The insulin enhancer binding site 2 (IEB2; FAR) box of the insulin gene regulatory region binds at least three factors that can be distinguished by their DNA binding characteristics

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Located at approximately 230 bp upstream from the transcription start site, the insulin enhancer binding site 2 (IEB2) or FAR region of the insulin gene is one of several important sequences involved in regulating transcription of the gene. The present study was undertaken to characterize the transcription factors binding at the IEB2/FAR region of the rat insulin II gene and to compare these with factors known to bind to the equivalent sequence in the rat I and human insulin genes. An endocrine-enriched factor, EFD3, was identified, which bound to the sequence CAGGAG. A second factor (D4) was identified as the widely expressed factor USF (upstream stimulating factor), while a third factor (D5) remained largely uncharacterized. The binding affinities of these three factors differed in the three genes, suggesting that the role of the IEB2/FAR sequence may vary subtly between the rat insulin II, rat insulin I and human insulin genes.

INTRODUCTION

Expression of the insulin gene is dependent on multiple *cis*-acting elements located upstream from the transcription start site [1]. In the rat insulin I gene two closely related 8 bp sequences, located at positions −104 [insulin enhancer binding site 1] (IEB1)/NIR box] and −223 (IEB2/FAR box), contain the E-box sequence CATCTG. Transfection studies have demonstrated that transcription of the insulin gene is virtually abolished when block mutations are made at both IEB1 and IEB2 sites [2], although transgenic mice studies suggest that sequences in addition to these are also important [3]. The factor IEF1 (insulin enhancer factor 1), first identified by DNA binding assays [4], is capable of recognizing both IEB sites. IEF1 appears to be a heterodimer of the widely distributed helix–loop-helix (HLH) proteins E12/E47, which are differential splice products of the E2A gene [5–7], and an HLH protein(s) with a pattern of expression restricted to endocrine cells [8,9]. The IEB2/FAR box interacts synergistically with an adjacent TAAT motif (the FLAT element) to function as a tissue-specific mini-enhancer [10]. There is a similar TAAT element adjacent to the IEB1/NIR box [11], but it has yet to be determined whether these sequences act synergistically.

The IEB1/NIR sequence is highly conserved among various insulin genes. In the rat insulin II gene it forms an integral part of the rat insulin promoter element 3 (RIPE3), which binds at least three distinct factors [12]. In contrast, the sequence of the IEB site at the FAR position (IEB2) differs; i.e. CATCTG in the rat insulin I gene, CACCCAGGAG in the rat insulin II gene and CACCGG in the human insulin gene. The functional significance of this sequence divergence is unknown. In the human insulin gene the IEB2 sequence binds the ubiquitous HLH protein USF (upstream stimulatory factor) and an uncharacterized factor, D2 [13]. The nature of the factors binding at the IEB2 sequence in the rat insulin II gene is unknown.

The IEB2/FAR element clearly has an important role in regulating transcription of the rat insulin I gene, while recent studies suggest that the equivalent sequences in the human and rat insulin II gene may also have regulatory functions [14,15]. The aim of the present study was to investigate binding of proteins to the IEB2 site in the rat insulin II gene. We report here that at least three different factors bind at this site.

EXPERIMENTAL

Chemicals and reagents

Oligonucleotides were purchased from Alta Bioscience, University of Birmingham, Birmingham, U.K. 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 the 43 kDa form of human USF was obtained from Dr. M. Sawadogo, MD Anderson Cancer Center, University of Texas, Houston, TX, U.S.A.

Cell lines

HIT T15 is a cell line derived from simian virus 40 (SV40)-transformed islets of Langerhans isolated from syrian hamsters. HIT M2.2.2 is a subclone of the HIT cells. βTC3 cells were derived from an insulinoma obtained by targeted expression of the SV40 T antigen in transgenic mice. 3T3, BHK and NRK

Abbreviations used: IEB1 and IEB2, insulin enhancer binding sites 1 and 2; EFD3, endocrine factor D3; USF, upstream stimulating factor; IEF1, insulin enhancer factor 1; HLH, helix-loop-helix; ML-U, adenovirus major late promoter upstream sequence; SV40, simian virus 40; SRF, serum response factor; EMSA, electrophoretic mobility shift assay.

