A tissue-specific nuclear factor binds to multiple sites in the human insulin-gene enhancer

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Sequence-specific binding of proteins from an insulin-secreting cell line (RINm-5F) to the human insulin-gene 5' region were examined by gel-retardation and methylation-interference analysis. Specific binding of a nuclear factor to sites between nucleotides –210 to –217 and –77 to –84 was detected. The same binding activity was shown at an upstream site (–313 to –320) with low affinity. Studies using mutated binding-site probes delineated a sequence 5'-C(T/C)CTAATG-3' for high-affinity interactions. This binding activity was also present in another insulin-producing cell line (HIT.T15), but not in extracts from cell lines that did not express the insulin gene (HeLa, HL60). Cross-species comparisons show that this sequence element is highly conserved and may thus play an important role in the cell-specific regulation of insulin-gene transcription.

INTRODUCTION

The temporal and spacial control of gene expression occurs mainly at the level of transcription initiation and is mediated by *cis*-acting elements flanking the gene (Maniatis *et al.*, 1987) classified according to their properties as enhancers and promoters (Banerji *et al.*, 1981; Khoury & Gruss, 1983). Enhancers and promoters contain multiple binding sites for cell-specific and ubiquitous protein-transcription factors, and it is the interaction of these factors with their cognate binding sites which controls the transcriptional activity and cell-type specificity of a gene (Davidson *et al.*, 1986; Nomiya *et al.*, 1987).

Transfection studies on deletion mutants of the rat insulin-1-gene 5' region have mapped the cell-specific enhancer and promoter to within 300 bp 5' of the transcription start site (Edlund *et al.*, 1985; Walker *et al.*, 1983). Further studies have shown that the enhancer and promoter contain multiple protein-binding sites (Ohlsson & Edlund, 1986; Moss *et al.*, 1988; Ohlsson *et al.*, 1988) some of which are important for maintaining transcriptional activity (Karlsson *et al.*, 1987; Moss *et al.*, 1988). The human insulin gene is transcriptionally active in rodent cell lines (Walker *et al.*, 1983) and in transgenic mice (Selden *et al.*, 1986; Bucchini *et al.*, 1986); furthermore, deletion of sequences between nucleotides –260 and –171 lead to loss of transcriptional activity (Walker *et al.*, 1983; A. R. Clark, D. S. W. Boam & K. Docherty, unpublished work). This may reflect the deletion of binding sites for important transcription factors.

In the present study we have used a gel-electrophoresis mobility-shift assay and methylation-interference analysis to explore the binding of proteins in nuclear extracts from a β -cell line (RINm-5F) to sites in the 5' region (–59 to –336) of the human insulin gene. Sequence-specific binding of a nuclear factor to three separate sites in this region was detected, two of high affinity and an upstream site of very low affinity. This binding activity

was only found in cell lines which express the insulin gene. We discuss whether this factor, IUF-1, may play a role in the tissue-specific control of insulin-gene expression.

MATERIALS AND METHODS

Cell culture

HL60 (a pluripotent promyelocytic cell line), and the rodent β -cell lines RINm-5F, HIT.T15 (at two different passage numbers, namely 70 and 80) cells were grown in RPMI 1640 supplemented with 10% foetal-calf serum (FCS). HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. To all media the following were added: glutamine (2 mM), streptomycin (40 μ g/ml) and penicillin (40 units/ml).

Preparation of nuclear and cytoplasmic extracts

Nuclear extracts were prepared and fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation as described by Ohlsson & Edlund (1986).

Labelling of DNA

DNA fragment probes representing the regulatory region of the human insulin gene were derived from a restriction-endonuclease-*Pst*I fragment of the human insulin gene 5' flanking region (–336 to +111) subcloned into plasmid pUC18 (pUins301). Plasmids were linearized by digestion with either *Bam*HI or *Bgl*II, then labelled using DNA polymerase I Klenow fragment and 200 μ Ci of [α - 32 P]dGTP (3000 Ci/mmol; Amersham International). The Klenow enzyme was inactivated by heating at 70 °C for 15 min and, after redigestion with an appropriate restriction enzyme, labelled fragments were separated on a 5%-polyacrylamide gel and eluted as described by Maxam & Gilbert (1980).

