Delineation of the insulin-responsive sequence in the rat cytosolic aspartate aminotransferase gene: binding sites for hepatocyte nuclear factor-3 and nuclear factor I

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Expression of the rat cytosolic aspartate aminotransferase gene is stimulated by glucocorticoids and repressed by insulin in the liver. The regulation by insulin and part of the glucocorticoid effect are mediated by a distal region in the promoter. A 142 bp fragment (−1844 to −1702) confers hormonal sensitivity to the heterologous thymidine kinase promoter in transient-transfection assays in H4IIEC3 hepatoma cells. Footprinting and gel-shift assays showed that several nuclear proteins bind to this region at conserved CCAAT-enhancer binding protein (C/EBP), activator protein (AP-1) and E-box sequences. Hepatocyte nuclear factor-3α (HNF-3)α and β bind to sequences upstream of a glucocorticoid-responsive element (GRE) half-site as demonstrated by supershift experiments. Nuclear factor I (NFI)-like proteins bind downstream of the GRE half-site. These sites around the GRE motif overlap with five insulin responsive element (IRE) -like sequences (TG/ATTT). The effect of insulin was not prevented by any single mutation in the IRE-like sites. However, mutation of two IRE sites (namely IREc and d) prevented the insulin effect although only marginally affecting the glucocorticoid effect. The results suggest that the effect of insulin is due to a complex interplay of factors requiring the synergistic contribution of at least two sites and underline the contribution of HNF-3 and NFI-like proteins.

Key words: glucocorticoids, forkhead proteins, transcription factors, gene regulation.

INTRODUCTION

Aspartate aminotransferase (EC 2.6.1.1.) is a ubiquitous pyridoxal phosphate-dependent enzyme. It is present in vertebrate cells as two isoenzymes, one cytosolic and the other mitochondrial, which play major roles in amino acid metabolism and in the malate–aspartate shuttle, necessary for the survival of all cells [1]. The isoenzymes also participate in ureogenesis and gluconeogenesis, two pathways restricted to certain tissues, particularly the liver. In keeping with the complex metabolic functions of aspartate aminotransferase, the expression of its gene is regulated differentially depending upon the organ. The cytosolic aspartate aminotransferase (cAspAT) activity is stimulated by glucocorticoids in the liver and in the kidney [2–4]. In the liver, and in differentiated hepatoma cells, glucagon (or cAMP) potentiates this effect whereas insulin decreases it [5]. These effectors are probably responsible for the increase in the enzyme activity in the liver during starvation or after a protein-rich diet. In a previous paper, we showed that these regulations occur at the transcriptional level [6]. Analysis of the promoter of the gene encoding cAspAT allowed us to delineate two regions important for hormonal regulation. Glucocorticoids act at two sites, a proximal one, with an unusual structure comprising two overlapping glucocorticoid-responsive elements (GRE) [7] and a distal one, also responsible for the inhibition by insulin [6].

Although many genes are regulated by insulin at the transcriptional level, no unique consensus insulin-responsive sequence (IRS) or element (IRE) has been reported, in contrast with consensus-responsive elements described for other hormones. In fact, positive and negative regulation by insulin can be distinguished. In the former case, several distinct factors have been implicated in different genes. In the case of negatively regulated genes, several sequences mediating the insulin effect have been described [8]. In particular, a common motif named IRS or IRE has been found in the promoters of the genes encoding phosphoenolpyruvate carboxykinase (PEPCK), insulin-like growth-factor-binding protein-1 (IGFBP-1), tyrosine aminotransferase (TAT), glucose-6-phosphatase (G6Pase) and apolipoprotein CIII [9–13]. The actual function of this consensus motif and the proteins that are relevant for the negative insulin effect are still under investigation. Hepatocyte nuclear factor-3 (HNF-3) as well as other proteins named insulin-responsive factors have been suggested to play regulatory roles in some gene promoters but not in others [8]. Another intriguing feature is that several of these genes are also positively regulated by glucocorticoids.

Because of the complexity of insulin action at the gene level, it is important to delineate the actual contribution of the regulatory sequences in several promoters to identify common themes in signalling by this hormone. In the present study, we have

Abbreviations used: cAspAT, cytosolic aspartate aminotransferase; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer-binding protein; G6Pase, glucose-6-phosphatase; GRE, glucocorticoid-responsive element; HNF-3, hepatocyte nuclear factor-3; IGFBP-1, insulin-like growth-factor-binding protein-1; IRE, insulin-responsive element; IRS, insulin-responsive sequence; NFI, nuclear factor I; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase.

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investigated in more detail the distal region of the *cAspAT* gene promoter, which mediates the insulin response and part of the glucocorticoid action. We show that in addition to other transcription-factor-binding sites, this region includes several IRE-like sequences, two of which bind HNF-3 [14] and Nuclear Factor I (NFI) [15]. Mutation of both IREs is required to inhibit the insulin effect.

EXPERIMENTAL

Cell culture

The rat-hepatoma clones H4IIEC3 and Fao are derived from the Reuber H35 hepatoma [16]. Cells were maintained as described [17] and treated, as indicated, with the various hormones [6].

Nuclear extracts

Nuclear extracts from H4IIEC3 cells were prepared according to the technique of Shapiro et al. [18]. For most of the gel retardation experiments, a rapid technique derived from Shreiber et al. [19] was used [20]. Nuclear extracts from rat liver were prepared according to Gorski et al. [21].

Plasmids

The p-2405/-26CAT plasmid previously described [6] was used for PCR experiments. The plasmid ΔMTV-CAT, derived from the plasmid MMTV-CAT by deletion of the sequence from position −190 to −88 of the mouse mammary tumour virus (MMTV) long terminal repeat, was a gift of Dr. R. Evans (The Salk Institute, San Diego, CA, U.S.A.) and is described elsewhere [22]. The pTkCAT plasmid was previously described [23]. The various plasmids were obtained by subcloning fragments of the *cAspAT* gene promoter obtained by PCR into the *Hin*dIII site of ΔMTV-CAT or pTkCAT vector. The mutations were created by double PCR experiments, using a procedure previously described [6]. For all the fragments subcloned into the *Hin*dIII site, a *Hin*dIII site was designed into the primers used. The plasmids used in the present study are named by a prefix containing the boundary of the promoter sequence followed by the parent plasmid (e.g. the plasmid constructed from the pTkCAT vector and the region −1844 to −1702 of the promoter is denoted −1844/−1702TkCAT). Plasmids or oligonucleotides in which a mutation was introduced into the promoter region are denoted with the suffix 'm' (e.g. a mutation in the putative E-box sequence is denoted E-boxm).