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cells, and the mouse pituitary corticotrophic cell line, AtT20, were used as non-insulin-expressing cell lines.

Preparation of nuclear extracts

Nuclear extracts were prepared by a modification of the method of Schreiber et al. [17]. Cells were centrifuged for 15 s at 12000 *g* and resuspended in 400 μl of ice-cold buffer containing 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF. 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 EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 mM *p*-aminobenzoic acid, 10 μg/ml aprotinin and 5% (v/v) glycerol. After vigorous shaking at 4 °C for 1–2 h, the nuclear extract was then centrifuged for 5 min at 12000 *g* (4 °C) in a microcentrifuge. The supernatant was collected, divided into small volumes and stored at −70 °C.

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 room temperature in buffer containing 25 mM Tris/HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol and 5% (v/v) glycerol. For competition experiments, the nuclear extract was incubated with 2.5–5.0 pmol (approx. 50–100-fold excess) of unlabelled oligonucleotide for 15 min at room temperature before addition of the probe. In some experiments nuclear extracts were preincubated for 15 min at room temperature with 1 μl of anti-USF antibody or preimmune serum before addition of the probe.

RESULTS

The relative locations of the IEB sequence in the three insulin genes and the sequence of the oligonucleotides used in this study are shown in Figure 1. To investigate binding of proteins to the IEB2 region of the rat insulin II gene, electrophoretic mobility shift assays were performed using an oligonucleotide probe, DR2, containing the IEB2 sequence, and a variety of cell nuclear extracts. Previous studies have demonstrated that DNA binding proteins have differential affinities for competitor polynucleotides that are used to sequester non-specific DNA binding factors [18]. In the present study three dissimilar polynucleotides, poly(dI-dC), poly(dG-dC) and poly(dA-dT), were used as non-specific competitors. Three complexes (D3, D4 and D5) were observed using oligonucleotide DR2 as probe and an HIT T15 nuclear extract (Figure 2a). Complexes D3 and D4 were formed most efficiently with poly(dA-dT), and complex D5 with poly(dG-dC). The differential binding properties of the factors responsible for complexes D3, D4 and D5 were clearly illustrated by increasing the ratio of poly(dI-dC) relative to poly(dA-dT) in the binding reaction (Figure 2b). Complex D3 was observed with extracts from endocrine cell lines such as βTC3, HIT M2.2.2, HIT T15 and AtT20 (Figure 2c), but not with nuclear extracts from 3T3 (Figure 2c) or BHK cells, nor with the rat kidney cell line NRK (results not shown). Complex D4 was observed with extracts from the β-cell lines HIT M2.2.2 and HIT T15, while a new complex D4′, with a slightly faster mobility than D4, was observed with extracts from βTC3 and AtT20 cells (Figure 2c), and the fibroblast cell lines 3T3 and BHK (results not shown).

