MOLECULAR AND CELLULAR BIOLOGY, Sept. 2010, p. 4234–4244 Vol. 30, No. 17
0270-7306/10/$12.00 doi:10.1128/MCB.01396-09

Islet β-Cell-Specific *MafA* Transcription Requires the 5′-Flanking Conserved Region 3 Control Domain▽

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Received 22 October 2009/Returned for modification 28 December 2009/Accepted 12 June 2010

MafA is a key transcriptional activator of islet β cells, and its exclusive expression within β cells of the developing and adult pancreas is distinct among pancreatic regulators. Region 3 (base pairs −8118 to −7750 relative to the transcription start site), one of six conserved 5′ *cis* domains of the *MafA* promoter, is capable of directing β-cell-line-selective expression. Transgenic reporters of region 3 alone (R3), sequences spanning regions 1 to 6 (R1-6; base pairs −10428 to +230), and R1-6 lacking R3 (R1-6$^{\Delta R3}$) were generated. Only the R1-6 transgene was active in MafA$^+$ insulin$^+$ cells during development and in adult cells. R1-6 also mediated glucose-induced *MafA* expression. Conversely, pancreatic expression was not observed with the R3 or R1-6$^{\Delta R3}$ line, although much of the nonpancreatic expression pattern was shared between the R1-6 and R1-6$^{\Delta R3}$ lines. Further support for the importance of R3 was also shown, as the islet regulators Nkx6.1 and Pax6, but not NeuroD1, activated *MafA* in gel shift, chromatin immunoprecipitation (ChIP), and transfection assays and *in vivo* mouse knockout models. Lastly, ChIP demonstrated that Pax6 and Pdx-1 also bound to R1 and R6, potentially functioning in pancreatic and nonpancreatic expression. These data highlight the nature of the *cis*- and *trans*-acting factors controlling the β-cell-specific expression of *MafA*.

MafA is an essential transcriptional activator in adult pancreatic islet β cells, due in part to regulating genes associated with cell identity, including *insulin*. MafA is produced only in insulin$^+$ cells within the pancreas, with production first detected around embryonic day 13.5 (E13.5), during the secondary and principal wave of insulin$^+$ cell production (33). This expression pattern is not novel just among islet-enriched transcription factors but even for *insulin*, as both are produced earlier, with transcription factors also more broadly expressed. Thus, *insulin* is expressed prior to E13.5 in a distinct population of cells which lack important regulatory molecules necessary for islet β-cell function (40, 41). Adult islet *MafA* levels appear to be a sensitive barometer of β-cell function, since many key metabolic and cellular effectors, such as glucose (20, 26, 53, 58), fatty acids (18), and insulin (52), greatly impact *MafA* expression.

The functions of islet-enriched transcription factors in pancreatic function and formation have been examined in detail by use of gene knockouts in mice. For example, global Pdx-1 null mice are apancreatic because of the role of Pdx-1 in early endocrine and exocrine progenitor development (24, 39), while later β-cell-specific removal results in cell dysfunction and diabetes (1, 10). In contrast, all other factors act later and more specifically, as exemplified by the reduction in distinct islet cell populations in *Pax6*$^{-/-}$ (i.e., α and β cells) (47, 51) and *Nkx6.1*$^{-/-}$ (β cells) (48) mice and the general loss of these populations in *NeuroD1*$^{-/-}$ mice (36).

MafA is an atypical islet-enriched transcription factor in not being essential to β-cell development, presumably due to compensation by the closely related MafB protein, which is silenced in β cells and exclusively expressed in α (glucagon hormone$^+$) cells soon after birth (2, 3). However, MafA is critical to adult β-cell function, with defects in knockout mice affecting multiple gene products associated with cell maturation (e.g., glucose transport, *insulin* transcription, glucose sensing, and the insulin secretory machinery [2a, 55, 57]). These results are further supported by the observation that human embryonic stem cells differentiated to produce insulin and many islet-enriched transcription factors were neither glucose responsive nor capable of protecting against streptozotocin-induced hyperglycemia until they became MafA$^+$ (7, 28).

The *cis*-acting sequences directing β-cell-enriched transcription have been defined most extensively for the *insulin* and *Pdx-1* genes. Control is mediated by sequences that are well conserved between mammalian genes, residing approximately between bp −250 and +1 (relative to the transcription start site) in the *insulin* gene and between bp −2761 and −2457 (termed area I) and bp −2153 and −1923 (area II) in *Pdx-1*. Notably, while each of these regions has a β-cell-line-specific activation pattern *in vitro*, none independently recapitulates the expression pattern of the endogenous gene in transgenic

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▽ Published ahead of print on 28 June 2010.

assays *in vivo*. Thus, *insulin*-transgenic reporters driven by roughly 700 bp of the proximal promoter are expressed selectively in islet β cells, but also in the brain (12). The proper β-cell-specific pattern was observed with a transgene containing approximately 8.5 kb of the *insulin* promoter (19). Similarly, only an area I/area II-driven transgene reiterated the endogenous *Pdx-1* expression pattern in developing and adult islet β cells (54). Early exocrine and endocrine *Pdx-1* expression is mediated by sequences within areas I, II, and III, with area III (bp −1879 to −1600) binding to the PTF1a transcription factor, a factor essential for acinar and endocrine progenitor cell development, contributing to activation (56).

There are six areas of high sequence identity within 10 kbp of the mammalian *MafA* gene (termed regions 1 through 6 [R1 to R6]), but just R3 (bp −8118 to −7750) is able to direct β-cell-line-selective reporter transcription (44). R3 is also the only conserved sequence domain in the chicken *MafA* promoter, with an 88% level of identity to the human gene over the 370-bp control domain. Interestingly, this identity is much greater than that in other islet β-cell control regions, such as *insulin* (63% identity between human and mouse I or mouse II genes [21]) or *Pdx-1* (78% identity between area II of the human and mouse genes [14]). We first sought to determine the significance of R3 in directing *MafA* expression to insulin$^+$ cells *in vivo*. Here we demonstrate that a bp −10428- to +230-driven transgene spanning R1 through R6 (termed R1-6:*eGFP*) recapitulated the endogenous pancreatic *MafA* expression pattern in mice during development and in adults, but transgenes driven by R3 alone or R1-6 lacking R3 (R1-6$^{\Delta R3}$) did not. Interestingly, although the nonpancreatic expression pattern of MafA has not been analyzed in mammals, R1-6:*eGFP* and R1-6$^{\Delta R3}$:*eGFP* were expressed in many tissues in the chicken (e.g., eye, nervous system, and limbs [29]). In addition, islet R1-6:*eGFP* activity was stimulated by glucose, the most important effecter of β-cell function. The essential role of R3 in driving *MafA* expression in β cells was also highlighted by our ability to link Pax6 and Nkx6.1, but not NeuroD1, to control in biochemical and transfection-based assays. Consistent with a direct and significant role in endogenous gene transcription, MafA was not present in the remaining insulin$^+$ cells of *Pax6* and *Nkx6.1* mutant mice. In addition to R3 binding, Pdx-1 and Pax6 factors associated with other conserved regions of the *MafA* promoter in scanning chromatin immunoprecipitation (ChIP) assays. Collectively, these results indicate that correct spatial and inducible regulation of *MafA* involves cross talk between *trans*-acting factors binding to multiple conserved *cis* elements located within distinct regions of the promoter, with R3 acting to direct expression specifically to the β cell.

MATERIALS AND METHODS

DNA sequence analysis. Transcription factor binding sites within conserved chicken, human, mouse, and rat R3 sequences of *MafA* were found using the TRANSFAC (22) and rVISTA (32) bioinformatic programs.

Transfection constructs. Noncomplementary mutations (G to T and C to A) were produced in the mouse R3:pTk chloramphenicol acetyltransferase (CAT) reporter (44) by use of a QuikChange mutagenesis kit (Stratagene) and the following oligonucleotides (the mutated bases are underlined): Pax6 mutant, TCTCTGCGCGTTTGAGTGACAGCGAACCCCAC (positions −7917 to −7886); NeuroD1 mutant, CTGGAGAGAAACGAGTTCCCTCTCC (positions −8046 to −8021); and Nkx6.1 mutant, CGAGGGCTGATTTCCTTAGA AAGCACCCAT (positions −8112 to −8083). Restriction enzyme digestion and partial DNA sequencing were used to verify each construct.

Transient transfections. Monolayer cultures of pancreatic islet β (βTC3) cells were maintained as described previously (14). The day before transfection, 10^6 cells were plated in 35-mm-diameter plates and then transfected with 1 μg of R3:pTk CAT and 1 μg of pRSV-LUC (2 μg in total), using Lipofectamine reagent (Invitrogen Life Technologies). Cellular extracts were collected at 40 to 48 h posttransfection, and luciferase (LUC; Promega, Madison, WI) and CAT (38) assays were performed. The pRSV-LUC activity was used to normalize the CAT activity from R3:pTk. Each experiment was performed on several separate occasions, with at least two independently prepared plasmids.