DNase I footprinting

A plasmid containing the −1838 to −1702 sequence of the *cAspAT* gene promoter was used for the footprinting experiments. It was end-labelled at sites in the polylinker (located at either end of the sequence) using the Klenow fragment of DNA polymerase I. The standard reaction was performed according to Vaulont et al. [24] with the following modifications: the nuclear proteins, 30–60 μg, were incubated with approx. 1 ng of labelled probe (30000–60000 c.p.m.) for 15 min on ice. After adjusting the concentration of $CaCl_2$ to 2.5 mM and incubating for 1 min at 20°C, DNase I was added, and the digestion was carried out at room temperature for 1 min. An A + G ladder of the fragments, prepared according to the technique of Maxam and Gilbert, was used to read the sequence [24a].

Electrophoretic-mobility-shift (or gel-shift retardation) assays

Probes were double-stranded oligonucleotides labelled using the Klenow fragment of DNA polymerase I. Protein–DNA binding was performed under the same conditions as those described for the footprinting experiments except that 2 μg of poly(dI-dC) was used as carrier and that 1.5 to 7 μg of nuclear-extract protein were added to the probe. After 15 min on ice, the samples were directly loaded onto a 6 % (w/v) polyacrylamide gel in 0.5 × TBE (1 × TBE = 45 mM Tris/borate/1 mM EDTA). The gel (0.2 cm in thickness and 16 cm in length) was pre-electrophoresed at 100 V for 1 h in the cold room (4 °C). Electrophoresis was performed at 260 V for 90 min in 0.5 × TBE buffer. For competition experiments, the cold oligonucleotides were preincubated 15 min on ice with the nuclear extracts prior to the addition of the probe. For supershift experiments with antibodies, the antibodies or sera (1 μl) were preincubated with nuclear extracts 2 h on ice prior to the addition of the radiolabelled probe. Antibodies directed against CCAAT-enhancer-binding protein α (C/EBPα) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies directed against NFI were provided by Dr. N. Tanese (New York University Medical Center, New York, NY, U.S.A.). Antisera against HNF-3α and β were made by Dr. R. Costa (University of Illinois, Chicago, IL, U.S.A.) and antiserum against HNF-3γ, prepared by Dr. S. Duncan (Rockefeller University, New York NY, U.S.A.), was also provided by Dr. R. Costa.

Transfection experiments

One day prior to the transfection, Fao or H4IIEC3 cells (0.3 to 0.5×10^6 cells/6 cm dish) were seeded into the usual culture medium containing foetal calf serum. Four ml of fresh medium with serum were added to the cells 2–3 h before the transfection. Various liposomal transfection reagents were tested [DOTAP and fugene™ 6 reagents (Boehringer Mannheim) and Dac30 reagent (Eurogentec, Liège, Belgium)]. Similar results were obtained in all cases and we routinely used the fugene™ 6 transfection reagent, according to the manufacturer's recommendations: 5 μl of fugene were mixed with 95 μl of complete medium. One μg of DNA in, at most, 10 μl of water were added, and the mixture was allowed to stand at room temperature for 30 min. It was then added to the cells for 6 h. The medium was then replaced by 4 ml of fresh medium containing serum, and the hormones (0.1 μM dexamethasone, 0.1 μM insulin) were added 16 h later. The cells were then cultured for 24 h.

Chloramphenicol acetyltransferase (CAT) assay

CAT activity was determined using the two-phase assay developed by Neumann et al. [25] as described elsewhere [23]. For each reaction, 30–60 μl of the cell-extract supernatant were added to 70–40 μl of a buffer solution to give the following final concentrations: 250 mM Tris/HCl pH 7.5, 5 mM EDTA, 1 mM chloramphenicol, 30 μM acetyl-CoA to which 0.5 μCi [^{3}H]acetyl-CoA were added (NEN). The reaction was performed for up to 3 h at 37°C. The solution was then transferred to a minivial and layered with 4 ml of Econofluor (NEN). After mixing vigorously, the two phases were allowed to separate for at least 30 min, and radioactivity was counted in a scintillation counter. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not the unreacted acetyl-CoA, is allowed to diffuse into the Econofluor phase. In these experiments, blanks were obtained by assaying CAT activity in cells that have undergone the same treatment in the absence of a CAT plasmid. Proteins were assayed according to the technique of Bradford [26].

RESULTS

The −1844 to −1702 fragment of the *cAspAT* gene promoter confers hormonal regulation to a heterologous promoter

A previous study using stable transfections in rat-hepatoma-Fao cells showed that a 265 bp fragment (−1983 to −1718) of the *cAspAT* gene promoter was essential for the positive stimulation of gene expression by glucocorticoids and for the partial inhibition of this stimulation by insulin [6]. This result prompted us to examine which DNA sequences are involved in these regulatory effects. We first introduced the −1984 to −1702 fragment in front of a heterologous promoter from the thymidine kinase gene, the *Tk* promoter, driving the *CAT* reporter gene, to ensure that it was indeed responsible for the action of the hormone. The construction was transiently transfected into the well-differentiated rat-hepatoma cell line H4IIEC3. Addition of glucocorticoids led to a 3–4-fold increase of the CAT activity, confirming that this distal sequence carries part of the glucocorticoid effect on the *cAspAT* gene promoter. Insulin was capable of inhibiting this glucocorticoid stimulation by 50% (Figure 1A). The same results were also obtained when the *cAspAT* gene sequence was introduced into a more complex heterologous promoter, the ΔMTV-CAT plasmid, in both cell lines, Fao and H4IIEC3 (results not shown). All subsequent deletions of the gene were introduced into the pTkCAT plasmid. Three 5′-deleted constructs (−1844/−1702TkCAT, −1801/−1702TkCAT and −1768/−1702TkCAT) were tested in H4IIEC3 cells. The longest one responded to the hormones with the same efficiency as the −1984/−1702TkCAT plasmid. In contrast, the −1768/−1702TkCAT plasmid completely lost the response to both hormones whereas the −1801/−1702TkCAT plasmid displayed a weak 1.7-fold glucocorticoid stimulation which was still partially inhibited by insulin (Figure 1A). With the smallest construct, we consistently observed a small increase in CAT activity in the presence of insulin. This increase was also present with the parent pTkCAT plasmid (results not shown) and was probably an intrinsic property of this plasmid. Therefore we focused our study on the 142 bp sequence (−1844 to −1702) to determine which sites were able to bind nuclear proteins, using *in vitro* footprinting and gel-retardation assays as well as transfection experiments.

