The *PAX6* gene is activated by the basic helix–loop–helix transcription factor NeuroD/BETA2

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PAX6 is a transcription factor that plays an important role during pancreatic morphogenesis. The aim of the present study is to identify the upstream activator(s) of the *PAX6* gene possibly involved in the early stages of pancreatic differentiation. Recently, individual elements regulating *PAX6* gene activity in the pancreas have been identified in a 1100 bp *Spe*I/*Hin*cII fragment 4.6 kb upstream of exon 0. Preliminary sequence analysis of this region revealed some potential DNA-binding sites (E boxes) specific for the binding of basic helix–loop–helix transcription factors. By using electrophoretic mobility shift assays, we demonstrated that both nuclear protein extracts from insulin-secreting RINm5F cells and *in vitro*-translated NeuroD/BETA2 can bind specifically to these E boxes. Furthermore, by transient transfection experiments we demonstrated that the expression of basic helix–loop–helix transcription factor NeuroD/BETA2 can induce activation of the *PAX6* promoter in the NIH-3T3 cell line. Thus we show that NeuroD/BETA2 is involved in the activation of the expression of PAX6 through E boxes in the PAX6 promoter localized in a 1.1 kb sequence within the 4.6 kb untranslated region upstream of exon 0.

Key words: basic helix–loop–helix transcription factor, insulin, NeuroD/BETA2, *PAX6*, pancreas differentiation.

INTRODUCTION

Molecular and genetic analysis of tissue and organ development has provided evidence that conserved regulatory proteins control morphogenesis. Members of the *PAX* gene family are a remarkable example of such regulators. *PAX* genes encode key regulators that are involved in the embryonic development of many organs, including eye, brain, kidney, thyroid gland, immune system and pancreas [1–4]. During embryogenesis, *PAX* genes exhibit highly restricted temporal and spatial expression patterns [4]. Two members of the *PAX* gene family, *PAX4* and *PAX6*, play important roles in islet differentiation [4–9]. *PAX6* expression occurs very early in the foregut of mouse embryo (embryonic day 9.0), but from day 15.5 its expression expands to all pancreatic endocrine cells. In newborn animals, the PAX6 protein is detected in all cells of the islets of Langerhans [7,10]. The role of the *PAX6* gene in the development of the pancreas has been analysed. The absence of PAX6 leads to islet disorganization, indicating an involvement of PAX6 in the early stage of islet morphogenesis [7,10].

The vertebrate *PAX6* gene is related to the *Drosophila* pair-rule gene [11] and encodes two DNA-binding domains, a paired domain [12,13] and a paired-like homoeodomain [14]. The *PAX6* gene shows complex spatiotemporal expression that is confined exclusively to the developing eye, the central nervous system and the pancreas [15–17].

Little is known about the molecular mechanisms that control the expression of the *PAX6* gene in general, and in the developing pancreas in particular. Recently, individual elements that regulate *PAX6* gene activity in the pancreas have been identified. These elements are located at 5′ and 3′ of the PAX6 coding region. Furthermore, preliminary sequence analysis of this region revealed some potential DNA-binding sites (E boxes) specific for the binding of bHLH (basic helix–loop–helix) transcription factors [18,19]. Here we show that NeuroD/BETA2 binds as a heterodimer with E47, the PAX6 E boxes localized in a 1100 bp element responsible for the regulation of *PAX6* gene activity in the pancreas, and that it is able to activate *PAX6* gene expression in a non-endocrine cell line.

EXPERIMENTAL

Cell culture and transfection

The insulin-secreting RINm5F cell line and fibroblast NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5 % CO_2. NIH-3T3 cells were plated 24 h before transfection into multiwell plates at a density of 5×10^5 cells per well, and were transfected by LIPOFECTAMINE (Gibco) according to the standard conditions for mammalian cells described by the manufacturer. The amount of each expression or reporter plasmid used in transfection was 700 ng per well, with the final 2.1 μg of DNA equilibrated with empty pcDNA3 vector. To test co-transfection efficiency, cells were co-transfected with internal control vector pCMV-Luc (Promega), encoding luciferase.

Cells were harvested 48 h after transfection and extracts were assayed for β-galactosidase activity using a β-galactosidase (GAL) fluorescent activity detection kit from Sigma, and for luciferase activity using Dual Luciferase Reporter Assay System kit from Promega. β-Galactosidase values (emission at 455 nm) were normalized for luciferase activity.

Preparation of nuclear extracts

Nuclear proteins were prepared as described elsewhere [20]. The nuclei from RINm5F cells were isolated through cell disruption in lysis buffer [10 mM Hepes, pH 7.9, 10 mM KCl, 0.1mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT (dithiothreitol) and 0.5 mM PMSF]. The nuclear pellet was resuspended in cold nuclear extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM

Abbreviations used: bHLH, basic helix–loop–helix; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay.

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Table 1 Oligonucleotides used in EMSAs

The binding sites are in bold, and the mutated positions are in italic.

Name	Sequence
E1	ACGGAAACGA**CAAATG**TGCTGTTTTAT
E1mut	ACGGAAACGA***AGAACT***TGCTGTTTTAT
E2–E3	TAAGGAAGTC**CAGTTG**G**CAGGTG**TCAA
E2mut–E3	TAAGGAAGTC***AGGTCT***G**CAGGTG**TCAA
E2–E3mut	TAAGGAAGTC**CAGTTG**G***AGGGCT***TCAA

$MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and 0.5 mM PMSF) and centrifuged at 10000 ***g*** for 5 min at 4 °C, and the supernatant (nuclear extract) was stored at − 80 °C.

