Volume 290, number 1,2, 27–30 FEBS 10157 September 1991

ADONIS 0014579391008686

Two proteins act as the IUF1 insulin gene enhancer binding factor

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Received 8 July 1991

IUF1 is a pancreatic β cell-specific factor which binds to the sequence 5'-CPyCTAATG-3' (CT box) within the human insulin gene enhancer. Here we show that IUF1 is composed of 2 binding activities that can be separated by DEAE ion exchange chromatography. South Western blot analysis indicates that these distinct binding activities have apparent molecular weights of 115 kDa and 46 kDa.

Insulin gene; Transcription factor; Gene expression

1. INTRODUCTION

Regulation of expression of the insulin gene involves discrete promoter and enhancer sequences located upstream of the transcriptional start site [1]. A number of transcriptionally important DNA sequences have been identified in the rat I insulin gene [2–4], the rat II insulin gene [5,6], and the human insulin gene [7–9]. These include both positively and negatively acting sequences [5,9–11].

Mutational analysis has shown that the most important positively acting enhancer elements are two closely related sequences 5'-GCCATCTGC-3' and 5'-GCCATCTGG-3' located at −108 and −233 in the rat insulin 1 gene, respectively [12]. Designated IEB1 and IEB2 (or Nir and Far), these sequences bind a factor of wide tissue distribution, which contains a helix loop helix motif [13–15].

Located on the 3' side of the IEB1 and IEB2 sequences are the CTI and CTII boxes. These have the consensus sequence 5'-CPyCAATG, and both bind a β cell-specific factor, IUF1 [8]. When linked to a heterologous promoter, a DNA fragment containing the putative rat I IUF1 binding site has no enhancer activity by itself, but does appear to potentiate the activity of the IEB site in β cells [16]. By screening a cDNA expression library from the insulin-secreting RIN cell line, Karlsson et al. [17] isolated a cDNA encoding a 38 kDa protein, Isl-1, which binds within the sequence TTAATAATCTAATTA. This sequence contains, in addition to the CT motif (TCTAAT), 3 copies of the motif TAAT, known to be important in the DNA-binding of homeodomain-containing proteins.

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Here we characterise further the insulin enhancer binding activity, IUF1, using a nuclear extract from a transplantable rat insulinoma [18]. The tumour is composed principally of β cells, and serves as a convenient source of gram-quantities of nuclear extract.

2. EXPERIMENTAL

2.1. *Nuclear extracts*

Nuclear protein extracts were prepared from a transplantable rat insulinoma, and from HITM2.2.2 (an SV40 transformed hamster β cell line), HeLa and BHK cell lines as previously described [19].

2.2. *DEAE ion exchange chromatography*

DEAE ion exchange chromatography was performed on a Spherogel TSK DEAE-5PW column (7.5 mm × 7.5 cm) (Beckman Instruments, High Wycombe, UK) equilibrated in 20 mM HEPES (pH 7.8), 10% glycerol (v/v), 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF (column buffer). Rat insulinoma nuclear protein extract (500 μl) was loaded on to the column, and bound protein was then eluted with a 0–0.5 M NaCl gradient in column buffer over 40 min at a flow rate of 0.5 ml/min. Fractions were collected at 2 min intervals. Electrophoretic mobility shift assays (EMSAs) were performed as previously described [19].

2.3. *South Western blotting*

Oligodeoxynucleotides (oligos) were synthesised on a laboratory PCR Mate and purified using OPC cartridges. Double stranded oligo concatemers were prepared by sequential 5'-phosphorylation, using T4 polynucleotide kinase, and ligation according to Vinson et al. [24]. 500 ng oligo concatamers were subsequently labelled by nick translation using [α-^{32}P]dCTP to a specific activity of approx. 10^8 dpm/μg. The labelled oligo concatamers were centrifuged through a 1 ml Sephadex G-50 column equilibrated in 10 mM Tris, 1 mM EDTA (pH 7.4).

Nuclear proteins (approx. 30 μg) were subjected to SDS polyacrylamide gel electrophoresis (SDS PAGE) (10% (w/v) acrylamide) and transferred onto a nitrocellulose filter (0.45 μm, Schleicher and Schuell, from Anderman and Co., East Molesley, Surrey) using a semi-dry blotting system (39 mM glycine, 48 mM Tris, 0.037% SDS (w/v), 20% methanol (v/v), at 8 mA/cm^2 filter for 1.5 h. The nitrocellulose filter was air dried for 30–60 min then subjected to a denatura-

