Original Article

Differences of Pancreatic Expression of 7B2 Between C57BL/6J and C3H/HeJ Mice and Genetic Polymorphisms at its locus (*Sgne1*)

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C57BL/6 (B6) mice develop glucose intolerance with age, whereas C3H/He (C3H) mice do not. In this study, we examined whether this differential glucose homeostasis was associated with differences of proteolytic activation of pancreatic prohormones. Radioimmunoassays showed comparable levels of fasting plasma insulin between the two strains but a significantly lower glucagon level in B6 mice. Pulse-chase analysis of glucagon biosynthesis in isolated pancreatic islets revealed that proglucagon was less efficiently processed in B6 mice. Because proprotein convertase (PC)2 and its 7B2 helper protein are required for this processing, we quantified islet mRNA levels by RT-PCR and protein levels by immunoblotting. The levels of proPC2 mRNA were similar between the two strains, but B6 protein extracts contained less of the mature PC2. In contrast, 7B2 mRNA and protein levels were both significantly lower in B6 pancreas. Sequencing of the 7B2 gene promoter and cDNA in the two strains revealed seven single nucleotide polymorphisms and one dinucleotide insertion/deletion in the cDNA as well as a single nucleotide polymorphism and two insertions/deletions in the promoter. Differential expression of 7B2 may contribute to the difference between B6 and C3H mice not only in glucagon production and secretion but also in glucose tolerance. *Diabetes* 55:452–459, 2006

Impaired glucose tolerance is a risk factor for diabetes. Its prevalence increases with age. The mechanism underlying this increase is not fully understood. A role of gastrointestinal and pancreatic hormones in the process has been proposed (1). The production and secretion of these hormones are partly regulated by the cellular prohormone activation system. This system consists primarily of the proprotein convertases (PCs) 1 and 2, carboxypeptidase E, the granin-like proteins proSAAS, and 7B2 (2,3). PC1 and PC2 are neuroendocrine members of a family of calcium-dependent subtilases that cleave prohormones and proneuropeptides after paired basic residues (4,5). Carboxypeptidase E removes the COOH-terminal basic residues exposed by PC cleavage (6). PC1 and PC2 display both overlap and specificity in their enzymatic cleavage of pancreatic prohormones (7–9). ProSAAS and 7B2 are resident acidic proteins of neuroendocrine secretory granules (3,10). ProSAAS is a PC1-specific competitive inhibitor (11–13). 7B2 facilitates the exit of proPC2 from the endoplasmic reticulum; its COOH-terminal peptide is a potent PC2 inhibitor (2,3). Genetic deficiencies for PC1, PC2, carboxypeptidase E, and 7B2 in mice have been shown to cause multiple impairment of pancreatic prohormone processing (14–17).

To verify the possible implication of these proteins in genetic susceptibility to age-induced glucose intolerance, we compared their islet expression in C57BL/6 (B6) mice, which develop glucose intolerance with age, and in the C3H/He (C3H) strain, which does not (18,19). We found that B6 mice have lower circulating glucagon, express less 7B2, and contain less active PC2 in pancreatic islets. In addition, we have identified 7B2 gene polymorphisms between the two mouse strains.

RESEARCH DESIGN AND METHODS

Mice, antibodies, and PCR primers. C57BL/6J and C3H/HeJ (8–12 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in a pathogen-free facility, on a 12-h light/dark cycle, with free access to food and water. Experimental manipulations of these animals were approved by the animal care committee of the Ottawa Health Research Institute and performed in accordance with the guidelines of the Canadian Council for Animal Care. The antibodies and the PCR primers used in this study are described in Tables 1 and 2, respectively.

Pancreatic islet isolation and culture. Islets of Langerhans were isolated from mouse pancreata by injecting a collagenase buffer (collagenase type XI, 1.5 mg/ml; Sigma-Aldrich, Oakville, ON, Canada) into the pancreatic duct as previously described (20). Briefly, mice were anesthetized and killed by cervical dislocation. Pancreata were digested by injection of a collagenase solution, and islets were handpicked under a microscope. They were either flash frozen for later extraction of RNA and proteins or cultured for 40 h at ~100 islets per 1 ml of RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in a 95% air/5% CO_2 humidified atmosphere. Half of the medium was changed 24 h later. For pulse-chase studies, each islet cohort was transferred into 0.5 ml serum-free RPMI-1640 containing 2.6 mmol/l glucose for 30 min to reduce the intracellular content of methionine and then transferred into 0.2 ml of the same medium containing 250 μCi ^{35}S-methionine and either 2.6 or 16.7 mmol/l glucose. After a 20-min incubation (pulse), the radioactive medium was replaced with fresh, nonradioactive, complete medium and incubation was resumed for 180 min (chase). Spent media and islets were collected at the two time points and kept frozen at −80°C until analysis.

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EGFP, enhanced green fluorescent protein; GADPH, glyceraldehyde-3-phosphate dehydrogenase; MF, mean fluorescence; PC, proprotein convertase.

TABLE 1
Antibodies used in this study

Anti-	Type	Conjugated	Source	Host	Antigens	Dilution IB	Dilution IP
7B2	Polyclonal	—	In-house	Rabbit	$7B2^{23-39}$	1:1,000	1:100
PC2	Polyclonal	—	Dr. N.G. Seidah	Rabbit	$PC2^{529-637}$	1:4,000	—
PC2	Polyclonal	—	Affinity Bioreagents	Rabbit	$PC2^{622-638}$	1:4,000	1:200
Glucagon	Polyclonal	—	Peninsula	Rabbit	$Glucagon^{1-37}$	1:1,000	1:200
β-Actin	Monoclonal	—	Cedarlane	Mouse	Chicken actin	1:250	—
IgG	Polyclonal	HRP	Amhersham	Goat	Rabbit IgG	1:4,000	—

HRP, horseradish peroxidase; IB, immunoblotting; IP, immunoprecipitation.

