Insulin upstream factor 1 and a novel ubiquitous factor bind to the human islet amyloid polypeptide/amylin gene promoter

Deborah BRETHERTON-WATT, Nicola GORE and David S. W. BOAM*

Division of Biochemistry, School of Biological Sciences, 2.205 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, U.K.

The islet amyloid polypeptide (IAPP) gene is expressed primarily in the islet β-cell and the peptide is co-secreted with insulin. To investigate mechanisms important in its regulation, we have used the electrophoretic mobility-shift assay and methylation interference to determine systematically sites of DNA–protein interactions in the human IAPP promoter. We identified β-cell-specific DNA–protein complexes at three sites, each of which contained a consensus binding site for insulin upstream factor 1 (IUF-1). This complex was displaced with an antiserum to IUF-1, confirming that IUF-1 binds to the human IAPP promoter *in vitro*. We have also identified a DNA–protein complex within the region $-220/-250$ in both β- and non-β-cell lines. This region contains a motif with partial identity with the binding site for the ubiquitous transcription factor upstream stimulatory factor (USF), which binds to the human insulin promoter. However, purified USF was not able to bind to this putative site in the IAPP promoter and an oligonucleotide containing a functional USF-binding site was unable to displace binding from the IAPP oligonucleotide. Methylation interference revealed that the DNA–protein complex binds to a sequence that overlaps the USE-like sequence, and may therefore be a novel helix–loop–helix protein. These results suggest that, although both IAPP and insulin are β-cell peptides, IAPP contains regulatory regions both common to and distinct from insulin.

INTRODUCTION

Islet amyloid polypeptide (IAPP/amylin) is a 37-amino acid peptide of the calcitonin gene family isolated from the amyloid deposits of non-insulin-dependent diabetic pancreas and insulinoma [1,2]. The major site of IAPP synthesis and release is the islet β-cell, where it is co-stored with insulin in the secretory granules [3,4] and released with insulin in response to a variety of secretagogues, both *in vitro* [5] and *in vivo* [6,7]. Expression of IAPP and insulin, however, is not obligatorily co-regulated: whereas insulin gene expression is restricted solely to the β-cell, IAPP production is found at other sites, e.g. IAPP mRNA has been detected in stomach and lung [8] and IAPP has been found in islet δ-cells [9]. In addition, the normal co-expression of IAPP and insulin is not found in certain neuroendocrine tumours [10,11] and cell lines [12].

The tissue-specific expression and inducibility of genes encoding most peptide hormones are regulated at the transcriptional level by binding of *trans*-acting proteins to *cis*-acting DNA sequences flanking their target genes (for review, see ref. [13]). These transcription factors often belong to large highly conserved protein families and share common elements in their actions. The insulin and IAPP genes are both expressed in the pancreatic β-cell and may therefore be expected to share common regulatory elements.

Two main types of *cis*-acting elements are thought to be important in tissue-specific expression of the insulin gene: E-boxes and TAAT boxes. E-boxes contain consensus binding sites for helix–loop–helix (HLH) proteins, which have been widely implicated in tissue-specific and developmental control of gene expression, including immunoglobulin and muscle-specific genes. The rat insulin gene 1 contains two E-boxes, NIR/IEB 1 and FAR/IEB 2 [14,15]. Both NIR and FAR boxes bind a protein, insulin enhancer factor 1 (IEF-1), a heterodimer of the ubiquitous E12/E47 HLH protein and a β-cell-specific factor, IESF-1 [16]. The human insulin gene FAR box, however, binds the ubiquitous HLH activator, upstream stimulatory factor (USF) [17] instead of IEF-1. The other important motifs, the TAAT boxes, are common recognition sites for homeodomain proteins. Rat insulin gene 1 contains a promoter proximal TAAT box [18], in addition to three copies of the TAAT sequence immediately downstream of the FAR box. This FAR-linked (A+T)-rich (FLAT) region, also called the E2 domain after the distinctive footprint produced by DNase digestion [19], can act as a transcriptional proto-enhancer [20] and a glucose-responsive element [21]. The human insulin gene contains three copies of the sequence CTAATG, the CT boxes. The CT-2 motif lies within the human insulin E2 domain, and the CT-1 box has recently been identified as a glucose-responsive element [22]. These motifs bind a β-cell-specific protein, insulin upstream factor 1 (IUF-1) [23]. The similar mobility and cell specificity of IUF-1 to the rat promoter proximal TAAT-box-binding protein, IPF-1, suggest that they are the same protein [24].

The regulatory region of the human IAPP gene contains motifs similar to both the rat and human insulin gene 5′-regions (Figure 1). A promoter-proximal TAAT motif is located at $-87/-82$ bp and a FLAT-like element is located upstream of a NIR/FAR-related sequence at $-140/-130$. Another FAR-like motif, but in reverse orientation, is seen in an 8 bp element at $-245/-238$ [25,26]. In order to study more fully the regulation of the human IAPP gene, we used a series of synthetic overlapping oligonucleotides, corresponding to the 5′-regulatory region of

Abbreviations used: IAPP, islet amyloid polypeptide; IUF-1, insulin upstream factor 1; HLH, helix–loop–helix; IEF-1; insulin enhancer factor 1; USF, upstream stimulatory factor; FLAT, FAR-linked (A+T)-rich; IPF-1, TAAT-box-binding protein; EMSA, electrophoretic mobility-shift assay; USE, upstream-stimulatory-factor-binding site.

