Pax6 Is Crucial for β-Cell Function, Insulin Biosynthesis, and Glucose-Induced Insulin Secretion

Yvan Gosmain,* Liora S. Katz,* Mounia Heddad Masson, Claire Cheyssac, Caroline Poisson, and Jacques Philippe

Diabetes Unit, Division of Endocrinology, Diabetes, and Nutrition, University Hospital, University of Geneva Medical School, 1211 Geneva 14, Switzerland

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The Pax6 transcription factor is crucial for endocrine cell differentiation and function. Indeed, mutations of *Pax6* are associated with a diabetic phenotype and a drastic decrease of insulin-positive cell number. Our aim was to better define the β-cell Pax6 transcriptional network and thus provide further information concerning the role of Pax6 in β-cell function. We developed a Pax6-deficient model in rat primary β-cells with specific small interfering RNA leading to a 75% knockdown of Pax6 expression. Through candidate gene approach, we confirmed that Pax6 controls the mRNA levels of the insulin 1 and 2, *Pdx1*, *MafA*, *GLUT2*, and *PC1/3* genes in β-cells. Importantly, we identified new Pax6 target genes coding for GK, Nkx6.1, cMaf, PC2, GLP-1R and GIPR which are all involved in β-cell function. Furthermore, we demonstrated that Pax6 directly binds and activates specific elements on the promoter region of these genes. We also demonstrated that Pax6 knockdown led to decreases in insulin cell content, in insulin processing, and a specific defect of glucose-induced insulin secretion as well as a significant reduction of GLP-1 action in primary β-cells. Our results strongly suggest that Pax6 is crucial for β-cells through transcriptional control of key genes coding for proteins that are involved in insulin biosynthesis and secretion as well as glucose and incretin actions on β-cells. We provide further evidence that Pax6 represents a key element of mature β-cell function. **(*Molecular Endocrinology* 26: 696–709, 2012)**

Type 2 diabetes mellitus is a common disease characterized by insulin resistance and insulin deficiency as well as hyperglucagonemia. Although the altered insulin secretion process in response to glucose observed in diabetes has been well characterized, much less is known about the molecular mechanisms leading to decreased insulin biosynthesis. To have a better understanding of β-cell function and alteration in diabetes, it will be necessary to define the transcriptional network that coordinates the overall insulin response to glucose, from the transcriptional activation of the insulin genes to enhanced insulin secretion.

Pax6 is a transcription factor which has been implicated in the development of the eye, the central nervous system (1), and the pancreas (2). In the mouse pancreas, Pax6 is restricted to endocrine cells both during development and in adulthood (3).

Mice with a targeted disruption of Pax6 die soon after birth with an absence of glucagon-producing cells and a markedly decreased number of insulin-positive cells (4). Thus, Pax6 appears to be critical for endocrine cell development. Mice with a conditional inactivation of Pax6 in β-cells have a diabetic phenotype with hypoinsulinemia (5). Because the total number of pancreatic endocrine cells and cell specification are not altered in Pax6 mutant mice, Pax6 is thought to be required for maintaining β-cell differentiation rather than for their genesis (5). Indeed, despite a strong reduction of Pdx1-positive cell number and lack of *GLUT2* expression, several transcription factors essential for endocrine development were maintained. Importantly, Pax6 is known to directly bind and activate the insulin, *Pdx1*, *MafA*, and *PC1/3* gene promoters, suggesting an essential role in insulin biosynthesis and more widely β-cell function (4, 6–9).

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* Y.G. and L.S.K. contributed equally to this work.

Abbreviations: ChIP, Chromatin immunoprecipitation; GSIS, glucose-stimulated insulin secretion; KRB-2.8 mM G, Krebs buffer + 2.8 mM glucose + 0.1% BSA; siRNA, small interfering RNA.

Of interest, we recently reported that Pax6 is essential for α-cell function by regulating the transcription of the *glucagon* gene but also the *MafB*, *cMaf*, and *NeuroD1/Beta2* genes (10), all critical for *glucagon* gene expression and α-cell differentiation as well as the genes coding for the processing enzyme PC2 and the molecular chaperone 7B2, which act in concert to cleave proglucagon to produce glucagon (11). These results indicate that Pax6 is critically involved in glucagon biosynthesis and α-cell differentiation. We now postulate that Pax6 also represents a key component of the transcriptional network of pancreatic β-cells leading to differentiation and adequate function.

The aim of the present study was to better define the role of Pax6 in β-cell function and identify the gene targets of Pax6. Because the presence of insulin is the most obvious evidence for the differentiation of β-cells, we investigated whether Pax6 could regulate genes coding for proteins involved in insulin gene transcription and more widely in β-cell differentiation and function. We developed a Pax6-deficient model, a partial knockdown with specific Pax6 small interfering RNA (siRNA) in primary rat β-cells.

We now report that Pax6 controls the expression of the insulin 1 and 2, *PC1/3*, *PC2*, *Pdx1*, *MafA*, *cMaf*, *GLUT2*, *GK*, *Nkx6.1*, *GLP-1R* and *GIPR* genes in β-cells. As it was previously described for the insulin, *MafA*, *cMaf*, and *Pdx1* genes, Pax6 is able to bind and activate the promoter regions of the *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* genes through specific sites as assessed by EMSA, chromatin immunoprecipitation (ChIP), mutational analyses, and transfection experiments. Furthermore, we clearly demonstrate that Pax6 knockdown in primary β-cells leads to an alteration of β-cell function mainly through disruption of glucose-stimulated insulin secretion (GSIS) as well as decreases of insulin biosynthesis and GLP-1 action.

Taken together, we identified new Pax6 target genes in adult pancreatic β-cells that are involved in β-cell differentiation and function and more widely illustrate the coordinate actions of transcriptional factors such as Pax6 on the overall β-cell response to glucose.

Results

Identification of Pax6 target genes in β-cells

To better understand the role of Pax6 in β-cells, we developed a Pax6-deficient model in adult primary rat β-cells. As we previously described for α-cells, we generated a Pax6 knockdown in primary β-cells by using specific siRNA directed against *Pax6* (10). We performed these experiments with Pax6 or corresponding scramble siRNA for 96 h and verified the efficiency of *Pax6* gene silencing in our system. Transient transfection of Pax6 siRNA led to a 75% decrease of Pax6 mRNA levels (Fig. 1A) followed by a corresponding decrease in Pax6 protein content (Fig. 1B), thus achieving knockdown of Pax6 in differentiated β-cells. We assessed the efficiency of siRNA transfection in rat primary β-cells and observed a rather homogenous effect on Pax6 expression and a good correlation between *Pax6* gene silencing and transfection efficiency (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), indicating that Pax6 siRNA affects *Pax6* gene expression in a majority of cells.

We then measured the level of expression of key genes involved in β-cell differentiation and function by real-time RT-PCR through a candidate gene approach. We analyzed the mRNA levels for *insulin 1* and *2*, *Pdx1*, Pax4, MafA, Nkx6.1, which represent specific markers of differentiated β-cells, as well as for *Nkx2.2*, *cMaf*, *GK*, *GLUT2*, *GLP-1R* and *GIPR*, *PPARγ*, *Isl1*, *NeuroD1/Beta2*, *Sox4*, which are all involved in β-cell differentiation and function, and *PC1/3* and *PC2* (responsible for proinsulin processing) in pancreatic β-cells treated with either Pax6-specific or scramble siRNA.

The specific silencing of Pax6 in β-cells led to a significant decrease of *insulin 1* and 2, *Pdx1*, *MafA*, *cMaf*, *PC1/3*, *GLUT2*, *Nkx6.1*, *PC2*, *GK*, *GLP-1R*, and *GIPR* mRNA levels after 96 h, indicating that these genes are potential Pax6 target genes (Fig. 1C). By contrast Pax4, Nkx2.2, PPARγ, Isl1, NeuroD1/Beta2, Kir6.2, and SUR1 mRNA levels were unaffected by Pax6 knockdown. We also assessed the expression of the insulin receptor (IR), IRS1, *TCF7L2*, *Ngn3*, *Sox4*, and *Sox9* genes and found no significant variation in their mRNA levels (data not shown).

We then quantified proteins encoded by the potential Pax6 target genes after 96 h of scramble and siPax6 transfection in primary β-cells. In agreement with the results obtained for mRNA, Pax6 knockdown led to a significant decrease of Pdx1, MafA, cMaf, Nkx6.1, PC1/3, PC2, GLUT2, GK, GLP-1R, and GIPR protein amounts, whereas Nkx2.2 and GAPDH used as controls were not affected (Fig. 2).