Construction of mutant PAX6 constructs

The PAX6 regulatory region in construct 406/Spe was mutated in the E box regions, using the GeneTailor site-directed mutagenesis system kit (Invitrogen) according to the manufacturer's protocol. Primers used in the mutagenesis reaction were: 5′ CTCATGGCACGGAAACGAAGAACTTGCTGTTTTAT 3′ (forward) and 5′ TCGTTTCCGTGCCATGAGTGAGGAAGT 3′ (reverse) for E1 box mutation; 5′ AATCAATCATAAGGAAGTGAGGTCTGCAGGTGTCA 3′ (forward) and 5′ CACTTCCTTATGATTGATTGTGATGCTTCGTGC 3′ (reverse) for E2 box mutation; 5′ CATAAGGAAGTGCAGTTGGAGGGCTTCAATCTTGG 3′ (forward) and 5′ CCAACTGGACTTCCTTATGATTGATTGTGATGC 3′ (reverse) for E3 box mutation. Mutated bases are underlined.

EMSAs (electrophoretic mobility shift assays)

An E1–E3 box oligonucleotide probe (182 bp) was prepared by PCR from a 406/Spe vector construct using the primers 5′ ACTTCCTCACTCATGGCACG 3′ (lower strand) and 5′ CTCCGTGACCCTTATAATTGGA 3′(upper strand). After PCR, amplified fragments were purified from agarose gel.

E1, E2–E3, E1mut, E2mutE3 and E2–E3mut double-stranded oligonucleotides were purchased from MWG-biotech. Sequences are reported in Table 1. Oligonucleotide O-r used in competition assays had the sequence 5′ ATAAGGCTAGGCTATCGGATCCTAGCTGATTCGAATGCCCTGAATTCCTTAA 3′.

Oligonucleotides were 5′-end-radiolabelled with [γ-^{32}P]ATP (Perkin Elmer) using T4 polynucleotide kinase (BioLabs) according to standard procedures [21]; generally 4 pmol of each DNA strand was labelled with 10 μCi of high specific radioactivity ATP. Labelled probes were purified from free [γ-^{32}P]ATP using Microspin S-300 HR columns (Pharmacia Biotech). The binding reaction mixture, containing 10 % (v/v) glycerol, 20 mM Tris/HCl, pH 7.5, 75 mM KCl, 0.5 mM DTT, 0.05 μg/μl BSA, an excess of the indicated competitor DNA and 0.5–1 μg of nuclear protein extract or 5 μl of *in vitro* translated/transcribed proteins, was incubated for 30 min at room temperature. Approx. 100 fmol of the probe was added for an additional 30 min. DNA–protein complexes were resolved by electrophoresis on non-denaturing 7 % (w/v) polyacrylamide gel in 0.5 × TBE buffer (1 × TBE = 45 mM Tris/borate and 1 mM EDTA) at 10 V/cm for 6 h at 4 °C. Finally, the gel was dried and autoradiographed.

For supershift assays, 4 μl of anti-NeuroD/BETA2 and/or anti-E47 antibodies (Santa Cruz Biotechnology) was added to the mixture before the DNA probes and preincubated with proteins for 30 min. DNA probes were then added to the whole mixture, which was incubated at room temperature for another 1 h. Complexes were analysed on a 5 % (w/v) polyacrylamide gel in 0.5 × TBE.

In vitro transcription/translation

The expression vectors pCS2-mND and pcDNA-mE47 were transcribed and translated *in vitro* using the TNT-coupled transcription/translation reticulocyte lysate system (Promega) according to the manufacturer's instructions, including SP6 or T7 polymerase and [^{35}S]methionine (Amersham Pharmacia Biotech). Translation products were analysed by SDS/PAGE.

South-western analysis

Samples of 20 μg of nuclear protein were run on a 12 % (w/v) polyacrylamide SDS/PAGE gel at 30–35 mA for 2–3 h. The proteins were then electroblotted on a PVDF membrane and the membrane was incubated in soaking buffer (1 % Triton X-100, 20 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, 5 mg/ml BSA, 2 mM DTT and 0.1 mM PMSF) at 4 °C overnight for protein renaturation. The membrane containing renaturated proteins was prehybridized with binding buffer [20 mM Tris/HCl, pH 7.5, 75 mM KCl, 5 mM DTT, 50 μg/ml BSA, 5 % (v/v) glycerol, 1 mM EDTA, 0.1 mM PMSF and 5 % (w/v) non-fat milk] at 4 °C for 1 h. Radiolabelled probe (400 ng) was then added to the binding buffer and incubated at room temperature for 8 h. Where present, aspecific competitors were added to the binding buffer 30 min before the probe. After washing three times with binding buffer, the membrane was dried at room temperature and exposed to X-ray film.

ChIP (chromatin immunoprecipitation) assay

RINm5F cells (5×10^8) grown to 90 % confluence in 100 mm plates were washed once with PBS at room temperature and fixed with 1 % (v/v) formaldehyde in PBS at 37 °C for 10 min. Cells were quickly rinsed twice with ice-cold PBS and scraped into 1 ml of ice-cold PBS. After spinning at 700 ***g*** at 4 °C, cell pellets were resuspended in 3.5 ml of lysis buffer (1 % SDS, 5 mM EDTA, 50 mM Tris/HCl, pH 8.1, plus protease inhibitor cocktail) and incubated on ice for 10 min. After lysis, the sample was sonicated three times for 15 s each (Sonifer Power Supply, Branson, 2.8 mA, microtip) and then centrifuged for 10 min at 14000 ***g*** at 4 °C. A 100 μl aliquot of the supernatant was set aside as the input fraction. NaCl and Triton X-100 were added to the remaining chromatin preparation to final concentrations of 150 mM and 1 % (v/v) respectively, followed by incubation for immunoclearing with Protein A–Sepharose (150 μl of 50 % slurry in 10 mM Tris/HCl, pH 8.1, 1 mM EDTA) for 2 h at 4 °C. After incubation, the sample was quickly centrifuged at 9000 ***g*** and the supernatant was collected. Immunoprecipitation was performed by adding 50 μl of polyclonal anti-NeuroD/BETA2 antibody (Santa Cruz Biotechnology) or an aspecific antibody (human IgG) and by incubating the mixture overnight at 4 °C; 150 μl of Protein A–Sepharose was then added and the slurry was incubated for a further 1 h at 4 °C. The Sepharose beads were harvested by centrifugation at 8000 ***g*** and washed sequentially for 10 min in 1 ml each of TSE I (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, 150 mM NaCl), TSE II (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, 500 mM NaCl), TSE III (0.25 M LiCl, 1 % Nonidet P40, 1 % deoxycholate, 1 mM EDTA, 10 mM Tris/HCl, pH 8.1) and TE buffer (10 mM Tris/HCl, pH 8.1, 1 mM EDTA). DNA was