* To whom correspondence should be addressed.

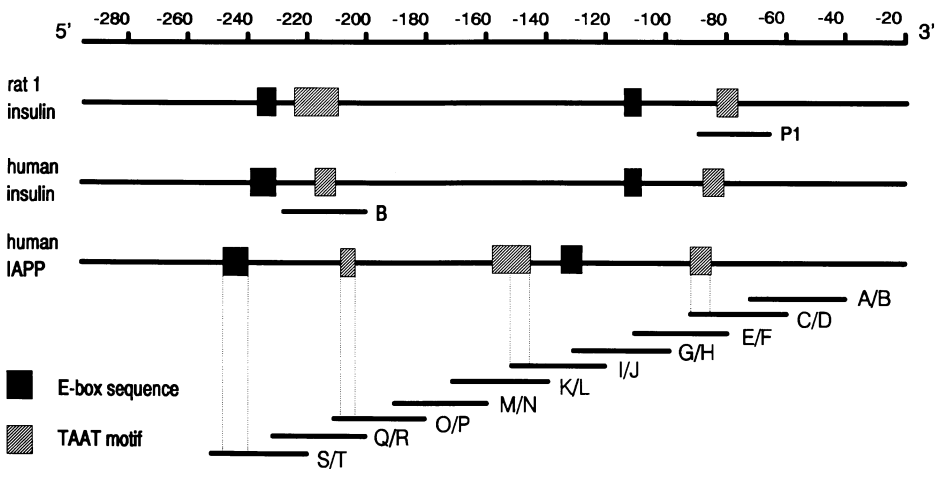

Figure 1 Comparison of the 5′-regulatory region of human IAPP with rat 1 and human insulin genes

Relative positions of synthetic oligonucleotides representing the human IAPP promoter (−250/−40) are indicated, with regions of homology with the human and rat insulin gene 1 promoters indicated.

the human IAPP gene, as probes in an electrophoretic mobility-shift assay (EMSA), to identify sites of DNA–protein interaction in the human IAPP promoter.

MATERIALS AND METHODS

Cell culture

HIT.T15$_{m2.2.2}$ cells were grown in RPMI 1640 medium, and βTC_3 and αTC_1 cells in Dulbecco's modified Eagle's medium. All media were supplemented with 10% foetal calf serum, 2 mM L-glutamine, 40 μg/ml streptomycin and 40 units/ml penicillin (Gibco BRL, Uxbridge, Middx, U.K.). HIT$_{m2.2.2}$ cells were found to undergo spontaneous dedifferentiation to a fibroblast-like phenotype after 12–14 continuous passages in culture, and RNA and nuclear extracts were therefore prepared from this cell line to represent both the β- (passage $n+5$–9) and non-β-cell type (passages $n+16$–25). Northern-blot analysis confirmed the loss of insulin and IAPP mRNA associated with this dedifferentiation process (results not shown).

Preparation of nuclear extracts

Nuclear extracts were prepared from the above cell lines by a modification of the method described by Dignam et al. [27]. After $(NH_4)_2SO_4$ precipitation, proteins were recovered and redissolved in buffer [20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol], dialysed against 2×100 vol. of this buffer, divided into aliquots and stored at −80 °C.

Nuclear extracts from HeLa, MPC-11 and BJA-B cell lines were prepared in the same way. Protein concentration was determined using a protein assay system (Bio-Rad Laboratories, Munich, Germany).

Probes

Overlapping 30-mer oligodeoxynucleotides (referred to subsequently as oligonucleotides) representing the regulatory region of the human IAPP gene [26] and the upstream-stimulatory-factor-binding site (USE) were synthesized and reverse-phase-purified (Genosys Biotechnologies, Cambridge, U.K.) (Table 1).

Complementary oligonucleotides were annealed by heating to 95 °C for 5 min in 10 mM Tris/HCl, pH 7.9, containing 2 mM $MgCl_2$, 50 mM NaCl and 1 mM EDTA, and cooling slowly to room temperature. Annealed oligonucleotides were end-labelled with 150 μCi of [γ-^{32}P]dATP (7000 Ci/mmol; ICN, High Wycombe, Bucks., U.K.) using T4 polynucleotide kinase, and labelled product was recovered from a Sephadex G-50 spun column [28]. For methylation interference, single-stranded oligonucleotides were end-labelled before annealing, using the same procedure.

EMSA

All assays were carried out, unless otherwise indicated, as follows: 5 μg of nuclear extract was added to a mixture containing 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5% (v/v) glycerol, 50 mM NaCl and 1 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia/LKB Biotechnology, Uppsala, Sweden) as a non-specific competitor. All components were added on ice including, where appropriate, unlabelled competitor DNAs or antiserum (sheep anti-IUF-1 antibody, from Dr. K. Docherty, University of Aberdeen, Scotland, U.K.). After a 10 min preincubation period, labelled oligonucleotide probes (15000 c.p.m.; 5–20 fmol) were added and the mixture was incubated for a further 20 min at room temperature. Samples were run at 150 V on a native polyacrylamide gel (6%; 29:1 acrylamide/bisacrylamide; 0.5×50 mM Tris/50 mM boric acid/1 mM EDTA).

