exclusively detected in glucagon-producing islet cells but not in insulin- or somatostatin-producing cells (Fig. 1). MafB was also occasionally detected in pancreatic polypeptide⁺ cells (data not shown), which presumably represent rare glucagon⁺pancreatic polypeptide⁺ cells (40). In contrast, c-Maf was not detected in islet cells, even though our specific antibody provided the expected c-Maf expression pattern in lens cells (41) (data now shown). Western blot analysis performed with in vitro–transcribed and –translated MafA and MafB also confirmed that anti-MafB BL658 only recognized MafB and that anti-MafB P-20 used previously cross-reacted with MafA (data not shown) (28). Anti-MafB BL658 antibody was used for all subsequent

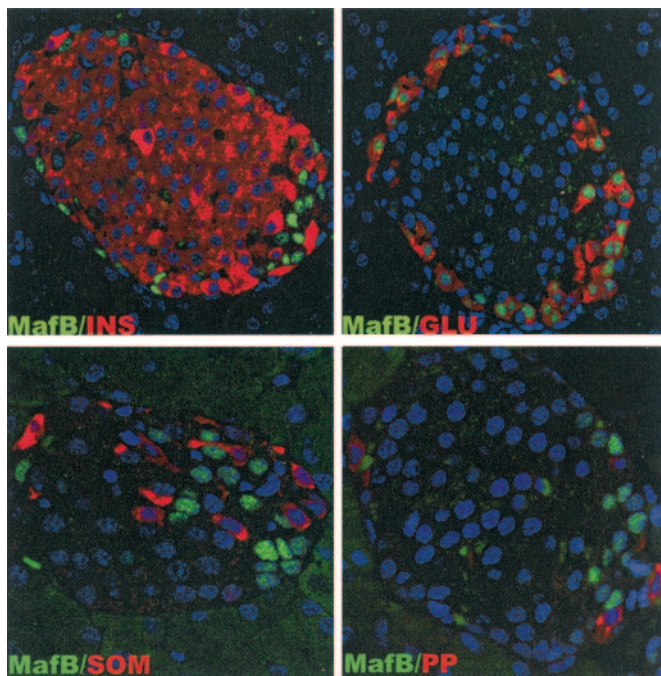


FIG. 1. MafB is expressed in α -cells but not in β - and δ -cells in the adult pancreas. Double immunofluorescence staining of adult pancreas sections with anti-MafB BL658 (green) and either anti-insulin (INS), α -glucagon (GLU), anti-somatostatin (SOM), or anti-pancreatic polypeptide antibody (red). MafB was detected in essentially all α -cells and rarely in pancreatic polypeptide cells. The nuclei are stained in blue.

studies. Importantly, these results indicated that MafB could be a key transcriptional regulator in islet α -cells.

MafB binds the glucagon gene promoter. Gel mobility shift assays were used to determine whether MafB was capable of specifically binding to the Maf protein binding site found within the G1 control region of the glucagon gene (42), located between nucleotides -77 and -51 (Fig. 2A). A unique binding complex was formed in the in vitro-transcribed and -translated MafB binding reaction, when compared with the control in vitro-transcribed and -translated sample. In competition analysis, wild-type G1 reduced the formation of this complex, whereas a MafB binding-defective mutant did not. In addition, the unique binding complex was super shifted with anti-MafB, an effect prevented by preincubation with the anti-MafB peptide antigen (Fig. 2A). Collectively, these results demonstrated that MafB was capable of selectively binding to the G1 control region of the glucagon gene in vitro.

To determine whether MafB also interacts with proximal control sequences of the endogenous glucagon gene, chromatin immunoprecipitation assays were performed in α TC-6 cells. Sequences spanning nucleotides -353 to $+7$ of the glucagon gene were PCR amplified from chromatin precipitated with anti-MafB, whereas chromatin treated with normal IgG or in the absence of antibody was not (Fig. 2B). Additionally, anti-MafB was incapable of precipitating control region sequences for PEPCK, a gene not expressed in α -cells (data not shown). These results demonstrated that MafB binds directly to regulatory sequences containing the G1 element in the endogenous glucagon gene.

