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A RIPE3b1-like factor binds to a novel site in the human insulin promoter in a redox-dependent manner

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Abstract In the human insulin gene, a regulatory sequence upstream of the transcription start site at −229 to −258 (the E2 element) binds a ubiquitous factor USF. The present study led to the identification of a second factor, D0, that binds to an adjacent upstream site, the C2 element, that has previously not been described. The results demonstrate that D0 exhibits similar properties to RIPE3b1, a factor shown to be an important determinant of insulin gene β-cell-specific expression. Binding of D0 to the C2 element was abolished by the oxidising agent diamide, and the alkylating agent *N*-ethylmaleimide. The results indicate that expression of the insulin gene may be regulated by a redox-dependent pathway involving RIPE3b1 or a RIPE3b1-like factor.

Key words: Insulin gene; Rat insulin protomer element 3b1

1. Introduction

Transcription of the insulin gene is regulated for the most part by sequences located within a region spanning 350 base pairs from the transcription start site [1]. A number of *cis*-acting elements have been characterised [2]. In the human insulin gene there are at least three A elements that bind a homeodomain factor, which plays an essential role in development of the islets of Langerhans [3], and in the regulation of the insulin gene by glucose [4].

Other important regulatory sequences in the human insulin promoter include the C1 element at −116 and two E box elements at −101 and −233. The equivalent C element in the rat insulin II gene has been extensively studied, and shown to bind a factor, RIPE3b1 [5–7], which has yet to be cloned and sequenced. RIPE3b1 may be important in the regulation of the insulin gene by glucose [8,9]. The proximal E element binds a basic helix-loop-helix factor, IEF1, that is a heterodimer of NeuroD/Beta2 and the ubiquitous E2A gene product E47 [10,11]. The distal E element is more complex binding at least three factors, one of which has been identified as the ubiquitous factor USF [12,13].

Since the distal E element lies within a transcriptionally important region of the human insulin promoter [14,15], the current study was undertaken to characterise further the proteins that bind at this site. The results show that there is another protein binding site adjacent to the E2 element. We show that this site, the C2 element, binds a RIPE3b1-like factor, the DNA binding activity of which is regulated in a redox-dependent manner.

2. Materials and methods

2.1. Chemicals and reagents

Oligodeoxynucleotides were purchased from Alta Bioscience, University of Birmingham, Birmingham, UK and Cruachem Ltd, West of Scotland Science Park, Acre Rd, Glasgow, UK. Single-stranded complementary oligonucleotides were annealed as previously described [16] and used as double-stranded probes labelled with [γ-^{32}P]ATP, or competitors, in electrophoretic mobility shift assays. An antibody specific for E47 was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

2.2. Preparation of nuclear extracts

Nuclear extracts were prepared by a modification of a previously described method [17]. Cells were centrifuged for 30 s at 12000×*g* and resuspended in 400 μl of ice-cold buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM dithiothreitol and 1×Protease Inhibitor Cocktail Solution (Boehringer Mannheim). Cells were allowed to swell for 15 min on ice before adding 25 μl of 10% (v/v) Nonidet P-40. The cells were then vortexed for 15 s and centrifuged for 30 s at 12000×*g*. The pellet was resuspended in 50 μl of ice-cold buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM dithiothreitol, 1×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 12000×*g* and 4°C in a microcentrifuge. The supernatant was collected, divided into small volumes and stored at −70°C.

2.3. Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed as previously described [16]. Nuclear extracts (2–4 μg of protein) were incubated with the ^{32}P-labelled probe for 15 min at 4°C in buffer containing 25 mM Tris-HCl, pH 7.5, 50 mM KCl, 250 ng poly(dA-dT).poly(dA-dT), 1 mM dithiothreitol, and 5% (v/v) glycerol. In redox experiments, dithiothreitol was omitted from the buffer. For competition experiments, the nuclear extract was incubated with 2.5–10.0 pmol (approximately 50–200-fold excess) of unlabelled oligonucleotide for 15 min at 4°C before addition of the probe. In some experiments nuclear extracts were preincubated for 15 min at 4°C with 2 μl of anti-E47 antibody or preimmune serum before addition of the probe.