Methylation interference

End-labelled oligonucleotides, in a volume of 0.1 ml, were methylated by the addition of 0.01 vol. of dimethyl sulphate (Aldrich Chemical Co., Gillingham, Dorset, U.K.) at room temperature for 2–5 min [29]. Reactions were stopped by the addition of 2-mercaptoethanol (final concentration 33 mM) and purified on a Sephadex G-50 spun column [28]. Methylated probe (10^6 d.p.m.; 1 pmol) was incubated as for gel-shift reactions with 10 μg of protein. After incubation for 30 min at room temperature, reaction mixtures were electrophoresed on a 6% polyacrylamide gel as described above. After autoradiography for 30 min, bound and free DNA bands were excised and eluted overnight in Tris/EDTA, pH 8.0, and repurified on Sephadex

Table 1 Oligonucleotide sequences used in EMSA

Putative TAAT motifs and E-box-like sequences are in bold; mutated residues are underlined. For oligonucleotides O/P and S/T these motifs are in reverse orientation.

Name	Sequence			Position
Human IAPP				
A/B	CTGAGCTGCC	TGATGTCAGA	GCTGAGAAAGG-3′	−70/−40
C/D	AAA**TTAATG**A	CAGAGGCTCT	CTGAGCTGCCT-3′	−90/−60
E/F	CTTTCTATCT	ATAGGGATGG	AAA**TTAATG**AC-3′	−110/−80
G/H	CTTCTGCTGC	CTGTGAGGTA	CTTTCTATCTA-3′	−130/−100
I/J	ATGTA**ATAAT**	**G**A**CCCATCCG**	CTTCTGCTGCC-3′	−150/−120
K/L	TAATATTTAC	TGATGAG**TTA**	**ATG**TA**ATAATG**-3′	−170/−140
M/N	ACACTGTGTA	TTTGCTACGT	TAATATTTACT-3′	−190/−160
O/P	ACAC**CATTAA**	CTGCACAAGG	ACACTGTGTAT-3′	−210/−180
Q/R	AAACTTCTGC	TGTGTATGAC	ACAC**CATTAA**C-3′	−230/−200
S/T	CTTACA**AAGA**	**TGGC**AAATTC	AAACTTCTGCT-3′	−250/−220
Human insulin				
B	CCCCTGGTTA	AGACT**CTAAT**	**G**ACCCGCTGG-3′	−230/−201
Bm1	CCCCTGGTTA	AGAC<u>C</u>**CTAAT**	**G**ACCCGCTGG-3′	
Bm2	CCCCTGGTTA	AGACT**CTA**<u>C</u>**T**	**G**ACCCGCTGG-3′	
Rat insulin 1				
P1	GCCC**TTAATG**	GGCCAAACGG	CA-3′	−85/−64
P1m2	GCCC**TT**<u>CC</u>**TG**	GGCCAAACGG	CA-3′	
Human USE				
USE	GATCGGTGTAGGC**CACGTG**ACCGGGTGTCCCTGA-3′			

G-50 spun columns. After lyophilization, samples were re-suspended in 100 μl of 1 M piperidine and heated to 95 °C for 30 min. Piperidine was removed by repeated washing with water followed by lyophilization. Samples were run on a 20% polyacrylamide sequencing gel with G+A markers [29].

Overexpression and purification of USF

USF_{43} was expressed from the USF expression plasmid pET3d-USF [30] in host strain BL21lysS, using a procedure modified from Pognonec et al. [31]. Briefly, cells were sonicated in lysis buffer [20 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM PMSF, 5 μg/ml leupeptin, 1% (w/v) aprotinin and 0.1% Nonidet P40] and subjected to two freeze-thaw cycles. Soluble proteins were then precipitated with 76% satd. $(NH_4)_2SO_4$, pelleted and stored. Pellets and supernatants from each step were dialysed against buffer containing 20 mM Hepes, 0.1 M potassium acetate, 20% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% Nonidet P40 and protease inhibitors as above. Further purification was carried out on a heparin–Ultrogel (IBF Biotechnics) column, protein being eluted with a 100–500 mM potassium acetate gradient. A protein that migrated as a single 43 kDa band on SDS/PAGE and formed a specific retarded band in the gel-shift assay was obtained (results not shown). Purity was estimated at 90% or higher.