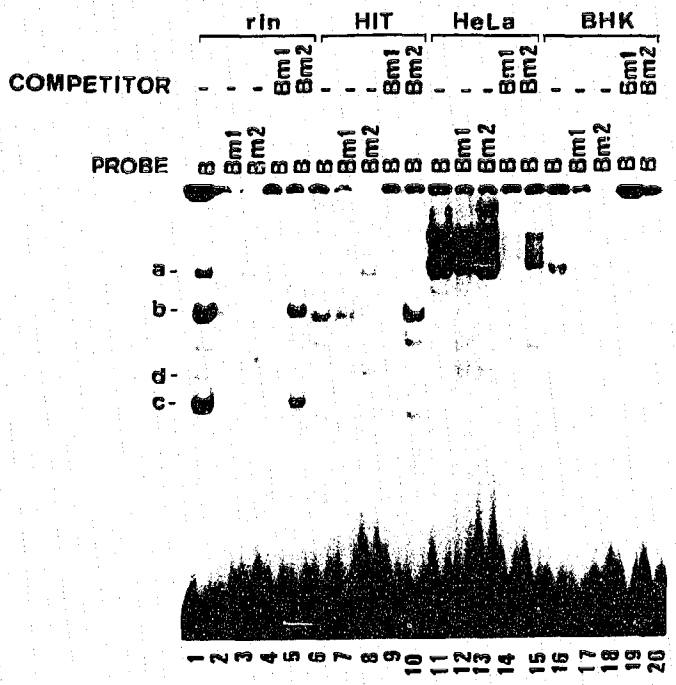

Fig. 1. Electrophoretic mobility shift assay of nuclear extracts from rat insulinoma (rin), HIT, HeLa and BHK cells. Each extract was assayed with radiolabelled oligo B, Bm1 and Bm2. Assays were also performed with radiolabelled oligo B, and excess unlabelled oligo Bm1 or Bm2 as competitor.

tion (6 M guanidine hydrochloride)–renaturation cycle [24]. Binding sites on the filter were blocked by incubation in binding buffer (25 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 50 mM KCl, 1 mM DTT) containing 5% (w/v) non-fat dried milk powder at 4°C for 30 min, then rinsed briefly in binding buffer plus 0.25% (w/v) non-fat dried milk powder. The filter was incubated overnight at 4°C in binding buffer containing 0.25% (w/v) non-fat dried milk powder, approx. 10^6 dpm/ml of ^{32}P-labelled oligo concatamers and 8 μg/ml poly(dI·dC)·poly(dI·dC). The filter was washed in binding buffer plus 0.25% non-fat dried milk powder (3 × 5 min at 4°C), and dried and autoradiographed (Fuji RX film).

3. RESULTS

IUF1 binding activity was characterised using 3 double-stranded 30-mers corresponding to sequences in the human insulin gene: oligo B 5′-CCCCTGGTTAAGACTCTAATGACCCGCTGG-3′, which contains a functional IUF1 binding site (underlined); oligo Bm1 5′-CCCCTGGTTAAGA/CCCTAATGA/CCCGCTGG-3′, which contains a T/C transversion which does not affect IUF1 binding; and oligo Bm2 5′-CCCCTGGTTAAGA/CTCTACTGA/CCCGCTGG-3′, which contains an A/C transversion which abolishes IUF1 binding [8,9].

In the rat insulinoma extract, 3 major retarded bands (a, b and c) and several minor bands, including band d, were observed using oligo B in an EMSA. In the same extract oligo Bm1 generated band b, and bands a, c and d, which were much fainter. Oligo Bm2 generated bands a and d. In competition studies using oligo B as probe, oligo Bm1 in excess abolished all bands, while oligo Bm2 abolished band a, but had no effect on bands b and c. Taken together, these data suggested that the major bands b and c were related to binding at the CT site in oligo B. Methylation interference analysis confirmed that bands b and c represented protein binding at identical sites (the CT motif) within oligo B (data not shown). The minor bands resulted from proteins binding at other sites in oligo B. Retarded bands b and c were unaffected by salt (NaCl) concentrations over the range 50–800 mM, while the minor bands were abolished at salt concentrations above approx. 100 mM (data not shown). Similar results were obtained with the HIT nuclear extract (Fig. 1, tracks 6–10) with minor differences in the mobility of bands. These could result from species differences in similar binding proteins.

