Transcriptional and Translational Regulation of β-Cell Differentiation Factor Nkx6.1*

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In the mature pancreas, the homeodomain transcription factor Nkx6.1 is uniquely restricted to β-cells. Nkx6.1 also is expressed in developing β-cells and plays an essential role in their differentiation. Among cell lines, both β- and α-cell lines express nkx6.1 mRNA; but no protein can be detected in the α-cell lines, suggesting that post-transcriptional regulation contributes to the restriction of Nkx6.1 to β-cells. To investigate the regulator of Nkx6.1 expression, we outlined the structure of the mouse nkx6.1 gene, and we identified regions that direct cell type-specific expression. The nkx6.1 gene has a long 5'-untranslated region (5'-UTR) downstream of a cluster of transcription start sites. The nkx6.1 gene sequences from −5.6 to +1.0 kilobase pairs have specific promoter activity in β-cell lines but not in NIH3T3 cells. This activity is dependent on sequences located at about −800 base pairs and on the 5'-UTR. Electrophoretic mobility shift assays demonstrate that homeodomain transcription factors PDX1 and Nkx2.2 can bind to the sequence element located at −800 base pairs. In addition, dicistronic assays establish that the 5'-UTR region functions as a potent internal ribosomal entry site, providing cell type-specific regulation of translation. These data demonstrate that complex regulation of both Nkx6.1 transcription and translation provides the specificity of expression required during pancreas development.

The development and differentiation of organs like the pancreas require the coordinate activation of unique sets of transcription factors (1, 2). Genetic studies in mice have recently revealed the critical role of several pancreatic transcription factors in the differentiation of the insulin-producing β-cells during pancreatic development (3–12).

Among the known pancreatic transcription factors, the homeodomian factor Nkx6.1 is unique in its absolute restriction in the mature pancreas to the β-cells. In the developing fetus, however, Nkx6.1 is initially expressed in almost all the epithelial cells of the pancreatic buds. Starting around embryonic day 13 (E13), Nkx6.1 expression becomes restricted to β-cells and β-cell precursors (13). Targeted disruption of the nkx6.1 gene causes a severe defect in β-cell differentiation in mice. The nkx6.1 null mutants have normal numbers of insulin-expressing cells through E12.5, but new β-cell formation is blocked after E12.5. Hence, Nkx6.1 is a necessary component of the signals triggering the major wave of β-cell differentiation and proliferation after E12.5. Nkx6.1 functions at one step in the hierarchy of transcription factors controlling pancreatic development and differentiation. Although Nkx6.1 represses the transcription of target genes (14), its downstream genetic targets have not been identified. Upstream of Nkx6.1, two homedomain transcription factors are known to control its pancreatic expression, Nkx2.2 in the fetal pancreas after E12.5 and PDX1 in adult β-cells (9, 15). However, the mechanisms by which these factors control Nkx6.1 expression and the potential roles of other factors are unknown.

Control of cell type-specific gene expression frequently operates at the level of gene transcription, but post-transcriptional mechanisms including controls at the level of translation initiation may play essential roles as well. Generally, cap-dependent ribosomal scanning identifies translation start sites and initiates translation on the majority of cellular mRNAs. This process is severely hampered on long 5'-untranslated regions (5'-UTR) containing multiple upstream reading frames and secondary structure (16). Translation of such mRNAs may initiate through a cap-independent mechanism utilizing an internal ribosomal entry site (IRES) in the 5'-UTR. Cellular mRNAs containing IRESs can be very specifically regulated, providing a post-transcriptional mechanism to control their expression (17, 18). By using this mechanism, the translation of a number of mammalian growth factor RNAs is specifically regulated during differentiation or cell growth (19–22). Furthermore, during Drosophila embryonic development, the 5'-UTRs of mRNAs encoding homeodomain transcription factors Antp and Ubx are known to regulate protein expression in a spatiotemporal manner, although there are no reports of the existence of IRESs in any mammalian homeodomain transcription factor genes (23, 24).

