Developmental Biology 365 (2012) 290–302

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Characterization and regulation of the *hb9/mnx1* beta-cell progenitor specific enhancer in zebrafish

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ARTICLE INFO

Article history:
Received for publication 6 February 2012
Revised 27 February 2012
Accepted 1 March 2012
Available online 9 March 2012

ABSTRACT

Differentiation of insulin producing beta-cells is a genetically well defined process that involves functions of various conserved transcription factors. Still, the transcriptional mechanisms underlying specification and determination of beta-cell fate are poorly defined. Here we provide the description of a beta-cell progenitor specific enhancer as a model to study initial steps of beta-cell differentiation. We show that evolutionary non-conserved upstream sequences of the zebrafish *hb9* gene are required and sufficient for regulating expression in beta-cells prior to the onset of *insulin* expression. This enhancer contains binding sites for paired-box transcription factors and two E-boxes that in EMSA studies show interaction with Pax6b and NeuroD, respectively. We show that Pax6b is a potent activator of endodermal *hb9* expression and that this activation depends on the beta-cell enhancer. Using genetic approaches we show that *pax6b* is crucial for maintenance but not induction of pancreatic *hb9* transcription. As loss of Pax6b or Hb9 independently results in the loss of *insulin* expression, the data reveal a novel cross-talk between the two essential regulators of early beta-cell differentiation. While we find that the known pancreatic E-box binding proteins NeuroD and Ngn3 are not required for *hb9* expression we also show that removal of both E-boxes selectively eliminates pancreatic specific reporter expression. The data provide evidence for an Ngn3 independent pathway of beta-cell specification that requires function of currently not specified E-box binding factors.

Introduction

The *Hb9/Hlxb9/Mnx1* gene encodes a homeobox transcription factor that in vertebrates has conserved functions in motoneuron differentiation and pancreas development (Broihier and Skeath, 2002; Ferrier et al., 2001; Grapin-Botton et al., 2001; Tanabe et al., 1998; Wendik et al., 2004). Furthermore, in humans, the heterozygous loss of *HLXB9* function is the major cause for sacral agenesis in patients with Currarino syndrome (Ross et al., 1998). Consistent with these diverse functions, *Hb9* genes in all vertebrates show a complex pattern of expression that is conserved in motoneurons, pancreatic endoderm, differentiating beta-cells and different mesodermal structures including the posterior notochord (Harrison et al., 1999; Li and Edlund, 2001; Li et al., 1999; Ross et al., 1998; Saha et al., 1997; Tanabe et al., 1998; Thaler et al., 1999; Wendik et al., 2004). While *Hb9* genes have been subject to various promoter studies (Lee et al., 2004; Nakano et al., 2005; Woolfe et al., 2005), not much is known about the molecular mechanism underlying their complex regulation. Previous studies focused on highly conserved non-coding sequence motifs identified in comparative genome promoter studies. The conservation is most striking in two 5 prime sequences, a distal 320 bp motif termed the A-box and a more proximal motif of about 150 bp termed the B-box (Nakano et al., 2005) (Fig. 1A). As these motifs show 74–94% identity in diverse species such as zebrafish and human it was suggested that they might have functions in gene regulation. Consistent with this notion, studies in mouse showed that the B-box and a second more proximal, mammalian-specific conserved motif termed MN^E are sufficient and required for targeting reporter expression to motoneurons (Lee and Pfaff, 2003; Lee et al., 2004; Nakano et al., 2005). However, sequence motifs related to MN^E have not been detected outside of mammals (Lee and Pfaff, 2003; Lee et al., 2004; Nakano et al., 2005) and mechanisms underlying *hb9* regulation in non-mammalian organisms and in endodermal and mesodermal structures remain to be investigated.

Here we use the zebrafish as a model to study *hb9* regulatory elements with a focus on pancreas-specific regulation. *Hb9* genes in zebrafish and mouse are expressed during two distinct phases of pancreas development (Harrison et al., 1999; Li et al., 1999; Wendik

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doi:10.1016/j.ydbio.2012.03.001

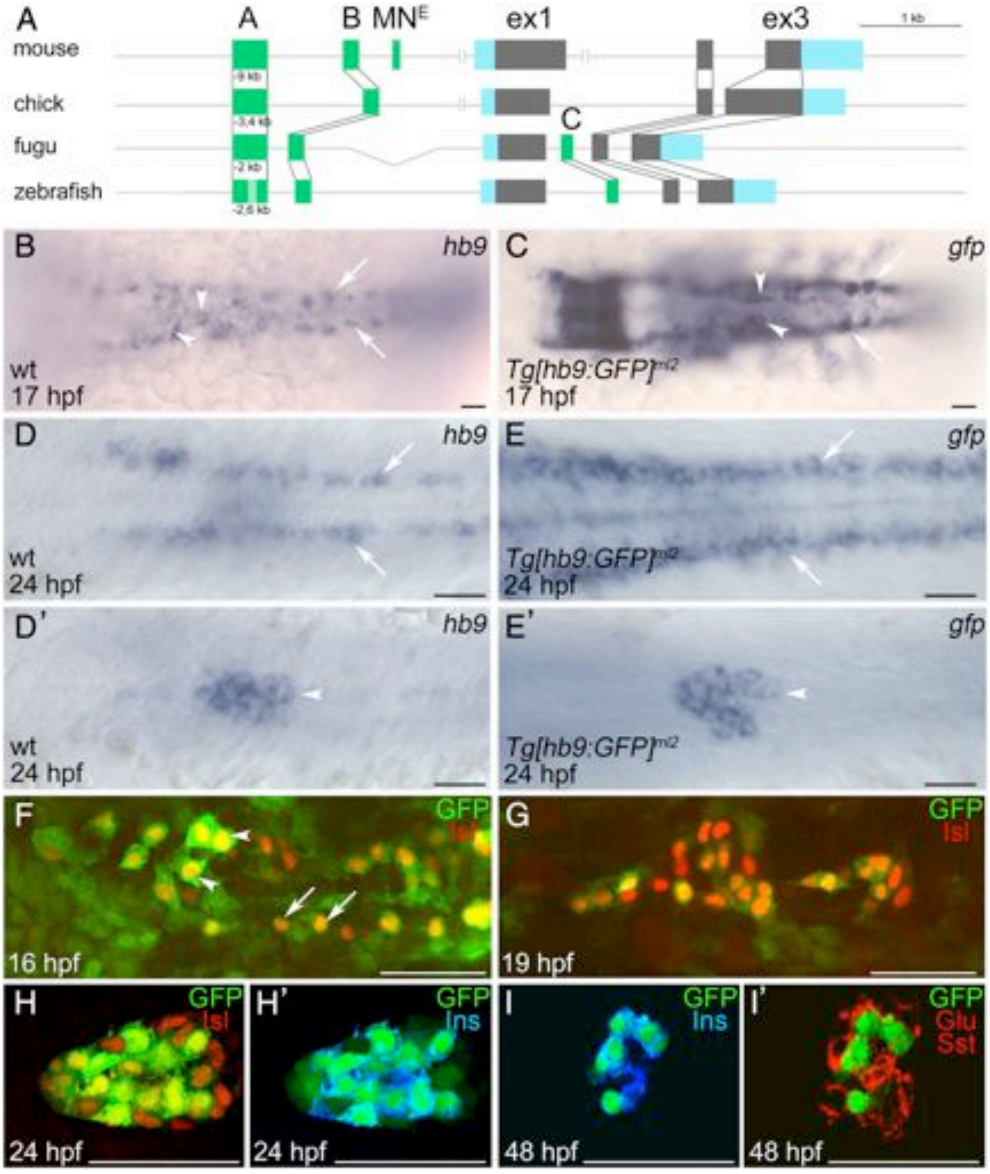

Fig. 1. Motoneuron and beta-cell specific GFP expression in *Tg[hb9:GFP]*^{ml2} embryos. (A) Schematic representation of the *hb9* gene from different species. Highlighted are conserved non-coding regions (dark green), and exons (coding sequences in grey, untranslated regions in blue). (B–I′) *In situ* hybridization analysis comparing mRNA expression of *hb9* (B, D, D′) and *gfp* (C, E, E′) in *Tg[hb9:GFP]*^{ml2} embryos at 17 hpf (B, C) and 24 hpf (D, E) reveals similar pattern in motoneurons and ventral interneurons (B–E, white arrows) and pancreas (B, C, white arrowheads). Asterisk in C marks ectopic *gfp* expression in rhombomere. D′, E′ show different focal planes of the embryos shown in D, E. (F–I) Confocal image projections of *Tg[hb9:GFP]*^{ml2} embryos labelled with GFP (green) and islet antibodies (red) at 16 hpf (F), 19 hpf (G) and 24 hpf (H) show overlapping expression of GFP and islet proteins in motoneurons (arrows) and in a subset of endocrine cells (arrowheads). (H–I′) Confocal image stacks of *Tg[hb9:GFP]*^{ml2} embryos labelled with antibodies for GFP and pancreatic hormones at 24 hpf (H′) and 48 hpf (I, I′) show that GFP overlaps with insulin (blue in H′, I) but not with glucagon (Glu) and somatostatin (Sst) (Glu + Sst in red, I′). Embryos are shown in dorsal (B–E) or ventral (F–I) view with anterior to the left. Scale bars in all figures correspond to 50 μm.

et al., 2004). Before onset of pancreatic organ morphogenesis, *Hb9* is expressed in the entire prospective pancreatic endoderm. With the onset of organ morphogenesis, expression is first downregulated and later reactivated specifically in differentiating beta-cells. Knockout studies in mouse revealed independent requirements for the early and late pancreatic *Hb9* expression in initiating pancreas morphogenesis and in regulating beta-cell maturation, respectively. In particular, one of the pancreatic anlagen, the dorsal bud, is missing in the mutants and the second, ventral bud, is formed but fails to establish mature beta-cells as revealed by reduced expression of the early beta-cell differentiation marker *insulin* and loss of the late differentiation marker *Glut2* (Harrison et al., 1999; Li et al., 1999). In zebrafish, morpholino knock down of *hb9* results in a strong reduction or even loss of embryonic *insulin* expression while early pancreas morphogenesis appears unaffected (Wendik et al., 2004). The data suggest that only the later beta-cell specific expression of *hb9* is associated with an evolutionary conserved function in beta-cell differentiation and maturation. Consistent with the requirement for *hb9* upstream of *insulin*, the onset of *hb9* expression in beta-cells precedes that of *insulin* (Wendik et al., 2004). This makes *hb9* one of the earliest specific markers for differentiating beta-cells and suggests that *hb9* expression is linked with initial steps of beta-cell specification.

