FEBS 21615 FEBS Letters 445 (1999) 315–320

Pax6 and Pdx1 form a functional complex on the rat somatostatin gene upstream enhancer

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Received 29 December 1998

Abstract The somatostatin upstream enhancer (SMS-UE) is a highly complex enhancer element. The distal A-element contains overlapping Pdx1 and Pbx binding sites. However, a point mutation in the A-element that abolishes both Pdx1 and Pbx binding does not impair promoter activity. In contrast, a point mutation that selectively eliminates Pdx1 binding to a proximal B-element reduces the promoter activity. The B-element completely overlaps with a Pax6 binding site, the C-element. A point mutation in the C-element demonstrates that Pax6 binding is essential for promoter activity. Interestingly, a block mutation in the A-element reduces both Pax6 binding and promoter activity. In heterologous cells, Pdx1 potentiated Pax6 mediated activation of a somatostatin reporter. We conclude that the β/δ-cell-specific activity of the SMS-UE is achieved through simultaneous binding of Pdx1 and Pax6 to the B- and C-elements, respectively. Furthermore, the A-element appears to stabilise Pax6 binding.

Key words: Pax6; Pdx1; Somatostatin; Pancreatic islet; Transcription

1. Introduction

Cell-specific expression of the glucagon, insulin, somatostatin, and pancreatic polypeptide genes by the four classical cell types of the islets of Langerhans is regulated at the transcriptional level [1–5]. The promoters of the glucagon, insulin, and somatostatin genes are composed of a combination of positively and negatively acting *cis*-elements [6–12]. Isolated positively acting *cis*-elements confer tissue- (islet-) specific or cell-type- (α-, β-, δ-) specific expression [13–17].

Thus, activity of the somatostatin promoter in islet β- and δ-cell lines relies on a composite enhancer element termed the somatostatin-upstream enhancer (SMS-UE) or TSE-I (tissue-specific element I) spanning nucleotides −115 to −75 (see Fig. 1) [11,18–20]. Additional positively acting A/T-rich *cis*-elements (TAAT1/TSE-II and TAAT2) further upstream are also contributing to promoter activity [18,21,22]. The activity of these elements is dependent on the integrity of a cyclic AMP response element (CRE) located at position −48 to −41 [11,18]. Additionally, negatively acting factors suppress activity in non-islet cells [12]. The SMS-UE is a tripartite element consisting of the subdomains SMS-UE-A, -B, and -C [11,19]. A so called PISCES motif (pancreatic islet cell enhancer sequence) is found in the C-element of the SMS-UE [13]. Interestingly, PISCES motifs are also found in promoter elements within the glucagon and insulin genes [13]. These elements, however, display different cell-type-specific activities [20]. Thus, while the glucagon gene G1-element shows preferential activity in α-cells, the glucagon gene G3-element has a broad islet cell-specific activity. In contrast, the insulin gene C2-element and the SMS-UE show β/δ-cell-specific activity [20]. To begin to unravel the mechanism by which the SMS-UE confers cell-type specificity, we analysed the factors binding to this element in a somatostatin-producing cell line. We found by antibody supershift experiments that the major complexes formed on the A-element were produced by Pdx1 and Pbx proteins. On the B- and C-elements we observed a complex composed of Pdx1 and Pax6 binding simultaneously. This complex was also formed by recombinant Pdx1 and Pax6. By mutational analysis of the SMS-UE, we found a correlation between formation of the Pdx1-Pax6 complex and promoter activity. Interestingly, small mutations that abolished Pdx1 and Pbx binding to the A-element did not reduce promoter activity. However, a block mutation that also reduced Pax6 binding to the SMS-UE did inhibit promoter activity. Furthermore, Pdx1 enhanced Pax6 mediated activity of a somatostatin reporter in heterologous cells. Together, these data suggest that the Pdx1-Pax6 complex is functional and required for cell-specific activity of the SMS-UE.