To understand the mechanisms that regulate β-cell-specific expression of Nkx6.1, we outlined the structure of the mouse nkx6.1 gene and identified a promoter that directs cell type-

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2 The abbreviations used are: 5'-UTR, 5'-untranslated region; IRES, internal ribosomal entry site; DMEM, Dulbecco’s modified Eagle’s medium; RACE, rapid amplification of cDNA ends; TK, herpes simplex virus thymidine kinase; CMV, cytomegalovirus; kb, kilobase pairs; bp, base pairs; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; RSV, Rous sarcoma virus.

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specific expression in β-cells. A promoter element found approximately 800 bp upstream of the transcription initiation sites contains binding sites for Nkx6.2 and PDX1 and functions as an important transcriptional enhancer. In addition, a potent IRES in the 5′-UTR further restricts Nkx6.1 expression to β-cells. These findings establish that gene regulation through an IRES plays a similar role in development of the mammalian pancreatic islet as in Drosophila development.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfections**—βTC3 cells and αTC1.6 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2.5% fetal bovine serum and 15% horse serum. HIT-T15 M2.22 cells and INR1 cells were grown in DMEM/H16 supplemented with 10% fetal bovine serum. NIH3T3 cells were grown in DMEM supplemented with 10% calf serum. COS7 cells were grown in DMEM with 10% fetal bovine serum with 4 mM glutamine, and INS-1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum with 50 μg 2-mercaptoethanol, 1 mM pyruvic acid, 10 mM HEPES.

For transient mammalian cell transfections, βTC3 cells, αTC1.6 cells, and NIH3T3 cells were plated in 6-well tissue culture plates 24 h before transfection. For the standard reporter gene analysis, 1.8 μg each luciferase reporter plasmid and 0.2 μg of the CMVβ-Gal plasmid were co-transfected into the cells using Superfect® (Qiagen) under conditions recommended by the manufacturer. Forty eight hours after transfection, cells were harvested, and luciferase and β-galactosidase assays were performed as described previously (14). Luciferase activity was corrected for transfection efficiency by use of the co-transfected CMVβ-Gal plasmid. For evaluation of PDX1 and Nkx6.2 effects on the reporter gene constructs, 50 ng of expression vector (pBAT12-TPF1, pBAT12Nkx6.2, or expression vector without insert, pBAT12(14)) were co-transfected into NIH3T3 cells with 2.0 μg each luciferase reporter plasmid. Cells were harvested 48 h later and assayed for luciferase activity. All reporter gene analyses were performed on at least three occasions, and data are expressed as mean ± S.E.

**RNA Isolation and Northern Blot Analyses**—Total RNA from cell lines was isolated using TRIzol® (Life Technologies, Inc.) per the manufacturer’s instructions. Northern blots were performed by standard procedures using 10 μg of total RNA (25). A fragment of hamster nkx6.1 cDNA was used as a probe for Northern analysis and was prepared by digesting pBAT12-Nkx6.1 (26) with KpnI and NotI and labeling the liberated fragment with [32P]dCTP.

5′-Rapid Amplification of cDNA Ends (RACE)—The 5′ end of mouse nkx6.1 cDNA was identified by 5′-RACE, using a modification of the protocol described by the RACE-PCR system, version 2.0 (Life Technologies, Inc.). For mouse cDNA, 2.5 pmol of specific primer HW8 (5′-GGCG TTC GCT TTG TTG ATG TAG GA-3′) was annealed to 1 μg of total RNA from βTC3 cells. Reverse transcription was carried out using SuperScript II reverse transcriptase (Life Technologies, Inc.). After first strand cDNA synthesis, the original mRNA template was removed by treatment with RNase, and homopolymeric dCTP tails were then added to the 3′ end of the cDNA using terminal deoxynucleotidyltransferase. By using these products as a template, we carried out 35 cycles of PCR using the 5′-RACE Abridged Anchor Primer (Life Technologies, Inc.) and HW9 (5′-CCG CTG GGG TAG TTG CCA AG-3′) as primers. For the nested PCR, we used Abridged Universal Amplification Primer (Life Technologies, Inc.) and HW11 (5′-GGG GAT CCG CCT ATG TCG CTC GGA -3′) as primers, and we performed 35 cycles of PCR. The PCR products were subcloned into pBluescript KS(−) and sequenced.