Electrophoretic mobility shift assays. Nuclear extracts were prepared from INS-1 and βTC3 cells as previously described (49). Gel shift reactions were performed at 4°C (20-μl final volume), with roughly 10 μg of βTC3 or INS-1 nuclear extract and 400 fmol of ^{32}P-radiolabeled (via polynucleotide kinase) probe. The binding buffer varied between transcription factors, as follows: for Nkx6.1, 10 mM HEPES (pH 7.9), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 3% Ficoll, 1 μg of poly(dI-dC), and 10 μg of bovine serum albumin; for NeuroD1, 25 mM HEPES (pH 7.9), 9% glycerol, 100 mM NaCl, 0.2 mM EDTA, 5 mM DTT, and 1 μg of poly(dI-dC); and for Pax6, 20 mM Tris (pH 8), 200 mM NaCl, 2 mM EDTA, 20% glycerol, 4 mM DTT, 1 μg of poly(dA-dT), and 1 μg of poly(dI-dC). Competition analysis was performed with a molar excess of unlabeled competitor to labeled probe (for the wild type, 5- to 50-fold excess; and for mutants, 25- to 50-fold excess). Antibody supershift analyses were performed by preincubating nuclear extract protein with Pax6 (Covance Research)-, NeuroD1 (Santa Cruz Biotechnology)-, or Nkx6.1 (Beta Cell Biology Consortium)-specific antibodies for 20 min at 4°C prior to addition of the radiolabeled probe. Samples were electrophoresed in 6% nondenaturing polyacrylamide gels at 150 V for 2 h in 1× TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA). Gels were then dried and visualized by autoradiography.

The following double-stranded mouse R3 oligonucleotides (the mutated bases are underlined) were used for binding reactions: for the NeuroD1 site (positions −8046 to −8021), CTGGAGAGAACAGATGTTCCCTCTCC; for the NeuroD1 site mutant, CTGGAGAGAAACGAGTTTCCCTCTCC; for Pax6-like site 1 (positions −7993 to −7962), CTCCACTCAGCCTTGTTTAG GAGAGAAAAGAG; for Pax6-like site 2 (positions −7917 to −7886), TCTC TGCGCGTTTGAGTGACGATAAACCCCAC; for mutant site 2, TCTCTGC GCGTTTGAGTGACAGCGAACCCCAC; for Pax6-like site 3 (positions −7849 to −7820), TATCATTTTATTGTCATATTTCACGGCCGT; for Pax6-like site 4 (positions −7822 to −7793), CGTAACGTTAATGGAAGATGCTT GCTGCAG; for the Nkx6.1 site (positions −8112 to −8083), CGAGGGCTGA TTTAATTAGAAAGCACCCAT; for the Nkx6.1 site mutant, CGAGGGCTG ATTTCCTTAGAAAGCACCCAT; for the rat *insulin 2* E1 element, TCTGGC CATCTGCTGATCC (50); for the Pax6 consensus, AATTCGTGAACTCAAG CGTGAAAATGG (46); and for the Nkx6.1 consensus, GATCTGACCATTTA ATTACCCTTCGTTGACAAGG (35).

ChIP assays. βTC3 cells (~0.5×10^8) were formaldehyde cross-linked and the sonicated chromatin-DNA complexes isolated as described previously (14). Anti-Pax6, anti-NeuroD1, anti-Pdx-1 (a gift from Chris Wright, Vanderbilt University), anti-Nkx6.1 (Beta Cell Biology Consortium), normal rabbit IgG (Santa Cruz Biotechnology), or no antibody was added to the sonicated chromatin, and the antibody-protein-DNA complexes were isolated by incubation with salmon sperm DNA-protein A-agarose (Upstate). PCR was performed on 1/10 of the purified anti-Pax6-, anti-NeuroD1-, or anti-Pdx-1-immunoprecipitated DNA, using either Ready-to-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) or a Master Mix PCR system (Eppendorf) with 15 pmol of mouse primers. The following primer sets were used: for region 1, −9471 (5′-TGGTGGGCAG TTTATAGGGTCAGT-3′) and −9124 (5′-GCCACCTACAGCTCACACAAA CTT-3′); for R3, −8120 (5′-CACCCCAGCGAGGGCTGATTTAATT-3′) and −7750 (5′-AGCAAGCACTTCAGTGTGCTCAGTG-3′); for R4 and R5 −6348 (5′-TGTCCATTCCCTGTTCCTCTCCCT-3′), −6041 (5′-TGTGTGGTAGTC AAGACAGGCCAA-3′), −4661 (5′-ACCTCTTGCCCTATGGCTGATGAT-3′), −4330 (5′-TGCACATTGATCTGGTGAGGTGGA-3′), −1873 (5′-TGCC CAGACATGTAGCTCATCCTT-3′), and −1607 (5′-TGGTAGCCACAGCCA TCAGTGTAA-3′); for R6, −506 (5′-GAATTCCTGAACCCATCCCAACCA-3′) and −251 (5′-AGACCAAGTGGCAGATTCTGAGGT-3′); and for phosphoenolpyruvate carboxykinase (*PEPCK*), −434 (5′-GAGTGACACCTCA CAGCTGTGG-3′) and −96 (5′-GGCAGGCCTTTGGATCATAGCC-3′). Cycling parameters were 1 cycle of 95°C for 2 min and 30 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s. The PCR products were resolved in a 1.4% agarose gel in Tris-acetate-EDTA (TAE) buffer and then visualized by ethidium bromide staining. Each experiment was repeated on at least three independent occasions.

MafA transgene construction and generation of transgenic mice. Sequences spanning positions −8120 to −7750 (R3), −10428 to +230 (R1-6), and −10428 to +230 lacking positions −8120 to −7750 (R1-6$^{\Delta R3}$) were cloned into hsp6811*eGFP*pA, which contains a tripartite nuclear localization signal (NLS) and the enhanced green fluorescent protein (EGFP) gene cloned into hsp6811pA (58). The minimal heat shock promoter was removed by restriction digestion, and R1-6 was inserted directly upstream of the mouse β-globin intron by homologous recombination (30, 44). The recombination arms were made with the following PCR primers: for the 5′ arm, −10393 (5′-GGACCCCTCTGCCA GGCTTTGTCC-3′) and −10194 (5′-ATCTCAAAATCATCCATGGTGCCA-3′); and for the 3′ arm, +57 (5′-CGGGCGGGAGAGCCCGGAGCGCGG-3′) and +230 (5′-CTGTGCTCAGGGGACGCCGCCGGC-3′). R3 was cloned upstream of the minimal heat shock promoter in hsp6811*eGFP*pA by use of PCR primers −8120 (5′-CACCCCAGCGAGGGCTGATTTAATT-3′) and −7750 (5′-AGCAAGCACTTCAGTGTGCTCAGTG-3′). To create the R1-6$^{\Delta R3}$:*eGFP* construct, positions −10393 to −8820 were amplified by PCR and cloned upstream of positions −7749 to +120 in the hsp6811*eGFP*pA construct lacking the minimal heat shock promoter. Primers used to amplify positions −10393 to −8820 were as follows: −10393, 5′-GCGCGCACTAGTGGACCCCTCTGCCA GGCTTTGTCC-3′; and −8820, 5′-GCGCGCGAATTCTGGTCTTCAAGGC AGCCGCTGCTG-3′. R1-6:*eGFP*, R1-6$^{\Delta R3}$:*eGFP*, and R3:*eGFP* constructs were verified by restriction digestion and DNA sequencing. Transgenic screening was performed by PCR, using DNA (~1 μg) obtained from either neonatal or adult mouse tails by use of a Puregene DNA purification kit (Gentra Systems, Minneapolis, MN). The PCRs (1 cycle of 95°C for 3 min; 25 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 1 min; and 1 cycle of 68°C for 5 min) were performed with primers spanning the unique NLS and *eGFP* sequences in the transgene (forward, 5′-AAAGAATTCGCCCTTCCATGGTGC; and reverse, 5′-TGTAGTTGCCGTCGTCCTTGAAGA).

Tissue preparation. Embryonic pancreases were isolated from transcription factor mutant mice (*NeuroD1*$^{-/-}$ mice [6, 36] at E15.5 and *Pax6*$^{Sey/Sey}$ mice [47] at P2), while embryonic (E12.5, E13.5, E15.5, and E18.5) and adult (P33) samples were collected from R1-6:*eGFP*, R1-6$^{\Delta R3}$:*eGFP*, and R3:*eGFP* transgenic mice. The day of vaginal plug discovery was designated stage E0.5. The transgenic and *NeuroD1*$^{-/-}$ embryonic tissues were fixed in 4% paraformaldehyde for 2 h at 4°C, washed with phosphate-buffered saline (PBS), dehydrated in increasing concentrations of ethanol, and embedded in paraffin. The newborn *Pax6*$^{Sey/Sey}$ tissue was fixed overnight in 4% paraformaldehyde at 4°C, immersed in 30% sucrose in PBS overnight at 4°C, and embedded in tissue freezing medium (Tissue-Tek; Triangle Biomedical Sciences) for cryoprotection. Whole-mount and bright-field images of the digestive organs from R1-6:*eGFP*, R1-6$^{\Delta R3}$:*eGFP*, and R3:*eGFP* mice were taken on a Stemi 2000-C microscope (Zeiss) with a Nikon 5700 digital camera.