2. Materials and methods

2.1. Plasmids

pCMX-STF1 [23] containing the rat Pdx1 cDNA under control of the CMV promoter was a gift from J. Leonard, pSG5-Pax6 containing the quail Pax6 cDNA under control of the SV 40 promoter was a gift from S. Saule. The somatostatin reporters were constructed by a multistep procedure. Initially a *Bgl*II restriction site was inserted between the *Sal*I and *Pst*I sites in pCAT-Basic (Promega) creating pCAT-Basic-B2. A 124 bp *Bgl*II-*Xba*I fragment containing the somatostatin gene sequences from −71 to +53 was isolated from p1.4-CAT [18] and inserted into *Bgl*II and *Xba*I digested pCAT-Basic-B2, creating p-71-CAT-Basic-B2. Double stranded oligonucleotides representing wild-type or mutant somatostatin gene sequences from −72 to −122 (see Fig. 1) were inserted into *Bgl*II digested p-71-CAT-Basic-B2. The 4X(GAL4-SMS-UE)-CAT reporter was designed using multimerised oligonucleotides (see Fig. 6A) inserted in the correct orientation into the *Bgl*II site of the somatostatin minimal reporter construct, pΔ2.5-CAT [18]. GAL4-Pdx1, GAL4-Cdx2 fusion constructs were generated by PCR and inserted in frame into pSG424 [24] using the *Bam*HI and *Xba*I cloning sites. The Isl-1 cDNA [25] was inserted into the *Eco*RI site of pSG424. GAL4-VP16 has been described previously [26]. All constructs were verified by dideoxy cycle sequencing.

2.2. Transfections

Transient transfection assays were done using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. Briefly, ap-

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PII: S0014-5793(99)00144-1

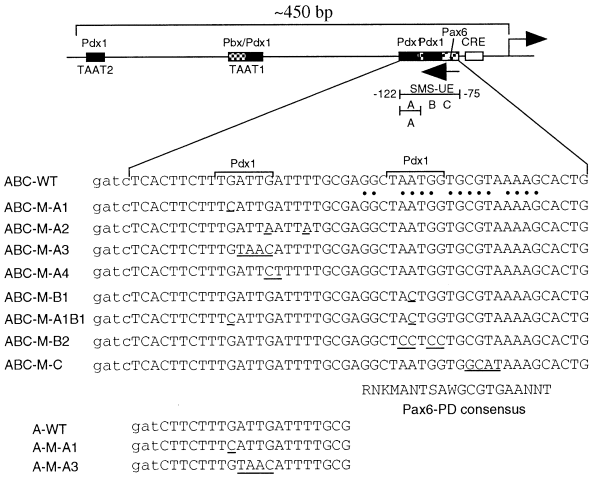

Fig. 1. Schematic representation of the rat somatostatin promoter and synthetic oligonucleotides used in EMSA. Alignment with the Pax6 paired domain consensus binding site [42] is shown and Pdx1 binding sites are indicated. The Pax6 binding site is indicated by a hatched box, Pdx1 binding sites as black boxes, Pbx binding sites as shaded boxes, and the cAMP response element (CRE) as an open box. The arrow indicates the orientation of the Pax6 binding site. The sequence of the SMS-UE with Pdx1 binding sites is shown aligned with the Pax6 paired domain consensus binding site. Mutations deviating from the wild-type rat somatostatin gene sequence are underlined.

proximately 2.5×10^5 NIH 3T3 cells were seeded 4–8 h before transfection in a 24-well tissue culture dish (Nunc). 150 ng of reporter plasmid was cotransfected with 40 ng pGL2-RSV plasmid [27] as an internal standard. 10 ng of SV40-Pax6 expression plasmid and 1 ng of CMv-Pdx1 expression plasmid was used. The same amount of empty expression vectors were used for control. All transfections were adjusted to a total of 500 ng DNA per well using pBluescript SKII$^+$ (Stratagene). 5–10 min before adding the transfection mix to the cells they were washed using 1 ml opti-MEM per well. The cells were incubated overnight in a total volume of 500 μl of the transfection mix, after which the same volume of normal culture medium with 20% serum was added. Cells were cultured for an additional 24 h before harvesting. Luciferase and CAT activities were measured as previously described [28]. Quantification of CAT assays was done using a PhosphoImager (Molecular Dynamics). Dual luciferase was measured using the Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer (Turner Designs). All reporter activities were normalized to the internal standard. For βTC3-som transfections 3 μl of lipofectamine was used and the culture period after transfection was 48 h.