**RNA Protection Assay**—The fragment from nucleotides −159 to nucleotide +100 of the nkx6.1 gene was amplified by PCR and subcloned into pBluescript KS(−). Labeled antisense RNA probe was generated using this fragment as a template. RNase protection assays were carried out using HybSpeed RPA kit (Ambion) per manufacturer’s protocol. Hybridization of the riboprobe to RNA was performed in a 10-ml reaction containing 8 × 10⁷ cpm of probe and 10 μg of total RNA from βTC3 cells and 40 μg of yeast tRNA. The control sample contained 50 μg of yeast tRNA alone.

**Cloning of the Mouse nkx6.1 Gene Promoter**—A λ DASH mouse genomic library was screened for the nkx6.1 gene using a mouse nkx6.1 partial cDNA probe corresponding to the coding region of exon1. The λDASH clone encoding the longest 5′ region of the nkx6.1 gene was subcloned into the EcoRI site of pBluescript KS(−). This plasmid contains an approximately 10-kb fragment of nkx6.1 gene (pBNkx6.1-10 kb). This clone was characterized by restriction enzyme analysis and sequencing.

**Reporter Gene Constructs and Assay**—To generate reporter plasmids, fragments of the 5′ region of the nkx6.1 gene (obtained either by restriction digestion or PCR) were ligated upstream of the luciferase gene in the plasmid pFOXLuc1 (14). Mutagenesis of the reporter gene constructs was performed using the Quick Change® mutagenesis kit (Stratagene). All constructs were confirmed by sequencing.

**In Vitro Transcription and Translation and Electrophoretic Mobility Shift Assay (EMSA)**—Nkx2.2 and PDX-1 proteins were produced in vitro using T7TNT Quick Coupled Lysate System® (Promega). Single-stranded wild-type oligonucleotides (5′-GATCTAGCCCCCTCATAAGTGATAAATGTGATC-3′) corresponding to the sequence between nucleotides −487 and −441 nucleotides, were 5′-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The labeled oligonucleotide was column-purified and annealed to an excess of complementary strand. EMSA buffers and electrophoresis conditions were as described previously (27). One μl of the in vitro reaction mixture was used for the EMSAs. The following oligonucleotides were used as competitors in EMSA reactions (top strands shown): M1, GATCTAGCCCCCTCATAAGTGATC-3′, and M2, GATCTAGCCCCCTCATAAGTGATC-3′. The ECL Plus® system (Amersham Pharmacia Biotech).

**Dicistronic Plasmids and Dicistronic Assay**—For generating the basic dicistronic construct, the RSV promoter region driving the CAT gene was inserted upstream of the luciferase gene in pFOXLuc1 to obtain pFOXRSV-CAT-Luc. The 5′-UTR of nkx6.1 was inserted bi-directionally into the region between the CAT and luciferase genes to obtain pFOXRSV-CAT-5′-UTR-Luc and pFOXRSV-CAT-5′-UTR-R-Luc. Next, by using PCR-based site-directed mutagenesis, the cloning sites and 5′-
UTR of the luciferase gene were removed. The constructs were confirmed by sequencing. These dicistronic reporter genes were co-transfected with pBluescript KS(+)
into mammalian cells, and 48 h after the transfection cells were harvested and assayed for luciferase and CAT activities, as described previously (28). Luciferase enzyme activity from each transfection was normalized to the activity of CAT and was used as an index of IRES activity.

RESULTS

Expression of Nkx6.1 in Cell Lines—To identify cell lines that express Nkx6.1, we performed Northern blot analysis with a hamster nksz.1 cDNA probe and Western blot analysis with antisem directed against the carboxyl-terminal end of the hamster Nkx6.1 protein. Total RNA and nuclear extracts for these assays were prepared from βTC3 cells and HIT-T15, from α-cell lines INR-1 and αTC1.6, and from non-pancreatic cell lines NIH3T3 and COS7. As shown in Fig. 1, nksz.1 mRNA is expressed in all four pancreatic islet cell lines but not in NIH3T3 cells. However, the Nkx6.1 protein can be detected only in βTC3 cells. Although the expression level of nksz.1 mRNA is higher in βTC3 cells than the other cell line, this difference alone cannot explain the greater difference in the expression of Nkx6.1 protein. These results suggest that the expression of Nkx6.1 is regulated both transcriptionally and post-transcriptionally.