Immunofluorescence. Confocal image analyses were performed on paraffin and frozen sections as described previously (34, 42). Briefly, paraffin (6 μm) and frozen (10 μm) block sections were mounted on glass slides. The slides were incubated with primary antibodies overnight at 4°C (rabbit anti-MafA [1:1,000; Bethyl Laboratories, Montgomery, TX], chicken anti-GFP [1:5,000; Abcam, Cambridge, MA], and guinea pig anti-insulin [1:1,000; Linco Research Immunoassay, St. Charles, MO]) and then with Cy2-, Cy3-, or Cy5-conjugated donkey anti-guinea pig, anti-chicken, and anti-rabbit IgG secondary antibodies (1:500; Jackson ImmunoResearch, West Grove, PA). Fluorescent images were captured on either a Zeiss LSM 510 confocal microscope, using an optical depth of 1.5 μm, or a Zeiss Axioskop 2 microscope. Nuclear counterstaining was performed with YoPro1 (Molecular Probes, Eugene, OR). Adobe Photoshop, version 7.0 (Adobe Systems, Inc.), was used to process the images. GFP- and MafA-stained cells were counted for GFP$^+$, GFP$^+$ MafA$^+$, and MafA$^+$ cells in transgenic sections from embryonic (for E12.5, every 24 μm; for E13.5, every 72 μm; and for E15.5 and E18.5, every 96 μm) and adult (every 250 μm) tissues. The transgenic analysis was performed on a minimum of three independent animals from each line for each time point.

Islet isolation and culture. Islets were isolated from R1-6:*eGFP* mice as previously described (5). Briefly, mice were sacrificed by cervical dislocation, and collagenase P (Roche) was then injected at 0.6 mg/ml into the pancreas via the bile duct and islets were incubated for 10 to 20 min. Islets were then handpicked five times under a dissecting microscope and washed three times in RPMI 1640 containing 5 mM glucose, 1% penicillin-streptomycin, and 10% fetal bovine serum. Islets were maintained overnight in RPMI containing 5 mM glucose at 37°C. After being cultured overnight, approximately 100 islets were incubated for 24 h at 37°C in RPMI 1640 containing 5 or 11 mM glucose. Total RNA was isolated from the glucose-treated R1-6:*eGFP* mouse islets (~180 islets per condition) by use of an RNeasy kit (Qiagen, Valencia, CA) and then subjected to DNase I treatment (Versagene RNA DNase kit; Gentra Systems, Minneapolis, MN). Reverse transcription PCR reagents (TaqMan; Applied Biosystems, Foster City, CA) were used to generate cDNAs from DNase I-treated RNA.

Real-time PCR. SYBR green master mix reagents (Applied Biosystems) were used in the PCR mixes for R1-6:*eGFP* islet and Nkx6.1 ChIP DNAs, and reactions were performed and analyzed using an Applied Biosystems Prism 7900 sequence detection system and software. The following oligonucleotide primers were used for detection: for *insulin*, 5′-CCACCCAGGCTTTTGTCAAA-3′ (5′) and 5′-CCCAGCTCCAGTTGTTCCAC-3′ (3′); for *MafA*, 5′-CCTGTAGAGG AAGCCGAGGAA-3′ (5′) and 5′-CCTCCCCCAGTCGAGTATAGC-3′ (3′); for *eGFP*, 5′-ATCGAGCTGAAGGGCATCGACTT-3′ (5′) and 5′-TGTCGGC CATGATATAGACGTTGTGG-3′ (3′); for γ-*actin*, 5′-GCACCCGGTGCTTC TGAC-3′ (5′) and 5′-CCAGATGCATACAAGGAC-3′ (3′); for *GAPDH*, 5′-A ACTTTGGCATTGTGGAAGG-3′ (5′) and 5′-GGATGCAGGGATGATGTT CT-3′ (3′); for 18S rRNA, 5′-AGTCCCTGCCCTTTGTACACA-3′ (5′) and 5′-GATCCGAGGGCCTCACTAAAC-3′ (3′); for *MafA* bp −9433 to −9319 (R1), 5′-CTGTATTTGGCCCTCCTCTCCG-3′ (5′) and 5′-GCGGGCAAAGT CAGGACACA-3′ (3′); for *MafA* bp −7988 to −7875 (R3), 5′-CACTCAGCC TTGTTTAGGAGAGAAAAGA-3′ (5′) and 5′-CCGAGCTGCGTGGGGTTT AT-3′ (3′); for *MafA* bp −6282 to −6186 (R5), 5′-GCACCCAGCCTCACTCT AGGACTT-3′ (5′) and 5′-CTGCCAATGCCACCACAGGA-3′ (3′); for *MafA* bp −4633 to −4541, 5′-GCCCTATGGCTGATGATCCACA-3′ (5′) and 5′-TG AGGGTGTGCTCGCTTGGA-3′ (3′); for *MafA* bp −1678 to −1560, 5′-CCA GTTGCTTTTCACGGCCTC-3′ (5′) and 5′-CGGGGAGCCATTGGAATGT C-3′ (3′); and for *MafA* bp −346 to −241 (R6), 5′-GAGACACCGAAGGAGT ACCCTGGA-3′ (5′) and 5′-AGAGGGCGCGAGACCAAGTG-3′ (3′). Islet RNA levels were calculated by the comparative ΔC_T method, in which γ-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rRNA were used for normalization (31). ChIP data are expressed as the percent recovery of coimmunoprecipitated DNA relative to input DNA (before immunoprecipitation).

RESULTS

R3 is necessary but not sufficient for β-cell-specific expression *in vivo*. To determine the significance of R3 in *MafA* expression *in vivo*, transgenic mouse *eGFP* reporter lines were created using R3 alone (bp −8120 to −7750), R1-6 (bp −10428 to +230), and R1-6 lacking R3 (Fig. 1A to C). The expected β-cell-selective activity pattern was found for each of the *MafA*:*eGFP* transgenes in cell-line-based experiments (data not shown).

Whole-mount immunofluorescence was performed at E13.5 to determine the gross expression profiles of the R3:*eGFP*, R1-6:*eGFP*, and R1-6$^{\Delta R3}$:*eGFP* transgenes. Very similar expression patterns in the eyes, whiskers, and limbs were obtained between the R1-6:*eGFP* and R1-6$^{\Delta R3}$:*eGFP* lines (Fig. 1D), although expression was lost in the nervous system in R1-6$^{\Delta R3}$:*eGFP* mice. Such complementary results imply that this reflects endogenous *MafA* expression, which has not yet been determined for mammalian nonpancreatic tissues. Notably, the R1-6:*eGFP* and R1-6$^{\Delta R3}$:*eGFP* expression pattern corresponds well with that found for chicken *MafA* (29). In contrast, GFP fluorescence was not detected for the R3:*eGFP* lines ($n = 3$) in any organ at either embryonic or adult time points (data not shown).

Light punctate staining in whole-mount images was first observed at E15.5 in R1-6:*eGFP* pancreases and became more pronounced by E18.5, as expected for a β-cell-expressed gene product (Fig. 2). Two R1-6:*eGFP* (labeled A and E) founder lines were established, and fluorescent nodes were also seen throughout the late developing and adult pancreases for both lines, with the E line having a stronger signal. In contrast to the case for R1-6:*eGFP* mice, GFP fluorescence was not observed in the adult pancreas for any of the R1-6$^{\Delta R3}$:*eGFP* lines ($n = 3$) (Fig. 3). These results demonstrate that R3 is essential for MafA expression in the pancreas, with other distinct sequences