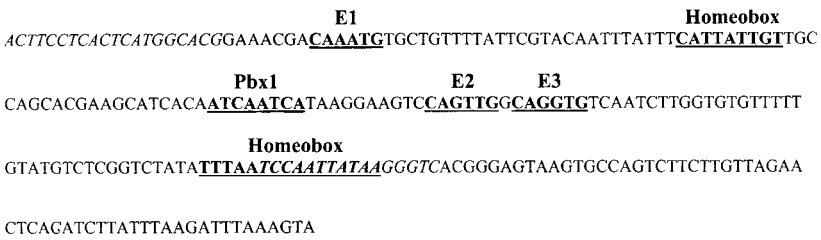

Figure 1 Sequence of a 233 nt element contained in the 1100 bp regulatory region controlling the expression of the *PAX6* gene in the pancreas

The sequence contains potential binding sites for bHLH (E boxes), a binding site for Pbx1 and two possible motifs for homoeoboxes. The primer sequences used in PCR amplification for EMSA probe synthesis and for ChIP analysis are in *italics*.

eluted from beads with 100 μl of elution buffer (1 % SDS/0.1 M $NaHCO_3$) at room temperature for 10 min and then heated at 56 °C for 6 h to reverse the formaldehyde cross-link. Extracted DNA was purified using a Wizard SV gel and PCR clean-up system (Promega).

PCR was performed using the primers 5′ ACTTCCTCACTCATGGCACG 3′ (lower strand) and 5′ CTCCGTGACCCTTATAATTGGA 3′(upper strand), and was conducted as follows: 1 min at 94 °C, 45 s at 50 °C and 1 min at 74 °C for 35 cycles, using a Progene DNA Thermal Cycler. The PCR products were separated on a 1.5 % (w/v) agarose gel and visualized by ethidium bromide staining.

DNA constructs

Expression vector pCS2-mND containing mouse NeuroD/BETA2 full-length cDNA was kindly donated by Dr J. E. Lee (University of Colorado, Boulder, CO, U.S.A.). Plasmid pcDNA-mE47 containing the complete coding sequence of mouse E47 in the pcDNA3 vector was provided by Dr A. Cano (Instituto de Investigaciones Biomedicas "Alberto Sols", Madrid, Spain). Constructs 406/Sal and 406/Spe were gifts from Dr. P. Gruss (Max-Planck-Institut of Biophysical Chemistry, Göttingen, Germany).

RESULTS

Characterization of binding of nuclear proteins to a *PAX6* regulatory element

Previous studies showed that a pancreas-specific regulatory element is located on a 1100 bp fragment 4.6 kb upstream of exon 0 of the *PAX6* gene [19]. Moreover, a sequence comparison performed among corresponding DNAs of mouse, human and *Fugu* (Japanese pufferfish) revealed a sequence in the *PAX6* regulatory region that might be responsible for controlling the expression of the gene in pancreas. This sequence contains potential binding sites for Pbx1, two possible motifs for homoeobox-binding sites and three E boxes for bHLH binding sites, indicated as E1, E2 and E3 respectively (Figure 1).

To characterize DNA-binding proteins that interact with this regulatory sequence, we performed EMSAs using a 182 bp radiolabelled probe including the three E boxes and flanking sequences (E1–E3 sequence). Nuclear extracts from insulin-secreting RINm5F cells generated two major DNA–protein complexes (Figure 2). The specificity of the DNA–protein interaction was examined by preincubating the extracts with an excess of unlabelled double-stranded competitor DNA. As shown in Figure 2, the two complexes differed in their sensitivity with respect to competitor DNA. The addition of 1 μg of salmon sperm DNA

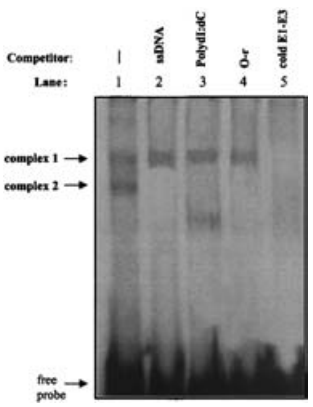

Figure 2 EMSAs of DNA–protein complexes with labelled E1–E3 oligonucleotide

Two complexes, indicated as complex 1 and complex 2, were generated from RINm5F nuclear proteins. The following competitors were used: lane 2, 1 μg of salmon sperm DNA (ssDNA); lane 3, 1 μg of poly(dI · dC); lane 4, unlabelled random oligonucleotide (O-r) at 100-fold molar excess; lane 5, unlabelled ('cold') E1–E3 oligonucleotide at 30-fold molar excess over labelled oligonucleotide.

and poly(dI · dC) or a 100-fold molar excess of a non-related double-stranded competitor oligonucleotide (O-r) induced complete disappearance of the faster band (complex 2), whereas no competing effect with the slower complex (complex 1) was observed. Moreover, unlabelled wild-type oligonucleotide displaced both complexes, indicating sequence-specific protein binding (Figure 2, lane 5).

