Foxa3 (HNF-3γ) binds to and activates the rat proglucagon gene promoter but is not essential for proglucagon gene expression

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Members of the Forkhead box a (Foxa) transcription factor family are expressed in the liver, pancreatic islets and intestine and both Foxa1 and Foxa2 regulate proglucagon gene transcription. As Foxa proteins exhibit overlapping DNA-binding specificities, we examined the role of Foxa3 [hepatocyte nuclear factor (HNF)-3γ] in control of proglucagon gene expression. Foxa3 was detected by reverse transcriptase PCR in glucagon-producing cell lines and binds to the rat proglucagon gene G2 promoter element in GLUTag enteroendocrine cells. Although Foxa3 increased rat proglucagon promoter activity in BHK fibroblasts, augmentation of Foxa3 expression did not increase proglucagon promoter activity in GLUTag cells. Furthermore, adenoviral Foxa3 expression did not affect endogenous proglucagon gene expression in islet or intestinal endocrine cell lines. Although Foxa3$^{-/-}$ mice exhibit mild hypoglycaemia during a prolonged fast, the levels of proglucagon-derived peptides and proglucagon mRNA transcripts were comparable in tissues from wild-type and Foxa3$^{-/-}$ mice. These findings identify Foxa3 as a member of the proglucagon gene G2 element binding-protein family that, unlike Foxa1, is not essential for control of islet or intestinal proglucagon gene expression *in vivo*.

Key words: diabetes, enteroendocrine, intestine, islet, pancreas.

INTRODUCTION

The control of blood glucose involves a complex interplay of neural and hormonal signals involving the central nervous system, adipose tissue, muscle, liver and the endocrine pancreas. Glucose is maintained in a normal physiological range via homoeostatic mechanisms integrating glucose sensing, glucose production and glucose utilization. Genetic loss-of-function defects at multiple control points in these pathways gives rise to increased glucose production, and/or defective glucose clearance, and ultimately Type II diabetes. Conversely, loss of function mutations in this pathway associated with decreased glucose production and/or enhanced insulin sensitivity and glucose clearance would be predicted to protect individuals from developing diabetes, and possibly increase the risk of developing hypoglycaemia. Despite intense investigation, relatively few genes have been identified that contribute to the development of Type II diabetes in human subjects [1].

The islets of Langerhans occupy a central role in the hormonal control of blood glucose. Although insulin gene mutations may be associated with defective proinsulin processing, these mutations are rare and do not contribute meaningfully to the prevalence of Type II diabetes [2–5]. Similarly, transcription factors that control insulin gene expression are also logical candidates for genetic defects leading to diabetes; however, only a few mutations in the *pdx-1* and *BETA2* genes have been identified in diabetic individuals [6–8].

As peptide products of the proglucagon gene, glucagon and glucagon-like peptide (GLP)-1 play central physiological roles in glucose homoeostasis [9,10]. Hence, proglucagon gene transcription factors or the receptors for the proglucagon-derived peptides also represent potential targets for genetic defects associated with abnormal glucose homoeostasis in human subjects. For example, increased glucagon action due to enhanced proglucagon gene transcription or increased glucagon receptor signalling might increase glycaemia and diabetes susceptibility in affected individuals. Although a Gly-40Ser mutation in the human glucagon receptor gene has been identified in a few subjects with diabetes, this mutation does not lead to decreased glucagon receptor signalling; hence the contribution of the Gly-40Ser mutation to diabetes susceptibility in affected individuals remains unclear [11–13].

The control of proglucagon gene transcription is complex, and reflects the integrated actions of numerous proglucagon gene transcription factors, including *isl*-1, *pax*-6, *brn*4, *cdx*-2 and members of the hepatocyte nuclear factor (HNF)-3 [Forkhead box a (Foxa)] gene family [14–20]. The Foxa3 gene family encodes a series of related proteins with overlapping DNA-binding specificities originally characterized as transcription factors important for hepatocyte gene expression [21]. The Foxa3 family is comprised of three members, Foxa1, Foxa2 and Foxa3 (formerly HNF-3α, β and γ) [22], that are expressed during embryonic development [23–25], suggesting that these genes may play important roles in patterning and tissue formation. The best-characterized Foxa gene, Foxa2, is expressed in the liver, gut and endocrine pancreas and binds to promoter elements in genes that subserve important metabolic functions *in vivo* [22].

Consistent with the importance of Foxa3 proteins for metabolic regulation, Foxa3-binding sites have been localized to DNA regions in the insulin-like growth factor-binding protein 1 ('IGFBP-1'), phosphoenolpyruvate carboxykinase ('PEPCK') and tyrosine aminotransferase ('TAT') promoters [26–28] that function as insulin- and glucocorticoid-response elements and Foxa2 has been shown to modify the glucocorticoid-regulation

Abbreviations used: EMSA, electrophoretic mobility-shift assay; Foxa, Forkhead box a; GLI, glucagon-like immunoreactivity; GLP, glucagon-like peptide; HNF, hepatocyte nuclear factor; RT-PCR, reverse transcriptase PCR.

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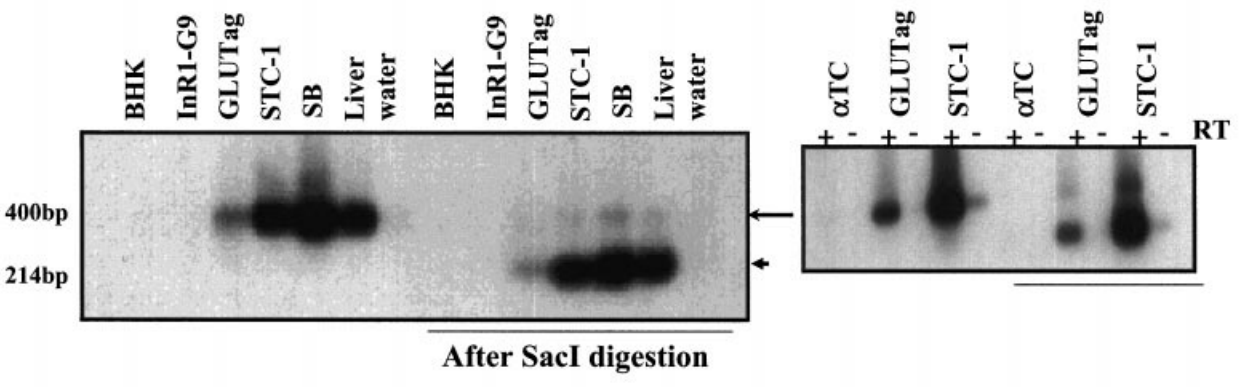