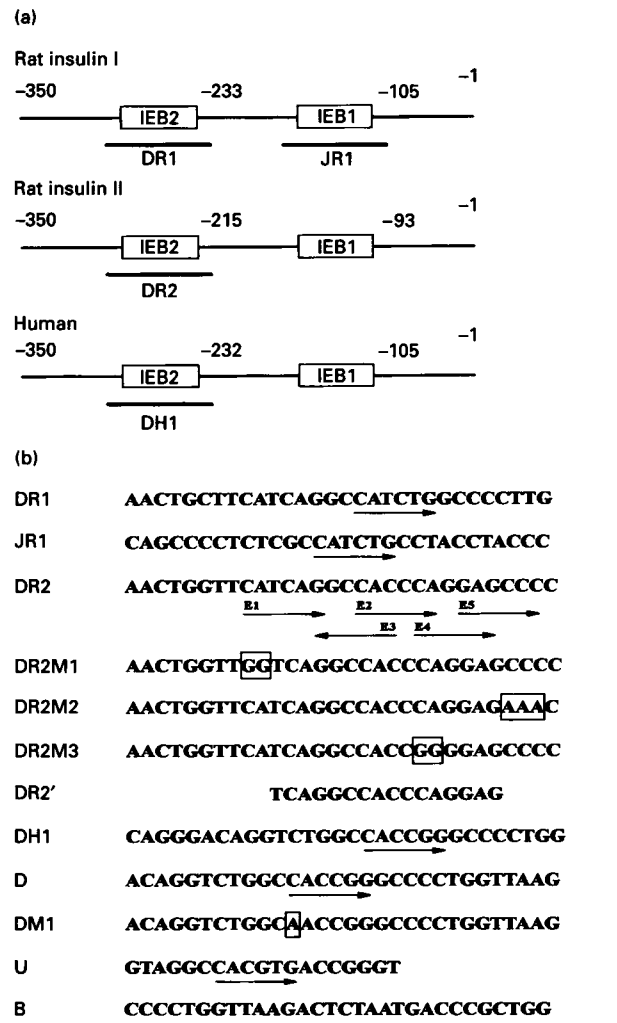

Figure 1 IEB sequences in the insulin gene enhancer

(**a**) Arrangement of IEB sites in the rat I, rat II and human insulin gene enhancers 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. E-box-related sequences are indicated by arrows. Sequences E1–E5 represent putative binding sites within oligonucleotide DR2. Boxed sequences represent base pair substitutions. Oligonucleotides B, D, Dm1 and U (USF) have been described previously [13,14].

These results indicated that at least three proteins bind to the IEB2 region in the rat insulin II gene. All three factors displayed differential binding for a range of non-specific competitors. The major binding activity, henceforth referred to as endocrine factor D3 (EFD3), was enriched in nuclear extracts from endocrine cell lines.

The binding activity EFD3 shared some characteristics with the factor IEF1. To determine whether IEF1 and EFD3 represent similar factors, an oligonucleotide, JR1, containing the IEB1 sequence from the rat insulin I gene, was used. Three complexes (J1, J2 and J3) were observed using oligonucleotide JR1 as probe, poly(dA-dT) as non-specific competitor and an HIT T15 nuclear extract (Figure 3a). Weak competition for formation of complex J1, previously identified as IEF1 [13], was observed by prior incubation of the extract with a 200-fold excess of oligonucleotide DR2. Further experiments directly compared the effect of non-specific competitor DNA and salt (KCl) con-