Oligodeoxynucleotides

30-mer oligodeoxynucleotides representing contiguous regions of the regulatory region of the human insulin

Abbreviations used: FCS, foetal-calf serum; DMEM, Dulbecco's modified Eagle medium; DMS, dimethyl sulphate; IUF-1, Insulin Upstream Factor 1; IEF-1, Insulin Enhancer Factor-1.

gene were labelled with polynucleotide kinase and 150 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol; Amersham International) as described by Maxam & Gilbert (1980). Labelled oligo(deoxy)nucleotides were annealed to a 2-fold excess of complementary strand by heating to 100 °C for 5 min in 10 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/2 mM-MgCl₂/1 mM-EDTA, followed by slow cooling to 25 °C over 2–3 h. Unlabelled competitor fragments were annealed in an identical manner. Double-stranded oligonucleotides were purified by polyacrylamide-gel electrophoresis.

Gel-retardation assay

Gel retardation assays were performed as described by Singh *et al.* (1986). Unless otherwise stated, all incubations contained 2 μg of poly(dI-dC) (Pharmacia) as a non-specific competitor. Incubations with restriction-fragment probes (10000 d.p.m.) were run on 4%-(w/v)-polyacrylamide gels pre-run in 0.5 $\times$ TBE (50 mM-Tris/50 mM-boric acid/1 mM-EDTA) at 10–15 V/cm for 30 min. Competitor DNAs were added to the binding reactions simultaneously with other components. Binding reactions with oligonucleotide probes (10000 c.p.m., 0.05 ng) were incubated as described above, but samples were run on 8%-polyacrylamide gels. Unretarded probe is seen at the bottom of gels in Figs. 2, 4, 5 and 6 (below).

Methylation interference

End-labelled oligonucleotides [(0.5–1 $\times 10^7$) dpm in 100 μl] were partially methylated by addition of 1 μl of dimethyl sulphate (DMS) followed by incubation at room temperature for 2–5 min as described by Maxam & Gilbert (1980). Reactions were stopped by addition of 10 μl of 3.3 M- β -mercaptoethanol, followed by purification on a Sephadex G-25 spun column (Maniatis *et al.*, 1982). Binding reaction mixtures contained 1–2 ng of methylated probe [(4–5) $\times 10^5$ d.p.m.], 100 μg of protein and 6–10 μg of poly (dI-dC) in a final volume of 100 μl . The mixture was incubated for 30 min at room temperature, then electrophoresed on an 8%-polyacrylamide gel as described above. After autoradiography

Fig. 1. Organization of the 5' region of the human insulin gene

Positions of synthetic oligodeoxynucleotide (Oligos) probes (A–E) corresponding to sequences between nucleotides –171 and –260 are also shown.

for 60 min, the bound and free DNA revealed were excised and eluted overnight at 37 °C. The eluates were re-purified on Sephadex G-25 spun columns. After heating at 90 °C for 15 min, 10 μl of 10 M-piperidine was added and samples heated for a further 30 min. Samples were freeze-dried and run on 20%-polyacrylamide sequencing gels with G+A markers as described by Maxam & Gilbert (1980).

RESULTS

Identification of a binding site, in the 5' region of the human insulin gene, for a sequence-specific protein factor

In the present study, binding of factors present in a RINm-5F-cell crude nuclear protein extract to sites in the putative 5' regulatory region of the human insulin gene were analysed by the gel-electrophoretic-mobility-shift assay (Singh *et al.*, 1986). To facilitate analysis of protein binding within this region, we constructed short double-stranded 30-mer synthetic oligodeoxynucleotides, representing overlapping sequences between nucleotides –260 and –171 (Fig. 1).