Figure 1 Foxa3 expression in intestinal endocrine cells

RT-PCR analysis of Foxa3 gene expression. The Foxa3-specific RT-PCR products (400 bp designated by a long arrow) generated using primers directed against murine Foxa3 contain a unique internal *Sac*I restriction site that generates fragments of 214 bp (short arrow) and 186 bp (not shown) following enzymic digestion. The PCR products were separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with a Foxa3-specific oligonucleotide probe complementary to sequences contained within the 214 bp (and 400 bp) DNA fragment. The oligonucleotide primers used were specific for mouse Foxa3; BHK and InR1-G9 are hamster fibroblasts and islet cells, respectively, whereas GLUTag and STC-1 are mouse intestinal endocrine cell lines and αTC cells are mouse islet glucagonoma cells [46,47,64]. SB, murine small bowel.

of insulin-like growth factor-binding protein 1 expression [26]. Foxa2 may also regulate islet growth and function via transcriptional control of the pdx-1 and GLUT-2 promoters [29–31].

Although mutations in Foxa2 (HNF-3β) have been identified in Japanese subjects with mature-onset diabetes of the young, the physiological significance of these mutations has not yet been reported [32] and Foxa2 mutations are rare in patients with Type II diabetes [33]. Furthermore, mice with homozygous mutations in the Foxa2 gene die *in utero*, hence the potential importance of Foxa2 for glucose homoeostasis in the adult remains unclear [34,35]. Remarkably, despite the putative importance of Foxa2 for hepatic gene expression and metabolic control [36], mice with a liver-specific deletion of Foxa2 are euglycaemic [37].

In contrast, tissue-specific deletion of Foxa2 in pancreatic β cells produces growth retardation and hyperinsulinaemic hypoglycaemia, attributed in part to reduced expression of β cell ion channels that regulate insulin secretion [38]. Similarly mice with targeted disruption of the structurally related Foxa1 gene exhibit neonatal growth retardation and die postnatally prior to 4 weeks of age [39,40]. Foxa1$^{-/-}$ mice exhibit hypoglycaemia associated with appropriately suppressed levels of insulin, and increased levels of cortisol and growth hormone but inappropriately low levels of circulating glucagon. Furthermore, the levels of pancreatic proglucagon mRNA transcripts are reduced in hypoglycaemic Foxa1$^{-/-}$ mice, demonstrating an essential role for Foxa1 in proglucagon gene expression [39,40].

Much less is known about the transcriptional targets for Foxa3 activity. The Foxa3 gene is expressed in several tissues, including the pancreas and gut [41], and reverse transcriptase PCR (RT-PCR) experiments have identified RNA transcripts for all Foxa genes, including Foxa3 (HNF-3γ), in mouse islets [42,43]. In contrast to the lethal phenotypes detected in mice with targeted inactivation of the Foxa1 and Foxa2 genes, disruption of the murine Foxa3 gene results in apparently normal mice without obvious major metabolic perturbations [44]. As Foxa proteins compete for binding to related DNA elements in several different promoters [22,36], we hypothesized that Foxa3, like Foxa1 and Foxa2 [45], might represent a candidate regulator of proglucagon gene expression and glucose homoeostasis. Accordingly we examined the importance of Foxa3 for proglucagon gene transcription using a combination of cell lines *in vitro* and Foxa3-knockout mice *in vivo*.

EXPERIMENTAL

Cell lines and promoter analysis

Islet hamster InR1-G9 glucagon-producing cells, mouse intestinal glucagon-producing endocrine cells (GLUTag and STC-1), mouse islet αTC cells and BHK fibroblast cells were propagated as described previously [46–48]. The rat proglucagon gene promoter plasmids −[476]GLU-Luc, −[220]GLU-Luc, −[93]GLU-Luc, −[82]GLU-Luc, [3G2-82]Luc and [3G2M4-82]Luc, as well as CMV-Luc and the promoterless SkLuc vector, have been described [18,39,49–51]. The adenovirus containing rat Foxa3 (HNF-3γ) [52] was a generous gift from Dr K. Sato (Tottori University, Nishimachi, Yonago, Japan). Cell transfections employed 2 μg of reporter plasmids using the Lipofectamine transfection reagent. All transfections were carried out in quadruplicate on at least three separate occasions and luciferase activity was measured as described previously [18,39,49–51].

Adenovirus expression experiments

Successful homologous recombination resulted in a recombinant virus encoding Foxa3 as described previously [52]. After plaque purification, viruses were amplified in HEK 293 cells and purified by CsCl gradient centrifugation to enrich the viral stocks as described previously [53]. Titering of the virus was calculated by measuring the absorbance of the viral solution at 260 nm, using a conversion factor of 1×10^{12} virions/absorbance unit.