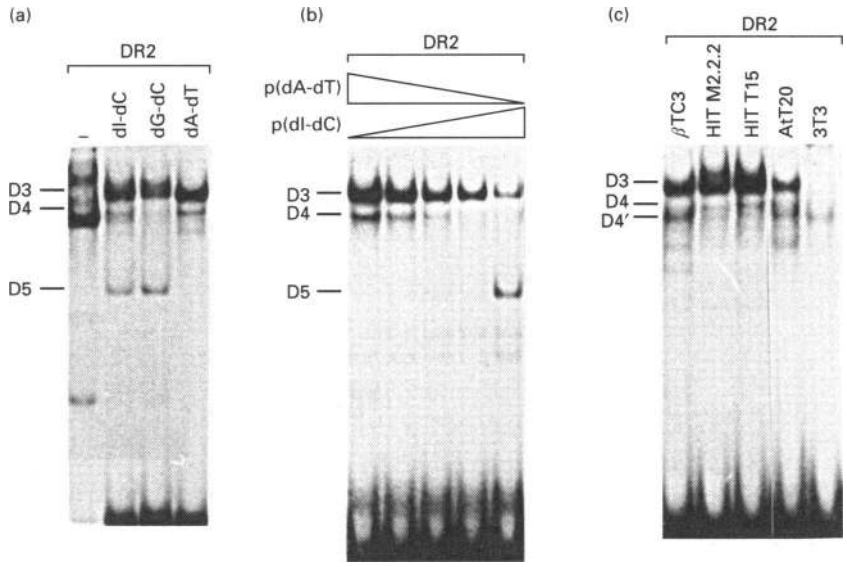

Figure 2 Binding of nuclear extract proteins to oligonucleotide DR2

(**a**) EMSA using oligonucleotide DR2 as probe and an HIT T15 nuclear extract. The nuclear extract was preincubated for 15 min at room temperature with no addition (–) or with 500 ng of the indicated polynucleotide before addition of the probe. The positions of the three major complexes D3, D4 and D5 are indicated. (**b**) EMSA using DR2 as probe and an HIT T15 nuclear extract. Poly(dI-dC) [p(dI-dC)] was used at 0, 125, 250, 375 and 500 ng per lane. Poly(dA-dT) [p(dA-dT)] was used at 500, 375, 250, 125 and 0 ng per lane. (**c**) EMSA using oligonucleotide DR2 as probe, poly(dA-dT) as polynucleotide and the indicated nuclear extracts. The positions of the major complexes D3, D4 and D4′ are indicated.

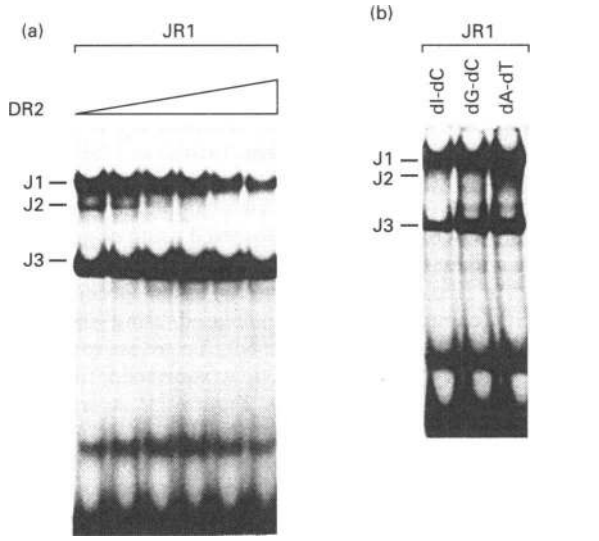

Figure 3 The DR2 oligonucleotide does not compete for binding of IEF1 to oligonucleotide JR1

(**a**) EMSA using oligonucleotide JR1 as probe, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. The competitor oligonucleotide DR2 was used at 0-, 5-, 25-, 50-, 100- and 200-fold excess. The positions of the three major complexes J1, J2, J3 are indicated. (**b**) EMSA using oligonucleotide JR1 as probe, an HIT T15 nuclear extract and the indicated polynucleotides.

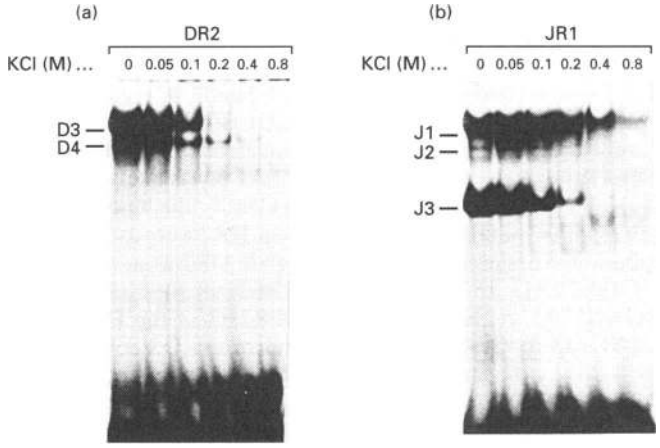

Figure 4 Effect of salt concentration on the binding of nuclear extract proteins

EMSA using oligonucleotide DR2 (**a**) or oligonucleotide JR1 (**b**) as probe, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. The KCl concentrations used in the binding buffer are indicated.