Fasting glucose, insulin, and glucagon. Mice (n = 4–8) were deprived of food overnight. Blood was then collected by cardiac puncture. Plasma was separated by centrifugation and assayed for glucose using the Beckman Coulter glucose analyzer and for insulin and glucagon using specific radioimmunoassay kits (Linco Research, St. Charles, MO).

Immunoblotting. Islet proteins were extracted in radioimmunoprecipitation assay buffer (50 mmol/l Tris-HCl, 1% Nonidet P-40, 0.25% sodium-deoxycholate, 150 mmol/l NaCl, and 1 mmol/l EDTA) and fractionated by SDS-PAGE. They were electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore). After a 10-min rinse in PBS with 0.05% Tween 20, the membranes were incubated at room temperature for 1 h in PBS with 0.05% Tween 20 containing 5% skimmed milk (blocking buffer) then for 90 min in blocking buffer containing a rabbit or mouse primary antibody. They were washed five times for 5 min with PBS containing 0.05% Tween 20 and then incubated for 1 h in blocking buffer containing horseradish peroxidase–conjugated secondary antibody against either rabbit or mouse IgGs. After five washes as above, they were probed for horseradish peroxidase reaction using the Western Lightning Chemiluminescence Reagent Plus kit as specified by the manufacturer (Perkin-Elmer, Markham, ON, Canada). Semiquantitative densitometry of immunoreactive bands was conducted on the ChemiGenius Bioimaging System using the Genetools software (Syngene, Frederick, MD).

Immunoprecipitation. Islets were lysed in 0.5 ml radioimmunoprecipitation assay buffer and spent media diluted to 0.75 ml with the same buffer. Media and lysates were supplemented with 0.04 volumes of 25X protease inhibitor cocktail (stock solution: one tablet per 50 ml; Boerhinger Manheim, Laval, Quebec, Canada). Each sample was then processed at 4°C, with mixing during incubations. To remove nonspecific immunoglobulin-binding proteins, the sample was supplemented with 0.1 μl normal rabbit serum and 15 μl of a 50% (wt/vol) protein A-agarose slurry (Sigma). After a 45-min incubation, the resin was sedimented by centrifugation at 3,000*g*. The supernatant was collected and supplemented with 5 μl of a diluted specific antibody (Table 1) and incubated for 1 h. Protein A-agarose (15 μl) was added, and incubation was resumed for 16 h. The resin with bound immune complexes was sedimented by centrifugation as above and rinsed three times with radioimmunoprecipitation assay buffer, twice with a buffer containing 1 mmol/l NaCl, 10 mmol/l Tris-HCl, and 1 mmol/l EDTA, pH 8, and twice with PBS containing 1 mmol/l EDTA. The pellet was suspended in 25 μl tricine buffer (450 mmol/l Tris-HCl [pH 8.45], 12% glycerol, 4% SDS, 0.05% phenol red, 0.05% Coomassie brilliant blue G-250, and 5% β-mercaptoethanol), boiled for 5 min, and sedimented as above. The supernatant was subjected to SDS-PAGE. The gel was fixed for 30 min in a 20% methanol/10% acetic acid solution and soaked for 30 min in Amplify fluor solution (Amersham, Oakville, ON, Canada). It was then dried and exposed to a phosphorimaging screen. The electrophoregram was analyzed on a Typhoon 8600 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Real-time quantitative RT-PCR. Total RNA was extracted using RNeasy extraction kit (Qiagen, Mississauga, ON, Canada) as prescribed by the manufacturer. An aliquot was reverse transcribed into cDNA using random hexameric primers and the Superscript II RNase H^- reverse transcriptase (Life Technologies, Burlington, ON, Canada) as previously described (21). The cDNA was used as a template in fluorometric quantitative PCR in a 20-μl reaction mixture containing 1 × LightCycler DNA Master SYBR Green I buffer (Roche, Laval, Quebec, Canada) (as source of $MgCl_2$, dNTP, SYBR-Green dye, and *Taq* polymerase) supplemented with 0.2 μl TaqStart antibody (BD Biosciences Clontech, Palo Alto, CA) and primers to a final concentration of 0.25 μmol/l each. PCR was conducted using the Roche LightCycler. The primer sequences and amplification conditions for each cDNA fragment are described in Table 2. Standards consisted of varying amounts of prequantified amplicons of 7B2, PC2, or glyceraldehyde-3-phosphate dehydrogenase (GADPH) cDNA. The concentrations of the experimental amplicons were derived at the maximum of the log-linear amplification using the second derivative method and normalized for GADPH mRNA content (21). For quality control, 5 μl of the SYBR Green–stained amplicons were run through a 2% agarose gel electrophoresis and visualized under ultraviolet to ascertain that only a single band of the expected size was amplified.

7B2 cDNA and promoter sequences. Genomic DNA was extracted from homogenates of B6 or C3H mouse thymus by proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Total RNA was prepared from mouse brain using the RNeasy extraction kit (Qiagen) and reverse transcribed to cDNA using an oligodT primer as described above. Part of the first exon and 1.5 kb of the upstream region of the 7B2 gene was amplified from genomic DNA (150 ng in a 50-μl reaction mixture) by long-range PCR using primer pair no. 1 (Table 2) and the Elongase enzyme mix as prescribed by the manufacturer (Invitrogen, Burlington, ON, Canada). The exon sequences (1,392 bp) were amplified from brain cDNA also by long-range PCR, using primer pair no. 3 (Table 2). The amplicons were sequenced in their entirety in both directions.