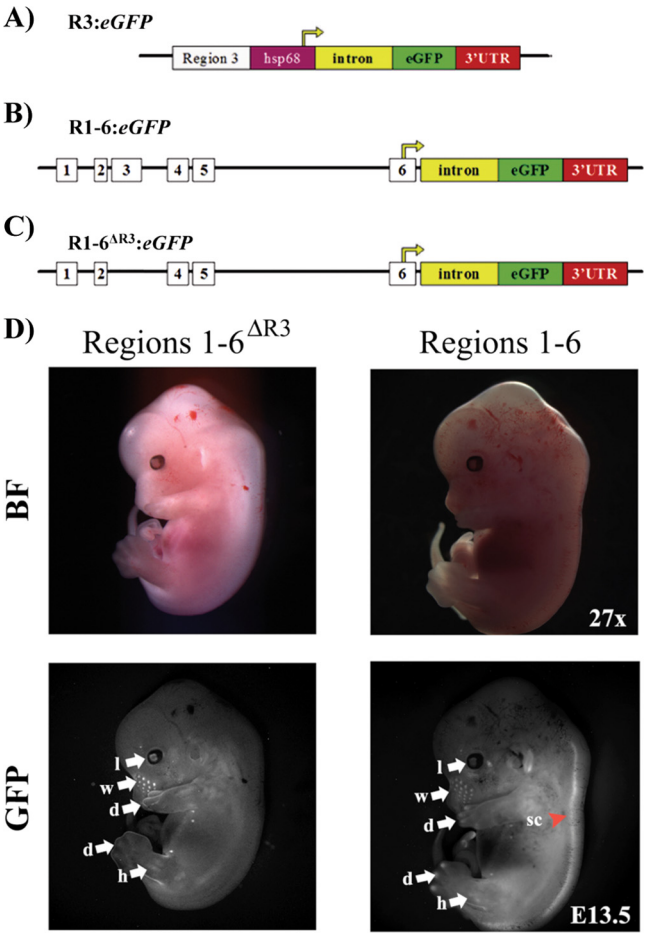


FIG. 1. The *MafA* R1-6:*eGFP* and R1-6$^{\Delta R3}$:*eGFP* transgenes are expressed in similar tissue-specific manners. (A) Diagrams of the R3 (A), R1-6 (B), and R1-6$^{\Delta R3}$ (C) transgenic constructs. The start of transcription is from the hsp68 minimal heat shock promoter in R3:*eGFP*, while the endogenous start site in region 6 is utilized in the R1-6:*eGFP* and R1-6$^{\Delta R3}$:*eGFP* lines. Nuclear GFP was produced from all of the constructs (data not shown). (D) Whole-mount immunofluorescence of E13.5 embryos, performed with both R1-6:*eGFP* (E line is shown) and R1-6$^{\Delta R3}$:*eGFP* transgenic mouse lines. The expression patterns in these transgenic mouse lines were similar in most tissues, including the lens and limb buds (arrows), yet were absent in the spinal cord in R1-6$^{\Delta R3}$:*eGFP* mice (arrowhead). l, lens; d, digit tips; w, whisker hair shaft; h, hind limb; sc, spinal cord; BF, bright field; GFP, GFP fluorescence.

found between bp −10428 and +230 apparently necessary for expression in the pancreas and elsewhere.

Sequences spanning R1 to R6 recapitulate endogenous MafA expression in β cells. Pancreases were obtained from E12.5, E13.5, E15.5, E18.5, and P33 animals to precisely characterize the age-dependent expression pattern of the R1-6 transgene. GFP$^+$ cells in the pancreatic epithelium were detected by antibody staining starting at E13.5, coincident with the onset of MafA expression (Fig. 4A). No GFP$^+$ cells were detected at E12.5 (data not shown). The inability to detect GFP fluorescence in whole mounts presumably reflects the very few MafA$^+$ cells normally produced at E13.5 (33). The GFP$^+$ cell number increased during development, coinciding with the massive wave of β-cell formation during the secondary transition (Fig. 4B to D). Costaining of GFP with either insulin or MafA demonstrated that R1-6:*eGFP* activity was present in a majority of MafA$^+$ cells throughout the lifetime of the animal and was localized exclusively to insulin$^+$ cells (Fig. 4E) (33, 37). These results established that the correct temporal and spatial signals for *MafA* expression in the pancreas are contained within the roughly 10-kbp promoter region of the R1-6 transgene.

MafA is regulated transcriptionally by glucose. The most important regulator of insulin expression in islet β cells is glucose, which activates *insulin* gene transcription, protein synthesis, and secretion. Glucose-induced transcription is dependent upon MafA, in a process mediated by increased *MafA* mRNA and protein levels upon acute stimulation (20, 26, 53, 58). To determine if MafA transcription was also regulated by glucose, islets from 6-month-old R1-6:*eGFP* mice were cultured with stimulating (11 mM) and nonstimulating (5 mM) glucose concentrations for 24 h. Endogenous *insulin* mRNA levels were induced approximately 2.6-fold under these conditions, whereas *MafA* and *eGFP* reporter levels were both increased roughly 4.5-fold (Fig. 5). An identical regulatory pattern was obtained with 3-month-old R1-6:*eGFP* islets (data not shown). These collective results demonstrate that glucose signaling induces *MafA* transcription through factors binding within the 10 kb 5′ of the transcription start site.

Pax6, NeuroD1, and Nkx6.1 bind specifically to R3 sequences *in vitro*. MafA is expressed exclusively in the wave of pancreatic insulin$^+$ cells produced after E13.5 that eventually populate the mature islet (33). However, it is significant only to β-cell maturation and adult function, not to earlier cell development steps (2a, 57). We concluded from the R1-6:*eGFP* and R1-6$^{\Delta R3}$:*eGFP* transgenics and earlier *MafA*-driven cell line transfection studies (44) that R3 is the primary regulatory domain driving pancreas-specific expression. A TRANSFAC-based bioinformatic approach was previously used to identify potential R3 transcription regulators, with Pdx-1, FoxA2, and Nkx2.2 associated with activation (Fig. 6A) (44). Here we tested the role in R3 activity of Pax6, NeuroD1, and Nkx6.1, which are key regulators of β-cell development and function.

R3 gel shift probes were made for each of the potential Pax6, NeuroD1, and Nkx6.1 binding sites (Fig. 6A). Only one of the four Pax6-like sites was found to bind to this factor in βTC3 nuclear extracts (bp −7917 to −7886), as demonstrated by both competition with a consensus Pax6 element and incubation with a Pax6 antibody (Fig. 6B). In addition, NeuroD1 was shown to interact with the basic helix-loop-helix (bHLH)-like site found within bp −8046 to −8021 by competition and antibody supershift analyses (Fig. 6C). Not only were competition experiments performed with wild-type and R3 mutant sites, but an identical pattern was obtained with the bHLH sites from the *insulin* and *G6PC2* genes (data not shown). Several complexes were formed with the probe for bp −8112 to −8083 in R3, using βTC3 and INS-1 β-cell extracts (Fig. 6D), with binding of the principal specific complex disrupted by the Nkx6.1 antibody.

Pax6, NeuroD1, and Nkx6.1 bind to endogenous R3 sequences and function as positive regulators. ChIP assays were used to determine whether Pax6, NeuroD1, and Nkx6.1 interactions with R3 could be observed *in situ*. R3 was selectively amplified by PCR from formaldehyde cross-linked β-cell chromatin precipitated with antibodies specific to NeuroD1, Pax6,

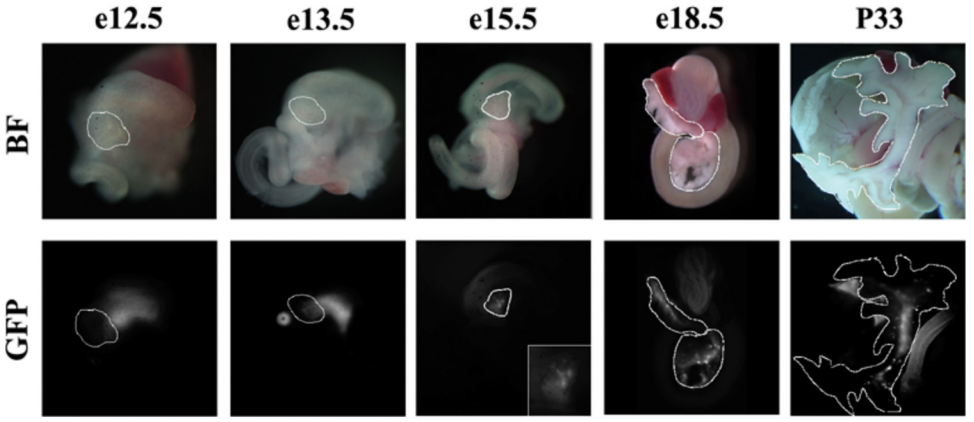


FIG. 2. The *MafA* R1-6:*eGFP* transgene is active within the pancreas. Digestive organs, including the stomach, pancreas, spleen, and small intestine, were collected from the R1-6:*eGFP* A and E (shown) lines. The same developmental and adult GFP fluorescence patterns were obtained for both lines, with GFP not detected in the pancreatic buds until E15.5. The dorsal pancreatic bud is magnified (×4) in the E15.5 GFP inset. Pancreases are outlined in white. The punctate pattern in E15.5, E18.5, and P33 pancreases is characteristic of insulin$^+$ cell expression. Low levels of EGFP were also detected in the E12.5 and E13.5 duodenum and stomach. BF, bright field; GFP, GFP fluorescence.

and Nkx6.1 (Fig. 7) but not from chromatin treated with IgG or in the absence of antibody treatment. In contrast, no signal was detected with promoter sequences from the *PEPCK* gene, which is not transcribed in β cells. These results demonstrate that NeuroD1, Pax6, and Nkx6.1 bind within R3 in β cells.