centration on the binding activities of EFD3 and IEF1. Formation of complex J1 was observed with all three polynucleotides using oligonucleotide JR1 as probe (Figure 3b). Using oligonucleotide DR2 as probe and an HIT T15 nuclear extract, formation of the D3 complex was abolished in 200 mM salt (Figure 4a). By comparison, formation of complex J1 was still detected in 800 mM salt using oligonucleotide JR1 as probe (Figure 4b). These results suggest that the factor responsible for the formation of complex D3 is most likely not the insulin gene regulatory protein IEF1.

Several lines of evidence suggest that the factors responsible for the formation of complexes D4 and J2 may be similar: (i) competition for formation of complex J2, but not complex J3, was observed with oligonucleotide DR2 (Figure 3a); (ii) formation of complexes D4 and J2 was observed in the presence

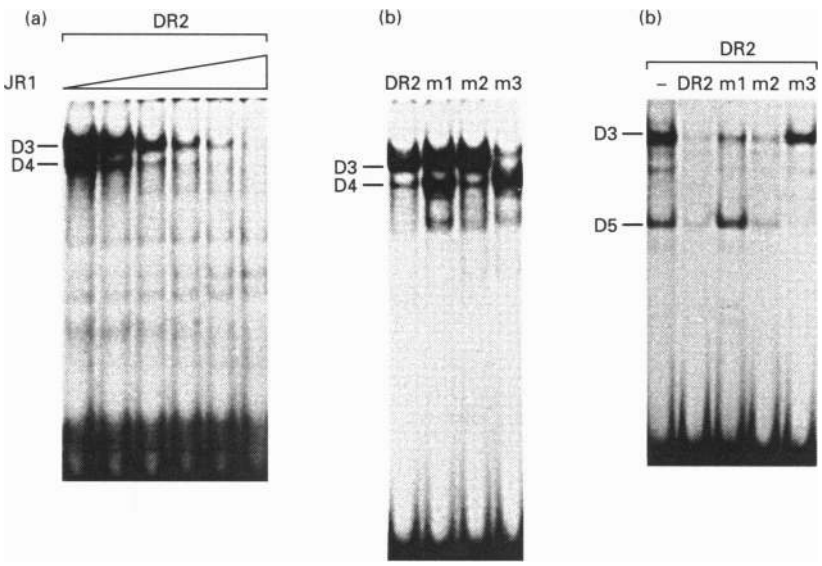

Figure 5 EFD3 binds to the E-box-related sequence CAGGAG

(**a**) EMSA using oligonucleotide DR2 as probe, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. The competitor oligonucleotide JR1 was used at 0-, 5-, 25-, 50-, 100- and 200-fold excess. The positions of the two major complexes D3 and D4 are indicated. (**b**) EMSA using oligonucleotides DR2, DR2m1, DR2m2 and DR2m3 as probes, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. (**c**) EMSA using oligonucleotide DR2 as probe, poly(dG-dC) as polynucleotide and an HIT T15 nuclear extract. The indicated unlabelled competitor oligonucleotides were used in approximately 100-fold excess.

of poly(dA-dT) as non-specific competitor; and (iii) formation of both complexes was abolished at salt concentrations above 400 mM (Figure 4b).

