

# Inhibitory effect of Pax4 on the human insulin and islet amyloid polypeptide (IAPP) promoters

Susan C. Campbell, Helen Cragg, Lucy J. Elrick, Wendy M. Macfarlane, Kathleen I.J. Shennan, Kevin Docherty\*

Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK

Received 1 November 1999

Edited by Philip Randle

**Abstract** Pax4 is a paired-box transcription factor that plays an important role in the development of pancreatic  $\beta$ -cells. Two Pax4 cDNAs were isolated from a rat insulinoma library. One contained the full-length sequence of Pax4. The other, termed Pax4c, was identical to Pax4 but lacked the sequences encoding 117 amino acids at the COOH-terminus. Pax4 was found to inhibit the human insulin promoter through a sequence element, the C2 box, located at –253 to –244, and the islet amyloid polypeptide promoter through a sequence element located downstream of –138. The inhibitory activity of Pax4 was mapped to separate regions of the protein between amino acids 2–230 and 231–349.

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**Key words:** Insulin gene; Diabetes mellitus; Amylin; Transcription

## 1. Introduction

The *pax* gene family encodes highly conserved DNA-binding transcription factors that control the expression of genes during embryogenesis [1,2]. Members of the family are all characterised by the presence of a DNA-binding motif, known as the paired domain, with some members of the family also containing a partial or full homeodomain [3]. Currently nine members of the family have been identified in mammalian genomes. These have been classified into four subgroups: Pax1/9, Pax2/5/8, Pax3/7 and Pax4/6. Pax4 and Pax6 are both involved in pancreatic islet development and are essential for the differentiation and maturation of pancreatic endocrine cells. Pax4 gene expression is essential for the differentiation of  $\beta$ -cells and Pax4-deficient mice lack functional  $\beta$ - and  $\delta$ -cells [4]. In contrast to Pax4, Pax6 gene expression is essential for the differentiation of  $\alpha$ -cells and Pax6 knockout mice lack islet  $\alpha$ -cells [5].

Although it is known that Pax4 is essential for the differentiation of  $\beta$ -cells, very little is known about the specific targets of Pax4 within the  $\beta$ -cells of the islets of Langerhans. In the present study we report the cloning of a full-length rat Pax4 cDNA and a shorter isoform, Pax4c, from a rat insulinoma library and investigate the role of Pax4 and Pax4c in

controlling insulin and islet amyloid polypeptide (IAPP) promoter activity in  $\beta$ -cells.

## 2. Materials and methods

### 2.1. Cloning of rat Pax4 cDNA

A partial mouse Pax4 cDNA [6] (GenBank accession number Y09584) labelled with [ $\alpha$ - $^{32}$ P]dCTP was used to screen a rat insulinoma library from CRI-G1 cells. Positive clones were sequenced on both strands (Alta Bioscience, University of Birmingham, UK).

### 2.2. In vitro transcription and translation

The pBluescript SK (+/–) phagemid (Stratagene, Amsterdam, The Netherlands) containing the Pax4 cDNA cloned into the *EcoRI* and *XhoI* restriction sites was linearised with the *ApaI* restriction enzyme at the 3' end of the Bluescript polylinker. Transcription reactions were carried out using T3 RNA polymerase (Boehringer, Mannheim, Germany). The RNA template was translated in a rabbit reticulocyte lysate following the manufacturer's specifications (Promega, Southampton, UK), and the products analysed by SDS-PAGE.

### 2.3. Plasmids

The control luciferase reporter construct pGL-LUC is based on the plasmid pGL2 (Promega), with the thymidine kinase promoter (TKp) from the herpes simplex virus cloned 5' to the firefly luciferase gene. The construct pGL-LUC200 varies from this in that it contains 200 base pairs of the human insulin gene promoter between –65 and –265 upstream of the TKp.

The constructs pBCins-169, pBCins-229, pBCins-258 and pBCins-341 all contain fragments of the human insulin gene promoter between –169 and +112, –229 and +112, –258 and +112 and –341 and +112 cloned upstream of the chloramphenicol acetyl transferase (CAT) reporter gene [7]. The Pax4 cDNA was cloned into the *EcoRI* and *XhoI* restriction sites of the pCR3 vector (Invitrogen NV, Leek, The Netherlands) to generate pCR3-PAX4. The Pax4c cDNA was cloned into the *EcoRI* and *ApaI* restriction sites of the pCR3 vector to generate pCR3-Pax4c.