MafB activates glucagon gene transcription. To test how MafB influences glucagon transcription, we first analyzed how the binding defective -77 - to -51 -bp mutant in

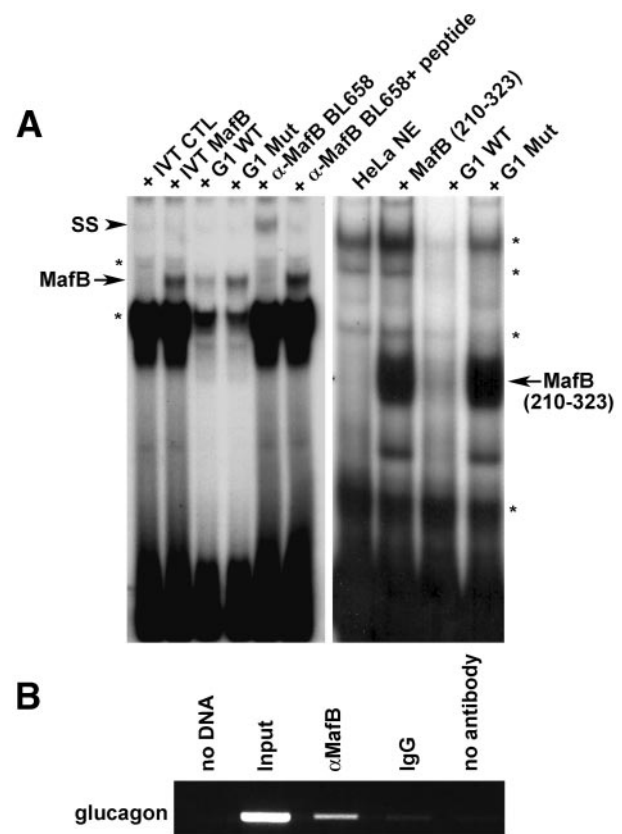


FIG. 2. MafB binds to the glucagon promoter in vivo. **A:** Gel shift binding reactions in the left panel were performed with the glucagon G1 element ($-77/-51$) probe in the presence of in vitro-transcribed and -translated MafB or a no DNA control (CTL). G1 element probe binding was also evaluated in the presence of the G1 wild-type (WT) and mutant (Mut) competitor and the MafB BL658 antibody with or without the blocking peptide. The specific MafB complex is super shifted (SS) using anti-MafB BL658, an effect prevented by the blocking peptide. The specificity of MafB (210–323) binding to G1 in the right panel was determined with G1 wild-type and mutant G1 competitors in HeLa nuclear extracts prepared from cells transfected with or without MafB (210–323). Nonspecific binding complexes are marked with an asterisk. **B:** The cross-linked DNA precipitated from α TC-6 cells with anti-MafB BL658 was analyzed by PCR with glucagon control region-specific primers. A representative experiment is shown ($n = 3$). As controls, PCRs were run with no DNA (–), input chromatin (1:100 dilution), and DNA obtained after being treated with rabbit IgG or with no antibody.

the G1 element affected a reporter driven by rat glucagon sequences spanning -476 to $+10$ base pairs. The activity of the binding-deficient mutant was reduced to roughly 30% of wild-type activity in α TC-6 cells (compare mutant G1 with -476 GLU in Fig. 3A). Consistent with a role in positive control, the MafB dominant negative-acting mutant, MafB (210–323), inhibited -476 GLU activity in α TC-6 cells (Fig. 3A). MafB (210–323) lacks an NH_2 -terminal transactivation domain but still retains the ability to bind specifically, as determined in gel shift competition assays with wild-type and mutant G1 (Fig. 2A). Furthermore, wild-type MafB expression resulted in a 50-fold activation of -476 GLU in non-glucagon-expressing HeLa cells, an activity severely attenuated in -476 GLU G1 mutant (Fig. 3B).