3. Results

Two important criteria for optimal binding of individual transcription factors to DNA in vitro are the type of competitor polynucleotide used to sequester non-specific DNA binding, and the length of DNA probe used. To investigate binding of proteins to the E2 region of the human insulin promoter, electrophoretic mobility shift assays were performed using two oligonucleotide probes of different lengths,

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Abbreviations: 2-ME, 2-mercaptoethanol; DM, diamide; DTT, dithiothreitol; EFD3, endocrine factor D3; EMSA, electrophoretic mobility shift assay; HLH, helix-loop-helix; IEF1, insulin enhancer factor 1; IUF1, insulin upstream factor 1; NEM, *N*-ethylmaleimide; RIPE3, rat insulin promoter element; USF, upstream stimulating factor

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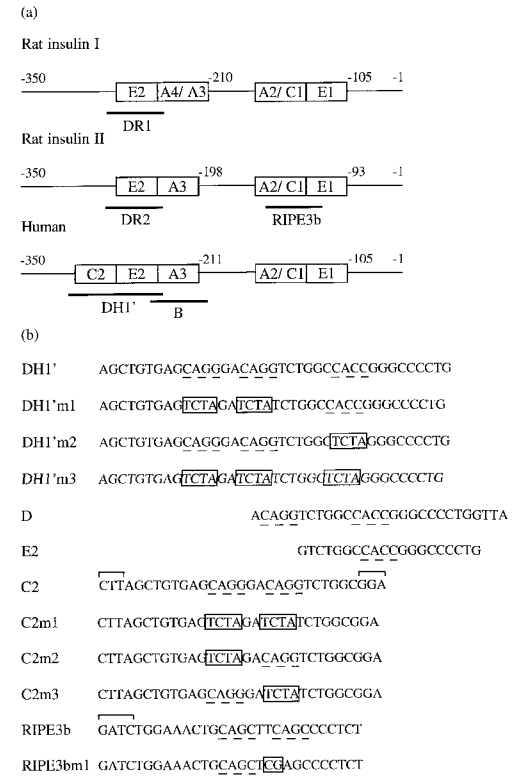

Fig. 1. CA(G/C)(G/C) sequences in the insulin gene promoter. a: Arrangement of regulatory sites in the rat I, rat II and human insulin promoters with the position relative to the transcription start site indicated as negative numbers. The relative locations of the oligonucleotides used in this study are represented by bold lines. b: Sequences of oligonucleotides used in this study. CA(G/C)(G/C) sequences are indicated by dashed lines. Boxed sequences represent base pair substitutions. Bracketed sequences represent linker sequences. Oligonucleotides DR1, DR2, DR2m3 and B have been described previously [12].

D and DH1′ (the sequences of oligonucleotides used in this study are given in Fig. 1), and a range of dissimilar polynucleotides. Two major complexes, D0 [12] and USF [13], and two minor complexes, X1 and X2, were observed using oligonucleotides D and DH1′ as probes and an HIT T15 nuclear extract (Fig. 2). Formation of complexes D0 and X2, were most efficient using the longer oligonucleotide DH1′ as probe and poly(dA-dT) as non-specific competitor. Since oligonucleotides D and DH′ both contain the E2 element, these results suggested that sequences outside the E2 element might be involved in binding of the unidentified factor D0 to DNA.

Adjacent to the E2 element in the human insulin promoter is a region that contains a direct repeat of the sequence CAGG, and is henceforth referred to as the C2 region. To investigate whether factor D0 recognises this site, two mutant oligonucleotides of DH1′ were used. Oligonucleotide DH1′m1 contained critical mutations within the C2 site, i.e. CAGG to TCTA, while E2 completely lacked the C2 region. Using oligonucleotide DH1′ as probe, competition for the D0 complex was observed with oligonucleotide DH1′ but not with oligonucleotides DH1′m1, E2 nor an unrelated oligonucleotide Oct1 (Fig. 2c). Confirmation that the D0 complex bound at a site distinct to the E2 element was obtained using several further mutant oligonucleotides of DH1′ as probes and an HIT T15 nuclear extract. Oligonucleotide DH1′m2 contained critical mutations at the E2 site, i.e. CACC to TCTA, and DH1′m3 at both the C2 and E2 sites. Oligonucleotide C2 completely lacked the E2 site, while C2m1 contained critical mutations within the C2 site, i.e. CAGG to TCTA. Formation of the D0 complex was observed using DH1′, DH1′m2 and C2 as probes, whereas mutations within DH1′m1, DH1′m3 and C2m1 abolished binding of the D0 complex (Fig. 3a). In contrast, formation of the USF complex was observed with oligonucleotides DH1′, DH1′m1 and E2, but not with any other oligonucleotide (Fig. 3a). This result indicated that D0 binds to the C2 region, and not to the E2 site.