DNA–protein interaction experiments

For electrophoretic mobility-shift assays (EMSAs), nuclear protein extracts were prepared from BHK and GLUTag cell lines [54]. Synthetic oligonucleotides corresponding to specific rat glucagon gene G2 sequences (G2-1, 5′-AGG CAC AAG AGT AAA TAA AAA GTT TCC GGG CCT CTG-3′; G2-2, 5′-CCT CAG AGG CCC GGA AAC TTT TTA TTT ACT CTT GTG-3′)

or G2 mutant sequences (the mutated sequences are underlined; G2M4-1, 5′-AGG CAC AAG AGT CCC TAA AAA GTT TCC GGG CCT CTG-3′; G2M4-2, 5′-CCT CAG AGG CCC GGA AAC TTT TTA GGG ACT CTT GTG-3′) were annealed, separated by electrophoresis with a 5 % non-denaturing polyacrylamide gel, and end-labelled with T4 polynucleotide kinase and [γ-^{32}P]dATP. The labelled oligonucleotides were purified through a microspin G-25 column (Pharmacia, Toronto, Canada). EMSAs were performed by incubating 5×10^4 c.p.m. of end-labelled DNA probe with 5–10 μg of nuclear protein in a buffer containing 1–2 μg of poly(dI/dC), 1 μg of BSA, 10 mM Tris/HCl (pH.8.0), 40 mM KCl, 6 % (v/v) glycerol, 1 mM dithiothreitol and 0.05 % Nonidet P-40 in a total volume of 20 μl for 30 min at 30 °C. For supershift experiments, nuclear proteins were premixed with 10–20 μg of either non-immune goat antiserum or anti-HNF-3γ serum from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), in reaction buffer on ice for 20 min prior to addition of labelled probe. EMSAs with competitor oligonucleotides were performed using 5×10^4 c.p.m. of end-labelled DNA probe and unlabelled oligonucleotides in 10–50-fold molar excess. The reaction mixtures were then loaded on to a 5 % non-denaturing polyacrylamide gel, and following electrophoresis the gel was dried, autoradiographed and analysed by PhosphorImaging techniques.

DNA, RNA and protein analysis

RNA was prepared from cells and tissues by the acid ethanol precipitation method [55]. For RT-PCR, first-strand cDNA synthesis was generated from total RNA using the SuperScript Preamplification System from Life Technologies (Toronto, Canada). Target cDNA was then amplified using synthesized specific oligonucleotide primer pairs for mouse Foxa3 (5′-AAGGCAAAGAAAGGAAACAGCGCCA-3′ and 5′-GATGCATTAAGCAGAGAGCGGGA-3′) and glyceraldehyde-3-phosphate dehydrogenase (5′-TCCACCACCCTGTTGCTGTAG-3′ and 5′-GACCACAGTCCATGACATCACT-3′; ACGT, Toronto, Canada). The conditions for PCR were: one initial cycle of 2 min at 94 °C, then 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, followed by extension at 72 °C for 5 min. PCR products were electrophoresed on 1.2 % agarose gel, transferred to a nylon membrane (Schleicher & Schuell) and hybridized with an internal oligonucleotide DNA probe. RNase protection analysis was carried out as described previously [41] using a 288 bp fragment of the mouse proglucagon cDNA (positions 236–523) and a 154 bp fragment of the mouse GAPDH gene as the probes. Nuclear proteins from cell and tissue extracts were prepared as described previously [18].

Analysis of glucagon-like immunoreactive proteins

Adult mice ($n = 5$–6 per group) were fasted for 24 or 48 h followed by refeeding of some mice for 6 h. Peptides were extracted from pancreas, jejunum (top half of the small intestine), ileum (bottom half of the small intestine) and colon by homogenization in 1 M HCl containing 5 % HCO_2H, 1 % trifluoroacetic acid and 1 % NaCl and passage through a cartridge of C_{18} silica (SepPak; Waters Associates, Milford, MA, U.S.A.) as described previously [56,57]. Aliquots of peptide extracts were dried *in vacuo* for determination of total protein by Lowry assay and total glucagon-like immunoreactivity (GLI) by radioimmunoassay. The K4023 antiserum (Biospacific, Emeryville, CA, U.S.A.) used for GLI radioimmunoassay cross-reacts with all N-terminal products of proglucagon processing (e.g. intestinal glicentin and oxyntomodulin, as well as pancreatic glucagon), and correlates with the production of GLP-1 and GLP-2 from proglucagon in the intestine.

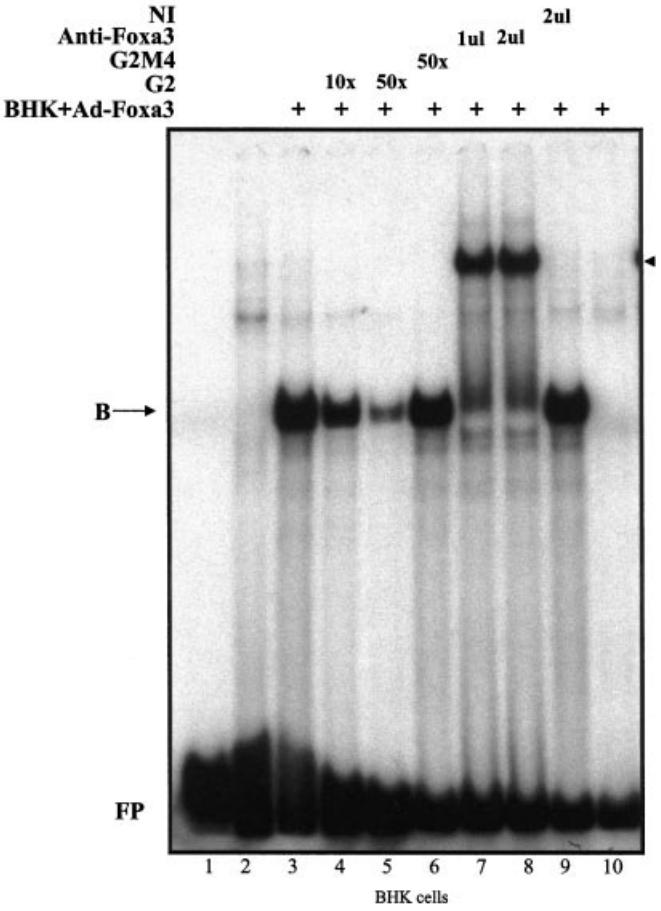

Figure 2 EMSA with the proglucagon gene G2 element and BHK fibroblast nuclear extracts

Nuclear extracts from wild-type hamster BHK cells, or BHK cells infected with an adenovirus containing Foxa3 (BHK + Ad-Foxa3) were incubated with the rat G2 glucagon gene promoter element with or without wild-type G2 or mutant G2 competitor oligonucleotide (G2M4) and with Foxa3-specific antisera (Anti-Foxa3) or non-immune antisera (NI) and analysed by SDS/PAGE as described in the Experimental section. The major specific DNA–protein complex detected using BHK cells infected with adenoviral Foxa3 is designated by an arrow (B), whereas the supershifted Foxa3 complex is designated by an arrowhead. FP, free probe.