To further investigate MafB activation of the glucagon gene, an adenovirus containing MafB coding sequences (Ad-MafB) was used to overexpress MafB in β TC-3 insulinoma cells and the nonislet NIH3T3 cell line. MafB protein and mRNA were effectively induced in a dose-

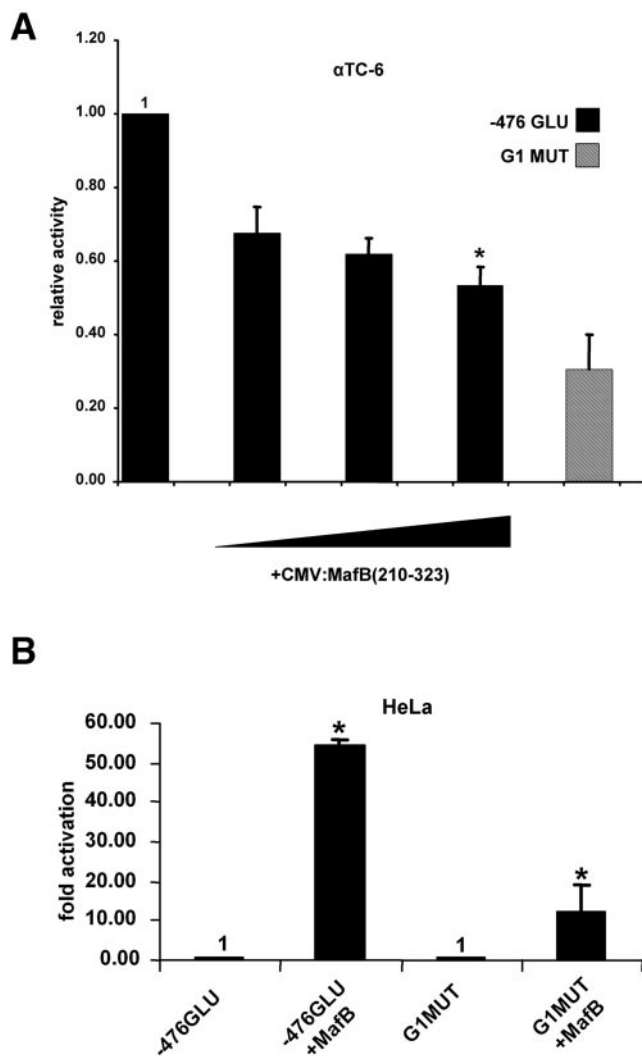


FIG. 3. MafB stimulates glucagon gene transcription. *A:* α TC-6 cells were transfected with wild type (solid bar) or the mutant G1 (hatched bar) of -476 GLU along with a dominant negative-acting MafB (CMV: MafB [210–323]) or the pcDNA3.1 vector control. MafB (210–323) reduced wild-type -476 GLU activity to roughly that of the mutant G1. CMV:MafB (210–323) was present in two- to eightfold the level of -476 GLU. Normalized luciferase activities \pm SE are presented relative to -476 GLU ($n = 3$). * $P < 0.05$ vs. -476 GLU alone. *B:* HeLa cells were transfected with wild type or the mutant G1 of -476 GLU and CMV: MafB or pcDNA3.1. Normalized luciferase activities \pm SE are reported relative to -476 GLU or the mutant G1, respectively, GLU ($n = 5$). * $P < 0.05$ vs. -476 GLU alone or -476 GLU +MafB.

dependent manner in Ad-MafB-infected cells, resulting in substantial overexpression compared with control cells infected with the adenovirus encoding GFP (Ad-GFP) (Fig. 4A). MafB levels in uninfected β TC-3 cells were low, with expression comparable with endogenous α TC-6 cell levels in Ad-MafB 1 \times -infected samples (Fig. 4A; data not shown). In accordance with this pattern, binding of MafB to the G1 element was also augmented in Ad-MafB-infected cells (data now shown). The effect of adenoviral delivery of MafB on glucagon gene activation was determined by quantitative real-time PCR. Infection with Ad-MafB resulted in a modest, dose-dependent two- to eightfold increase in endogenous glucagon mRNA levels in β TC-3 cells, whereas endogenous insulin, Pdx1, and γ -actin expression was not influenced significantly (Table 1). In contrast, Ad-MafB did not increase endogenous glucagon mRNA levels in either NIH3T3 (Table 1) or α TC-6 cells

