0888-8809/02/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 16(1):170–183

Hepatic Nuclear Factor-3 (HNF-3 or Foxa2) Regulates Glucagon Gene Transcription by Binding to the G1 and G2 Promoter Elements

BENOIT R. GAUTHIER, VALERIE M. SCHWITZGEBEL, MAIA ZAIKO, ALINE MAMIN, BEATE RITZ-LASER, AND JACQUES PHILIPPE

Unité de Diabétologie Clinique, Centre Médical Universitaire, 1211 Genève 4, Switzerland

Glucagon gene expression in the endocrine pancreas is controlled by three islet-specific elements (G3, G2, and G4) and the α-cell-specific element G1. Two proteins interacting with G1 have previously been identified as Pax6 and Cdx2/3. We identify here the third yet uncharacterized complex on G1 as hepatocyte nuclear factor 3 (HNF-3)β, a member of the HNF-3/forkhead transcription family, which plays an important role in the development of endoderm-related organs. HNF-3 has been previously demonstrated to interact with the G2 element and to be crucial for glucagon gene expression; we thus define a second binding site for this transcription on the glucagon gene promoter. We demonstrate that both HNF-3α and -β produced in heterologous cells can interact with similar affinities to either the G1 or G2 element. Pax6, which binds to an overlapping site on G1, exhibited a greater affinity as compared with HNF-3α or -β. We show that both HNF-3β and -α can transactivate glucagon gene transcription through the G2 and G1 elements. However, HNF-3 via its transactivating domains specifically impaired Pax6-mediated transactivation of the glucagon promoter but had no effect on transactivation by Cdx2/3. We suggest that HNF-3 may play a dual role on glucagon gene transcription by 1) inhibiting the transactivation potential of Pax6 on the G1 and G3 elements and 2) direct activation through G1 and G2. (*Molecular Endocrinology* 16: 170–183, 2002)

GLUCAGON IS A 29-amino acid peptide that raises blood glucose levels through a concerted action on hepatic glycogenolysis and gluconeogenesis (1). Expression of the glucagon gene is restricted to the α-cells of the endocrine pancreas, the L cells of the intestine, and certain areas of the brain (2, 3). Previous studies have shown that tissue-specific expression of the glucagon gene is conferred by four *cis*-acting elements (G1, G2, G3, and G4) located within the proximal promoter region of the gene (4, 5). Elements G2, G3, and G4 confer islet-specific expression while G1 restricts glucagon gene transcription to the α-cells. Some of the transcription factors regulating the activity of the glucagon gene have been identified. Cdx2/3, Brn-4, hepatocyte nuclear factor-3β (HNF-3β), Pax2, NeuroD/Beta2, and E47 interact with the G1, G2, G3, and G4 element, while Pax6 binds both the G1 and G3 elements (6–13). Recently, the heterodimeric Pbx-Prep1 homeodomain protein was also shown to interact with G3 and with a novel binding element identified as G5 (14). Optimal and regulated expression of the glucagon gene in α-cells of the pancreas results from the combinatorial interactions of these factors binding to their cognate site(s).

Initial studies characterized G1 as a 41-bp element harboring two A/T-rich sequences that form a nearly perfect repeat and are putative binding sites for homeodomain-containing DNA binding proteins (4, 15). We further demonstrated that the direct repeat was critical for α-cell-specific expression of the glucagon gene (5). A third proximal A/T-rich sequence was shown to interact with the homeobox protein Isl-1, which is expressed in all four principal cell types of the endocrine pancreas (16). In the presence of nuclear extracts derived from a glucagon-producing cell line, InR1G9, at least three protein complexes interact with an oligonucleotide comprising the A/T-rich direct repeat (G1-56 element). The paired-homeodomain transcription factor, Pax6, can bind as a monomer to the distal AT-rich site or form an heterodimer with the caudal related protein Cdx2/3 that will interact with both AT-rich sites (6, 8–10, 12). The third complex forming on the G1 element remains to be characterized.

In this study, the nuclear protein forming the third protein(s) complex on the distal AT-rich site of G1 is molecularly identified as HNF-3β, a member of the HNF-3/forkhead transcription factor family, which plays an important role in the development of endoderm-related organs (17–22). We previously demonstrated that the G2 element interacts with HNF-3 (23); we now delineate G1 as a second HNF-3 binding site on the glucagon gene promoter. We demonstrate that HNF-3α and -β are able to interact with G2 and the distal AT-rich site of G1 with similar affinities. However, in InR1G9 cells, HNF-3β, as opposed to HNF-3α, appears to be the predominant binding activity detected on the G1 element. Transactivation

Abbreviations: CAT, Chloramphenicol acetyltransferase; DN-HNF-3β, dominant negative form of HNF-3β; GST, glutathione-*S*-transferase; HNF, hepatocyte nuclear factor; PAP, alkaline phosphatase.

experiments demonstrate that HNF-3β and -α can activate glucagon gene transcription through the G2 and G1 elements. Pax6, which also binds the distal AT-rich site of G1, exhibits a greater affinity as compared with HNF-3α or -β. However, HNF-3 specifically impairs Pax6-mediated transactivation of the glucagon promoter through G1 and G3 but has no effect on transactivation by Cdx2/3. We show that this inhibition is conferred by the two transactivation domains of HNF-3. We suggest that HNF-3 may play a dual role on glucagon gene transcription by 1) inhibiting the transactivation potential of Pax6 on the G1 and G3 elements and 2) direct activation through G1 and G2.

RESULTS

We have previously reported that G1 is a proximal upstream promoter element critical for α-cell-specific expression of the glucagon gene. At least three protein complexes can be resolved by EMSA when an oligonucleotide corresponding to G1 (G1-56) is incubated with nuclear protein extracts derived from the hamster glucagon-producing cell line InR1G9 (Fig. 1B and Ref. 5). The fastest migrating complex, B1, was identified as the paired homeodomain transcription factor Pax6, while the slowest migrating complex, B3, was shown to be an heterodimer between Pax6 and the caudal related factor Cdx2/3 (Fig. 1B). As shown in Fig. 1A, binding of Pax6 to G1 requires the distal A/T-rich region while Cdx2/3 binds to the proximal A/T-rich site of G1 (6, 10, 12). Several lines of evidence suggested that the third, yet uncharacterized, complex, B2, which as Pax6 forms on the distal A/T-rich site of the G1 element belongs to the HNF-3 family of transcription factors: 1) the G2 element, which binds HNF-3 β, can effectively compete for factors interacting with G1 (4); 2) nuclear protein extracts derived from HNF-3-expressing but not from other cell lines produced in EMSA a complex of similar electrophoretic mobility as B2 in the presence of G1 (5); and 3) sequence comparison analysis revealed that the distal A/T-rich region of G1 harbors an inverted DNA-binding site for the family of HNF-3 transcription factors. This family of nuclear activators consists of three genes (α, β, and γ) encoding proteins that bind to DNA via a highly conserved winged helix domain (24). To determine whether the B2 complex detected in nuclear protein extracts derived from the glucagon-producing cell line, InR1G9, corresponded to an HNF-3 family member, we performed EMSA using specific antisera against each of the members (Fig. 1B). Whereas incubation of binding reactions with preimmune serum or antibodies raised against HNF-3α and -γ had little or no effect on the formation of the various complexes (Fig. 1B, lanes 1, 2, 5 and 6), the addition of HNF-3β antibodies completely abolished B2 (Fig. 1B, lane 4). Antibodies against HNF-3β also abolished the Pax6/Cdx2/3 heterodimer and altered the migration pattern of Pax6 (10), indicating a cross-reactivity of the Ig to Pax6. Complexes containing either Pax6 or HNF-3β were recognized by HNF-3β antisera while Cdx2/3 or HNF-3α were unaffected by the antibody, confirming a nonspecific cross-reactivity of the antibody to Pax6 (data not shown). We conclude that B2 represents essentially HNF-3β. Similar results were obtained with the mouse α-cell line αTC-1 (data not shown). Glucagon gene expression has also been reported in enteroendocrine cells of the intestine (3). We therefore investigated whether or not HNF-3 (α, β, or γ) binding activity on G1 could be detected using nuclear protein extracts isolated from the intestinal cell line GLUTag (25). For this purpose, we used an oligonucleotide harboring only the distal A/T-rich site of G1 (G1-54) to which B2 but not Pax6 or the heterodimer Pax6/Cdx2/3 can interact (Fig. 1, A and C, lane 1). This oligonucleotide produced one predominant complex in the presence of InR1G9 nuclear protein extract that was mostly eliminated by HNF-3β antibodies, while only a slight decrease in band intensity was observed with the HNF-3α antibody (Fig. 1C, lanes 5 and 6). Nuclear protein extracts from GLUTag cells generated two retarded complexes on G1-54 (Fig. 1C, lane 8). The first complex was abolished when antibodies to HNF-3α were added to the binding reaction (Fig. 1C, lane 9) while the second complex, of weaker intensity, was supershifted in the presence of HNF-3β antibodies (Fig. 1C, lane 10). A similar binding pattern was generated using nuclear protein extracts from HepG2 that express both HNF-3α and -β (Fig. 1C, lanes 12–15). Of note, the mobility of mouse and human HNF-3α and -β complexes on G1 was slightly slower as compared with the hamster factors. Interestingly, the addition of HNF-3α antibodies to InR1G9, GluTag, or HepG2 nuclear extracts caused the appearance of new faster migrating complexes that was not observed with G1-56 (Fig. 1C, lanes 5, 9, and 13). Potentially, sequestration of HNF-3α may allow a yet unknown factor to interact with G1 in the absence of Pax6. Taken together, these results indicate that HNF-3α and -β, but not -γ, can interact with the G1 element of the glucagon gene promoter. However, the binding activity of HNF-3β is favored in pancreatic endocrine cells while the reverse is observed in enteroendocrine cells. Thus, we define G1 as a second HNF-3 binding site on the glucagon gene promoter.