To obtain an approximate estimate of the molecular masses of nuclear factors that bound specifically to our probe, South-western analyses were performed. After SDS/PAGE separation of RINm5F nuclear extracts, proteins were electroblotted on a PVDF membrane. By probing the membrane with the E1–E3 radiolabelled oligonucleotide in the absence of unlabelled competitor, at least four major bands in a range of molecular masses between 100 and 35 kDa (Figure 3, lane B) were identified. In the presence of an excess of poly(dI · dC) DNA competitor (10 μg), only one major band at approx. 75 kDa and a signal at approx. 60 kDa (Figure 3, lane A) were visible. In the presence of a 30-fold molar excess of unlabelled probe, no signals could be observed (Figure 3, lane C).

Identification of factors binding to *PAX6* regulatory elements

In view of the data reported above, Western blot analysis of RINm5F nuclear proteins was carried out using anti-NeuroD/BETA2 antibody. As reported in Figure 3 (lane D), a signal

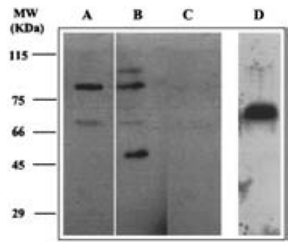

Figure 3 South-western analysis (lanes A–C) and Western analysis using anti-NeuroD/BETA2 antibody (lane D) of 20 μg of RINm5F nuclear proteins after electroblotting on a PVDF membrane

South-western hybridization was performed with 400 ng of labelled E1–E3 probe. Lane A, 10 μg of poly(dI · dC) was added to binding buffer 30 min before addition of the probe as an aspecific competitor; lane B, no competitor was added; lane C, 30-fold molar excess of unlabelled probe. The positions of molecular mass (MW) markers are indicated on the left.

corresponding to the 60 kDa band of the South-western blot was observed.

NeuroD/BETA2 belongs to the bHLH family of transcription factors. Members of this family of transcription factors are E box-binding proteins that play important roles in the cell-specific expression of many different genes [22,23]. NeuroD/BETA2 is a B-type bHLH transcription factor of 60 kDa that is known to be involved in the first stages of pancreatic development [24] and to bind to the insulin E box sequences to activate gene transcription in mature secreting cells [25].

To establish if NeuroD/BETA2 is able to interact with the E boxes of the *PAX6* regulatory element, the DNA–protein complexes generated in gel shift assays were analysed in the presence of a specific antibody against mouse NeuroD/BETA2 (Figure 4). This antibody recognized protein in complex 1, as shown by the disappearance of the corresponding band in Figure 4. In contrast, the less specific complex 2 was not affected even by a large quantity of anti-NeuroD/BETA2 antibody (Figure 4A, lanes 3). These experiments were performed in the absence as well as in the presence of unlabelled DNA competitor poly(dI · dC) (Figure 4A, lanes 3 and 4 respectively). The results clearly indicate that a specific recognition of complex 1 formation occurred.

The class B bHLH factors appear to bind poorly to DNA as homodimers, whereas they bind E boxes with high affinity once they form heterodimers with class A proteins [26,27]. To activate insulin gene expression, NeuroD/BETA2 binds E box sequences as a heterodimer with E47 (Pan1), a class A protein that is ubiquitously expressed [25]. The DNA–protein complexes generated in gel shift assays were analysed using a specific antibody against mouse E47. In the presence of anti-E47 antibody, complex 1 disappeared completely, indicating that in complex 1 NeuroD/BETA2 probably heterodimerizes with E47 in binding the probe (Figure 4A, lanes 7 and 8). We also tried to identify the 75 kDa band observed in South-western experiments by performing Western blot analysis on RINm5F nuclear proteins using an anti-E47 antibody. No positive results were obtained (not shown).

The binding of NeuroD/BETA2 and E47 to the E1–E3 region of the *PAX6* gene regulatory element was confirmed by gel shift assays using *in vitro* translated NeuroD/BETA2 and E47 proteins and the E1–E3 box oligonucleotide as a probe. Duplicate reactions were carried out in the presence of [^{35}S]methionine and assessed by SDS/PAGE to confirm protein integrity (results not shown). As shown in Figure 4(B), NeuroD/BETA2 alone or in combination with E47, in the presence of 1 μg of poly(dI · dC) DNA, bound to this probe, forming DNA–protein complexes. The retarded band observed with NeuroD/BETA2 reacted with antibody raised

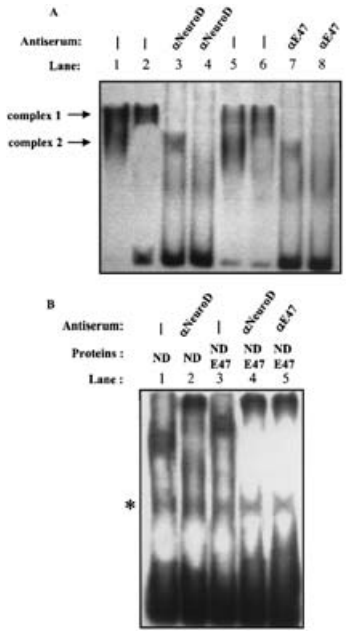

Figure 4 Identification by supershift analysis of factors binding to the *PAX6* regulatory element

