Regulation of Pax4 Paired Homeodomain Gene by Neuron-restrictive Silencer Factor*

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An elucidation of the key regulatory factors in pancreas development is critical for understanding the pathogenesis of diabetes mellitus. This study examined whether a specific regulatory mechanism that exists in neuronal development also plays a role in the pancreas. In non-neuronal cells, neuron-restrictive silencer factor (NRSF) actively represses gene transcription via a sequence-specific DNA motif known as the neuron-restrictive silencer element (NRSE). This DNA motif has been identified in many genes that are specific markers for cells of neuronal and neuroendocrine lineage. We identified several genes involved in pancreas development that also harbor NRSE-like motifs, including pax-1, Beta2/NeuroD, and pax4. The paired homeodomain transcription factor Pax4 is implicated in the differentiation of the insulin-producing β-cell lineage because disruption of the pax4 gene results in a severe deficiency of β-cells and the manifestation of diabetes mellitus in mice. The NRSE-like motif identified in the upstream pax4 promoter is highly conserved throughout evolution, forms a DNA-protein complex with NRSF, and confers NRSF-dependent transcriptional repression in the context of a surrogate gene promoter. This cis-activating NRSE element also confers NRSF-dependent modulation in the context of the native pax4 gene promoter. Together with earlier reports, these new findings suggest an important functional role for NRSF in the expression of the pax4 gene and infer a role for NRSF in pancreatic islet development.

Pancreas development appears to follow a precise pattern of gene expression events characterized by temporal and spatial specificity. As such, a hierarchical model of transcription factor gene expression has evolved based on phenotypic observations from specific knock-out and transgenic animal studies. An interpretation of these data is illustrated in Fig. 1 (see “Results”) (1). Several transcription factors that are essential for β-cell differentiation are also involved in the neuronal development program. For example, the expression of Beta2/NeuroD is necessary for terminal differentiation of all endocrine cell types similar to its requirement for the differentiation of neurons in the brain (1). Similarities between islet and neuronal cells also exist at the level of physiology, such as in their electrical excitability and secretory vesicle functions. In addition to these similarities in differentiation and phenotypic profiles, the likelihood of the existence of additional molecular parallels is strong. Here we investigated whether the NRSE/NRSF† transcriptional repressor mechanism is involved in islet cell gene expression coincident with its role in neuron-specific gene regulation.

The cis-regulatory NRSE is a 21-bp motif that confers transcriptional repression of genes in many non-neuronal cell types (2, 3). Inhibition of NRSF binding to the NRSE in differentiating neurons permits transcriptional expression of these genes and facilitates terminal differentiation of neuronal lineages through neuron-specific gene regulation (4). Expression of a dominant-negative form of NRSF in the developing chick spinal cord resulted in precocious expression of neuronal target genes in neural progenitors (5). In contrast, constitutive expression of NRSF in differentiating neuroblasts led both to a down-regulation of endogenous NRSF target genes and the defective development of neuronal axon pathfinding (6).

The earlier discovery that pancreatic β-cells are devoid of NRSF-binding activity (7) reconciles observations of the co-expression of several genes in both neuronal cells and β-cells, such as the N-methyl-D-aspartate receptor-1, SCG10, synapsin, and MAPK8/1 genes, which are all NRSF target genes (7, 8). Here we report the identification of a functional NRSE regulatory DNA element in the promoter of the pax4 gene and describe the potential importance of this element in the regulation of the expression of pax4 in the pancreas.

EXPERIMENTAL PROCEDURES

Cell Lines—All cell culture reagents were purchased from Invitrogen except fetal bovine serum (Omega Scientific, Tarzana, CA). INS-1 cells were cultured in RPMI medium with 2 mM t-glutamine and supplemented with 10% fetal bovine serum, 10 mmol/liter HEPES, 1 mmol/liter sodium pyruvate, and 50 μmol/liter 2-mercaptoethanol (9). PC12 cells were cultured in FK12 medium with 10% fetal bovine serum. 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All media contained 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified incubator gassed with 5% CO2. Cell cultures were passaged by trypsinization and subcultured every 5 days.