Expression vectors. The B6 and C3H cDNA amplicons were cloned into an eukaryotic expression vector between *Spe*I and *Bam*H1 restriction sites under the human cytomegalovirus promoter enhancer; the final vectors were named pB6/7B2cDNA and pC3H/7B2cDNA, respectively. To construct vectors for promoter activity assays, another PCR was conducted on genomic DNA from

TABLE 2
PCR primers used in this study

Pair no.	Name	Sequence (5′→3′)*	Thermocycle	Amplicon (bp)
	7B2P-F:	TGGTGGTGGTGGGGAATCTA		
1	7B2ex1-R:	GTCACCTCCCTCCGAAACCT	35× (94° −10 s/60° −30 s/68° −195 s)	1,734
	7B2P/Xho-F:	ACACTCTGAGGCCAGCAACCTATTTTTCCA		
2	7B2P/Bam-R	AGAGGATCCGGCGAGCTTCTTGCAATCTA	35× (94° −30 s/55° −30 s/68° −75 s)	970
	7B2/Spe-F	GACTAGTATGCCATCTACTTGTGGCAATTGCTTG		
3	7B2/Bam-R	AGGATCCGCACATACATTTATTAAGAAGCAGCAGAG	35× (94° −30 s/65° −30 s/68° −180 s)	1,406
	7B2-F	GGGGGATTTTTTTGATGTGGA		
4	7B2-R	CCCAAACAGCATAACCCAAAA	40× (95° −0 s/55° −5 s/72° −10 s)	122
	GAPDH-F	TACGGCTACAGCAACAGGGT		
5	GAPDH-R	GGGTGCAGCGAACTTTATTG	40× (95° −5 s/55° −5 s/72° −15 s)	235

*Restriction sites are underlined.

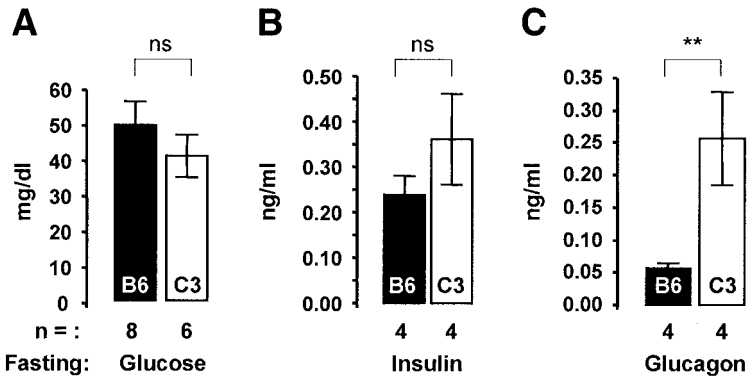

FIG. 1. Fasting plasma glucose, insulin, and glucagon. C3, C3H; ns, not statistically significant. **$P < 0.01$ by Student's t test.

B6 and C3H mice using primer pair no. 2 (Table 2). The 0.9-kb amplicon contained the entire exon 1 and 0.6 kb of the proximal upstream region. It was inserted between *Xho*I and a *Bam*HI restriction sites into the promoterless pd2EGFP vector (BD Biosciences Clontech), upstream to the gene for a short-lived (half-life: 2 h) enhanced green fluorescent protein (EGFP). The final promoter assay vectors were named pB6/7B2-d2EGFP and pC3H/7B2-d2EGFP, respectively.

Transfection and expression analysis. For transfection, human embryonic kidney HEK293 cells, mouse pancreatic αTC1-c19 MIN6 and βTC3 cells were cultured in a 5% CO_2/95% air atmosphere at 37°C in DMEM/10% fetal bovine serum. For each transfection, cells (0.75×10^6 per well) were seeded in triplicate in six-well plates and grown overnight. They were transfected with 4 μg of expression plasmid using Lipofectamine 2000 liposomes, as specified by the manufacturer (Life Technologies) and incubated at 37°C for 48 h.

HEK293 cells were transfected with pB6/7B2cDNA and pC3H/7B2cDNA vectors and were metabolically labeled and analyzed for 7B2 biosynthesis by immunoprecipitation as described above. αTC1-c19 MIN6 and βTC3 cells were transfected with pB6/7B2-d2EGFP and pC3H/7B2-d2EGFP promoter assaying vectors and rinsed once with PBS, harvested by trypsinization in 2 ml of complete medium, and sedimented at 250 g for 5 min at 4°C. They were resuspended in 1 ml ice-cold PBS and analyzed for EGFP fluorescence using the Beckman-Coulter Epics XLTM flow cytometer at excitation and emission wavelengths of 448 and 508 nm, respectively. Mock-transfected cells were used to establish the window of autofluorescence and the gate of EGFP-specific fluorescence. The relative promoter activity was defined as the ratio of the mean fluorescence (MF) of gated cells transfected with a promoter-containing vector (MFprom+) over the MF of gated cells transfected with the promoterless vector (MFprom–). The results are expressed as means of MFprom+/MFprom– derived from two experiments ± SE.

RESULTS

B6 mice have lower levels of glucagon in plasma. We first compared the fasting levels of plasma glucose, insulin, and glucagon between B6 and C3H young mice. The levels of glucose or insulin were not significantly different between the two strains, in spite of trends toward higher glucose and lower insulin in B6 mice (Figs. 1*A* and *B*). In contrast, the level of plasma glucagon was fivefold lower in B6 mice ($n = 4$, $P < 0.01$) (Fig. 1*C*), an indication of impaired production or release of this hormone. The insulin/glucagon molar ratio was 3.4-fold higher (4.8 in B6 mice vs. 1.4 in C3H mice), suggesting that hypoglucagonemia contributes to normoglycemia in these mice. The relative hypoglucagonemia also was observed in 12-month-old B6 mice, after glucose intolerance has developed (not shown).