To confirm these results in another model, we used the β-TC3 insulin-producing cells. These experiments were performed similarly to those in primary β-cells with Pax6 and scramble siRNA incubated with β-TC3 cells during 96 h achieving a 75% decrease in Pax6 mRNA and protein levels. Pax6 target genes identified in primary β-cells were also specifically affected in β-TC3 cells, whereas *Pax4*, *Nkx2.2*, *Isl1*, *NeuroD1/Beta2*, *Kir6.2*, and *Sur1* mRNA levels were unchanged (Supplemental Fig. 2). We also assessed the viability of β-cells after 96 h of *Pax6* gene

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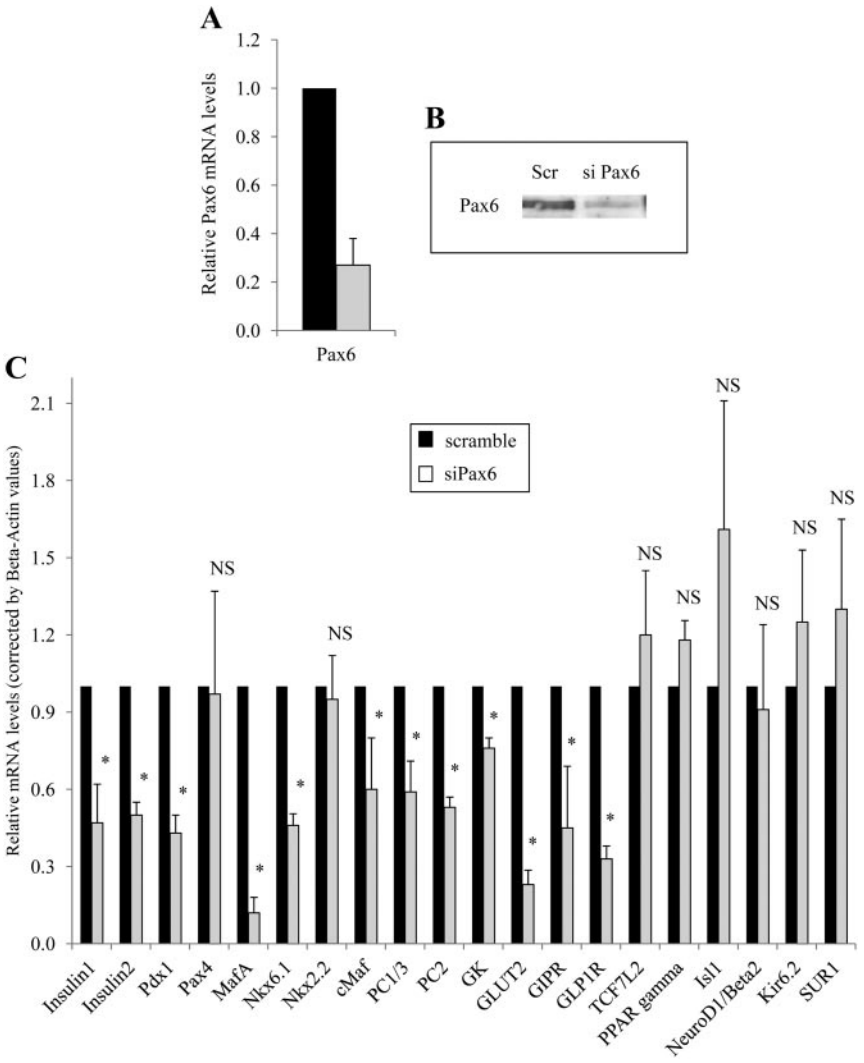

FIG. 1. Specific inhibition of *Pax6* gene expression by siRNA identifies target genes in primary rat β-cells. Primary β-cells (~40,000 cells/condition) were transfected with 100 nM Pax6 siRNA cocktail (siPax6-614 and siPax6-1007) or corresponding scramble (+) during 96 h. A, Effects of Pax6 siRNA on *Pax6* gene expression by real-time RT-PCR. Data are corrected by β-actin mRNA values. B, Western blot analyses of Pax6 protein content from transfected primary β-cells with scramble (Scr) or specific Pax6 siRNA (siPax6) after 96 h. C, Quantitative analyses of the expression of key pancreatic endocrine genes coding for insulin and proteins involved in β-cell differentiation and function or insulin gene transcription 96 h after scramble or Pax6 siRNA transfection. Data are expressed relative to β-actin mRNA values; corrected for transfection efficiency as the means ± SEM for at least five different experiments. *, Statistical significance with $P < 0.05$ value using a Student's *t* test; NS, no significant effect.

silencing in different models and observed that there was no significant changes in apoptotic cell number between scramble and siPax6 conditions (Supplemental Fig. 3).

Our data thus indicate that Pax6 specifically controls the expression of key genes involved in insulin gene transcription and processing as well as insulin secretion.

Identification of functional Pax6-binding sites on Pax6 target gene promoters

Among the different genes we identified as potential targets of Pax6, the genes coding for insulin 1 and 2, Pdx1, MafA, PC1/3, and GLUT2 have previously been proposed to be regulated by Pax6 in β-cells and cMaf and PC2 in α-cells. Furthermore, these studies have indicated that Pax6 directly binds to the promoter of the insulin, *Pdx1*, *MafA*, and *PC1/3* genes to activate their transcription, although no precise Pax6-binding site has been defined in the *PC1/3* gene promoter, and the molecular mechanisms involved for Pax6 regulation on the *GLUT2* gene remain unknown. We also previously described the direct control of Pax6 on the *cMaf* gene promoter (10) and the regulation of the *PC2* gene by Pax6 through a Maf regulatory element element on which either cMaf or MafA can bind (11). We did not investigate further these genes except for the *GLUT2* and *PC1/3* genes.

To further characterize the effects of Pax6 on *GLUT2*, *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene expression, we searched for potential binding sites on their promoter regions. *In silico* analyses of the 5′-flanking region of these genes revealed several highly conserved Pax6 putative binding sites in each promoter (Fig. 3). To further analyze the respective binding sites of Pax6 on the identified gene promoters, we performed EMSA experiments with probes containing each putative Pax6-binding sites (Fig. 4). Proteins-DNA complexes were observed on one putative binding site of the *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* promoters with extracts from BHK-21 cells overexpressing mPax6. To verify the nature of these complexes, a Pax6 antibody was used and able to supershift the complexes, indicating that Pax6 indeed interacts with each of these promoter regions. Cold wild-type and mutant oligonucleotides were also added at a 200-fold excess to further verify specificity; although wild-type Pax6-binding sites disrupted the complexes, mutant sites did not, indicating that Pax6 binds specifically the *PC1/3* (+243/+261 bp), *GK* (−34/−16 bp), *Nkx6.1* (+377/+395 bp), *GLP-1R* (−233/−216 bp), and *GIPR* (−366/−348 bp) gene promoter regions. Other potential sites identified in the

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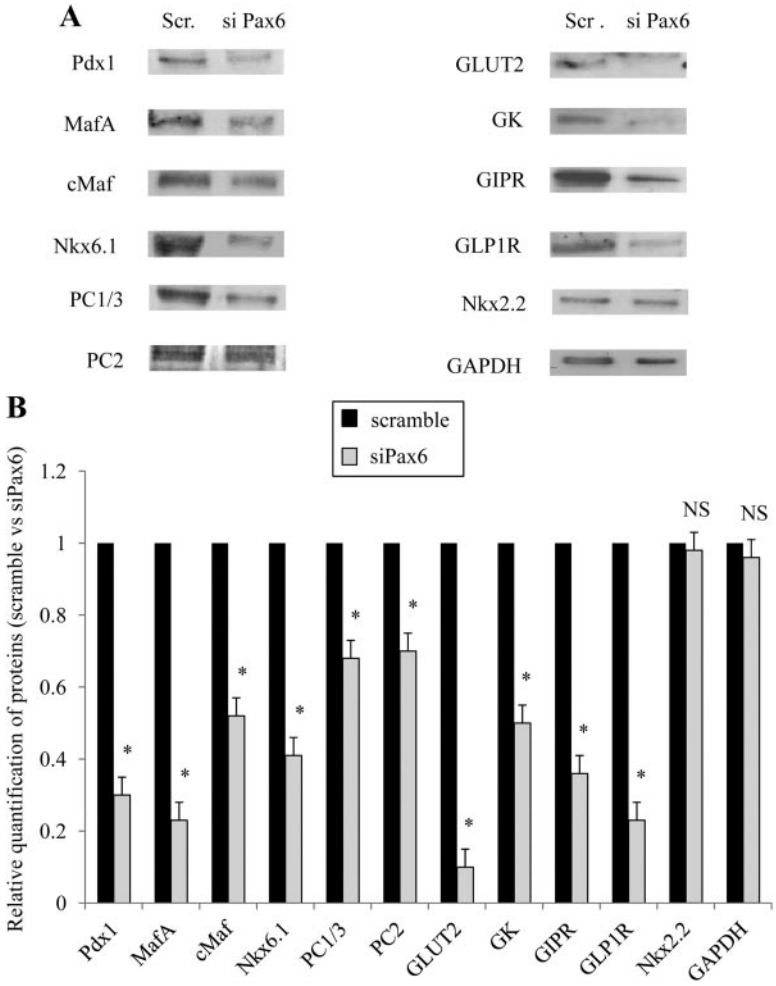