Footprinting experiments

In vitro DNase I footprinting analyses were carried out to identify protein binding sites. A promoter fragment from −1838

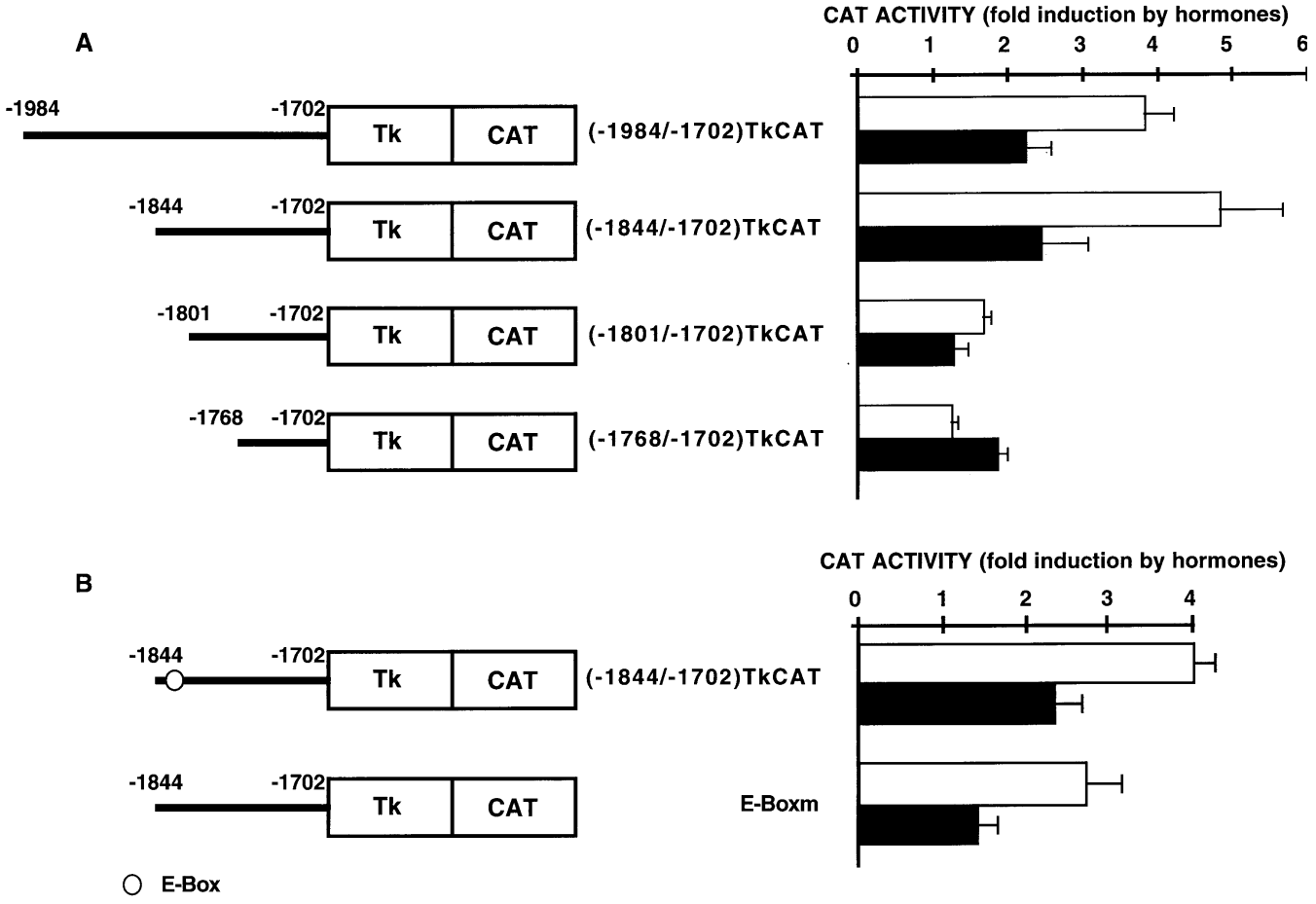

Figure 1 Effect of dexamethasone and insulin on constructs containing *cAspAT* gene fragments upstream of the thymidine kinase promoter

(**A**) Fragments of the *cAspAT* gene were inserted upstream of the thymidine kinase (Tk) promoter driving *CAT* gene activity and transfected into H4IIEC3 cells. (**B**) The wild-type −1844/−1702TkCAT plasmid and its mutant in the E-box (E-Boxm) were transfected into the H4IIEC3 cells. Results are expressed as the fold induction over the basal activity by either dexamethasone alone (□) or dexamethasone plus insulin (■). Values represent the mean ± S.E.M. of 4–8 independent experiments in duplicate or triplicate.

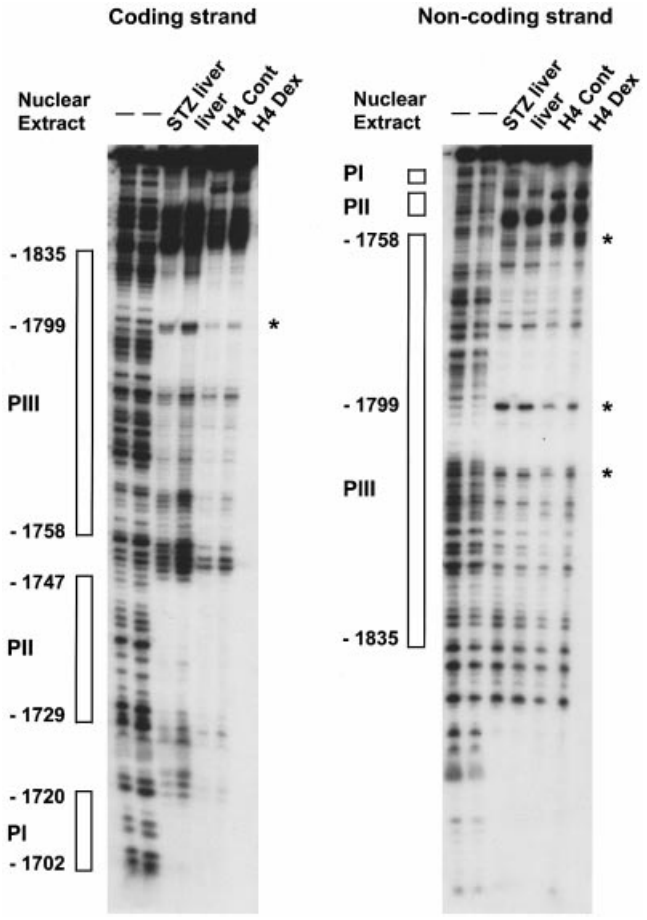

Figure 2 DNase I footprinting of the interactions of proteins with the (−1838 to −1702) *cAspAT* gene fragment

The probe was labelled on the coding strand at the *Bam*HI site and on the non coding strand at the *Eco*RI site. Boxes to the left represent the observed footprints with the numbers indicating their limits. Hypersensitive sites are indicated by stars. Nuclear extracts were prepared from the livers of rats treated (STZ liver) or not (liver) by streptozotocin and from H4IIEC3 cells treated (H4 Dex) or not (H4 Cont) by dexamethasone.