Structure of the Mouse nksz.1 Gene—As an initial step toward characterizing the 5′ end of the nksz.1 gene, we cloned and sequenced exon 1 and the 5′-flanking region of the mouse nksz.1 gene. 5′-RACE performed on RNA from βTC3 cells identified a cluster of seven transcription start sites clustered at 1 kb upstream from the translation start site with no additional intervening introns (Fig. 2B). RNase protection analysis with βTC3 RNA confirmed the position of four of these start sites.

Deletion Analysis of the Mouse nksz.1 Promoter—When ligated upstream of the luciferase reporter gene and transfected into mammalian cell lines, a large fragment of the mouse nksz.1 gene including 5.6 kb of genomic DNA sequence upstream of the mouse nksz.1 coding region is shown. Seven transcription start sites identified by 5′-RACE is shown in boldface, and four transcription sites identified by RNase protection are labeled with asterisks. The translation start site is indicated by italics. Two potential CCAAT boxes and other promoter elements are indicated in underlined and boldface. Several proximal TAAT sequences are shown in boldface. The sequence of the mouse nksz.1 promoter region and Exon1 are available in the GenBank™ data base under accession number AF290166.
NIH3T3 (Fig. 3), demonstrating that this fragment of the gene contains sequences that are important for β-cell-specific expression. Deletion of the sequence between −5600 and −2570 bp increases luciferase activity modestly in both cell lines. This increase is lost, however, when equal molar quantities of the plasmid are used for the transfections (data not shown).

Further deletion of the region between −893 and −645 bp causes a significant decrease in promoter activity in βTC3 cells but not in NIH3T3 cells, implicating this region in β-cell-specific expression. Deletion of sequences within the proximal 334 bp of the promoter causes the progressive diminution of promoter activity in both cell types showing that this region is important for basal promoter activity, although the greater decrease in activity in β-cells suggests some degree of β-cell-specific function for these sequences.

To map more precisely the β-cell-specific enhancer sequences within the region between −893 and −645 bp, we generated a series of small deletions within this region. As shown in Fig. 4A, the sequences between −840 and −771 bp are necessary for this β-cell-specific activity. This sequence also can function weakly as a β-cell-specific enhancer when linked to a heterologous promoter (Fig. 4B). This sequence contains two potentially important binding sites for β-cell transcription factors, the Nkx2-2-binding site core sequence TAAGTG (30) and the PDX-1-binding site core sequence TAAT (31). Mutation of the Nkx2-2-binding site core sequence TAAGTG (30) and the PDX-1-binding site core sequence TAAT (31) causes a more modest decrease in promoter activity (Fig. 4moter activity, although mutation of the Nkx2-2-binding site core sequence TAAT (31). Mutation of the Nkx2-2-binding site core sequence TAAGTG (30) and the PDX-1-binding site core sequence TAAT (31). Mutation of the Nkx2-2-binding site core sequence TAAGTG (30) and the PDX-1-binding site core sequence TAAT (31).

To address whether PDX-1 and Nkx2-2 bind to these sites, we performed an EMSA using a double-stranded oligodeoxynucleotide corresponding to nucleotide −817 to −788 (B1) as a probe. In vitro translated Nkx2-2 and PDX-1 can bind to this site (Fig. 5A), and they are competed by the unlabeled oligonucleotide. An unlabeled oligonucleotide containing a mutation in the Nkx2-2-binding site core (mutant M2) can still compete for PDX-1 binding but not for Nkx2-2 binding. Interestingly, an unlabeled oligonucleotide containing a mutation in the PDX-1-binding core (mutant M1) cannot compete for either PDX-1 or Nkx2-2 binding.