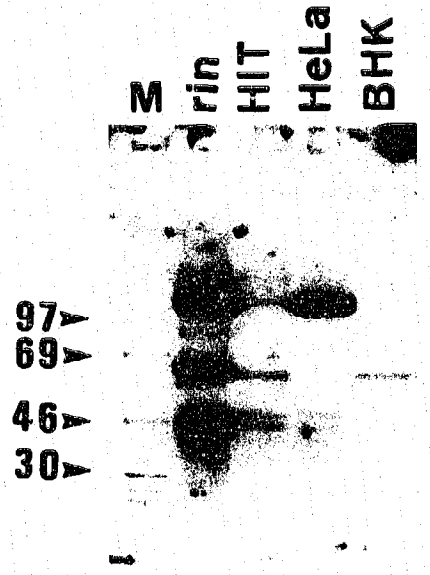

Fig. 2. South Western blot analysis of nuclear extracts from rat insulinoma (rin), HIT, HeLa and BHK cells. Blots were probed with radiolabelled oligo B. ^{14}C protein molecular weight markers are shown in track M.

In the HeLa nuclear extract, oligo B generated 2 major slow mobility bands, while oligo Bm1 generated 3 major slow mobility bands, and oligo Bm2 four major slow mobility bands (Fig. 1, tracks 11–15). In BHK nuclear extract, oligos B, Bm1 and Bm2 generated a single slow mobility band, which ran as a doublet (tracks 16–20). These results confirmed that the IUF1 binding activity was not present in HeLa or BHK cells, but that they contained proteins which bound to oligo B at uncharacterised sites. IUF1 binding activity was also absent from nuclear extracts prepared from the mouse corticotrophic cell line AtT20 (data not shown).

The proteins binding to oligo B in the various cell extracts were further characterized by probing a South Western blot with oligo B. In the insulinoma and HIT cell extracts 3 major proteins of M_r 115 kDa, 65 kDa, and 46 kDa were observed (Fig. 2). In the HeLa extract, a single protein of M_r 115 kDa was observed together

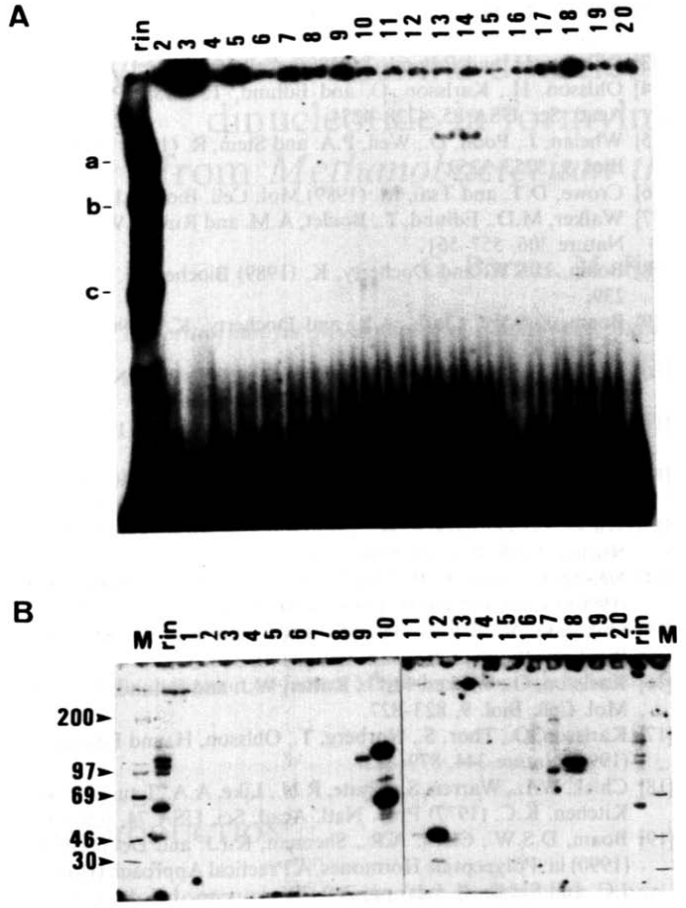

Fig. 3. (A) Electrophoretic mobility shift assay of rat insulinoma nuclear extract DEAE column fractions. Rat insulinoma extract was applied to the DEAE column and eluted with a 0–0.5 M NaCl gradient as described in section 2. Twenty fractions (1 ml) were collected, and 10 μl of each fraction assayed using radiolabelled oligo B. Tracks are: rin, rat insulinoma nuclear extract; 2–20, DEAE column fraction 2–20. (B) South Western blot analysis of rat insulinoma nuclear extract DEAE column fractions. The twenty DEAE column fractions from above were precipitated with ice-cold trichloracetic acid and subjected to South Western blot analysis using radiolabelled oligo B.Track M, ^{14}C protein molecular weight markers.

with at least 4 minor proteins of M_r 30–40 kDa, while the BHK extract contained a single prominent protein of M_r 65 kDa (Fig. 2).