Beta-cell formation requires activities of a conserved network of cross-regulating transcription factors which interact during pancreas development to establish the characteristic expression programmes for the distinct pancreatic cell types (Edlund, 2002; Habener et al., 2005; Kinkel and Prince, 2009; Schwitzgebel, 2001; Wilson et al., 2003). Genetic studies in mouse identified more than a dozen transcription factors that contribute to this network and that are required for proper differentiation of beta-cells. So far only a few of these

factors have been studied in the context of zebrafish pancreas development. Among these, the homeobox-containing proteins Nkx6.1 and Nkx6.2 are required for defining a beta-cell progenitor pool (Binot et al., 2010), while Nkx2.2a and Pax6b are essential for beta-cell fate determination (Pauls et al., 2007; Verbruggen et al., 2010). Ngn3/Neurog3, a basic helix–loop–helix (bHLH) factor that in mouse is a key regulator of endocrine cell differentiation was reported to be expressed in the larval but not the embryonic zebrafish pancreas (Apelqvist et al., 1999; Gradwohl et al., 2000; Jenny et al., 2002; Kinkel and Prince, 2009; Moro et al., 2009; Rukstalis and Habener, 2009; Schwitzgebel et al., 2000; Zecchin et al., 2007). The bHLH factor NeuroD/Beta2, that is expressed, similar to the situation in mouse (Huang et al., 2002; Itkin-Ansari et al., 2005; Naya et al., 1997), in all endocrine precursors, was found to be missing in *nkx6.1/nkx6.2* double morphants (Binot et al., 2010) and reduced in *nkx2.2a* single morphant zebrafish embryos (Pauls et al., 2007). Notably, reduction of *neuroD* expression in these morphants correlates with the loss of *insulin* expression, which is consistent with a possible function of NeuroD in beta-cell fate determination downstream of the *nkx* genes.

We have previously generated a *hb9:GFP* transgenic zebrafish line *Tg[hb9:GFP]*^{ml2} (also known as *Tg[mnx1:GFP]*^{ml2}) in which 3.1 kb of *hb9* genomic sequences, including the A- and B-box, are sufficient to recapitulate several aspects of *hb9* expression (Flanagan-Steet et al., 2005; Kimmel and Meyer, 2010). In contrast to the currently available *Hb9* mouse promoter lines (Arber et al., 1999; Nakano et al., 2005; Thaler et al., 1999; Wichterle et al., 2002), these fish also show reporter expression in the pancreas, specifically recapitulating the functionally conserved *hb9* expression in beta-cells. Here we provide the first characterization of an *hb9* beta-cell enhancer element and of upstream transcription factors mediating the beta-cell specific expression. We show that in zebrafish the *hb9* beta-cell enhancer element localizes to 478 bp of non-conserved distal sequences and we provide molecular and genetic evidence for a direct binding of the endocrine progenitor factors NeuroD and Pax6b on this element. Our data reveal distinct functions of the 478 bp motive in establishing and maintaining beta-cell specific expression, further indicating that the *hb9* pancreas enhancer represents a promising model for detailed analyses of the molecular regulation of beta-cell fate specification and determination.

Materials and methods

Transgenic lines and fish maintenance

The *Tg[ins:dsRed]* transgenic line (Shin et al., 2008) was used for injection of DNA constructs. AB strain and *Tg[hb9:GFP]*^{ml2} transgenic line were used for mRNA and morpholino injections. The *pax6b*^{sa0086} allele (Verbruggen et al., 2010) was obtained from the Sanger Center.

DNA constructs and promoter analysis

The zebrafish *hb9* promoter fragments were initially isolated from PAC clones that were identified by hybridizing with a radioactive labelled *hb9* cDNA. Southern analysis of the PAC DNA revealed the presence of the 5′ UTR of *hb9* on a 5.3 kb EcoRI fragment. This fragment was sub-cloned into pBluescript and sequenced on an ABIPrism 310 Genetic Analyzer (ABI Prism® BigDye™ Terminator, Applied Biosystems). Sequencing of the amplified *hb9* promoter revealed an 8 bp deletion at −1558 bp and 2 substitutions at −1046 and −717. Conserved promoter regions of the *hb9* gene were identified by comparing the zebrafish, mouse, human, chick and Fugu locus by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW2 Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Potential binding sites were identified by Match-1.0 Public, Patch 1.0 and P-Match-Public 1.0 Public programmes (www.gene-regulation.com).

hb9pG1 vector was generated by cloning the 3.1 kb *hb9* promoter into the pG1 vector (Gilmour et al., 2002) by using EcoRI/BamHI restriction enzymes. The *hb9*-deletion constructs were generated based on *hb9pG1* vector by using the indicated restriction enzymes. *hb9*^{NheI}*:GFP_pG1* was generated by introducing a NheI restriction site into the P^{E}/A-box boundary with the Site-Directed Quick-Change Mutagenesis Kit (Stratagene). From this constructs *hb9*$^{\Delta NheI}$*:GFP* and *hb9*$^{\Delta A}$*:GFP* were generated by cutting with EcoRI/NheI and NheI/EagI respectively and blunting with T4 Polymerase (Fermentas, EP0061) and religation. For generation of *hb9*$^{\Delta 1}$*:GFP* and *hb9*$^{\Delta 2}$*:GFP* constructs PCR was used to amplify the desired elements and PCR-fragments were cloned upstream of A-Box into *hb9*^{NheI}*:GFP_pG1*. *hb9*$^{\Delta 1}$*:GFP* was generated with hb9_EcoRI_F (GAATTCCTGAGGAAAACGCTTTCG) and NheI_R (TCGATCATGCTAGCACAAATGGGCCGTGC) primers, *hb9*$^{\Delta 2}$*:GFP* was generated with SalI_F (GTCGACATTTAAATTAGCCTGGCATCTGG) and dr_hb9_NheI_R (GCTAGCCAGTTTGCTTTGATAAGCC) primers. Quick-Change Mutagenesis Kit (Stratagene) was used for the generation of *hb9*^{Em}*:GFP*. The following primers were used: NrD_mut_F (CATTTAAATTAGCCTGGAGATCTGACATCGTCAATCACG), NrD_mut_R (CGTGATTGACGATGTCAGATCTCCAGGCTAATTTAAATG), E47mut_F (GTGTTCGGTTAAGTAAACTAGTGTTTTCGGGCTGGTTC) and E47mut_R (GAACCAGCCCGAAAACACTAGTTTACTTAACCGAACAC).

For generation of *hb9*^{PE229}*:gfp* DNA the P^{E229} fragment was PCR-amplified by using SalI_F and NheI_R primers and cloned into *hb9pG1*, linearized with SalI/StuI. For generation of *hb9*$^{\Delta AB}$*:GFP* plasmid *hb9*^{NheI}*:GFP_pG1* was digested with NheI/StuI and religated. For cloning of reporter vectors *P*^{E}*+hsp:GFP*, *A+hsp:GFP* and *P*^{E}*+A+hsp:GFP* zebrafish *hsp70* promoter was first amplified by PCR with the following primers: hsp70_StuI_F (AGGCCTTCAGGGGTGTCGCTTGGT) and hsp70_BamHI_R (GGATCCTTGTACAAACTTGGCAGGAA). *P*^{E}*+hsp:GFP* construct was generated by cloning *hsp70* PCR fragment into *hb9*^{NheI}*:GFP_pG1* after cutting of last with NheI/BamHI and blunt of NheI-site. For generation of *A+hsp:GFP hsp70* was cloned into *hb9*$^{\Delta NheI}$*:GFP_pG1* after serial cutting with AvrII/BamHI and blunt of AvrII site. For generation of *P*^{E}*+A+hsp:GFP* reporter construct *hsp*-PCR fragment was cloned into *hb9pG1* after digest with AvrII/BamHI. Before injection promoters, except vectors showed on Fig. 3, A, were cloned into pT2KXIG (Kawakami, 2004) vector by restriction with XhoI/BamHI (vector) and SalI/BamHI (promoter).

Generation of transgenic zebrafish

Tg[hb9:GFP]^{ml2} transgenic line was generated as described by Flanagan-Steet et al. (2005). For microinjection of *hb9:GFP*, *hb9*$^{\Delta A}$*:GFP*, *hb9*$^{\Delta NheI}$*:GFP*, *hb9*$^{\Delta 1}$*:GFP*, *hb9*$^{\Delta 2}$*:GFP*, *hb9*^{PE229}*:GFP*, *hb9*$^{\Delta AB}$*:GFP*, *P*^{E}*+hsp:GFP*, *A+hsp:GFP*, *P*^{E}*+A+hsp:GFP* and *hb9*^{Em}*:GFP* constructs the intact Tol2 vectors, containing corresponding *hb9* promoter were injected into 1-cell stage *Tg[ins:dsRed]* zebrafish embryos together with *transposase* mRNA. GFP-positive embryos were raised to adulthood and screened for transgene transmission to their progeny.

Microinjection of DNA, mRNA and morpholinos

For microinjection of constructs shown on Fig. 3, A, DNA were linearized with NotI and purified DNA were injected into one-cell stage zebrafish embryos. Knock-down experiments were performed with published splicing morpholinos against *pax6b* (Mo2Pax6b (in this text called MO^{pax6b}), 5′-TTGATTTGCACTCACGCTCGGTATG) (Verbruggen et al., 2010), ATG morpholino for *neuroD* (nrdMO) (in this text called MO^{neuroD}: TGACTTCGTCATGTCGGAACTCTAG) (Sarrazin et al., 2006), and ATG morpholino for *ngn3* (MO^{ngn3}: GGATCTTGGAGTCATTCTCTTGCAA). Morpholino was injected into 1-cell stage AB-strain or *Tg[hb9:GFP]*^{ml2} zebrafish embryos.