RESULTS AND DISCUSSION

The proximal rat proglucagon gene promoter is functionally organized into discrete subdomains, with at least four enhancer-like sequences, designated G1–G4, identified within the first 300 bp upstream of the transcription start site [58,59]. The results of previous studies have shown that the rat proglucagon gene G2 element binds Foxa (HNF-3) proteins, with Foxa2 (HNF-3β) the predominant HNF-3-binding protein identified in glucagon-producing islet cells [20,60]. Furthermore, Foxa1 also binds to the G2 element and activates proglucagon gene transcription [39]. As Foxa proteins exhibit overlapping DNA-binding specificity, we examined whether Foxa3 might also interact with the G2 element. However, we did not detect binding of the Foxa3

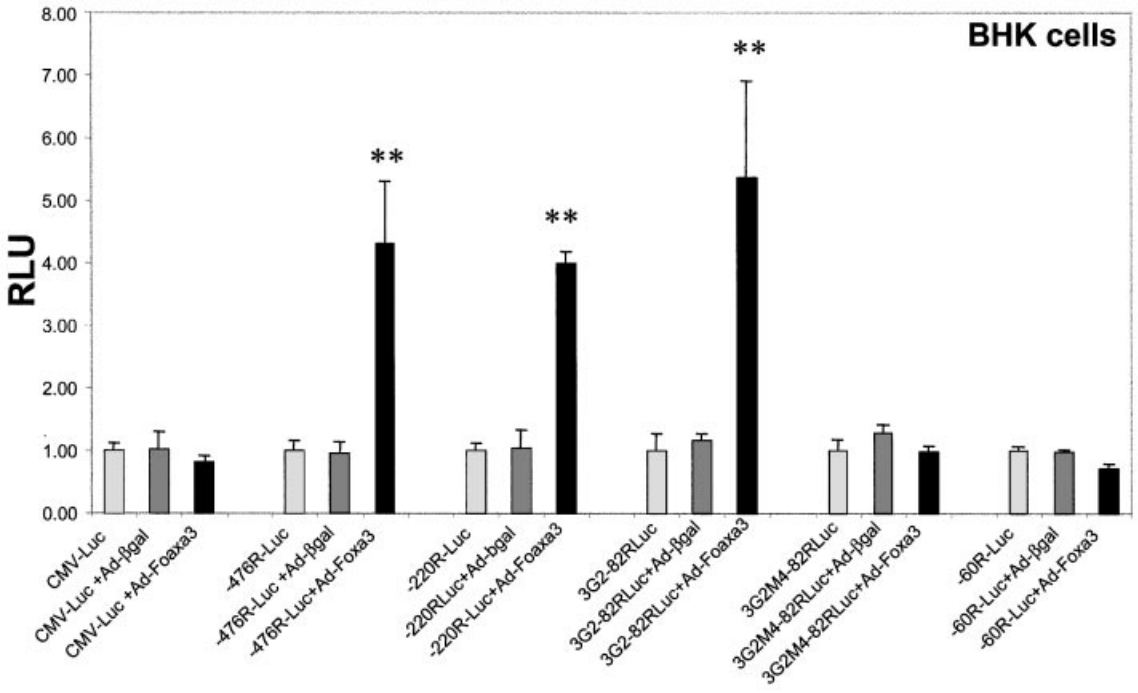

Figure 3 Foxa3 transactivates the rat proglucagon gene promoter in BHK fibroblasts

The reporter plasmids (2 μg) were transfected in quadruplicate and 24 h later cells were mock-infected, or infected with a virus encoding Foxa3 (Ad-Foxa3) or β-galactosidase (Ad-β-gal). The results, expressed as relative luciferase activity (RLU), are normalized to the luciferase activity obtained following transfection of the reporter plasmids alone in the absence of virus. -476R-Luc, -220R-Luc and -60R-Luc contain 476, 220 and 60 nucleotides, respectively of the proximal rat proglucagon gene 5′-flanking region extending to $+58$ bp in exon 1, fused to the luciferase cDNA reporter [18,47,49]. 3G2 and 3G2M contain three copies of the wild-type or mutant rat proglucagon gene G2 element ligated in tandem immediately adjacent to the first 82 bp of the rat proglucagon gene promoter fused to the luciferase cDNA. $^{**}P < 0.01$, compared with transfection activity obtained without Foxa3.

(HNF-3γ) protein to the glucagon gene G2 element using islet InR1-G9 extracts (results not shown).

As the proglucagon and Foxa3 genes are expressed in the small and large intestine [41], and recent studies have detected the presence of Foxa1 and Foxa2 in GLUTag enteroendocrine cells [45], we examined whether Foxa3 may play a role in enteroendocrine proglucagon gene transcription. RT-PCR analysis using Foxa3-specific oligonucleotide primers detected Foxa3 mRNA transcripts in both mouse STC-1 and GLUTag intestinal endocrine cell lines (Figure 1). In contrast, Foxa3 was not detected in RNA isolated from the islet glucagon-producing αTC cell line (Figure 1), in keeping with the lack of detectable Foxa3–DNA complexes in InR1-G9 islet cells [20]. Consistent with the nucleotide sequence of mouse Foxa3, digestion of the Foxa3 RT-PCR products with *Sac*I yielded two smaller fragments of 214 and 186 bp (Figure 1).

