Neurogenin3 and Hepatic Nuclear Factor 1 Cooperate in Activating Pancreatic Expression of Pax4

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Stuart B. Smith‡, Rosa Gosál‡, Hirotaka Watada†, Juehu Wang, Steven C. Griffen‡, and Michael S. German***†‡

From the Diabetes Center and **Department of Medicine, University of California San Francisco, San Francisco, California 94143

During fetal development, paired/homeodomain transcription factor Pax4 controls the formation of the insulin-producing β cells and the somatostatin-producing δ cells in the islets of Langerhans in the pancreas. Targeting of Pax4 expression to the islet lineage in the fetal pancreas depends on a short sequence located ~2 kb upstream of the transcription initiation site of the Pax4 gene. This short sequence contains binding sites for homeodomain transcription factors PDX1 and hepatic nuclear factor (HNF)1α, nuclear receptor HNF4α, and basic helix-loop-helix factor Neurogenin3. In the current study we demonstrate that the HNF1α and Neurogenin3 binding sites are critical for activity of the region through synergy between the two proteins. Synergy involves a physical interaction between the factors and requires the activation domains of both factors. Furthermore, exogenous expression of Neurogenin3 is sufficient to induce expression of the endogenous Pax4 gene in the mouse pancreatic ductal cell line mPAC, which already expresses HNF1α, whereas expression of both Neurogenin3 and HNF1α are necessary to activate the Pax4 gene in the fibroblast cell line NHE13T3. These data demonstrate how Neurogenin3 and HNF1α activate the Pax4 gene during the cascade of gene expression events that control pancreatic endocrine cell development.

The mammalian endocrine pancreas consists of four different cell types, α, β, δ, and PP cells, expressing glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. During fetal development, the process that guides pancreatic precursor cells to form each of the distinct endocrine cell types involves the sequential expression of a number of pancreatic transcription factors, including Nkx2.2, Nkx6.1, Pdx1, NeuroD1, Neurogenin3, Pax4, and Pax6 (for review see Refs. 1 and 2). Genetic studies in mice have begun to outline a hierarchy of transcription factors that dictate the differentiation of the endocrine lineages (3–10).

Basic helix-loop-helix factor Neurogenin3 has been shown to control the differentiation of all pancreatic endocrine cell types. Neurogenin3 is expressed early in endocrine progenitor cells in the fetal pancreas but not in mature endocrine cells (11–13). Targeted disruption of the neurogenin3 gene in mice results in the total agenesis of endocrine cells (10), and transgenic over-expression of Neurogenin3 using the pax4 promoter drives the precocious differentiation of a large population of endocrine cells that are largely α cells (11, 14). The preponderance of α cells suggests that additional factors are important for diverting endocrine precursor cells from the α cell fate to the alternate endocrine lineages.

One such factor may be the paired homeodomain transcription factor, Pax4. Mice homozygous for a targeted disruption of the pax4 gene develop α cells but few β or δ cells, demonstrating its role in endocrine cell type differentiation (7). Like Neurogenin3, Pax4 expression in the pancreas crests during the period of peak β-cell differentiation in the fetus and is absent from mature islets, demonstrating that it functions only during the development of the endocrine cells (15).

Investigation of the mechanisms that control Pax4 expression has provided insight into the factors that lie upstream of Pax4 in the pathway for differentiation of insulin-producing β cells. A previous study of the human Pax4 gene promoter identified a 50-bp region (that is highly conserved in the mouse promoter) (16) located between ~1910 and ~1960 bp relative to the transcription start site that is critical for promoter activity (16–18). Removal of this region ablated promoter activity in transgenic mice, and two copies of the region activated the heterologous HSV-TK promoter 12-fold in β cells (18). The 50-bp sequence contains several transcription factor binding motifs, including an E box capable of binding bHLH family members such as Neurogenin3 along with sites capable of binding factors including hepatic nuclear factor (HNF)1α, HNF4α, and Pdx1.

In the current study we investigated the role of the human Pax4 promoter binding factors in controlling Pax4 gene expression. Mutation of the binding sites in the promoter demonstrated that the most influential promoter elements are the HNF1α binding site and the E box. In co-transfection experiments, a combination of HNF1α and Neurogenin3 strongly

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†‡ Recipient of a Juvenile Diabetes Research Foundation postdoctoral fellowship.