Our results suggest that HNF-3β is the predominant HNF3 binding activity in α-cell lines. However, recently HNF-3α was demonstrated to interact with the G2 element of the glucagon gene promoter, and the HNF-3α null mutation in mice leads to a marked decrease in glucagon content and hypoglycemia (26, 27). We therefore determined whether or not expression levels, compartmentalization of HNF-3α and -β proteins, or differences in affinity for the G1 and G2 binding sites could account for these discrepancies. Western blot analysis revealed that both factors are expressed and properly localized to the nucleus in InR1G9 cells (Fig. 2A). The same results were obtained

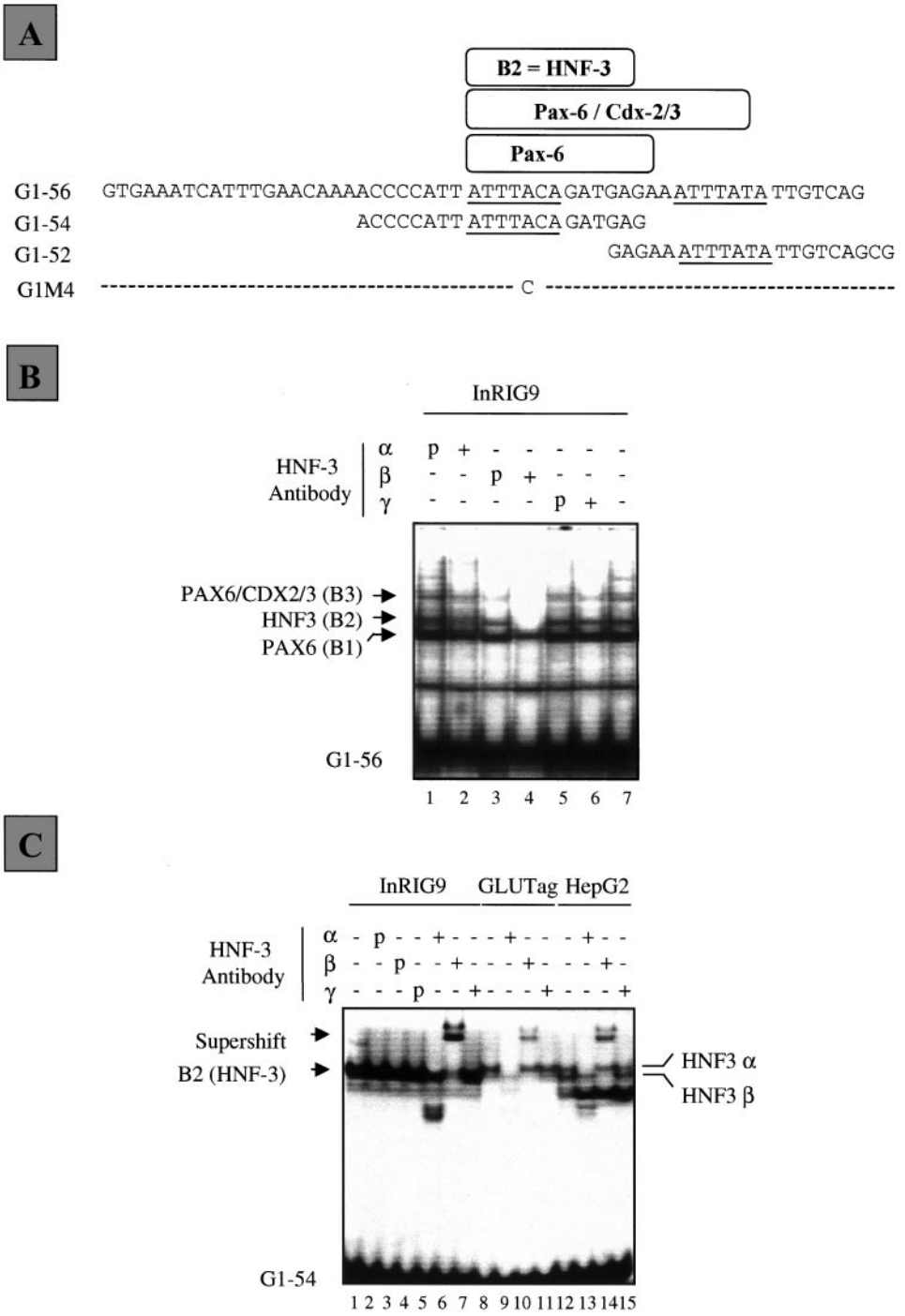

Fig. 1. HNF-3β Is the Predominant Member of the HNF-3 Family, Which Interacts with the G1 and G2 Element of the Glucagon Gene Promoter in InR1G9 Cells

A, Schematic representation illustrating oligonucleotides for the glucagon gene G1 element and protein complexes formed on G1. The two A/T-rich elements are *underlined*. B, EMSAs were performed using a ^{32}P-labeled G1-56 oligonucleotide and nuclear protein extracts (6 μg) isolated from glucagon-producing InR1G9 cells. The three main complexes observed in EMSA in the presence of InR1G9 extracts are shown in lane 7. The corresponding binding factors are depicted on the *left* of the figure. Samples were incubated either with preimmune serum (p; lanes 1, 3, and 5) or with the indicated polyclonal antibody (+; lanes 2, 4, and 6). The identification of HNF-3β as the binding factor forming B2 is demonstrated by the disappearance of this complex in the presence of the antibody raised against HNF-3β (lane 4). C, EMSAs were also performed with 6 μg of nuclear protein extracts prepared from the intestinal cell line GLUTag and the hepatoma cell line HepG2 in the presence of a ^{32}P-labeled G1-54 oligonucleotide. This oligonucleotide is only recognized by proteins forming complex B2 since it is lacking sequences that are required for the binding of Pax6 and Cdx2/3. The corresponding binding factors are depicted on the *right* of the figure.

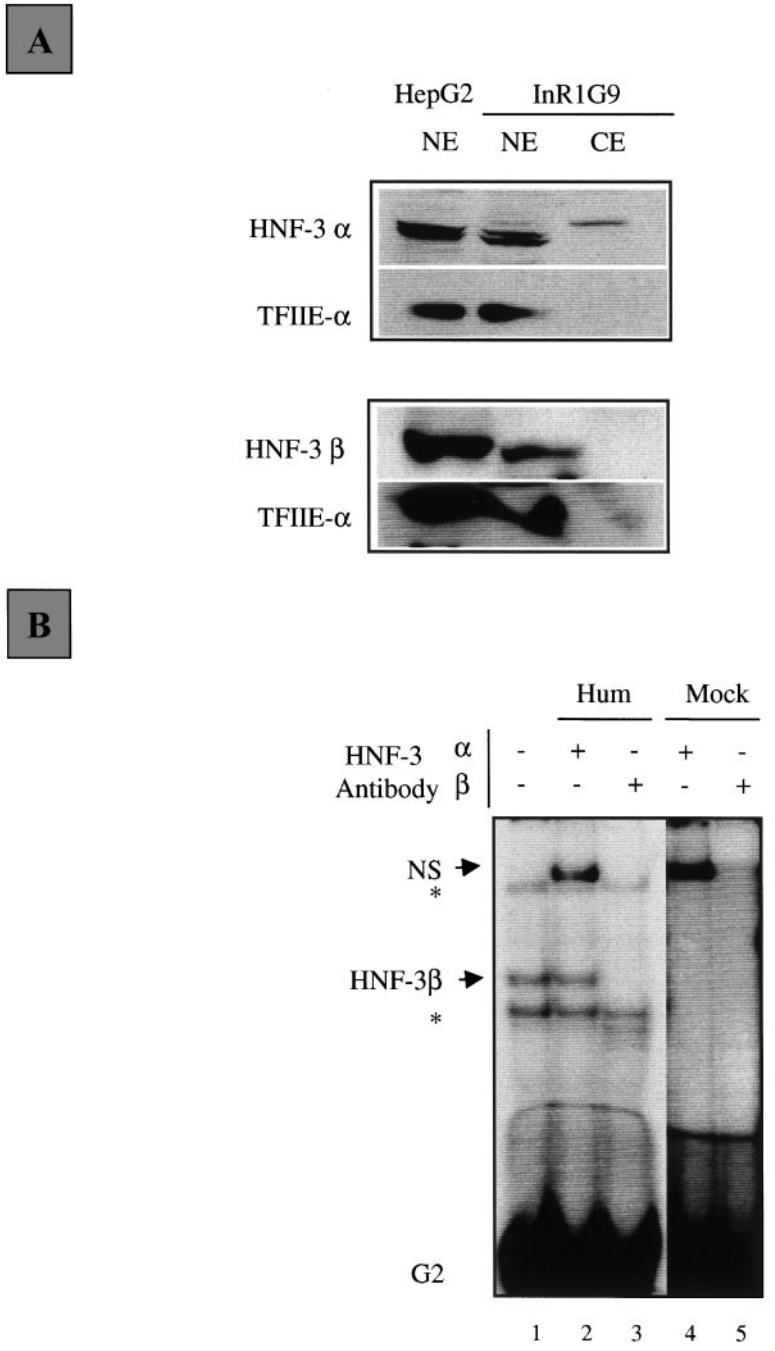

Fig. 2. HNF-3α and -β Proteins Are Both Expressed in InR1G9 Cells and Are Localized to the Nucleus