As previous experiments demonstrated that Foxa proteins can either activate (Foxa1) or inhibit (Foxa2) proglucagon gene transcription via the G2 promoter element [20,39], we examined whether Foxa3 was also capable of interacting with the proglucagon gene G2 element. The BHK fibroblast cell line was infected with an adenovirus encoding a functionally active rat Foxa3 cDNA [52], following which G2-binding activity was examined using BHK nuclear extracts. A single major G2 complex was detected with the G2 probe in BHK cells infected with the Foxa3 virus, but not in uninfected cells, which was progressively diminished following incubation with increasing amounts of unlabelled excess G2 (Figure 2, lanes 3–5). In contrast, the mutant G2 oligonucleotide G2M4 that fails to bind Foxa proteins [20,39,60] did not compete for Foxa3 binding (Figure 2, lane 6). The BHK G2 complex was markedly attenuated and supershifted by antisera directed against Foxa3, but not by non-immune sera, consistent with the presence of Foxa3 in this DNA–protein complex (Figure 2, lanes 7–9).

To ascertain the functional significance of the Foxa3–G2 interaction, we studied the activity of a series of G2-containing proglucagon promoter-luciferase plasmids in BHK cells in the presence or absence of adenovirus-derived Foxa3 or the control adenoviral β-galactosidase. BHK cells transduced with adenoviral Foxa3 exhibited significantly increased proglucagon promoter activity from plasmids containing the G2 element in its native context within the proximal rat proglucagon gene 5′-flanking region (Figure 3). Similarly, Foxa3 increased the activity of a reporter gene containing the G2 element upstream of a minimal rat proglucagon promoter, 3G2-82RLuc (Figure 3). In contrast, no activation of proglucagon promoter activity was observed in cells infected with the control adenoviral β-galactosidase virus, or in cells infected with Foxa3 in the presence of reporter genes containing the G2M4 mutant sequence that is devoid of Foxa3-binding activity (Figure 3). These findings demonstrate that adenoviral transduction of Foxa3 in heterologous BHK cells is associated with enhanced G2 binding activity (Figure 2) and increased proglucagon promoter activity in a G2-dependent manner (Figure 3).

In contrast to data implicating Foxa1 and Foxa2 in islet hormone gene expression, much less is known about Foxa3 in the endocrine pancreas or gut. Foxa3 mRNA transcripts have been localized to islet β cells and β cell lines; however, Foxa3 has not been implicated in the control of insulin gene transcription or β cell development [42,43,61]. We assessed the role of Foxa3 in

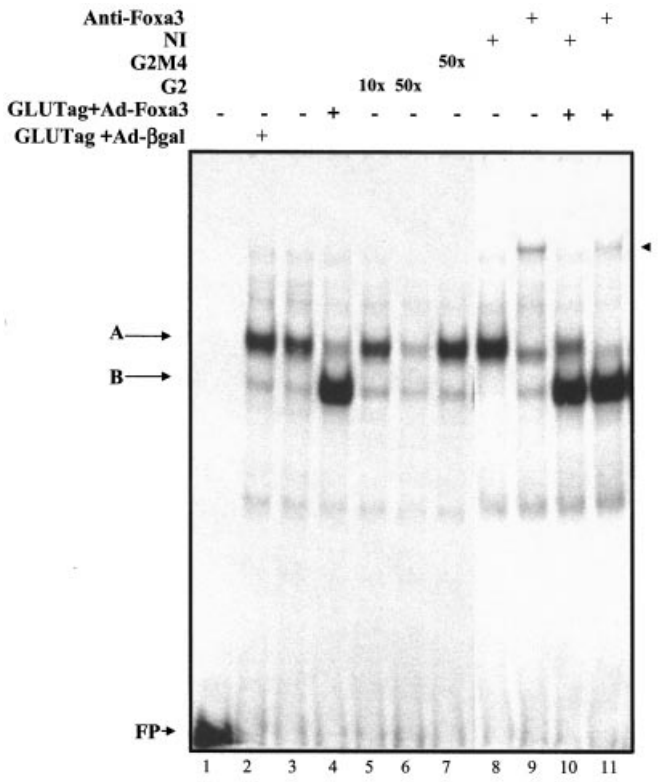

Figure 4 EMSA with the proglucagon gene G2 element and GLUTag gut endocrine cell nuclear extracts

Nuclear extracts from wild-type GLUTag cells, GLUTag cells infected with an adenovirus containing Foxa3 (GLUTag + Ad-Foxa3) or with a control adenoviral β-galactosidase, were incubated with the rat G2 glucagon gene promoter element with or without wild-type G2 or mutant G2 competitor oligonucleotide (G2M4) and with Foxa3-specific antisera (Anti-Foxa3) or non-immune antisera (NI) and analysed by SDS/PAGE as described in the Experimental section. The major specific DNA–protein complex detected in GLUTag cells infected with AD-Foxa3 is designated by an arrow and labelled B, whereas the major endogenous G2 DNA–protein complex detected in wild-type GLUTag cells in the absence of exogenous adenoviral Foxa3 infection is designated A. The supershifted complex detected with the Foxa3-specific antisera is shown by the arrowhead. FP, free probe.

proglucagon gene expression due to findings that both Foxa1 and Foxa2 regulate proglucagon gene transcription through binding to common elements in the proximal promoter [20,39,45,60].

To ascertain whether endogenous Foxa3 interacts with the G2 element in proglucagon-expressing cells, we assessed Foxa3–G2 binding in nuclear extracts from mouse enteroendocrine cells. GLUTag cells were chosen for these studies in contrast to STC-1 cells, as GLUTag cells exhibit a more differentiated enteroendocrine cell phenotype and express the proglucagon gene in a regulated manner [46,62]. A major G2 complex, designated A, was obtained with GLUTag nuclear extracts in the absence of adenoviral Foxa3 transduction, that was markedly diminished following competition with 10–50-fold excess unlabelled G2, but not by the mutant oligonucleotide G2M (Figure 4). Adenoviral transduction of GLUTag cells with Foxa3 markedly diminished formation of complex A and produced a more rapidly migrating complex, designated B (Figure 4, lane 4). Formation of complex B was completely attenuated by competition with even 10-fold-excess unlabelled G2. Antisera against Foxa3 supershifted complex A, but had much less effect on complex B in GLUTag cells compared with BHK cells (compare Figures 2 and 4). Taken together, these findings demonstrate the presence of endogenous Foxa3 as a minor component of the endogenous G2–A complex in intestinal endocrine cells that express the mouse proglucagon gene.