Fig. 2. A nuclear factor binds with specificity to the insulin-gene 5' region

(a) Gel retardation assay showing binding of nuclear proteins from RINm-5F cells to five labelled oligodeoxynucleotides (A–E) spanning nucleotides –260 to –171 of the human insulin gene. (b) Specificity of binding to oligonucleotide B. Indicated molar excesses of unlabelled oligonucleotides A–E were included in binding reactions containing labelled oligonucleotide B and RIN nuclear extract.

When nuclear proteins from the $(\text{NH}_4)_2\text{SO}_4$ (0.2 g/ml) fraction were incubated with each of the ^{32}P -labelled oligodeoxynucleotides in a gel retardation assay, two prominent retarded bands were seen with probe B, and minor bands were generated by probes C, D and E (Fig. 2a). No binding activity in 0.3 g/ml- $(\text{NH}_4)_2\text{SO}_4$ or cytoplasmic (S100) extracts was observed (results not shown); therefore only 0.2 g/ml- $(\text{NH}_4)_2\text{SO}_4$ extracts were used in following experiments.

To determine whether the DNA-protein complex formed by probe B was specific, excess unlabelled oligonucleotides were included in binding reactions with probe B. Only oligonucleotide B efficiently competed for binding (Fig. 2b). The overlapping oligonucleotides D and E did not compete for the DNA-protein complex formed by probe B, which implies localization of the putative binding site near or between the 3' end of oligonucleotide D (-219) and the 5' end of oligonucleotide E (-212).

To characterize the binding site of the RIN-cell nuclear factor within oligonucleotide B, methylation interference

analysis of the major retarded complex was performed (Scheidereit & Beato, 1987). Fig. 3(a) shows that methylation in a core sequence of CTAATG interfered with protein binding (Fig. 3b).

In an attempt to delineate the 5' boundary of the RIN-cell nuclear-factor-binding site on the coding strand and to assess further the specificity of this interaction, two-point-mutated variants of oligonucleotide B were constructed: Bm1 and Bm2 (Fig. 4a). Oligonucleotide Bm1 contains a point mutation where T(-216) has been substituted by a C residue outside the core binding site. Oligonucleotide Bm2 contains a point mutation where A(-212) is substituted for by a C residue within the core binding site. Oligonucleotide Bm1 formed a complex with the RIN-cell nuclear factor of mobility identical with that seen with wild-type oligonucleotide B (Fig. 4b) and was able to compete for the complex formed by oligonucleotide B (Fig. 4c). Oligonucleotide Bm2 failed

Fig. 3. Identification of a protein-binding site within oligonucleotide B, by methylation interference analysis

(a) Methylation interference analysis of the DNA-protein complex formed by a factor in a RINm-5F-cell nuclear extract with oligonucleotide B. Lane F, free DNA; lane B, bound DNA eluted from the major retarded band (see Fig. 2a); lane GA, G + A chemical sequencing ladder. (b) The nucleotide sequence of the coding and non-coding strands of oligo B is shown with fully protected (●) and partially protected (○) G and A residues marked.

Fig. 4. Analysis of the sequence specificity of the DNA-protein complex formed between oligo B and a RIN-cell nuclear factor

(a) Sequence of wild-type oligonucleotide (Oligo) B and its point-mutated variants Bm1 and Bm2. Substituted residues in Bm1 and Bm2 are underlined. (b) Comparison of the ability of mutant oligonucleotides Bm1 and Bm2 to form complexes with a RIN-cell nuclear factor. Probes used were: lane 1, oligonucleotide B; lane 2, oligonucleotide Bm1; lane 3, oligonucleotide Bm2. (c) Gel retardation competition assay demonstrating the capacity of oligonucleotides Bm1 and Bm2 to act as competitors correlates with their ability to form DNA-protein complexes. Labelled oligonucleotide B was used as a probe, with 5 μg of RIN-cell nuclear extract. The '50', '100' and '500' values refer to molar excesses of unlabelled oligonucleotide.