The pCR3-Pax4/FLAG construct was made by introducing the FLAG amino acid sequence (Asp Tyr Lys Asp Asp Asp Lys), between the NH<sub>2</sub>-terminal methionine and amino acid 2 of Pax4. The region of Pax4 between 1 and 457 bp was amplified from pCR3-Pax4 by PCR, using a specific 5' primer containing the sequence for the FLAG epitope. The amplified fragment was then cloned into the pCR3 vector (Invitrogen). The pCR3-Pax4 vector was then digested with *BglII* and *XhoI* to remove the Pax4 fragment between 427 and 1050. The pCR3-Pax4(1–457)/FLAG vector was also digested with *BglII* and *XhoI*, and Pax4(427–1050) was ligated into pCR3-Pax4(1–457)/FLAG, generating pCR3-Pax4/FLAG.

IAPP gene promoter constructs pTKCAT, pTAC-2798, pTAC-477, pTAC-391, pTAC-222 and pTAC-138 were constructed as previously described [13].

The GAL-Pax4 plasmids were constructed by first amplifying, using the polymerase chain reaction, the fragment of Pax4 corresponding to the desired amino acids. The amplified fragments were digested with *BamHI* and *XbaI*, and cloned in frame with the GAL4 sequences corresponding to amino acids 1–147 of the GAL4 DNA-binding domain in the plasmid pSG424 [8]. The GAL-VP16 construct contains amino acids 413–490 of VP16 cloned in frame with the GAL4 DNA-

\*Corresponding author.

E-mail: k.docherty@abderdeen.ac.uk

binding domain in pSG424 [9]. The reporter plasmid pGAL4TKCAT contains five copies of the GAL4-binding site cloned upstream of the herpes virus thymidine kinase promoter in the eukaryotic expression vector pBL2CAT [10].

#### 2.4. Cell culture

MIN6, a  $\beta$ -cell line derived from transgenic mice expressing the SV40 large T antigen under the control of the rat insulin gene promoter [11], was grown in DMEM containing 5 mM glucose, supplemented with 15% myclone foetal calf serum (Life Technologies, Paisley, UK). COS-7 and BHK cells were grown in DMEM containing 16 mM glucose, supplemented with 10% (v/v) foetal calf serum (Life Technologies).

#### 2.5. Transfections

Cells at about 80% confluence in six-well plates were transfected by mixing 2  $\mu$ g DNA and 24  $\mu$ l of a 1 nM lipid suspension containing a 2:1 mixture of dioleoyl-L- $\alpha$ -phosphatidylethanolamine (DOPE), (Sigma, Poole, UK) and dimethyl-dioctadecylammonium bromide (DDAB), (Fluka) in 1 ml of serum-free Optimem (Life Technologies). The lipid complexes were allowed to form for 20 min at room temperature before being added to the washed cells. Following 5 h incubation, 1 ml of complete medium containing 20% foetal calf serum was added to the cells. After 12 h the medium/DNA complexes were replaced by complete medium and the cells left for a further 24 h. The cells were then harvested and the cell pellet resuspended in 100  $\mu$ l of 100 mM  $\text{KH}_2\text{PO}_4$  pH 7.8/1 mM dithiothreitol solution and lysed by freeze/thawing three times. Cell debris was removed by centrifugation at 13 000 rpm for 1 min.

#### 2.6. Reporter gene assays

For luciferase assay, 30  $\mu$ l of cell extract was added to 350  $\mu$ l of buffer A pH 7.8 (15 mM  $\text{MgSO}_4$ , 30 mM glycylglycine, 2 mM  $\text{Na}_2\text{ATP}$ ) containing 0.45 mM coenzyme A and 2.56 mM Triton X-100. To this, 150  $\mu$ l of buffer G (30 mM glycylglycine) containing 0.5 mM luciferin (Sigma) was injected and the luminescence read at 560 nm using a Berthold Luma LB9501. CAT assays were performed using the Quan T-CAT Assay following the manufacturer's specifications (Amersham, Slough, UK). Protein content of the cell extract was measured using Bio-Rad protein assay reagent.