The IEB2 region of the rat insulin II gene enhancer contains four E-box-related sequences (E1, E2, E3 and E4; Figure 1b) and a putative binding site, E5, for the GGGCCC binding factor [19]. Competition of complex D3 by oligonucleotide JR1 (Figure 5a) suggested that the recognition site of EFD3 was likely to be one of these E-box-related sequences. To identify the binding site of EFD3, three mutant oligonucleotides of DR2 were used. Critical mutations were present in oligonucleotide DR2m1 at the E1 site, i.e. CATCAG to GGTCAG; DR2m2 at the E5 site, i.e. GGAGCCC to GGAGAAA; and DR2m3 at the E4 site, i.e. CAGGAG to GGGGAG. Formation of the D3 complex was abolished by the mutation within DR2m3, but unaffected by the DR2m1 and DR2m2 mutations (Figure 5b). In addition, competition for complex D3 was observed with oligonculeotides DR2m1 and DR2m2, but not DR2m3 (Figure 5c). Therefore EFD3 binds to the E-box-related sequence at the E4 site, i.e. CAGGAG, in the IEB2 region of the rat insulin II gene enhancer. It also binds, with lower affinity, to the IEB1 site in rat insulin I gene enhancer. In contrast, competition of the D5 complex was observed with oligonucleotides DR2m2 and DR2m3 but not with DR2m1 (Figure 5c), suggesting that factor D5 binds to the E1 site containing the sequence CATCAG within oligonucleotide DR2.

The DNA binding activity of factor D4 was dramatically increased with both oligonucleotides DR2m1 and DR2m3 as probes (Figure 5b). Oligonucleotide DR2m3 contains a double substitution at the E4 site, i.e. CACCCA to CACCGG. This sequence is similar to that of the IEB2 site in the human insulin gene, which has previously been shown to bind USF [13]. To confirm the identify of complex D4, an oligonucleotide, U, containing the USF binding site from the adenovirus major late promoter (ML-U), was used. Competition for complex D4 was observed with oligonucleotide U (Figure 6a) using oligonucleotide DR2 as probe. In addition, competition of complex D4 was observed with oligonucleotide D, containing the human IEB2 sequence, but not with Dm1 which contains a critical mutation within the human IEB2 sequence [14], nor with an unrelated oligonucleotide B (Figure 6a). An anti-USF antibody also competed for formation of complex D4 (Figure 6d). To directly compare the binding characteristics of USF with those of complex D4, the effect of different polynucleotides on USF binding activity was examined. Two complexes (U1 and U2), as previously described [13], were observed using oligonucleotide U as probe and an HIT T15 nuclear extract (Figure 6). The binding activities of factor D4 and of both the tissue-specific complex U1 and USF (complex U2) were increased using poly(dA-dT) as non-specific competitor (Figure 6c). To investigate further the binding activity of complex D4, a shortened form of oligonucleotide DR2 was used (see Figure 1). A single retarded complex with a similar mobility to that of complex D4 was observed using oligonucleotide DR2′ as probe and an HIT T15 nuclear extract (Figure 6b). Taken together, these results indicate that complex D4 represents a factor with USF binding activity, which recognizes E-box-related sequences at sites E2/E3 within the IEB2 region of the rat insulin II gene.

We next investigated the binding of factors D3 and D4 to the IEB2 sites in the rat I and human insulin genes using oligonucleotides containing the rat I (DR1) and human (DH1) IEB2 regions. Using oligonucleotide DR1 as probe, a complex D6, previously identified as IEF1, with a similar mobility to complex D3, was observed (Figure 7). A previously undetected retarded complex D0, with a similar mobility to complexes D3 and D6, was observed using oligonucleotide DH1 as probe. This factor is most likely related to complex D3, as oligonucleotide DH1 competed for binding of complex D3 to oligonucleotide DR2 (results not show). The efficiency of USF binding to the IEB2 sites differs between the insulin genes as the intensity of the complex (D7/D4/D1), identified as USF, varied between the three probes (Figure 7). Two other complexes, D8 and D9, with