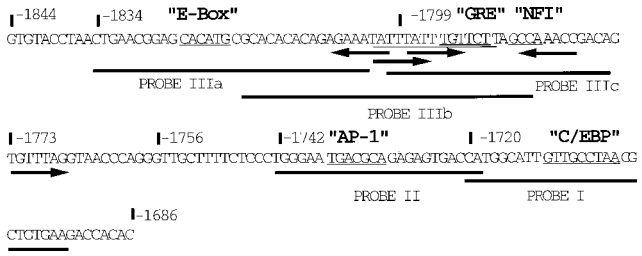

Figure 3 Sequence of the (−1844 to −1686) fragment of the *cAspAT* gene

The sequence of the (−1844 to −1686) fragment of the *cAspAT* gene and the positions of the five probes used in the gel-shift retardation experiments are shown. The positions of the probes are indicated by the thick lines. The sequences similar to consensus motifs for several transcription factors are underlined and five IRE-like motifs are shown by arrows.

to −1702 was analysed. Three protected regions (named PI to PIII) were identified with nuclear extracts prepared from either H4IIEC3 cells or rat liver. Two of the regions (PI and PII) were

Table 1 Motifs and sequences for the binding of transcription factors

The origin of the motif sequence (consensus or gene) is indicated as well as the position of the corresponding site in the *cAspAT* gene and in the footprint.

motif	sequence	gene	position	footprint
E-Box	CAnnTG	consensus	-1824/-1819	PIII
	CACATG	cAspAT		
1/2GRE	TGTTCT	consensus	-1724/-1789	PIII
	TGTTCT	cAspAT		
AP-1	TGACTCA	consensus		
	TGACGCA	cAspAT	-1736/-1730	PII
	TGAGTCA	collagenase		
	TGACTAA	T-antigen		
C/EBP	T(T/G)NNG(C/T)AA(T/G)	consensus	-1703/-1711	PI
	TTAGGCAAC	cAspAT		
NFI	TGGCNNNNNGCCA	consensus		
	TTGTTCTTAGCCA	cAspAT	-1795/-1783	PIII
HNF-3	CTAAGC(A/C)AAA(T/C)A	consensus		
	TAGGAT			
	ACAAATAAATA	cAspAT	-1792/-1802	PIII
	AAGAATCAATA		-1788/-1798	PIII
	CTAAGTCAATA	TTR		
	CAAAACAAACT	IGFBP-1		
	TTGTCAAAACA	PEPCK		
	TAGAACAAACA	TAT		
IRE	T(G/A)TTT(TG/GT)	PEPCK consensus		
	TATTTCT	IREa cAspAT	-1801/-1807	PIII
	TATTTAT	IREb	-1802/-1796	PIII
	TATTTGT	IREc	-1798/-1792	PIII
	GGTTTGG	IREd	-1779/-1785	PIII
	TGTTTAG	IREe	-1773/-1767	PIII

clearly detected whereas protection of the PIII sequence was only partial (Figure 2). The PI region corresponds to the sequence −1702 to −1720 while the PII footprint spans the sequence from −1729 to −1747. The large, partially protected PIII footprint corresponds to the sequence −1758 to −1835 and contains a hypersensitive site located at −1799. No obvious difference was seen with nuclear extracts prepared from cells that had been treated with hormones (dexamethasone, insulin or both) for 2 h or extracts that had been prepared from control or streptozotocin-treated rats [3]. However, this is not uncommon and has been reported for other genes [27]. Analysis of the sequence (Figure 3) revealed that several motifs resembled consensus sequences known to bind nuclear factors (Table 1). A C/EBP motif [28] is present in the sequence PI. A putative activator protein (AP-1) motif, which binds dimers of the Jun–Fos transcription-factor family, is suspected in region PII. Finally, both an E-box sequence, which binds transcription factors containing a helix-loop-helix dimerization domain, and an HNF-3 binding sequence can be deduced from the analysis of region PIII. The existence of an HNF-3 binding site was also supported by the presence of the hypersensitive site at −1799 in the footprinting experiments, a characteristic feature for HNF-3 binding [29]. A sequence corresponding to the half-site of the consensus NFI-binding site is also present in this PIII region as well as a GRE half-site and several sequences with homology to IREs. In order to confirm the observations made in the DNase I protection experiments, gel-shift retardation assays were per-

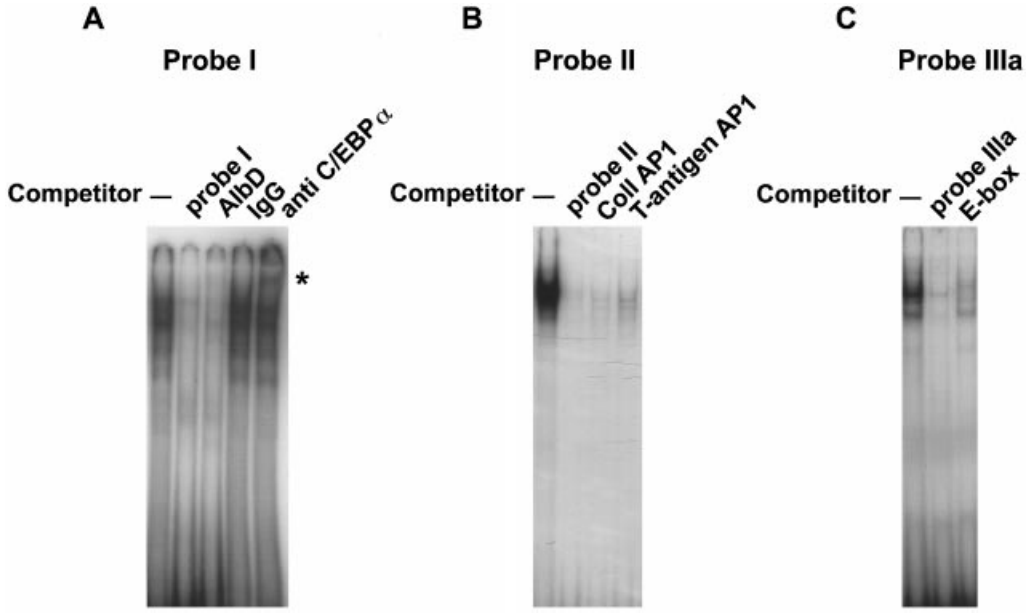

Figure 4 Analysis of protein binding to the *cAspAT* gene by electrophoretic-mobility-shift-assay experiments