Because the insulinoma extract was available in relatively large quantities, it was possible to further characterise these DNA binding proteins by DEAE ion exchange chromatography. Twenty fractions were collected from the DEAE ion exchange column, and each fraction analysed by EMSA using oligo B as probe (Fig. 3A). The 3 major binding activities observed in the unfractionated extract were separated on the column; activities corresponding to band a in fractions 13 and 14, band b in fraction 11, and band c in fraction 10. South Western (SW) blot analysis of the rat insulinoma nuclear extract revealed the major proteins of 46 kDa and 65 kDa, and a cluster of 3 proteins of M_r 95, 100 and 115 kDa (Fig. 3B). The 115 and the 65 kDa protein eluted in fraction 10. The 46 kDa protein eluted in fractions 11 and 12; approx. 10% in fraction 11 and 90% in fraction 12. The 95 kDa protein eluted in fractions 17 and 18, and the 100 kDa protein in fraction 18.

The column fractions were also analysed using oligo Bm2. The two minor EMSA bands a and d (see Fig. 1) were localised to fractions 13/14 and 10 respectively (data not shown). SW blot analysis with oligo Bm2 revealed 2 proteins of M_r 65 kDa and 115 kDa in the rat insulinoma extract: the 46 kDa protein observed with probe B did not bind to oligo Bm2. The 65 kDa protein eluted in fraction 10, while the 115 kDa protein was separated into 2 components which eluted in fractions 12/13 and 18 (Fig. 4). Thus, the 65 kDa protein in fraction 10 binds both oligos B and Bm2. It appears to be a protein of restricted tissue distribution, which binds non-specifically to DNA sequences, since it was also shown to bind to a variety of other short unrelated oligos (data not shown).

4. DISCUSSION

The pancreatic β cell-specific nuclear factor, IUF1, binds to the sequence 5′-CPyCTAATG-3′ (CT box) at positions, −313, −217 and −84 in the human insulin gene [8]. The two proximal CT boxes are paired to IEB motifs with a short separating sequence, and it has been suggested that the close juxtaposition of the IEB and CT boxes may constitute a β cell-specific proto-enhancer [9]. The IEB box is the dominant positive-acting enhancer element while the CT box may serve to potentiate activity of this motif [16]. The function of the distal CT box is unknown, although it is located within a region of the gene which exhibits positive enhancer activity (Clark and Docherty, in preparation).

This study reveals that the β cell-specific IUF1 activity is comprised of 2 components designated b and c. Competition studies in EMSA with oligos containing mutations in the IUF1 binding site, along with methylation interference analysis, confirmed that both components involved protein–DNA interactions at the CT motif. These components could be separated from each

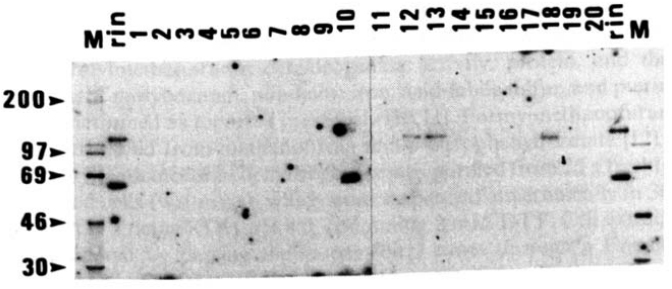

Fig. 4. South Western blot analysis of rat insulinoma nuclear extract DEAE column fractions. The twenty DEAE column fractions (see legend to Fig. 3) were subject to South Western blot analysis using radiolabelled oligo Bm2.

other by DEAE chromatography. The high mobility β cell-specific EMSA component (band c) co-fractionated with two major proteins of 115 kDa and 65 kDa. Other oligos bound to the 65 kDa protein suggesting that it was unlikely to contribute to β cell-specific interactions with oligo B. The 115 kDa protein interacted with sequences containing the CT box, i.e. it bound to oligo B but not to oligo Bm2, which suggests that this protein may be responsible for the β cell-specific EMSA complex c. Besides this protein there were several other proteins of M_r 95–110 kDa, which were not β cell-specific. They did not bind to the CT box within oligo B (i.e. bound oligo B and Bm2), and eluted from the DEAE column in fractions 13/14 and 18.

Assignment of the molecular size of the β cell-specific EMSA complex b by SW blotting was more difficult. Activity in the EMSA assay overlapped with that of a 46 kDa protein, however the maximal activities did not coincide. Such a discrepancy could arise if the 46 kDa protein were associated with a negative factor yielding a complex of slightly more acidic nature. This possibility and the possibility that the 46 kDa protein represents the mature form of the product of the Isl-1 cDNA [17] warrants further investigation.

Acknowledgement: This work was supported by a grant from the Wellcome Trust.

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