2.3. Cell culture and cell extracts

NIH-3T3-B (obtained from R. Schumacher), αTC1.9 [2], and βTC3-som were cultured in DMEM 1000 mg/l glucose containing 10% fetal calf serum, 100 U penicillin/ml, and 100 μg/ml streptomycin, as previously described [29]. βTC3-som cells are a subculture of βTC3 cells [30] which arose spontaneously in our laboratory. It is characterized by somatostatin production in approximately 30–50% of the cells in the otherwise homogenous insulin-producing cell line (H.V. Petersen, unpublished result). βTC3-som cells uniformly express Pdx1 and Pax6 (not shown). Nuclear extracts were prepared by the method of Schreiber et al. [31] except that buffer C contained 500 mM KCl instead of 400 mM NaCl. Furthermore, leupeptin (1 μg/ml), aprotinin (1 μg/ml), 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and 1 mM sodium-orthovanadate were included. The recombinant proteins were produced by transiently transfecting 2×10^6 NIH 3T3 cells in six-well tissue culture dishes (Nunc) with 3–6 μg of the relevant expression plasmid. Whole cell extract was prepared as follows: cells were washed in PBS and lysed in whole cell extract buffer (2.5 mM DDT, 20 mM HEPES pH 7.8, 10 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 0.2% Triton X-100 supplemented with leupeptin (1 μg/ml), aprotinin (1 μg/ml), AEBSF (1 mM), and 1 mM sodium-orthovanadate) using 175 μl per well. After shaking for 10 min at room temperature, 33 μl 2.1 M KCl, 46% glycerol buffer was added and shaking was allowed to proceed for 10 min at room temperature. The contents of the wells were adjusted to 15% glycerol using a 50% stock, collected and centrifuged at $20\,000\times g$ for 10 min at 4°C. The supernatant was collected and stored at −80°C. Protein content was measured using the Bradford assay (Bio-Rad).

2.4. Antisera and electrophoretic mobility shift assays

The 1859 anti-Pdx1 antiserum has been described previously [32]. The bg11 anti-Pax6 antiserum was a gift from S. Saule and has been described previously [33]. Pbx1 antiserum was purchased from Santa Cruz, Inc. 5 μg of nuclear extract was used in standard EMSA. The reaction buffer contained 12.5% glycerol, 30 mM Tris-HCl, 75–100 mM KCl, 20 mM NaCl, 1 mM MgCl, 20–50 μg/ml poly-dIdC and 20–50 μg/ml poly-dGdC, 2–4 μg/ml herring sperm DNA, 0.1% Nonidet NP-40, 3 mM DTT. For recombinant proteins up to 10 μg of whole cell extract was used, and all reactions were adjusted to the same protein concentration using extracts from untransfected cells. Antisera were used in a 1/10 dilution in the presence of 15 mM NaN_3 using 2–3 μl per binding reaction as described previously [28]. Binding reactions were separated on a 5% polyacrylamide gel as previously described [28]. The gels were dried and analyzed by autoradiography. Gel images were scanned using Adobe Photoshop 3.0 and figures prepared in Canvas 5.0.2.

3. Results

3.1. Pdx1 and Pbx proteins can bind the isolated SMS-UE-A element

The SMS-UE of the somatostatin gene contains a Pax6 binding site [20,34] and a Pdx1 binding site [21,23] that have been shown to confer preferential transcriptional activity in β- and δ-cell lines [20]. As the binding site of the Pax6 paired domain and Pdx1 binding site only comprise the SMS-UE-B and -C elements it seemed possible that an SMS-UE-A element binding factor contributed to promoter activity along with Pax6 and Pdx1. We therefore initially identified the factors capable of binding to the A-element. The sequence of the A-element (TTGATTGATTT) resembles the TAAT2 element (ATGATTAATTA, differences underlined) which is known to bind Pdx1, either as a monomer or as a complex with one of the Pbx proteins [22]. By performing EMSA using the A-element probe (see Fig. 1) with nuclear extracts isolated from βTC3-som, an insulin- and somatostatin-producing subculture of the islet cell line βTC3, we found that one major complex was formed with βTC3-som extract that was absent from extracts prepared from the glucagon-producing cell line αTC1.9. In addition, two minor complexes were present in both αTC1.9 and βTC3-som extracts (Fig. 2). Addition of antisera specific for either Pdx1 or Pbx showed that the β-cell-specific complex was due to Pdx1 binding while the two minor complexes were Pbx immunoreactive (Fig. 2). In contrast to the TAAT2 element, Pbx/Pdx1 dimer formation was not observed on the A-element and this was found to be due mainly to the nucleotide differences at position seven and to a minor degree also at position 11 between the two sites (F.G. Andersen, manuscript in preparation). A point mutation at position three of the Pbx site (M-A1) as well as a block mutation at positions 4–7 (M-A3) abolished both Pdx1 and Pbx binding to the A-element (Fig. 2, lanes 10 and 11).