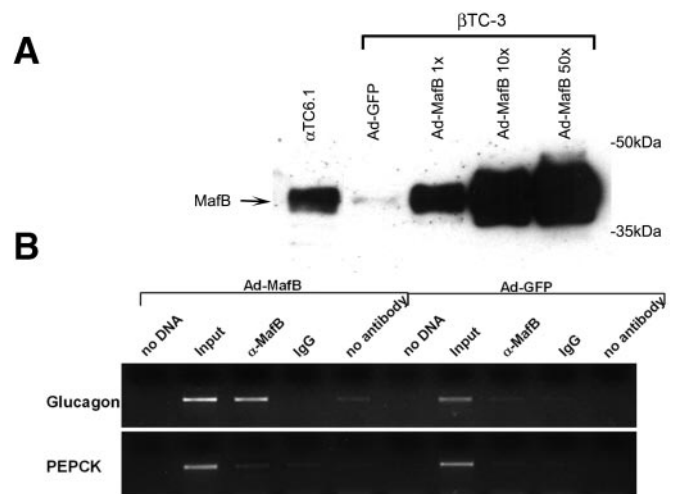


FIG. 4. Adenovirus overexpression of MafB activates endogenous glucagon gene transcription in β TC-3 cells. *A:* Western blot analysis with anti-MafB BL658 of nuclear extracts (15 μ g) from α TC-6 cells alone and β TC-3 cells infected with Ad-GFP (MOI 100) or Ad-MafB (1 \times = MOI 2; 10 \times = MOI 20; and 50 \times = MOI 100). The endogenous MafB protein level in β TC3 cells is represented in the Ad-GFP lane. MafB levels were very similar between α TC-6 cells and 1 \times Ad-MafB-infected β TC-3 cells. *B:* The cross-linked DNA from Ad-GFP-infected (MOI 100) or Ad-MafB-infected (50 \times = MOI 100) β TC-3 cells was precipitated with anti-MafB BL658 or IgG and analyzed by PCR with glucagon and PEPCK control region-specific primers. As controls, PCRs were run with no DNA (-), input chromatin (1:100 dilution), and DNA obtained after being treated with rabbit IgG or with no antibody.

(data not shown). Chromatin immunoprecipitation experiments also revealed that MafB occupancy of endogenous glucagon G1 control region spanning sequences was increased in β TC-3 cells infected with Ad-MafB (Fig. 4B), confirming that the amplification of glucagon gene expression was, at least in part, due to direct transactivation by MafB. Collectively, these results suggest that MafB is a significant activator of glucagon gene expression. In addition, that high-level, α -cell-like transcription of the glucagon gene involves cooperation between MafB with other distinct cell enriched factors.

TABLE 1
Ad-MafB activates endogenous glucagon gene transcription in β TC-3 cells and not NIH3T3

	Ad-GFP	Ad-MafB		
		1 \times	10 \times	50 \times
βTC-3				
MafB	1.00	45.52	1,230.57	18,260.62
Glucagon	1.00	2.02	3.48	7.83
Insulin I	1.00	1.29	1.37	1.30
Insulin II	1.00	1.09	1.04	1.00
pdx1	1.00	1.49	0.83	0.81
γ -Actin	1.00	1.17	0.78	0.88
NIH3T3				
MafB	1.00	27.61	143.99	1,486.43
Glucagon	ND	ND	ND	ND
Insulin I	ND	ND	ND	ND
pdx1	ND	ND	ND	ND
γ -Actin	1.00	0.98	0.85	0.96

Data are expressed as the normalized fold difference relative to GFP alone (defined as 1). MafB and glucagon mRNA levels in β TC-3 and NIH 3T3 cells infected with Ad-GFP (MOI 100) or Ad-MafB (1 \times = MOI 2; 10 \times = MOI 20; 50 \times = MOI 100). All of the expression data were derived by real-time PCR analysis. A representative experiment is shown ($n = 3$). ND, not detected.