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Papendick was purchased from Promega (Madison, WI). Double-stranded oligonucleotides containing two tandem copies of the pax4 NRSE (2 × 5′-CCCGT GCTGT CCTGC CCATG GTGCT GAAAC TATCT G-3′) or the mutated pax4-NRSE (2 × 5′-CCCGT GCTGT CAGGC CCATG AACGAC TATCT G-3′) were synthesized, and end-labeled using the Klenow fragment of DNA polymerase I in the presence of [γ-32P]ATP. Free

The abbreviations used are: NRSE, neuron-restrictive silencer element; NRSF, neuron-restrictive silencer factor; MODY, maturity onset diabetes of the young.

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nucleotides were separated by centrifugation through a G-50 column. DNA-protein-binding reactions were carried out in a 20-μl final volume of reaction buffer containing 10 mM Tris (pH 7.6), 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl2, 5% glycerol, and 250 μg of poly(dI-dC)/ml. The nuclear extract (10 μg of protein) was added to the reaction buffer in the absence or presence of unlabeled competitor DNA (SCG10–36, 5'-GCCAT CCGGAG AGTGC CTCGT C-C3') or antibody and pre-incubated for 10 min on ice. Radiolabeled probe (20,000 cpm) was then added, and the mixture was incubated for a further 20 min on ice. Electrophoresis to resolve DNA-protein complexes was performed in 4% non-denaturing polyacrylamide gels in 0.25 Tris borate-EDTA buffer at 150 V for 2–3 h. Antibodies for NRSF were a kind gift from Gail Mandel, Stony Brook, NY (P73 and F3).

**RESULTS**

To determine whether NRSF participates in islet-cell differentiation, we screened data bases for NRSE-like elements in regulatory regions of the transcription factor genes indicated in Fig. 1 (see Table I). Our focus on *pax4* in this study was based on several criteria: (a) the NRSE-like motif is present in the 5'-regulatory region where most functionally characterized NRSEs are located, (b) the similarity to the consensus NRSE is strong such that all nucleotides known to be required for functional integrity are conserved, (c) the core sequence is conserved 100% throughout evolution in a region of the promoter that otherwise displays relatively low nucleotide identity (Fig. 2A), and (d) Pax4 is a critical component of terminal cell fate directing the formation of β-cells in the developing islet such that Pax4-null mice display a failure of β-cell development (10). We aligned the sequence with the corresponding regions of several species (Fig. 2A). A high degree of conservation was maintained in all species examined, although the flanking sequences were poorly conserved. Moreover, the core NRSE motif is entirely conserved in the *pax4* promoter throughout evolution. Three of the four mismatches from the consensus NRSE were located at positions most frequently altered in phylogeny and believed not to be essential for NRSF binding (11).
The functional analysis of the putative \textit{pax4} NRSE was initially demonstrated by showing that the \textit{Pax4} promoter \textit{NRSE} binds to NRSF. We performed electromobility shift assays using nuclear cell extracts from HeLa cells, known to express elevated levels of NRSF. An oligonucleotide comprising a pair of tandem 36-bp repeats from the \textit{pax4} gene promoter was used as probe (Fig. 2B). In the presence of an unlabeled competitor oligonucleotide (see “Experimental Procedures”) a prominent complex formed in the binding reaction was abolished. When a mutated SCG10 oligonucleotide bearing no binding affinity for NRSF was used as a competitor, the protein-DNA complex was unaffected. Furthermore, in the presence of either of two NRSF-specific antisera, the same complex was substantially diminished, an effect that was not observed in the presence of a control antiserum (Fig. 2B). To further confirm NRSF binding to the \textit{Pax4} NRSE, we compared the electromobility shift assay profile of the \textit{pax4} probe with a mutant \textit{pax4} probe containing mismatched nucleotides in positions known to be essential to NRSE function (Fig. 2C). In all lanes, the complexes with the fastest mobility remained clearly visible sug-

\begin{figure}[h]
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\caption{The \textit{pax4} NRSE sequence and binding activity. A, alignment of the consensus sequence derived from 29 functional NRSEs with the putative NRSE in the \textit{pax4} gene promoter. Nucleotides shown in bold type are conserved in most functional NRSEs and are conserved in the \textit{pax4} promoter in all species analyzed. \textit{Asterisks} mark nucleotides that are most commonly altered in functional NRSEs. \textit{Shaded regions} illustrate nucleotide positions that are conserved throughout the evolutionary gap represented by the selected species. \textit{B and C}, gel shifts demonstrating sequence-specific binding activity of NRSF to the \textit{pax4} NRSE. A $^{32}$P-labeled probe consisting of two tandem repeats of the \textit{pax4} NRSE was incubated with HeLa cell nuclear extracts. Details are described under “Experimental Procedures.”}
\end{figure}
gesting that these interactions represent nonspecific binding under our experimental conditions. In contrast, the low mobility complex identified as the NRSF-NRSE complex in Fig. 2B was not observed in the presence of the mutated pax4 NRSE probe. The loss of this complex suggested that the protein component of this complex is NRSF.