(**A**) Labelled probe I was incubated with H4IIEC3 nuclear extracts and with either none (lane 1) or 100-fold molar excesses of the indicated unlabelled competitors: probe I (lane 2), a C/EBP consensus oligonucleotide (AlbD) derived from the albumin-gene promoter (lane 3), preimmune IgG (lane 4), antibodies directed against C/EBPα (lane 5). The supershifted band in lane 5 is indicated by an asterisk. (**B**) Labelled probe II was incubated with H4IIEC3 nuclear extracts and with either none (lane 1) or 100-fold molar excesses of unlabelled probe II (lane 2), an AP-1 site derived from the collagenase gene (lane 3) or from the T-antigen gene (lane 4). (**C**) Labelled probe IIIa was incubated with H4IIEC3 nuclear extracts and with either none (lane 1) or with 100-fold molar excesses of unlabelled probe IIIa (lane 2) or an E-box site derived from the adenovirus genome (lane 3).

formed using the sequences protected from DNase I digestion and which contained putative consensus sequences known to bind transcription factors.

Identification of C/EBP, AP-1 and E-box binding sites by gel-shift retardation assays

Three different probes used for the gel-shift experiments (probes I–IIIa) are positioned on the sequence shown in Figure 3. They correspond to the protected regions that were identified in Figure 2. Complexes between these oligonucleotides and proteins from nuclear extracts were obtained with all the probes. Competition experiments were then performed to determine if consensus oligonucleotides corresponding to known transcription-factor-binding sites could displace the proteins from the probes (see Table 1 for the competitors used).

For probe I (-1721 to -1694), four retarded bands were observed (Figure 4A, lane1). These bands were specific since they decreased in the presence of an excess of the unlabelled oligonucleotide I (lane 2). Addition of a C/EBP consensus oligonucleotide (AlbD) derived from the albumin-gene promoter [30] completely abolished the binding (lane 3). Moreover, antibodies directed against C/EPBα induced a supershift of the higher bands (lane 5, asterisk).

Probe II (-1742 to -1718), the sequence of which has a 6 to 7 bp homology with an AP-1 consensus sequence, was able to form one major complex (Figure 4B, lane 1) which was competed for by an excess of unlabelled probe (lane 2). The radiolabelled probe was also competed for when the AP-1 oligonucleotides corresponding to sequences in the collagenase [31] and the T-antigen [32] genes were added to the reaction (lanes 3 and 4).

Several complexes, including a major one, were observed with probe IIIa (-1835 to -1804) (Figure 4C, lane1). The latter could be competed for by an excess of the unlabelled oligonucleotide (lane 2) and by an E-box consensus sequence found in the adenovirus genome [33] (lane 3).

The E-box sequence is not responsible for *cAspAT* gene regulation

Since both the dexamethasone and insulin effects are lost when only the short -1768 to -1702 fragment of the *cAspAT* gene is tested, we focused our study on the 5′-part of the gene fragment between -1844 and -1768. First we tested, in transient transfections, a mutation in the putative E-box (E-boxm, Table 2). The results are shown in Figure 1B; mutation of the E-box site partially decreased the glucocorticoid stimulation, but the inhibition by insulin was still present.

Identification of HNF-3 and NFI binding sites by gel-shift retardation assays

We next focused on the region which shows sequence similarity to the HNF-3 and NFI sites and the IRS/IREs. Two probes (IIIb and IIIc, Figure 3) were designed, based on the sequence similarities between the *cAspAT* promoter, HNF-3 and NFI consensus sites and the IRS derived from other gene promoters. Indeed, five sequences bearing sequence similarities to the IRS/IREs described in the promoters of the *PEPCK*, *IGFBP-1*, *TAT* and *G6Pase* genes [12] were found in this region of the *cAspAT* promoter (Figure 3). Probe IIIb (-1817 to -1784) included IREs a, b and c, a GRE-consensus half-site (1/2 GRE) and two overlapping sequences with similarity to the HNF-3 consensus-binding site. Probe IIIc (-1800 to -1775) included IREs c and d, the GRE half-site and a perfect half-site for NFI. Gel-shift experiments using probe IIIb revealed a large retarded band

Table 2 Probes and oligonucleotides used for the electrophoretic-mobility-shift assays and transfection experiments

For the *cAspAT* sequences, the wild-type is shown above with the consensus motif that binds a transcription factor underlined. The mutated sequence is shown below with the mutation in bold characters. In each case, only the 5′ to 3′ strand is written.

Name	Sequence
Probe I	CATGGCATTGTTGCCTAACGCTGTGAA
Probe II	TGGGAATGACGCAGAGAGTGACCA
Probe IIIa	CTGAACGGAGCACATGCGCACACACAGAGAAA
Probe IIIb	GCACACACAGAGAAATATTTATTTGTTCTTAGCC
Probe IIIc	TTTATTTGTTCTTAGCCAAACCGACA
Probe IIIcm	TTTATTTGTTCTTAGCC**GCGT**CGACA
−1830 to −1801	CGGAGCACATGCGCACACACAGAGAAATAT
E-Boxm	CGGAG**TC**CA**GA**CGCACACACAGAGAAATAT
−1800 to −1771	TTATTTGTTCTTAGCCAAACCGACAGTGTT
GREm	TTATTT**AC**T**A**TTAGCCAAACCGACAGTGTT
−1815 to −1785	AGAGAAATATTTATTTGTTCTTAGCCAAAC
IREam	AGAG**CCC**TATTTATTTGTTCTTAGCCAAAC
−1815 to −1785	AGAGAAATATTTATTTGTTCTTAGCCAAAC
IREbm	AGAGAAATA**GGG**ATTTGTTCTTAGCCAAAC
−1815 to −1785	AGAGAAATATTTATTTGTTCTTAGCCAAAC
IREcm	AGAGAAATATTT**CG**TTGTTCTTAGCCAAAC
−1795 to −1765	TGTTCTTAGCCAAACCGACAGTGTTTAGGT
IREdm	TGTTCTTAGCC**GCGT**CGACAGTGTTTAGGT
−1808 to −1765	AGAAATATTTATTTGTTCTTAGCCAAACCGACAGTGTTTAGGT
IREcm/dm	AGAAATATTT**CG**TTGTTCTTAGCC**GCGT**CGACAGTGTTTAGGT
AlbD, C/EBP oligonucleotide	AAAGATGGTATGATTTTGTAATGGGGTAGGA
Collagenase AP-1oligonucleotide	CTAGTGATGAGTCAGCCGG
T-antigen AP-1 oligonucleotide	CGAGGAAGTGACTAACTGAGCACAG
Adenovirus E-box oligonucleotide	TGTAGGCCACGTGACCGGGTGTT
TTR, HNF-3 oligonucleotide	TGACTAAGTCAATAATCAGAAT