Element mutations were made in the mouse *MafA* region 3:pTk reporter to specifically disrupt Pax6, NeuroD1, or Nkx6.1 binding. The loss of binding led to a significant decrease in R3-driven reporter activity in transfected βTC3 cells (Fig. 7E). These results suggest that Pax6, NeuroD1, and Nkx6.1 directly activate *MafA* R3 in β cells.

Loss of only endogenous Pax6 and Nkx6.1 affects MafA expression in insulin$^+$ cells *in vivo*. *Pax6$^{Sey/Sey}$* mutant mice have a noticeable reduction in all islet cell types, but most significantly in glucagon- and insulin-producing cells (47). The Pax6 protein produced from the *Pax6^{Sey}* allele lacks the homeo- and transactivation domains, effectively producing a null animal (15). Immunofluorescence staining revealed that MafA was not present in the insulin$^+$ cells remaining at P2 in the *Pax6$^{Sey/Sey}$* pancreas (Fig. 8A), although many other islet-enriched transcription factors were still present (e.g., Nkx2.2, Nkx6.1, and Pdx-1 [4, 51]). Similarly, MafA was not found in E18.5 insulin$^+$ cells in the pancreases of *Nkx6.1$^{-/-}$* mice (33), which specifically lack second-wave β cells (48).

NeuroD1$^{-/-}$ mice are diabetic due to reduced β-cell numbers (36), although the severity is strain dependent (23). Interestingly, MafA was still detected in insulin$^+$ cells of NeuroD1 mutants at E15.5 (Fig. 8B). Cumulatively, these

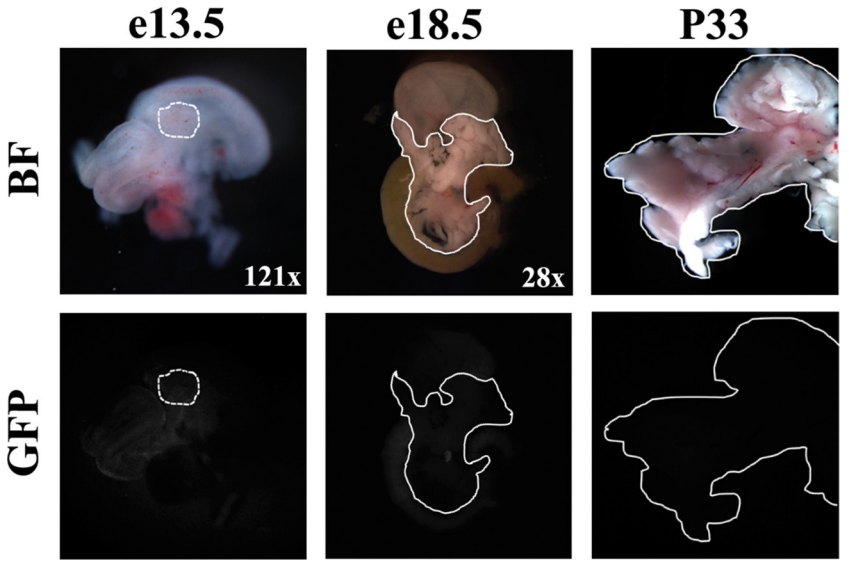


FIG. 3. R3 is necessary for pancreatic transgenic expression. Gut tissues were collected from E13.5 and E18.5 embryos and P33 animals from the R-6$^{\Delta R3}$:*eGFP* line and examined via whole-mount immunofluorescence. No GFP expression was detected within the developing or adult pancreas, which is outlined in white. BF, bright field; GFP, GFP fluorescence.

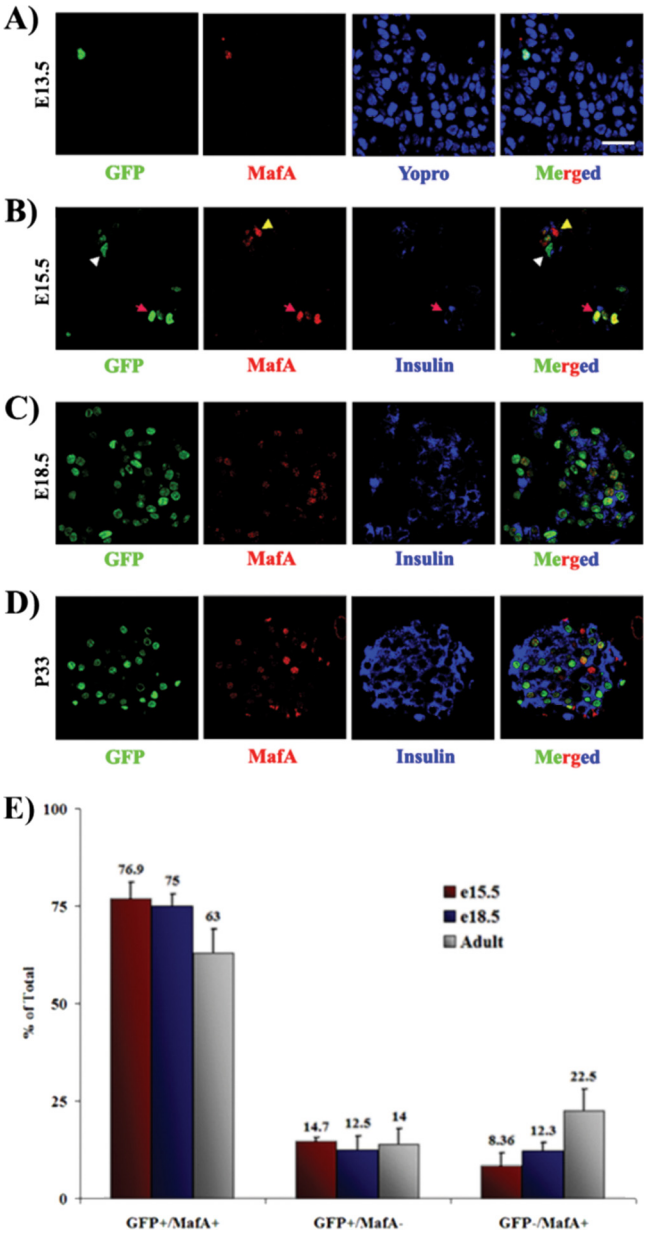


FIG. 4. *MafA* R1-6 drives GFP transgene expression, predominantly in MafA$^+$ cells, in the developing and adult pancreas. Sections from E13.5 (A), E15.5 (B), E18.5 (C), and P33 (D) mice were collected, and sections were stained for GFP (green), MafA (red), and/or insulin (blue). GFP was first detected in MafA$^+$ cells at E13.5. GFP was usually coexpressed with MafA (red arrowheads), although the occasional single GFP$^+$ (white arrowheads) or MafA$^+$ (yellow arrowheads) cell was observed, as illustrated in panel B. These images are representative of both the R1-6:*eGFP* A and E lines. Nuclei in panel A were counterstained in blue. Bar, 20 μm. (E) The mean percentages ± standard deviations of pancreatic GFP$^+$ MafA$^+$, GFP$^+$, and MafA$^+$ cells to the total number of GFP$^+$ and MafA$^+$ cells at E15.5 (red bars), E18.5 (blue bars), and P33 (gray bars) are shown for both the A and E lines. Cells were counted from at least three independently isolated pancreases per time point.

results indicate that Pax6 and Nkx6.1, but not NeuroD1, are essential to *MafA* transcription *in vivo*.

Pax6 and Pdx-1 are also bound to non-R3 conserved sequences within the 10-kbp promoter region. The R1-6:*eGFP*

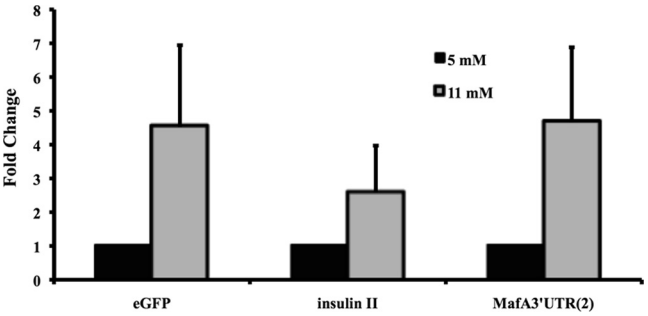
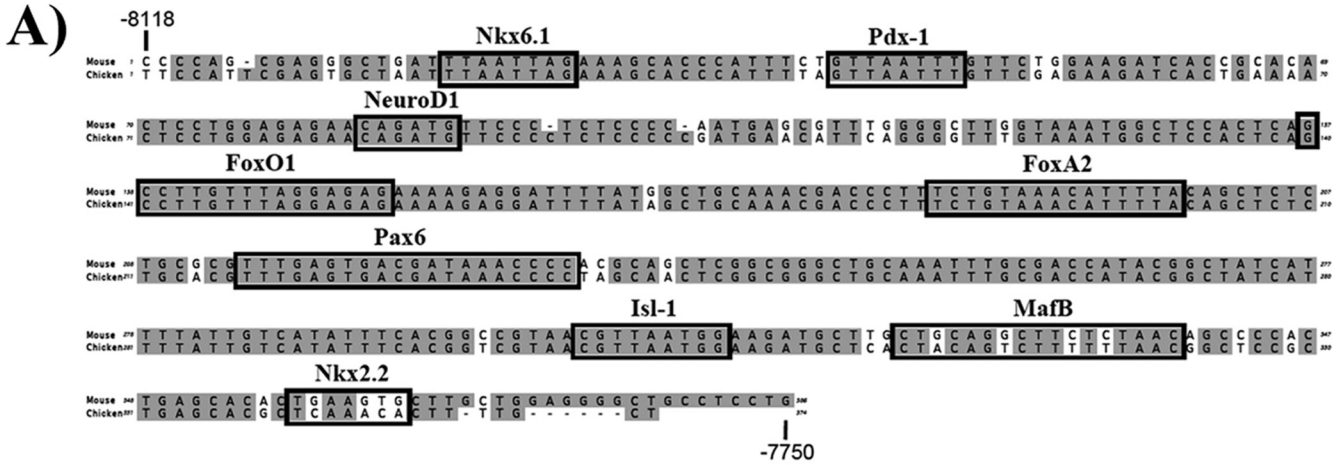