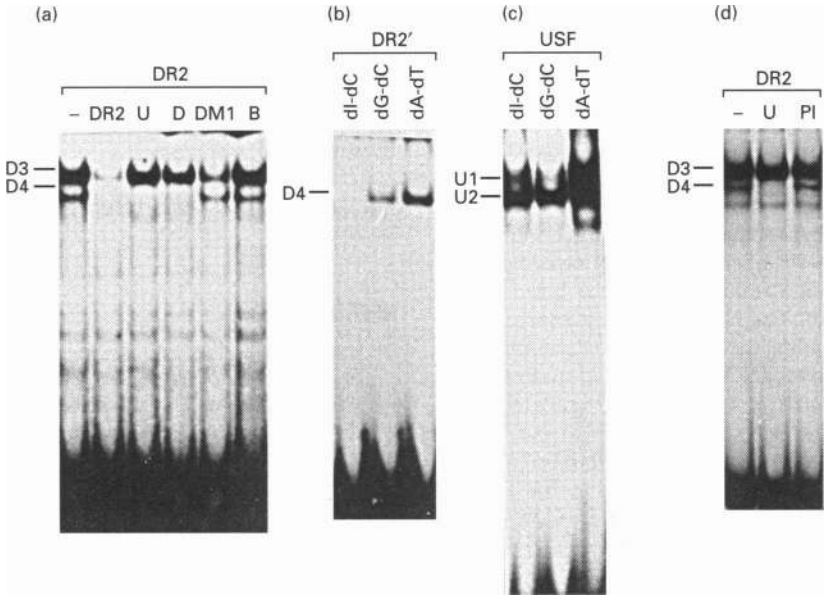

Figure 6 The major binding complex D4 is a USF-like factor

(**a**) EMSA using oligonucleotide DR2 as probe, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. The indicated unlabelled competitor oligonucleotides were used in approximately 50-fold excess. The positions of the two major complexes D3 and D4 are indicated. (**b**) EMSA using oligonucleotide DR2′ as probe and an HIT T15 nuclear extract. The indicated polynucleotides were used at 500 ng per lane. (**c**) EMSA using oligonucleotide USF as probe and an HIT T15 nuclear extract. The indicated polynucleotides were used at 500 ng per lane. The positions of the two major complexes U1 and U2 are indicated. (**d**) EMSA using oligonucleotide DR2 as probe, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. Nuclear extracts were preincubated with 1 μl of anti-USF antibody (U) or preimmune serum (PI).

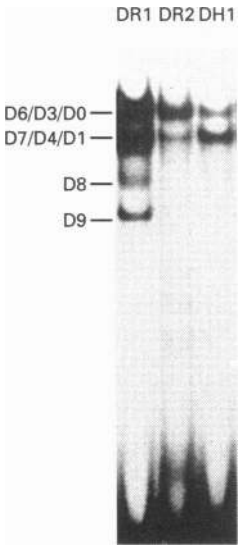

Figure 7 Binding of nuclear extract proteins in the IEB2/FAR site in the rat I, rat II and human insulin gene enhancer regions

EMSA using oligonucleotides DR1, DR2 and DH1 as probes, poly(dA-dT) as polynucleotide and an HIT T15 nuclear extract. The positions of the major complexes D6/D3/D0, D7/D4/D1, D8 and D9 are indicated.

no counterparts in the other two insulin genes, were observed using oligonucleotide DR1 as probe (Figure 7). These results suggest that a similar set of factors recognize the IEB2 regions of the insulin genes investigated. However, the DNA binding affinity of the individual factors varies between the different genes.

DISCUSSION

In this study it has been demonstrated that at least three distinct factors recognize the IEB2 region of the rat insulin II gene enhancer region. A factor, D4, is identified as the HLH factor USF, while a second, largely uncharacterized, factor, D5, binds to the sequence CATCAG. A third factor, named EFD3, binds to an E-box-related sequence, CAGGAG, and exhibits a similar tissue distribution and gel shift mobility as IEF1, an important insulin gene regulatory protein [4]. However, several lines of evidence support the conclusion that EFD3 and IEF1 are different proteins. First, an oligonucleotide, DR2, containing the binding site for EFD3, only weakly competes for the IEF1 complex J1. Secondly, unlike EFD3, binding of IEF1 to the probe is not affected by the type of non-specific competitor used. Thirdly, whereas formation of IEF1 is relatively salt-independent, binding of EFD3 to the probe was abolished at ionic concentrations greater than 200 mM KCl.