To determine whether the lower level of plasma glucagon in B6 mice relative to C3H mice was due to reduced proglucagon gene expression in pancreatic islets, we examined the level of proglucagon mRNA by quantitative RT-PCR and the biosynthesis of glucagon-related proteins by pulse-chase analysis using isolated islets. There was no difference between the two strains in the level of proglucagon mRNA (not shown). Metabolic pulse labeling of proglucagon, used as a measure of translation of its mRNA, did not reveal any significant difference between the two strains, at either low or high glucose (Fig. 2, *odd-numbered lanes*). During chase, most of the proglucagon was converted to mature glucagon (Fig. 2, *even-numbered lanes*); however, under high glucose, there was some oxyntomodulin in B6 islets but not in C3H islets (Fig. 2, *lanes 4* vs. *8*), suggesting impaired proglucagon processing in B6 islets.

Reduced activation of proPC2 in B6 pancreatic islets. Conversion of proglucagon to glucagon in pancreatic islet α-cells is primarily mediated by PC2 (15,22). We therefore compared B6 and C3H islets for PC2 gene expression. We analyzed islet mRNA by real-time RT-PCR for PC2 mRNA and islet lysates by immunoblotting for the various molecular forms of PC2. Immunoblotting was conducted using antibodies raised against a PC2 (529-637) recombinant protein or a PC2 (622-638) synthetic peptide. The anti-PC2 (529-637) antibody recognized the 75-kDa precursor and the 69-kDa proPC2 intermediate (proPC2int, cleaved within the prodomain) and the 64-kDa mature forms of PC2, whereas the anti-PC2 (622-638) antibody primarily recognized the 64-kDa mature form. There was no difference in the level of PC2 transcripts between the two strains (not shown). Islet levels of proPC2 and proPC2int were not noticeably different between the two mouse strains, but B6 islets contained less mature PC2 and more low molecular proteins reacting with both antibodies (Fig.

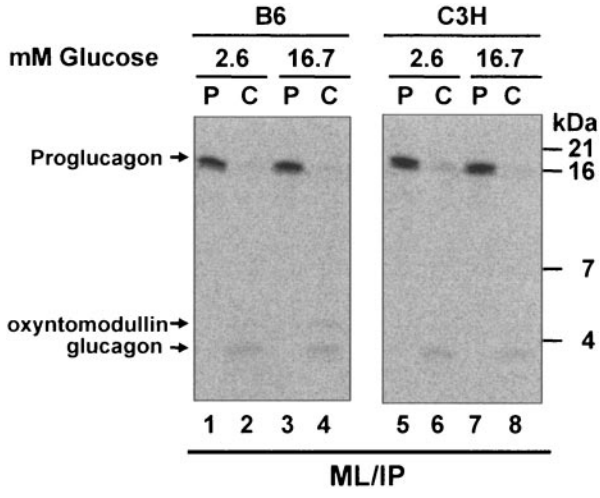

FIG. 2. Pulse-chase analysis of glucagon biosynthesis. Islets were isolated from B6 or C3H mice ($n = 6$) and pooled. Immunoprecipitated glucagon-related peptides were analyzed by 15% SDS-PAGE and phosphorimaging. C, chase; ML/IP, metabolic labeling and immunoprecipitation; P, pulse.

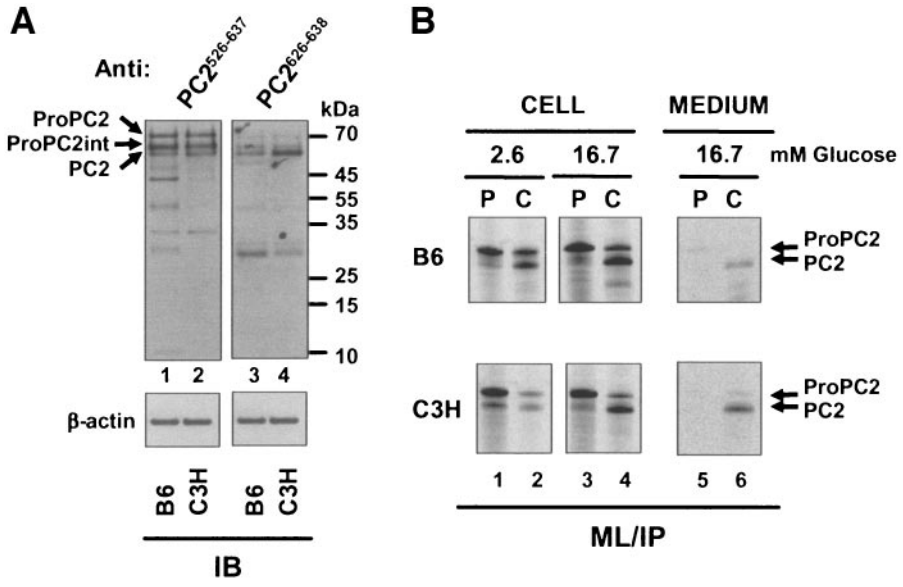

FIG. 3. PC2 expression. Islets were isolated from B6 or C3H mice (n = 3) for separate RNA and protein extraction. *A*: PC2-specific immunoblot (IB) using antibodies against PC2$^{529-637}$ and PC2$^{622-638}$. PC2 band intensities were normalized for that of β-actin. *B*: Pulse-chase analysis of PC2 biosynthesis in cultured islets. Immunoprecipitates obtained using the anti-PC2$^{622-638}$ antibody from islet extracts and culture media were analyzed by 12% SDS-PAGE and phosphorimaging. ProPC2int was detected by immunoblotting of islet extracts but not by immunoprecipitation of pulse-labeled islet extracts (*B*). ML/IP, metabolic labeling and immunoprecipitation.