A, Cytoplasmic (CE) and nuclear (NE) fractions from InR1G9 and HepG2 were immunostained with HNF-3α and -β antibodies. To confirm the purity of the nuclear and cytoplasmic extracts, membranes were also immunostained with polyclonal antibodies raised against the nuclear protein TFIIE-α. B, Nuclear protein extracts derived from human islets (Hum) were used in EMSA in the presence of a ^{32}P- labeled G2 oligonucleotide. The complex harboring HNF-3β is indicated by an *arrow* while two uncharacterized complexes are shown as *asterisks* (*). A nonspecific complex, which is obtained with the antibody raised against HNF-3α (lanes 2 and 4), is depicted as NS. Mock represents EMSAs performed in the presence of antibodies without nuclear protein extracts.

with nuclear extracts derived from the hepatoma cell line HepG2, which exhibits HNF-3α and -β binding activity to G1 in EMSA. To determine the relative binding activities of HNF-3α and -β from normal pancreatic endocrine cells, nuclear protein extracts were prepared from human islets, and binding assays were performed with the G2 element. Three complexes were detected, one of which was recognized by antibodies raised against HNF-3β, but not -α (Fig. 2B). The remaining complexes have yet to be characterized in

these human pancreatic islet nuclear protein extracts. Of note, the addition of the HNF-3α, but not -β, antibody resulted in the formation of a nonspecific low mobility complex in the presence of G2 (Fig. 2B, lanes 2, 4, and 5). To evaluate the relative binding affinity of HNF-3α and -β for either G1 or G2, gel shift competition experiments were performed with nuclear protein extracts derived from BHK-21 cells overexpressing HNF-3α or -β. The HNF-3α complex formed on labeled G1-54 was effectively competed off by a 50-fold molar excess of cold G1-54 and G2 oligonucleotides, respectively (Fig. 3A, *left panel*). In the presence of a labeled G2 element, the HNF-3α complex was partially competed by a 100-fold molar excess of either G2 or G1-54 (Fig. 3A, *right panel*). Similar results were obtained in the presence of HNF-3β (Fig. 3B), indicating that both HNF-3α and -β display very similar binding characteristics to either the G1 or G2 element of the glucagon gene promoter. Taken together, these results indicate that HNF-3α is expressed in InR1G9 cells but that HNF-3β is the predominant EMSA binding activity on the G1 and G2 elements from both InR1G9 cells and normal human islets. The fact that the marked decrease in glucagon content observed in HNF-3α homozygous mutant mice cannot be corrected by the substitution of HNF-3β cannot be explained by the relative affinities of HNF-3α and -β for the G1 and G2 sites.

Since the distal A/T-rich site of G1 is a target for both HNF-3 (α or β) and Pax6, we compared the relative binding affinity of the three factors for this element. Pax6 and HNF-3 (α or β)-containing nuclear protein extracts from BHK-21 cells were incubated simultaneously with labeled G1-56 before the addition of cold competitor. As shown in Fig. 4, Pax6 was effectively competed off by a 50- to 100-fold molar excess of unlabeled G1-56 while a 100-fold molar excess of the same oligonucleotide slightly competed for HNF-3α or -β binding. G1 is therefore a better target site for Pax6 than for either HNF-3α or -β.

Although Cdx2/3 was shown to preferentially form a complex with the proximal A/T-rich site of G1, it may also interact with the most distal site where HNF-3 binds (10). We thus determined the DNA binding specificity of Cdx2/3 and HNF-3α and -β for G1. The G1-56, as well as the proximal (G1-52) and the distal (G1-54) A/T-rich sites of G1, were used as competitors. As expected, G1-54, but not G1-52, competed effectively for the binding of HNF-3α and -β to labeled G1-56 at a 100-fold molar excess (Fig. 5, A and B). Inversely, Cdx2/3 was completely competed off by a 100- to 500-fold molar excess of unlabeled G1-52, whereas G1-54 appeared to compete only at higher molar fold excess (500-fold). Unlabeled G1-56 appeared to compete equally well for both HNF-3 and Cdx2/3 (Fig. 5, A and B). We conclude that the distal A/T-rich region of G1 displays a greater binding affinity for HNF-3 than for Cdx2/3 while the opposite is observed for the proximal A/T-rich site.

To determine the functional impact of the individual or combined G1 and G2 elements on the transcriptional regulation of the glucagon gene by HNF-3 (α and β), we performed transient transfection studies in the nonislet cell line BHK-21. HNF-3α increased chloramphenicol acetyltransferase (CAT) activity of reporter constructs containing either G1 (G1–31Glu) or G2 (G2–31Glu) in a dose-dependent manner up to 4-and 5-fold higher than control levels, respectively (Fig. 6A). An additive effect on CAT activity, as compared with individual sites, was observed when reporter constructs harboring both elements (G2–138Glu, 16-fold; −292Glu, 16-fold; and −350Glu, 10-fold) were cotransfected with increasing amounts of HNF-3α. Similar quantitative results were also obtained for HNF-3β (Fig. 6B). We then assessed the consequences of a point mutation (A to C; at position −84) within the first 350 bp of the glucagon promoter which abrogate HNF-3 binding to G1 *in vitro* but leaves binding to G2 intact (G1M4–350Glu) (Fig. 1A and Ref. 5). This mutation resulted in an impaired induction of CAT activity at levels quantitatively similar to those observed with constructs containing either G1 or G2 (Fig. 6, A and B). Taken together, these results indicate that both G1 and G2 binding sites are required for maximal activation of the glucagon gene promoter by HNF-3 (α or β) in BHK-21 cells and that each element contributes to half of the full activation.

To analyze the effect of ectopic expression of HNF-3 (α and β) in the glucagon-producing cell line InR1G9, we cotransfected expression vectors for either HNF-3α or -β along with CAT reporter gene constructs driven by the G1, G2, or both G1 and G2 elements of the glucagon gene promoter. Overexpression of HNF-3 (α or β) had no significant effect on basal activity of the various constructs (Fig. 7, A and B). Interestingly, similar results have been obtained in InR1G9 cells with Pax6 and Cdx2/3, which are key transcription factors involved in the regulation of glucagon gene transcription (12). These results suggest that in pancreatic α-cells, glucagon gene expression is governed by a complex regulatory mechanism involving multiple proteins that may tether the effect of individual factors such as HNF-3, Pax6, or Cdx2/3. We therefore pursued our study in an heterologous system in which the functional impact of individual components can be assessed.

To establish whether Cdx2/3 and HNF-3 (α and β) were capable of functionally interacting on the G1 element, combinations of HNF-3α or -β and Cdx2/3 expression vectors were cotransfected along with the reporter construct G1–31Glu; no significant increase in CAT activity, as compared with Cdx2/3 alone, was observed in BHK-21 cells (Fig. 8A). Higher amounts of HNF-3α or -β (up to 2 μg) produced similar results (data not shown). We conclude that HNF-3 (α and β) and Cdx2/3 do not have additive or synergistic activity on glucagon gene transcription. To assess the functional relevance of HNF-3 (α and β) and Pax6 binding to the same A/T-rich site of the G1 element, BHK-21