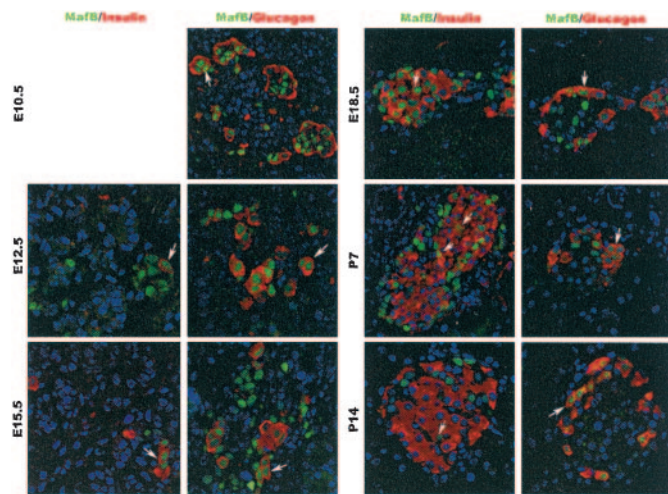


FIG. 5. MafB is expressed in both insulin- and glucagon-expressing cells during pancreas development. Double immunofluorescence staining of embryonic and postnatal mouse pancreatic sections with anti-MafB serum (green) and either anti-insulin or anti-glucagon (red). MafB was found in glucagon⁺ and insulin⁺ cells by E10.5 and E12.5 (arrows), respectively. Postnatally, MafB becomes restricted to α -cells. Nuclei are stained in blue.

MafB is expressed in α - and β -cells during development. MafB expression was next analyzed during pancreas development by immunohistochemistry (Fig. 5). Expression was initially observed in glucagon⁺ cells at E10.5. MafB was also detected in the early insulin-producing cells at E12.5, and expression persisted in glucagon- and insulin-producing cells throughout pancreas development (Fig. 5) but was not found in either somatostatin or pancreatic polypeptide cells (data not shown). These results establish that MafB is selectively produced in insulin⁺ and glucagon⁺ islet progenitor cells during pancreas development.

MafB becomes progressively restricted to α -cells postnatally because expression in β -cells is downregulated within 2 weeks of birth and is undetectable in these cells by 3 weeks (Fig. 5; data not shown). To further characterize the transition between differentiating insulin-producing cells (MafB⁺) and mature β -cells (MafB⁻), colocalization of MafB with Pdx1 was examined at E18.5 and P4. Pdx1 is principally expressed in insulin⁺ cells and not glucagon⁺ cells at these times (43). As expected, only a few Pdx1⁺MafB⁺ cells were detected (Fig. 6A). In contrast to the pancreas, MafB was expressed in essentially all Pdx1⁺ cells along the villi of the duodenum at P4 and at lower levels in many Pdx1⁺ cells in the duodenal crypts (Fig. 6A). This pattern indicates that MafB may also be contributing in the duodenum to transcription of large Maf-regulated genes like *pdx1*.

MafB is expressed in endocrine progenitor cells within the developing pancreas. MafB expression was also detected in some surrounding non-hormone-expressing cells between E12.5 and E18.5 (data not shown), potentially representing the MafB⁺Neurogenin3⁺ islet progenitor cells detected by microarray analysis (44). Costaining of MafB and Neurogenin3⁺ (Ngn3⁺) supported this hypothesis because MafB was found in a fraction of the Ngn3⁺ cells at early endocrine differentiation stages (Fig. 6B). The presence of MafB in Ngn3⁺ cells, a transcription factor essential for the production of all islet cell types (45), suggests that MafB may be present in proliferating islet progenitor cells. Coexpression of MafB and ki67, a