RESULTS

DNA–protein interactions within the human IAPP promoter

In order to identify β-specific complexes, we compared binding patterns of the oligonucleotides in nuclear extracts from a β- (βTC_3) and non-β- (HeLa) cell line (Figure 2). All oligonucleotides were capable of forming retarded complexes with the nuclear extracts tested. However, only oligonucleotides C/D, I/J, K/L and O/P were found to bind predominantly β-cell-specific complexes (Figure 2, lanes 3, 9, 11 and 15), with a weak β-cell-specific doublet binding to oligonucleotide E/F (lane 5). With oligonucleotide I/J, this β-cell-specific complex was also a doublet with a predominant lower band (lane 9), while three β-cell-specific complexes bound to oligonucleotide K/L, with the middle band predominant (lane 11). Oligonucleotides C/D and O/P both formed a single β-cell complex (lanes 3 and 15).

A β-cell-specific complex binds to multiple sites within the human IAPP promoter

The β-cell-specific complex binding to probe C/D was investigated further, using additional β- and non-β-cell nuclear extracts (Figure 3a). The complex was not detectable with extracts of HeLa, MPC-11 and BJA-B (Figure 3a, lanes 5–7), but low levels were detected with αTC_1 extract (lane 2). High levels of this complex were detected with $HIT_{m2.2.2}$ cells (lane 3): the slightly higher mobility compared with βTC_3 extract (compare lanes 1 and 3) is probably due to species differences in the source cell lines [23]. After dedifferentiation of the $HIT_{m2.2.2}$ cells, the levels of this complex were significantly decreased (compare lanes 3 and 4), concomitant with a reduction in insulin and IAPP mRNA levels (results not shown).

Similar patterns of β-cell-specific expression to that seen with C/D were observed with oligonucleotides I/J, K/L and O/P (results not shown). Each of these oligonucleotides contain the sequence A/T TAATG (a reverse motif in oligonucleotide O/P) similar to the IUF-1/IPF-1-binding site, CTAATG (Table 1 and Figure 1) of the human and rat gene 1 insulin promoters [18,23]. In order to confirm the common identity of this complex binding to the different probes, cross-competition experiments were carried out, displacement of the labelled complex indicating competition of unlabelled oligonucleotides for limited protein-binding sites. Figure 3(b) shows EMSA using probe C/D and βTC_3 nuclear extract. At 400 × molar excess (as shown), displa-

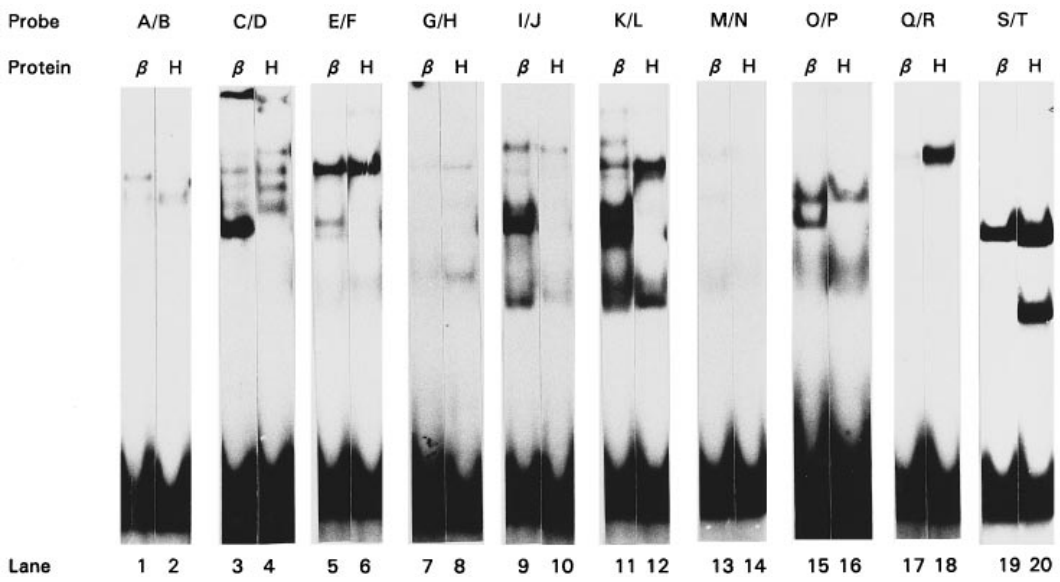

Figure 2 EMSA showing binding of nuclear proteins (5 μg) from βTC_3 (β) and HeLa (H) cells to labelled 30-mer oligonucleotide probes (A/B–S/T) representing overlapping regions of human 5′ IAPP from $-250/-40$ bp

cement of C/D was seen only with oligonucleotides C/D, I/J, K/L and O/P (Figure 3b, lanes 3, 6, 10 and 12), i.e. only those oligonucleotides containing the TAAT motif, which were also shown to bind a β-cell-specific complex. At lower levels of competitor ($5\times$ or $20\times$ molar excess), no displacement was seen (results not shown). At $100\times$ molar excess, displacement was seen only with oligonucleotides C/D and I/J.

When oligonucleotides I/J or O/P were used as the probe, displacement of the β-cell complex was also seen only with those oligonucleotides containing a TAAT motif (results not shown). However, O/P was found to bind this complex weakly and displace poorly. Again, displacement of both probes was seen only at levels of competitor oligonucleotides in excess of $100\times$ molar concentration.

These results suggest that a similar β-cell-specific factor binds to multiple sites within the IAPP promoter, although the affinity of the binding may vary at the different sites.