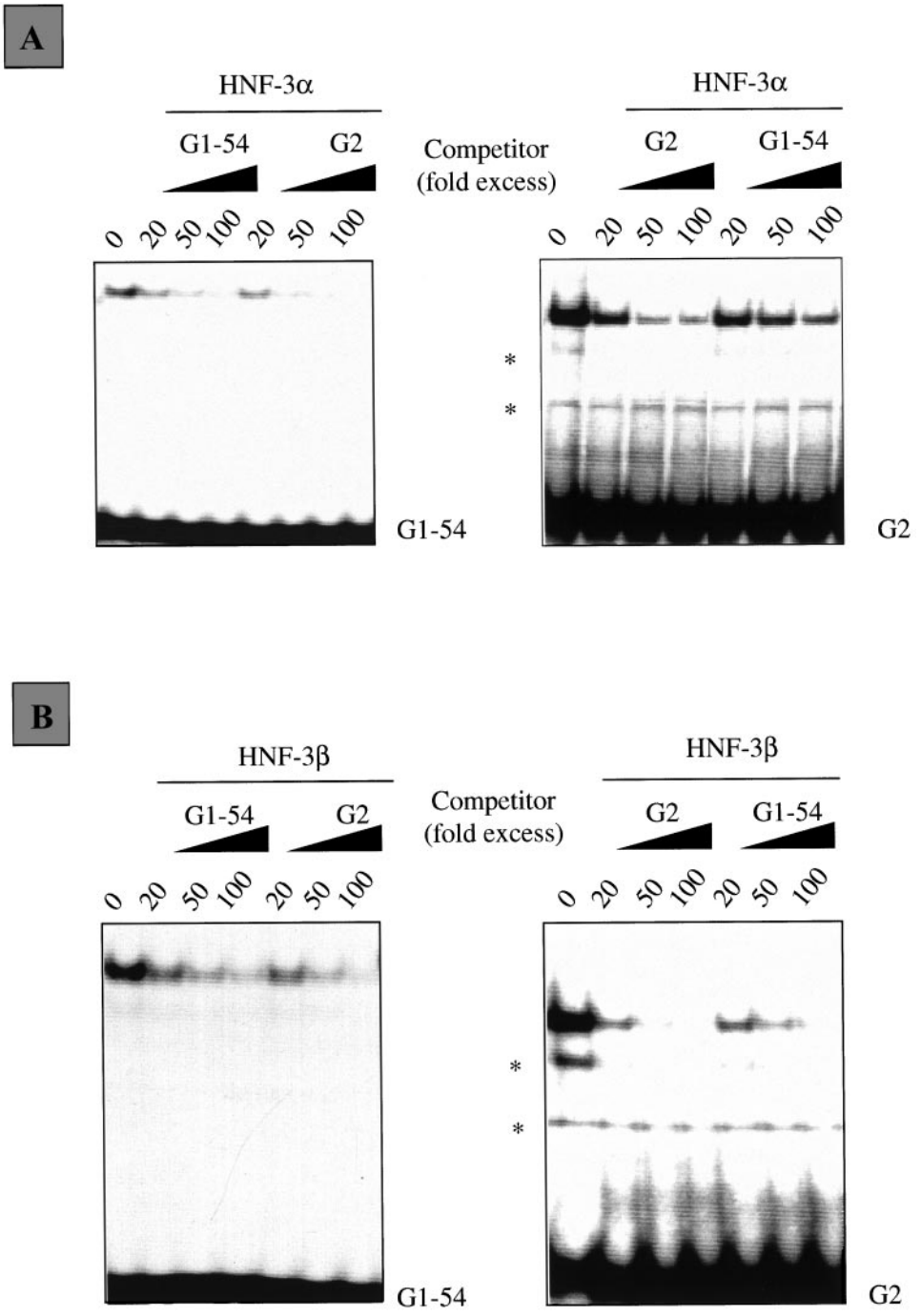

Fig. 3. HNF-3α and -β Display Similar Affinities for Either the G1 or G2 Element of the Glucagon Gene Promoter

DNA-protein complexes formed using nuclear extracts of BHK-21 cells overexpressing either HNF-3α (panel A) or -β (panel B) in the presence of the oligonucleotides G1-54 or G2 were subjected to competition assays against unlabeled G1-54 and G2. The fold molar excess of competitor is indicated *above* each lane. Two nonspecific complexes using the G2 oligonucleotide in the presence of either HNF-3α or -β is depicted by an *asterisk* (*).

cells were cotransfected with fixed amounts of either G1–31Glu or -138Glu and Pax6 expression vector along with increasing amounts of either HNF-3α or -β. As previously reported, transfection of 0.25 μg of Pax6 induced a 15- to 30-fold increase in CAT activity as compared with the control sample (Fig. 8B and Ref. 12). The addition of 30 ng of either HNF-3α or -β to transfectants containing 0.25 μg of Pax6 resulted in a drastic decrease in Pax6-induced activity (Fig. 8B). These data suggest that even though Pax6 displays a better affinity for G1 (Fig. 4), HNF-3α and -β can hinder Pax6-mediated transactivation through the G1 ele-

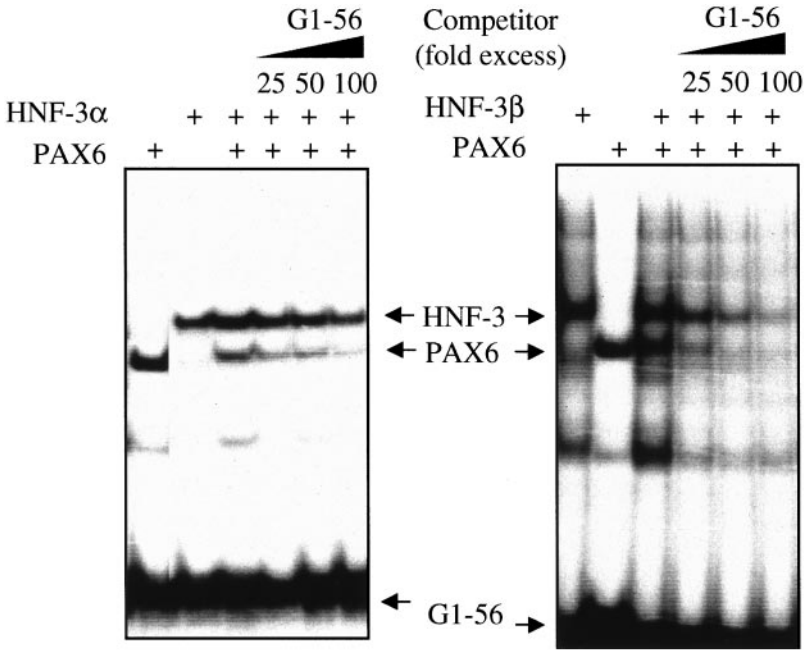

Fig. 4. Pax6 Exhibits a Greater Affinity for the G1 Element of the Glucagon Gene Promoter as Compared with Either HNF-3α or -β

Labeled G1-56 oligonucleotide was mixed with nuclear extracts derived from BHK-21 cells overexpressing Pax6, HNF-3α (*left panel*), or HNF-3β (*right panel*). Competition assays were performed using increasing fold molar excess of cold G1-56 as depicted *above* each panel. The addition of either Pax6 or HNF-3-containing BHK-21 nuclear protein extracts in each sample is indicated by a +.

ment of the glucagon promoter. Similar results were also obtained with the reporter construct G2–138Glu, which harbors both HNF-3 binding sites (Fig. 8B). However, the presence of G2 resulted in a small increase in the CAT activity of G2–138Glu at higher amounts of HNF-3α or -β (0.5 μg), indicating that these transcription factors have the potential to increase glucagon gene expression, albeit to lower levels, once they have suppressed Pax6 transactivation.

To determine whether HNF-3 (α and β) interaction with the G1 element is necessary for the inhibition of Pax6-mediated activation, we conducted cotransfection experiments with a reporter construct harboring the G3 element (G3–31Glu), which binds Pax6 but not HNF-3. Cotransfection with Pax6 resulted in a 14-fold induction in CAT activity of the reporter construct, which was inhibited by the presence of either HNF-3α or -β (Fig. 9A). Similar results were obtained with the mutant reporter construct G1M4–350Glu (Fig. 9B). In an attempt to investigate a possible mechanism for HNF-3-mediated repression of Pax6 transactivation, we tested for direct protein-protein interaction between these two transcription factors (Fig. 9C). In a glutathione-*S*-transferase (GST) pull-down experiment, HNF-3α and -β were found to bind efficiently to glutathione-Sepharose beads containing GST-Pax6 but not GST alone (Fig. 8B). We thus suggest that HNF-3 (α or β) can directly interact with Pax6 and attenuate its transactivation potential on the glucagon gene promoter.

To delineate the region of HNF-3 involved in conferring suppression of Pax6-mediated transactivation, we performed transient transfection experiments using a dominant negative (DN) form of HNF-3β, which lacks the two transactivating domains. Nuclear protein extracts derived from BHK-21 cells transfected with the expression vector harboring the DN-HNF-3β cDNA generated a faster migrating complex as compared with the wild-type HNF-3β in the presence of G1, consistent with the nuclear localization of the truncated protein (Fig. 10A) (28). Although a significant decrease in CAT activity was observed at 0.5 μg of DN-HNF-3β, the truncated protein was unable to effectively inhibit Pax6 transactivation of the −138Glu reporter construct as compared with the wild-type HNF-3β for similar amounts of transfected cDNA (compare Fig. 8B to Fig. 10 B).

DISCUSSION

HNF-3 has previously been shown to interact with the enhancer-like G2 element of the rat glucagon gene promoter (11). This study confirms and extends these findings to the G1 promoter element and thus defines HNF-3 as an essential regulator of glucagon gene expression in pancreatic α-cells. We show that two members of the HNF-3 family, HNF-3α and -β, can interact with similar affinities to either the G1 or G2