To determine whether both CAGG sequences are involved in binding of the D0 complex to the C2 region, two further mutant oligonucleotides of C2 were used. Oligonucleotide C2m2 contained critical mutations at the upstream CAGG sequence, i.e. CAGG to TCTA, while C2m3 contained the same mutation but at the downstream CAGG site. Using

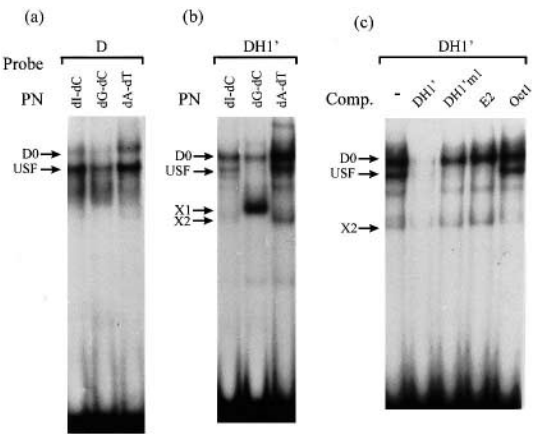

Fig. 2. Binding of nuclear extract proteins to oligonucleotides D and DH1′. EMSA using oligonucleotides D (a) and DH1′ (b) as probes and an HIT T15 nuclear extract. The nuclear extract was preincubated for 15 min at 4°C with 500 ng of the indicated polynucleotide before addition of the probe. (c) EMSA using DH1′ as probe and an HIT T15 nuclear extract. The indicated unlabelled competitor oligonucleotides were used at approximately 50-fold excess. The positions of the major complexes D0, USF, X1 and X2 are indicated.

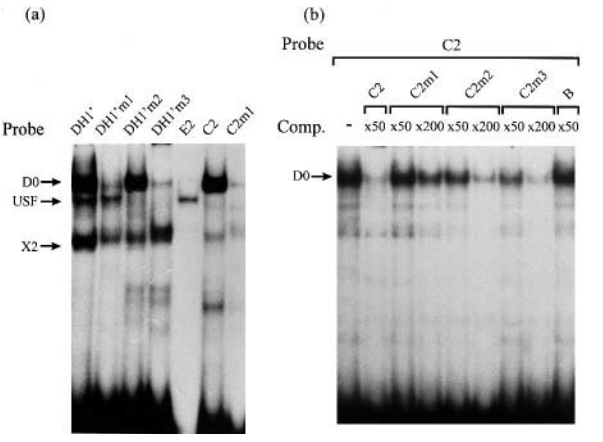

Fig. 3. Complex D0 binds to the sequence CAGGGACAGG. a: EMSA using oligonucleotides DH1′, DH1′m1, DH1′m2, DH1′m3, E2, C2 and C2m1 as probes and an HIT T15 nuclear extract. b: EMSA using oligonucleotide C2 as probe and an HIT T15 nuclear extract. The unlabelled competitor oligonucleotides were used at approximately 50- and 200-fold excess as indicated. The positions of the major complexes D0, USF and X2 are indicated.

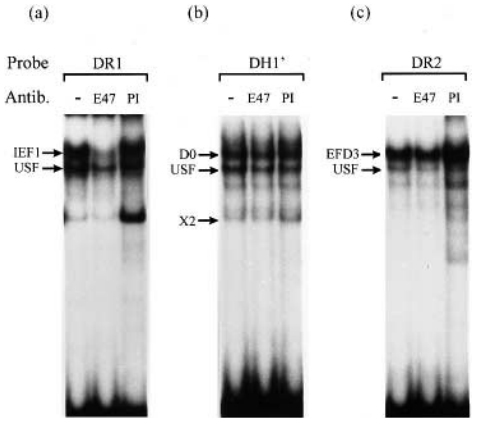

Fig. 4. The E47 antibody does not compete for binding of complex D0 to oligonucleotide DH1′. EMSA using oligonucleotides DR1 (a), DH1′ (b) and DR2 (c) as probes, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. Nuclear extracts were preincubated with 2 μl of anti-E47 antibody (E47) or preimmune serum (PI). The positions of the major complexes IEF1, D0, X2, EFD3 and USF are indicated.

oligonucleotide C2 as probe and an HIT T15 nuclear extract, competition for complex D0 was not observed with oligonucleotide C2m1 at either 50- or 200-fold excess (Fig. 3b). In contrast, competition for the D0 complex was observed with both C2m2 and C2m3 as competitors at 200-fold excess. This result suggests that both CAGG sequences might be involved in binding of D0 to the C2 region.