To determine whether enhanced expression of Foxa3 was associated with increased proglucagon promoter activity and proglucagon gene expression in GLUTag cells, we assessed the transcriptional activity of transfected rat proglucagon promoter plasmids, and the levels of GLUTag proglucagon mRNA transcripts in the presence or absence of adenoviral-derived Foxa3. In contrast to the results obtained using BHK cells, we did not observe increased proglucagon promoter activity in GLUTag cells transduced with the Foxa3 adenovirus (Figure 5A), even with increasing doses of adenoviral Foxa3 (results not shown). The lack of Foxa3-dependent proglucagon promoter activation was not attributable to a selective defect in nuclear localization of Foxa3 in GLUTag versus BHK cells (Figure 5B). Furthermore, transduction of enteroendocrine (GLUTag and STC-1) or islet (InR1-G9 and αTC1) glucagon-producing cells with adenoviral Foxa3 had no effect on the levels of endogenous proglucagon mRNA transcripts in these cell lines (results not shown).

These results demonstrate that although Foxa3 is capable of binding to the G2 element and activating G2-dependent transcription in fibroblasts, it does not regulate proglucagon gene expression in cell lines expressing the endogenous proglucagon gene *in vitro*.

Although cell-transfection studies implicate a possible role for Foxa2 in control of proglucagon gene expression [45], mice with a disruption of the Foxa2 gene exhibit defective floor plate and notochord formation and early embryonic lethality [34,35], precluding the analysis of metabolic homoeostasis in the adult mouse globally deficient in Foxa2. Hepatic Foxa2 does not appear essential for glucose homoeostasis as recently demonstrated through tissue-specific gene ablation of Foxa2 specifically in hepatocytes [37]. In contrast, specific deletion of Foxa2 in pancreatic β cells results in growth retardation and hyperinsulinaemic hypoglycaemia [38], implicating an essential role for Foxa2 in the control of glucose-stimulated insulin secretion *in vivo*.

To ascertain whether Foxa3 is essential for regulation of proglucagon gene expression in normal islet or intestinal endocrine cells *in vivo*, we assessed proglucagon gene expression in Foxa3$^{-/-}$ mice. Although Foxa3$^{-/-}$ mice exhibit mild hypoglycaemia in response to a prolonged fast [61], circulating levels of fed and fasted circulating pancreatic glucagon were normal [61]. To determine whether the levels of pancreatic or intestinal proglucagon-derived peptides were dependent on the presence of Foxa3, we analysed the profile of intestine GLI after 24–48 h of fasting, and after 6 h of refeeding. No alterations in the levels of glucagon-like immunoreactive peptides were detected in the pancreas, jejunum, ileum or colon of fasted or fasted/refed Foxa3$^{-/-}$ mice (Figure 6). Furthermore, the levels of proglucagon mRNA transcripts were comparable in the pancreas, ileum and colon of wild-type versus Foxa3$^{-/-}$ mice (Figure 7). These results clearly demonstrate that although control of proglucagon gene expression is abnormal in Foxa1$^{-/-}$ mice [39,40], genetic disruption of Foxa3 is not associated with detectable changes in pancreatic or intestinal proglucagon gene expression *in vivo*.

Although we did not detect Foxa3 expression in InR1-G9 or αTC-1 islet A cell lines, Foxa3 is expressed in two murine proglucagon-expressing enteroendocrine cells and binds to the proglucagon gene G2 element in GLUTag nuclear extracts. The results of our studies clearly demonstrate that both virally derived and endogenous Foxa3, like Foxa1 and Foxa2 [39,45,60], binds to the proglucagon gene G2 promoter element, and hence