For induction experiments single-cell injection was performed at 16–32 cell stage in AB-strain, *Tg[hb9:GFP]*^{ml2} and *Tg[hb9*$^{\Delta NheI}$*:GFP]*. The following mRNA were injected: Casanova (cas_pCS2+), nucRFP

(h2b-RFP Red_pCS2+; obtained from W. Driever), Pax6b-HA (Pax6b-HA_pCS2+) and NeuroD-Flag (NeuroD-Flag_pCS2+). For mRNA synthesis vectors were restricted with NotI and mRNA was produced by using Sp6 mMessage mMachine Kit, Ambion.

EMSA

The following oligos were used for EMSA (only forward primers are listed, reverse primers present the same sequence but reverse and complement):

E^1 (CATTTAAATTAGCCTGGCATCTGGACATCGTCAATCACG)
E^{1m} (CATTTAAATTAGCCTGGAGATCTGACATCGTCAATCACG)
E^2 (GTGTTCGGTTAAGTAACACCTGGTTTTCGGGCTGGTTC)
E^{2m} (GTGTTCGGTTAAGTAAACTAGTGTTTTCGGGCTGGTTC)
P^{1+2+3} (GCATCTGGACATCGTCAATCACGGGCTCGTGACACCGAG)
P^{4+5+6}
(AGAGGGGACTCGTGGGTCATCTCGTTTAAAGCGCATTTTAAATTAC)
P^{7+8}
(TGTGTTCGGTTAAGTAACACCTGGTTTTCGGGCTGGTTCATCAGT)
P^{9+10} (TGAATCACTTAAGAGATAATCAGGCTTATCAAAGC)
P^{11} (ACTTTGTATTATGCAATGTTTTCAAAA)
P^{12} (GAAAGGAAAAAGCATGAAGCACTGAATC)
$P6^{13}$ (GTGTCCTTTAATGACTTTTATAGTTGG)
P^{14} (CCCATTTGTGCAATTATGATCGAAAATATTTA).

EMSA was performed according to a published protocol with some modifications (Hartl et al., 2006). Oligos were end-labelled using Adenosine 5′-triphosphate ([γ-^{32}P], PerkinElmer) and polynucleotide kinase (Fermentas, EK0031). Pax6b-HA protein and NeuroD-Flag/E47 complex were synthesised *in vitro* by TNT Quick-coupled Transcription/Translation kit (Promega) using the following plasmids: E47_pcDNA3 (obtained from S. Pfaff), Pax6b-HA_pCS2+, and NeuroD-Flag_pCS2+. After oligo labelling and protein synthesis the binding reaction was performed using 25,000 cpm for each oligo, 2 μl protein in the presence of 0.1 mg/ml of poly(dI-dC) and 1× binding buffer (5× buffer: 60 mM HEPES pH 7.9, 20 mM Tris–HCl pH 7.9, 300 mM KCl, 60% (v/v) glycerol, 5 mM DTT and 5 mM EDTA). The reaction was incubated for 45 min at room temperature. For competition assays, the unlabelled competitors were added at a 100-fold higher concentration and incubated at room temperature for 15 min, then the labelled probes were added and incubated for an additional 40 min. Binding was analysed by running a native 5% polyacrylamide gel, followed by overnight exposure of film (GE Healthcare, Amersham Hyperfilm™ MP) at −80 °C before development.

Whole-mount in situ hybridization and immunohistochemistry

Whole mount *in situ* hybridization using antisense RNA-probes labelled with digoxigenin (DIG) was performed as previously described (Hauptmann and Gerster, 2000). The following probes were used: *insulin* (obtained from W. Driever), *hb9* (Wendik et al., 2004) and *gfp* (pG1) (Gilmour et al., 2002). RNA probes were prepared by using T7 or Sp6 RNA-Polymerases and DIG-Labelling Mix (Roche) according to the supplied protocol. All probes were detected using Anti-digoxygenin-AP FAB fragments (Roche).

For immunohistochemistry embryos were fixed for 1 h at RT in 4% PFA and 1% DMSO. After fixation embryos were blocked with blocking buffer for 1 h at RT and incubated with primary antibodies overnight at +4 °C. The following day embryos were washed 3 times for 15 min in 1× PBST and incubated in blocking buffer for 1 h at RT. Embryos were then incubated with secondary antibodies overnight at +4 °C. Blocking and staining solutions contained 1% sheep serum, 1% BSA, 1% triton X-100, and 1% DMSO in PBS. Primary antibodies: anti-GFP (1: 200, rabbit IgG, Torrey Pines Biolabs), Islet-I Homeobox (1: 200, mouse IgG, 39.4D5, DSHB) and polyclonal guinea pig anti-insulin (1: 200, Dako), polyclonal rabbit anti-human glucagon (1:200, Dako) and polyclonal rabbit anti-somatostatin (1:200, Dako); secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 633 goat anti-guinea pig IgG (1: 1000, Invitrogen) and mouse Goat IgG, Cy3-conjugated (1:1000, Chemicon).

Imaging

Confocal image stacks were generated with Zeiss LSM Exciter5 microscope equipped with the following lasers: 25 mW Argon laser (468, 488, 514 nm), 1 mW 543 nm HeNe-laser, 5 mW 633 nm HeNe-laser and a 25 mW 405 nm diode-laser. Fluorescence detection was performed with the following dichroic beam splitters and filters: HFT405/488/543/633, NFT545, BP 505–530 (GFP and Alexa 488), LP 560 (Cy3) and LP 650 (Alexa 633). A Leica DM 6000 microscope was used for DIC images of whole mount *in situ* staining. AdobePhotoshop was used for image arrangement and adjustment.

Results

3.1 kb of 5′ flanking region of the zebrafish hb9 gene contains motoneuron and beta-cell specific regulatory elements

Tg[hb9:GFP]^{ml2} transgenic fish that contain 3.1 kb of 5 prime upstream non-coding region of the zebrafish *hb9* gene fused to the *gfp* cDNA show GFP expression in motoneurons and in the pancreas. In order to clarify whether the reporter expression recapitulates the endogenous *hb9* expression, we compared *hb9* and *gfp* mRNA expression at different stages of development (Fig. 1, Supplemental Fig. 1). Whole mount mRNA *in situ* hybridisation analyses of *Tg[hb9:GFP]^{ml2}* embryos between 10 and 24 h post fertilization (hpf) revealed similarities but also differences between *hb9* and *gfp* expression. In particular we found that *gfp* expression in *Tg[hb9:GFP]^{ml2}* does not recapitulate *hb9* expression in early endoderm, notochord, hypochord and swim bladder and that *gfp* is expressed ectopically in various tissues including hindbrain, eyes and anterior somites (Supplemental Fig. 1). The differences in expression suggest that additional enhancer and silencer elements outside the 3.1 kb fragment must contribute to the complex *hb9* expression. However, consistent with the expected overlapping expression in motoneurons and beta-cells, we observed similar patterns of robust *gfp* and *hb9* expression in the spinal cord and the primary pancreatic islet (Figs. 1B–E′). Immunofluorescence analyses of 12–24 somite stage *Tg[hb9:GFP]^{ml2}* embryos (16–24 hpf) with an anti-islet antibody, a marker for primary neurons and pancreatic endocrine cells, confirm that GFP is expressed in spinal motoneurons (Fig. 1F) and in a subpopulation of the early endocrine cells (Figs. 1F, G). To further define which endocrine cell types are expressing GFP, we stained 24 hpf and 48 hpf *Tg[hb9:GFP]^{ml2}* embryos with antibodies against insulin and with a mixture of the alpha and delta-cell markers anti-glucagon and anti-somatostatin (Figs. 1H–I′). Overlapping expression of GFP and insulin but not GFP and glucagon/somatostatin at 48 hpf shows that pancreatic expression in *Tg[hb9:GFP]^{ml2}* is specific to beta-cells (Figs. 1I, I′). In summary, this shows that cis-regulatory enhancer elements controlling the highly conserved *hb9* expression in motoneurons and beta-cells are present within the 3.1 kb of the 5 prime upstream non-coding region.

In sequence alignments of 5 prime *Hb9* regions from zebrafish, fugu (*Takifugu rubripes*), chicken, mouse and human, the A-box and B-box are found 2.6 and 2 kb upstream of ATG, respectively (Fig. 1A). To localize the specific regulatory elements required for pancreatic and motoneuron gene expression, we generated *hb9:GFP*-reporter constructs lacking the A and/or B box and flanking genomic regions (Fig. 3A, Supplemental Fig. 2A), and analysed their activities in embryos injected with the corresponding DNA. Such transient reporter assay results in mosaic F0 embryos in which only a small

portion of the cells can show reporter expression due to the mosaic distribution of the injected DNA. To distinguish beta-cell specific expression from potential nonspecific expression in the surrounding cells, the DNA constructs were injected into *Tg[ins:dsRed]* transgenic embryos in which beta-cells express dsRED, and analysed for overlapping expression of GFP and dsRED. In independent control injections with the 3.1 kb *hb9:GFP* construct, we observed GFP/dsRED double labelled cells in 25–40% of the injected embryos (Supplemental Figs. 2B–D). Motoneuron specific expression, as judged by the presence of GFP labelled peripheral axonal projections, was observed in 80–100% of the embryos. No GFP expression could be observed after injection of constructs that were either missing the A- and B-Box-containing distal half or that were missing the proximal part between the B-box and HindIII restriction site (Fig. 2A, Supplemental Fig. 2A). Most importantly, GFP expression in motoneurons was observed following injection of all constructs containing proximal sequences together with the B-box, while beta-cell specific GFP expression was only observed with constructs that contained the most distal part including the A-box (Fig. 2A, Supplemental Fig. 2A).