A component of this β-cell complex is IUF-1

We then compared this IAPP–oligonucleotide β-cell complex with that formed with oligonucleotides corresponding to the IUF-1/IPF-1-recognition motifs of the human and rat 1 insulin genes (Table 1, Figure 1). Oligonucleotide B contains the human insulin CT-2-recognition motif for IUF-1 [23], and oligonucleotide P1 corresponds to the rat gene 1 promoter proximal IPF-1-binding site [18]. When oligonucleotides B and P1, both containing an intact (C/T)TAATG motif, were used as probes, they bound complexes of similar mobility to the C/D complex (Figure 3c, lanes 1, 2 and 3). In addition, an intact TAAT motif was required to displace C/D binding in cross-competition experiments: oligonucleotides P1, B and Bm1 (containing a mutation outside the putative binding site) were able to specifically displace binding of the β-complex to the C/D oligonucleotide (Figure 3c, lanes 5, 7 and 8), whereas the lower-mobility ubiquitous complexes were not displaced. In contrast, oligonucleotides Bm2 and P1m2, which contained mutated TAAT motifs, were unable to displace (lanes 6 and 4). Conversely, oligonucleotides C/D and I/J were able to displace binding to oligonucleotide probes B and P1 at $100–400\times$ molar excess (results not shown). Oligonucleotide O/P was a weak displacer of B and P1 at this concentration. The similar mobility of the complexes and the ability of wild-type and mutant oligonucleotides corresponding to the human and rat 1 insulin genes IUF-1/IPF-1-binding sites to cross-compete with sites in the human IAPP promoter make it likely that the same complex is responsible for binding to both promoters, and that at least a component of this complex is IUF-1.

To confirm that IUF-1 binds to these sequences of the IAPP promoter, an antibody-blocking experiment was performed. An antibody to IUF-1 was able to partially displace this complex not only from oligonucleotides B and P1 but also from C/D, I/J and O/P (Figure 3d): this antibody blocks IUF-1 binding rather than causing a supershift in the protein–DNA complex (K. Docherty, personal communication). Decreasing amounts of the IUF-1 antiserum were found to displace binding to oligonucleotides B and C/D in a parallel fashion (results not shown). This result confirms that IUF-1 is, at least partially, responsible for this β-cell complex found at multiple sites within the IAPP promoter.

IAPP ($-250/-220$) contains a binding site for a novel ubiquitous factor

When incubated with a range of nuclear extracts from different cell lines, oligonucleotide S/T formed a single prominent complex with all extracts tested (Figure 4a). We investigated this complex further as oligonucleotide S/T contains the human IAPP gene partial FAR box [26]. Whereas the rat insulin gene 1 FAR motif binds the β-specific IEF-1, in the human insulin gene the FAR motif binds a ubiquitous protein, USF. It was possible therefore that the ubiquitous factor binding to oligonucleotide S/T was also USF. However, although pure recombinant USF_{43} bound strongly to its consensus binding site (oligonucleotide USE, Table 1 and Figure 4b, lane 12), it did so with a mobility distinct from the S/T DNA–protein complex (lane 1). USF was unable to bind to the S/T oligonucleotide (lane 11) and neither was