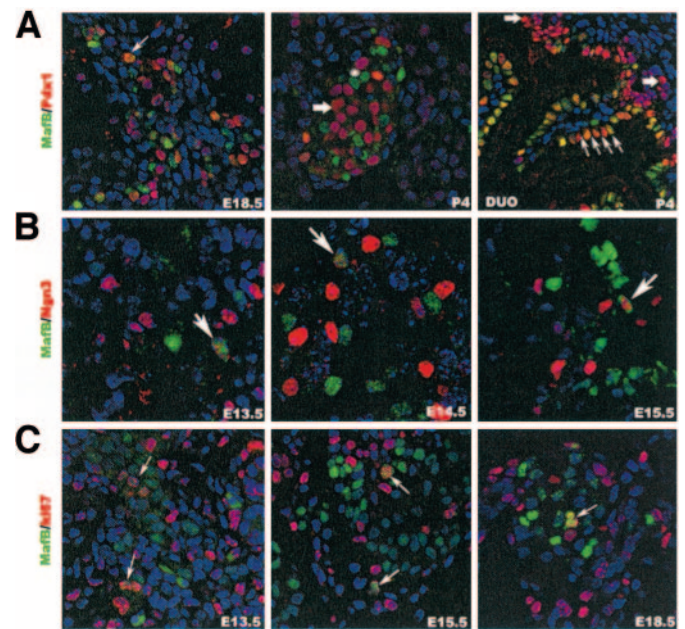


FIG. 6. Colocalization analysis of MafB with Pdx1, Ngn3, and ki67. **A:** Double immunofluorescence staining of E18.5 and P4 pancreatic and P4 duodenum (DUO) sections with anti-MafB (green) and anti-Pdx1 (red). Pdx1 and MafB expression domains overlapped in developing pancreata (arrows represent MafB⁺Pdx1⁺ cells) but became separated postnatally (thick arrows indicate cells expressing Pdx1 only, whereas a MafB⁺ cell is marked with an asterisk). MafB and Pdx1 were coexpressed in the villi of the duodenum. Nuclei were stained in blue. **B:** Double immunofluorescence staining of E13.5, E14.5, and E15.5 pancreatic sections with anti-MafB (green) and anti-Ngn3 (red) antibodies. MafB was only detected in some Ngn3⁺ cells (arrows). **C:** Double immunofluorescence staining of E13.5, E15.5, and E18.5 pancreatic sections with anti-MafB (green) and anti-ki67 (red). A small fraction of MafB⁺ cells coexpressed the ki67 proliferation marker (arrows).

marker for dividing cells, was found in some MafB⁺ cells throughout development (Fig. 6C). At E13.5, the MafB⁺ki67⁺ cells are readily detectable, but these cells become less abundant at E15.5 and E18.5. Because glucagon and insulin are not expressed in replicating cells during pancreatogenesis (46,47), these observations suggest that MafB is expressed in both dividing and nondividing islet progenitor cells.

MafB expression is maintained in insulin- and glucagon-producing cells of Pax4- and Pax6-deficient animals. To provide insight into when MafB acts during pancreatic development, expression was analyzed in Pax4- and Pax6-deficient mice. The vast majority of β - and δ -cells (22) are lost in *pax4* mutant mice, whereas glucagon- and insulin-producing cell numbers are severely reduced in both Pax6^{-/-} (19,20) and Pax6^{sey/sey} (12) animals. The Pax6^{sey/sey} mutation prevents translation of a functional protein (38). MafB expression was observed in the insulin⁺ and glucagon⁺ cells remaining in Pax4 and Pax6^{sey/sey} mutants at E15.5 (Fig. 7), indicating that neither Pax4 nor Pax6 is required for MafB expression. These results may also indicate that MafB can participate in gene regulation early in α - and β -cell development, before Pax4 and Pax6 expression.

DISCUSSION

Large Maf proteins are involved in regulating the transcription of genes important to cell type-specific expression and differentiation. MafA has been identified as a β -cell-specific activator of the insulin gene (28–30) and was