FIG. 2. Specific inhibition of *Pax6* gene expression decreases protein content of corresponding putative Pax6 target genes in primary β-cells. A, Western blot analyses of putative Pax6 targets from total cell extracts of transfected primary β-cells with scramble (Src) or specific Pax6 siRNA (siPax6) after 96 h. B, Histograms represent protein amounts after 96 h transfection with specific Pax6 or scramble siRNA. Each Western blotting was performed on three different transfection experiments. Data are presented as the means ± SEM. *, Statistical significance with $P < 0.05$ using Student's *t* test; NS, no significant effect.

search, in the *GLUT2* (−945/−927 and −409/−391 bp), PC1/3 (−78/−60 bp), *Nkx6.1* (−804/−786 and −521/−500 bp), and *GLP-1R* (−729/−711 and −664/−646 bp) promoters were also studied in EMSA experiments but displayed no specific Pax6 binding (data not shown). The absence of functional Pax6-binding site on the *GLUT2* proximal promoter suggests that Pax6 regulates GLUT2 by indirect mechanisms.

To determine the *in vivo* binding of Pax6 on the promoter regions of the identified genes, we performed ChIP assays on rat islets and β-TC3 cells (Fig. 5, A and B). We designed primers for PCR amplification corresponding to mouse and rat promoter regions, including the Pax6-binding sites revealed by *in silico* analyses as well as EMSA experiments. We used as a positive control the *insulin 1* gene promoter. We found that Pax6 binds the promoter regions of the *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene in rat islets as well as in insulin-producing cells, confirming the potential direct involvement of Pax6 on these promoters. We used as a negative control, a part of exon 1 of the *CNAP1* gene, which was previously described as a locus that should not be enriched by ChIP (ChIP-IT; Active Motif, Carlsbad, CA).

Analyses of PC1/3, GK, Nkx6.1, GLP-1R, and *GIPR* gene promoter activities

We next investigated the respective transcriptional effects of Pax6 on the identified target genes. We performed transient transfections in BHK-21 cells where Pax6 is absent with the *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters and the expression plasmid containing mouse Pax6 cDNA (Fig. 6). Pax6 overexpression led to a significant activation of the insulin 1 gene promoter as previously described (4), as well as the *Pdx1* and *MafA* gene promoters (data not shown) (7, 8). We thus assessed the effect of Pax6 overexpression on the newly identified target gene promoters; each promoter was specifically activated from 2- to 3.5-fold, indicating that Pax6 transactivates these promoters (Fig. 6A). We also performed mutations of characterized Pax6-binding sites on the *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters. We verified the consequences of these mutations on Pax6 binding in EMSA experiments and showed that an excess of the mutated probes was unable to compete for Pax6 binding (Fig. 4). We then assessed the effects of these mutations in BHK-21 cells and observed that mutations of Pax6-binding sites on each promoter led to an absence of Pax6 transactivation, reflecting the functionality of these elements (Fig. 6A).

To formally prove that the identified Pax6-binding sites are involved in the transcriptional control of the target genes, we performed transient transfections of native and Pax6 mutated promoter constructs in HIT-T15 insulin-producing cells. We observed that mutations of functional Pax6-binding sites on each promoter led to a significant decrease of promoter activities (Fig. 6B). Although mutations of Pax6-binding sites significantly decreased the *GLP-1R* and *GIPR* promoter activities by only 25%, the effects observed on *PC1/3*, *GK*, and *Nkx6.1* were greater than 60%. These observations suggest that Pax6 activation of transcription may be more

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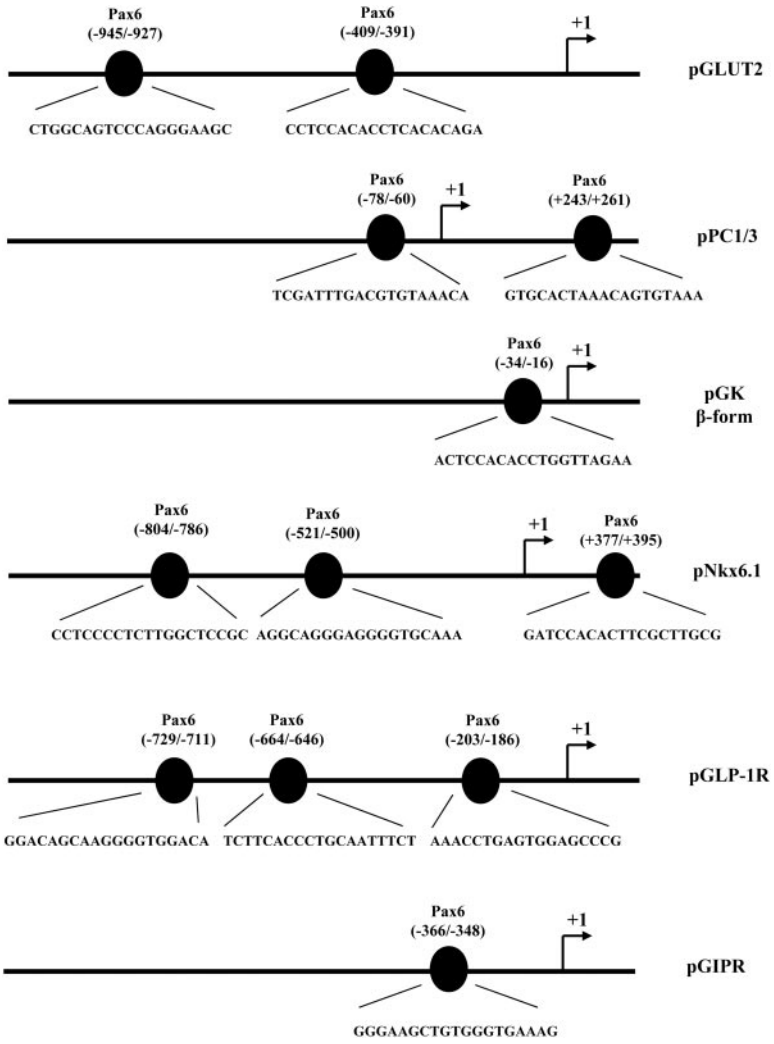

FIG. 3. Organization of Pax6 target gene promoters. Schematic representation of the *GLUT2*, *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters. Putative Pax6-binding sites are identified using *in silico* analyses of binding site search with the Genomatix software (Matinspector, available at http://www.genomatix.de) and represented by a *black circle*. Sequences of the Pax6 putative binding site is mentioned in *capitals*.

important for *PC1/3*, *GK*, and *Nkx6.1* gene expression compared with the *GLP-1R* and *GIPR* genes.

Overall, these data provide further evidence that Pax6 acts directly on +243/+261, −34/−16, +377/+375, −233/−216, and −366/−348 sites of the *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters, respectively, to control their transcription.

Inhibition of *Pax6* gene expression significantly affects insulin biosynthesis

To further assess the functional consequences of Pax6 knockdown in primary β-cells on insulin biosynthesis, we measured insulin content after 96 h of scramble and si-Pax6 transfections. We found that a 75% decrease of *Pax6* gene expression was associated with a 46% decrease in insulin content (Fig. 7A).

We next assessed the effects of *Pax6* gene silencing on insulin secretion. We first analyzed the KCl-stimulated insulin secretion in the Pax6-deficient cells as a control, because the *SUR1* and *Kir6.2* genes are not affected by the decreases of Pax6 expression. Interestingly, the effects of KCl both in the presence of scramble and Pax6 siRNA were quantitatively similar (4.11 ± 1.05 *vs.* 3.12 ± 0.42) (Supplemental Table 1), reflecting that KCl-stimulated secretion is not affected in our model (Fig. 7B). This observation illustrates that the general insulin secretory process is not altered after *Pax6* gene silencing.