Fig. 5. Analysis of protein binding in three restriction fragments of the insulin gene 5' flanking region between -339 and -59

(a) Comparison of the sequences of: (i) the identified RIN-cell-factor-binding site (-210 to -215) with two other potential binding sites (ii) -77 to -82 and (iii) -313 to -318, within the human insulin-gene regulatory region. (b) Capacity of oligonucleotides A, B and C to compete for complexes formed by three labelled restriction fragment probes (-336/-261, -260/-171 and -166/-59). Competitor oligonucleotides were included at a 500-fold molar excess. The most prominent retarded bands are marked. (c) Scanning-laser-densitometry analysis of complex IIIc, generated by probe -336/-261 in competition assays with oligonucleotides A, B and C in an experiment typical of that shown above (b). Values refer to peak area in arbitrary units. Ablation of peak IIIc exclusively by oligonucleotide B was reproducibly seen in a number of separate experiments of identical design. (d) Competition analysis of multiple DNA-protein complexes, formed by probes -59/-166 and -171/-260, with oligonucleotides Bm1 and Bm2. Competition-gel-retardation assays were identical with those described for (b). A 500-fold molar excess of competitor oligos was used. Competitors: lane 0, no competitor; lane 1, oligo B; lane 2, oligonucleotide Bm1; lane 3, oligonucleotide Bm2.

to form a complex or compete for the complex formed by oligonucleotide B. Although it can be concluded that the mutation in oligonucleotide Bm2 abolishes binding to the RIN-cell nuclear factor, experiments with oligonucleotide Bm1 indicate either that T(-216) does not participate in factor binding or that the T-to-C mutation does not disrupt binding.

A RIN-cell nuclear factor binds to three sites in the human insulin gene 5' flanking region with variable affinities

Inspection of the sequence of the human insulin gene 5' region revealed the presence of two further sequences identical with the core binding sequence, CTAATG, at nucleotides -82 to -77 and -318 to -313, but with slightly diverging 5' flanking sequences (Fig. 5a).

To determine whether the core binding site CTAATG is sufficient to bind the RIN cell factor or requires 5'-flanking residues, and also to investigate the putative binding sites at nucleotides -82 to -77 and -320 to -313 (Fig. 5a), three restriction fragment probes spanning nucleotides -336 to -59 (see Fig. 1) were used in

gel retardation assays. Each of the fragments used contains a single putative binding site. All probes generated multiple retarded bands (Fig. 5b). To investigate the nature of these multiple complexes, competition experiments using oligonucleotides A, B and C were performed. Oligonucleotide B exclusively competed for three out of five bands generated by probe -260/-171 (IIb, IIIb and Vb) and two major bands generated by probe -166/-59 (IIa and IVa). In contrast, with probe -336/-261, only the minor band, IIIc, was competed for by oligonucleotide B: this is more clearly demonstrated by scanning laser densitometry of the lanes shown in Fig. 5b (Fig. 5c). None of the retarded bands generated by the restriction-fragment probes were competed for by oligonucleotide A; however, oligonucleotide C competed for band Ib (Fig. 5b). No competition was also observed with oligonucleotide E; however, bands IVb, Ic and IIc could be competed for by oligonucleotide D (results not shown).

To determine whether the DNA-protein complexes competed for by oligonucleotide B interacted with the core binding sequence defined above, competition gel

Fig. 6. Cell-type specificity of a protein factor which binds to oligonucleotide B

(a) A 5 μ g portion of nuclear protein extracts (0.2 g/ml) from different cell lines were incubated with 32 P-labelled oligonucleotide B in standard gel retardation binding reaction mixtures, with the following (0.2 g) nuclear extracts: lane 1, RINm5F; lane 2, HIT.T15 (P70); lane 3, HIT.T15 (P80); lane 4, HeLa; lane 5, HL60. (b) Analysis of binding activity in HIT.T15 nuclear extracts. 32 P-labelled oligonucleotide B was incubated with 5 μ g of HIT.T15 (passages 70 and 80) nuclear extract in the presence of a 500-fold molar excess of competitor oligonucleotide: lane 0, no competitor; lane 1, oligonucleotide B; lane 2, oligonucleotide Bm1; lane 3, oligonucleotide Bm2.

retardation assays were performed on probes -260/-171 and -166/-59 with the mutant-binding-site oligonucleotides Bm1 and Bm2. Oligonucleotide Bm1 showed the same capacity to compete for DNA-protein complexes as oligonucleotide B (Fig. 5d), whereas oligonucleotide Bm2 showed no activity as a competitor. Thus these data indicate that the competed-for bands shown in Figs. 5(b) and 5(d) resulted from the binding of a factor to the CTAATG element with the same specificity as that originally observed with oligonucleotide B.