3*A*). These observations suggested that B6 probably contained less PC2 activity due to increased degradation.

We also conducted a pulse-chase analysis of the activation of proPC2 in cultured islets under basal (2.6 mmol/l) and stimulating (16.7 mmol/l) glucose concentration. Immunoprecipitation was conducted on islet extracts and culture media using the anti-PC2 (622-638) antibody. The results are shown in Fig. 3*B*. Densitometry was performed to evaluate conversion of proPC2 to PC2. Intracellularly, there was no significance between the two strains: there was more proPC2 than PC2 during pulse at both glucose levels (Fig. 3*B*, *lanes 1* and *3*). After chase, there were comparable amounts of the two forms at low glucose (Fig. 3*B*, *lane 2*) but two- to threefold more PC2 than proPC2 at high glucose (Fig. 3*C*, *lane 4*). However, relative to B6 islets, C3H islets released two to four more mature PC2 in the culture medium during chase (Fig. 3*B*, *lane 6*).

B6 pancreatic islets express less 7B2. Biosynthesis and trafficking of PC2 is facilitated by 7B2, which serves as a specific helper protein and transient inhibitor (2,3). In the absence of 7B2, newly synthesized proPC2 folds improperly, is retained in the endoplasmic reticulum, and is eventually degraded. It was therefore possible that the apparent degradation of PC2 in B6 islets was due to a relative 7B2 deficit. To verify this possibility, we compared B6 and C3H islets for the levels of 7B2 mRNA and protein as well as for its biosynthesis. Quantitative RT-PCR for the mRNA showed that B6 islets contained approximately fourfold less 7B2 mRNA than C3H islets (Fig. 4*A*, $P < 0.01$, $n = 3$). Semiquantitative immunoblotting revealed that, relative to C3H islets, B6 islets contained approximately threefold less 7B2 (Fig. 4*B*, $P < 0.001$, $n = 3$). Pulse labeling and immunoprecipitation showed that newly biosynthesized 7B2 by B6 islets (cell extracts and spent media

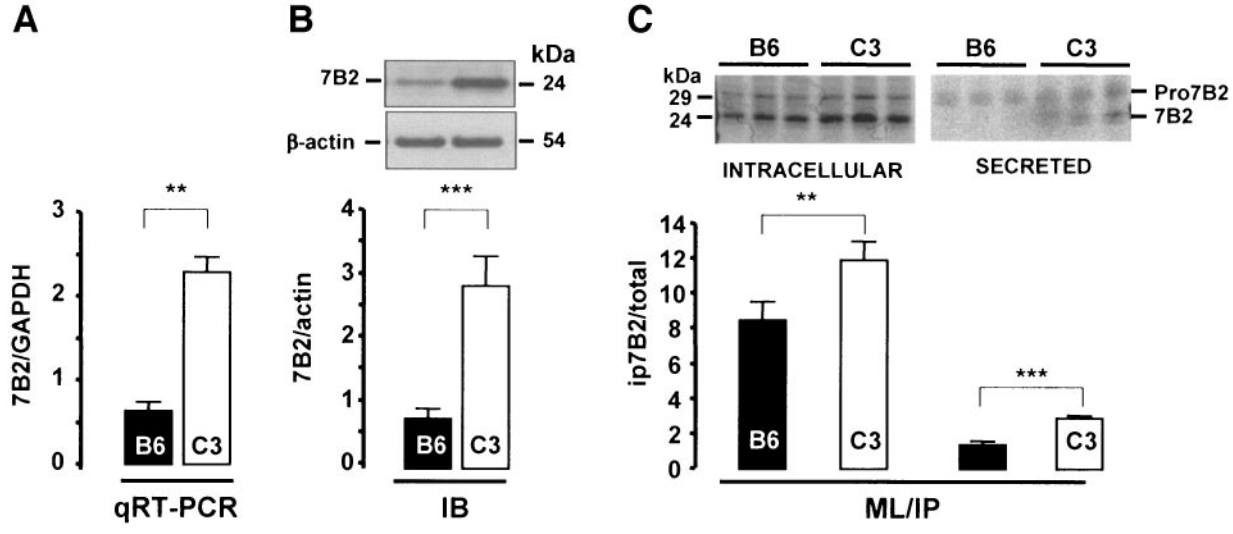

FIG. 4. 7B2 expression. Islets were isolated from B6 or C3H (C3) mice (n = 3) for separate RNA and protein extraction. *A*: qRT-PCR for 7B2 mRNA. The concentrations of 7B2 mRNA were normalized for that of GADPH. *B*: Immunoblotting (IB) for 7B2. 7B2 band intensities were normalized for that of β-actin. *C*: Pulse-chase analysis of 7B2 biosynthesis in cultured islets. Intracellular and secreted 7B2 immunoprecipitates were analyzed by 12% SDS-PAGE and phosphorimaging. Pro7B2 and 7B2 signal intensities were summed and normalized for the total intensity of the lane. Significance: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Student's t test. ML/IP, metabolic labeling and immunoprecipitation.