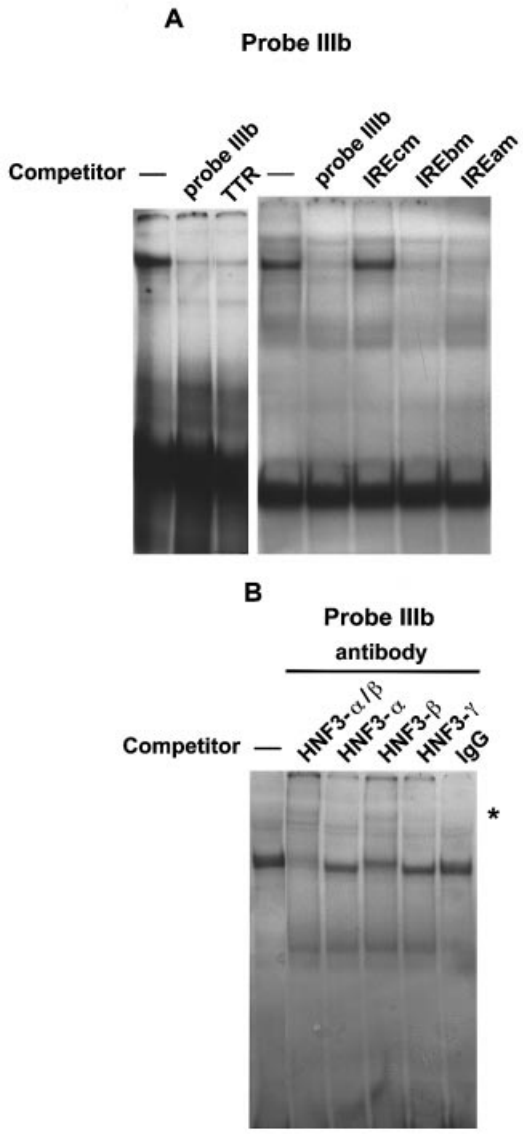

Figure 5 Analysis of protein binding to the −1817 to −1784 fragment of the *cAspAT* gene promoter by electrophoretic-mobility-shift-assay experiments

(**A**) Labelled probe IIIb (−1817 to −1784) was incubated with H4IIEC3 nuclear extracts (lanes 1 and 4) alone or in the presence of a 100-fold molar excess of the indicated unlabelled competitors: probe IIIb (lanes 2 and 5), HNF-3 binding site from the transthyretin (TTR) gene promoter (lane 3) or probe IIIb mutated in IREc (IREcm, lane 6), IREb (IREbm, lane 7) or IREa (IREam, lane 8). (**B**) Probe IIIb was incubated with H4IIEC3 nuclear extracts alone (lane 1) or with antibodies directed against the following HNF-3s: HNF-3α (lane 3), HNF-3β (lane 4), HNF-3α + HNF-3β (lane 2), HNF-3γ (lane 5) or preimmune IgG (lane 6). The supershifted band with anti-HNF-3β antibodies is indicated with an asterisk.

which could consist of several complexes (Figure 5A, lanes 1 and 4). This band was completely displaced by an excess of unlabelled oligonucleotide (lanes 2 and 5) as well as by an oligonucleotide corresponding to the HNF-3 site of the transthyretin promoter [14] (lane 3). Three oligonucleotides similar to probe IIIb, containing mutations in either the IRE a, b or c (Table 2) were used in competition experiments (Figure 5A). Mutation in the IREc (IREcm, lane 6) but not in the IREa or b sites (IREam, IREbm, lanes 8 and 9) prevented displacement of the radioactive probe from the proteins. Thus the formation of the complexes was dependent on the integrity of the IREc. Antibodies directed against various isoforms of the HNF-3 transcription factor (α, β and γ) were used in supershift experiments (Figure 5B). The antibody directed against HNF-3β led to the displacement of the fast migrating band of the complex and to the appearance of a super-shifted band (lane 4), whereas the anti-HNF-3α (lane 3) or γ (lane 5) antibodies were able to displace the slow migrating band. There are two putative overlapping HNF-3 sites in the probe IIIb sequence: one covers mainly IREb and c and the other covers IREc and the 1/2 GRE. The former is consistent with the presence of a DNase I hypersensitive site at −1799 (Figure 2). The results observed with the antibodies are very similar to results found in the gel-shift experiments using a TAT oligonucleotide and a nuclear extract from the FTO2B-hepatoma cells [34]. The fact that a clear super-shift is observed only with the anti-HNF-3β antibody is in agreement with the fact that HNF-3β is the predominant member of the HNF-3 family in H4IIEC3 cells [35,36]. When antibodies against both HNF-3α and β were used together, all of the major bands decreased (lane 2), suggesting that complexes with different isoforms of HNF-3 were present.

Probe IIIc, spanning IREs c and d, the 1/2 GRE, the HNF-3 and the NFI sites, was able to form a set of three specific retarded bands (Figure 6A, lane 2). All the complexes were competed for by an excess of the unlabelled probe (lane 1). When the antibody directed against HNF-3β was used, the major, fastest migrating, complex (arrow) was largely displaced and a faint super-shifted