(**A**) E1–E3-binding protein complexes were specifically detected by antibodies directed against bHLH proteins NeuroD/BETA2 (lanes 3 and 4) and E47 (lanes 7 and 8). Labelled E1–E3 probe was incubated with nuclear extracts from RINm5F cells, and protein complexes formed are marked by arrows. The antibodies were incubated with nuclear extracts before the addition of the labelled E1–E3 probe. Lanes 1, 3, 5 and 7 correspond to free competitor binding reactions. In lanes 2, 4, 6 and 8, 1 μg of poly(dI · dC) DNA was added. (**B**) Binding to the E1–E3 probe of *in vitro* transcribed and translated NeuroD/BETA2 (ND) and E47 proteins in EMSAs. The labelled oligonucleotide was incubated in the presence of 1 μg of poly(dI · dC) with 5 μl of *in vitro* translation reaction containing either protein NeuroD/BETA2 alone (lanes 1 and 2) or in combination with *in vitro* translated E47 (lanes 3–5). In lanes 2, 4 and 5, the indicated antibodies were incubated in the binding mixture with translated proteins prior to addition of the labelled probe.

against NeuroD/BETA2 (Figure 4B, lane 2), while the retarded band observed with NeuroD/BETA2 in combination with E47 was recognized by both antibodies (anti-NeuroD/BETA2 and anti-E47; Figure 4B, lanes 4 and 5). The fast DNA–protein complex marked by an asterisk in Figure 4(B) and generated using *in vitro* translated E47 or NeuroD/BETA2 was neither supershifted, nor was its amount decreased, by using anti-E47 or anti-NeuroD/BETA2 antibodies, indicating the presence of a non-specific endogenous binding factor in the reticulocyte lysate.

To determine which of the three E boxes contained in the E1–E3 *PAX6* region is recognized by NeuroD/BETA2, E1 and E2–E3 oligonucleotides were synthesized (see the Experimental section and Table 1) and used as probes in gel shift assays for RINm5F cell nuclear extracts. As reported in Figure 5(A), the formation of three complexes could be observed for the E1 probe, and two for the E2–E3 probe. The nature of these complexes was studied by evaluating competition with an excess of poly(dI · dC) DNA and with a non-correlated double-stranded competitor oligonucleotide (O-r). All complexes were resistant to the competition of poly(dI · dC) up to a concentration of 100 μg/ml (Figure 5A, lanes 5 and 11) and of a 100-fold molar excess of a unlabelled double-stranded competitor DNA, indicating sequence-specific protein binding (Figure 5A).

More information about the nature of the observed DNA–protein complexes was obtained from supershift experiments using anti-NeuroD/BETA2 and anti-E47 antibodies. As shown

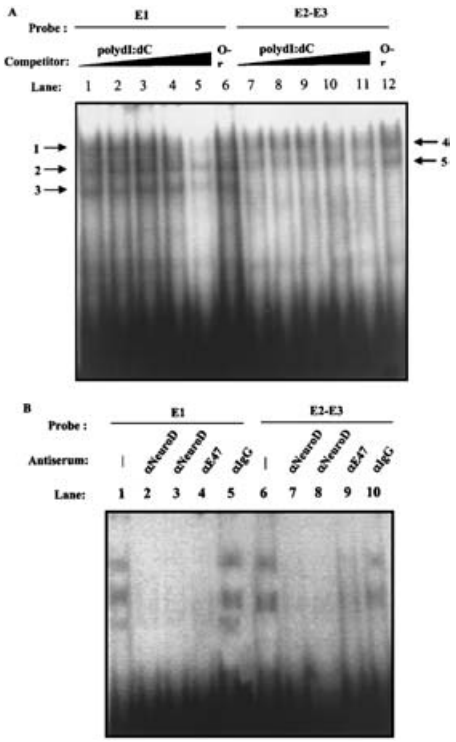

Figure 5 Analysis of DNA–protein complexes binding to the *PAX6* gene E box probes E1 and E2–E3

(**A**) Three complexes (denoted 1, 2 and 3) were generated by the E1 oligonucleotide, and two complexes (denoted 4 and 5) were generated by the E2–E3 oligonucleotide. In samples in lanes 1 to 5 and in lanes 7 to 11, 100, 400, 800, 1000 and 2000 ng of poly(dI · dC) DNA was added respectively to the binding reaction. In lanes 6 and 12, a 100 fold-molar excess of unlabelled random oligonucleotide (O-r) was added. (**B**) Supershift analysis of the DNA–protein complexes generated by the E1 and E2–E3 probes. The amount of anti-NeuroD/BETA2 antibody added was 4 μl in lanes 2 and 7, and 1 μl in lanes 3 and 8. In lanes 5 and 10, an IgG was used as a negative control.

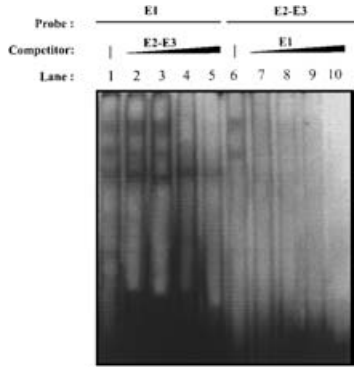

Figure 6 Competition experiment for E1 and E2–E3 oligonucleotides

E1 and E2–E3 labelled probes were incubated with 5-, 10-, 50- and 100-fold molar excesses of unlabelled oligonucleotides E2–E3 and E1 respectively.

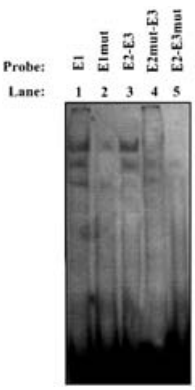

Figure 7 EMSA analysis for mutant E box probes

The sequences of the oligonucleotides used as labelled probes are reported in Table 1.

in Figure 5(B), the addition of specific anti-NeuroD/BETA2 and anti-E47 antibodies completely disrupted the binding of all retarded complexes observed for the E1 and E2–E3 probes. In contrast, binding to the E1 or E2–E3 probes was not affected by antibodies directed against an uncorrelated protein (IgG). These results indicate that NeuroD/BETA2 protein in combination with E47 binds to both probes E1 and E2–E3, forming three distinct retarded complexes with E1 and two complexes with E2–E3, probably of different stoichiometry.