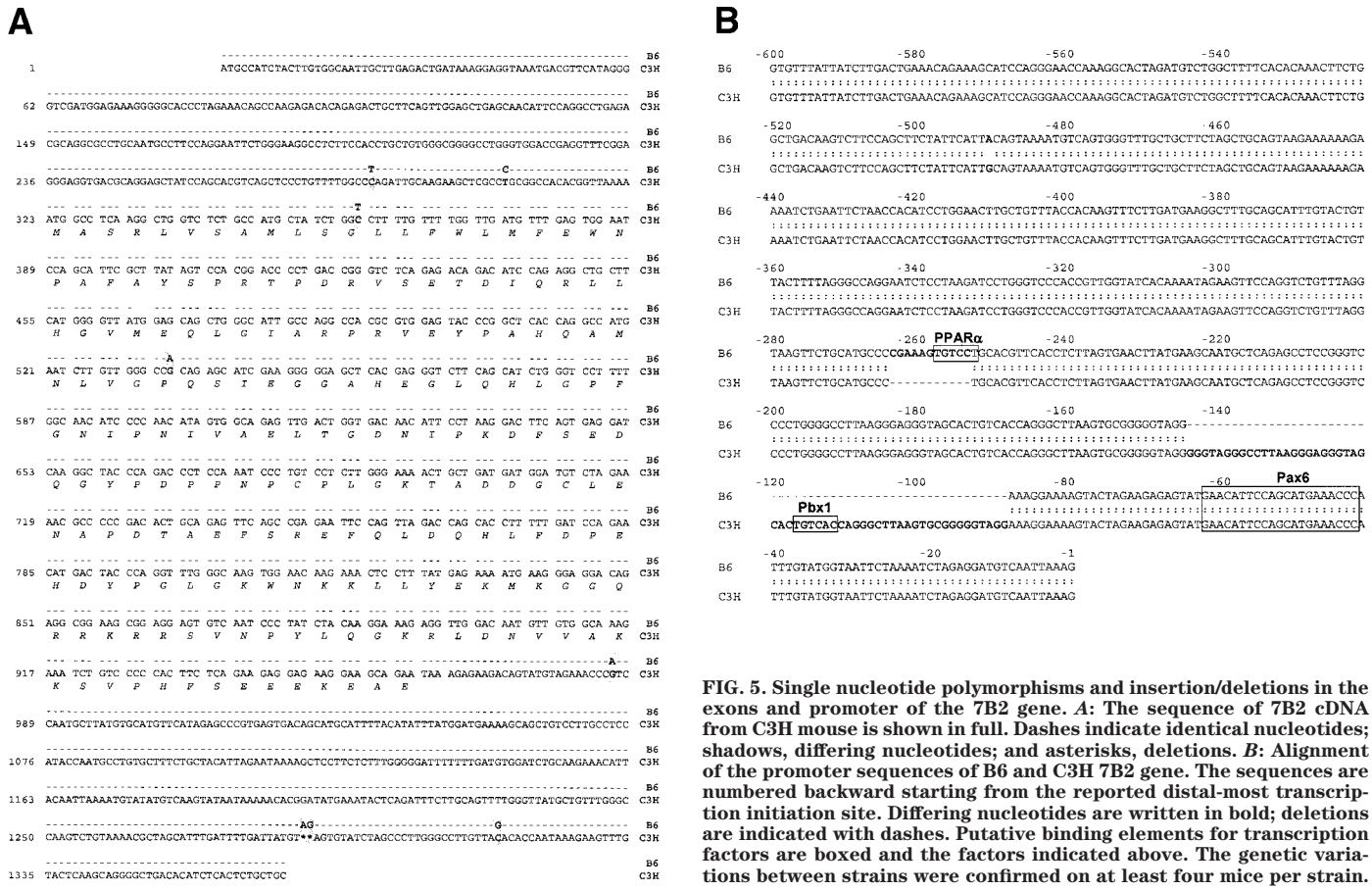

FIG. 5. Single nucleotide polymorphisms and insertion/deletions in the exons and promoter of the 7B2 gene. *A*: The sequence of 7B2 cDNA from C3H mouse is shown in full. Dashes indicate identical nucleotides; shadows, differing nucleotides; and asterisks, deletions. *B*: Alignment of the promoter sequences of B6 and C3H 7B2 gene. The sequences are numbered backward starting from the reported distal-most transcription initiation site. Differing nucleotides are written in bold; deletions are indicated with dashes. Putative binding elements for transcription factors are boxed and the factors indicated above. The genetic variations between strains were confirmed on at least four mice per strain.

combined) represented only 60% of that produced by C3H islets (Fig. 4*C*, triplicate experiments, $P < 0.05$). Note that very little mature 7B2 was found in the B6 spent media, suggesting that release of the content of secretory granules (where mature 7B2 is normally found) may be impaired in these islets. These results suggest that the difference in islet 7B2 levels between the two strains was primary due to a difference in 7B2 gene transcription or mRNA stability.

7B2 genetic polymorphism. To determine whether the differential expression of 7B2 between the two mouse strains had a genetic basis, we amplified by PCR the corresponding full-length cDNA from reverse-transcribed brain RNA and 0.6 kb of the promoter region from genomic DNA. Sequence comparison of the cDNA amplicons revealed two transitions in the 5′-untranslated region, two synonymous transitions in the coding region, one transition, one transversion, as well as one dinucleotide insertion-deletion in the 3′-untranslated region (Fig. 5*A*). A similar comparison of the promoter amplicons showed a purine transversion at position −491, an 11-bp insertion/deletion at position −253 to −264, and a 56-bp insertion/deletion at position −88 to −146 (Fig. 5*B*).