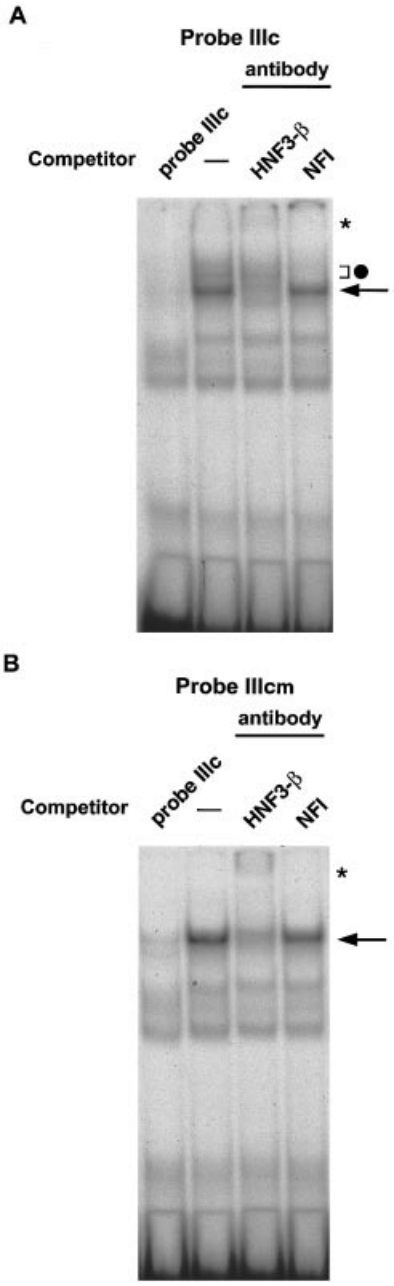

Figure 6 Analysis of protein binding to the −1800 to −1775 fragment of the *cAspAT* gene promoter by electrophoretic-mobility-shift-assay experiments

(**A**) Labelled probe IIIc (−1800 to −1775) was incubated with H4IIEC3 nuclear extracts alone (lane 2), in the presence of a 100-fold molar excess of unlabelled probe IIIc (lane 1) or with antibodies directed against HNF-3β (lane 3) or against NFI (lane 4). The arrow and the dot represent respectively the HNF-3 and the two NFI complexes. The supershifted band with anti-HNF-3β antibodies is indicated with an asterisk. (**B**) Probe IIIc mutated in the IREd site (IIIcm) was incubated with H4IIEC3 nuclear extracts alone (lane 2), in the presence of a 100-fold molar excess of unlabelled probe IIIc (lane 1) or with antibodies directed against HNF-3β (lane 3) or against NFI (lane 4). The supershifted band with anti-HNF-3β antibodies is indicated with an asterisk.

band appeared (lane 3, asterisk). The two, less abundant, slow-migrating complexes (dot) disappeared when an anti-NFI antibody was used (lane 4). When probe IIIc was mutated (probe IIIcm) to inactivate the NFI site (IREdm, Table 2), it could only bind the fastest migrating band (corresponding to HNF-3-like proteins) (Figure 6B, lane 2). Anti-HNF-3β antibodies still displaced most of this abundant band as a supershifted band (lane 3, asterisk), whereas anti-NFI antibodies did not change the migration pattern (lane 4). These results confirm that the mutation inactivated the NFI-binding site. Binding studies using a probe containing IREe did not reveal strong complex formation with this sequence, suggesting that it is a poor target for nuclear proteins, at least under the conditions used here (results not shown).

Insulin responsive elements are necessary for the hormonal regulation of the *cAspAT* gene

Fragments of the −1844 to −1702 *cAspAT* gene promoter bearing mutations in the IRE or GRE sequences (Table 2) were placed in front of the *Tk* promoter driving the expression of the *CAT* gene, and the resulting constructs were transiently transfected into H4IIEC3 cells. Both the IREcm and the GREm mutated sequences responded less efficiently to dexamethasone (2–3-fold induction in the mutants versus 5–6-fold for the wild-type), but insulin was still inhibitory (Figure 7A). The impaired stimulation by the glucocorticoid could be explained by the fact that HNF-3 binding in the vicinity of the GRE is, indeed, necessary for a maximal hormonal effect. HNF-3 may act as an accessory factor for the glucocorticoid receptor, as has been shown for the *TAT*, *PEPCK* and *IGFBP-1* genes [29,34,37]. Mutation of IREd alone (IREdm), which overlaps the NFI-conserved half-site, did not significantly modify the response to insulin, but the stimulation by glucocorticoids was slightly blunted (4-fold induction). Mutations in both IRE c and d (IREcm/dm) led to a decreased glucocorticoid effect (2–3-fold induction), whereas insulin became completely ineffective. In order to focus on the insulin effect, the results were expressed as the percent decrease by insulin of the glucocorticoid stimulatory effect. Using this presentation, it is clear that the IREcm/dm mutant completely lost the negative insulin effect (Figure 7B).

DISCUSSION

Although the negative regulation of gene expression by insulin has been widely studied, the transcription factor(s) responsible for the insulin effect remain elusive. Several transcription factors, such as members of the HNF-3 and C/EBP families, bind to the IRS/IRE in the genes encoding PEPCK, IGFBP-1 and TAT [8]. However, their binding is not correlated with insulin inhibition, suggesting that either an unknown transcription factor interferes with the binding of HNF-3 or a complex involving several factors is required for this effect [8,38]. In the present paper, we show that the region of the *cAspAT* gene, which is responsible for the dual, antagonistic effects of glucocorticoids and insulin is located within a 142 bp region (−1844 to −1702).

Glucocorticoid induction

The −1844 to −1702 fragment of the *cAspAT* gene promoter confers sensitivity to glucocorticoids when it is introduced upstream of the basal *Tk* promoter driving the CAT activity (4–6-fold induction). Careful study of the sequence reveals only one consensus half-site of the GRE. Conserved bases important for the second half-site are not present with the correct spacing in the sequence adjacent to this half-site. Mutation of the half-site (GREm) leads only to a partial loss of the sensitivity to glucocorticoids (2–3-fold induction). The effect of glucocorticoids is also decreased by mutations in the neighbouring HNF-3 site (IREc), as well as in the more distant E-box site. It is, therefore,