3.2. Pax6 and Pdx1 forms a ternary complex with the SMS-UE

To test whether a Pax6 and Pdx1 containing complex could form on the SMS-UE, we performed EMSA using the ABC-

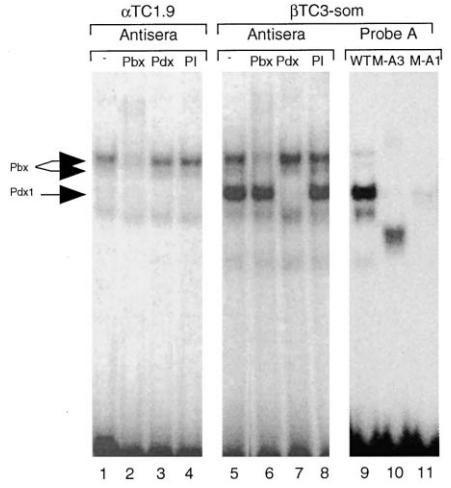

Fig. 2. Pdx1 and Pbx proteins bind the SMS-UE A-element. ^{32}P-labelled A-oligonucleotide (wild-type or mutant as indicated) was incubated with nuclear extract from αTC1.9 or βTC3-som cells and anti-Pdx1 or anti-Pbx antisera as indicated. Pdx1 and Pbx immunoreative complexes are indicated. Note that both mutations (M-A1 and M-A3) abolish binding of both Pdx1 and Pbx proteins.

probe spanning the entire SMS-UE (Fig. 1) in the presence of βTC3-som nuclear extract. Two major complexes S1 and S2 were observed, however, on shorter exposures (not shown) the S1 complex was resolved into two complexes of slightly different mobilities, S1a and S1b (Fig. 3, lane 1). Inclusion of Pax6 or Pdx1 antisera in the binding reaction identified the S1a complex as Pdx1 and the S1b complex as Pax6 (Fig. 3, lanes 3–5). The S2 complex contained both Pdx1 and Pax6 (Fig. 3, lanes 3–5). The Pax6 antiserum failed to completely shift the S2 complex (Fig. 3, lane 4). This is due to the presence of a Pdx1 dimer generated by co-occupancy of the A- and B-elements (see Fig. 2, lane 2 and Fig. 4, lanes 12–14). However, full length recombinant Pdx1 often failed to generate this complex, most likely due to insufficient amounts of Pdx1 produced in the transfected fibroblasts. The identification of complex S2 as a Pax6-Pdx1-DNA ternary complex was corroborated by the finding that recombinant Pdx1 and Pax6 formed a complex with a mobility identical to the S2 complex (Fig. 3, lane 9). In agreement with a previous study [28] we observed that rat Pdx1 migrates slightly faster than mouse Pdx1 (Fig. 3, compare lanes 1 and 7).

3.3. *Sequence requirements for Pax6 and Pdx1 binding to the SMS-UE: intact A-, B-, and C-elements are required for optimal Pax6 binding*

In order to examine the functional relevance of Pax6 and Pdx1 binding to the SMS-UE, we introduced a set of mutations in either the A-, B-, or C-elements (see Fig. 1) and tested the effect of these on binding of recombinant proteins as well as promoter activity. Plasmids encoding wild type Pax6 and a truncated version of rat Pdx1 (Pdx1(ΔN)) deleting most of the activation domain of Pdx1 [35] were used to generate recombinant proteins in NIH-3T3 cells. Pdx1(ΔN) was used to ensure different migration of Pax6 and Pdx1 monomers. The M-A1 mutation which abolished Pdx1 binding to the A-element (see Fig. 2) had no effect on Pax6 binding or Pax6-Pdx1-DNA ternary complex formation (Fig. 4, lanes 7 and 8). In contrast, the M-A3 mutation, which also abolished Pdx1 binding to the A-element (see Fig. 2), reduced Pax6 binding as well as Pax6-Pdx1-DNA complex formation (Fig. 4, lanes 13–16). This was also clearly seen using βTC3-som nuclear extract (data not shown). The M-A4 mutation led to a similar reduction in complex formation as the M-A3 mutation (Fig. 4, lanes 17–20). Conversely, the M-A2 mutation resulted in increased Pax6 and Pdx1 monomer, and Pax6-Pdx1-DNA complex formation (Fig. 4, lanes 9–12). The importance of a functional Pdx1 binding B-element for ternary complex formation was demonstrated using the M-B1 mutation (Fig. 1). Specific targeting of the Pdx1 consensus site, leaving the Pax6 consensus unaffected, resulted in reduced Pdx1 binding and abolishment of the Pax6-Pdx1-DNA complex, without influencing Pax6 monomer formation (Fig. 4, lanes 21–24). The M-A1B1 mutation targeting both the A- and B-element Pdx1 sites resulted in severe reduction of Pdx1 complex formation, but left Pax6 monomer binding unchanged (Fig. 4, lanes 25–28). The M-B2 mutation abolished the Pax6 complex as well as Pdx1 binding to the B-element and consequently Pax6-Pdx1-DNA ternary complex formation (Fig. 4, lanes 29–32). Similarly, when using βTC3-som nuclear extract no Pax6 immunoreactive complex were observed (data not shown). Finally, the M-C mutation abolished formation of the Pax6 complexes without affecting Pdx1 binding (Fig. 4, lanes 33–36). The same effect was seen using βTC3-som nuclear extract (data not shown).