Inhibition of *Pax6* gene expression significantly affects proinsulin processing

Pax6 was previously reported to regulate proinsulin processing through the control of PC1/3 (9). In addition, we described that Pax6 controls the processing of proglucagon in rat primary α-cells through PC2 (11). We now report that *PC2* gene expression is also controlled by Pax6 in primary β-cells as well as PC1/3. To further assess the functional consequences of Pax6 knockdown on proinsulin processing in primary β-cells, we measured the secreted proinsulin and insulin by β-cells in the medium after 96 h of Pax6 and scramble siRNA transfections. Cells were incubated in medium containing 2.8 and 16.7 mM glucose. We observed that the proinsulin/insulin ratio was drastically lower at 16.7 mM compared with 2.8 mM glucose in control conditions (Fig. 7C). By contrast, after *Pax6* gene silencing, the proinsulin/insulin ratio was significantly increased compared with the controls (by 4.85-fold) (Fig. 7D), indicating that Pax6 knockdown affects insulin gene transcription and insulin content as well as proinsulin processing. These results highlighted the crucial role exerted by Pax6 on insulin biosynthesis in primary β-cells.

Silencing of *Pax6* gene expression decreases the glucose- and GLP1-induced effects on insulin secretion

We next assessed the effects of Pax6 knockdown on GSIS. We measured insulin in the incubation medium of rat primary β-cells at basal condition (2.8 mM glucose) and after 1 h of incubation in 16.7 mM glucose after 96 h of scramble or siPax6 transfection (Fig. 8). Although basal values were not significantly different between

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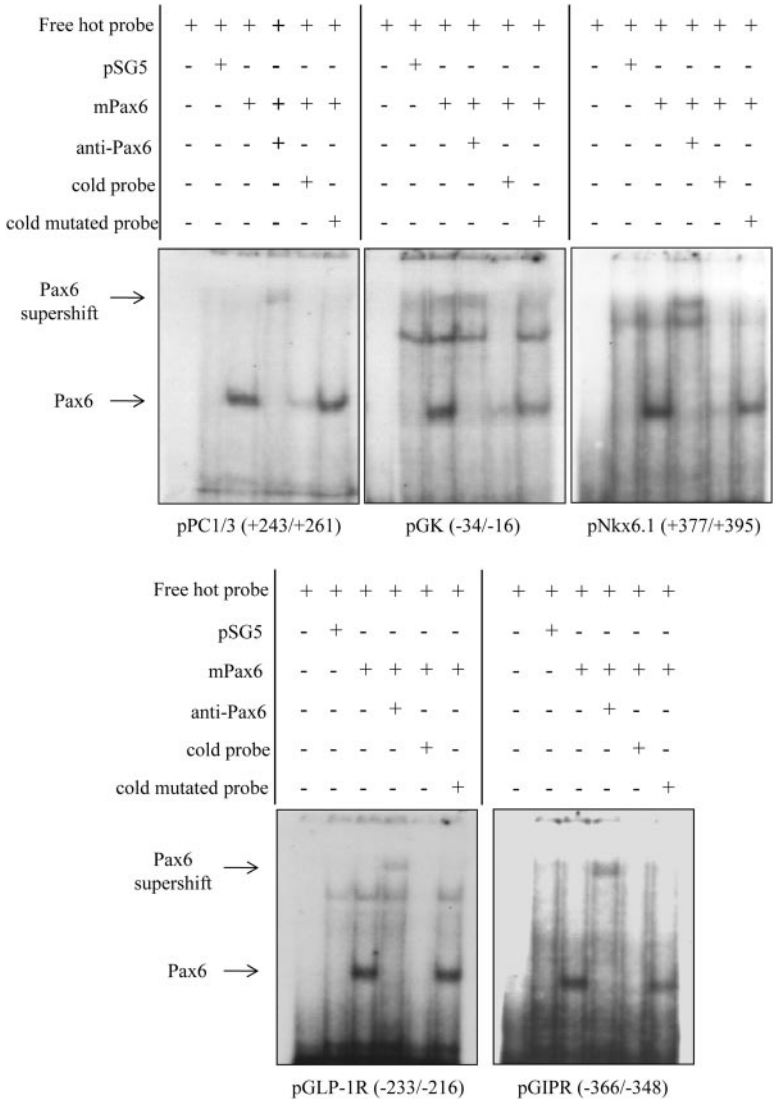

FIG. 4. Analyses of Pax6 binding on the *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters by EMSA experiments. Representative gels for Pax6 binding on *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters. EMSA were performed with 5′ end-labeled PC1/3 (+243/−261 bp), GK (−34/−16 bp), Nkx6.1 (+377/+395 bp), GLP-1R (−233/−216 bp), and GIPR (−368/−348 bp) oligonucleotides (listed in Supplemental Tables 2 and 3) in the presence of nuclear extracts from BHK-21 cells (pSG5, negative control) or BHK-21 cells overexpressing mouse Pax6 (p46 isoform). EMSA experiments were performed at least twice for each probe.

scramble and siPax6 conditions, incubation at 16.7 mM glucose significantly increased insulin secretion (4.86 ± 1.6-fold induction) in control conditions but did not induce any significant change in the presence of Pax6 siRNA (1.48 ± 1.03-fold induction, no significant effect) (Fig. 8 and Supplemental Table 1). Because Pax6 regulates GLP-1 and GIP receptor gene expression, we also assessed the effects of GLP-1 and GIP on rat primary β-cells after 96 h of scramble and siPax6 transfection. We measured insulin in low glucose [2.8 mM glucose (non-stimulatory condition)] and high glucose media (16.7 mM glucose). Addition of either GLP-1 or GIP to high-glucose medium led to a robust stimulation of insulin secretion (Fig. 8). Furthermore, these effects were drastically decreased in the presence of Pax6 siRNA compared with control condition.

Because GSIS was strongly affected by Pax6 silencing, we quantified specifically the GLP-1 and GIP stimulatory effects on insulin secretion. Both GLP-1 and GIP increased insulin secretion in the presence of scramble and Pax6 siRNA and thus potentiated GSIS. However, the potentiation effect of incretins on GSIS was only slightly decreased by Pax6 siRNA after GLP-1 stimulation and not altered for GIP, indicating that the decrease in GLP-1R and GIPR expression after Pax6 knockdown are not sufficient to completely abolish the incretin's actions (Supplemental Table 1).

Knockdown of *Pax6* gene expression affect *GSIS* through *Pdx1* and *MafA* gene silencing

Because Pax6 knockdown abolishes the GSIS, we attempted to assess the specific contribution of Pax6 in this process. Pdx1 and MafA, which are critical for insulin secretion, are indeed affected after 96 h of *Pax6* gene silencing (Fig. 2). We thus performed transient transfections of Pax6 siRNA and corresponding scramble in rat primary β-cells for 16, 24, and 48 h (Fig. 9). We measured the mRNA levels and protein of Pax6, Pdx1, and MafA at each time point. Pax6 mRNA levels were significantly decreased at 16 h already, whereas Pdx1 and MafA mRNA were significantly affected only after 24 h (Fig. 9A). By contrast, the Pax6 cell content was not significantly affected after 16 h of gene silencing but decreased by 50% at 24 h to reach maximal inhibition at 48 h (Fig. 9B). MafA and Pdx1 proteins were also significantly affected only after 24 h. Indeed, MafA and Pdx1 protein amounts decreased significantly by 50 and 25% at 24 h and by 70 and 50% at 48 h, respectively.

We next analyzed the glucose effect on insulin secretion after 16, 24, and 48 h of Pax6 silencing (Fig. 9C). Although the GSIS was not affected at 16 h, it was decreased by 57 and 72% after 24 and 48 h of *Pax6* gene silencing. We previously demonstrated that GSIS was completely abolished after 96 h Pax6 silencing (Fig. 8). These observations strongly suggest that disruption of GSIS requires a marked decrease of Pax6, Pdx1, and

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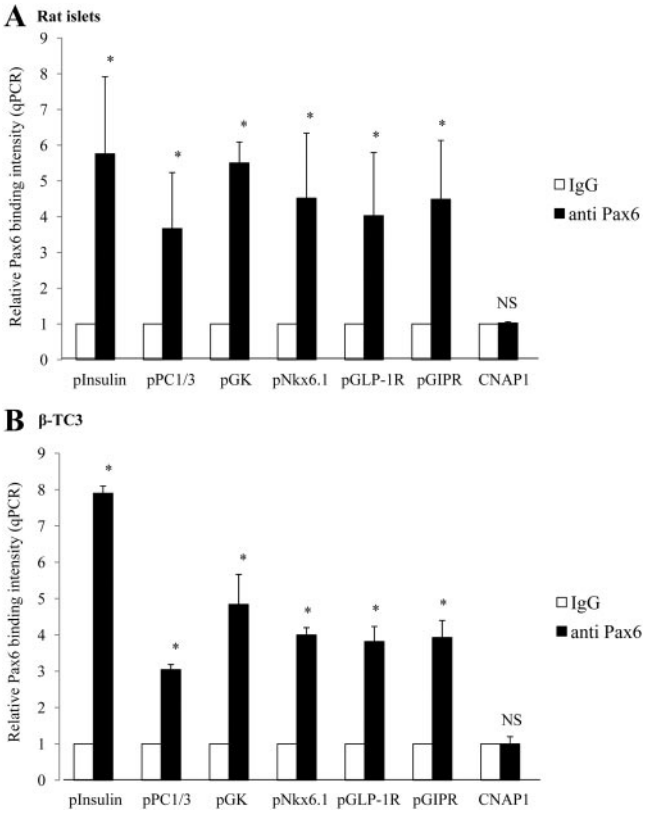