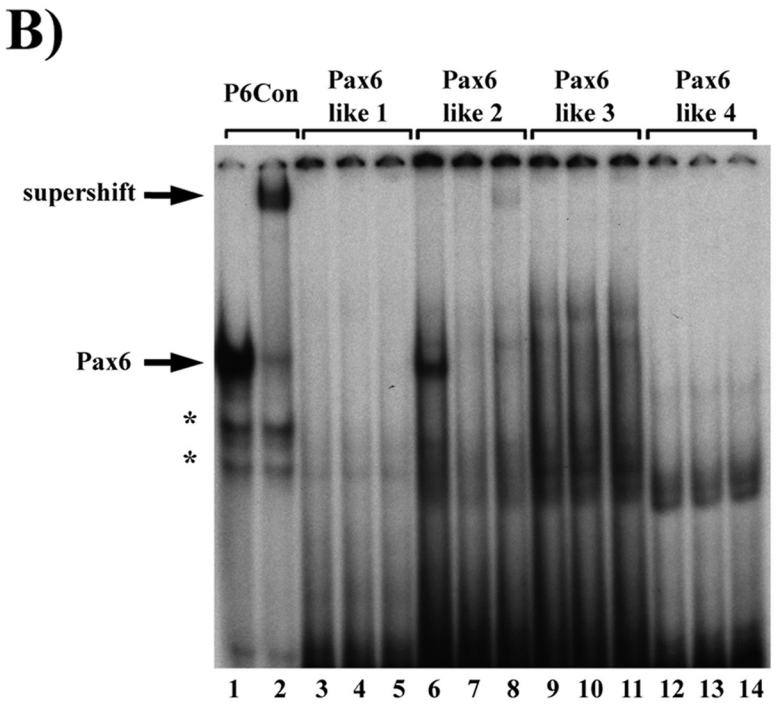
FIG. 5. *MafA* transcription is induced in response to stimulating glucose treatment. Mouse islets were collected from the R1-6:*eGFP* line and cultured at a high (11 mM) or low (5 mM) glucose concentration. Real-time PCR was performed with primers specific to *eGFP* coding sequences, *insulin II* coding sequences, or the *MafA* 3′-untranslated region (UTR). Normalized mRNA values ± standard deviations are presented as fold changes between low and high glucose stimulation.

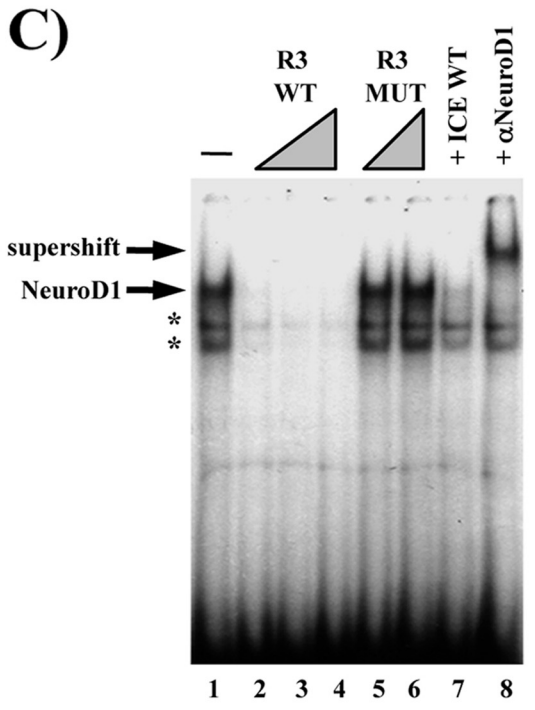
and R1-6$^{\Delta R3}$:*eGFP* transgenic experiments demonstrated that R3 was essential to β-cell-specific transcription in mice (Fig. 1 to 4). Moreover, MafA expression was severely and selectively compromised upon reducing the expression of various transcriptional effectors of β-cell development and function *in vivo* (Fig. 8) (44). These data raised the possibility that *MafA* expression was also mediated by the actions of such effectors on non-R3 promoter sequences. As a consequence, a scanning ChIP analysis was performed to assess Pdx-1, Pax6, and Nkx6.1 binding throughout the *MafA* promoter region. As expected, antibodies to Pdx-1, Nkx6.1, and Pax6 precipitated R3, not *PEPCK*, promoter sequences (Fig. 9). In addition, Pdx-1 binding was detected in R6, and Pax6 binding was detected in regions 1 and 6 (Fig. 9A). Taken together, these results imply that β-cell-specific transcription of the *MafA* gene *in vivo* is regulated principally by factors binding within R3, with further influence by (at least) Pax6 and Pdx-1 acting upon conserved *cis*-acting elements residing outside R3.

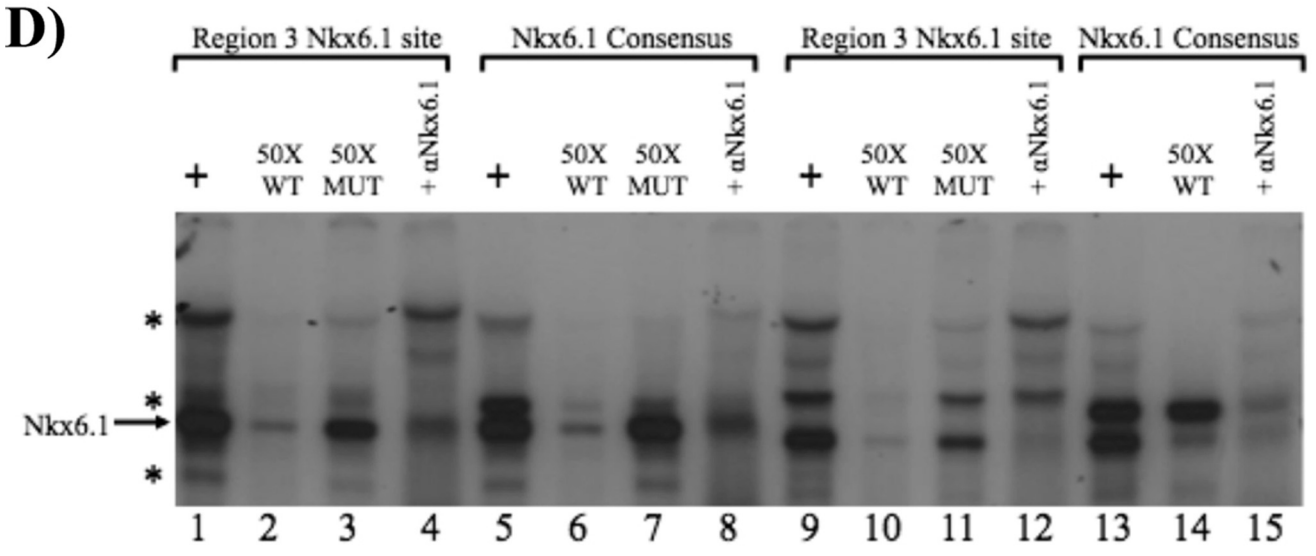
DISCUSSION

MafA is expressed late in pancreatic development and exclusively within insulin$^+$ cells destined to populate the islet, a unique expression pattern in relation to all other islet transcription factors. Previously, *MafA* R3 was shown to be sufficient to direct β-cell-line-specific expression. Here we showed, upon analyzing the R1-6:*eGFP*, R1-6$^{\Delta R3}$:*eGFP*, and R3:*eGFP* transgenic constructs in mice, that R3 is essential for pancreatic *MafA* transcription. Hence, we found that only R1-6:*eGFP* recapitulated endogenous MafA expression in developing and adult pancreatic β cells, whose activity in islets was increased in response to glucose. Further support for a novel role for R3 in *MafA* expression came upon demonstrating that the β-cell developmental regulators Pax6, NeuroD1, and Nkx6.1 were associated with R3 activation *in vitro* and that MafA expression was lost in the insulin$^+$ cell population remaining in Pax6 and Nkx6.1 mutant mice. The loss of MafA in these islet-enriched factor mutants is consistent with its late expression during β-cell formation and the absence of a developmental phenotype in *MafA*$^{-/-}$ mice. In addition, our ability to observe R3