7B2 cDNA expression and promoter activity in transfected cells. To determine whether the sequence variations between the two 7B2 mRNAs may differentially affect their translation, we conducted a theoretical analysis of their folding online (available from http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi), using the MFOLD algorithm. The results indicated that the overall free energy of folding (−ΔG) was comparable between the two mRNA (−474.8 vs. −482.9 kcal/mol for B6 and C3H mRNA, respectively). However, the B6 mRNA sequence upstream of the initiator codon was involved in more complex secondary structure than the corresponding C3H sequence (not shown). It has been suggested that such structures could impede translation initiation (23,24). It was therefore possible that B6 mRNA was less efficiently translated than C3H mRNA. To verify this possibility, we transiently transfected the pB6/7B2cDNA and pC3H/7B2cDNA expression vectors into HEK293 cells, which lack endogenous 7B2. After pulse labeling, secreted 7B2 immunoreactive proteins were analyzed. The B6 expression vector induced slightly less expression of 7B2 than the C3H vector (Fig. 6*A*), but the difference was not significant.

To verify whether the genetic differences observed in the promoter region could contribute to the difference of pancreatic 7B2 expression between B6 and C3H mice, we cloned this region into the pd2EGFP plasmid, upstream of a reporter gene encoding a short-lived EGFP. We transiently transfected the plasmids into the mouse pancreatic α-cell line αTC1-Cl9 and the β-cell lines βTC3 and MIN6. The results are shown in Fig. 6*B*. Compared with the promoterless pd2EGFP vector, both 7B2 promoter-containing vectors induced 1.5-fold ($P < 0.01$) and 6.6-fold ($P < 0.001$) more EGFP expression in βTC3 and MIN6 cell lines, respectively, than in αTC1 cell line. However, in each cell line, the activity of the two promoters was comparable. These results suggest that the nucleotide differences observed in the promoter and coding regions cannot alone account for the differential expression of 7B2 in the endocrine pancreas of the two mouse strains. Alternatively, the established cell lines used in these assays and

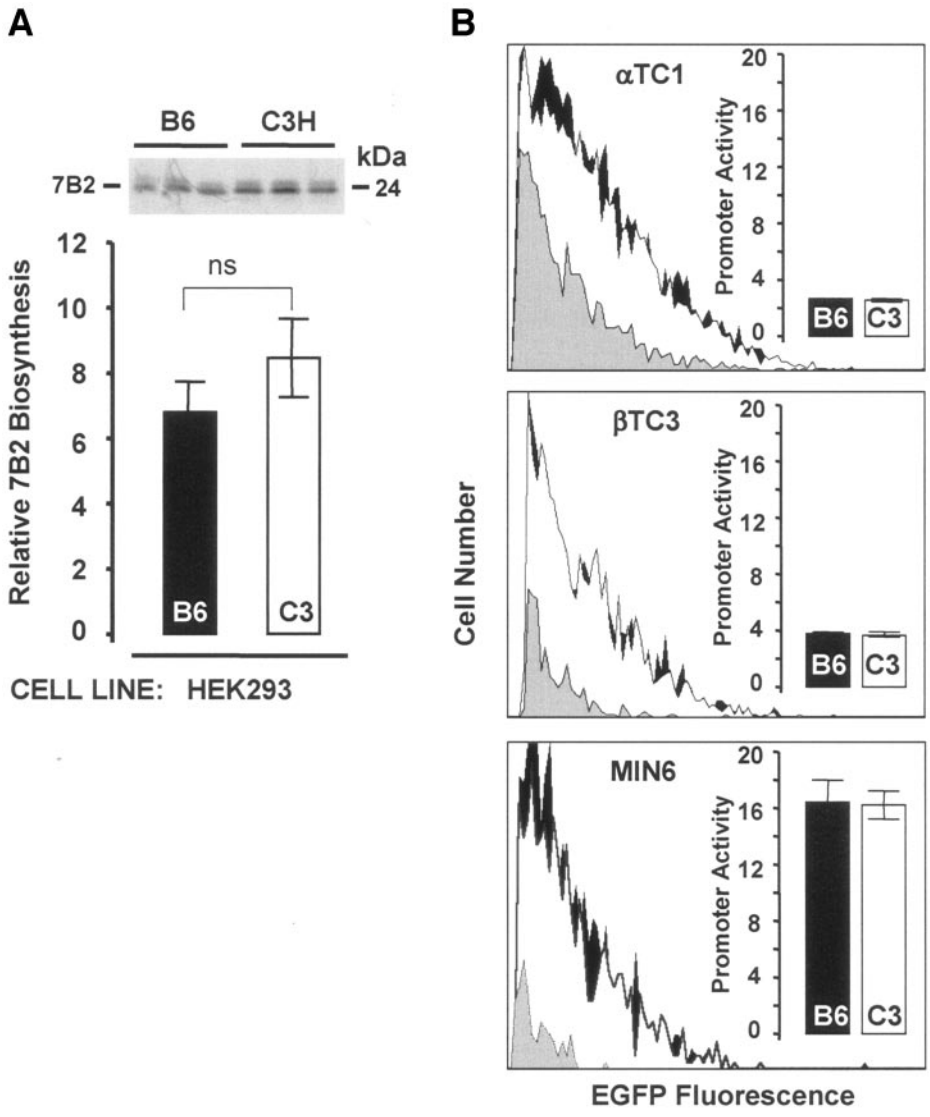

FIG. 6. Comparative analysis of 7B2 expression in transiently transfected cells. *A*: Pulse-chase analysis of 7B2 cDNA expression in HEK293 cells. Secreted 7B2 immunoprecipitates were analyzed by 12% SDS-PAGE and phosphorimaging. Pro7B2 and 7B2 signal intensities were summed and normalized for the total signal intensity in the lane. ns, statistically not significant. *B*: 7B2 promoter activity. Values are expressed as fold increase of mean EGFP-specific fluorescence detected in cells transfected with the 7B2 promoter-containing pB6/7B2-d2EGFP (black) and pC3H-d2EGFP (white) vectors over that detection in cells transfected with the promoterless pd2EGFP vector (gray) ± SE in two experiments conducted in triplicate. The cryptic promoter activity of the pd2EGFP vector varied among cell lines.

the assay conditions are inadequate to make the differences apparent.