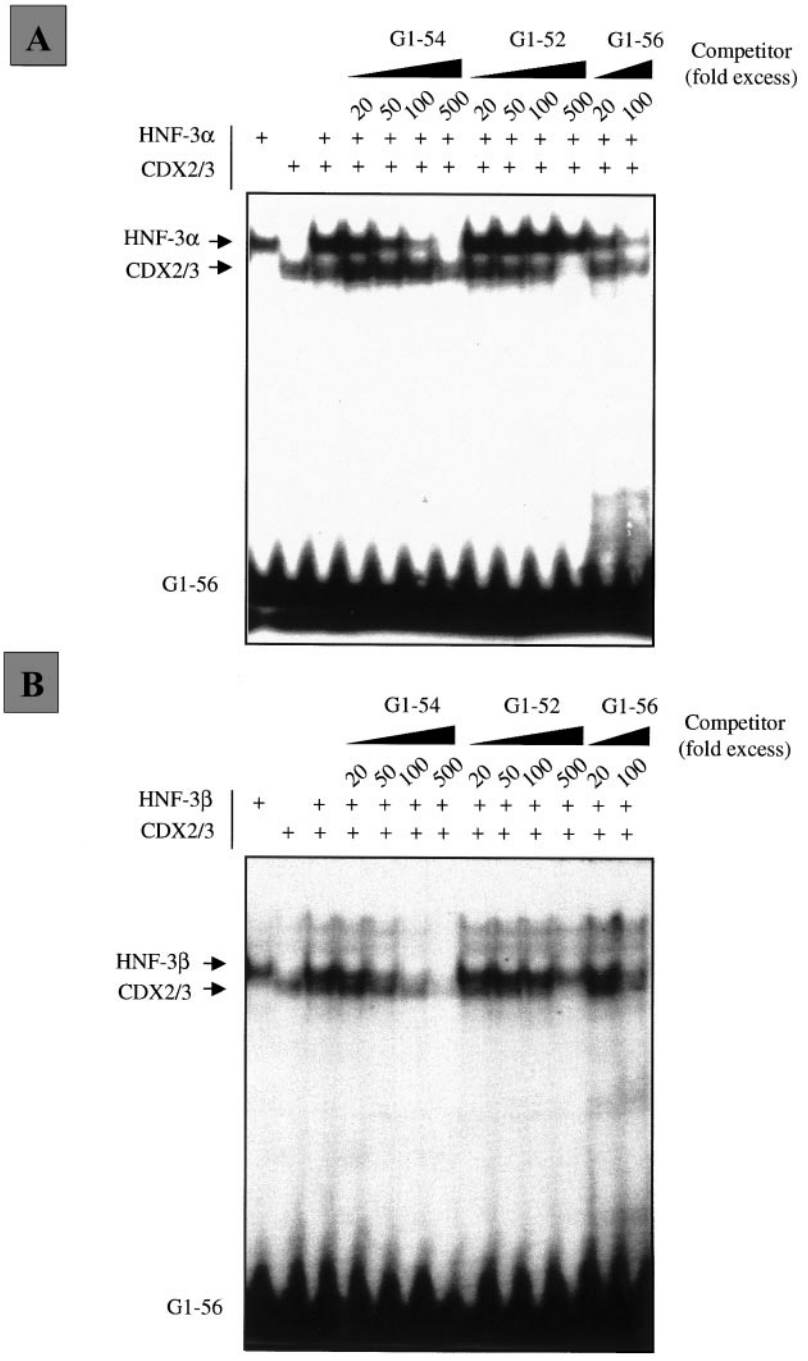

Fig. 5. HNF-3 α and β Exhibit Greater Affinities for the Distal A/T-Rich Site of the G1-54 Element as Compared with Cdx2/3

Labeled G1-54 oligonucleotide was mixed with 6 μg of nuclear extracts derived from BHK-21 cells overexpressing Cdx2/3, HNF-3α (panel A) or HNF-3β (panel B). Competition assays were performed using increasing fold molar excess of cold G1-54, G1-52, or G1-56 as depicted above each panel. The addition of either Cdx2/3 or HNF-3- derived BHK-21 nuclear protein extracts in each sample is indicated by a +.

element. These two sites contain an identical core sequence (5′-GTAAATAA-3′), albeit on opposite strands, which is reminiscent of the HNF-3 consensus DNA binding element (WTRTTKRYTY, where W = A or T; K = G or T; Y = pyrimidine; and r = purine) (29). However, it appears that HNF-3β, as opposed to HNF-3α, is the predominant binding activity detected on both elements in the glucagon-producing cell lines InR1G9 and α-TC-1 and in normal human islets, even though both factors are expressed in these cells. Two independent studies have recently demonstrated that, in HNF-3α-deficient mice, glucagon-producing pancreatic α-cells developed normally, but glucagon mRNA steady state levels were reduced by 50–70%,

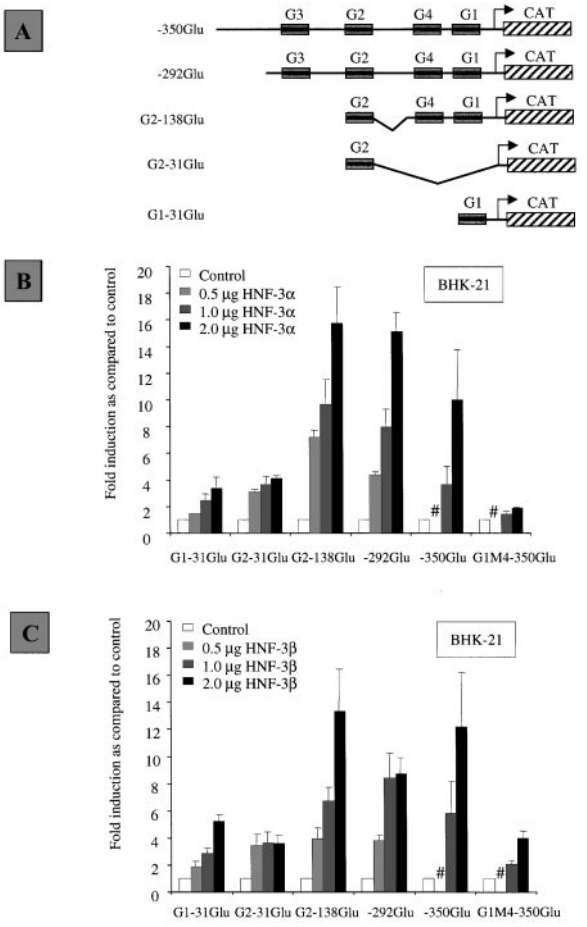

Fig. 6. The G1 and G2 Elements of the Glucagon Promoter Respond Similarly to Increasing Amounts of HNF-3α and -β in BHK-21 Cells

A, Schematic diagram of the chimerical constructs used in transfection experiments. A point mutation was introduced into the G1 element of the −350Glu construct to generate G1M4–350Glu. Transient cotransfection studies using BHK-21 cells were performed with increasing amounts of either HNF-3α (panel B) or HNF-3β (panel C). Data are presented as fold stimulation of basal CAT activity (reporter plasmid transfected along with an empty pSG5 expression vector). The effect of 0.5 μg of either HNF-3α or -β on CAT activities of the −350Glu and G1M4–350Glu constructs was not determined and is depicted as #.

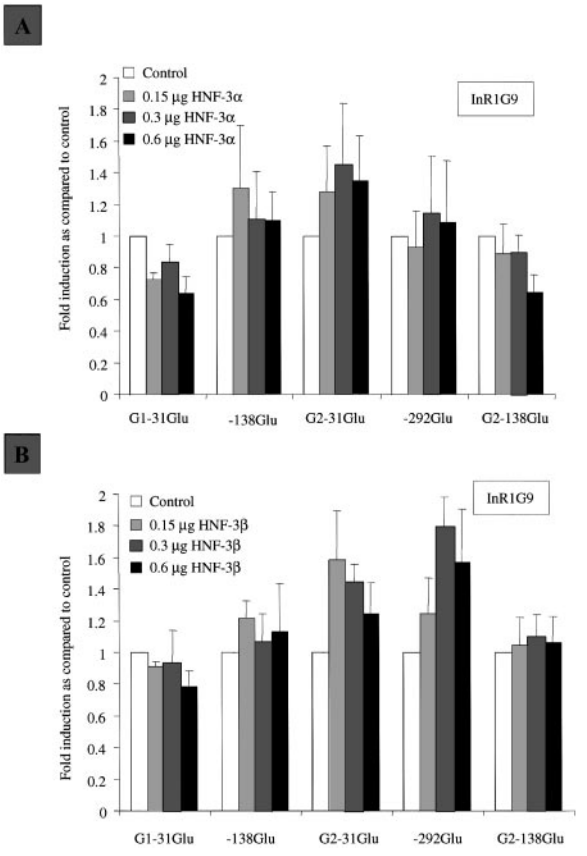

Fig. 7. HNF-3α and -β Do Not Transactivate the Glucagon Gene Promoter in InR1G9 Cells

Transient cotransfection studies using InR1G9 cells were performed with increasing amounts of either HNF-3α (panel A) or HNF-3β (panel B) and 10 μg of various glucagon reporter constructs. Data are presented as fold stimulation of basal CAT activity (reporter plasmid transfected along with an empty pSG5 expression vector).

implying a direct role of HNF-3α in the regulation of this gene, which cannot be substituted by HNF-3β (26, 27). Discrepancies observed between our results and transgenic animals indicate that although HNF-3α and -β may be concomitantly expressed in similar cells and share identical consensus DNA binding sites, their binding activities may be regulated by cellular constraints *in vivo*. Such constraints may be imposed by chromatin, which is intimately related to the expression of eukaryotic genes *in vivo*. HNF-3α is of particular interest as it has been shown to induce chromatin modifications on several genes such as the albumin, ER, and the α-fetoprotein gene (30–33). A similar situation could occur for the glucagon gene in which HNF-3α may act directly or indirectly as a cellular determinant that establishes a promoter environment favorable for transcriptional activation by HNF-3β or other critical transcription factors. In mice lacking HNF-3α, chromatin restructuring may be less efficient through HNF-3β, thus resulting in lower levels of glucagon gene expression. Alternatively, HNF-3β, which plays a determinant function during embryonic endoderm development, may be down-regulated and replaced by HNF-3α in glucagon-producing cells after terminal differentiation in mice, as recently suggested by Kaestner and co-workers for liver gene expression (34). In contrast, HNF-3β may be reactivated in immortalized cell lines becoming the predominant HNF-3 activity as observed in InR1G9 and α-TC-1 cells. The analysis of the functional impact of HNF-3β on glucagon gene expression *in vivo* awaits new experimental strategies since homozygous mice bearing a targeted null mutation for this transcription factor die early in