#### 2.7. Electrophoretic mobility shift assays (EMSA)

For the preparation of nuclear extracts, cells were centrifuged for 30 s in a microcentrifuge and resuspended in 400  $\mu$ l of ice-cold buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM dithiothreitol and 1 $\times$ Protease Inhibitor Cocktail Solution (Boehringer, Mannheim). Cells were allowed to swell on ice for 15 min before adding 25  $\mu$ l of 10% (v/v) Nonidet P-40. The cells were then vortexed for 15 s and centrifuged for 30 s in a microcentrifuge. The pellet was resuspended in 50  $\mu$ l of ice-cold buffer containing 20 mM HEPES, pH 7.0, 0.4 M NaCl, 1 mM dithiothreitol, 1 $\times$ Protease Inhibitor Cocktail Solution and 5% (v/v) glycerol. After vigorous shaking for 90 min, the nuclear extract was then centrifuged for 30 s at 4°C in a microcentrifuge. The supernatant was collected, divided into small volumes and stored at –70°C. EMSAs were performed as previously described [12]. Nuclear extracts (5  $\mu$ g of protein) were incubated with radiolabelled probe for 20 min at room temperature in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM dithiothreitol, 1 mM EDTA, and 5% (v/v) glycerol. In some experiments nuclear extracts were preincubated for 15 min at 4°C with 3  $\mu$ l or 6  $\mu$ l of anti-FLAG M5 antibody (Sigma) or pre-immune serum before addition of the probe. The oligonucleotides DH1 and C2 used in the EMSAs correspond to the human insulin gene promoter sequences 5'-AGCTGTGAGCAGGGACAGGTC-TGGCCACCGGGCCCTG-3' (located between –238 and –275) and 5'-AGCTGTGAGCAGGGACAGGTCTGGC-3' (located between –238 and –262) respectively.

### 3. Results

A mouse partial Pax4 cDNA sequence was used as a probe to screen the rat insulinoma library CRI-G1. The secondary library screen identified 14 positives clones, and after sequencing, one of the clones was found to contain the full-length sequence of Pax4, which is 1047 bp in length with a single

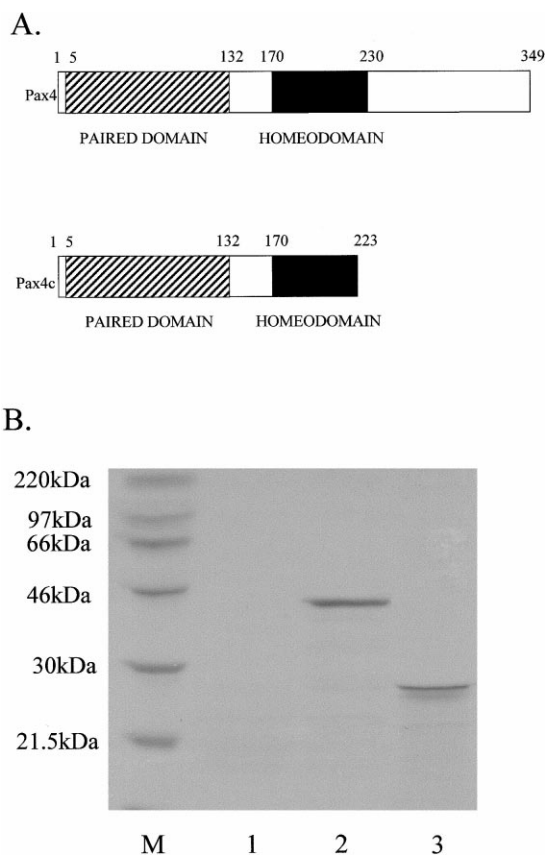


Fig. 1. In vitro translation of Pax4 and Pax4c. A: Schematic diagram of Pax4 and Pax4c. B: RNA was transcribed from Pax4 and Pax4c sub-cloned into pBluescript, using T3 RNA polymerase and translated using rabbit reticulocyte lysate in the presence of [ $^{35}$ S]methionine. The translation products were analysed by SDS-PAGE. Lane 1 shows the translation process in the absence of RNA. Lane 2 shows the translation process using 4  $\mu$ l of Pax4 RNA. Lane 3 shows the translation process using 4  $\mu$ l of Pax4c RNA.