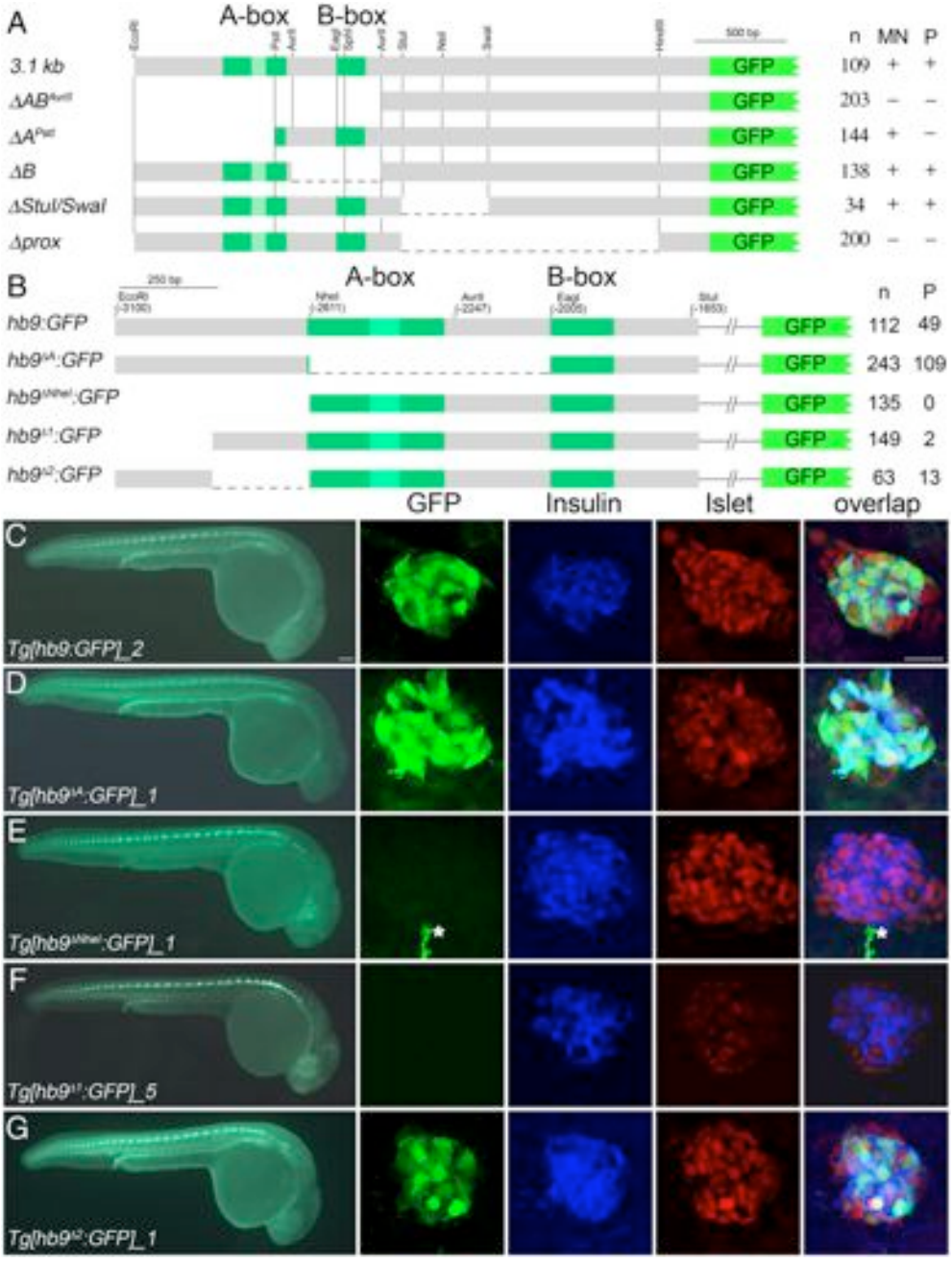

Fig. 2. Pancreas specific GFP expression in *Tg[hb9:GFP]* embryos requires distal non-conserved *hb9* promoter sequences. (A) Scheme of non-Tol2 *hb9*-reporter constructs (left) and results of the transient reporter assay (right, indicated are the numbers (n) of embryos showing presence (+) or absence (−) of GFP signal in spinal cord (SC) and pancreas (P)). (B) Scheme of Tol2 *hb9*-reporter constructs (left) and results of transient reporter assays (right, indicated are the numbers of GFP positive embryos (n) and of embryos with GFP positive beta-cell (P)). (C–G) GFP expression in representative transgenic lines at 28 hpf in whole embryos (left panel) and in fixed tissue (right panel) stained with antibodies for GFP (green), insulin (blue) and islet (red). The right panel shows confocal image projections and the corresponding overlays (white asterisk in E marks a motor axon). Pancreatic GFP expression is found for reporter constructs lacking either the A-box (*Tg[hb9*$^{\Delta A}$*:GFP]*, C) or the 249 bp flanking the A-box (*Tg[hb9*$^{\Delta 2}$*:GFP]*, F), but not for constructs lacking the most distal 229 bp (*Tg[hb9*$^{\Delta Nhe1}$*:GFP]*, D; *Tg[hb9*$^{\Delta 1}$*:GFP]*, E). Scale bar corresponds to 100 μm (whole embryos) and 20 μm (confocal image stacks). For further details on the GFP expression in all transgenic reporter lines see Supplementary Table 1.

In summary, the distal sequences that include both conserved motifs can direct tissue specific *hb9* expression in the pancreas and motoneurons and the proximal non-conserved sequences seem to be important either for basal expression or to enhance expression levels. Furthermore, our analyses suggest a conserved role for the B-box in regulating motoneuron specific expression and provide a first hint for the presence of an *hb9* beta-cell specific enhancer distal to the B-box.

478 bp of non-conserved sequences are required and sufficient for beta-cell specific expression

To better characterize the beta-cell enhancer we next generated constructs in which distal sequences were deleted (Fig. 2B). For these experiments, we used Tol2-mediated transgenesis in order to increase the likelihood of having beta-cell expression in our transient assays and to enhance recovery of stable transgenic lines. Surprisingly,

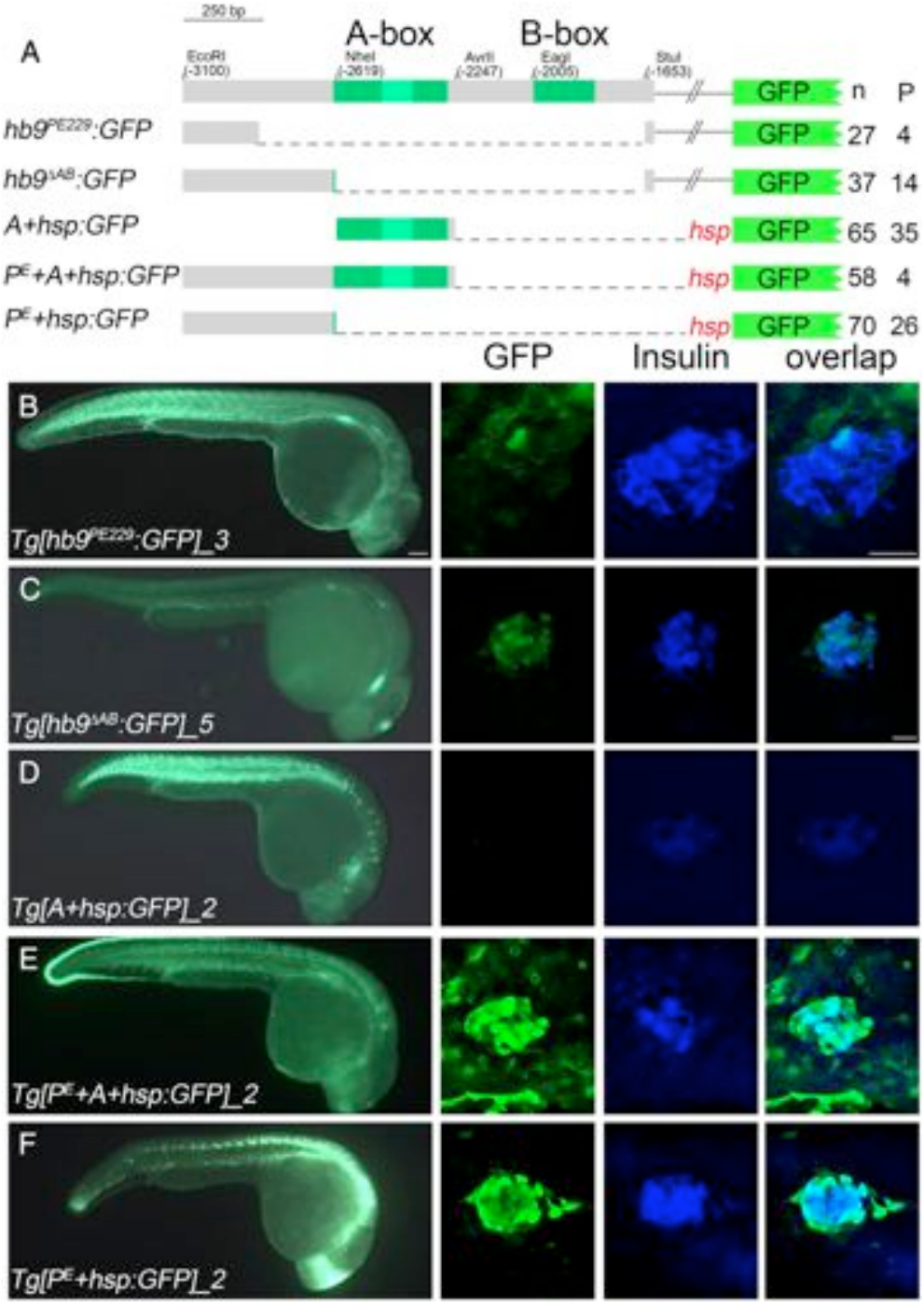

Fig. 3. 478 bp of proximal *hb9* promoter sequences are sufficient for beta-cell specific regulation. (A) Overview of transient GFP reporter experiments with a scheme of deletion constructs on the left and corresponding numbers of GFP positive embryos (n) and embryos expressing GFP in the pancreas (P) on the right side. (B–F) GFP expression in representative transgenic lines at 28 hpf in whole embryos (left panel) and in fixed tissue (right panel) stained with antibodies for GFP (green) and insulin (blue). The right panel shows confocal image projections and the corresponding overlays (right-most panel). Scale bar corresponds to 100 μm (whole embryos) and 20 μm (confocal image stacks). For more details see also Supplementary Table 2.

loss of the A-box (*hb9$^{\Delta A}$:GFP*) did not lead to loss of reporter expression in beta-cells as identified by insulin antibody staining. Analyses of transiently expressing embryos revealed a similar frequency of pancreas specific GFP expression for *hb9$^{\Delta A}$:GFP* and *hb9:GFP* constructs (Fig. 2B). To confirm the transient expression data, we isolated a total of 12 independent stable integrations for *Tg[hb9$^{\Delta A}$:GFP]* and 2 independent alleles for *Tg[hb9:GFP]* (Figs. 2C, D, Supplemental Table 1). From those, nine of the *Tg[hb9$^{\Delta A}$:GFP]* and both *Tg[hb9:GFP]* showed robust GFP expression in beta-cells and in motoneurons (Figs. 2C, D). The data suggest that the A-box is not required for pancreas and for motoneuron specific reporter expression. Notably, these transgenic lines showed a highly variable reporter expression in gut endoderm, epidermis, somites and nervous system, indicating that our reporter constructs are strongly influenced by positional effects (Supplemental Table 1).