Previous studies have shown that sequence-specific DNA binding proteins can be sequestered by binding to non-specific competitors [18]. For example, two factors, the serum response factor (SRF) and FACT1 (YY1), bind to sites in the skeletal α-actin promoter [20]. Of these, *in vitro* binding of the SRF to the promoter is attenuated by a range of different polyanion competitors, while FACT1 is unaffected. By comparison, in the present study all three factors that bind to the IEB2 region showed differential binding with a range of dissimilar polynucleotides. EFD3 and factor D4 (USF) bound more efficiently

in the presence of poly(dA-dT). In contrast, binding of factor D5 was attenuated by poly(dA-dT).

More than 20 members of the HLH family of transcription factors, that are capable of recognizing the E-box motif CANNTG, have so far been identified. These factors bind DNA either as homodimers or as heterodimers by interacting with other HLH proteins [21]. Structurally related to *Drosophila hairy* and *Enhancer of split*, the HLH factor Hes-1 [22] preferentially recognizes a different consensus sequence, CACNAG, known as the N-box. Altered binding specificity for the N-box has been shown to be due to a proline residue within the basic DNA binding region of the protein. The Hes-1 protein is expressed in a wide variety of tissues, including the epithelial cells of embryonic respiratory and digestive organs [22,23]. Functional studies show that Hes-1 acts as a negative regulator of transcription, repressing transcription either by binding directly to the N-box or by preventing other HLH proteins from binding to their E-box site [22]. The sequence of the binding site for EFD3, CAGGAG, resembles the N-box consensus sequence CACNAG. This raises the possibility that EFD3 might represent a protein with similar gene regulating properties to those of the Hes family of transcription factors [22,24,25]. It is worth noting, therefore, that the IEB sequence has been shown to be involved in both positive and negative regulation of the insulin gene [26,27]. This has led to suggestions that this element is recognized by both positively and negatively acting transcription regulators [28,29]. So far, identifying factors involved in the negative activity associated with the IEB sequence has proved elusive. For example, in one study, multiple IEB sites linked to a heterologous promoter were shown to repress transcription of the reporter gene [27]. No binding activity except for IEF1 could be detected binding to multiple IEB sites *in vitro*. However, EFD3 would have remained undetected in such studies because of its similar band shift mobility to IEF1, and unless suitable polynucleotides were used, it would be sequestered by interaction with non-specific competitor DNA.

In the human insulin gene, the IEB2 region has previously been shown to be involved in transcription [14]. Factors binding to this region have recently been identified as USF and an uncharacterized factor D2 [13]. Another factor, D0, is now identified as recognizing this region. Factor D0 was not detected previously due to differences in the *in vitro* binding conditions. D0 may be similar to EFD3, as oligonucleotide DH1 competed for binding of complex D3 to oligonucleotide DR2 (results not shown). The IEB2 sequence of the human insulin gene is a higher-affinity USF binding site than the equivalent sequence in the rat insulin II gene. Mutations within the rat insulin II IEB2 oligonucleotide, i.e. DR2m1 and DR2m3, which make this sequence more similar to the human sequence, exhibit higher-affinity USF binding than the wild-type oligonucleotide (DR2). The USF binding site CACCGG is reconstituted in oligonucleotide DR2m3. However, no such site is present in DR2m1, and increased binding of USF might be due to recreating additional contacts in flanking sequences outside the E-box-related sequence to help stabilize USF binding to a non-ML-U sequence.

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In conclusion, three factors which recognize the IEB2 region of that rat insulin II gene have been identified. Distribution of the major binding activity, EFD3, appears to be restricted to endocrine cell lines. The role of this factor is not known, but it may be involved in the negative regulation of insulin gene transcription.

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