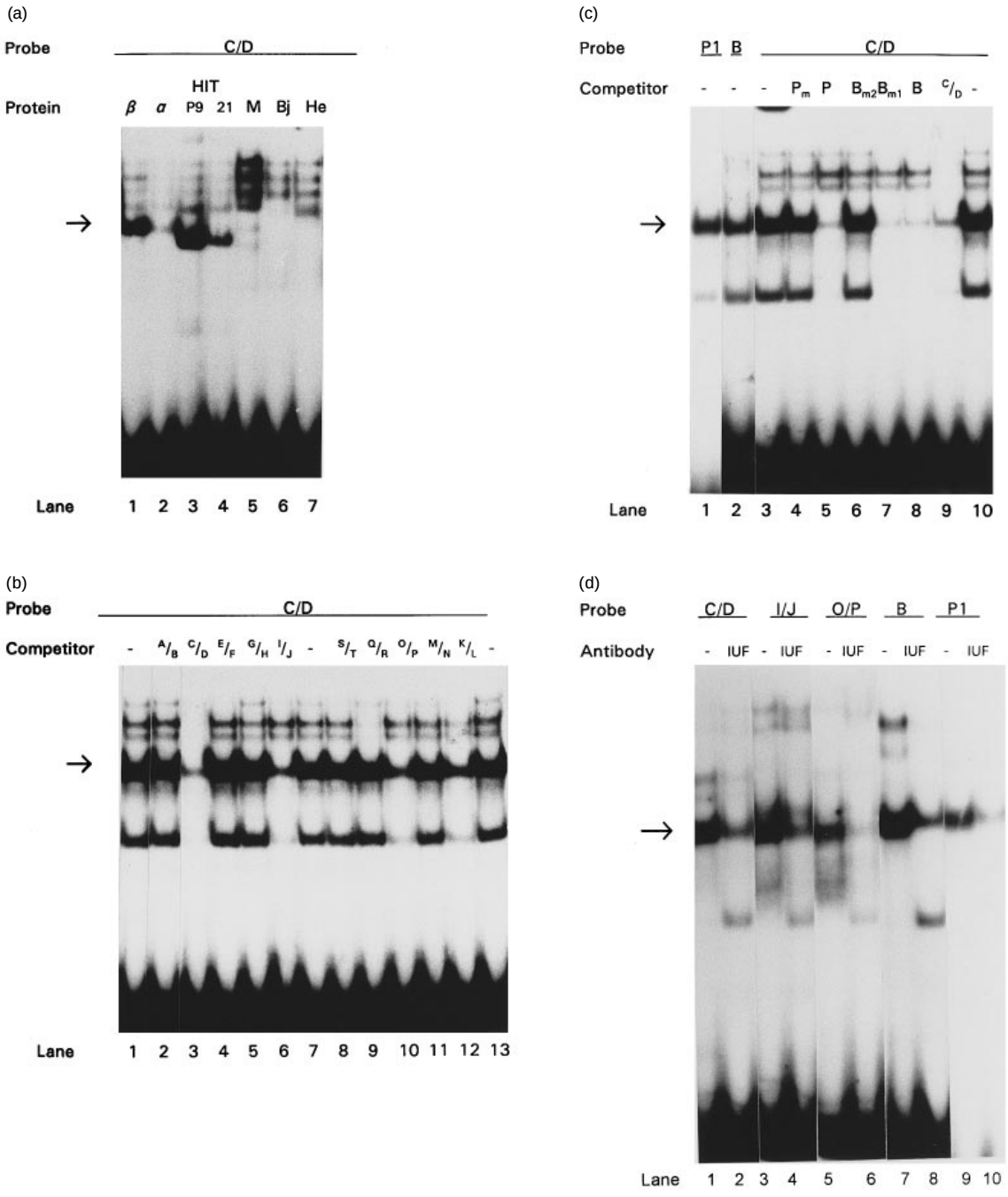

Figure 3 EMSA showing that a β-cell-specific complex is IUF-1

(**a**) Cell specificity of binding to oligonucleotide C/D (−90/−60) was determined using 5μg of nuclear protein from various cell lines: β, βTC_3; α, αTC_1; P9, $HIT_{m2.2.2}$ passage 9 (β-cell-like); 21, $HIT_{m2.2.2}$ passage 21 (non-β-cell-like); M, MPC-11; Bj, BJA-B; He, HeLa. The β-cell IUF-1 complex is indicated. (**b**) Specificity of IUF-1 binding to probe C/D. Unlabelled oligonucleotides (400 × molar excess) representing regions of the IAPP promoter were included in the reaction mixtures containing 5 μg of βTC_3 nuclear extract. The IUF-1 complex is indicated. (**c**) Specificity of IUF-1 binding to probe C/D. Excess (400 ×) unlabelled oligonucleotides representing regions of the rat 1 (oligonucleotide P1) and human (oligonucleotide B) insulin promoters were used. Mutated oligonucleotides P1m2 (Pm), Bm1 and Bm2 show that the IUF-1 complex requires an intact TAAT motif. Lanes 1 and 2 show that oligonucleotides P1 and B bind protein complexes of identical mobility to oligonucleotide C/D. All reactions were carried out with 5 μg of βTC_3 nuclear extract. The IUF-1 complex is indicated. (**d**) EMSA showing that anti-IUF-1 serum is able to block binding to the TAAT motifs of oligonucleotide C/D (lanes 1 and 2), I/J (lanes 3 and 4), O/P (lanes 5 and 6), B (lanes 7 and 8) and P1 (lanes 9 and 10). Lanes 1, 3, 5, 7 and 9 (−) contained 2 μl of preimmune sheep serum; lanes 2, 4, 6, 8 and 10 contained 2 μl of sheep anti-IUF-1 serum. The IUF-1 complex is indicated.

oligonucleotide USE able to displace the S/T DNA–protein complex from either βTC_3 or HeLa nuclear extract (lanes 3 and 8). Oligonucleotide I/J, which also contains a FAR-like sequence (Table 1) was also unable to displace this complex binding to S/T (lanes 4 and 9).