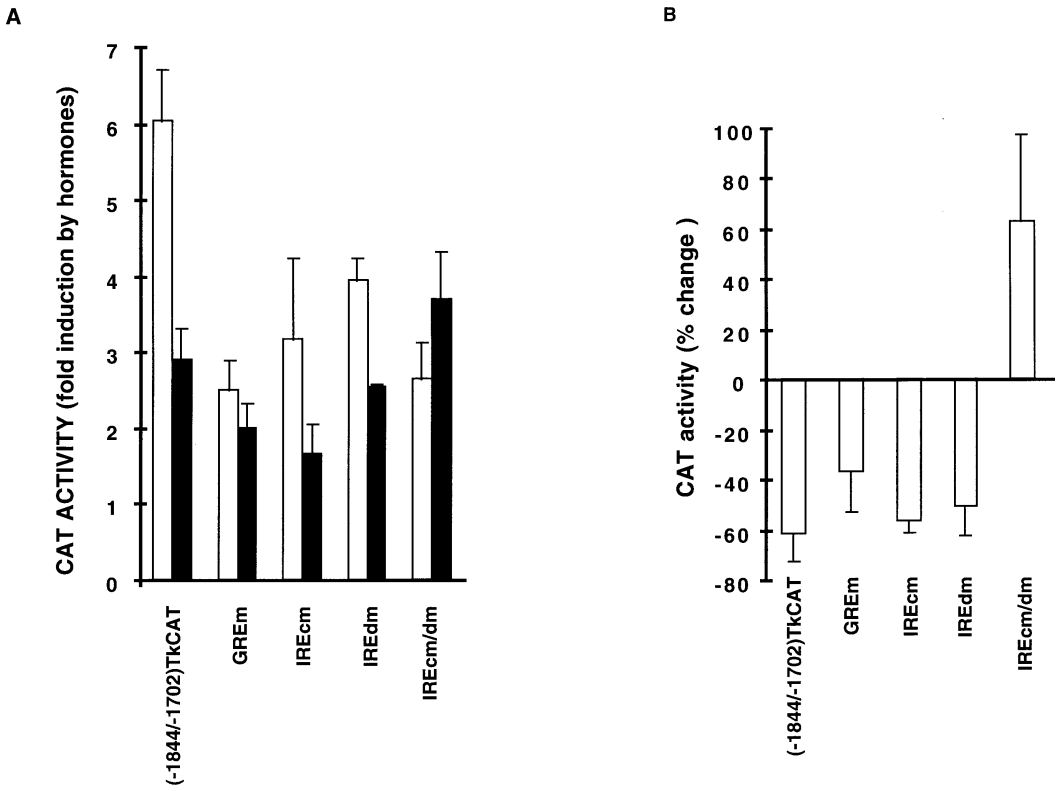

Figure 7 Analysis of the effect of mutations in the −1808 to −1770 fragment of the *cAspAT* gene on the regulation of the gene activity by glucocorticoids and insulin

Mutations in the IREs and in the GRE half-site were made in the −1844 to −1702 fragment and introduced in front of the *Tk* promoter driving the expression of the *CAT* gene. Regulation by the hormones was studied after transfection into H4IIEC3 cells. (**A**) The CAT activities, expressed as the fold-stimulation by hormones above the basal level, are the mean ± S.E.M. of 3 to 14 independent experiments, each in triplicate. Dexamethasone (□), dexamethasone plus insulin (■). To focus on the intensity of the negative insulin effect, another representation of the data is shown in (**B**). In this case, the percent inhibition by insulin of the increase in CAT activity elicited by glucocorticoid is shown for the various constructs. It corresponds to $\{1-[(DI-B)/(D-B)]\} \times 100$ where B, D and DI are respectively the basal, dexamethasone and dexamethasone plus insulin CAT activities in arbitrary units.

likely that the glucocorticoid effect is mediated by a large complex, possibly including the glucocorticoid receptor and other nuclear proteins. This is supported by recent studies showing that the glucocorticoid receptor can interact with several nuclear proteins on regulatory sequences, thus mediating a hormonal effect even in the absence of a canonical GRE [39–41].

Insulin action

In several genes negatively regulated by insulin (*PEPCK*, *IGFBP-1*, *TAT*, *G6Pase*), the IRE/IRS contains a common core motif [T(G/A)TTT(T/G)(G/T)] [12]. However, the actual factor mediating insulin-inhibitory action (named insulin-responsive factor [37]) is still unknown. In 3T3-L1 adipocytes, NFI was shown to mediate repression of the *GLUT4* promoter by insulin [42]. Modifications in the phosphorylation status of the NFI proteins, occurring after insulin treatment, may be a potential mechanism [42]. However, an unknown protein also binds to the IRE, which displays no similarity to other IREs [43]. In the *cAspAT* gene promoter, deletion studies led us to focus our studies on the central part of the fragment (−1818 to −1755) which shows sequence similarity to the IRS as well as to HNF-3 and NFI binding sites.

Dramatic effects on the response of the *cAspAT* gene to insulin were obtained only when several sites were mutated at once (Figure 7). Indeed, mutation of either IRE c or d alone partially reduced the dexamethasone stimulation, with no change in the inhibition by insulin. In contrast, the inhibitory effect of insulin disappeared when a double IREc/d-mutated sequence was transfected into the H4IIEC3 cells, whereas there was only a partial decrease in the glucocorticoid effect. Thus the sequences that are necessary for the effect of insulin are not required for a complete glucocorticoid effect. IREc overlaps the HNF-3 binding sequence whereas IREd partially overlaps the NFI half-site. Mutation of IREd prevents the formation of the two slow migrating complexes displaced by anti-NFI antibodies. However, a concomitant mutation in IREc is also required to prevent the insulin effect. Indeed, the −1818 to −1789 bp region of the *cAspAT* gene shows similarity to the complex regions found in the promoters of several genes regulated positively by glucocorticoids and negatively by insulin. The IRS is located in the vicinity of the element necessary for full glucocorticoid induction and the HNF-3 transcription factor seems necessary for this effect. The HNF-3-like motif in the *cAspAT* gene contains two adjacent sequences bearing a 6 out of 7 or 7 out of 7 bp identity with the 7 bp core motif T(G/A)TTT(T/G)(G/T) found in the *PEPCK* and *IGFBP-1* IRS (see Table 1).

In conclusion, this paper describes the identification of a complex IRE in the rat *cAspAT* gene promoter. It requires the binding of several proteins, which are members of the HNF-3 and NFI families to mediate insulin signalling. However, additional studies are required to determine whether other nuclear

proteins are involved and to determine the mechanism of signalling through these complex IREs. Recently, several studies have shown that FKHR, a forkhead-transcription factor, may be involved in the insulin regulation of *IGFBP-1* promoter activity [44,45], and that insulin stimulates its phosphorylation through the phosphatidylinositol-3 kinase pathway [46]. Protein kinase B phosphorylates the forkhead-transcription factor as well as other proteins of the forkhead family [46–49]. This phosphorylation leads to inactivation of the transcription factor [50], possibly through its export out of the nucleus [48]. It is striking that, among the genes which are regulated similarly by glucocorticoids and insulin, several encode enzymes of the gluconeogenic pathway (PEPCK, G6Pase, cAspAT). It is tempting to hypothesize that a mutation inactivating the transcription factor involved in insulin-gene regulation might lead to a dysfunction of hepatic-glucose production, a component of the multifactorial disease, type II non-insulin-dependent diabetes.

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