3.4. *Mutations that disrupt the Pax6-Pdx1 complex formation impair promoter activity*

To analyse the functional importance of ternary complex formation we introduced the mutations used in Fig. 2C into a somatostatin reporter construct (−122/+53) linked to CAT. We then assayed promoter activity by transiently transfecting βTC3-som cells with wild type and mutant constructs. The M-A3, M-B2, and M-C mutations that all affected Pax6 binding and reduced or abolished ternary complex formation impaired SMS-UE mediated activity to a level seen with a −71 reporter construct (Fig. 5A). The M-B1 mutation which compromises

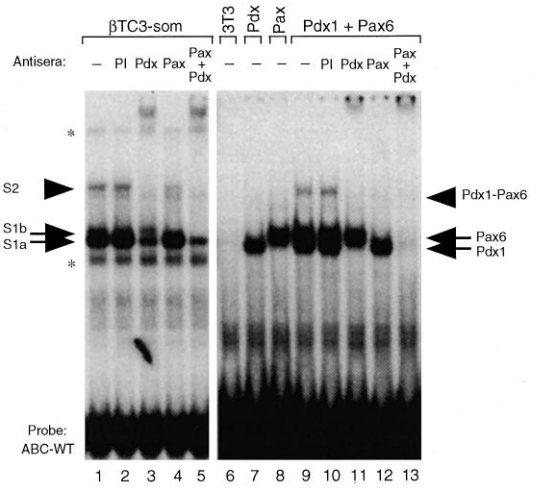

Fig. 3. Pax6 and Pdx1 forms a ternary complex with the somatostatin upstream enhancer. ^{32}P-labelled ABC-oligonucleotide was incubated with nuclear extract from βTC3-som cells (A) recombinant Pdx1 and Pax6 (B), and anti-Pdx1 or anti-Pax6 antisera as indicated. The S1a (Pdx1) and S1b (Pax6) complexes are indicated by arrows. Arrowheads indicates the S2 complex (Pdx1-Pax6-DNA ternary complex). Asterisks indicate non-specific binding. Note that recombinant rat Pdx1 migrates slightly faster than Pdx1 from the mouse cell line βTC3-som.

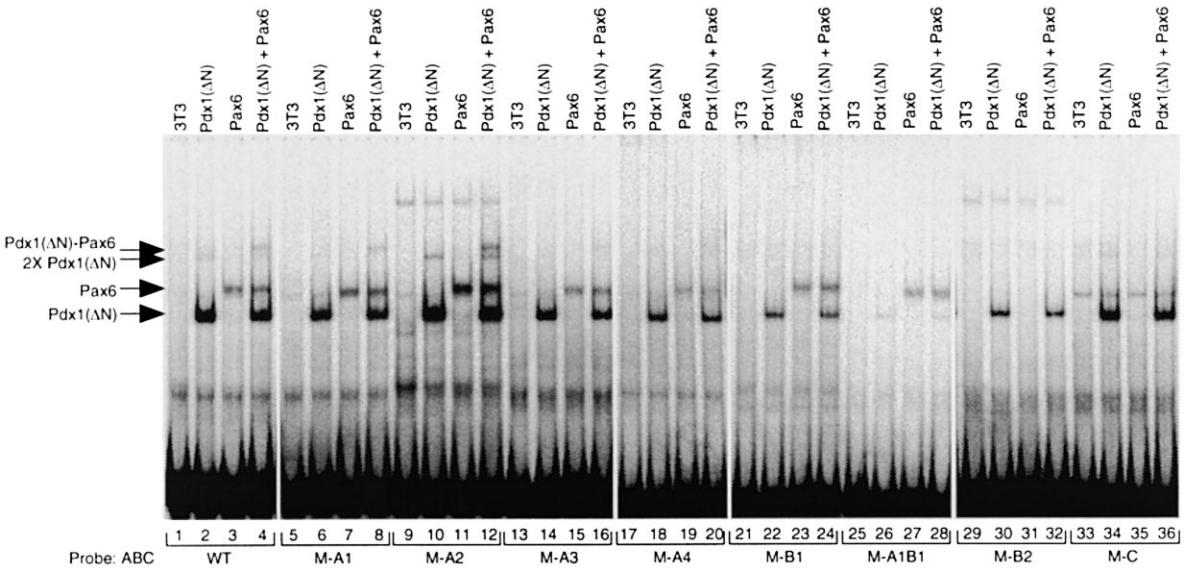

Fig. 4. Mutations in the A-, B-, and C-elements of the SMS-UE affects ternary complex formation. ^{32}P-labelled ABC-oligonucleotide (wild-type or mutant as indicated) was incubated with recombinant N-terminally truncated Pdx1 (Pdx1(ΔN)) and Pax6 as indicated. Pdx1 and Pax6 monomers are indicated by arrows. Arrowhead indicates the Pdx1-Pax6-DNA ternary complex.