The lack of beta-cell specific GFP expression in *ΔA^{PstI}:GFP* transient line (Fig. 2A) but not in *hb9$^{\Delta A}$:GFP* (Fig. 2B) suggests that the non-conserved distal part of the 3.1 kb fragment must mediate beta-cell specific expression. Consistent with this notion, we found that the loss of the distal 478 bp in *hb9$^{\Delta NheI}$:GFP* injected embryos abolishes beta-cell specific expression in transient reporter assays and also in *Tg[hb9$^{\Delta NheI}$:GFP]* stable transgenic allele (Figs. 2B, E, Supplemental Table 1). We therefore conclude that the non-conserved distal promoter sequences contain essential elements for pancreas specific expression.

To define the position of these elements more precisely, we generated constructs lacking either the distal or proximal halves of the non-conserved sequences between −3097 and −2868 bp (*hb9$^{\Delta 1}$:GFP*) and between −2868 and −2619 (*hb9$^{\Delta 2}$:GFP*), respectively (Fig. 2B). In transient reporter assays, *hb9$^{\Delta 1}$:GFP* DNA failed to induce pancreatic GFP expression (1.3% of the embryos showed overlapping GFP and dsRED expression, n = 149, Fig. 2B) while the *hb9$^{\Delta 2}$:GFP* (overlapping expression in 21% of embryos, n = 63, Fig. 3B) induced pancreas specific GFP expression, although the frequency was reduced as compared to the *hb9:GFP* control construct (43%, n = 112, Fig. 2). Results with stable transgenic lines were consistent with the transient assays as 6 out of 7 *Tg[hb9$^{\Delta 1}$:GFP]* alleles were negative for GFP expression in the pancreas while 7 out of 10 *Tg[hb9$^{\Delta 2}$:GFP]* alleles showed specific expression in beta-cells (Figs. 2F, G, Supplemental Table 1).

To determine if the distal sequences are sufficient to activate *gfp* expression in beta-cells, we generated two constructs in which the most distal 229 bp (*hb9^{PE229}:GFP*) or 478 bp (*hb9$^{\Delta AB}$:GFP*) were directly fused to the proximal part of the *hb9* promoter (Fig. 3A). Both constructs induced pancreas specific expression in transient assays, indicating that the distal sequences contain the pancreas enhancer. However, in stable transgenic lines only *Tg[hb9$^{\Delta AB}$:GFP]* alleles showed beta-cell specific GFP expression (Fig. 3C, Supplemental Table 2). While four of the nine *Tg[hb9^{PE229}:GFP]* alleles showed weak expression in the pancreas, immunofluorescence analyses revealed that in three alleles GFP expression did not overlap with insulin (Fig. 3B, Supplemental Table 2). These data suggest that the most distal 229 bp are required but not sufficient for beta-cell specific gene expression. They further suggest that the 478 bp of non-conserved distal *hb9* sequences, that we hereafter refer to as P^{E}, contain multiple elements that together regulate beta-cell specific expression.

We next asked if the distal 478 bp contain all sequence motifs sufficient for beta-cell specific expression. To answer this question, we generated constructs in which the 478 bp P^{E} fragment was cloned in front of the zebrafish *hsp70* promoter linked to a *gfp* cDNA (*P^{E}+hsp:*

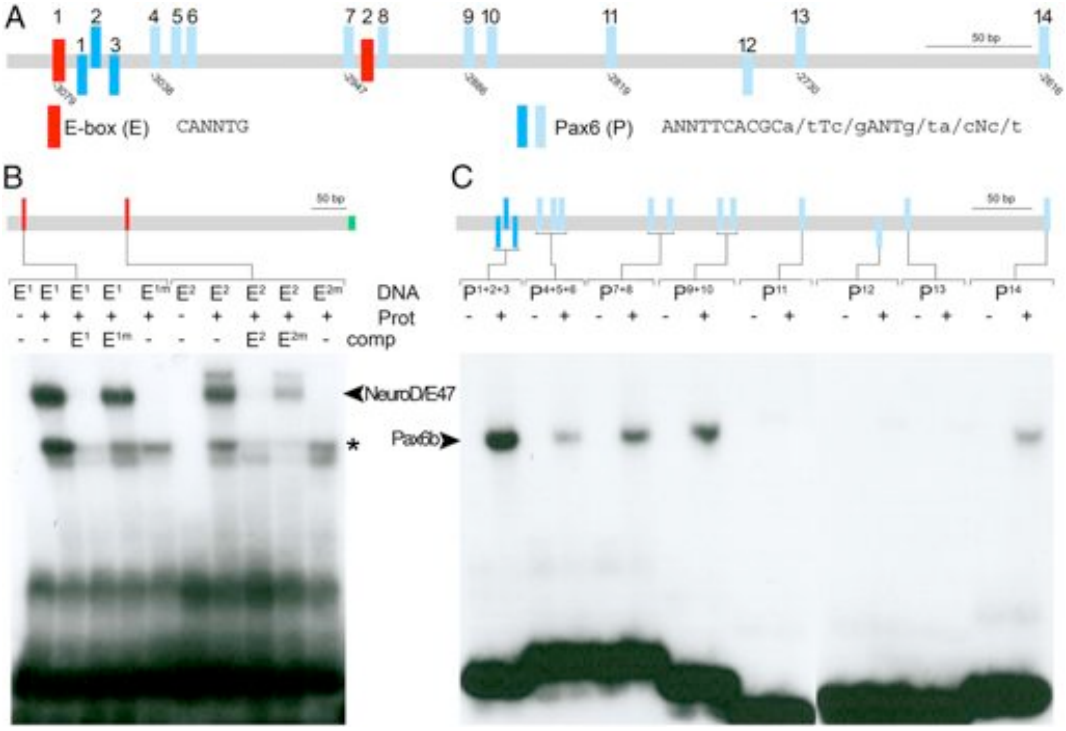

Fig. 4. *In vitro* binding of NeuroD and Pax6b to P^{E} specific sequence motives. (A) Schematic representation of potential binding sites for the transcription factors NeuroD (E-box in red) and Pax6 (blue) within the *hb9* pancreas enhancer as revealed by bioinformatic sequence analyses. Pax6 sites with a core match between 0.76 and 0.9 are shown in light blue, and sites with a core match between 0.9 and 1 are shown in dark blue (core = TCACG). Orientation of Pax6 sites is indicated by position relative to the line (above: positive DNA strand; below: negative DNA). (B) EMSA studies show binding of *in vitro* synthesized NeuroD/E47 dimers (Prot) to oligonucleotides (DNA) spanning the E-boxes (E^{1}, E^{2}) but not to corresponding oligonucleotides with mutated E boxes (E^{1m} and E^{2m}). Competitor assays with unlabelled oligonucleotides (comp, as indicated) revealed efficient competition by E^{1} and E^{2} but not by E^{1m} and E^{2m}. Notably, unlabelled E^{2m} reduced binding of labelled E^{2} to NeuroD/E47, suggesting that E^{2} binds NeuroD/E47 with lower affinity as compared to E^{1}. Bands indicated by the asterisk presumably correspond to monomeric DNA–protein complexes. (C) EMSA studies with *in vitro* synthesized Pax6b protein (Prot) and oligonucleotides spanning individual or multiple potential Pax6b sites (as indicated). EMSA signals could be detected for P^{1+2+3}, P^{4+5+6}, P^{7+8}, P^{9+10}, P^{14} but not for P^{11}, P^{12} and P^{13} (positions of protein-bound DNAs are indicated by arrowhead).

GFP, Fig. 3A). Based on the weak beta-cell specific reporter expression in *Tg[hb9*$^{\Delta AB}$*:GFP]* embryos we further asked if the A-box contributes to pancreas expression. To test this possibility we generated constructs in which *hsp:GFP* was combined with either the A-box only (*A+hsp:GFP*) or with P^E and A-box (*P^E+A+hsp:GFP*, Fig. 3A). In transient assays 54% of the *P^E+hsp:GFP* (n=65), 37% of the *P^E+A+hsp:GFP* (n=70) but only 7% of the *A+hsp:GFP* (n=58) injected embryos showed beta-cell specific expression (Fig. 3A). Consistent with an A-box independent beta-cell specific regulation by the *P^E*, pancreatic GFP expression in three out of four *Tg[P^E+hsp:GFP]* lines was very similar to that in the two *Tg[P^E+A+hsp:GFP]* alleles while it was missing in all three *Tg[A+hsp:GFP]* alleles (Figs. 3D–F, Supplemental Table 2). Overall, the data show that the P^E, and in particular the distal half of the P^E, plays a critical role in beta-cell regulation and that the P^E alone is sufficient for regulating beta-cell specific expression.

Direct binding of Pax6b and NeuroD to multiple sites within the hb9 P^E

Bioinformatic analyses of the P^E sequence revealed several potential binding sites for transcription factors with well described function in early endocrine cell differentiation, including consensus sequences for Pax6 (Epstein et al., 1994; Zhou et al., 2002), E-box-binding proteins such as Ngn3/NeuroD, and RAR (Mangelsdorf and Evans, 1995), among others. For our initial studies we focused on the endocrine progenitor factors Pax6b and NeuroD, for which 14 and 2 potential binding sites were found in the P^E, respectively (Fig. 4A). To determine if these consensus sites are recognized by the corresponding transcription factors, we first tested direct binding in electrophoretic mobility shift assay (EMSA) with *in vitro* translated proteins and radioactively labelled synthetic double-stranded oligonucleotides. To confirm the specificity of the observed interaction, we performed competition assays with unlabeled self-competitors and also analysed whether the translated proteins recognized mutated consensus motifs.