When co-transfected into NIH3T3 cells, PDX-1 can activate the β-cell-specific enhancer linked to the rat prolactin promoter (Fig. 5B). Nkx2-2, however, cannot activate the mini-enhancer.
by itself or in combination with PDX-1 (Fig. 5B), although it can activate the intact *nkx6.1* promoter (data not shown). Neither factor affects the expression of luciferase from the parent vector containing the prolactin promoter alone. Recently Sepulveda *et al.* (32) demonstrated that the closely related cardiac homeodomain factor Nkx2.5 cooperates with GATA-4, a zinc finger transcription factor, to activate the α-actin promoter. Interestingly, for this interaction, a GATA4-binding site is not necessary. To test the possibility that Nkx2.2 also cooperates with GATA factors in pancreatic β-cells to activate the *nkx6.1* promoter, we co-transfected vectors expressing either GATA4 or GATA6 along with the Nkx2.2 expression vector and a reporter plasmid containing either the β-cell-specific mini-enhancer linked to the rat prolactin promoter or the intact *nkx6.1* promoter driving luciferase. However, neither GATA4 nor GATA6 produced any additional activation of the *nkx6.1* promoter or mini-enhancer (data not shown).

There are additional PDX-1 and Nkx2.2-binding sites in the *nkx6.1* promoter outside of the β-cell-specific enhancer region. There are multiple TAAT sequences that fit the PDX-1 binding consensus within the proximal 900 bp of the promoter (see boldface sequences in Fig. 2B) as well as several copies of the (C/T)AAG sequence that forms the core of the Nkx2.2-binding sequence. One of these sites, located at −460 bp, can function as a high affinity Nkx2.2-binding site (Fig. 5C).

It should be noted as well that there are other potential binding sites for β-cell transcription factors in the *nkx6.1* promoter, including two copies of the HNF6-binding site consensus sequence (33) (see Fig. 2B). The functional importance of these sites is difficult to ascertain since they fall within a region of the proximal promoter that is also important for expression in NIH3T3 cells.

**Complex Function of the *nkx6.1* Gene 5'-UTR**—Whereas its promoter plays a critical role in expression of the *nkx6.1* gene, Fig. 3 demonstrates that sequences within the 5'-UTR are at least as important. In βTC3 cells, the deletion of the 5'-UTR causes a nearly complete loss of luciferase expression from the *nkx6.1* promoter constructs. In NIH3T3 cells, however, removal of the 5'-UTR increases luciferase activity.

When moved from its normal position downstream of the transcription start site, the function of the 5'-UTR changes. As shown in Fig. 6, when positioned downstream of the *nkx6.1* or herpes simplex virus thymidine kinase (TK) minimal promoter, the 5'-UTR enhances the expression of luciferase in α- and β-cell lines but not in the NIH3T3 cell line. In contrast, the 5'-UTR produces no activity in α- and β-cells and significant repression in NIH3T3 cells when placed upstream of the *nkx6.1* or TK promoters.

These results demonstrate that the 5'-UTR can function as a position-independent repressor in non-islet cells, and as an activator in islet cells when located in its normal position downstream of the promoter. Cell type-specific function ap-

**Fig. 5.** PDX-1 and Nkx2.2 binding to the *nkx6.1* promoter. A, EMSA using *in vitro* translated Nkx2.2 and PDX-1 is shown. 32P-Labeled oligonucleotides encoding the B1 enhancer element (sequences are shown in Fig. 4B) were incubated with 1 μl of each *in vitro* translated protein for 15 min at room temperature and then subjected to electrophoresis on a 5% polyacrylamide gel. Unlabeled competitor oligonucleotides (sequences are shown in Fig. 4B) were added at 20-fold molar excess. B, a reporter plasmid containing five tandem copies of the B1 enhancer element upstream of the prolactin minimal promoter driving luciferase and pBAT12 expression plasmids expressing the Nkx2.2 and PDX-1 cDNAs under the control of the CMV promoter were co-transfected into NIH3T3 cells. Relative luciferase activities are calculated with the activity of cells transfected with the pBAT12 expression vector without cDNA insert set at 1. All data are shown as mean ± S.E. C, an EMSA using *in vitro* translated Nkx2.2 is shown. 32P-Labeled oligonucleotides encoding the B1 enhancer element (lanes 1–3) or the related sequence at −460 (lanes 4 and 5) in the *nkx6.1* promoter (see “Experimental Procedures” for sequence) were incubated with 1 μl of the *in vitro* translated protein for 15 min at room temperature and then subjected to electrophoresis on a 5% polyacrylamide gel. The control lanes (2 and 4) contain *in vitro* translated luciferase protein. N.S. indicates a nonspecific protein-DNA complex produced by proteins present in the rabbit reticulocyte lysate mix.
pears to be dependent on an intact 5′-UTR, since the 5′-UTR loses all specificity when cut in half (Fig. 6).