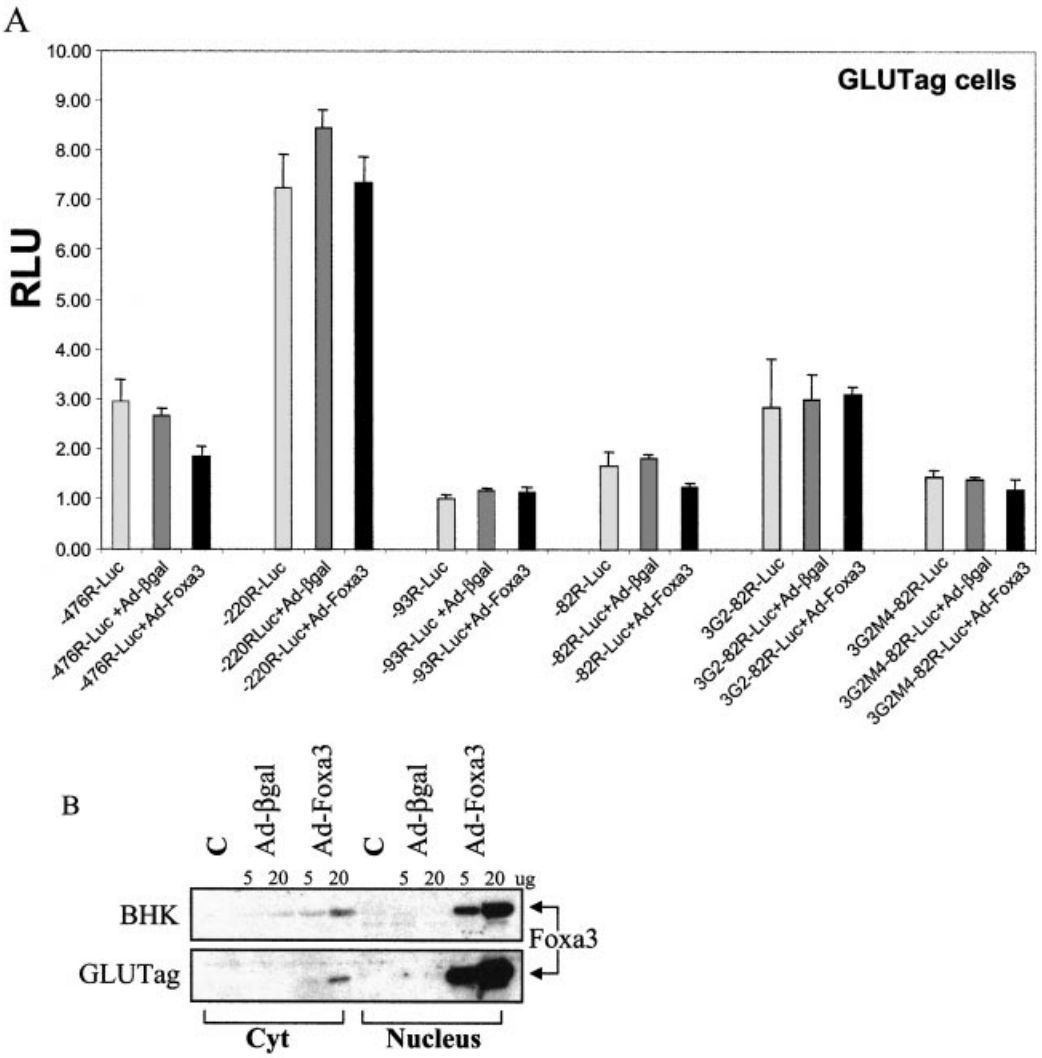

Figure 5 (A) Transcriptional properties of Foxa3 in mouse GLUTag enteroendocrine cells and (B) Western blot analysis of Foxa3 in transduced cell lines

(**A**) The reporter plasmids were transfected in quadruplicate and 24 h later cells were mock-infected, or infected with a virus encoding Foxa3 (Ad-Foxa3) or β-galactosidase (Ad-βgal). The results, expressed as relative luciferase activity (RLU), are normalized to the luciferase activity obtained following transfection of the reporter plasmid -93R-Luc alone in the absence of virus. -476R-Luc, -220R-Luc, -93R-Luc and -82R-Luc contain 476, 220, 93 and 82 nucleotides, respectively, of the proximal rat proglucagon gene 5′-flanking region extending to $+58$ bp in exon 1, fused to the luciferase cDNA reporter. 3G2 and 3G2M contain three copies of the wild-type or mutant rat proglucagon gene G2 element ligated in tandem immediately adjacent to the first 82 bp of the rat proglucagon gene promoter fused to the luciferase cDNA. (**B**) Various amounts of purified protein (5 or 20 μg) from adenoviral Foxa3 or adenoviral β-galactosidase (Ad-βgal) HEK 293 cell lysates were used to infect BHK fibroblasts or GLUTag endocrine cells, following which cytoplasmic (Cyt) or nuclear extracts were isolated and analysed by Western blotting for expression of the Foxa3 protein. C, control non-infected cells.

Foxa3 represents a candidate proglucagon gene transcription factor. Although Foxa3 increased proglucagon promoter activity in BHK fibroblasts, no promoter activation was detected following enhanced Foxa3 expression in GLUTag cells. These results are similar to those obtained in transfection studies of Foxa1 or Foxa2, which fail to augment proglucagon promoter activity in transfected InR1-G9 islet cells [45], yet activate the same promoter constructs in transfected fibroblasts. Similarly, the transcription factors Pax-6 and Cdx-2/3 activate proglucagon promoter activity in BHK fibroblasts but not in transfections of islet cell lines [63]. These findings may be potentially explained by the already 'maximal' expression of the endogenous factors in endocrine cells or, alternatively, the existence of highly complex regulatory mechanisms involving multiple interacting transcription factors that serve to constrain the effects of a single overexpressed factor in an endocrine cell *in vitro* [45].

Whether Foxa3 interacts with one or more accessory transcription factors present in GLUTag cells that restrict its activity remains unknown. Nevertheless, expression of adenoviral Foxa3 resulting in marked augmentation of the G2–B complex in either islet or intestinal cell lines does not affect the levels of proglucagon gene expression *in vitro*. These results are consistent with our finding that the levels of both pancreatic and intestinal proglucagon mRNA transcripts are normal in Foxa3 mutant mice. These data strongly argue against a role for Foxa3 as a transcriptional regulator of proglucagon gene expression.

One potential explanation for findings of normal proglucagon gene expression in the absence of Foxa3 may be due to

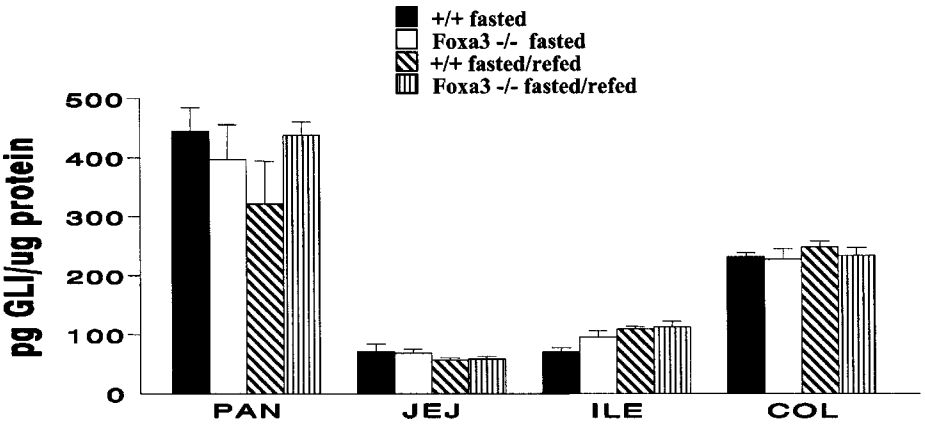

Figure 6 GLI peptides in tissues of wild-type and Foxa3$^{-/-}$ mice

Groups of 4–6 mice were subjected to a 48 h fast alone (fasted), or a 48 h fast followed by 6 h of refeeding (fasted/refed). +/+, Wild-type. PAN, pancreas; JEJ, jejunum; ILE, ileum; COL, colon.