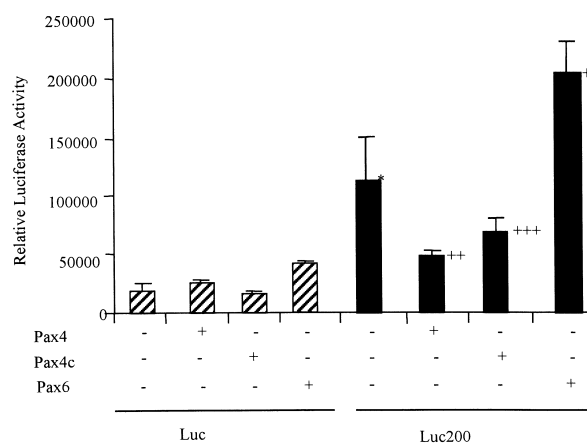


Fig. 2. Effect of Pax4 and Pax4c on pGL-LUC and pGL-LUC200 constructs in MIN6 cells. MIN6 cells were co-transfected with pGL-LUC and Pax4; with pGL-LUC and Pax4c; with pGL-LUC and Pax6; with pGL-LUC200 and Pax4; with pGL-LUC200 and Pax4c; or with pGL-Luc200 and Pax6, as indicated. Luciferase activity is defined as relative luciferase activity where activity is normalised against protein concentration. The data are representative of three separate experiments and show the mean  $\pm$  S.D. of four separate determinants.

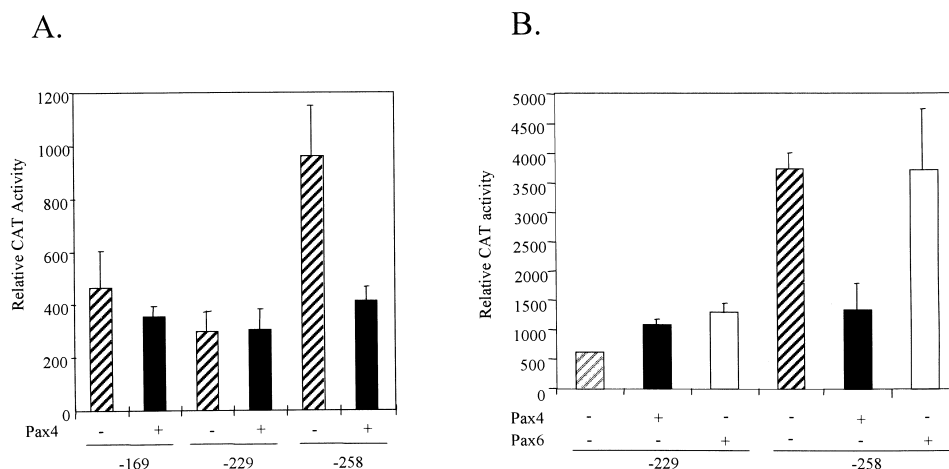


Fig. 3. Effect of Pax4 and Pax6 on human insulin gene promoter/CAT constructs. A: MIN6 cells were co-transfected with pBCins-169 and Pax4; with pBCins-229 and Pax4; or with pBCins-258 and Pax4, as indicated. CAT activity is defined as relative CAT activity where activity is normalised against protein concentration. The data are representative of three separate experiments and show the mean  $\pm$  S.D. of four separate determinants. B: MIN6 cells were co-transfected with pBCins-229 and Pax4; with pBCins-229 and Pax6; with pBCins-258 and Pax4; or with pBCins-258 and Pax6, as indicated. CAT activity is defined as relative CAT activity where activity is normalised against protein concentration. The data are representative of three separate experiments and show the mean  $\pm$  S.D. of four separate determinants.