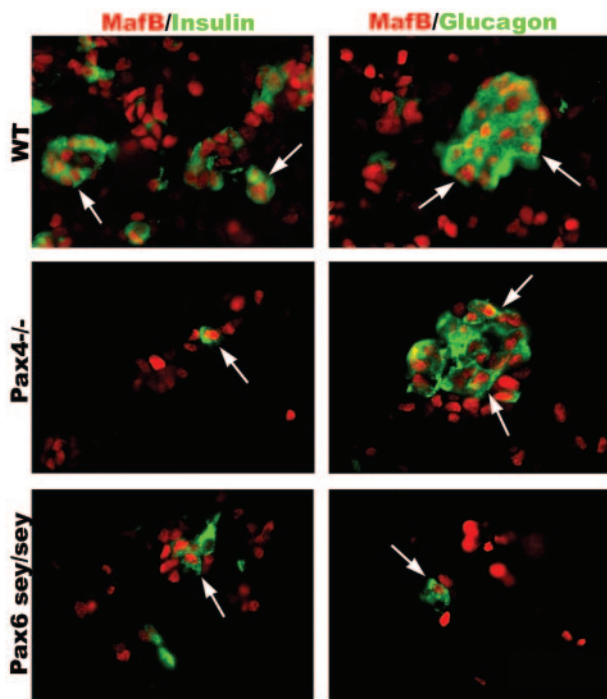


FIG. 7. MafB is expressed in Pax4 and Pax6 *sey/sei* mutant animals. Double immunofluorescence staining of E15.5 pancreatic sections of wild-type, Pax4, and Pax6 *sey^{NEU}/sey^{NEU}* mutant mice with anti-MafB (red) and either anti-insulin or anti-glucagon (green). MafB expression was detected in the insulin⁺ and glucagon⁺ cells produced in both the Pax4 and Pax6 mutants.

recently found to be critical to glucose-stimulated insulin secretion in vivo (33). In addition to MafA, the closely related MafB protein is also capable of activating insulin gene expression (28). In this study, we have shown that MafB can also specifically activate glucagon gene expression in cell culture-based assays and directly binds to the control region of the endogenous glucagon gene. In addition, MafB was coexpressed with insulin and glucagon during pancreatogenesis and in early replicating non-hormone-producing cells. Collectively, our data suggest that MafB may be involved in both α - and β -cell formation and islet α -cell function.

Immunohistochemical analysis with the newly available peptide-specific antibody BL658 revealed that MafB is only present in islet α -cells within the adult pancreas (Fig. 1). The previous detection of MafB in islet β -cells most likely represents cross-reactivity with MafA (28). The unique presence of MafB in α -cells strongly indicates a key role in α -cell function. Our transfection and gel shift results strongly suggest that MafB activates glucagon gene expression through the G1 element at bp -77 to -51 . However, c-Maf has also recently been proposed to stimulate glucagon expression (48). Because this suggestion was primarily based on studies in cell lines and due to our inability to detect c-Maf in islet cells (data not shown), we do not believe that this factor regulates glucagon expression in vivo. c-Maf mRNA levels are a small fraction of MafA (1 of 100) and MafB (1 of 50) in the islet (28). Presumably, the c-Maf mRNA present in islet samples (28) was either due to relatively low levels of expression in islet cells and/or due to contamination from blood cells present in these highly vascularized structures (37). Most significantly, MafB was shown to specifically bind to sequences associated with control of the endogenous glucagon gene in the islet α -cell line, α TC-6 (Fig. 2).

Adenoviral-mediated overexpression of MafB in β TC-3 cells resulted in activation of endogenous glucagon gene expression (Table 1). Although commonly considered to represent a β -cell line, β TC-3 cells also express low levels of both glucagon and MafB. Strikingly, Ad-MafB failed to activate *insulin* and *pdx1* in β TC-3 cells, two large Maf protein target genes associated with β -cell identity (49). This may indicate that large Maf protein activity is not limiting to transcription of these genes in this β -cell line. In contrast to *insulin* or *pdx1*, *glucagon* is expressed very inefficiently in β TC-3 cells and hence may be more sensitive to MafB-mediated activation. Similarly, *glucagon* expression was not induced by Ad-MafB in α TC-6 cells, presumably because of high-level expression in this α -cell line. On the other hand, the inability of MafB to activate β -cell genes may reflect an unappreciated specificity of action between large Maf proteins, which are generally thought to be functionally interchangeable in vitro (28). Glucagon mRNA expression induced by Ad-MafB in β TC-3 cells was also very low relative to α -cells (data not shown), suggesting that other cell-enriched factors act together with MafB to stimulate high-level gene transcription (e.g., Brn4) (50). This proposal is consistent with both our inability to activate *glucagon* in either α TC-6 or NIH3T3 cells (Table 1; data not shown) and the absence of glucagon in developing insulin⁺/MafB⁺ cells (Fig. 5).