Next we asked whether the pax4 NRSE confers transcriptional regulation. After inserting the tandem NRSE sequence into the multiple cloning site of a luciferase reporter plasmid we performed assays to determine whether cis-regulatory functions are encoded in the pax4 NRSE (Fig. 3A). Forty-eight h after transfection into 3T3 cells reporter gene activity was determined. Relative to the control vector, expression of the pax4 NRSE construct was attenuated by ~60% (Fig. 3B). Because 3T3 cells express NRSF, these findings supported the predicted model that the pax4 NRSE mediates NRSF-dependent transcriptional repression. Consistent with this model, the mutant-NRSE plasmid construct displayed no attenuation in activity suggesting that the intact NRSE motif is necessary for the observed transcriptional repression in 3T3 cells.

To further determine whether NRSF is the mediating factor in repression of the pax4 NRSE construct, we transfected the same reporter genes into endocrine cell lines devoid of NRSF-binding activity. We then tested whether exogenous expression of NRSF in these cells, by transfection with an expression plasmid, reproduces the attenuation of reporter gene activity. In both PC12 and INS-1 cells, the NRSE reporter constructs were co-expressed with either the pcDNA3.1 control vector or an NRSF expression vector (Fig. 3C). In both PC12 and INS-1 cells, reporter gene expression was attenuated by ~50% in the presence of NRSF. In contrast, no significant difference in gene expression level was observed with the mutant-NRSE construct in the presence or absence of NRSF. These findings indicate that these two endocrine cell types provide a permissive environment for the transcription of the NRSE-containing gene promoter. Furthermore, these data demonstrated that the pax4 NRSE confers NRSF-specific transcriptional repression in the context of this reporter gene system.

Finally, we determined whether the pax4 NRSE confers transcriptional regulation in the context of the native pax4 promoter. We took a reporter gene construct in which 2153 bases of the pax4 gene promoter were placed upstream of the
luciferase reporter gene and transfected 3T3, PC12, and INS-1 cells. Compared with the control empty vector, gene expression levels were elevated 1–2-fold in PC12 and 3T3 cells, whereas an 8-fold increase was observed in INS-1 cells (Fig. 4A). Several reports of studies of the pax4 promoter describe a pancreatic β-cell-specific enhancer positioned ~2 kb upstream of the transcriptional start site (Fig. 4B) (12–14). This 250-bp enhancer region confers β-cell specificity via its putative-binding sites for the transcription factors Beta2/NeuroD, Isl1, HNF1α, HNF4α, HNF3β, and PDX-1 (12–14). The pax4 NRSE sequence is located ~1300 bp upstream from the transcription start site and is thereby ~450 bp downstream of the enhancer region. This explains the mere 1–2-fold enhancement of gene expression in the PC12 cells, independent of the lack of NRSF activity. In contrast, INS-1 cells, which express these β-cell definitive transcription factors, correspondingly display an elevated level of expression in the absence of NRSF-mediated repression.

To determine whether NRSF represses pax4 promoter activity in INS-1 cells, we introduced increasing concentrations of NRSF by transfection of the NRSF expression plasmid. This experiment produced a biphasic functional response in pax4 gene promoter activity. Transfection of 1 μg of NRSF induced a small but statistically significant decrease in reporter gene activity. However, increasing concentrations of NRSF caused a dose-dependent increase in reporter gene activity resulting in

**Fig. 4. Transcriptional responses of the pax4 gene promoter to NRSF.** A, luciferase expression was monitored in 3T3, PC12, and INS-1 cells from a reporter construct in which 2153 bases of the pax4 gene promoter were placed upstream of the luciferase gene. Data are presented as -fold expression over empty vector. B, schematic representation of the regulatory region of the pax4 promoter. C, reporter gene expression levels of the pax4 promoter construct co-transfected with increasing concentrations of the NRSF expression vector in INS-1 cells. Firefly luciferase reporter activity was normalized to Renilla luciferase activity from a co-transfected internal control plasmid (pRL-CMV). Each experiment was performed at least three times in quadruplicate. Results are expressed as mean ± S.E.
an ~60% increase in expression above control levels (Fig. 4C). Collectively, these data provide compelling evidence that pax4 gene expression is regulated in part by NRSF through its binding to, and regulating the activity of, the NRSE located in the 5′ upstream promoter region.