open reading frame of 349 amino acids (GenBank accession number AF198155). Two of the clones encoded Pax4c, which is similar to Pax4 but lacked the 117 amino acids at the COOH-terminus (GenBank accession number AF198156). A schematic structure of Pax4 and Pax4c is shown in Fig. 1A. When the Pax4 and Pax4c genes were transcribed and translated in vitro they generated single products of approximately 38 kDa and 25 kDa respectively (Fig. 1B). To determine if Pax4 and Pax4c had a role in regulating the insulin gene the effect of overexpressing Pax4 and Pax4c on insulin gene transcription was investigated. MIN6 cells were co-transfected

with the control construct pGL-LUC or pGL-LUC200, which contains a  $-65$  to  $-265$  fragment of the human insulin gene promoter and Pax4 or Pax4c. When overexpressed with pGL-LUC, Pax4 and Pax4c had no effect on transcriptional activity, but when overexpressed with pGL-LUC200 there was a decrease in transcriptional activity (Fig. 2). In order to map the site within the insulin promoter through which Pax4 was mediating its effect, additional insulin gene promoter constructs were used. These comprised deletions of the human insulin gene promoter cloned upstream of the CAT reporter gene [7]. Pax4 had no effect on transcriptional activity when

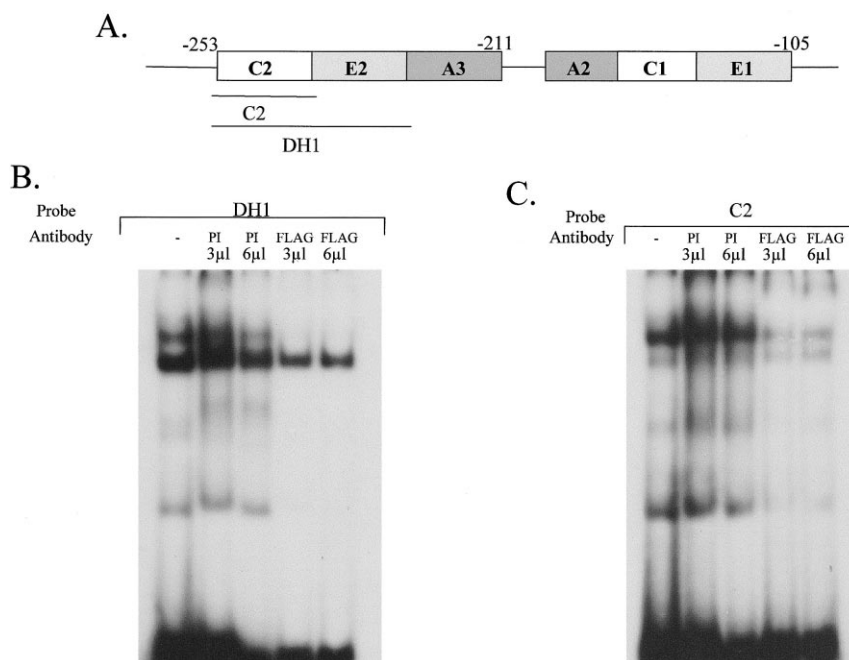


Fig. 4. EMSA analysis of Pax4 binding to the human insulin gene promoter. Pax4 binding to the insulin gene promoter was analysed by binding to the oligonucleotide C2 or DH1, from nuclear cell extracts prepared from BHK cells overexpressing Pax4. Competition was performed by prior incubation of the extract for 20 min with the indicated amount of preimmune rabbit serum (PI) or anti-FLAG antibody.

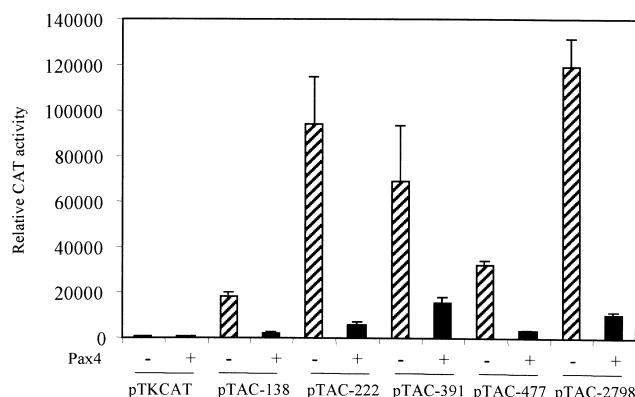


Fig. 5. Effect of Pax4 on human IAPP gene promoter/CAT constructs. A: MIN6 cells were co-transfected with pTKCAT and Pax4; with pTAC-138 and Pax4; with pTAC-222 and Pax4; with pTAC-391 and Pax4; with pTAC-477 and Pax4; or with pTAC-2798 and Pax4, as indicated. CAT activity is defined as relative CAT activity where activity is normalised against protein concentration. The data are representative of three separate experiments and show the mean  $\pm$  S.D. of four separate determinants.