FIG. 5. *In vivo* interactions of Pax6 on the Insulin, *PC1/3*, *PC2*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters. Quantitative ChIP analyses in rat islet cells (A) and β-TC3 insulin-producing cell line (B). Histograms represent relative binding of Pax6 on the insulin (pInsulin), *PC1/3* (pPC1/3), *PC2* (pPC2), *GK* (pGK), *Nkx6.1* (pNkx6.1), *GIPR* (pGIPR), *GLP-1R* (pGLP-1R) gene promoters, and the negative control *CNAP1* gene. Binding was analyzed by real-time PCR, and amount of amplified DNA for different target gene promoters were corrected by total precipitated DNA in each condition. Binding intensity data are expressed relative to IgG immunoprecipitation (nonspecific binding) and are presented as the mean ± SEM for at least three independent experiments for β-TC3 and two independent experiments for rat islets cells. An antihistone H4 immunoprecipitation was also performed as a positive control for each promoter (data not shown). *, Statistical significance with $P < 0.05$ value using a Student's *t* test, NS, no significant effect.

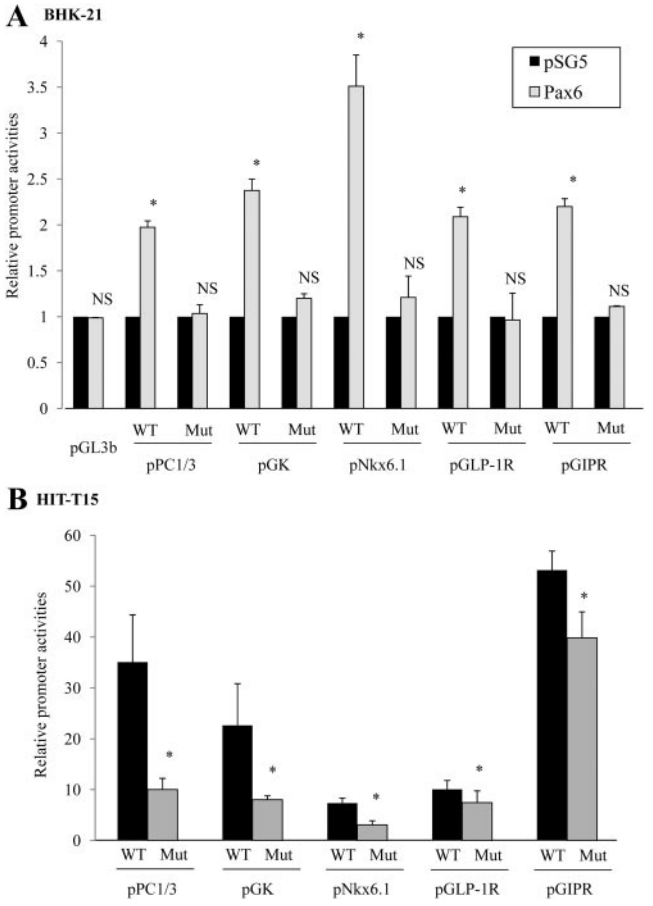

FIG. 6. Mutations of Pax6-binding sites affect the promoter activities of Pax6 target genes. The native and Pax6 mutant constructs of PC1 (−1500/+354 bp), GK (−1118/+14 bp), Nkx6.1 (−90/+1000 bp), GLP-1R (−490/+10 bp), and GIPR (−155/+166 bp) gene promoters were transfected in BHK-21 and HIT-T15 insulin-producing cell lines for 48 h. Histograms represent normalized luciferase activities of native and mutated promoter constructs *vs.* human placental alkaline phosphatase activities to monitor transfection efficiency. A, The native PC1/3, GK, Nkx6.1, GLP-1R, and GIPR (*black bars*) and corresponding mutated-Pax6 +243/+261 PC1/3, −34/−16 GK, +377/+375 Nkx6.1, −233/−216 GLP-1R, and −368/−348 GIPR promoter constructs (*gray bars*) were cotransfected in BHK-21 cells with pSG5 or mouse Pax6 cDNA (mPax6). Overexpression of mouse Pax6 was systematically verified in each cotransfection experiment by Western blot analyses. Histograms represent the Pax6 transactivation effects on native and mutated promoter constructs with pSG5 condition used as references (*black bars*). B, The native and mutated promoter constructs were transfected in HIT-T15 cells. The relative activities of each promoter constructs were expressed compared with pGL3basic activities. A minimum of three independent transfections were performed, each of them carried out in duplicate. Data are presented as the means ± SEM. *, Statistical significance with $P < 0.05$ using Student's *t* test; NS, no significant effect. WT, Wild-type (native promoter); Mut, mutated construct (mutated promoter).

MafA and that the relative contribution of each factor on insulin secretion cannot be assessed. Pax6 thus acts synergistically with MafA and Pdx1 to control response of insulin secretion to glucose.

Taken together, we identify the *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* genes as new Pax6 targets in β-cells. We characterize the direct action of Pax6 on *PC1/3*, *GK*, *Nkx6.1*, *GLP-1R*, and *GIPR* gene promoters through identification of functional binding sites in each promoter regions. Furthermore, we clearly demonstrate that Pax6 knockdown leads to an alteration of β-cell function especially on insulin biosynthesis and GSIS (Table 1).

Discussion

β-Cell differentiation and function result from a combinatorial network of transcription factors, which include Pax4, Nkx2.2, Pdx1, MafA, Nkx6.1, Pax6, and NeuroD1/Beta2. Targeted disruption studies in mice have revealed the critical importance of these transcription factors and their potential interrelationships (12–15). Pax6 is one of the major factors of specification and maintenance of the differentiated islet cells, particularly α- and

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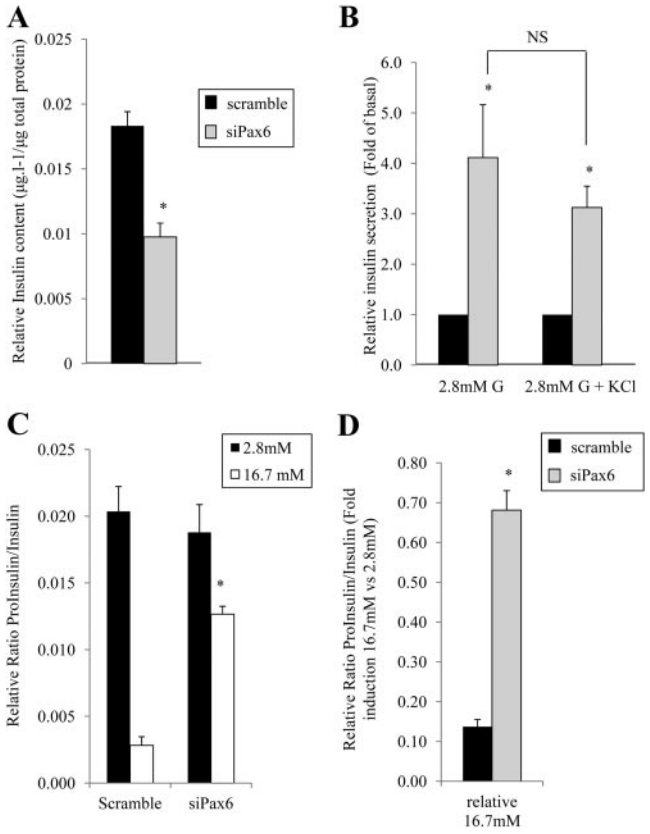

FIG. 7. *Pax6* gene silencing in rat primary β-cells affects insulin content and maturation. A, Insulin content after specific *Pax6* gene silencing. Histograms represent insulin content in primary β-cells 96 h after scramble or Pax6 siRNA transfection (40,000 cells/condition). Data are presented as the means ± SEM for at least three different experiments. *, Statistical significance with $P < 0.05$ value using a Student's *t* test; NS, no significant effect. B, KCl-stimulated insulin secretion. Insulin values from primary β-cells supernatants after 1 h of incubation in KRB-2.8 mM G and an additional hour in KRB-2.8 mM G + KCl (50 nM) in the presence of scramble or Pax6 siRNA. C, Effects of Pax6 silencing on proinsulin processing in rat primary β-cells. Data are represented by relative proinsulin/insulin ratio for scramble or Pax6 siRNA conditions after 1 h incubation of in 2.8 or 16.7 mM glucose medium. D, Data are also represented by the fold induction between 16.7 and 2.8 mM, reflecting the difference between the proinsulin/insulin ratio in scramble and Pax6 siRNA conditions. *, Statistical significance compared with scramble conditions with $P < 0.05$ value using a Student's *t* test. Data are presented as the means ± SEM for at least three independent experiments.