Identification of an IRES in the 5′-UTR—Several features of the 5′-UTR suggest that it may provide a poor template for protein synthesis after cap-dependent scanning: it is long (973 bp), G/C-rich (67.3%), and contains out of frame ATG codons with reasonable Kozac consensus sequences: These limitations could be overcome by an IRES. In addition, the presence of an IRES that functions in a cell type-specific manner could explain the functional characteristics of the 5′-UTR.

To test for this possibility, a dicistronic gene (pFoxRSV-CAT-Luc) was constructed placing the CAT gene and the luciferase gene in series under the control of RSV promoter. The 5′-UTR of the nkh6.1 was inserted between the two cistrons of this plasmid (pFOX-CAT-5′-UTR-Luc). In addition, the 5′-UTR was inserted in an inverted orientation (pFOX-CAT-5′-UTR-R-Luc) as a nonspecific control. These plasmids were transfected into two β-cell lines, βTC3 and INS1, the α-cell line αTC1.6, and two non-islet cell lines NIH3T3 and COS7. CAT and luciferase activity were assayed 48 h after transfection. The ratio of luciferase activity to CAT activity provides a gauge of IRES activity. All data are shown as mean ± S.E. The ratio of CAT/luciferase activity for the control dicistronic constructs without the 5′-UTR alone set at 1.

**Discussion**

In the present study, we have characterized the nkh6.1 promoter and mapped a region involved in its cell type-specific expression. In addition, we found that the expression of Nkh6.1 also is controlled at the post-transcriptional level, and an IRES in the 5′-UTR plays an important role in directing its expression to islet cells.

Like many transcription factor genes, the 5′-flanking region of the mouse nkh6.1 gene lacks a classic TATA box. The TATA box is typically located 30 bp upstream of the transcription initiation site and helps specify the transcription initiation site by directing the binding of TFIID. Characteristic of genes that lack TATA boxes, the nkh6.1 gene has multiple transcription initiation sites as mapped by 5′-RACE and RNase protection assay. Also characteristic of TATA-less genes, the transcription initiation sites lie just downstream of two CCAAT boxes, at least one of which is functional in both islet and non-islet cells.

Complex interactions among a number of transcription factors control the temporal expression of genes during the development of the pancreas. Tight control over the temporal and spatial expression of these factors is essential for proper development of the endocrine cells. Nkh6.1 is expressed in at least three different cell types during mouse pancreatic development as follows: initial broad expression in the epithelial cells that compose the dorsal and ventral buds, restricted expression after embryonic day 13 in islet cell precursors, and finally in mature β-cells. Studies of mice that lack an intact nkh2.2 gene demonstrate that Nkh2.2 is required for Nkh6.1 expression in the pancreas after E13; specific inactivation of the pdk-1 gene in insulin-expressing cells demonstrates that PDX-1 is required for maintaining Nkh6.1 expression in differentiated β-cells (9, 15). It can be concluded from these prior studies that Nkh6.1 expression is regulated directly or indirectly by these two factors. The promoter studies reported here support the conclusion that both factors drive Nkh6.1 expression directly, by binding to the nkh6.1 gene promoter.