A)
-8118
Nkx6.1
Pdx-1
NeuroD1
FoxO1
FoxA2
Pax6
Isl-1
MafB
Nkx2.2
-7750


B)
P6Con
Pax6 like 1
Pax6 like 2
Pax6 like 3
Pax6 like 4
supershift
Pax6
1 2 3 4 5 6 7 8 9 10 11 12 13 14


C)
R3 WT
R3 MUT
+ ICE WT
+ αNeuroD1
supershift
NeuroD1
1 2 3 4 5 6 7 8


D)
Region 3 Nkx6.1 site
Nkx6.1 Consensus
Region 3 Nkx6.1 site
Nkx6.1 Consensus
+
50X WT
50X MUT
+ αNkx6.1
Nkx6.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

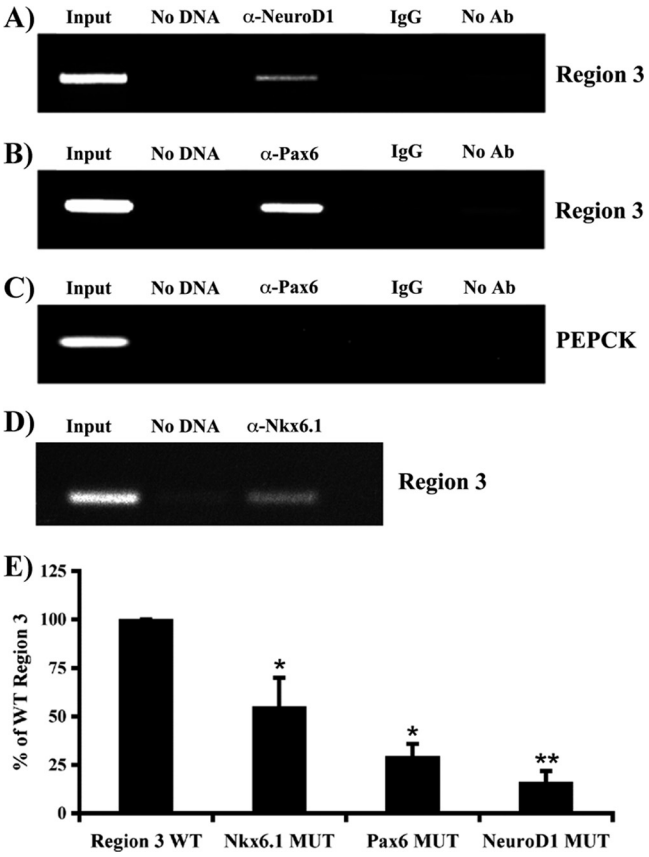


FIG. 7. Pax6, Nkx6.1, and NeuroD1 binding elements are necessary for R3 stimulation. Formaldehyde-cross-linked βTC3 chromatin was incubated with antibodies specific to NeuroD1 (A), Pax6 (B and C), and Nkx6.1 (D). Immunoprecipitated DNA was analyzed by PCR with primers specific to R3 (A, B, and D) or *PEPCK* (C). For controls, PCR was performed with total input DNA (1/100 dilution; input), no DNA, DNA immunoprecipitated with rabbit IgG (IgG), or DNA precipitated in the absence of antibody (no Ab). Only the anti-Pax6 results are shown for *PEPCK*, although the same pattern was seen for NeuroD1 and Nkx6.1. Each experiment was repeated with three separate chromatin preparations. (E) βTC3 cells were transfected with wild-type R3 (WT) and Pax6, NeuroD1, and Nkx6.1 binding-site mutants (MUT) of R3:pTk. Normalized region 3:pTk activity ± the standard error of the mean for each mutant is presented as a percentage of wild-type R3 activity. Wild-type and mutant R3:pTk constructs were active only in transfected β-cell lines, not in non-β cells (data not shown). Asterisks denote statistically significant differences between the mutant and wild-type activities, as assessed by Student's *t* test (*, $P < 0.001$; **, $P < 0.005$).

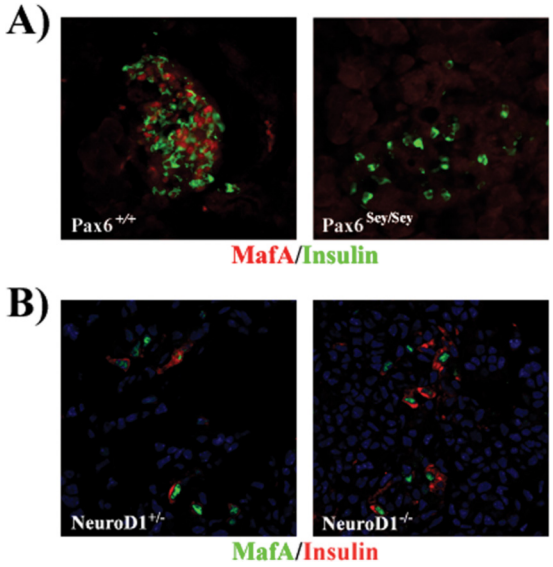


FIG. 8. MafA is not produced in insulin$^+$ cells remaining in Pax6 mutant mice but is present in those in NeuroD1 mutant mice. MafA and insulin expression was examined by immunofluorescence analysis of pancreatic sections from P2 *Pax6*$^{sey/sey}$ (A) and E15.5 *NeuroD1*$^{-/-}$ (B) mice and either wild-type or NeuroD1$^{+/-}$ control mice. *NeuroD1*$^{+/-}$ and wild-type mice were phenotypically indistinguishable (36), enabling us to use *NeuroD1*$^{+/-}$ mice as the control in this analysis. MafA was undetectable in the *Pax6* mutant and was present in *NeuroD1*$^{-/-}$ insulin-producing cells. Nuclei were counterstained in blue.

regulators binding to other conserved promoter sequences indicates that functional interactions between factors acting in distinct *cis*-sequence domains are critical to β-cell expression.

Since R3 was the only mammalian conserved region capable of directing β-cell-line selectivity and the sole conserved *MafA* promoter control region in chickens (44), we tested if a transgene driven by R3 alone could direct expression *in vivo*. While the R3:*eGFP* lines were inactive in all tissues (data not shown), only *Pdx-1* area II, not its area I and III control domains, independently directed transgenic reporter expression to a small fraction of Pdx-1$^+$ β cells (11, 46). In a manner resembling that for *MafA* R1-6, the area I/II transgene recapitulated the comprehensive expression pattern of *Pdx-1* in the secondary transition (E13.5 or later) (44), while area I/II/III lines were expressed in earlier pancreatic exocrine and endocrine

FIG. 6. Identification of Pax6, NeuroD1, and Nkx6.1 binding sites within R3. (A) The gray highlighted sequences are identical between mouse and chicken R3 sequences. The locations of characterized and TRANSFAC-localized potential islet-enriched factor binding sites are superimposed on the R3 sequences. The Pdx-1, FoxA2, Nkx2.2, MafB, and Isl-1 binding sites (black boxes) have been described previously (2, 8, 27, 44). The Pax6, NeuroD1, and Nkx6.1 binding elements identified in the gel shift studies below are also denoted with black boxes. (B) Gel shifts were performed with probes corresponding to the consensus Pax6 site (lanes 1 and 2) and the four Pax6-like sites (lanes 3 to 14). The probes were incubated in the presence of βTC3 nuclear extract (alone; lanes 1, 3, 6, 9, and 12), and the specificity and composition of the βTC3 protein-DNA complexes were assayed with a 100-fold molar excess of the consensus Pax6 site (lanes 4, 7, 10, and 13) or by preincubation with an anti-Pax6 antibody (lanes 2, 5, 8, 11, and 14). (C) The βTC3 complexes formed with the NeuroD1-like site probe (bp −8046 to −8021; lane 1) were analyzed with a 10- to 50-fold molar excess of unlabeled wild-type competitor (R3 WT; lanes 2 to 4), a 25- to 50-fold molar excess of the R3 E-box mutant (R3 MT; lanes 5 and 6), or a 10-fold molar excess of the *insulin* NeuroD1 element (ICE WT; lane 7). Addition of anti-NeuroD1 antibody identified the specific location of the NeuroD1-containing binding complex (lane 8). (D) The Nkx6.1 consensus (lanes 5 to 8 and 13 to 15) and R3 Nkx6.1-like (lanes 1 to 4 and 9 to 12) sites were incubated in the presence of either βTC3 (lanes 1 to 8) or INS-1 (lanes 9 to 15) cell extracts. The Nkx6.1 complex was assessed with a 50-fold excess of either the wild-type R3 Nkx6.1 site (WT; lanes 2, 6, 10, and 14) or a probe for a R3 Nkx6.1 binding mutant (MUT; lanes 3, 7, and 11). The specificity of the Nkx6.1 complex was determined by incubation with an Nkx6.1 antibody (lanes 4, 8, 12, and 15). Asterisks denote nonspecific binding complexes in panels B to D.