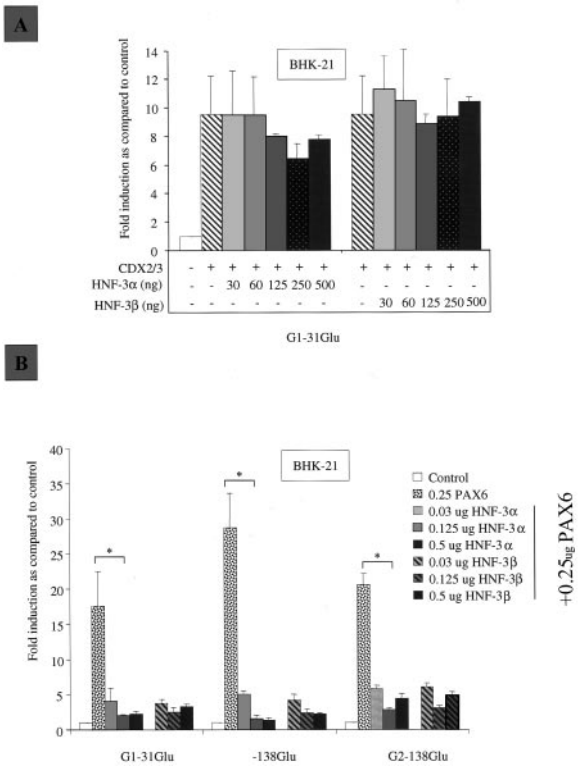

Fig. 8. HNF-3 Specifically Impairs Pax6-Mediated Transactivation but Has No Effect on Transactivation by Cdx2/3 in BHK-21 Cells

Transient cotransfection studies using BHK-21 cells were performed with various reporter construct (as depicted in the figure) along with either 0.25 μg of Cdx2/3 (panel A) or Pax6 (panel B) and increasing amounts of either HNF-3α or -β as indicated in the figure. Data are presented as fold stimulation of basal CAT activity (reporter plasmid alone).

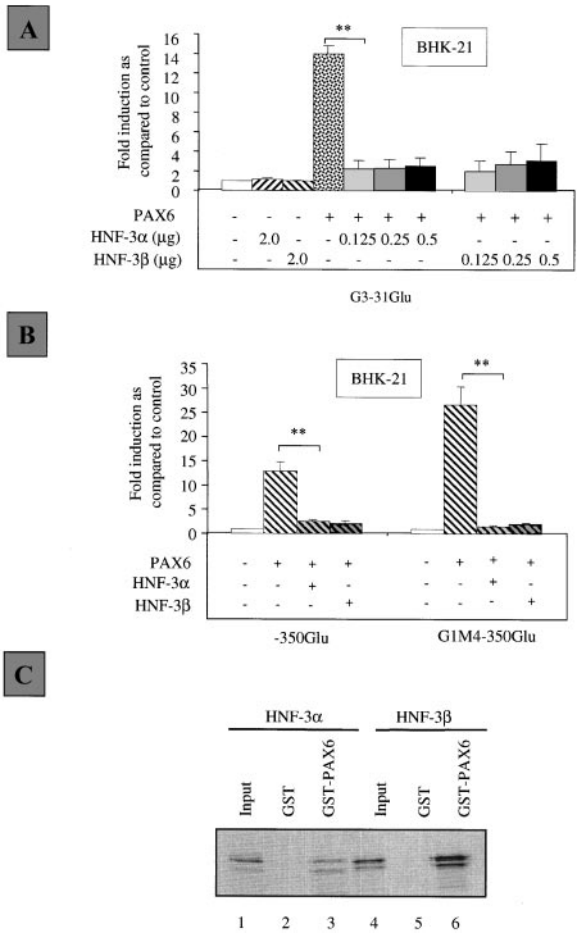

Fig. 9. HNF-3 Interacts Directly with Pax6 and Interferes with the Transactivation of the Glucagon Gene by Pax6

A, BHK-21 cells were cotransfected with the CAT reporter construct G3–31Glu along with 0.25 μg of Pax6 and increasing amounts of either HNF-3α or -β as indicated in the figure. Cotransfection experiments were also performed with 2 μg of either HNF-3α or -β. Values are plotted as fold induction of the control experiment performed in the presence of empty pSG5 expression vector. B, Similar transfections were performed with a glucagon gene promoter/CAT reporter constructs harboring a mutation in the G1 element (G1M4–350Glu). However, a single amount of either HNF-3α or -β (0.25 μg) was employed in these experiments. The wild-type construct, −350Glu, was used as control. C, HNF-3α and -β associates with Pax6 in a GST pull-down assay. L-[^{35}S]methionine-labeled HNF-3 α (lane 1, one-tenth of input) and β (lane 4, one-tenth of input) were incubated with 10 μg of bacterially expressed GST (lanes 2 and 5) or GST-Pax6 (lanes 3 and 6).

embryogenesis before the differentiation of pancreatic endoderm (17, 18, 21).

The proximal promoter element G1 is a well conserved regulatory element that confers α-cell-specific expression of the glucagon gene in the pancreas. Characterization of HNF-3 as the last major complex forming on G1 in InR1G9 cells will now permit a detailed analysis of the molecular mechanism governing cell-specific expression of glucagon in endocrine cells. Interestingly, neither HNF-3, Pax-6, nor Cdx2/3 is found exclusively in pancreatic endocrine cells; rather, they are all expressed at varying degrees in different tissues. Hussain and co-workers (35) have previously demonstrated that Brn-4, which is predominantly expressed in neuronal cells and α-cells of the endocrine pancreas, could interact with the distal G1 element and potentially confer α-cell-specific expression of the glucagon gene. The respective roles of Brn-4, Pax6, Cdx2/3, and HNF-3 in the cell-specific expression of the glucagon gene thus remain to be established.

HNF-3α and -β were equally capable of activating the glucagon gene promoter in a heterologous assay system, confirming that there are no differences in the ability of these factors to bind the G1 or G2 element. Both elements appear of similar functional importance, and each contributes to half of the full activation of the glucagon promoter in BHK-21 cells. However, in pancreatic α-cells, G1 by itself confers only weak transcriptional activation and is dependent on the upstream enhancer element G2 (or G3) for high levels of expression. Conversely, G1 is required for G2 to enhance transcription indicating a potential interaction

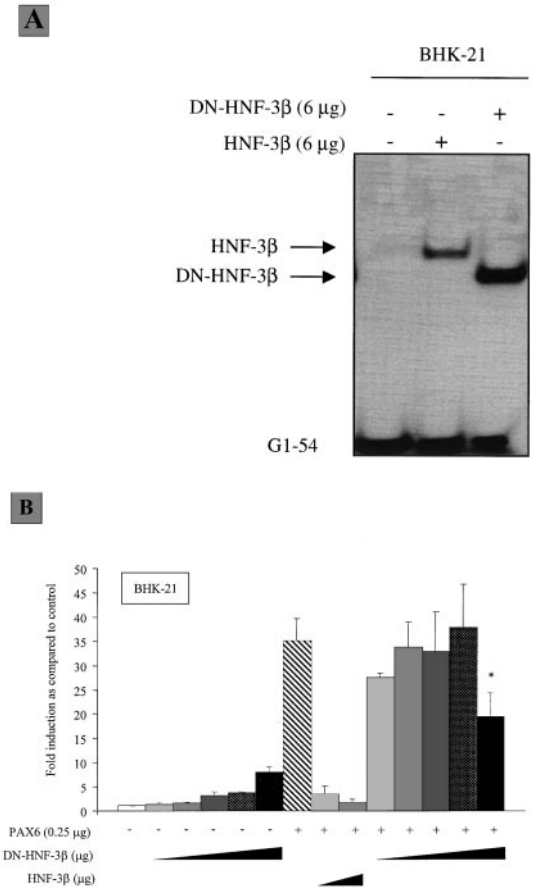

Fig. 10. DN-HNF-3β Is Unable to Inhibit Pax6-Mediated Transactivation of the −138Glu Reporter Construct

A, Labeled G1-54 oligonucleotide was mixed with 6 μg of nuclear extracts derived from BHK-21 cells overexpressing either HNF-3β or DN-HNF-3β. B, BHK-21 cells were cotransfected with the CAT reporter construct −138Glu along with 0.25 μg of Pax6 and/or increasing amounts of either HNF-3β (0.03 and 0.06 μg) or its dominant negative form (0.03, 0.06, 0.125, 0.25, and 0.5 μg) as indicated in the figure. Values are plotted as fold induction of the control experiment performed in the presence of empty pSG5 expression vector. *, Statistical significance as compared with the reporter construct in the presence of Pax6.

between these two sites (4). According to a current view of enhancer function, specific interactions between enhancer-binding proteins and factors that bind proximal promoter elements are important to achieve enhancer-promoter selectivity (36). HNF-3 may thus function as an accessory factor between the G1 and G2 elements to orchestrate enhanced transcription of the glucagon gene promoter in α-cells (37, 39). Interestingly, HNF-3-mediated induction of glucagon gene expression through the G1 element was much lower than that observed with either Pax6 or Cdx2/3. This binding site, which is located adjacent to the TATA box and thus in close proximity to the basal transcription machinery, may stimulate transcription of the glucagon gene by recruiting components of the basal transcription complex via HNF-3, Pax6, and Cdx2/3. However, it has been proposed that heterologous cells, such as BHK-21, are deprived of coactivator proteins that are required to interact with HNF-3 activation domains and allow proper stimulation of the basal transcription complex (40). Lower levels of glucagon gene promoter expression and the lack of synergism between G1 and G2 in the presence of HNF-3 may be partly explained by the absence of these coactivators in BHK-21 cells.