To evaluate the possible existence of affinity differences in the interactions with the E1 and E2–E3 boxes, we performed competition experiments of labelled E1 and E2–E3 boxes with unlabelled E2–E3 and E1 respectively of increasing concentrations (from 5× to 100×). As reported in Figure 6, proteins showed a higher affinity for the E1 box oligonucleotide than for the E2–E3 oligonucleotide. Indeed, complexes formed with the E1 box required a 100× molar excess of the E2–E3 box to be disrupted, whereas a 5× molar excess of unlabelled E1 box was sufficient to disrupt the E2–E3 complexes.

To determine which 6 bp E1, E2 and E3 sequences are critical for the formation of DNA–protein complexes, mutant E1mut, E2mut–E3 and E2–E3mut oligonucleotides were synthesized and used as probes in EMSA (Table 1). As shown in Figure 7, binding to the E1 box and the E1–E2 boxes requires their integrity. In fact, in gel shift assays no resolved retarded bands were observed with the labelled mutated probes E1mut, E2mut–E3 and E2–E3mut. In the same way, unlabelled probes E1mut, E2mut–E3 and E2–E3mut failed to compete efficiently with the labelled E1 and E2–E3 probes even when present at a large excess (results not shown).

The direct involvement of NeuroD/BETA2 and E47 in the interaction with the E1 and E2–E3 oligonucleotides was demonstrated by performing gel shift assays using *in vitro* translated proteins in the presence of 1 μg of poly(dI · dC). Both sequences were recognized by NeuroD/BETA2 alone or as a heterodimer with E47 protein (Figure 8, lanes 1–4). The specificity of complex formation was confirmed by the ability of anti-NeuroD/BETA2 and/or anti-E47 antibodies to disrupt all complexes (results not shown).

NeuroD/BETA2 binds the *PAX6* regulatory promoter region *in vivo*

The EMSA experiments indicated that NeuroD/BETA2 and E47 bind *in vitro* to the regulatory element localized in a 4.6 kb region upstream of exon 0 of *PAX6*. To demonstrate that NeuroD/BETA2 binds the 1100 bp *PAX6* promoter regulatory element *in vivo*, a ChIP assay was carried out using extracts from RINm5F cells. For this purpose, DNA-binding proteins of RINm5F cells were covalently linked to genomic DNA by treatment of the cells with formaldehyde. The DNA–protein complexes were then sheared

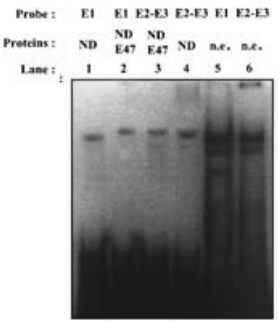

Figure 8 Binding to E1 and E2–E3 probes of *in vitro* transcribed and translated NeuroD/BETA2 and E47 proteins in EMSAs

NeuroD/BETA2 (ND) protein was incubated alone or in combination with E47 with labelled probes E1 and E2–E3. In lanes 5 and 6, nuclear extracts (n.e.) from RINm5F cells were used as a control.

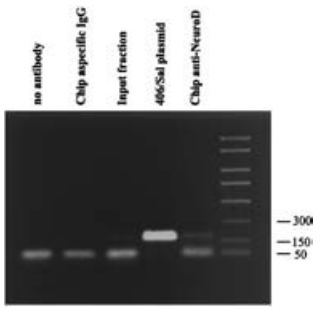

Figure 9 ChIP for the NeuroD/BETA2 transcription factor

ChIP was carried out as described in the Experimental section. The ChIP assay detects NeuroD/BETA2 protein bound to the endogenous regulatory region of the *PAX6* gene. After precipitation of DNA–protein complexes with anti-NeuroD/BETA2 or with aspecific human IgG (technical grade), PCR amplification was performed by using PAX6 primers that amplify a 182 bp region that contains the three E boxes. A positive signal was observed after immunoprecipitation with anti-NeuroD/BETA2 antibodies (lane 5), but not in controls in which immunoprecipitation was performed without antibody (lane 1) or with aspecific antibodies (lane 2). 'Input fraction' refers to non-immunoprecipitated DNA from RINm5F cells (lane 3), and 406/Spe plasmid refers to a PCR product obtained using the 406/Spe vector as a positive control (lane 4). The lower band present in all lanes corresponds to PCR primers.

by sonication, and specific DNA–protein complexes were immunoprecipitated with antibody against NeuroD/BETA2. Covalent linkage was reversed and the purified double-stranded DNA was used as a template to amplify, by PCR, a 182 bp region internal to the 1100 bp regulatory element that specifically covers the E boxes. In a 1.5 % (w/v) agarose gel, a band of the expected amplified product could be observed both in the input fraction used as positive control (Figure 9, lane 3) and in the chromatin fraction obtained after immunoprecipitation with anti-NeuroD/BETA2 antibody (lane 5). The corresponding control, in which immunoprecipitation was performed with aspecific antibodies (lane 2), did not show a PCR signal.