These data demonstrate clearly that the factor binding to the

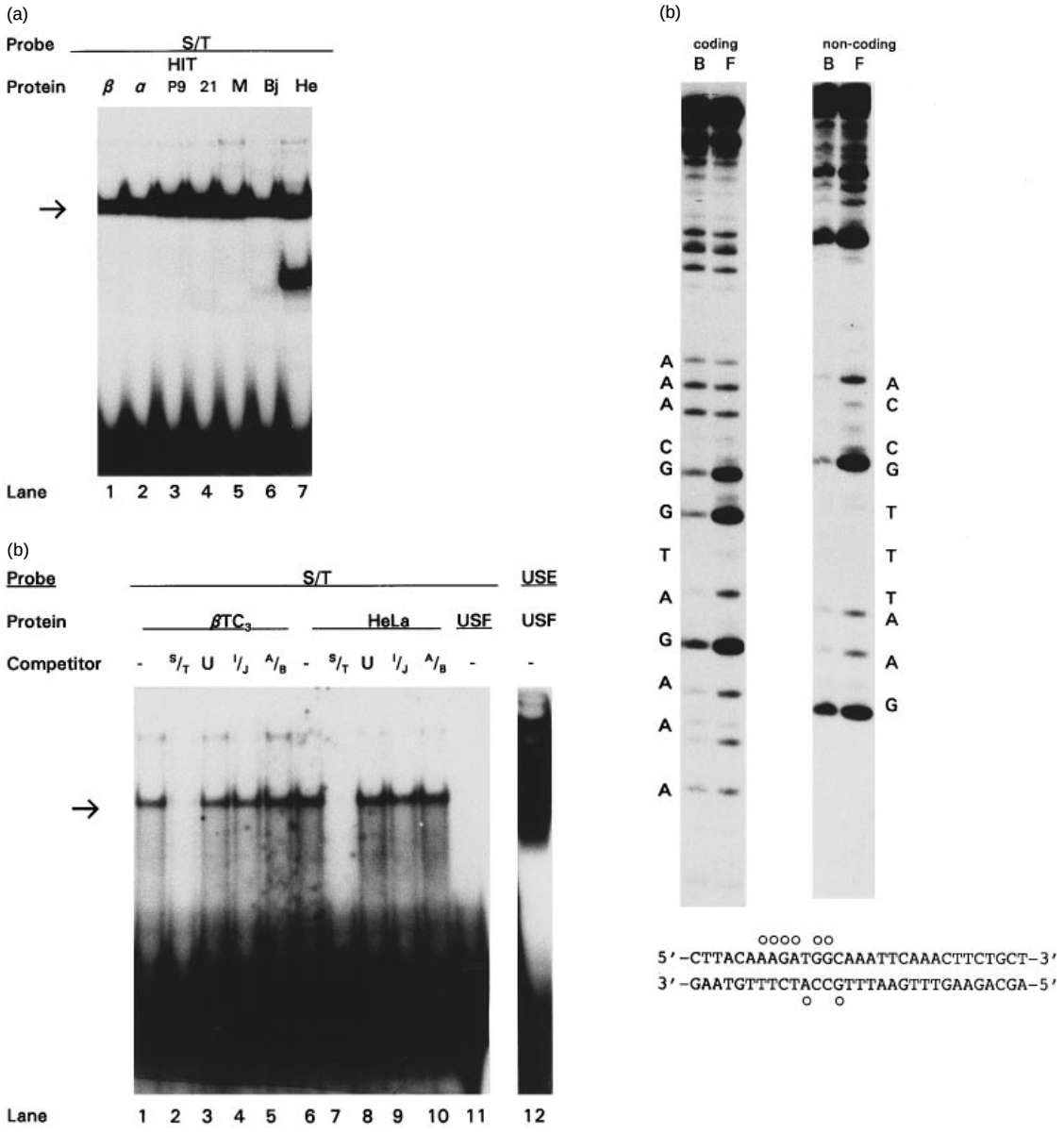

Figure 4 EMSA showing specificity of binding to probe S/T (−250/−220)

(**a**) Cell specificity of binding to probe S/T was determined using 5 μg of nuclear protein from various cell lines: β, βTC_3; α, αTC_1; P9, $HIT_{m2.2.2}$ passage 9 (β-cell-like); 21, $HIT_{m2.2.2}$ passage 21 (non-β-cell-like); M, MPC-11; Bj, BJA-B; He, HeLa. The main complex is indicated. (**b**) Specificity of binding to S/T. Excess unlabelled oligonucleotides were included in the reaction mixtures with 5 μg of βTC_3 and HeLa nuclear extract. Lanes 11 and 12 show USF binding to oligonucleotides S/T and USE respectively. (**c**) Methylation-interference analysis (top) of the DNA–protein complex formed by oligonucleotide S/T and HeLa nuclear extract. Lane B, bound fraction; lane F, free fraction. The sequence of oligonucleotide S/T is shown (bottom) with protected or partially protected residues indicated (○).

S/T oligonucleotide is quite distinct from USF. We therefore carried out methylation-interference analysis in order to determine the protein-binding site of this factor within the S/T oligonucleotide. Figure 4(c) shows that methylation interfered with protein binding in a region overlapping with the IAPP FAR-like sequence, GCCATCTT.

DISCUSSION

In this study, we have undertaken a systematic analysis of protein binding to *cis*-acting elements of the first 250 bp of the IAPP gene-regulatory region. This region was chosen because it contains the insulin-like NIR and FAR boxes and E2-like domain [14,19], and therefore probably contains the major sites controlling β-cell-specific expression of the human IAPP gene [25].