The concept that HNF-3 suppresses Pax6-mediated activation of the glucagon gene through the G1 and G3 elements of the promoter by physical protein interaction, rather than by competition for a common binding site, defines a novel function for this transcription factor. Pdx1 as been ascribed to physically interact with HNF-3 to up-regulate its own gene transcription. However, this interaction was shown to be dependent on DNA binding at two different promoter elements (41). Consistent with this inhibitory effect, we have previously demonstrated that a mutation abrogating binding of HNF-3, but not of Pax6, to G1 resulted in an increase in glucagon gene transcription in the pancreatic α-cell line InR1G9 (11). Interestingly, a recent study concluded that repression of glucagon gene transcription by insulin implicated the transcription factor Pax6 and a complex interaction between the proximal promoter elements G1 and G4 and the more distal enhancer-like elements G2 and G3 (42). It may thus be possible that the insulin-induced pathway utilizes HNF-3 as target protein to modulate Pax6 activity on glucagon gene transcription. In this regard, coincidence of insulin target sequences with HNF-3 binding sites has been reported for several genes such as PECK, IGF binding protein, tyrosine aminotransferase, and cholesterol 7α-hydroxylase (43–46). We have delineated the two transactivating domains of HNF-3 as the regions involved in conferring Pax6-mediated transactivation of the glucagon gene. Potentially, these regions may interact with either the paired or homeo DNA-binding domain of Pax6 to destabilize the interaction of this protein to the G1 or G3 element. GST pull-down experiments, performed in the absence of DNA, suggest that HNF-3-Pax6 protein interactions are probably DNA independent. Furthermore, heterodimers between Pax6 and HNF-3 were never observed in EMSA on either G1 or G3.

Full expression of the glucagon gene in pancreatic α-cells is dictated by the combinatorial effect of various transcription factors assembling on different *cis*-acting elements. We demonstrate that HNF-3α and -β bind independently to at least two DNA control elements, G1 and G2, and transactivate glucagon gene expression. Furthermore, HNF-3 (α and β) physically interacts with Pax6 to down-regulate glucagon gene transcription. HNF-3α or -β may mediate α-cell-specific expression by permitting access of both Pax6 and Cdx2/3 to a chromatin-free G1 element while conferring optimal expression by interacting with the enhancer-like G2 element. The significance of HNF-3

functional dichotomy on glucagon gene regulation remains to be further defined.

MATERIALS AND METHODS

Plasmid Sources and Construction

CAT reporter constructs harboring serial deletions or specific *cis*-acting elements of the glucagon gene promoter were described previously (4, 5, 11). cDNAs corresponding to *HNF-3*α and -β (kindly provided by Robert Costa, University of Illinois, Chicago, IL) were subcloned into the *Eco*RI site of pSG5 (Stratagene, Amsterdam, The Netherlands). Expression vectors harboring the hamster *Cdx-2/3* and quail *pax6* cDNAs were obtained from Michael S. German (University of California, San Francisco, CA) and Simon Saule (Institut Curie, Orsay Cedex, France) respectively. pEBOTd harboring the cDNA of DN-HNF-3β was kindly provided by Axel Kahn (INSERM, Paris, France). Plasmid G2–31GluCAT was constructed by ligation of a double strand oligonucleotide corresponding to the G2 *cis*-acting element of the glucagon gene promoter (5′-GATCCAGGCACAAGAGTAAATAAAAAGTTTCCGGGCCTCTGC-3′) (11) into the −31GLUCAT vector (47), which had been cut with *Bam*HI and blunt ended using the Klenow fragment of DNA polymerase I (Roche Diagnostics, Rotkreuz, Switzerland).

Cell Culturing

The Syrian baby hamster kidney BHK-21, the human hepatoma HepG2, the enteroendocrine GLUTag, and the glucagon-producing hamster InR1G9 (48) cell lines were grown and maintained in RPMI 1640 (Seromed, Basel, Switzerland) supplemented with 5% FCS, 5% newborn calf serum (Life Technologies, Inc.; Basel, Switzerland), 100 U/ml penicillin (Seromed), 100 μg/ml streptomycin (Seromed), and 2 mM glutamine (Life Technologies, Inc.; Basel, Switzerland). Human pancreatic islets were isolated from whole pancreata obtained from multiorgan cadaveric donors (20–65 yr) as described previously (49).

Transient Transfection and CAT Assay

The BHK-21 cell line was transiently transfected using the calcium phosphate precipitation technique (50). Each 10-cm Petri dish received a precipitate containing 10 μg of *cat* gene reporter construct, 0.5 μg of pSV$_2$PAP (internal control), and variable amounts of expression vectors for the various transcription factors (all cloned into pSG5). The final amount of DNA in each transfection was maintained constant by adding the expression vector pSG5 without an insert. Cells were harvested 48 h after transfection in 250 mM Tris-HCl and disrupted by three consecutive freeze-thaw cycles. CAT and alkaline phosphatase (PAP) activities were determined as previously described (5). PAP activity was used to standardize for transfection efficiency. The CAT/PAP activity values presented for each set of experiments correspond to the mean and SD of at least three individual transfections performed in duplicate. The values calculated were normalized as fold induction of the control sample obtained from cells transfected with the empty pSG5 expression vector. InR1G9 cells were transfected in suspension by the diethylaminoethyl-dextran method as described previously (4).

Nuclear protein extracts enriched for Pax6, Cdx2/3, and HNF-3α and -β were obtained by transfecting BHK-21 cells with 10 μg of various transcription factor cDNAs. Cells were harvested 48 h after transfection, and nuclear protein extracts were prepared as described by Schreiber *et al.* (51).

EMSA

Oligonucleotides used in EMSA are described in Fig. 1A. Double-strand forms were radioactively labeled by filling in the ends using the Klenow fragment of DNA polymerase I in the presence of [α^{32}P]-dCTP and purified using the QIAquick nucleotide removal kit (QIAGEN AG, Basel, Switzerland). DNA binding assays were performed as described previously (52) using nuclear extracts prepared by the method of Schreiber *et al*. (51).

Western Blot Assay for HNF-3 Proteins

Cytoplasmic and nuclear fractions were isolated from InR1G9 and HepG2 cells according to the protocol of Schreiber *et al.* (51). Approximately 25 μg of each protein extract were resolved on a 10% SDS-polyacrylamide gel and transferred electrophoretically to polyvinylidene difluoride membranes. Immunoblotting was performed with polyclonal antibodies to HNF-3α and -β (1:5,000) (R. H. Costa, University of Illinois, Chicago, IL) and TFIIE-α (1:1,000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and goat antirabbit IgG antisera conjugated with horseradish peroxidase (1:7,500) (Amersham Pharmacia Biotech, Lagerstrasse, Switzerland). Immunoreactive products were detected on x-ray films using enhanced chemiluminescence (SuperSignal West Pico), as directed by the manufacturer (Pierce Chemical Co., Rockford, IL).

GST Pull-Down Assay

L-[^{35}S]methionine-labeled HNF-3α and -β polypeptides were produced using the TNT rabbit reticulocyte lysate-coupled transcription-translation system (Promega Corp., Madison, WI) according to the manufacturer's protocol. The labeled proteins were incubated with either GST or GST-Pax6, and the binding reactions were treated as outlined by Ritz-Laser *et al.* (12).

Statistical Analysis

Results are expressed as mean ± SE. Where indicated, the statistical significance of the differences between groups was estimated by *t* test. * and ** indicate statistical significance with $P < 0.05$ and $P < 0.01$, respectively.

Acknowledgments

We thank Robert Costa for providing antibodies to HNF-3α and -β.

Received July 19, 2001. Accepted September 7, 2001.

Address all correspondence and requests for reprints to: Benoit Gauthier, Ph.D., Division de Biochimie Clinique, Centre Médical Universitaire, 1211 Genève 4, Switzerland. E-mail: benoit.gauthier@medecine.unige.ch.

This work was supported by the Swiss National Fund, the Institute for Human Genetics and Biochemistry, the Berger Foundation, and the Carlos and Elsie de Reuters Foundation.