The D0 complex shares similar binding characteristics with several other important insulin gene regulatory proteins; IEF1, RIPE3b1 and EFD3. IEF1 is a heterodimer of a class A protein E47 and a cell-specific HLH partner BETA2/ NeuroD [10,11], while RIPE3b1 and EFD3 have yet to be fully characterised. To investigate whether factors D0 and IEF1 are related, an oligonucleotide DR1, containing the E2 sequence from the rat insulin I gene was used. Two major complexes, IEF1 and USF were observed using oligonucleotide DR1 as probe, poly(dA-dT) as non-specific competitor and an HIT T15 nuclear extract. Confirmation of the identity of the IEF1 complex was obtained with an anti-E47 antibody that competed for binding of the IEF1 complex, but did not affect binding of USF (Fig. 4a). In contrast, the antibody failed to compete for formation of the D0 complex using oligonucleotide DH1′ as probe (Fig. 4b), or EFD3 using oligonucleotide DR2 as probe (Fig. 4c). These results indicated that the D0 complex was not the insulin gene regulatory protein IEF1.

To investigate whether factors D0 and RIPE3b1 are related, an oligonucleotide RIPE3b, containing the RIPE3b (or C1) sequence from the rat insulin II gene was used. A major complex R1 was observed using oligonucleotide RIPE3b as probe and an HIT T15 nuclear extract. Confirmation that the R1 complex represented a RIPE3b1-like factor was obtained using a mutant form of this oligonucleotide, RIPE3bm1, that has previously been shown to abolish binding of the RIPE3b1 complex. Using RIPE3bm1 as probe and an HIT T15 nuclear extract only weak formation of the R1 complex was observed (Fig. 5a). Competition for complex R1 was observed with oligonucleotides RIPE3b, C2 and DR2, but not with C2m1 and DR2m3 which contain critical mutations within their respective binding sites for D0 and EFD3. Lower complexes observed using RIPE3b as probe were competed for by the unrelated oligonucleotide B and most likely represent non-specific binding factors. Further experiments directly compared the effect of salt on binding of complexes to oligonucleotides DH1′, DR2 and RIPE3b. Formation of the major complexes D0, EFD3, USF and R1 were all abolished in 200 mM and 400 mM salt using oligonucleotides DH1′, DR2 and RIPE3b as probes, and an HIT T15 nuclear extract (Fig. 6a–c). In contrast, formation of complex X2 to oligonucleotide DH1′ (Fig. 6a) and the β-cell-specific factor IUF1 to oligonucleotide B (Fig. 6d) were still detected in 400 mM salt. Together these results suggest that the factors responsible for the formation of complexes D0 and EFD3 most likely represent RIPE3b1-like factors.

In vitro binding of many mammalian transcription factors is regulated by the redox state of the protein (reviewed in [18]). To investigate the effect of redox conditions on the binding activity of the D0 complex, the oxidising agent diamide was used. Using oligonucleotide C2 as probe, only weak formation of the D0 complex was observed when HIT T15 nuclear extract was treated with 10 mM diamide for 15 min at 4°C before addition of the probe (Fig. 7a). To determine whether this effect is reversible, HIT T15 extract pre-incubated with diamide was then treated with various concentrations of the reducing agent 2-mercaptoethanol for 15 minutes at 4°C before addition of the probe. Partial D0 binding activity was restored using 0.1% 2-ME, whereas higher concentrations of 0.25% and 0.5% 2-ME, or 20 mM dithiothreitol (data not shown) resulted in the full restoration of D0 binding activity. To determine whether this effect was specific, HIT T15 nuclear extract was treated with the alkylating agent *N*-ethylmaleimide (NEM). Treatment of the extract with 10 mM NEM resulted in an almost complete loss of binding activity of the D0 complex (Fig. 7b). In contrast to treatment with diamide, no restoration of binding activity for D0 was observed by further treatment with the reducing agents 2-ME or DTT. By comparison, binding of IUF1 to DNA was unaffected by treatment of nuclear extract with 2-ME, DM or DTT (Fig. 7c). These results indicated that the D0 complex binds to DNA in a redox-dependent manner.