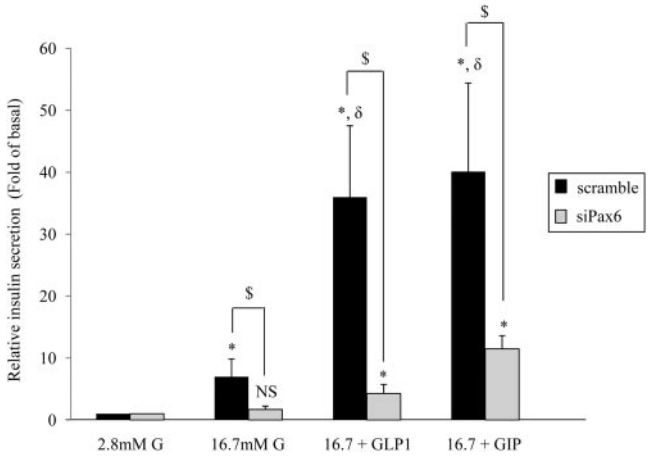

FIG. 8. Pax6 affects glucose- and incretin-stimulated insulin secretion in rat primary β-cells. Glucose, GLP-1, and GIP effects on insulin secretion after *Pax6* gene silencing in rat primary β-cells. Insulin values were determined from primary β-cells supernatants after 1 h of incubation in KRB-2.8 mM G and an additional 1 h in KRB-16.7 mM G or KRB-16.7 + GLP-1 (10 nM) or GIP (10 nM) in the presence of scramble or Pax6 siRNA. *, Statistical significance compared with 2.8 mM G condition with $P < 0.05$ value using a Student's *t* test; NS, no significant effect; δ, statistical significance compared with 16.7 mM G condition with $P < 0.05$ value using a Student's *t* test; $, statistical significance compared with scramble condition with $P < 0.05$ value using a Student's *t* test.

β-cells. The phenotype of Pax6−/− knockout mice suggests critical and nonredundant functions exerted by Pax6 in both cell types. Mice with a targeted disruption of Pax6 display a drastic decrease of all four types of differentiated endocrine cells without affecting endocrine cell mass, suggesting that Pax6 is required for endocrine cell differentiation (4, 5).

The finding that Pax6-deficient mice present a strong reduction of insulin-positive cells underlines the key role exerted by Pax6 in β-cell maturation. Indeed, Pax6 deficiency is characterized by decreased expression of the transcription factors Pdx1 and MafA implicated in the terminal differentiation process of endocrine cells and particularly β-cells as well as GLUT2 expression (5, 16). By contrast, Pax6 deficiency does not affect the expression of Ngn3, Isl1, and Nkx2.2, which are all involved in β-cell development and more widely in endocrine cell fate (5). Furthermore, Pax6 mutant mice present a diabetic phenotype characterized by hypoinsulinemia (5). Taken together, these observations suggest that Pax6 plays a key role in terminal differentiation of β-cells leading to mature insulin-producing cells.

Several Pax6 target genes have previously been identified in the eye and central nervous system as well as in the endocrine pancreas, including the β-cells. Indeed, the insulin, *Pdx1*, *MafA*, *PC1/3*, and *GLUT2* genes were previously described as Pax6 targets, although for the latter two genes, the mechanisms by which Pax6 regulates them was not entirely characterized. To better understand how Pax6 specifically controls β-cell differentiation and function, we investigated the consequences of Pax6 deficiency on the expression of genes coding for proteins known to play key roles in β-cells.

We show that in primary rat β-cells, Pax6 not only control the expression of the insulin 1 and 2, *Pdx1*, *MafA*, *PC1/3*, and *GLUT2* genes but also the *GK*, *Nkx6.1*, *cMaf*, *GLP-1R*, and *GIPR* genes. We found that Pax6 directly regulates these genes through activation of their promoters. Indeed, we characterized at least one func-

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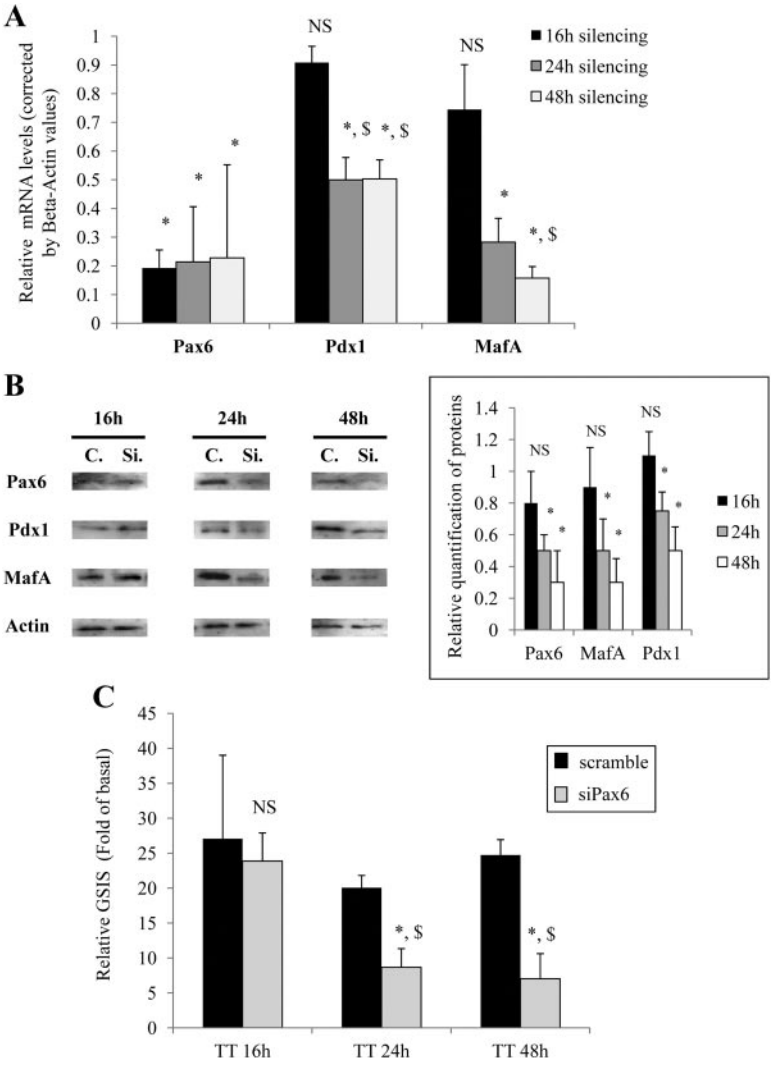

FIG. 9. Pax6 affects directly and indirectly GSIS in rat primary β-cells through Pdx1 and MafA silencing. Rat primary β-cells were transfected with scramble or Pax6 siRNA during 16, 24, and 48 h. A, Effects of *Pax6 siRNA* on *Pax6*, *Pdx1*, and *MafA* gene expression by real-time RT-PCR. Data are corrected by β-actin mRNA values. Histograms represent the relative amount of Pax6, Pdx1, and MafA mRNA levels after 16, 24, and 48 h of Pax6 siRNA transfections compared with scramble conditions. B, Western blot analyses of Pax6, Pdx1, and MafA protein cell contents from transfected primary β-cells with scramble (C.) or specific Pax6 siRNA (Si.) after 16, 24, and 48 h. Quantitative analysis of Western blottings is illustrated in the *inset*. Histograms represent the effects of Pax6 knockdown on Pax6, MafA, and Pdx1 protein amounts after 16, 24, and 48 h of gene silencing. C, Insulin secretion measurements after 16, 24, and 48 h of Pax6 or scramble siRNA. Cells were incubated for 1 h in KRB-2.8 mM G (basal) and for an additional 1 h in KRB-16.7 mM G (glucose stimulation). The GSIS values are represented by fold inductions compared with basal values. *, Statistical significance compared with scramble condition with $P < 0.05$ using Student's *t* test; NS, no significant effects; $, statistical significance compared with 16-h conditions with $P < 0.05$ value using a Student's *t* test. Data are presented as the means ± SEM for at least three different experiments.

tional Pax6-binding site on each proximal promoter by both ChIP and EMSA assays as well as site-directed mutagenesis.