Transactivation of the *PAX6* gene promoter by NeuroD/BETA2

We examined the ability of NeuroD/BETA2 to activate transcription of the *PAX6* gene by transiently co-transfecting NIH-3T3 cells with the mammalian expression vector pCS2-mND (which expresses mouse NeuroD/BETA2) and with promoter/*lacZ* fusion constructs 406/Sal and 406/Spe. Vectors 406/Sal and

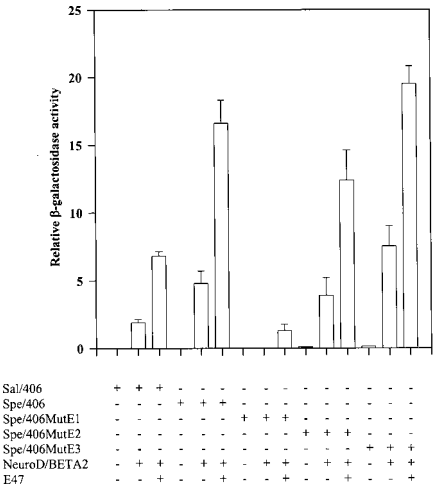

Figure 10 Transactivation of the *PAX6* promoter by NeuroD/BETA2 and E47 expression constructs

NIH-3T3 cells were transfected with PAX6–β-galactosidase reporter gene vectors Sal/406 or Spe/406, Spe/406mutE1, Spe/406mutE2 and Spe/406mutE3, and with expression plasmids pCS2-mND (for NeuroD/BETA2) and pcDNA-mE47 (for E47). β-Galactosidase activities were normalized with respect to the activity of luciferase after co-transfection of cells with the internal control vector pCMV-Luc. Results are expressed as relative β-galactosidase activity after normalization, and values are means $\pm$ S.E.M. of three individual transfection experiments done in triplicate.

406/Spe were generated using a reporter *lacZ* gene fused to 8 and 5 kb sequences respectively localized in the untranslated 5′ region downstream of exon 0 of the *PAX6* gene and containing the 1100 bp regulatory element. Previous studies demonstrated that both constructs are able to drive transgenic *lacZ* expression in the cornea and the pancreas of transgenic mice [19].

NIH-3T3 cells, which do not express PAX6, were transfected with either 406/Sal or 406/Spe vector alone or together with the pCS2-mND expression vector. The transfection efficiency was checked by co-transfection with a pCMV-Luc internal control vector, encoding luciferase, and β-galactosidase activity in cell extracts was normalized for luciferase activity.

Co-transfection with pCS2-mND and 406/Sal or 406/Spe significantly activated reporter gene expression (Figure 10), suggesting that NeuroD/BETA2 can activate reporter gene transcription by stimulating *PAX6* promoter elements. Cells that were transfected with 406/Sal or 406/Spe reporters alone did not show any stimulation of *lacZ* reporter expression, similar to non-transfected cells (Figure 10).

To verify the effect of the co-expression of E47 and NeuroD/BETA2 in *PAX6* promoter activation, NIH-3T3 cells were also co-transfected with expression vectors pCS2-mND and pcDNA-mE47 (expressing mouse E47 protein) and reporter vector 406/Sal or 406/Spe. Expression of NeuroD/BETA2 in combination with E47 led to a striking increase in the expression of the reporter gene (Figure 10), suggesting that NeuroD/BETA2 heterodimerized to E47 activates transcription of *PAX6*. In fact, NeuroD/BETA2 is known to bind DNA inefficiently as a homodimer, and levels of endogenous E47 in NIH-3T3 cells are probably rate-limiting under the conditions of transfection.

To investigate whether the putative NeuroD/BETA2-binding sites E1, E2 and E3 are essential for *PAX6* promoter/enhancer activation, four of the six bases of each E box were mutated to obtain constructs Spe/406mutE1, Spe/406mutE2 and Spe/406mutE3 respectively (Table 1). Constructs were generated by

PCR-based site-directed mutagenesis of the 406/Spe construct, and the mutations were the same as in the mutated oligonucleotides used in EMSAs and reported in Table 1. The constructs were examined for promoter activity by transient co-transfection of NIH-3T3 cells with expression vectors pCS2-mND and pcDNA-mE47, as shown in Figure 10. When compared with wild-type Spe/406, mutation in the E1 region resulted in a 92% decrease in transactivation when Spe/406mutE1 was co-transfected with pCS2-mND and pcDNA-mE47. These data suggest that E1 binding site is involved in *PAX6* promoter activation. In contrast, mutations in the E3 and E2 regions (the same mutations that abolished binding shown in Figure 7) did not substantially affect reporter gene transactivation, suggesting that the E3 and E2 regions alone are not essential for *PAX6* promoter/enhancer activitation. The E1 box site therefore constitutes the major site that is responsive to NeuroD/BETA2 and E47 proteins.

DISCUSSION

During development, the mammalian pancreas is formed from the epithelial cells of the embryonic gut at the foregut–midgut junction, and differentiates into two distinct compartments: the exocrine tissue, which produces digestive enzymes, and the endocrine islets of Langerhans, which produce specific hormones. The islets are arranged into a core of insulin-producing β cells surrounded by a mantle of glucagon-producing α cells, and smaller numbers of somatostatin- and pancreatic polypeptide-producing cells (δ and PP cells respectively). The co-ordinated regulation of gene expression required for normal pancreatic development is not completely understood, but it clearly requires the ordered activation of nuclear transcription factors by both intracellular and extracellular signals. Several transcription factors (i.e. Pdx1, ISL1, PAX6, PAX4, NeuroD/BETA2 and NKX2.2) are required for normal pancreatic endocrine development [28–31]. One of these factors, PAX6, has been identified as a regulator of pancreatic development [4,5,7]. PAX6 belongs to the PAX family of transcription factors and contains both a paired domain and a homoeodomain as DNA-binding elements. The role of PAX6 in the developing pancreas has been analysed in two different mutant mice [7,10]. One mutant was generated by replacing most of the coding sequence with the *lacZ* gene by homologous recombination in embryonic stem cells [7,10]. The second mutant harbours a point mutation that results in a truncated PAX6 protein [7,10]. At early stages of endocrine cell development, both PAX6 mutants appear to exhibit similar phenotypes, since glucagon-expressing cells, although greatly reduced in number, are detectable. Furthermore, the islets are disorganized, suggesting a role for PAX6 in islet morphogenesis [7,10].