overexpressed with pBCins-169 or pBCins-229, but when overexpressed with pBCins-258 there was a decrease in transcriptional activity, similar to that seen with the pGL-LUC200 construct (Fig. 3A). To determine if the negative effect Pax4 was having on the human insulin gene promoter was specific, another member of the Pax gene family, Pax6, the one most closely related to Pax4, was investigated. Unlike Pax4, Pax6 had no significant effect on transcriptional activity of the pBCins-258 construct (Fig. 3B). Pax4 binding to the insulin gene promoter was then examined by EMSA, using nuclear extracts from BHK cells overexpressing FLAG-tagged PAX4 constructs. Two probes were used, C2 which corresponded to sequences from  $-238$  to  $-262$  and DH1 which corresponded to sequences from  $-238$  to  $-275$  (Fig. 4A). PAX4 was shown to bind to both probes. Binding was inhibited by an anti-FLAG antibody, but not by preimmune serum confirming the specificity of the EMSA assay. We next investigated the effect of Pax4 on a series of deletion constructs corresponding to sequences within the human IAPP promoter [13]. Pax4 exhibited a strong repressive effect on the constructs containing sequences  $-2798$  to  $+450$ ,  $-477$  to  $+450$ ,  $-391$  to  $+450$ ,

$-222$  to  $+450$ , and  $-138$  to  $+450$  (Fig. 5). There was no effect on the control pTKCAT plasmid. These results indicated that PAX4 was repressing the activity of the IAPP promoter through a site located downstream of  $-138$ .

The repressive activity of Pax4 was further investigated by using deletions of Pax4 fused to the DNA-binding domain of GAL4 (amino acids 1–147) in the pSG424 vector. The GAL-Pax4 constructs were co-transfected into the cell lines MIN6 and COS-7 together with the reporter plasmid pGAL4TKCAT, which contains five copies of the GAL4-binding site upstream of the CAT reporter gene. The results showed that full-length Pax4 (amino acids 2–349) exhibited a strong repressive effect in both cell types (Fig. 6). This repressive effect was also seen with constructs containing amino acids 2–230 and 231–349 as well with amino acids 131–349. This suggested that two regions located at the NH<sub>2</sub>-terminus and the COOH-terminus contained repressor activity. The control VP16 construct exhibited positive activity in both cell types under similar conditions.

#### 4. Discussion

In this study we show that Pax4 acts as a transcriptional repressor of the human insulin and IAPP promoters. The repressive effect was mapped to within the region  $-229$  to  $-258$  of the human insulin promoter. EMSA analysis further showed that PAX4 could bind to the C2 element [14] located within this region at  $-244$  to  $-253$ . This element contains a sequence that is also present in a related element of the rat insulin I promoter that is known to bind Pax6 [15]. The consensus binding sequence for Pax4 has yet to be characterised, but based on the similarity in DNA-binding sites for other members of the *pax* gene family [16,17] it is likely to be similar to PAX6. In fact Pax4 has recently been shown to bind similar DNA-binding sites in vitro as the Pax6 protein [18]. Our data support the view that the negative Pax4-binding site is located within the C2 element of the human insulin promoter. The binding site within the human IAPP promoter remains to be mapped. However, the results of Pax4 on the IAPP promoter suggest that Pax4 may be having an effect by binding to the promoter downstream of  $-138$ . Interestingly, a negative element has been identified in the IAPP promoter located between  $-111$  and  $-102$  [13]. Based on the consensus binding