Insulin gene transcription was previously shown to be activated by Pax6. However, the insulin genes are also under the control of Pdx1 and MafA, two transcription factors regulated by Pax6 as shown by binding, transactivation, and gene knockout studies (12, 14, 17–20). Interestingly, insulin promoter studies have revealed a synergistic effect of Pdx1, MafA, and Pax6 of the rat insulin 1 promoter, suggesting cooperative interactions between these factors (6). Our results and previous studies thus indicate that Pax6 is a critical regulator of *insulin* gene transcription through a direct as well as an indirect effect through Pdx1 and MafA. The overall effect is quantitatively important, because 75% inhibition of Pax6 expression in primary β-cells results in a 60% decrease in insulin mRNA levels.

In addition to insulin, our results confirm the regulation of Pdx1 and MafA by Pax6. Indeed, Pax6 has previously been reported to directly bind and activate *Pdx1* and *MafA* gene promoters (7, 8). There is actually a close interrelationship between these factors, because Pax6 controls Pdx1 and MafA, whereas MafA controls Pdx1 and *vice versa* (21). The relationship goes beyond their respective control, because Pax6, Pdx1, and MafA are together involved in the regulation of several important genes expressed in β-cells. Indeed, in addition to the *insulin* gene, the *GLUT2*, *Nkx6.1*, and *GK* genes are known to be controlled by MafA and Pdx1, and our results show that Pax6 is also involved in their regulation (22, 23). Overall, we conclude that Pdx1, MafA, and Pax6 act coordinately to activate essential genes involved in β-cell differentiation and function. Among the major functions that Pax6, Pdx1, and MafA control, insulin biosynthesis through insulin gene transcription and proinsulin processing is one of the most significant. Proinsulin processing depends on both the PC1/3 and PC2, proconvertases encoded by genes that are down-regulated by the inhibition of Pax6 in primary β-cells.

We previously showed in α-cells that the *PC2* gene is also controlled by Pax6 but indirectly through MafB and cMaf (11). Indeed, ectopic expression of each large Maf protein rescues transcription of the *PC2* gene promoter in α-cells deficient for Pax6. It is likely that in β-cells Pax6 acts through MafA and or cMaf to regulate *PC2* gene expression. Pax6 also controls *PC1/3* gene expression as suggested previously (9). We now extend these data by showing that Pax6 directly binds to a specific element of

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TABLE 1. Involvement of Pax6 on β-cell functions

Gene	Function	Direct and indirect action of Pax6 location of binding sites and references
Pdx1	β-cell differentiation Insulin gene transcription Insulin secretion	Direct action −2071/−2052 Samaras *et al*. (7)
MafA	β-cell differentiation Insulin gene transcription Insulin secretion	Direct action −7917/−7886 Raum *et al*. (8)
Nkx6.1	β-cell differentiation Insulin gene transcription Insulin secretion	Direct action +377/+395
cMaf	β-cell differentiation	Direct action +373/+385 Gosmain *et al*. (10)
PC1/3	Proinsulin processing	Direct action +243/+261
PC2	Proinsulin processing	Indirect action through cMaf (−659/−629) Katz *et al.* (11)
GLUT2	Glucose transport and metabolism	Indirect action through Pdx1 (−645/−635) Waeber *et al*. (29)
GK	Glucose phosphorylation and metabolism	Direct action −34/−16
GLP-1R	Insulin secretion (GSIS) and Insulin biosynthesis	Direct action −233/−216
GIPR	Insulin secretion (GSIS)	Direct action −366/−348

Identified target genes and their functions, direct or indirect involvement of Pax6, as well as location of functional Pax6-binding sites on target gene promoters are listed in the table. Specific elements involved in indirect or direct actions of Pax6 are referenced.

the 5′-UTR (untranslated region) of the *PC1/3* promoter and thus directly activates the *PC1/3* gene transcription and that Pax6 is functionally critical for proinsulin processing.

Pax6 not only controls MafA but all the large Maf expressed in α- and β-cells, because we show that the *cMaf* gene is affected by Pax6 silencing in primary β-cells, and we previously reported the role of *Pax6* in *MafB* and *cMaf* gene expression in primary rat α-cells (10). These results are in agreement with the drastic decrease of large Maf-positive cells observed *in vivo* in Pax6-deficient mice and clearly position Pax6 upstream of the large Maf in islet cells (16).

In addition to insulin biosynthesis, Pax6, Pdx1, and MafA are also critically involved in the regulation of insulin secretion by glucose (14, 24–26). MafA-deficient mice present a specific defect in glucose-induced insulin secretion, whereas Pdx1 has a more general role on insulin secretion (27). These factors control several genes implicated in the insulin secretory process (23, 28). The *GLUT2* gene is indeed a direct target of Pdx1, and specific mutation of the GLUT2TAAT motif leads to a significant decrease in transcriptional activity (29). Because we were not able to identify any functional Pax6-binding sites on the *GLUT2* gene promoter, we propose that Pax6 is an indirect regulator of *GLUT2* through the positive action of Pdx1.

In addition to GLUT2, we show that Pax6 also controls *GK* gene expression directly. Thus, Pax6 regulates two critical genes coding for proteins involved in the early phase of glucose handling by β-cells. We further illustrate the functional consequences of Pax6 down-regulation by showing the marked reduction of GSIS. This reduction is secondary to the altered expression of Pax6, Pdx1, and MafA. We were unable to quantify the respective role of each of them through time-course studies. By contrast, insulin secretion induced by KCl was preserved, in agreement with the fact that *SUR1* and *Kir6.2* gene expression were not affected by inhibition of Pax6 (24). These data

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also indicate that Pax6 does not control the overall process of insulin secretion.

Not only Pax6 appears essential in the regulation of insulin secretion by glucose but also through its control of incretin biosynthesis and actions. Pax6 has previously been proposed to be required for the synthesis of GLP-1 and GIP by the enteroendocrine L and K cells, through activation of their gene expression (24, 30). Additionally, maturation of GIP and GLP-1 is dependent on PC1/3, another target of Pax6 (31). These data clearly illustrate the role of Pax6 in incretin biosynthesis. We extend these findings by showing that Pax6 controls the β-cell response to incretins by regulating their respective receptor genes through the binding to their promoters. Pdx1 and MafA have also been suggested to regulate the GLP-1 and GIP receptors. At present, however, there is no data available on the mechanisms by which these factors exert their effects. Functionally, we demonstrate that the glucose-dependent GLP-1 effect on insulin secretion is affected but only moderately, whereas the response induced by GIP is not. We propose that the effective decrease in receptor expression may not have been sufficient to markedly alter the incretin response, because both GLP-1 (proglucagon) and GIP mRNA and protein levels were decreased in these experiments.

As mentioned above, we also identified Nkx6.1 as a new Pax6 target gene. However, the pancreatic expression of Nkx6.1 appeared unchanged in Pax6 mutant mice (5). In this model, expression of Nkx6.1 was assessed by immunochemistry, which is not quantitative. We thus conclude that Pax6 activates *Nkx6.1* gene expression but is not critical, and its absence is not sufficient to abolish its expression. Of note, Nkx6.1-deficient mice present similarly to MafA-deficient mice, a specific defect in GSIS, further stressing the critical role of Pax6 and its target genes in GSIS (14, 27). The *Nkx6.1* gene may also represent a target of MafA, illustrating again the strong dependency of GSIS on these factors (23).

As for the transcription factors Pdx1, NeuroD1/Beta2, HNF-1α/β, and HNF-4α, Pax6 is associated with diabetes. Indeed, heterozygous mutations of Pax6 are associated with glucose intolerance or early onset diabetes mellitus in humans (32, 33). Furthermore, conditional inactivation of Pax6 in the mouse pancreas is characterized by a diabetic phenotype reflecting an essential role of Pax6 in the regulation of glucose homeostasis and endocrine cell functions (5). *Pax6* gene expression seems also to be modulated by nutrition. Indeed, the fasting state leads to a significant decrease of Pax6 mRNA levels compared with the postprandial state in mouse islets (13). *Pax6* gene expression is also sensitive to glucotoxicity and lipotoxicity (34, 35), because chronic hyperglycemia as well as excessive amounts of fatty-acids decrease the mRNA levels of *Pax6* in rodent islets as well as a number of key endocrine genes, including *insulin*, *Pdx1*, *Beta2*, *Nkx6.1*, *MafA*, *glucokinase*, and *GLUT2*, illustrating the marked deleterious effects observed on *β-cell* gene expression in the diabetic state (35–38).