¶ Recipient of a Juvenile Diabetes Research Foundation International postdoctoral fellowship and Juvenile Diabetes Research Foundation International advanced postdoctoral fellowship. Present address: Dept. of Medicine, Metabolism, and Endocrinology, School of Medicine, Juntendo University School of Medicine 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.

†† Present Address: Dept. of Medicine, University of California Davis, Sacramento, CA 95817.

‡‡ To whom correspondence should be addressed: Diabetes Center, University of California San Francisco, 513 Parnassus Ave., Rm. HSW1090, San Francisco, CA 94143-0534. Tel.: 415-476-9262; Fax: 415-731-3612; E-mail: mgerman@biochem.ucsf.edu.

The abbreviations used are: HNF, hepatic nuclear factor; GST, glutathione S-transferase; CMV, cytomegalovirus; HSV-TK, herpes simplex virus thymidine kinase.
activates the promoter, but neither factor can activate alone. The activation domains of both factors are necessary for this synergy, and the two proteins physically interact. Neurogenin3 alone is sufficient to induce the endogenous mouse Pax4 gene expression in a mouse pancreatic ductal cell line, which may be attributed to endogenous expression of HNF1α in these cells. Remarkably, the combination of Neurogenin3 and HNF1α can stimulate transcription of the endogenous Pax4 gene in the non-pancreatic mouse fibroblast cell line NIH3T3.

MATERIALS AND METHODS

Cell Culture and Transfection—βTC-3 and a TC1.6 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2.5% fetal bovine serum and 15% horse serum. NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were split into 6-well plates 24 h prior to transfection with 1 × 10⁶ cells/well for a TC1.6 and βTC-3 cells and 5 × 10⁵ cells/well for NIH3T3 cells. 2 µg of reporter construct was used per well; 20 µg of any co-transfected transcription factor cDNA was used per well. The cationic lipid TransFast (Promega) was used for all transfections according to the manufacturer’s instructions. Cells were harvested 48 h after transfection, and luciferase assays were performed with the Promega luciferase assay system according to the manufacturer’s instructions. Luciferase activity was corrected for protein concentration.

Construction and Purification of Recombinant Adenoviruses—Mouse neurogenin3 cDNA was cloned into the pShuttle vector and a recombinant adenovirus was constructed using the Adeno-X™ expression system (Clontech). A control adenovirus containing the bacterial β-galactosidase gene was a generous gift from Dr. C. Newgard (Duke University, Durham, NC). Adenoviruses were produced, purified, and desalted through a Sephadex G-25 column as described previously (19, 20).

Viral Infection—mPAC (clones L20 and L452) and NIH3T3 cells were seeded in 6-well plates at a density of 5 × 10⁵ (mPAC) or 5 × 10⁴ (NIH3T3) cells/well and treated the next day at a multiplicity of infection of 10 viral particles/cell with AdCMV-Bgal or AdCMV-Neurogenin3 at 37°C for 2 h. Cells were then washed with phosphate-buffered saline and cultured in fresh medium for 48 h prior to harvesting. Adenovirus-assisted transfection of NIH3T3 cells was carried out as previously described (21). Briefly, plasmid DNA (4 µg) was mixed with 4 µg of 25-kDa polyethylenimine (PEI; Aldrich) in Opti-MEM 1 serum-free medium and cultured in fresh medium for 48 h prior to harvesting. Adenovirus-assisted transfection of NIH3T3 cells was carried out as previously described (21). Briefly, plasmid DNA (4 µg) was mixed with 4 µg of 25-kDa polyethylenimine (PEI; Aldrich) in Opti-MEM 1 serum-free medium and cultured in fresh medium for 48 h prior to harvesting. Adenovirus-assisted transfection of NIH3T3 cells was carried out as previously described (21).