Pdx1 binding to the B-element and the M-A1B1 mutation which affects Pdx1 binding to the A- as well as the B-element, both without affecting Pax6 binding, resulted in an approximately 50% reduction in promoter activity (Fig. 5A). The M-A4 mutation also showed a 50% reduction in promoter activity, a slightly higher activity than observed for the M-A3 mutation although these two mutations appeared to affect Pax6 binding similarly. The M-A1 mutation did not affect promoter activity, suggesting that binding of Pdx1 and Pbx proteins to the A-element does not contribute to promoter activity in islet cells. A single mutation, M-A2, resulted in increased promoter activity (Fig. 5A) which correlated with increased formation of the Pax6-Pdx1-DNA ternary complex on this mutant, in addition to increased binding of Pax6 and Pdx1 monomers.

3.5. *Pdx1 potentates Pax6 mediated activation of a somatostatin reporter*

To further test whether Pax6 and Pdx1 were capable of interacting functionally on the somatostatin promoter we transfected NIH-3T3 cells with a somatostatin reporter construct in the presence or absence of Pax6 and Pdx1 expression

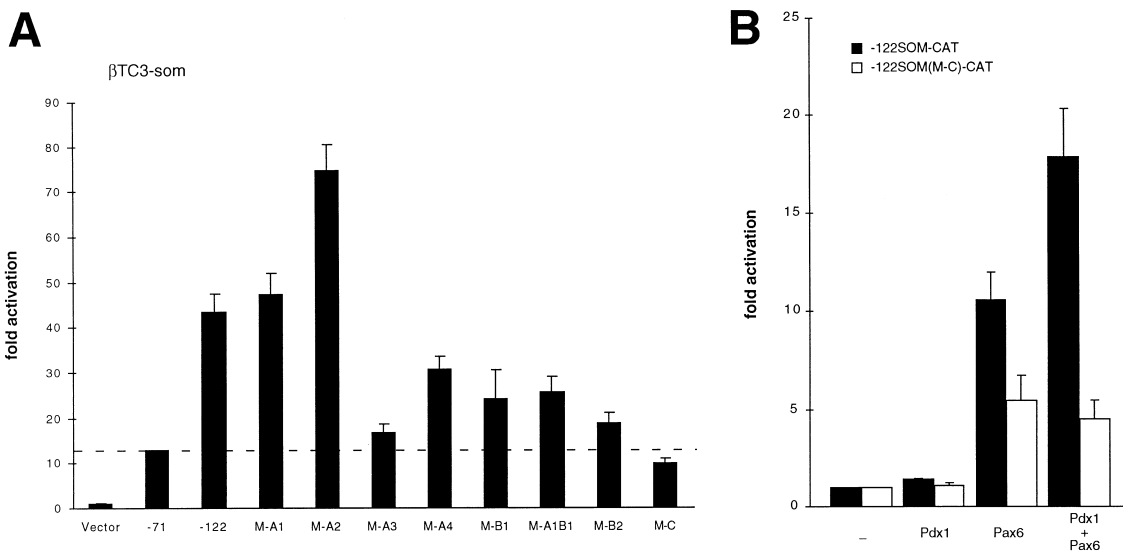

Fig. 5. Pax6 and Pdx1 cooperatively activate the somatostatin promoter. A: Mutations that impair ternary complex formation reduce somatostatin promoter activity in somatostatin-producing cells. βTC3-som cells were transfected with the indicated wild-type or mutant reporter plasmids. Note that the M-A1 mutation that compromises Pdx1 and Pbx binding to the A-element does not reduce promoter activity and that the M-A3, M-B2, and M-C mutations that affect formation of the Pax6-Pdx1-DNA complex all eliminate the activity of the SMS-UE. The average of at least three independent experiments is presented, error bars indicate SEM. B: Pdx1 potentiates Pax6 mediated activation of the rat somatostatin promoter. NIH-3T3 cells were transfected with −122SOM-CAT or −122SOM(M-C)-CAT and co-transfected with the indicated effector plasmids or empty expression vectors as control. The average of three independent experiments is presented, error bars indicate S.E.M.