Overall, our data indicate the critical role of Pax6 in the transcriptional control of key genes involved in insulin biosynthesis/secretion, which are also affected by hyperglycemia and excess of fatty free acids (39). Thus, changes in *Pax6* gene expression in this context could represent a substantial component of the pathophysiological mechanisms of glucolipotoxicity in pancreatic endocrine cells.

In conclusion, we have demonstrated that Pax6 acts as a critical transcription factor in β-cell through the control of insulin biosynthesis and secretion and more widely β-cell differentiation (Table 1). Pax6 represents a key component of the transcriptional network, along with Pdx1, MafA, and Nkx6.1, that controls β-cell physiology. Furthermore, we reinforce the idea that Pax6 is not only involved in the terminal maturation of β-cells but also in the control of glucose utilization and β-cell response to incretins, reflecting its crucial role in β-cell function.

Materials and Methods

Islets isolation

Islets of Langerhans were isolated using a modification of previously described procedures (40). β-Cells were separated from non-β-cells by autofluorescence-activated sorting as we previously described (10). We obtained two major populations characterized by immunohistochemistry, β-cells and an enriched α-cell fraction. Fraction 1 was composed almost exclusively by insulin-positive cells (>95%), whereas fraction 2 by a majority of glucagon-positive cells (~75 ± 5%). As noted, we obtained about 250,000 primary β-cells respectively per rat; 16 h after isolation, β-cells were cultured on extracellular matrix-coated plates derived from 804G cells (Laminin-5-rich extracellular matrix) in DMEM containing 10% fetal calf serum, 11.2 mM D-glucose, 2 mM L-glutamine, and antibiotics.

Cell culture

The insulin-producing β-TC3 cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 3 g/liter glucose, 15% fetal calf serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The nonislet Syrian baby hamster kidney (BHK21) cell lines were grown in RPMI 1640 (R-6504; Sigma, Basel, Switzerland) supplemented with 5% fetal bovine serum and 5% newborn calf serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

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Materials and plasmids

The cDNA coding for a full-length active form of mouse Pax6 was cloned into the pSG5-ATG expression vector. Promoters of the mouse *PC1/3*, *PC2*, *GLP-1R*, and *GIPR* genes were cloned from genomic DNA, and mouse Nkx6.1and rat GK promoters were generously provided respectively by M. S. German and M. Magnusson. Each promoter was subcloned in the reporter luciferase vector pGL3-basic vector (Promega, Madison, WI).

RNA interference

Two different specific sequences of siRNA were designed for Pax6 against rodent mRNA sequence (Stealth RNAi 614 and 1007; Invitrogen). Primary adherent β-cells were transfected with 100 nM mixed Pax6 siRNA as we previously described (10). Total RNA and cellular extracts were isolated 96 h after transfection.

Western blot analyses

Total extracts were isolated as described (41) from primary rat β-cells. Five to twenty micrograms of each protein extract were resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred electrophoretically to nitrocellulose membranes. After incubation with specific primary antibodies and secondary antibodies coupled to HRP, the signal was detected with Super Signal West Pico Trial kit (Pierce Chemical Co., Rockford, IL). Immunoblotting was performed with polyclonal antibodies to rabbit Pax6 (gift from S. Saule), PC1/3 (gift from D. F. Steiner), PC2 (gift from S. Seidah), MafA and cMaf (no. BL1069 and BL662; Bethyl, Montgomery, TX), Nkx6.1 (obtained from O. Madsen, Beta Cell Biology Consortium, Nashville, TN), Nkx2.2 (obtained from Beta Cell Biology Consortium), GIPR (gift from T. J. Kieffer), GLP1R (gift from B. Thorens), Pdx1 (gift from C. Wright), insulin receptor (gift from K. Kriauciunas), GK (gift from M. Magnuson), glyceraldehyde-3-phosphate dehydrogenase (no. MAB374; Millipore, Bedford, MA), and antimouse GLUT2 (gift from B. Thorens) diluted 1:1000 and goat antirabbit or antimouse or rabbit antisheep conjugated with HRP diluted 1:10,000 (nos. sc2030, NIF825, and sc-2770; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The signal was detected with Super Signal West Pico Trial kit (Pierce Chemical Co.). Protein loading was normalized by Coomassie coloration. At least three independent experiments were performed with cell extracts.

Measurements of insulin content and secretion

Primary rat β-cells were removed and frozen in 1 ml of acid/ethanol mixture after 96 h of scramble and siPax6 transient transfection to measure insulin and proinsulin contents. Insulin secretion was analyzed from supernatants of rat primary β-cells in the same conditions. Insulin and proinsulin were quantified by an ELISA kit (High Range Rat Insulin ELISA kit and Rat/Mouse Proinsulin ELISA kit; Mercodia, Winston Salem, NC) following the manufacturer's recommendations.

Insulin values were corrected by total protein amount in each condition.

For insulin secretion experiments, primary β-cells were first incubated during 2 h in depleted medium [Krebs buffer + 2.8 mM glucose + 0.1% BSA (KRB-2.8 mM G)] to stop the insulin secretion. Cells were then incubated for 1 h in KRB-2.8 mM G for basal values of insulin secretion and stimulated for an additional 1-h incubation with 16.7 mM glucose or 16.7 mM glucose combined with 10 nM GLP-1/GIP (nos. H-5552 and H-5645; Bachem, Torrance, CA) or KCl. In each experiment, we used 2.8 mM glucose condition (basal values) as the references, and stimulated-insulin secretion are represented by fold induction compared with basal values. All insulin secretion values were normalized to total protein amount.

RNA preparation and RT-PCR analyses

Total RNA was isolated from mouse islets and primary rat β-cells using TRIzol reagent according to the manufacturer's specifications (Invitrogen). Different targets were analyzed by real-time RT-PCR with QuantiTect SYBR Green kit (QIAGEN, Valencia, CA) and Light-Cycler (Roche Diagnostics, Indianapolis, IN) using specific primers (Supplemental Tables 2 and 3).

Promoter analyses

For all transfections, BHK-21 cells were transfected with Lipofectamine 2000 according to the manufacturer's specifications (Invitrogen). Promoter activities were analyzed as we previously described (10).

Electrophoretic mobility shift assays

EMSA reactions were performed as previously described (42). EMSA were performed using the sequence of the 5′-flanking region of mouse PC1/3, Nkx6.1, GIPR, GLP-1R, and rat GK genes corresponding to promoter regions containing highly conserved putative Pax6-binding sites as mentioned in Fig. 3B. Each probe was incubated in the presence of 10 μg of nuclear extracts from BHK-21 cells (pSG5, negative control) or BHK-21 cells overexpressing mouse Pax6 (p46 isoform). An anti-Pax6 antibody was used to test the specificity of Pax6 binding as well as native and mutated cold probes in 200-fold excess. Arrows indicate specific shifts and supershifts for Pax6 proteins on probes. Overexpression of mouse Pax6 was verified by Western blot analyses. Native and mutated Pax6 putative binding site oligonucleotides are listed in Supplemental Table 4.

ChIP assays

ChIP assays were performed as previously described (41); 50 μg of chromatin extract of rat islets and β-TC3 were used for experiments and incubated with 10 μg of anti-Pax6 (no. PRB-278P; Covance, Richmond, CA) and 5 μg of α-acetyl-histone H4 antibodies (no. 06-866; Upstate, Waltham, MA) as well as rabbit IgG (no. sc-2027; Santa Cruz Biotechnology, Inc.) (described by Active Motif as a locus that should not be enriched by ChIP). Binding was analyzed by real-time PCR with a Light-Cycler (Roche Diagnostics). An antihistone H4 immunoprecipitation was also performed as a positive control for each promoter (data not shown). The sets of PCR primers used for analysis of binding are indicated in Supplemental Table 5.

Site-directed mutagenesis

The promoter mutants were constructed using standard PCR as we previously described (10). The procedure used the PC1/3, GK, Nkx6.1, GLP-1R, and GIPR promoter DNA constructs as template and two synthetic oligonucleotide primers containing the desired mutation for the reaction. We performed mutation of each functional Pax6-binding sites core sequence. The oligo-

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nucleotide sequences and corresponding Pax6 mutation are listed in Supplemental Table 4.

Data analysis

Data are presented as means ± SEM and analyzed by Student's *t* test. A *P* value of less than 0.05 was considered to be statistically significant.

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Address all correspondence and requests for reprints to: Yvan Gosmain, Diabetes Unit, University Hospital, 1211 Geneva 14, Switzerland. E-mail: yvan.gosmain@hcuge.ch.

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