RT-PCR—48 h after virus infection, cells were collected and total RNA was isolated using the RNeasy kit (Qiagen). RNA samples were treated with DNase to remove contamination of genomic DNA. First-strand cDNA was prepared using 3 µg of total RNA, using the Superscript II RT kit and random hexamer primers (Invitrogen) in a total volume of 20 µl according to the manufacturer’s instructions. 1 µl of the cDNA product was used as a template for PCR reactions under the following conditions: an initial incubation at 95°C for 3 min, followed by 25 cycles (Neurogenin3/β-actin) or 35 cycles (HNF1a/Pax4) of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Primer sets were as follows: mouse Pax4 forward GTTGTGCGTATCCCGAAGCC; neurogenin3 forward CGCACCAGCGC-
CTATCTCCAAC, reverse CCACATATGAGGAGGAAGCCAC, TaqMan activities were determined (Fig. 1). Mutations in either the E box or infected into factors (E-box sequence). The resulting constructs were trans-

Nonidet P-40). Samples were then incubated for 1 h at 4°C with gentle rocking, and the beads were then washed three times with interaction buffer. The bound proteins were eluted with 25 µl of Laemmli buffer. 15-µl aliquots of the eluted proteins were fractionated on SDS-polyacrylamide gels, dried by heat and vacuumed and autoradiographed.

Plasmid Construction—Deletion mutants of Neurogenin3 and HNF1α were generated by PCR and cloned into BglII and BamHI sites of the eukaryotic expression vector pBat12 (15) or pCDNA3 (Invitrogen), both of which express the inserted cDNA under the control of the CMV promoter.

RESULTS

Mutational Analysis of the PAX4 Promoter—A previous study demonstrated the importance of a 50-bp region within the pax4 promoter for transcriptional activity of the entire promoter, both in transgenic animals and in pancreatic cell lines (18). Furthermore, two tandem copies of the region increased activity of a heterologous promoter 12-fold in the pancreatic β-cell line βTC3 but not in the NIH3T3 fibroblast line, thus exhibiting the same cell specificity as the native promoter. We set out to determine which transcription factor binding motifs within this region were most critical for promoter activity by introducing mutations into the promoter sequences previously shown to bind HNF4α, HNF1α, and pancreatic bHLH factors (E-box sequence). The resulting constructs were transfected into βTC3 cells, and their relative transcriptional activities were determined (Fig. 1). Mutations in either the E box or the HNF1α binding motif ablated the activity of the 50-bp minienhancer when linked to the heterologous HSV-TK promoter, whereas a mutation in the HNF4α site resulted in a less dramatic reduction in promoter activity of ~50% (Fig. 1). All the constructs tested showed no significant activity above background in either the pancreatic α-cell line αTC1.6 or NIH3T3 cells (data not shown).

Cooperativity between HNF1α and Neurogenin3—To compare the relative importance of the pancreatic transcription factors that bind to the critical elements within the minienhancer, NIH3T3 cells were co-transfected with the minienhancer HSV-TK reporter construct and CMV promoter-driven expression plasmids containing cDNAs encoding the transcription factors (Fig. 2). Because the previous study demonstrated that no individual transcription factor was sufficient to activate the heterologous promoter in NIH3T3 cells (18), we transfected the wild type PAX4 minienhancer luciferase reporter construct.

Plasmids containing the indicated transcription factor cDNAs ligated downstream of the CMV promoter were co-transfected with the reporter construct. Luciferase activities of all samples were determined 48 h after transfection and are expressed relative to the activity in cells transfected with the reporter construct and the empty CMV expression plasmid (pBAT12). Results are expressed as the mean ± S.E. of data from experiments performed in triplicate on at least three separate occasions. HNF1αDN encodes a dominant negative mutant of HNF1α.

b, RT-PCR was performed with primers for HNF1α with RNA isolated from the indicated cell lines. β-actin was amplified from each sample to demonstrate the relative integrity of the RNA.

Fig. 3. Synergistic activation of the PAX4 minienhancer in different cell lines. a, three different cell lines, the fibroblast line NIH3T3 and the two in the heterologous promoter in NIH3T3 and the two inlet lines a, αTC1.6 and βTC3, were transfected with the wild type PAX4 minienhancer luciferase reporter construct. Plasmids containing the indicated transcription factor cDNAs ligated downstream of the CMV promoter were co-transfected with the reporter construct. Luciferase activities of all samples were determined 48 h after transfection and are expressed relative to the activity in cells transfected with the reporter construct and the empty CMV expression plasmid (pBAT12). Results are expressed as the mean ± S.E. of data from experiments performed in triplicate on at least three separate occasions. HNF1αDN encodes a dominant negative mutant of HNF1α.