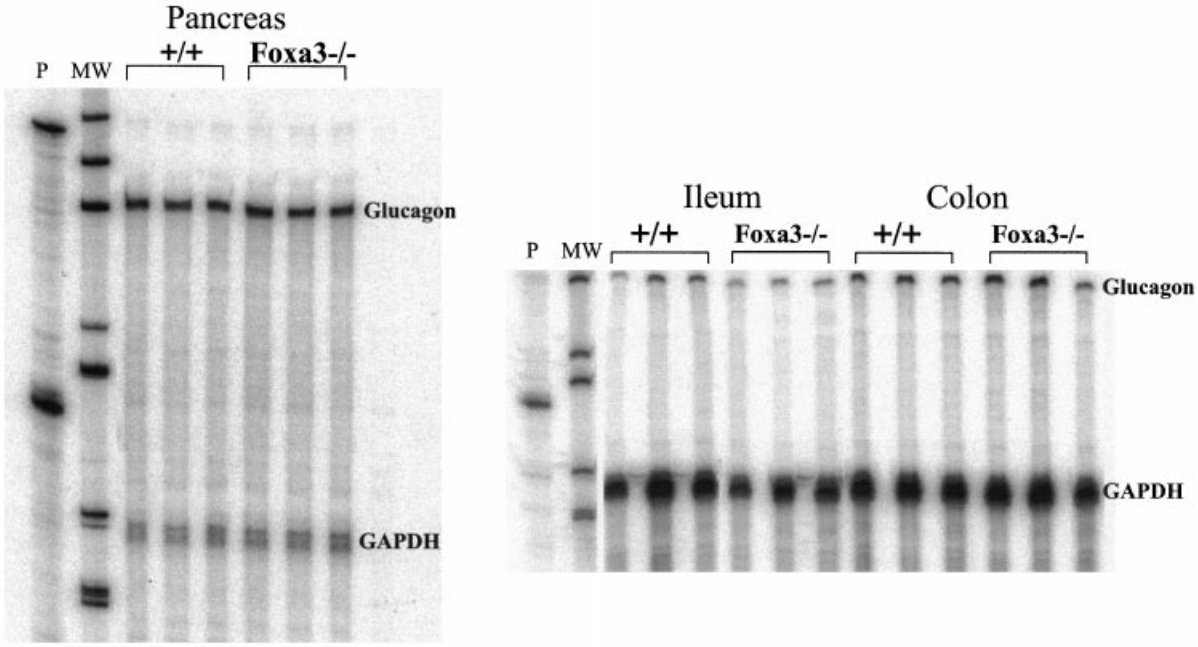

Figure 7 Total RNA (20 μg) from adult pancreas, ileum and colon from fasted (48 h) control wild-type (+/+) or mutant (Foxa3$^{-/-}$) mice was subjected to RNase protection analysis with probes for glucagon and GAPDH

Left-hand panel: adult pancreas. Right-hand panel: adult ileum and colon. Following normalization using the internal GAPDH signal, no significant differences were observed in wild-type versus Foxa3$^{-/-}$ mice. P, probe alone.

up-regulation of other transcription factors that compensate for Foxa3 deficiency. Indeed, both Foxa1 and Foxa2 are up-regulated in the liver of Foxa3$^{-/-}$ mice, and may modify the phenotype of complete Foxa3 deficiency [44]. Given the major difficulties in isolating pure populations of islet A cells or gut endocrine L cells, it is not possible to exclude the possibility that up-regulation of Foxa1 or Foxa2 in proglucagon-producing cells may account for normal levels of proglucagon gene expression in Foxa3 mutant mice.

The overlapping functions of Foxa proteins in metabolic control are illustrated by studies analysing gene expression in embryoid bodies with mutant Foxa alleles. Foxa2 and Foxa1 function as positive and negative regulators, respectively, of the maturity onset diabetes of the young ('MODY') genes HNF-4/HNF-1, providing a model whereby Foxa proteins regulate gene expression by competing for Foxa-binding sites [36]. Although the levels of islet Foxa3 are reduced in HNF-1α mutant mice, whether Foxa3 deficiency contributes to the phenotype in these mice cannot presently be ascertained [43].

In conclusion, although Foxa3 is expressed in islets and both islet and enteroendocrine cell lines and binds to the proglucagon gene G2 promoter, it does not exert a functional role in the

regulation of islet or intestinal proglucagon gene expression. Furthermore, our data demonstrating normal levels of tissue GLI in the fasting and fed state, taken together with an appropriate glucagon response to mild hypoglycaemia in fasted Foxa3 mice [61], suggest that Foxa3 is dispensable for biosynthesis and secretion of both intestinal and pancreatic proglucagon-derived peptides *in vivo*. Hence, the precise role of Foxa3 in the biology of islet or intestinal endocrine cells remains to be determined.

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to P.L.B. and D.J.D.D.J.D. is a Senior Scientist of the CIHR, Y.L. was a research fellow of the Canadian Diabetes Association and P.L.B. is supported by the Canada Research Chairs Program. Studies at the University of Pennsylvania were facilitated by the University of Pennsylvania Diabetes Center (P30-DK19525) and the Penn Center for Molecular Studies in Digestive and Liver Disease (P30-DK50306). This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (RO1 DK55342 and PO1 DK49210 to K.H.K.).

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Received 16 January 2002/19 April 2002; accepted 9 May 2002
Published as BJ Immediate Publication 9 May 2002, DOI 10.1042/BJ20020095