To test NeuroD binding activities, the potential binding sites (E-box motifs E^1 and E^2) were incubated with a mixture of NeuroD and its dimerization partner E47 (Fig. 4B). We found that NeuroD/E47 heterodimers bind to both E-boxes and that this interaction is specific, as NeuroD/E47 bound neither of the mutated E-boxes and unlabeled self-competitors efficiently prevented interaction with the labelled probes.

A slightly different strategy was used to test the 14 Pax6 paired box consensus sites (termed P^1–P^{14}). As ten of these sites are separated from their next neighbours by less than 12 nucleotides, we not only used DNA oligos containing individual paired box consensus sites but in some cases also oligos with additional neighbouring consensus sites (Fig. 4C). The EMSA studies revealed strongest signals for the oligonucleotides containing the most distal binding sites (P^{1+2+3}) and slightly weaker signals for P^{4+5+6}, P^{7+8}, P^{9+10} and P^{14}. The potential binding sites P^{11}, P^{12} and P^{13} were not recognized by Pax6b protein (Fig. 4C). Notably, all Pax6b-interacting oligonucleotides showed a similar shift independent of whether one, two or three potential Pax6 binding sites were present on the oligonucleotides. We therefore reasoned that a single Pax6b protein binds each oligonucleotide. EMSA studies with mutated versions of P^{1+2+3} and P^{9+10} reveal different binding affinities for the neighbouring Pax6 consensus motifs, which could explain these results (Supplemental Fig. 3). In particular we find that P^3 and P^9 are dispensable for efficient binding to Pax6b while intact P^{1+2} and P^{10} sites are required for efficient competition with labelled P^{1+2+3} and P^{9+10}, respectively (Supplemental Fig. 3). As steric hindrance might prevent simultaneous binding of two

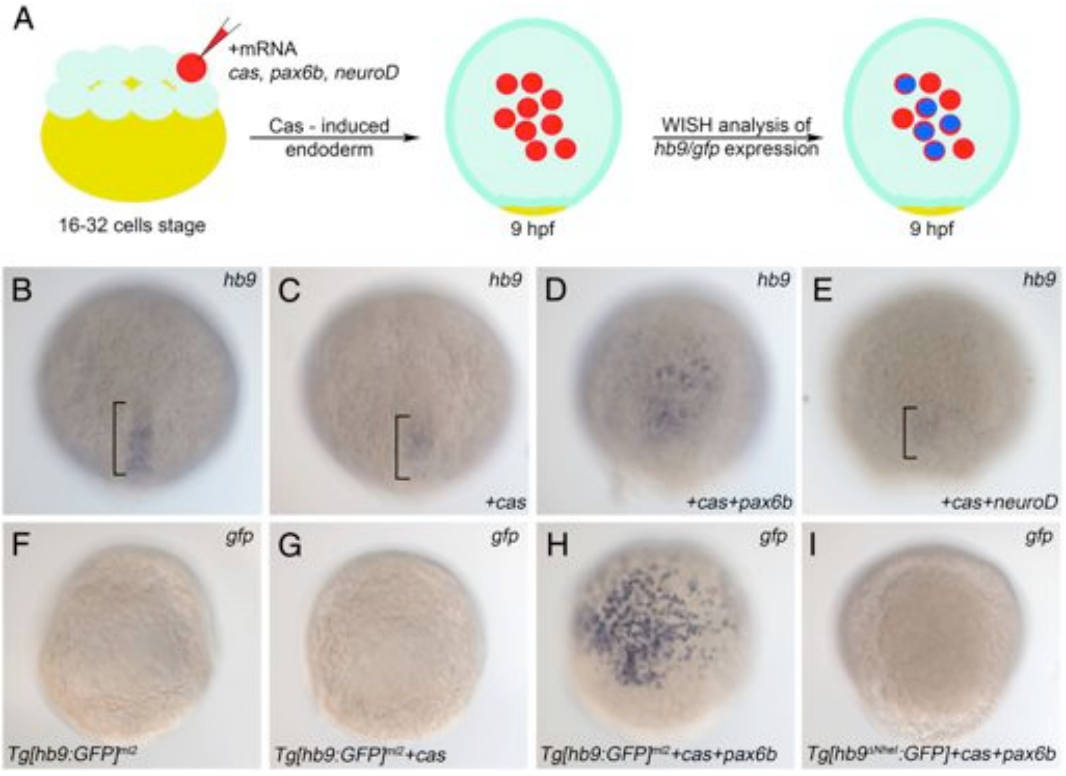

Fig. 5. Pax6b but not NeuroD is sufficient to induce endodermal *hb9* expression. (A) Schematic of the induction experiment: One cell of a 16–32 cell embryo stage was injected with mRNA encoding either Cas alone or a mixture of mRNAs encoding Cas together with NeuroD or Pax6b. Injected embryos were fixed 8 h later (9 hpf) and analysed by mRNA *in situ* hybridisation. (B–E) Expression of *hb9* mRNA in uninjected control embryos (B) and in embryos injected with *cas* (C), *cas/paxb6* (D) and *cas/neuroD* (E) mRNA. Note that only *cas/pax6b* mRNA injected embryos (D) show ectopic *hb9* signals. Signals in posterior notochord (bracket in B, C, E) represent endogenous *hb9* expression, while reduced signals in the *cas* (C), and *cas/neuroD* (E) injected embryos correlate with the developmental delay caused by the injection. (F–I) Expression of *gfp* mRNA in transgenic reporter lines in which *gfp* is controlled by P^E-containing (*Tg[hb9:GFP]^{ml2}* in F–H) or P^E-deleted (*Tg[hb9$^{\Delta Nhel}$:GFP]* in I) *hb9* promoter-sequences. Uninjected (F) and *cas*-mRNA injected *Tg[hb9:GFP]^{ml2}* embryos (G) show no *gfp* signals while injection of *cas/pax6b* mRNA results in a robust *gfp* induction (H). Lack of *gfp* signals in *cas/pax6b* injected *Tg[hb9$^{\Delta Nhel}$:GFP]* shows that *gfp* induction by Pax6b is P^E-dependent.

Pax6b factors on neighbouring P1 and P2 binding sites, the data suggest efficient interaction of Pax6b with either P1 or P2 and with P10.

In summary, our EMSA data show that at least two transcription factors with functions in endocrine progenitors, Pax6b and NeuroD, can directly bind to the binding sites in the *hb9* P^E. Furthermore, they reveal that the sequences with highest *in vitro* binding affinity for these proteins are localized in the distal part of P^E, which is most critical for beta-cell specific expression as based on our promoter analyses.

Ectopic induction of hb9 expression by Pax6b but not by NeuroD

Next we tested if overexpression of Pax6b and NeuroD encoding mRNA is sufficient to induce expression of either *hb9* mRNA in wild type embryos or *gfp* mRNA in our transgenic reporter lines. In order to target the injected mRNA specifically to endoderm we took advantage of a previously described approach for cell-autonomous induction of endoderm by Casanova/Sox32 (Kikuchi et al., 2001; Stafford et al., 2006). In these experiments, a mixture of mRNAs encoding Cas together with Pax6b or NeuroD and the lineage tracer nucRFP (data not shown) was injected into a single-cell of 16–32 cell stage embryos (Fig. 5A). Due to Cas activity, the progeny of the injected cells adopted an endodermal fate which can then be analysed for *hb9* or *gfp* mRNA expression. Based on the short half-life of injected mRNA we choose 9 hpf for the fixation of embryos. Our analyses revealed ectopic activation of *hb9* in 63% (n = 11, Figs. 5B–D, Supplemental Table 3) of the *pax6b* mRNA injected embryos while *neuroD* mRNA had no effect (n = 13, Fig. 5E, Supplemental Table 3). To test if the Pax6b responsive elements are present on the 3.1 kb promoter fragment, we next injected the *cas/pax6b* mixture into *Tg[hb9:GFP]*^{ml2} transgenic embryos. These embryos showed robust expression of GFP protein as well as *gfp* mRNA, while non-injected embryos as well as embryos injected only with *cas* mRNA were *gfp* negative (Figs. 5F–H, Supplemental Table 3). To clarify whether Pax6b responsiveness requires the P^E, we next injected *pax6b/cas* mRNA in *Tg[hb9*$^{\Delta NheI}$*:GFP]* transgenic embryos, in which the P^E is missing. No *gfp* expression could be observed in these embryos (Fig. 5I). Altogether, these data show that Pax6b is sufficient to activate endoderm specific *hb9* expression and that this activation is mediated through the P^E.

Pax6b is required for maintenance of hb9 expression in beta-cells

To test whether NeuroD and Pax6b are required for endogenous regulation of pancreas-specific *hb9* expression we performed knockdown analyses with published morpholinos. We used a *pax6b* splicing morpholino which has been shown to cause loss of *insulin* expression (Verbruggen et al., 2010) and a *neuroD* morpholino that was used to study *neuroD* function of the lateral line organ (Sarrazin et al., 2006). Analyses of 24 hpf embryos injected with *neuroD* morpholino revealed no difference in the staining intensities or the number of *hb9* expressing pancreatic cells between *neuroD* morphants (19.2 cells/embryo, n = 5) and wild type (wt) embryos (20.8 cells/embryo, n = 5) (Figs. 6A–D, G). Together with the gain-of-function analyses, this suggests that NeuroD is neither required nor sufficient for early *hb9* regulation.