**DISCUSSION**

We show that NRSF binds to the NRSE-like motif in the 5′-regulatory region of the pax4 gene and that it confers significant NRSF-mediated repression of gene transcription in the context of a constitutively active promoter. Furthermore, the NRSE displays cis-regulatory control of transcription in the context of the native pax4 gene promoter region in a clonal pancreatic β-cell line. These findings suggest that NRSF may be a relevant regulatory factor in pax4 gene expression and may, in turn, be important in β-cell differentiation.

Several reports provide support for a role of NRSF in both pax4 gene expression and β-cell differentiation. For example, two of the genes up-regulated during activin A-induced differentiation of AR42J cells to neuron-like cells include the intermediate neurofilament gene and the synaptophysin gene (15). Both genes are known targets of NRSF (11). Notably, the synaptophysin gene is devoid of any neuron-specific genetic elements other than the NRSE such that in a recent report Lietz et al. (16) demonstrated that in the absence of NRSF, the promoter of the synaptophysin gene is constitutively active (16). Thus, up-regulation of synaptophysin gene expression in the activin A-treated AR42J cells is likely to result from decreased NRSF activity; an event that is known to occur during neuronal differentiation (17). In the presence of hepatocyte growth factor, activin A treatment causes AR42J cells to differentiate into insulin-producing cells (18, 19). Under these conditions, pax4 gene expression is substantially enhanced (18). Moreover, activin A has been shown to increase pax4 gene expression in pancreatic β-cell lines, independent of changes in the expression of other transcription factors (20). We speculate that if activin A indeed increased synaptophysin gene expression via suppression of NRSF activity (16), it seems feasible that the activin A-dependent increase in pax4 gene expression in both β-cell lines and AR42J cells is also mediated through the down-regulation of the transcriptional NRSF repressor.

Unlike the promoter of the synaptophysin gene, the pax4 gene promoter appears to be regulated by multiple factors. We found that PC12 cells lacking NRSF were not capable of sustaining pax4 promoter activity. Indeed, several groups have shown that a specific region of the pax4 promoter harbors the binding sites for several different transcription factors (12–14). This region of the Pax4 promoter represents a sequence of DNA in which most if not all of the regulatory information for tissue-specific pax4 gene expression is contained. Notably, of the six transcription factor genes that distinguish susceptibility to MODY, four of the factors interact with the pax4 regulatory region (i.e. HNF4α-MODY1, HNF1α-MODY3, PDX1-MODY4, and Beta2/NeuroD-MODY6). Thus, the simultaneous expression of these transcription factors required for efficient pax4 transcription may play a role in regulating the tissue-specific expression of Pax4.

Our observation that elevated levels of NRSF result in a stimulation of pax4 promoter activity suggests a biphasic regulatory mechanism of NRSF. Notably, elevated levels of NRSF enhance expression of the genes encoding dynamin I (21), atrial natriuretic peptide (22), and corticotropin-releasing hormone (23) in contrast to its role in gene repression at lower levels of expression. However, an implied role for NRSF in regard to biphasic expression patterns requires further investigation.

The functional data presented in our study are derived from transient transfection experiments. Although this methodology is justified in analyses of NRSF function, it is noteworthy that NRSF-mediated gene repression may be dependent on rearrangement of the chromatin environment of the target gene (17, 24). Studies employing chromatin immunoprecipitation assays may yield more functionally relevant data. In this regard, studies of the regulation of the genome-integrated pax4 gene should prove to be more suited to the chromatin-responsive effects of NRSF and yield more predictive functional data.

We speculate that a possible role of NRSF in pax4 gene regulation may be to confer a temporary stop mechanism in an otherwise expression-permissive environment. This concept of developmental regulation is attractive, given that developmental processes are critically dependent on both spatial and temporal patterns of regulation of gene expression (1). The recent report suggesting that constitutive expression of pax4 in mouse embryonic stem cells significantly promotes the development of insulin-producing cells (25) favors this notion and supports the notion of a direct involvement of pax4 in pancreas development.

In conclusion, demonstration that a functionally responsive NRSE resides in the promoter region of the pax4 gene introduces the possibility that NRSF may play a direct functional role in the differentiation of pancreatic islet cell types.

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