A β-cell-specific complex was identified binding to oligonucleotides C/D, I/J, K/L and O/P, all of which contain a consensus (T/A)TAATG motif and a component of this complex has been identified as IUF-1, the protein factor binding to the human insulin gene CT motifs. However, a number of other proteins known to bind to TAAT sequences have recently been isolated and characterized. Isl-1 is found in β, α and other neuroendocrine cell lines [32] and can bind both TAAT motifs of rat insulin gene 1, the IPF-1- and IEF-2-binding sites, although

it is distinct from both proteins. German et al. [33] have also identified cdx-3 and lim-1 as two rat insulin gene 1 FLAT-binding homeodomain proteins. IDX-1 and STF-1, both of which bind to TAAT sequences in the somatostatin gene promoter, and IPF-1 have all been independently cloned [34–36]. Comparison of the cDNAs for these three proteins suggests that they are similar or identical [34]. Furthermore, the similarity between binding-site sequences, expression patterns and electrophoretic mobility all suggest that IUF-1 is closely related to or identical with these proteins. A number of proteins, whether β-cell-specific, neuroendocrine or ubiquitous, are therefore capable of interacting with these TAAT sequences. The multiple β-cell-specific complexes detected by EMSA with oligonucleotides I/J and K/L may be explained by multiple co-operative binding of IUF-1 and (possibly) other TAAT-binding proteins to repeated TAAT motifs found in these oligonucleotides (Table 1), which together represent a region of homology with the rat insulin gene 1 FAR-FLAT mini-enhancer [20].

The evidence that a factor binding at multiple TAAT motifs within the IAPP promoter is IUF-1 has been based on the cell specificity of the complex, cross-competition analysis and antibody-shift experiments. Physically, the binding of β-cell nuclear extract to C/D is characteristic of IUF-1, with the presence of the minor high-mobility band [23,37]. However, the presence of this band was found to vary with different preparations of nuclear extract and may therefore be a proteolytic cleavage product. In cross-competition experiments, an intact TAAT motif was found to be necessary for displacement of the β-cell complex. In addition, high levels of competitor oligonucleotide were necessary to displace this complex; self-displacement of IUF-1 from its consensus sequences, oligonucleotide B, also requires more than $100\times$ molar excess [37].

Although we found that an IUF-1-like complex binds at several sites within the IAPP promoter, the affinity of such binding varied. In particular, oligonucleotide O/P was both a weak binder and poor displacer of this complex. However, the affinity of IUF-1 binding may be affected by both the position of the binding site within the probe and the presence of flanking sequences. For example, oligonucleotide E/F, which also contains the TAAT motif as it overlaps with oligonucleotide C/D, bound this IUF-1-like complex only weakly and was unable to displace it from other oligonucleotides, and oligonucleotide Q/R which contains the reverse TAAT motif at an even more terminal position did not bind IUF-1 at all. It has been previously noted that DNA binding by IUF-1 is inefficient if its binding motif is situated at the end of a DNA probe [23]. In addition, non-specific DNA-binding proteins may interfere with DNA–protein interactions at the extreme termini of probes (D. S. W. Boam and I. Davidson, unpublished work).

We detected low levels of this IUF-1-like complex in αTC_1 extract, although IUF-1 is thought to be β-cell-specific [24]. However, IUF-1 has been detected in a glucagon-producing subclone of HIT.T15 cells [37]. In addition, STF-1/IPF-1 has recently been shown to form a weak complex in αTC_1 extract as a result of a small subpopulation of αTC_1 cells expressing insulin [38]. However, it cannot be discounted that the α-cell complex is not due to IUF-1, but a distinct TAAT-box-binding protein, although the similar mobility of the α- and β-complexes appear to make this unlikely.

After dedifferentiation of the $HIT_{m2.2.2}$ cells, levels of the IUF-1 complex were significantly decreased. This decrease was parallelled by a concomitant reduction in IAPP and insulin gene expression and suggests that IUF-1 is associated with the maintenance of a β-cell phenotype and expression of β-cell genes. STF-1/IPF-1 has recently been identified as an ontogenetic marker of commitment to a pancreatic and, ultimately, β-cell phenotype [39], and it is therefore possible that loss of STF/IPF/IUF-1 expression is associated with a loss of the β-cell phenotype. The recent demonstration that loss of IUF-1 expression in transgenic mice leads to a total absence of pancreatic development [40] would be consistent with this observation.

A highly expressed ubiquitous factor was shown to bind to oligonucleotide S/T. This oligonucleotide contains a partial FAR box located in a similar position, relative to the transcription start site, to that in the human insulin gene. As the human insulin FAR motif has recently been shown to bind the ubiquitous HLH transactivator, USF [17], we investigated further the identity of this factor. However, pure recombinant USF did not bind to oligonucleotide S/T and was unable to displace the S/T-bound complex from oligonucleotide S/T. In addition, USF bound to its consensus site with a different mobility from that of the S/T complex; this S/T-binding factor is not therefore USF. Methylation-interference analysis, however, showed that the S/T complex binds to a site overlapping the putative USF-binding site, i.e. the partial E-box. Whatever this, as yet unidentified, widely distributed protein is, it binds to an E-box-like motif and may therefore be a novel HLH-type protein. Whether this factor is able to contribute to the regulation of human IAPP gene expression remains to be determined: oligonucleotide S/T is known to lie within an area of negative regulation of the human IAPP gene ([25,41]; D. Bretherton-Watt, N. Gore and D. S. W. Boam, unpublished work). Further compensatory positive elements remain to be identified.

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