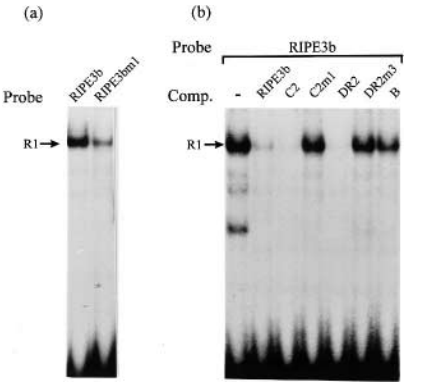

Fig. 5. The major binding complex D0 is a RIPE3b1-like factor. a: EMSA using oligonucleotides RIPE3b and RIPE3bm1 as probes and an HIT T15 nuclear extract. b: EMSA using RIPE3b as probe and an HIT T15 nuclear extract. The indicated unlabelled competitor oligonucleotides were used in approximately 50-fold excess. The position of the major complex R1 is indicated.

4. Discussion

This study has demonstrated that the insulin gene complex D0 binds to the sequence CAGGGACAGG, referred to as the C2 element, in the human insulin promoter. This sequence resembles the binding sites recognised by two other important insulin gene regulatory proteins, IEF1 (CATCTG) and RIPE3b1 (CAGCTTCAGC). However, several lines of evidence suggest that the D0 complex is not IEF1, but instead is related to the RIPE3b1-binding complex. Firstly, an antibody specific for IEF1 is unable to compete for the D0 complex. Secondly, oligonucleotide C2, is able to compete for binding of the RIPE3b1-like complex R1, whereas oligonucleotide C2m1 that contains critical mutations within the C2 element does not. And thirdly, whereas formation of IEF1 is relatively salt-independent [12], binding of the complexes D0 and R1 were abolished at ionic concentrations greater than 200 mM KCl.

This study has also demonstrated that factor EFD3 represents a RIPE3b1-like factor. EFD3 was initially characterised as a β-cell enriched factor that binds to the sequence CAGGAG at position −217 in the rat insulin II gene [12]. The role of this factor in transcription of the rat insulin II gene has not yet been studied in detail, although a two-fold reduction in activity was observed by linker scanning mutagenesis of this region (−229 to −218) [5]. In contrast, D0 binds at position −242 in the human insulin promoter, which lies adjacent to the E2 element. A previous study has shown that both the C2 and E2 elements are located within a transcriptionally important region of the promoter. Thus deletion of the region from −258 to −229 produced a 25-fold reduction in transcription activity [14]. The individual roles of the C2 and E2 sites within this region requires further investigation.

RIPE3b1 is a β-cell-specific factor that was first identified as binding to the RIPE3 element located between positions −126 to −86 in the rat insulin II gene [5–7]. The RIPE3 element behaves as a β-cell-specific enhancer element when linked to a heterologous minimal promoter [6,19] and is divided into two subelements, RIPE3a (−110 to −86) and RIPE3b (−126 to −101). RIPE3b1 and a ubiquitous complex, RIPE3b2, composed of at least three polypeptides (p58, p62 and p110) [20], recognise the RIPE3b element. Screening of a HIT cell cDNA library with a RIPE3b probe recently identified the Rip-1

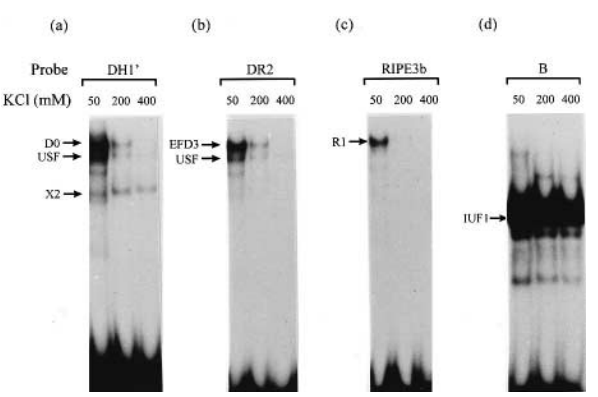

Fig. 6. Effect of salt concentration on the binding of nuclear extract proteins. EMSA using oligonucleotides DH1′ (a), DR2 (b), RIPE3b (c) or B (d) as probe and an HIT T15 nuclear extract. The salt concentrations used in the binding buffer are indicated. The positions of the major complexes D0, USF, X2, EFD3, R1 and IUF1 are indicated.