REFERENCES

1. Unger R, Orci L 1981 Glucagon and the A cell: physiology and pathophysiology. N Engl J Med 304:1518–1524
2. Philippe J 1991 Structure and pancreatic expression of the insulin and glucagon genes. Endocr Rev 12:252–271

3. Mojsov S, Heinrich G, Wilson I, Ravazzola M, Orci L, Habener J 1986 Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. J Biol Chem 261:11880–11889
4. Philippe J, Drucker D, Knepel W, Jepeal L, Misulovin Z, Habener J 1988 α-Cell-specific expression of the glucagon gene is conferred to the glucagon promoter element by the interactions of DNA-binding proteins. Mol Cell Biol 8:4877–4888
5. Morel C, Cordier-Bussat M, Philippe J 1995 The upstream promoter element of the glucagon gene, G1, confers pancreatic α cell-specific expression. J Biol Chem 270:3046–3055
6. Andersen F, Heller R, Petersen H, Jensen J, Madsen O, Serup P 1999 Pax6 and Cdx2/3 form a functional complex on the rat glucagon gene promoter G1-element. FEBS Lett 445:306–310
7. Dumonteil E, Laser B, Constant I, Philippe J 1998 Differential regulation of the glucagon and insulin I gene promoters by the basic helix-loop-helix transcription factors E47 and BETA2. J Biol Chem 273:19945–19954
8. Hussain M, Habener J 1999 Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator. J Biol Chem 274: 28950–28957
9. Jin T, Trinh D, Wang F, Drucker D 1997 The caudal homeobox protein cdx-2/3 activates endogenous proglucagon gene expression in InR1–G9 islet cells. Mol Endocrinol 11:203–209
10. Laser B, Meda P, Constant I, Philippe J 1996 The caudal-related homeodomain protein Cdx-2/3 regulates glucagon gene expression in islet cells. J Biol Chem 271: 28984–28994
11. Philippe J, Morel C, Prezioso V 1994 Glucagon gene expression is negatively regulated by hepatocyte nuclear factor 3β. Mol Cell Biol 14:3514–3523
12. Ritz-Laser B, Estreicher A, Klages N, Saule S, Philippe J 1999 Pax-6 and Cdx-2/3 interact to activate glucagon gene expression on the G1 control element. J Biol Chem 274:4124–4132
13. Ritz-Laser B, Estreicher A, Gauthier B, Philippe J 2000 The paired-homeodomain transcription factor Pax-2 is expressed in the endocrine pancreas and transactivates the glucagon gene promoter. J Biol Chem 275: 32708–32715
14. Herzig S, Fuzesi L, Knepel W 2000 Heterodimeric pbx-prep1 homeodomain protein binding to the glucagon gene restricting transcription in a cell type-dependent manner. J Biol Chem 275:27989–27999
15. Drucker D, Philippe J, Jepeal L, Habener J 1987 cis-Acting DNA sequence controls glucagon gene expression in pancreatic islet cells. Trans Assoc Am Physicians 100:109–115
16. Drucker D, Wang M 1995 The LIM domain homeobox gene isl-1 is a positive regulator of islet cell-specific proglucagon gene transcription. J Biol Chem 270: 12646–12652
17. Ang S, Wierda A, Wong D, Stevens K, Cascio S, Rossant J, Zaret K 1993 The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development 119:1301–1315
18. Ang S, Rossant J 1994 HNF-3β is essential for node and notochord formation in mouse development. Cell 78: 561–574
19. Duncan S, Navas M, Dufort D, Rossant J, Stoffel M 1998 Regulation of a transcription factor network required for differentiation and metabolism. Science 281:692–695
20. Levinson-Dushnik M, Benvenisty N 1997 Involvement of hepatocyte nuclear factor 3 in endoderm differentiation of embryonic stem cells. Mol Cell Biol 17:3817–3822
21. Sasaki H, Hogan B 1994 HNF-3β as a regulator of floor plate development. Cell 76:103–115
22. Zaret K 1999 Developmental competence of the gut endoderm: genetic potentiation by GATA and HNF3/fork head proteins. Dev Biol 209:1–10
23. Philippe J 1995 Hepatocyte-nuclear factor 3 β gene transcripts generate protein isoforms with different transactivation properties on the glucagon gene. Mol Endocrinol 9:368–374
24. Lai E, Clark K, Burley S, Darnell J 1993 Hepatocyte nuclear factor 3/forkhead or 'winged helix' proteins: a family of transcription factors of diverse biologic function. Proc Natl Acad Sci USA 90:10421–10423
25. Drucker D, Jin T, Asa S, Young T, Brubaker P 1994 Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line. Mol Endocrinol 8:1646–1655
26. Kaestner K, Katz J, Liu Y, Drucker D, Schutz G 1999 Inactivation of the winged helix transcription factor HNF3α affects glucose homeostasis and islet glucagon gene expression *in vivo*. Genes Dev 13:495–504
27. Shih D, Navas M, Kuwajima S, Duncan S, Stoffel M 1999 Impaired glucose homeostasis and neonatal mortality in hepatocyte nuclear factor 3α-deficient mice. Proc Natl Acad Sci USA 96:10152–10157
28. Vallet V, Antoine B, Chafey P, Vandewalle A, Kahn A 1995 Overproduction of a truncated hepatic nuclear factor 3 protein inhibits expression of liver-specific genes in hepatoma cells. Mol Cell Biol 15:5453–5460
29. Overdier D, Porcella A, Costa R 1994 The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino-acid residues adjacent to the recognition helix. Mol Cell Biol 14:2755–2766
30. Cirillo L, McPherson C, Bossard P, Stevens K, Cherian S, Shim E, Clark K, Burley S, Zaret K 1998 Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. EMBO J 17:244–254
31. Shim E, Woodcock C, Zaret K 1998 Nucleosome positioning by the winged helix transcription factor HNF3. Genes Dev 12:5–10
32. Crowe A, Sang L, Li K, Lee K, Spear B, Barton M 1999 Hepatocyte nuclear factor 3 relieves chromatin-mediated repression of the α-fetoprotein gene. J Biol Chem 274: 25113–25120
33. Robyr D, Gegonne A, Wolffe A, Wahli W 2000 Determinants of vitellogenin B1 promoter architecture. HNF3 and estrogen responsive transcription within chromatin. J Biol Chem 275:28291–28300
34. Sund N, Ang S, Sackett S, Shen W, Daigle N, Magnuson M, Kaestner, K 2000 Hepatocyte nuclear factor 3β (Foxa2) is dispensable for maintaining the differentiated state of the adult hepatocyte. Mol Cell Biol 20:5175–5183
35. Hussain M, Lee J, Miller C, Habener J 1997 POU domain transcription factor brain 4 confers pancreatic α-cell-specific expression of the proglucagon gene through interaction with a novel proximal promoter G1 element. Mol Cell Biol 17:7186–7194
36. Blackwood E, Kadonaga J 1998 Going the distance: a current view of enhancer action. Science 281:60–63
37. Braun H, Suske G 1998 Combinatorial action of HNF3 and Sp family transcription factors in the activation of the rabbit uteroglobin/CC10 promoter. J Biol Chem 273: 9821–9828
38. Nitsch D, Schutz G 1993 The distal enhancer implicated in the developmental regulation of the tyrosine aminotransferase gene is bound by liver-specific and ubiquitous factors. Mol Cell Biol 13:4494–4504
39. Wang J, Stromstedt P, Sugiyama T, Granner D 1999 The phosphoenolpyruvate carboxykinase gene glucocorticoid response unit: identification of the functional domains of accessory factors HNF3 β (hepatic nuclear factor-3 β) and HNF4 and the necessity of proper alignment of their cognate binding sites. Mol Endocrinol 13: 604–618

40. Lai E, Prezioso V, Smith E, Litvin O, Costa R, Darnell J 1990 HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev 4:1427–1436
41. Marshak S, Benshushan E, Shoshkes M, Havin L, Cerasi E, Melloul D 2000 Functional conservation of regulatory elements in the pdx-1 gene: PDX-1 and hepatocyte nuclear factor 3β transcription factors mediate β-cell-specific expression. Mol Cell Biol 20:7583–7590
42. Grzeskowiak R, Amin J, Oetjen E, Knepel W 2000 Insulin responsiveness of the glucagon gene conferred by interactions between proximal promoter and more distal enhancer-like elements involving the paired-domain transcription factor Pax6. J Biol Chem 275:30037–30045
43. De Fabiani E, Crestani M, Marrapodi M, Pinelli A, Golfieri V, Galli G 2000 Identification and characterization of cis-acting elements conferring insulin responsiveness on hamster cholesterol 7α-hydroxylase gene promoter. Biochem J 347:147–154
44. Nitsch D, Boshart M, Schutz G 1993 Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. Proc Natl Acad Sci USA 90:5479–5483
45. O'Brien R, Noisin E, Suwanichkul A, Yamasaki T, Lucas P, Wang J, Powell D, Granner D 1995 Hepatic nuclear factor 3- and hormone-regulated expression of the phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein 1 genes. Mol Cell Biol 15:1747–1758
46. Unterman T, Fareeduddin A, Harris M, Goswami R, Porcella A, Costa R, Lacson R 1994 Hepatocyte nuclear factor-3 (HNF-3) binds to the insulin response sequence in the IGF binding protein-1 (IGFBP-1) promoter and enhances promoter function. Biochem Biophys Res Commun 203:1835–1841
47. Philippe J, Morel C, Cordier-Bussat M 1995 Islet-specific proteins interact with the insulin-response element of the glucagon gene. J Biol Chem 270:3039–3045
48. Takaki R, Ono J, Nakamura M, Yokogawa Y, Kumae S, Hiraoka T, Yamaguchi K, Hamaguchi K, Uchida S 1986 Isolation of glucagon-secreting cell lines by cloning insulinoma cells. In Vitro Cell Dev Biol 22:120–126
49. Oberholzer J, Triponez F, Mage R, Andereggen E, Buhler L, Cretin N, Fournier B, Goumaz, C, Lou J, Philippe J, Morel P 2000 Human islet transplantation: lessons from 13 autologous and 13 allogeneic transplantations. Transplantation 69:1115–1123
50. Graham F, Van der Eb A 1973 A new technique for the assay of infectivity of human adenovirus DNA. Virology 52:456–467
51. Schreiber E, Matthias P, Muller M, Schaffner W 1988 Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping bandshift assay (PCBA). EMBO J 7:4221–4229
52. Philippe J 1991 Insulin regulation of the glucagon gene is mediated by an insulin-responsive DNA element. Proc Natl Acad Sci USA 15:7224–7227