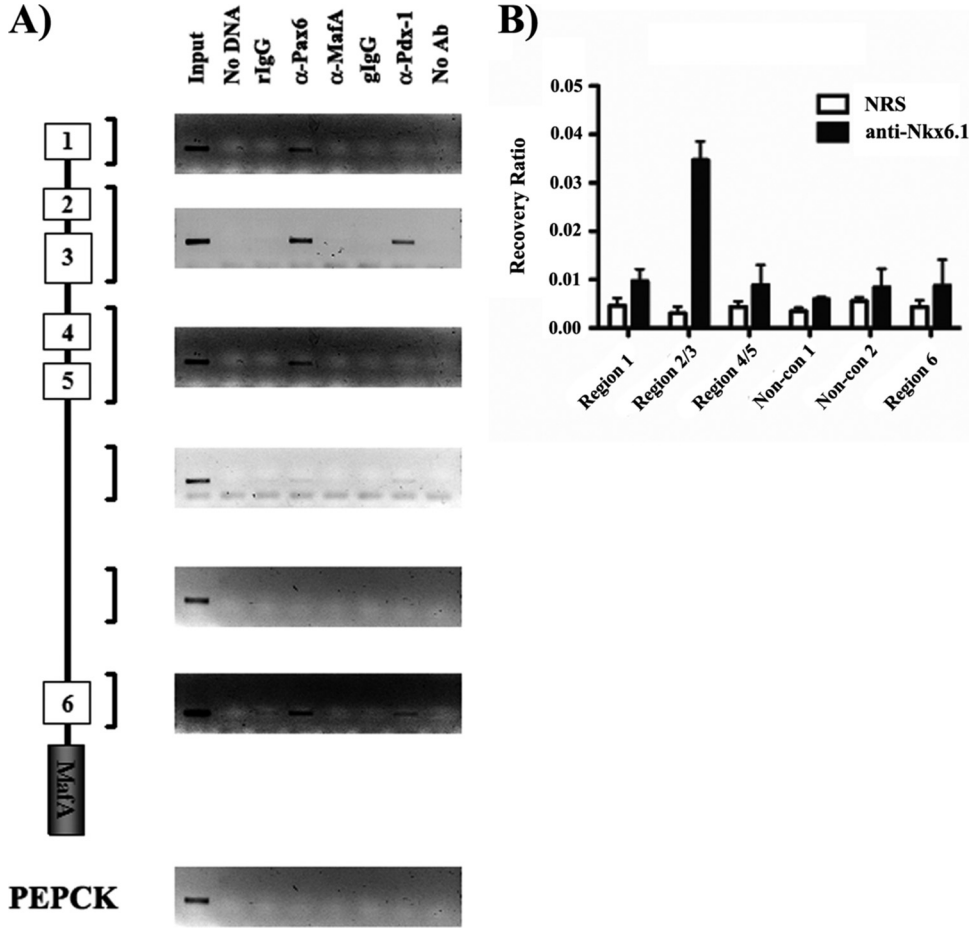


FIG. 9. Nkx6.1 binds only within R3, whereas Pdx-1 and Pax6 bind to multiple conserved regions of the MafA promoter. Scanning ChIP analysis was performed on roughly 10 kb of *MafA*, using antibodies specific to Pdx-1, MafA, and Pax6 (A) and to Nkx6.1 (B). (A) Immunoprecipitated DNA was analyzed with *MafA*-specific (R1, R2 and R3, R4 and R5, for distinct nonconserved sequences 1 and 2 and R6) and *PEPCK* (control)-specific primers. The PCRs were performed with total input DNA (1:100 dilution), no DNA, nonspecific rabbit IgG (rIgG), anti-Pax6, anti-MafA, nonspecific goat IgG (gIgG), anti-Pdx-1, or no antibody (no Ab). (B) Real-time PCR was performed on Nkx6.1-immunoprecipitated DNA. DNA samples were incubated with either Nkx6.1 or control IgG (NRS), with the data presented as recovery of the indicated region (region 1, regions 2 and 3, regions 4 and 5, or region 6), as a percentage of the input level. Each experiment was repeated with at least three independently isolated chromatin preparations.

Pdx-1$^+$ progenitors (11). These results thus established that functional interactions between *trans*-acting factors of distinct control domains may be crucial in spatiotemporal gene expression within the pancreas. *MafA* R1-6 also mediated glucose-inducible expression (Fig. 5). In contrast, this critical metabolic effector of *insulin* transcription and β-cell function impacted Pdx-1 activation quite differently, by influencing nuclear localization (9, 43).

Interestingly, the R1-6:*eGFP* and R1-6$^{\Delta R3}$:*eGFP* transgenes retained expression in many extrapancreatic tissues at E13.5, yet R1-6$^{\Delta R3}$:*eGFP* was inactive in both the pancreas and the nervous system (Fig. 1 and 3). We presume that the sequences spanning R1 to R6 are critical for *MafA* transcription in these tissues, as the expression pattern for both transgenic lines was analogous to that described for MafA in chickens, the only species for which the distribution has been examined thoroughly (29). The apparent coordinating role for R3 in tissues of such distinct developmental origins as the pancreas and nervous system is not surprising, as many transcription factors have been shown to be shared between them (25). This includes Nkx6.1, Pax6, and NeuroD1, which were all found here to profoundly affect R3-mediated activation *in vitro*, with MafA lost in the insulin$^+$ cells remaining in *Nkx6.1* (33) and *Pax6* mutant mice (Fig. 8).

In contrast to Nkx6.1, Pax6, and many other islet-enriched transcription factors linked to R3 control (e.g., Nkx2.2, Pdx-1, FoxO1, Isl-1, and MafB [2, 8, 27, 44]), MafA was still present in insulin$^+$ cells of *NeuroD1*$^{-/-}$ mice (Fig. 8B). This result was unexpected, since NeuroD1 bound R3 endogenously and the binding-site mutant profoundly reduced transfected R3-driven activity (Fig. 7), although *MafA* mRNA levels were also recently found to be unaffected after conditional removal of NeuroD1 from islet β cells *in vivo* (17). Two interpretations of these results are (i) that NeuroD1 does not directly regulate *MafA* transcription and (ii) that a closely related bHLH family member acts in a compensatory manner. An example of the latter is the case of the MyoD and Myf5 bHLH proteins, which play functionally redundant roles in skeletal muscle formation.

Hence, neither single mutant has a phenotype, while no skeletal muscle is formed in *MyoD*$^{-/-}$; *Myf5*$^{-/-}$ mice (45). MafB is also thought to act in a functionally redundant manner relative to MafA during β-cell development (3). The presence of the closely related NeuroD2 protein in β cells suggests a compensatory role in *NeuroD1*$^{-/-}$ mice (13, 23), but studies on *NeuroD1*$^{-/-}$; *NeuroD2*$^{-/-}$ mice need to be performed to test this hypothesis.

MafA is uniquely detected in the insulin^{+} cells produced during the secondary transition of pancreatic development, linking expression to β-cell maturation and function. The regulatory properties of R3 have been found to be fundamental to *MafA* transcription in the pancreas. This has been demonstrated using a variety of different transcriptional assays, and R3 appears to direct β-cell-specific *MafA* expression through coordinating actions with other conserved promoter regions. The activity of this transcription unit is exquisitely sensitive to the levels of glucose and many islet enriched-transcription factors, as, for example, expression was lost in the remaining insulin^{+} cells produced in all but *NeuroD1* knockout mice (2, 8, 33, 44). It will be interesting to determine if R1-6 activity is also responsive to other extracellular signals that impact β cells, such as insulin (52) and BMP4 (16). Furthermore, since much of the conserved 370 bp of R3 is untested and few characterized islet-enriched transcription factors remain unassociated with control, an in-depth characterization may identify novel factors intimately involved in β-cell formation and function.

ACKNOWLEDGMENTS

EL250 cells were kindly provided by D. Mortlock at Vanderbilt University Medical Center.

This work was supported by grants from the National Institutes of Health (DK50203 to R.S., DK60581 to R.G.M., VUMC9183 to B.S.-P., DK072504 to L.S., and DK083160 to C.S.H.), the American Lebanese Syrian Associated Charities (ALSAC) (to B.S.-P.), and the Vanderbilt Molecular Endocrinology Training Program (5T32 DK07563 to J.C.R.). The transgenic mice were developed with Vanderbilt Transgenic/ES shared resource grants CA68485 and DK20593. Partial support was also provided to the Molecular Biology Core Laboratory by the Vanderbilt University Diabetes Research and Training Center (Public Health Service grant P60 DK20593).

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