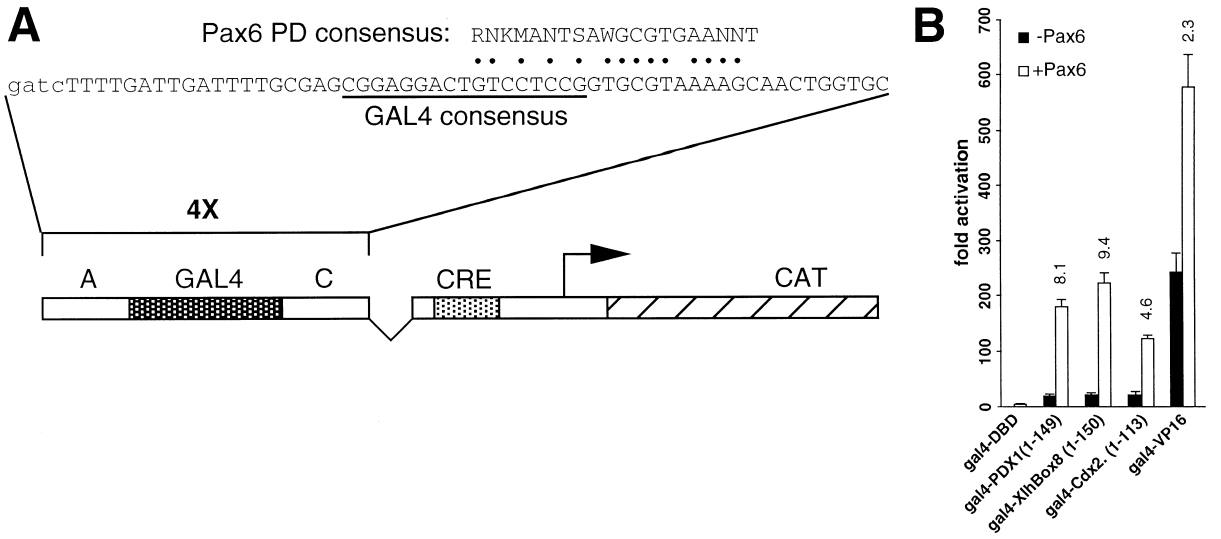

Fig. 6. The activation domains of Cdx2 and Pdx1 synergize effectively with Pax6 when coupled to a heterologous DNA-binding domain. NIH 3T3 cells were transfected with 4X(GAL4-SMS-UE)-CAT and co-transfected with the indicated effector plasmids or empty expression vectors as control. The average of at least three independent experiments is presented, error bars indicate S.E.M. The numbers above the bars indicate a cooperative index calculated as the ratio of CAT activity obtained with a GAL4 fusion construct and Pax6 divided by the sum of the levels obtained by either effector alone. A: Schematic representation of the synthetic 4X(GAL4-SMS-UE) reporter. Four copies of an oligonucleotide spanning the SMS-UE with the B-element replaced by a GAL4 binding site without disrupting the Pax6 consensus binding site was cloned in the *Bgl*II site a position −71 of the minimal somatostatin promoter construct pΔ2.5-CAT. B: The N-terminal regions of rat and *Xenopus* Pdx1 (XlHbox8) as well as Cdx2/3 can cooperate with Pax6. The average normalised activity of each GAL4 fusion construct, in the absence or presence of co-transfected Pax6, is presented relative to that of cells transfected with the GAL4 DNA-binding domain. A GAL4-VP16 fusion construct included for comparison showed modest cooperativity.

vectors. Transient transfection analysis revealed that Pdx1 could enhance Pax6 mediated transcriptional activation of the −122SOM-CAT reporter construct (Fig. 5B). Mutation of the Pax6 binding site (−122SOM(M-C)-CAT) reduced Pax6 mediated activity by 50% and abolished the potentiation by Pdx1 (Fig. 5B). A −71 somatostatin reporter was only activated 1.4-fold by Pax6 and Pdx1 did not potentate this activity (not shown).