Although there are several binding sites for Nkh2.2 and PDX-1 in the proximal Nkh6.1 promoter, only the sites located at −800 are required for expression specifically in the β-cell line. The more proximal sites may also contribute, but removal of more proximal sequences affects expression in NIH3T3 cells as well. The presence of binding sites for both factors in the β-cell-specific enhancer located at −800 bp is intriguing given the essential role of both factors in Nkh6.1 expression. Despite the juxtaposed binding sites, however, Nkh2.2 does not activate the β-cell-specific enhancer even in the presence of co-expressed PDX-1.

We recently found that Nkh2.2 by itself cannot activate transcription even from a construct with 7 tandem repeats of an ideal Nkh2.2-binding site. The NK2 domain just downstream of the homeodomain in Nkh2.2 inhibits the activation domain, and some modification of the NK2-specific domain may be required to allow Nkh2.2 to activate transcription (30). When Nkh2.2 is overexpressed as in the co-transfection experiments reported here, the non-islet cells may lack specific modifiers of...
the NK2 domain, or the capacity of the cells to modify the NK2 domain may be exceeded. Hence, we cannot rule out the possibility that Nkx2.2 cooperates with PDX1 in regulating the Nkx6.1 promoter in the normal cellular context.

In addition, a different array of factors may control Nkx6.1 expression at different points in development. The cells used for these experiments are probably more representative of mature β-cells than undifferentiated pancreatic epithelial cells or islet cell progenitors. During the differentiation of islet cells in the fetal mouse pancreas, the islet cell progenitors transiently express the basic helix-loop-helix transcription factor neurogenin3 prior to further maturation and expression of PDX-1 (34). Some of these early neurogenin3-expressing cells co-express Nkx6.1, but not PDX-1, demonstrating that cells at this stage in differentiation do not require PDX-1 for Nkx6.1 expression (34). Other homeodomain proteins capable of binding to the Nkx6.1 promoter in the normal cellular context.

In addition to controls at the level of the promoter, expression from the nkh6.1 gene is regulated by its long, complex 5′-UTR, which has similarities to the 5′-UTRs found in some Drosophila homeobox genes. For example, 

\[ \text{Nkh6.1} \text{ mRNA} \]

contains a 968-bp 5′-UTR and 2 upstream ATGs, and 

\[ \text{antp} \text{ mRNA} \]

contains a 1735-bp 5′-UTR and 15 upstream ATGs. Both of these 5′-UTRs contain IRESs that promote developmentally regulated translation. Similarly, the nkh6.1 5′-UTR functions as an IRES; while it can function in all the cells types tested, its activity is significantly higher in islet cell lines. These results demonstrate that the Nkh6.1 expression of the nkh6.1 5′-UTR shows cell type specificity. In addition, the 5′-UTR also inhibits transcription in the NIH3T3 cells, but not in islet cells, in a position-independent fashion. Taken together, the 5′-UTR contributes significantly to the cell type-specific expression of Nkh6.1.

The identified functions of the 5′-UTR, however, cannot completely explain the differences in expression of Nkh6.1 between α- and β-cell lines. No Nkh6.1 protein can be detected in α-cell lines, despite the presence of nkh6.1 mRNA, suggesting that post-transcriptional regulation of Nkh6.1 expression contributes to its restriction from α-cells. Although the IRES in the 5′-UTR provides a mechanism for cell type-specific translation of nkh6.1 mRNA, the activity of the IRES is similar in αTC1 cells and β-TC3 cells. It is possible that in the context of the intact gene, the IRES may function in a more tightly restricted fashion, or other posttranscriptional mechanisms may play additional roles in the cell type-specific expression of Nkh6.1.

It should be noted that further controls provide additional limits on the function of Nkh6.1 once the protein is expressed. When it binds to target genes, Nkh6.1 is a potent transcriptional repressor; but a sequence in the carboxyl-terminal end of the molecule prevents DNA binding by the homeodomain (14). Presumably only when this binding inhibition is relieved by interactions provided in the appropriate cellular environment can Nkh6.1 then turn off target genes. Together, several layers of regulation ensure that gene targeting by nkh6.1 is tightly restricted temporally and spatially.

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