In contrast, *pax6b* morphants showed a reduced number of *hb9* expressing pancreatic cells (14.3 cells/embryo, n = 9) and expression levels per cell appear much weaker as revealed by comparing staining intensities in the spinal cord and the pancreas of morphants and wild type embryos (Figs. 6A, B, D, F, G). We further noted that *pax6b* morpholino had only a minor effect on the pancreatic GFP protein expression levels in 24 hpf injected *Tg[hb9:GFP]*^{ml2} embryos (data not shown). GFP is a very stable protein that remains in the cells even when mRNA expression has stopped. We therefore reasoned that the difference between the GFP protein and *hb9* mRNA expression levels in the beta-cells of 24 hpf *pax6b* morphants could indicate a defect in maintenance rather than induction of *hb9* mRNA expression. To test this, we followed *hb9* expression and *gfp* mRNA in *pax6b−/−* mutants from 18 hpf until 36 hpf (Figs. 6H–O, Supplemental Fig. 4). At 18 hpf, *hb9* expression in *pax6b* mutants is similar to that in wild

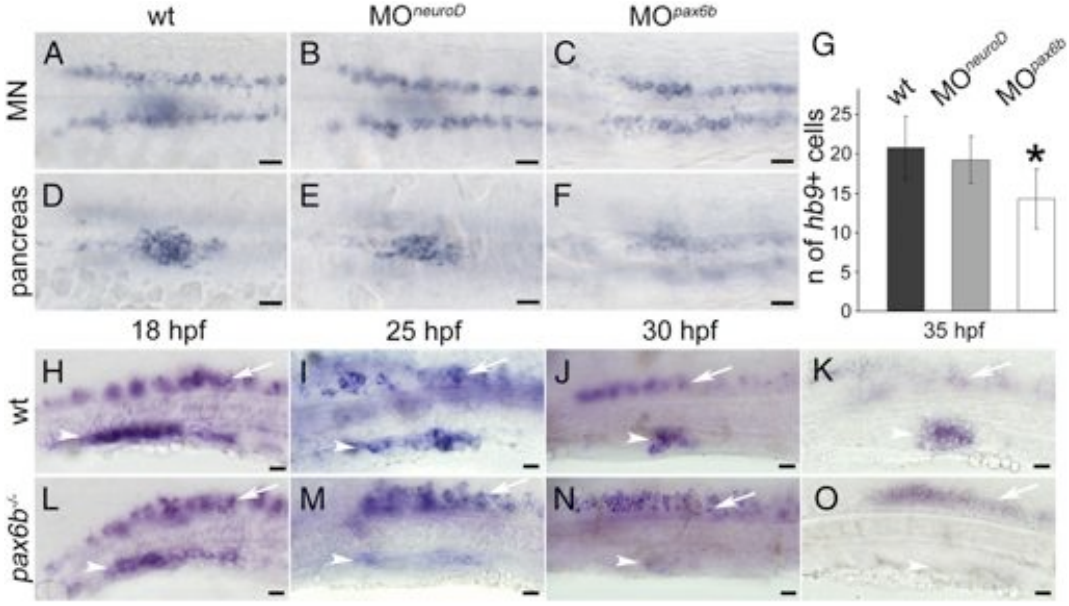

Fig. 6. *hb9* expression is initiated but not maintained in *Pax6b* deficient embryos. (A–F) Expression of *hb9* mRNA at 24 hpf in motoneurons (MN in A, C, E) and in the pancreatic islet (pancreas in B, D, F) of uninjected wild type (wt) embryos (A, B) and embryos injected with *neuroD* (C, D) or *pax6b* morpholinos (E, F). Note that pancreatic *hb9* expression is reduced in *pax6b* but not in *neuroD* morphants while motoneuron specific expression in the same embryos is not affected. Embryos are shown in dorsal view with anterior to the left. Scale bars in all figures correspond to 20 μm. (G) Number of *hb9* positive cells in the pancreas of the wild type (wt), *neuroD* (MO^{neuroD}) and *pax6b* (MO^{pax6b}) morphants. (p-value for neuroD morphants 0.19429, p-value for pax6b morphants 0.00467). (H–O) *hb9* expression in wild type embryos (H, I, J, K) and *pax6b* mutants (L, M, N, O) at 18 hpf (H, L), 25 hpf (I, M), 30 hpf (J, N) and 35 hpf (K, O). Note that *hb9* expression in pancreas (white arrowhead) decreases during development in mutants, whereas expression in motoneurons (white arrow) is not affected. Embryos are shown in lateral view with anterior to the left. Scale bars in all figures correspond to 20 μm.

type (Figs. 6H, L), whereas between 25 hpf and 30 hpf absence of Pax6b leads to the progressive reduction of *hb9* expression in the pancreas (Figs. 6I, J, M, N). At 35 hpf no *hb9* expression could be detected in the endocrine pancreas (Figs. 6K, O). Importantly, at all stages analysed, motoneuron *hb9* expression in *pax6b* −/− mutants was not affected (Figs. 6H–O), revealing the specific role for Pax6b in regulation of *hb9* in the endocrine pancreas. Taken together, our results show that Pax6b is required for maintenance but not initiation of *hb9* expression.

Induction of pancreatic hb9 expression requires E-box elements

While our studies suggest that NeuroD is not required for *hb9* expression, they do not exclude the option that other bHLH proteins regulate *hb9* transcription through interaction with the two E-box elements. A promising candidate for such factors is Ngn3 even though *ngn3* expression has not been reported in the early zebrafish pancreas (Kinkel and Prince, 2009; Zecchin et al., 2007). As expression levels of *ngn3* may be below detection limit, we used a genetic approach to determine *ngn3* functions in embryonic *hb9* transcription. However, comparison of pancreatic *hb9* or *gfp* expression revealed no significant differences between uninjected embryos, and embryos that were injected with *ngn3* morpholinos alone or with a combination of *neuroD* and *ngn3* morpholinos (Figs. 7A, D, C, F, G, data not shown). This shows that neither *ngn3* nor *neuroD* is required for early pancreatic *hb9* expression.

To test more directly for a function of the E-boxes we asked if they are required for beta-cell specific reporter expression. Injection of the *hb9^{Em}:GFP* reporter construct in which both E-boxes were mutated by site-directed mutagenesis resulted in only 1 out of 120 embryos (0.8%) showing GFP expression in the pancreas. Further, analyses of two *hb9^{Em}:GFP* transgenic fishlines revealed a complete absence of GFP expression in the endocrine pancreas (Fig. 7I). As motoneuron specific expression in these embryos was similar to that of *Tg[hb9:GFP]^{ml2}* embryos, our data suggest a specific requirement of one or several unspecified E-box-binding factors during initiation of beta-cell specific *hb9* expression.

Discussion

Vertebrate *hb9* genes show conserved expression and function in early beta-cell maturation. While *hb9* genes have been subject to several promoter analyses, the enhancer elements regulating pancreas specific expression have not been identified. By using transient and stable transgenic reporter assays, we now show that in zebrafish, a non-conserved 5 prime upstream region of 478 bp is sufficient and required for targeting expression specifically to beta-cell progenitors. We further identify NeuroD and Pax6b as transcription factors that directly bind *in vitro* to this enhancer element and we present *in vivo* gain and loss-of-function analyses that suggest essential function of currently not specified E-box-binding factors and of Pax6b during initiation and maintenance of beta-cell specific expression of *hb9*, respectively.

hb9 regulation by conserved and non-conserved enhancer elements

Hb9 genes throughout vertebrates show complex embryonic expression in various tissues of endodermal, ectodermal and mesodermal origin (Arber et al., 1999; Broihier and Skeath, 2002; Ferrier et al., 2001; Grapin-Botton et al., 2001; Ross et al., 1998; Tanabe et al., 1998; Thaler et al., 1999; Wendik et al., 2004). Here we show that 3.1 kb of zebrafish *hb9* upstream genomic DNA is sufficient to recapitulate the conserved expression of *hb9* in motoneurons and in differentiating beta-cells. These sequences include two highly conserved non-coding motifs, the A- and B-box. Our data show that in zebrafish, the B-box mediates reporter expression in motoneurons and some ventral interneurons while the A-box is dispensable for embryonic *hb9* regulation. Similar conclusions have been drawn from the analyses of the A- and B-boxes of the mouse *hb9* promoter, in which three copies of the B-box fused to a minimal promoter were sufficient to regulate expression in motoneurons, while a corresponding construct with three copies of the A-box failed to induce any specific reporter expression (Nakano et al., 2005). Interestingly, motoneuron specific *hb9* regulation in mouse also involves a second mammal-specific element termed MN^{E}, which has a distinct mode of molecular activity as compared to the B-box (Lee and Pfaff, 2003; Lee et al.,

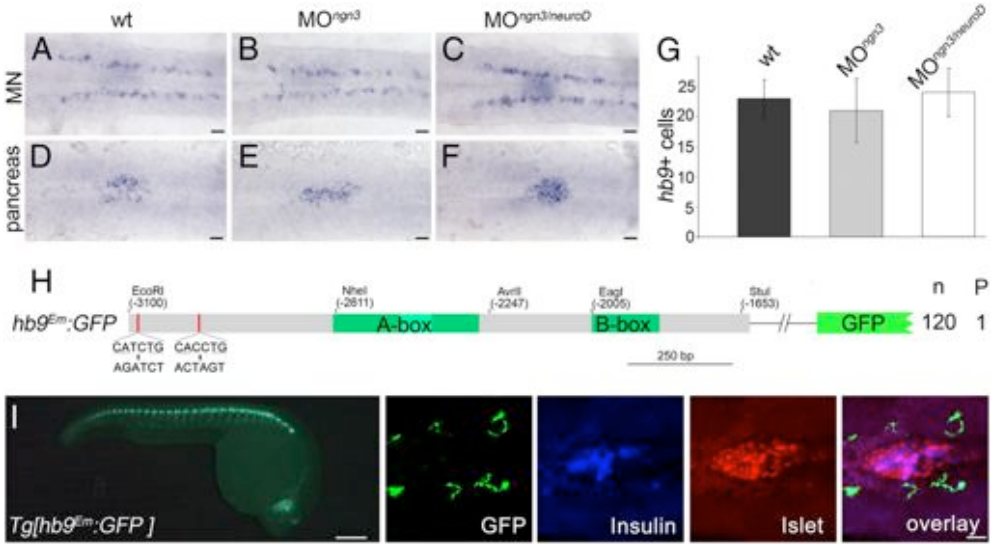

Fig. 7. E-box binding proteins but not Ngn3 and NeuroD are required for beta-cell specific *hb9* expression. (A–F) Similar expression of *hb9* mRNA at 24 hpf in motoneurons (MN in A–C) and in the pancreatic islet (pancreas in D–F) of uninjected wild type (wt) embryos (A, D) and embryos injected with *ngn3* (B, E) or a combination of *ngn3* and *neuroD* morpholinos (C, F). Different focus planes of the same embryos are shown in dorsal view with anterior to the left. (G) Countings of *hb9* positive cells in the pancreas of the wild type (wt, n = 21), *ngn3* (MO^{ngn3}, n = 32) and *ngn3/neuroD* (MO$^{ngn3/neuroD}$, n = 22) morphants at 24 hpf. (H) Schematic drawing of the *hb^{Em}:GFP* construct in which both E-boxes (red line) were mutated by site-directed mutagenesis (origin and mutated sequences are shown with consensus sequences being underlined). Numbers on the right summarize the results of transient reporter assays in which a single embryo out of 120 GFP positive embryos (n) showed reporter expression within the pancreas (P). (I) GFP expression in *Tg[hb9^{Em}:GFP]* embryos at 28 hpf in whole embryos and in embryos stained with antibodies for GFP (green, expression corresponds to motor axons), insulin (blue) and islet (red). The right images shown are confocal image projections with an overlay at the right. Scale bar corresponds to 100 μm (live embryo in I) and 20 μm (A–F, confocal images in I).