Inspection of the upstream 'CTAATG' box at nucleotides -319 to -313 shows that it is identical with that between nucleotides -216 and -210 (Fig. 5a), yet the apparent affinity with which these two sites bind a nuclear factor differs markedly. Only immediately upstream do the sequences diverge: the binding site at -216 to -210 is preceded by a C residue, whereas the upstream site at -319 to -313 is preceded by a G residue. We infer that a C-to-G nucleotide change at the 5' boundary of the CTAATG box results in a dramatic loss of binding activity. Furthermore, it should be noted that the point mutated oligonucleotide Bm1, which did not abolish binding activity, contains the sequence CCCTAATG, one identical with the downstream putative binding site between nucleotides -84 and -77. These experiments delineate a consensus binding site of $^5\text{C(T/C)CTAATG}^3$ where the 5' C residue appears to be very important for binding activity.

A nuclear DNA-binding factor is found only in cell lines which express the insulin gene

To determine whether the RIN-cell DNA-binding activity we have previously detected is present in other

cell lines which express, or do not express, the insulin gene, gel retardation analysis was used to look for DNA-binding activity in 0.2 g/ml- $(\text{NH}_4)_2\text{SO}_4$ nuclear extracts from other cell lines. Only extracts from RIN and two HIT cell lines formed retarded bands, whereas no retardation was seen with HeLa- or HL60-cell extracts (Fig. 6a). A very faint retarded band could be seen with the HeLa-cell extract, but this was not reproducible when different HeLa extracts were used (results not shown). Three retarded bands were generated by the HIT.T15 (passage 70) and two bands generated by HIT.T15 (passage 80) extracts, with mobility nearly identical with that of the bands generated by the RIN-cell extracts were observed, although the relative intensities of these bands varied according to the origin of the extract. To confirm that these bands were generated by the same binding activity seen in the RIN-cell extracts, competition experiments using the mutated oligonucleotides Bm1 and Bm2 were performed. Oligonucleotide Bm1 competed for binding of the factors, present in both HIT-cell extracts, to labelled oligonucleotide B, whereas oligonucleotide Bm2 did not compete for binding to labelled oligonucleotide B (Fig. 6b). This indicated that the nature of the DNA-protein interaction seen in the two HIT-cell extracts was similar to that seen in the RIN-cell extract. Furthermore, this DNA-binding activity seems to be found only in cell lines which express the insulin gene.

DISCUSSION

In the present study we used gel-retardation and methylation-interference analysis to identify binding sites for putative transcription factors within the human-insulin-gene regulatory region. We have identified three sites for what seems to be the same binding activity, but with differing affinities. The two high-affinity binding sites at -210 to -217 and -77 to -84 differ at only one position where T(-216) has been substituted for by C(-83), without any loss of binding activity. The upstream binding site, at nucleotides -313 to -320, is nearly identical with the binding site between -210 and -217, except for the substitution of C(-217) for G(-320). This change leads to an almost total loss of binding activity. Clearly the binding activity which we have identified here, and named IUF-1 (Insulin Upstream Factor-1), displays a high degree of selectivity in the sequence specificity of the interaction with its binding site, where recognition of some residues is more important than that of others. Evidence presented here indicates that interaction of IUF-1 with its binding site results in the formation of multiple complexes, each of different mobility in the gel-retardation assays. It is possible that this may reflect stoichiometric binding of a single factor, or the presence of distinct multiple molecular species with the same binding specificity, perhaps generated by phosphorylation (Montminy & Bilezikjian, 1987), O-glycosylation (Jackson & Tjian, 1988) or by differential splicing of a single primary transcript (Santoro *et al.*, 1988).