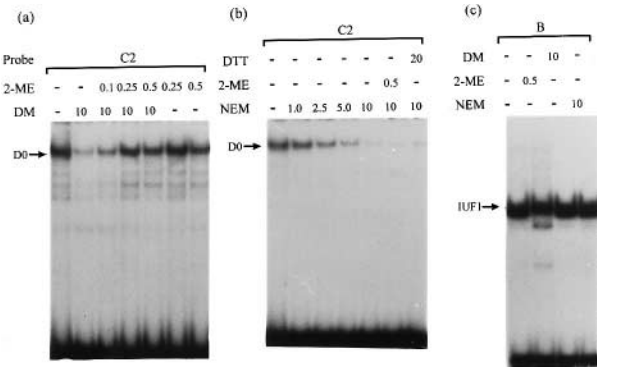

Fig. 7. Complex D0 binds to the insulin promoter in a redox-dependent manner. a: EMSA using oligonucleotide C2 as probe and an HIT T15 nuclear extract. Nuclear extracts were preincubated with 10 mM diamide as indicated for 15 min at 4°C and then treated with 0.1%, 0.25% or 0.5% 2-mercaptoethanol (2-ME) respectively for 15 min at 4°C before addition of the probe. b: EMSA using oligonucleotide C2 as probe and an HIT T15 nuclear extract. Nuclear extracts were preincubated with *N*-ethylmaleimide (NEM) at the indicated concentration for 15 min at 4°C and then treated with 0.5% 2-ME or 20 mM dithiothreitol (DTT) before addition of the probe. c: EMSA using oligonucleotide B as probe and an HIT T15 nuclear extract. Nuclear extracts were treated with 0.5% 2-ME, 10 mM diamide or 10 mM NEM as indicated for 15 min at 4°C before addition of the probe. The positions of the major complexes D0 and IUF1 are indicated.

protein which is most likely a component of the RIPE3b2-binding complex [20]. The RIPE3b1 factor has not yet been isolated.

In the rat I and rat II insulin gene promoters there is no equivalent sequence of the C2 element at a related position. However, a recent paper reported that in the rat I insulin gene at position −329 to −307 there is a related sequence (CACC) that binds the factor PAX6 [21]. In the pancreas, two Pax genes Pax4 and Pax6 are expressed, which possess a conserved sequence motif, the paired box, that encodes a DNA-binding domain [22]. Pax4 is required for normal development of pancreatic endocrine cells as mice homozygous for a null allele of Pax4 lack mature β- and δ-cells, while α-cells are present in higher numbers [23]. In contrast, the pancreas of Pax6 homozygous mutant mice lack glucagon-producing cells, suggesting that Pax6 is essential for the differentiation of α-cells [24]. To date, there have been no reports that Pax4 or Pax6 can recognise sites within the human insulin promoter. However, based on the observation that RIPE3b1 and PAX4/6 share similar binding sites and both have important roles in pancreatic islet hormone gene expression, a likely candidate gene for the factor RIPE3b1 is a member of the PAX family of vertebrate genes.

In recent years there have been major advances made in the identification and cloning of factors that bind to and activate the insulin gene. The new challenges now lie in understanding how the function of insulin gene factors are controlled within the β-cell. IUF1 (insulin upstream factor 1), for example, is a β-cell-specific factor that binds to at least three sites within the human insulin promoter [25]. Binding of IUF1 is triggered when islets of Langerhans are exposed to high glucose, and abolished by exposure to low glucose [4]. A recent study showed that glucose (a cellular stress) activates a specific kinase, SAPK2 (stress-activated protein kinase), within the β-cell that in turn activates IUF1 indirectly by a novel IUF1-acti-

vating enzyme [26]. Similarly, RIPE3b1 is a regulator of glucose-mediated transcription of the insulin gene [8,9]. In particular RIPE3b1 binding activity has been shown to be stimulated approximately eight-fold by glucose. This raises the question as to what mechanisms are involved in modulating DNA binding of RIPEb1.

Oxidative stress can also modulate expression of certain genes, by pathways that most likely involve protein kinases [18]. However, an additional effect of oxidative stress is to directly modulate redox-sensitive regulatory factors through oxidation of sulphydryl residues. For example, the binding of many factors, including USF [27], NF-κB [28], AP-1 [29] and PAX8 [30], is reduced or lost when critical cysteine residues are oxidised. In the present study we have demonstrated that binding of the RIPE3b1-like complex, D0, is reduced when the protein is treated with the oxidising agent, diamide. Binding of this factor can then be restored by treatment with a reducing agent such as 2-ME. Furthermore, this effect appears to be specific, as alkylation with NEM abolishes binding of the factor irreversibly. From these results we can thus suggest that one way in which insulin gene transcription might be controlled is by a redox-regulated pathway that affects binding of RIPE3b1 or a RIPE3b1-like factor. Experiments are currently under way to investigate whether changes in the redox environment of the β-cell can regulate expression of the insulin gene.

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