Pancreatic Expression of Pax4

NIH3T3 αTC1.6 βTC3

β-actin

HNF1α

HNF1αDN

αTC1.6

βTC3

NIH3T3

a, three different cell lines, the fibroblast line NIH3T3 and the two in the heterologous promoter in NIH3T3 and the two inlet lines a, αTC1.6 and βTC3, were transfected with the wild type PAX4 minienhancer luciferase reporter construct. Plasmids containing the indicated transcription factor cDNAs ligated downstream of the CMV promoter were co-transfected with the reporter construct. Luciferase activities of all samples were determined 48 h after transfection and are expressed relative to the activity in cells transfected with the reporter construct and the empty CMV expression plasmid (pBAT12). Results are expressed as the mean ± S.E. of data from experiments performed in triplicate on at least three separate occasions. HNF1αDN encodes a dominant negative mutant of HNF1α.

b, RT-PCR was performed with primers for HNF1α with RNA isolated from the indicated cell lines. β-actin was amplified from each sample to demonstrate the relative integrity of the RNA.
PAX4 minienhancer −1.5-fold; the combination of both factors only activated to a level marginally greater than the sum of the activation produced by each factor alone (Fig. 3a).

In βTC3 cells, expression of Neurogenin3/E47 alone was sufficient to induce activation of the minienhancer (−7-fold), but the addition of HNF1α produced no further activation (Fig. 3a). We hypothesized that the marked effect of Neurogenin3/E47 alone might be due to cooperation with endogenous HNF1α, which is present in α TC1.6 and βTC3 cells but not in 3T3 cells (Fig. 3b). To test this hypothesis, we co-transfected a plasmid expressing a dominant negative form of HNF1α (T547E548fsdelTG/ter) (23) along with the Neurogenin3/E47 expression plasmids into βTC3 cells. Addition of the plasmid expressing the dominant negative HNF1α mutant eliminated the stimulatory effect of Neurogenin3/E47.

Synergy Requires Activation Domains of Both Factors—To map regions of HNF1α and Neurogenin3 required for their synergistic interaction, cDNAs encoding truncated variants of both proteins were engineered and co-transfected as shown into NIH3T3 cells along with the minienhancer reporter construct (Fig. 4a). The carboxyl-terminal proximal region of Neurogenin3, which contains a transcriptional activation domain, is required for synergy, whereas the amino-terminal region is not. HNF1α contains three separate activation domains, all of which are required for synergy.
which are located on the carboxyl-terminal side of the basic helix-loop-helix motif (24). Deletion of activation domain II (construct Δ 288–317) appears to have had no effect on synergy (Fig. 4b), but the removal of activation domains I (construct 1–485) or III (construct Δ 288–496) diminished the ability of HNF1α to synergize with wild type Neurogenin3/E47. In addition, the HNF1 dimerization domain, amino acids 1–33, which is required for forming HNF1-HNF1 dimers, is also important for producing full synergistic activation.

To determine whether the synergy between Neurogenin3/E47 and HNF1α is associated with a physical interaction between the two factors, we tested the ability of the two proteins to interact in vitro. A chimeric protein consisting of the bHLH domain and carboxyl terminus of Neurogenin3 fused to GST was mixed with 35S-labeled HNF1α. The GST-Neurogenin3 fusion protein was able to bring down the labeled HNF1α protein, whereas the GST protein alone could not, demonstrating that the two transcription factors can physically interact in vitro.

Synergistic Activation of the Endogenous pax4 Gene—The human PAX4 minienhancer experiments demonstrate that Neurogenin3/E47 and HNF1α can synergize in an artificial system, but we next asked whether such an interaction could activate the endogenous mouse pax4 gene. Mouse pancreatic ductal cell lines (mPAC L4S2 and mPAC L20) and NIH3T3 cells were infected with an adenovirus expressing Neurogenin3 under the control of the CMV promoter. In both mPAC cell lines, which already express high levels of HNF1α, Neurogenin3 expression alone was sufficient to induce transcription of the endogenous pax4 gene as gauged by RT-PCR (Fig. 5a). In NIH3T3 cells, however, HNF1α was needed in addition to Neurogenin3 to significantly induce expression of the native pax4 gene (Fig. 5b).