The present study has shown that IUF-1 is distributed in a cell-type-specific manner. The relative intensities and electrophoretic mobilities of IUF-1 complexes seem to differ between RIN- and HIT-cell lines. This may reflect inter-species differences, although binding specificity is well preserved. It is unlikely that IUF-1 is a CCAAT-

Human	-217	-210	-84	-77
	CTCTAATG		CCCTAATG	
Dog	-173	-166	-59	-52
	CTCTAACG		CCCTCATG	
Mouse I	-216	-209	-83	-76
	CTATAATA		CCTTAATG	
Rat I	-216	-209	-83	-76
	ATCTAATT		CCTTAATG	
Rat II	-204	-197	-83	-76
	CTCTAATT		CCTTAATG	

Fig. 7. Comparisons of sequence of putative upstream and downstream IUF-1-binding sites in the 5' region of different mammalian insulin genes

For the upstream 'low-affinity' site in the human insulin gene (-312 to -320) there was no significant sequence similarity with any other species examined.

box-binding factor (Raymondjean *et al.*, 1988), although its binding site is similar, because we could not detect any binding activity in HeLa cells, which are known to contain CCAAT-box-binding proteins (Jones *et al.*, 1987). Moss *et al.* (1988) have reported tissue-specific DNA binding in HIT-M2.2.2-cell extracts to a region of the rat insulin I gene which contains a sequence partially identical with that of the IUF-1-binding site (Fig. 7). DNAase footprinting analysis of the rat insulin-I-gene enhancer shows protection of this sequence (Ohlsson & Edlund, 1986; Ohlsson *et al.*, 1988); however, these studies also showed that there was binding to the same site in BHK-cell extracts (Ohlsson & Edlund, 1986). This may be due to the presence of IUF-1-like activity in these cells, in which case it cannot be claimed that IUF-1 is truly exclusively found in insulin-producing cells.

The observations made in the present study are based upon the use of a human gene regulatory region and rodent-cell lines, since no corresponding human cell line is currently available. However, there is evidence that cell-type-specific transcription of the human insulin gene occurs in rodent β -cell lines (Walker *et al.*, 1983; A. R. Clark, D. S. W. Boam & K. Docherty, unpublished work) and in transgenic mice (Selden *et al.*, 1986; Bucchini *et al.*, 1986); therefore it is probable that some or all of the *trans*-acting factors responsible for activating rodent insulin-gene transcription also perform the same role vis-à-vis the human insulin gene.

In the present study we have not determined whether IUF-1 is transcriptionally active, although preliminary results from our laboratory show that the IUF-1-binding site contributes towards enhancer activity (A. R. Clark, D. S. W. Boam & K. Docherty, unpublished work). Several other lines of evidence indicate that IUF-1 may have a role in the control of insulin-gene transcription. Comparison of the 5' flanking regions of several mammalian insulin genes shows a high degree of sequence similarity and positional conservation of putative IUF-1-binding sites (Fig. 7). This may indicate evolutionary pressure for conservation of function.

Mutational analysis of rat insulin-I-gene transcription shows that the analogous proximal and distal high-affinity IUF-1 binding sites reside in mutationally sensitive regions of the rat insulin-I-gene 5' region (Karlsson *et al.*, 1987). Studies on the rat insulin-I gene have revealed the presence of another factor, Insulin Enhancer Factor 1 (IEF-1), which contributes significantly towards

transcriptional activity of the gene (Ohlsson *et al.*, 1988; Moss *et al.*, 1988; Karlsson *et al.*, 1987). Additional studies upon the rat insulin I gene suggest that the region containing a sequence partially identical with the IUF-1 site delineated here (Fig. 7) is devoid of intrinsic transcriptional activity, but serves to potentiate the activity of IEF-1 2-fold (Karlsson *et al.*, 1989). Those studies do not address the nature of the potentiation mechanism; therefore it still remains for a precise function for IUF-1 in the developmental and tissue-specific control of insulin gene transcription to be defined. Studies on transgenic mice bearing mutated insulin 5' regions should help to elucidate the physiological role of IUF-1 and other important factors.

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