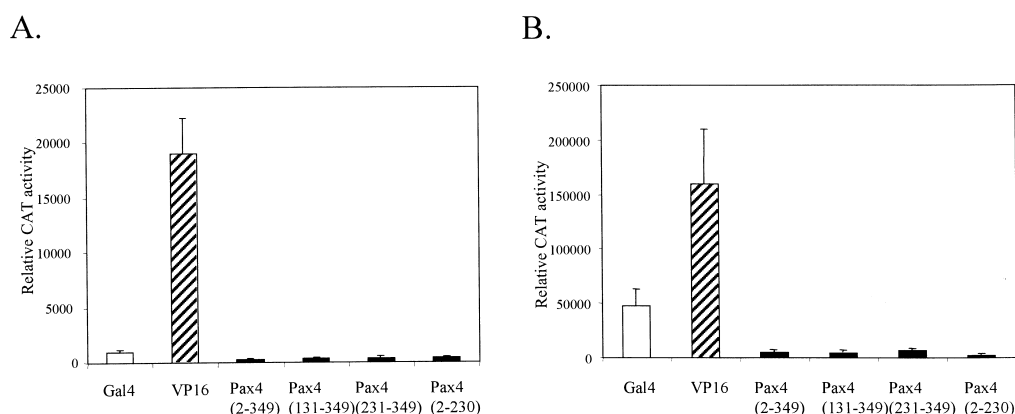


Fig. 6. The transcriptional activation properties of Pax4. MIN6 cells (A) or COS-7 cells (B) were co-transfected with the reporter plasmid pGAL4TKCAT and pSG424 (Gal4); with pGAL4TKCAT and Gal-VP16 (VP16); or with pGAL4TKCAT and the indicated Gal-Pax4 chimeric constructs. CAT activity is defined as relative CAT activity where activity is normalised against protein concentration. The data are representative of three separate experiments and show the mean  $\pm$  S.D. of four separate determinants.

site for *pax* gene family members and particularly Pax6, this element could also act as a possible binding site for Pax4.

Several other member of the *pax* gene family act as transcriptional repressors. Pax2, Pax5 and Pax8 have repressive domains that have been mapped to the COOH-terminus [19]. Pax3 also exhibits repression properties, due to a repression domain located in the first 90 NH<sub>2</sub>-terminal amino acids [20]. In the case of Pax4 at least two regions located within the NH<sub>2</sub>- and COOH-termini contain sequences that can act as transcriptional repressors when fused to a heterologous transcription factor. The fact that Pax4c, which lacks the –224 to –349 sequences at the COOH-terminus, acts as a transcriptional repressor suggests that the two repressor domains can act independently. Recently, a study demonstrated that the COOH-terminal region of Pax4 caused weak activation of the GAL4 reporter in 293 cells, 40% less than Pax6 [18]. Pax6 has a potent transactivation domain and no repression domain located in its COOH-terminus, however both the paired domain and homeodomain can exhibit some negative effects on transactivation function [21].

We isolated two Pax4 cDNAs from the rat insulinoma library. The larger form was similar to the full-length rat Pax4, while the shorter form, which lacked 117 amino acids at the COOH-terminus, was similar to the rat Pax4c [22]. There are two other rat Pax4 isoforms, Pax4b and Pax4d, which did not appear in any of the clones isolated from the rat cDNA library. This suggests that these isoforms may be expressed at low levels in rat  $\beta$ -cells. Northern blot analysis of RNA from the rat  $\beta$ -cell line BRIN D-11 [23] showed the presence of one major transcript and additional smaller transcripts (data not shown). Pax8 also has multiple isoforms that arise from alternative splicing of a primary transcript [24]. These Pax8 isoforms have different transactivating properties. In the case of Pax4, the Pax4 and Pax4c isoforms both exhibit strong repressive activity while co-expression of the two isoforms failed (data not shown) to demonstrate any regulatory (such as dominant negative) effect of one isoform on the other.

**Acknowledgements:** Supported by grants from the Wellcome Trust and the British Diabetic Association. S.C.C. was supported by a research studentship from the Medical Research Council. We thank Dr M. Carty and Dr W. Soeller of Pfizer Inc., Groton, CT, USA, for supplying the IAPP gene promoter constructs.

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