**DISCUSSION**

The current study demonstrates that the function of the critical −1910-bp element of the PAX4 gene promoter depends on two sites, a bHLH binding site (E box) and an HNF1α binding site. The pancreatic transcription factors Neurogenin3 (along with its heterodimeric partner E47) and HNF1 bind to these sites, physically interact, and synergistically activate both the promoter and the endogenous pax4 gene. As has been observed for many other transcription factor interactions (25–29), we demonstrated a physical interaction between the two proteins and showed that the activation domains of both proteins were required for synergy. Therefore, cooperativity between HNF1α and Neurogenin3 may result from the cooperative recruitment of transcriptional co-activators to the transcription complex on the pax4 gene.

Neurogenin3 is not the only pancreatic bHLH factor that can dimerize with E47 and bind the PAX4 promoter E box,2 and it seems possible that others, especially neuroD1, could substitute for Neurogenin3 on the PAX4 promoter in vivo. Two observations suggest that Neurogenin3 is the most important bHLH activator of the PAX4 promoter. First, Neurogenin3 and Pax4 have almost identical expression patterns during pancreatic development, with maximum expression between embryonic days 13 and 15 and no expression in mature islets (15). Second, in conjunction with heterodimeric partner E47, Neurogenin3 exhibits a higher affinity for the PAX4 promoter E box than for E boxes from other pancreatic promoters.3 Finally, the current study demonstrates that the Neurogenin3/E47 heterodimer is a better activator of the PAX4 minienhancer than any of the other bHLH dimers tested.

Similarly, HNF1β could possibly substitute for HNF1α. HNF1α and HNF1β are related proteins sharing 93% sequence identity in their DNA binding domains, 75% identity in their dimerization domains, and 47% identity in their carboxyl-terminal activation domains (30). HNF1α and HNF1β can form homodimers or heterodimers with each other, producing complexes with very similar or identical DNA binding characteristics. Both factors are found in the developing pancreas when Pax4 is present, and both factors can synergize with Neurogenin3 and activate the Pax4 promoter. Both factors are expressed broadly in the early pancreatic bud, although HNF1β may appear earlier (31, 32). Either or both may play a role in Pax4 expression.

The role of the HNF4α binding site adjacent to the HNF1 binding site in the PAX4 promoter remains uncertain. The wild type minienhancer is maximally stimulated by the combination of Neurogenin3/E47 and HNF1α, with no further activation produced from the addition of HNF4α 1 or HNF4α 2 (data not shown). Mutation of the HNF4α site reduced the activity of the

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2 S. Smith, H. Watada, and M. German, unpublished data.
3 S. Smith, H. Watada, and M. German, submitted for publication.

![Figure 5. Synergistic activation of the endogenous pax4 gene.](image)
minienhancer, but the same mutated minienhancer could still be potently activated by a combination of Neurogenin3 and HNF1α (data not shown). Furthermore, Neurogenin3 and HNF1α alone were sufficient to induce expression of the endogenous pax4 gene in NIH3T3 cells (which have no endogenous HNF4α), although the level of expression is far below the level seen in βTC3 cells. It is possible that the absence of an appropriate ligand or other interacting proteins normally present in vivo could reduce the activity of HNF4α in these assays. We cannot rule out a role for HNF4α in pax4 gene expression.

The pancreatic transcription factor PDX1 can also bind to the HNF1 site in the PAX4 promoter. We found no circum-
mstances, however, in which PDX1 could activate the PAX4 promoter by itself or in combination with other factors. In view of the fact that PDX1 is not co-expressed with Neurogenin3 in islet progenitor cells (11), some of which also co-express PAX4 (18), it seems unlikely that PDX1 plays a direct role in PAX4 expression during normal development.

These data strongly support a model in which HNF1 proteins and Neurogenin3 cooperate in activating PAX4 expression and β-cell differentiation in the developing pancreas and place these factors upstream of PAX4 on an increasingly detailed map of the hierarchy of factors that control islet cell determination and differentiation. Ultimately, our understanding of this pathway may lead to the development of methods for driving progenitor cells to differentiate into pancreatic islet cells for patients with diabetes. The ability of this cascade to function in cells as disparate as pancreatic duct cells and NIH3T3 fibroblasts provides encouraging evidence that this approach to islet cell production may eventually succeed.

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