During pancreas development, MafB was initially detected in glucagon⁺ cells by E10.5 and in insulin⁺ cells by E12.5 (Fig. 5). Interestingly, MafB becomes progressively restricted to α -cells postnatally because expression in β -cells is undetectable within ~ 2 weeks of birth. Although it is unclear why MafB is lost in β -cells during this time, this period coincides with profound morphological and physiological changes to islet cells. Thus, α -, β -, δ -, and pancreatic polypeptide cells arrange into the mature islet configuration, with β -cells in the core and other cells at the periphery, and migrate away from ductal structures. In addition, and perhaps most importantly, the glucose responsiveness of β -cells is established at this time (6).

The MafB expression pattern is unique because no other known pancreatic transcription factor is expressed in only α - and β -cell progenitors and then becomes restricted to α -cells postnatally. In general, the transcription factors important to the islet can be grouped according to their expression pattern within the developing pancreas. Thus, such transcription factors have been found in early non-hormone progenitor cells (i.e., Ngn3), cells that produce each of the endocrine hormones (NeuroD1, Isl1, and Pax6) as well as in a specific hormone-producing cell type and its progenitors (Nkx6.1, Nkx2.2, Pax4, ARX, and Brn4) (51,52). However, none of these patterns apply to MafB or the closely related MafA transcription factor, which is only found in insulin-producing cells of the secondary transition and mature islet β -cells (28,32). MafB was also the most highly expressed gene within the islet among the 27 found within the susceptibility locus for type 2 diabetes on human chromosome 20 (53). Significantly, the transient expression of MafB in differentiating and immature insulin-producing cells implies its likely involvement in regulation of genes important to β -cell development, whereas the persistent presence in glucagon⁺ cells indicates a more fundamental role for MafB in islet α -cells.

MafB and Ngn3 expression domains partially overlapped during early pancreas development (Fig. 5), possibly indicating that proliferating MafB⁺ cells represent MafB⁺/Ngn3⁺/hormone⁻ progenitor cells (Fig. 8). This

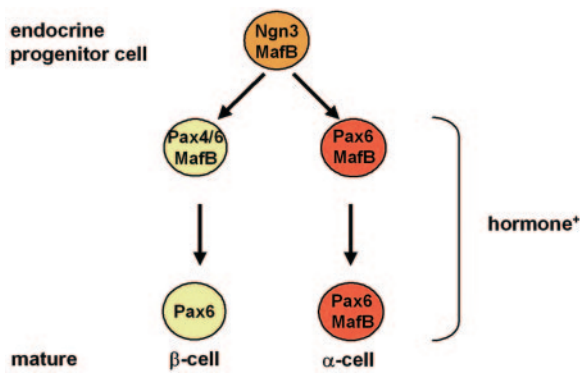


FIG. 8. Expression pattern of MafB during α - and β -cell differentiation. MafB is expressed in $ngn3^+$ cells (orange [44]), insulin $^+$ (green), and glucagon $^+$ (red) cells. Expression of MafB is maintained in the insulin- and glucagon-producing cells in Pax4 and Pax6 mutant mice. Developing β -cells produce both Pax4 and Pax6, whereas α -cells only express Pax6. MafB expression persists only in α -cells after birth.

proposal is consistent with the essential but transient nature of Ngn3 in islet cell formation. In addition, the maintenance of expression in the insulin $^+$ and glucagon $^+$ cells of Pax4- and Pax6-deficient mice implies an early role in transcriptional regulation in both α - and β -cell development. Alternatively, MafB could act in parallel with Pax4 and Pax6 in developing α - and β -cells. Unfortunately, MafB-deficient mouse lines have not been examined for islet phenotypes (34), an analysis that will be essential for fully understanding the importance of MafB in α - and β -cell formation and function. Based on our observations, however, defects in the formation of both insulin $^+$ and glucagon $^+$ cells in the developing pancreas of these animals are predicted, as well as long-term deficiencies in proper regulation of glucose metabolism.

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