2004; Nakano et al., 2005). While the B-Box is directly regulated by Hox/Pbx factors to mediate anterior–posterior positional information (Nakano et al., 2005), the MN^E is regulated by basic helix–loop–helix (bHLH) and Lim-homeodomain (Lim-HD) transcription factors that integrate signals of dorsal–ventral patterning and cell differentiation (Lee and Pfaff, 2003; Lee et al., 2004; Lee et al., 2008). The highly conserved function of Lim-HD, bHLH and Hb9 proteins in motoneuron differentiation implies that molecular interactions between these factors are not restricted to mammals (Briscoe et al., 2000; Lee and Pfaff, 2003; Lee et al., 2004; Shirasaki and Pfaff, 2002; Zhou and Anderson, 2002), even though MN^E related sequences are not detectable in non-mammalian system. Our transient reporter assays reveal that a critical region of 300 bp, including the B-box, is required for motoneuron specific expression in zebrafish (compare ΔA^{SphI} and ΔAB^{AvrII} in Fig. 2, Supplemental Fig. 1). While low sequence conservation may prevent bioinformatics-based detection of MN^E related sequences, it is still possible that this critical region similarly receives combined regulatory input from bHLH, Lim-HD and Hox/Pbx factors. Alternatively, in non-mammalian organisms, the regulatory function of the MN^E may be located to the enhancer of the *hb9* paralog *mnr2*, which is missing in mammals (Wendik et al., 2004). Preliminary sequence analyses of the two *mnr2* genes in zebrafish did not provide evidence for MN^E related sequences and it will be interesting to determine how conserved factors which regulate the MN^E, such as the bHLH proteins Olig2, Ngn2, NeuroD and the lim-HD factors Isl1/2 and Lhx3 (Lee and Pfaff, 2003), contribute to motoneuron specific *hb9*/*mnr2* regulation in non-mammalian organisms.

Recent high throughput analyses of cis-regulatory elements came to the conclusion that a lot of enhancer elements are not conserved between vertebrates and this includes both activator and repressor elements (Birney et al., 2007; McGaughey et al., 2009; Roh et al., 2007). Consistent with this, we were unable to recapitulate all sites of endogenous *hb9* mRNA expression with our transgenic *hb9*-reporter lines and most transgenic lines, including the initially reported *Tg[hb9:GFP]^{ml2}* and *Tg[hb9:GFP]^{ml3}* (Flanagan-Steet et al., 2005; Kimmel and Meyer, 2010) show ectopic reporter expression in *hb9*-negative tissues such as hindbrain and eye. This shows that *hb9* is regulated by additional activating and repressing elements outside of the analysed 3.1 kb region. As these more distal sequences lack homology to corresponding regions of other vertebrate *hb9* genes, this suggests functions for additional non-conserved element in *hb9* regulation. Among those, the as-yet unidentified enhancer regulating expression in caudal structures and notochord is of major interest when considering the connection of human HLXB9 and Currarino syndrome. In these patients, reduced HLXB9 function resulting from loss of one *HLXB9* allele is thought to cause formation of caudal teratomas, which in consequence lead to sacral agenesis (Ross et al., 1998).

Beta-cell specific regulation by the non-conserved P^E

Previous analyses of the mouse *hb9* gene failed to detect a beta-cell enhancer in a 9 kb fragment upstream of the *hb9* gene (Arber et al., 1999; Nakano et al., 2005; Wichterle et al., 2002). Notably, this fragment contained the A-box at the most distal position. In our studies we localize the zebrafish *hb9* beta-cell enhancer to a 478 bp sequence directly distal to the A-box, thus to a region that was not analysed in mouse. Our transient and transgenic reporter analyses demonstrate that these 478 bp, termed P^E, are required and sufficient for targeting expression to beta-cell progenitors. While sequence alignments of the P^E reveal no obvious homologies to the Medaka *hb9* gene, we also found that the zebrafish *hb9:GFP* construct is sufficient to regulate beta-cell specific expression in *hb9:gfp* transgenic Medaka embryos (D. Meyer, unpublished data). This demonstrates that the regulatory activity of this early beta-cell enhancer is conserved, at least in fish. The lack of pancreas specific GFP-expression in *Tg[hb9$^{\Delta 1}$:GFP]* but not in *Tg[hb9$^{\Delta 2}$:GFP]* further suggests that the distal part of the P^E contains essential elements while the proximal part is dispensable for beta-cell expression. Consistent with these data, the majority of the EMSA-confirmed Pax6b binding sites and both E-boxes localize to the distal half of the P^E. Nevertheless, the distal part without the A- and B-box containing sequences is not sufficient to trigger beta-cell specific reporter expression. Possibly, the proximal half of the P^E and the flanking conserved sequences both contain functionally redundant regulatory elements that interact with the distal elements.

Molecular regulation of the P^E

Our studies revealed specific requirements of E-box-binding factors and Pax6 during initiation and maintenance of beta-cell specific *hb9* expression. In particular, we show that the P^E contains two E-boxes and multiple Pax6-binding sites that interact *in vitro* with NeuroD/E47 and Pax6b proteins, respectively. From the 14 potential Pax6-type paired box binding sites revealed by bioinformatic analyses, at least five are recognized by Pax6b protein *in vitro*. Interestingly, four of these Pax6b-interacting sequences contain two or three nearly adjacent Pax6 consensus motifs and one of these double Pax6 consensus motifs has an intervening E-box. The functional relevance of these clusters is unclear, in particular as the presence of multiple Pax6 consensus motifs did not result in binding of multiple Pax6b proteins.

In order to determine the biological role of the E-boxes and the Pax6 binding sites, we tested if loss or gain of protein function affects *hb9* expression. We show that the E-boxes are essential to initiate beta-cell specific reporter expression in *hb9:GFP* transgenic embryos and that this induction is initiated independent of the E-box-binding proteins NeuroD and Ngn3. Consistent with the previously reported lack of *ngn3* mRNA expression in the early zebrafish pancreas (Kinkel and Prince, 2009; Moro et al., 2009), we also find that *ngn3* is dispensable for early beta-cell development in zebrafish. The results reveal major difference between mouse and zebrafish *ngn3* functions in pancreas development and they imply that in zebrafish at least one additional E-box binding factor acts upstream or in parallel to NeuroD during beta-cell differentiation. Notably, in human only a few of the homozygous NGN3 missense mutations are associated with neonatal diabetes as an indicator for missing beta-cells (Rubio-Cabezas et al., 2011). While the non-diabetes phenotypes could result from hypomorphic NGN3 alleles it is also possible that differentiation of beta-cells in human may require additional to-be identified E-box-binding factors similar to the situation in zebrafish.

Our data further suggest that the paired-box binding sites within the P^E mediate Pax6b-dependent *hb9* transcriptional activation. We show that injection of *pax6b* mRNA results in ectopic induction of *hb9* mRNA and of *gfp* mRNA in *hb9:GFP* transgenes, and that this induction is P^E dependent. The data strongly suggest a direct regulation of the P^E by Pax6b, while they also reveal that Pax6b is not required for the initiation but for the maintenance of pancreatic *hb9* expression. Interestingly, almost no beta-cells are observed in *pax6b*-mutants showing that besides Hb9, other factors are probably acting downstream from Pax6b to drive the differentiation of beta-cells.

Conclusion

Currently, the vertebrate *insulin* promoter is the major model for studying the molecular basis of beta-cell specific transcription. Our studies now introduce the *hb9*-P^E element as a novel model not only for studies of the regulation of early beta-cell specific transcription, but also for studying the molecular control of beta-cell differentiation. Our studies further revealed interactions of two classes of transcription factors with this novel beta-cell specific enhancer. We show that a currently unspecified combination of E-box-binding proteins is required to initiate P^E based expression and that Pax6b is a

potent activator of this enhancer that is required to maintain *hb9* expression. Our analyses suggest that these genes have independent function during initial steps of beta-cell differentiation in zebrafish. As Pax6b and Hb9 are both required to initiate expression of *insulin* this raises the question whether the observed function of Pax6b in maintaining endocrine *hb9* expression represents a conserved cross-regulation between two beta-cell fate-regulating transcription factors. Previously reported interactions between Pax6 and various co-factors in non-pancreatic tissues provide interesting opportunities to address questions on the molecular regulation of beta-cell restriction of transcription in the context of early induction in beta-cell progenitors or maintenance after maturation. Reported interactions of Pax6 with members of the Sox and Ets family of transcription factors and with the chromatin remodelling factor Brg1 provide interesting novel links for future studies on the genetic and epigenetic regulation of beta-cell restricted expression (Cvekl et al., 2004; Kamachi et al., 2001; Yang et al., 2006).

Acknowledgments

We thank Robin Kimmel and Elisabeth Ott for critical reading of the manuscript and all members of the institute for suggestions and fruitful discussions. Special thanks go to Frederic Biemar for help during the initial cloning of the *hb9* promoter, to Wolfgang Driever for general support during the starting phase of this project and for providing *Tg[ins:dsRed]* fish and *insulin in situ* probes, to Marianne L. Voz for providing *ngn3* morpholino and to Francesco Argenton and Samuel L. Pfaff for providing expression plasmid for NeuroD and E47, respectively. This project was supported by grant from the University of Freiburg (D.M.), FWF P-20492 (D.M.), Belgian State (SSTC, IUAP/PAI) and the European Union (B.P.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2012.03.001.

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