3.6. The activation domains of Pdx1 and Cdx2/3 can co-operate with Pax6 when fused to the GAL4 DNA binding domain

The activation domain of Pdx1 has been mapped to mainly three highly conserved sequences within the N-terminal 75 amino acids [35,36]. These sequences are also required by Pdx1 to synergistically activate insulin-enhancer mediated transcription together with products of the E2A gene. To determine whether the activation domain of Pdx1 was required for transcriptional co-operativity with Pax6, we used a fusion protein between the GAL4 DNA binding domain (GAL4-DBD) and the activation domain of Pdx1. As a reporter, we constructed a synthetic promoter containing four copies of the SMS-UE with the B-element replaced by a GAL4 binding site. The multimerised oligonucleotide was inserted in front of the somatostatin minimal promoter in pΔ2.5-CAT (Fig. 6A). This reporter could be activated by Pax6 (Fig. 5B) while the parent vector could not (data not shown). Activation by GAL4-Pdx1 and transcriptional synergy between GAL4-Pdx1 and Pax6 was readily observed on this reporter (Fig. 6B). This activity of the Pdx1 activation domain was conserved between *Xenopus laevis* and rat Pdx1 (Fig. 6B). The cooperativity observed between Cdx2/3 and Pax6 on the glucagon promoter (see accompanying manuscript) prompted us to examine whether the activation domain of Cdx2/3 could also synergise with Pax6 on this synthetic reporter. Use of GAL4-Cdx2/3 fusion constructs revealed that the N-terminal activation domain of Cdx2/3 [37] was indeed capable of cooperating with Pax6 on the synthetic promoter when fused to the GAL4-DBD (Fig. 6B). A GAL4-VP16 fusion construct was highly active on this reporter but only capable of modest cooperativity with Pax6 (Fig. 6B).

4. Discussion

Pdx1 has previously been reported to stimulate somatostatin gene transcription [21–23,38]. Three Pdx1 binding sites are located within approximately 400 bp of the somatostatin 5′-flanking region [21,23]. Nevertheless, the first 120 bp of the promoter is sufficient to confer islet cell-specific expression in transfection assays [11]. In such a construct mutational inactivation of the B-element Pdx1 binding site reduces promoter activity substantially [11]. Similarly, in a construct including 1400 bp of 5′-flanking sequence, inactivation of the Pdx1 site in the SMS-UE B-element reduces promoter activity by 70% [18]. However, as several conserved bases in the Pax6-PD consensus site are also affected by this mutation it cannot be excluded that the Pax6 binding is also affected. We now show that the mutational sensitivity of the C-element is due to the disruption of the Pax6 binding. Moreover, we show that this Pax6 binding site is overlapping with a Pdx1 binding site and that these two sites can be co-occupied. Furthermore, formation of the Pax6-Pdx1-DNA ternary complex is reduced when nucleotides outside the Pax6-PD consensus site are mutated (i.e. the M-A3 and M-A4 mutation in the A-element) or deleted (not shown). The reduced ternary complex formation correlates with reduced promoter activity. In contrast, a point

mutation (M-A1) which reduces Pdx1 and Pbx binding to the A-element (similar to the M-A3 mutation) has no effect on promoter activity, suggesting that binding of Pdx1 or Pbx to the A-element is not required for promoter activity. One possible explanation for the sensitivity of the A-element to the M-A3 and M-A4 mutations but not the point mutation (M-A1) could be that Pax6 contacts this element perhaps through its homeodomain. This interaction could be selectively affected by the M-A3 and M-A4 mutations. The increased binding of Pax6 to the M-A2 mutation and correlating increase in promoter activity supports that A-element sequences (which are located outside the paired domain recognition sequence) are involved in determining the affinity of Pax6 towards the SMS-UE. The mode of Pax6 binding to the SMS-UE is currently under investigation. Our data do not support a model involving binding of Pbx proteins to the A-element. It is, however, a possibility that binding of Pbx proteins to the A-element might modulate the activity of the SMS-UE in other cell types. We have shown that Pax6 mediated activation of a somatostatin reporter in heterologous cells can be potentiated by Pdx1. Together, our data suggest that the SMS-UE comprises a composite Pdx1/Pax6 binding site and that this contributes to the cell specificity of the somatostatin promoter. In contrast to the insulin and glucagon 5′-flanking regions which in transgenic mice direct expression to the appropriate cell type [2,3], the somatostatin 5′-flanking region (−4000/+53) drives expression of the non-transforming K1 mutant SV40 T-antigen to β-cells rather than δ-cells (M. Low, R. Ventimiglia, and R.H. Goodman, personal communication). As δ-cells are thought to arise from a Pdx1 positive, insulin-producing precursor [39,40], we propose that islet-specific activity of the somatostatin 5′-flanking region gene relies on co-operation between Pdx1 and Pax6. The expression of Pdx1 and Pax6 in newly formed δ-cells (data not shown, [41]) is consistent with such a model. However, additional mechanisms, possibly mediated by *cis*-elements upstream of −4000 bp or in the 3′-region, must be required to limit activity to δ-cells.

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