It is clear that further studies are required to dissect the possible multiple functions of the *PAX6* gene product. In this context, the dissection of *PAX6* promoters in a search for regulatory elements required for specific expression in the pancreas will provide valuable tools for the analysis of PAX6 function in the pancreas. Little is known about the molecular mechanisms that control the expression of the *PAX6* gene. Results from studies on the primary structure of *PAX6* in quail and *Caenorhabditis elegans* suggest that the expression of PAX6 is under the control of different regulators through alternative promoters [32–34]. Individual control elements have been identified that regulate *PAX6* gene activity in the pancreas (as well as in the lens, neural retina and telencephalon) [19]. These elements are individual modules located 5′ and 3′ to the *PAX6* coding region. For the pancreas, a specific regulatory element is located in a 1100 bp *Spe*/*Hin*cII fragment 4.6 kb upstream of exon 0. Interestingly, this regulatory element contains consensus binding sequences for transcription factor Pbx1, which is involved in the control of gene expression in the pancreas [35] and, additionally, two homoeobox protein-binding motifs. Furthermore, preliminary analysis of the sequence of this region revealed some potential DNA-binding sites (E boxes) specific for binding of bHLH transcription factors. In accordance with our previous results [36], we have identified a specific temporal hierarchy of activation of transcription factors that are involved during pancreatic development, starting from Neurogenin3 through NeuroD/BETA2 to PAX6. The direct involvement of NeuroD/BETA2 in the activation of the *PAX6* gene has been only hypothesized. On the basis of the structural characteristics of the minimal promoter sequence required for the expression of PAX6 in the pancreas [19], we identified the presence of a consensus E box sequence (CANNTG). In general, tissue-specific factors that bind E boxes have been shown to consist of heterodimers between a ubiquitous class A and a tissue-specific class B family member of the bHLH transcriptional activators [26]. Examples of class A bHLH family members are the E2A gene products (E12 and E47), ITF2 and HEB [37,38]. Class B members include NeuroD/BETA2, MyoD and myogenin [39–42]. Dimerization between bHLH proteins depends on the HLH region, while DNA–protein interactions are mediated by the basic region. These activators bind to the consensus sequence CANNTG, with heterodimerization increasing DNA binding and activation [26].

NeuroD/BETA2 is expressed in pancreatic islet endocrine cells [24], in the intestine [43], in the pituitary gland [44] and in a subset of neurons in the central and peripheral nervous systems [41]. This factor was isolated and characterized by exploiting its ability to activate neurite formation upon ectopic expression in *Xenopus* embryos [41] and insulin reporter gene transcription in transfected β cells [25]. Starting from this point, we performed experiments aiming at proving an involvement of NeuroD/BETA2 in the activation of *PAX6* through interaction with three E boxes present in the 1100 bp of the regulatory region of the *PAX6* promoter. EMSAs performed using a 182 bp fragment of the 1100 bp regulatory region revealed the presence of specific nuclear proteins that are able to interact with it. The involvement of NeuroD/BETA2 was demonstrated by supershift experiments performed using an anti-NeuroD/BETA2 antibody that specifically disrupted the DNA–protein complex. Results obtained using *in vitro* translated NeuroD/BETA2 confirmed these data.

In an attempt to identify the sequences of the E boxes involved, EMSAs were performed using probes containing E1 box and E2–E3 box sequences. Both probes were able to form complexes. A higher affinity for protein binding was apparent for the E1 box. Supershift experiments showed that, in the complexes formed with both E boxes, NeuroD/BETA2 and E47 are probably involved as a heterodimer. These observations were confirmed by EMSAs carried out with *in vitro* translated NeuroD/BETA2 and E47 proteins. Moreover, proteins bind to E box regions in a sequence-specific manner, as demonstrated by using mutant oligonucleotides in EMSAs.

We then carried out experiments specifically aimed at proving the direct activation by Neuro/BETA2 of regulatory elements of *PAX6* involved in pancreas development. In this respect, co-transfection of a cell line normally not expressing PAX6 (i.e. NIH-3T3) was performed using expression vectors harbouring the complete coding sequence of mouse NeuroD/BETA2 and the pancreatic regulatory element of the *PAX6* gene. The results clearly showed a specific activation induced by NeuroD/BETA2.

Interestingly, a more marked increase in reporter activation was observed with the 406/Spe than with the 406/Sal reporter vector. The former corresponds to a shorter sequence containing

the minimal *cis*-control element that is able to activate *PAX6* expression in the developing pancreas [19]. These findings can be ascribed to the higher specificity of the sequence considered. It is likely that, in the 406/Sal reporter vector, sequence(s) are present that might interfere with the binding of NeuroD/BETA2, ameliorating its *trans*-activation properties.

The involvement of the three putative NeuroD/BETA2 binding sites (E1, E2 and E3) in the activation of *PAX6* expression was examined using reporter constructs mutated in the relevant binding sites. Mutations in the E1 box region dramatically decreased reporter gene expression, suggesting that the E1 box sequence is important for *PAX6* gene promoter activation. However, although we observed sequence-specific binding of NeuroD/BETA2 to the E3 and E2 box regions *in vitro*, transient transfections using mutant constructs did not show significant reduction in transactivation level when compared with that of the wild-type construct.

In order to demonstrate the physiological relevance of our results, we demonstrated by a ChIP assay that NeuroD/BETA2 binds *in vivo* to the endogenous regulatory region of *PAX6*, by using sequence-specific primers for a 182 bp region that contains the three E boxes.

On the basis of the present results and those reported elsewhere [19], we can identify a 233 bp sequence as a region that is probably involved in the activation of the *PAX6* gene, primarily through the E1 box sequence. However, the presence of additional NeuroD/BETA2-responsive elements in the 1100 bp regulatory sequence cannot be excluded, and such elements may also play a role in *PAX6* gene activation.

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