DISCUSSION

B6 mice represent a model of spontaneous glucose intolerance. Proteolytic activation of prohormones regulating glucose homeostasis has not been systematically investigated in these mice. In this report, we present evidence showing reduced expression of 7B2 in the pancreas of the B6 mouse compared with the C3H mouse. The reduction is associated with a relative decrease in proPC2 activation and in the level of circulating glucagon. The lower level of fasting plasma glucagon in B6 mice is consistent with impaired processing of proglucagon in pancreatic α-cells, due to lower amount of active PC2. The glucose intolerance of B6 mice has been attributed to impaired glucose-induced secretion of insulin (25). Glucagon positively influences insulin release from islet β-cells in a paracrine fashion (26). Its lower level in B6 α-cells may contribute to the relative inefficiency of regulated insulin secretion in this mouse strain.

7B2 is widely expressed in neuronal and endocrine cells. In pancreatic islets, it is mostly found in the secretory granules of α- and β-cells (27–29). Pro7B2 acts as an intracellular PC2-specific helper protein (rev. in 2,3). Ablation of its gene in mouse leads to loss of PC2 activity and impaired processing of pancreatic prohormones, particularly proglucagon and prosomatostatin (17). A mild hypoglycemia is detectable in these mice, depending on sex and genetic background (17,30).

Unlike gene ablation, which abrogates 7B2 expression in all neuroendocrine cells, polymorphisms of its gene sequence may affect expression in some cells but not in others. The polymorphisms observed between B6 and C3H did not cause differential expression in transfected cells. They may be either irrelevant or relevant only in the large context of the locus where other *cis* elements may impact on their activity. It has been reported that the rat 7B2 promoter requires uncharacterized sequences in the first intron for increased basal activity and upregulation by forskolin or phorbol ester (29,31). We have sequenced 1.5 kb of the 3′ end of this intron and have identified a few single nucleotide polymorphisms (not shown). However, none were located within a recognizable regulatory element after a search for transcription factor binding sites using the Match and Patch online algorithms (available from www.gene-regulation.com/index.html). Moreover, the 7B2 promoter sequence analyzed in the current study

included the first exon that specifies the 5′-untranslated region of 7B2 mRNA. This region, which is relatively long (322 nucleotide), contains three out-of-frame AUGs before the initiator codon. These features are known to reduce the efficiency of mRNA translation (23,32). This region also contains a putative neuron-restrictive silencing element that may impair transcription in the presence of cognate binding factors (33). These multiple levels of regulation may have overcome whatever differential effect the deletion/insertions in the promoter have on 7B2 gene expression in B6 and C3H in transfected cells. Analysis of a minimal promoter free of these other regulatory elements may be necessary to reveal the effects of these genetic differences on 7B2 promoter activity.

It is also unclear how the genetic differences between B6 and C3H mice affect 7B2 expression in the various cell types of pancreatic islets. This expression will depend on the content and the level of cognate transcriptional factors found in these cells. In this context, we used the Match and Patch online algorithms to determine whether the 11-bp insertion and the 56-bp deletion in the B6 7B2 promoter contained potential binding elements for transcription factors involved in pancreatic development or functional differentiation. Putative binding elements for peroxisome proliferator–activated receptors α (TGTCCT) and Pbx1 (TGTCAC) were identified in the insertion and the deletion, respectively. Peroxisome proliferator–activated receptor α is thought to participate in islet compensatory response to abnormal glucose homeostasis (34). Pbx1 is a homeodomain transcription factor that has been shown to regulate the activity of Pdx1, another homeodomain transcription factor known to be critical for normal pancreatic development and differentiation (35,36). The presence of these binding elements suggests that the 7B2 gene is one of the downstream genes activated by these factors during islet biogenesis. It also implies that insertion or deletion of these elements may affect 7B2 expression in the developing pancreas. It is noteworthy that the two promoters also carry a conserved putative binding element for Pax6, a transcription factor known to play a role in α-cell differentiation and to regulate proglucagon gene expression (37,38).

To search for genes that might contribute to the relative glucose intolerance of B6 mice, Kayo et al. (39) conducted a quantitative trait locus analysis of the F2 and F3 progeny of C3H × B6 crosses, using intraperitoneal glucose tolerance as quantitative trait and genome-wide polymorphic markers. They identified two chromosomal regions that influence this trait: one in the distal region of chromosome 2 around the D2Mit48 marker, the other in the telomeric region of chromosome 13, around the D13Mit148 marker. The PC2 gene (locus symbol *Pcsk2*) is located near D2Mit48 and the PC1/3 gene (locus symbol *Pcsk1*) near the D13Mit148 (39). After sequencing the exons and proximal promoters of *Pcsk1* and *Pcsk2* in B6 and C3H mice, they observed no polymorphism that would support the candidacy of these genes as glucose tolerance effectors. We have confirmed that pancreatic islet expression of PC1/3 mRNA and protein is similar between the two strains (not shown). The 7B2 locus (*Sgne1*) is also located on mouse chromosome 2, 17 cM from *Pcsk2*. In this study, we have unveiled a number of *Sgne1* polymorphisms between B6 and C3H mice. More experiments are needed to establish whether this locus is an authentic quantitative trait locus for glucose tolerance.

In summary, we have described several nucleotide sequence variations in the 7B2 gene between the B6 and the C3H mouse. These variations are associated with relatively lower levels of 7B2 and PC2 in pancreatic islets as well as decreased plasma glucagon in the B6 mouse. Whether and how these variations contribute to the difference in glucose homeostasis between the two strains is still to be determined.

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