Identification and characterization of *cis*-acting elements conferring insulin responsiveness on hamster cholesterol 7α-hydroxylase gene promoter

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Bile acid biosynthesis occurs primarily through a pathway initiated by the 7α-hydroxylation of cholesterol, catalysed by cholesterol 7α-hydroxylase (encoded by *CYP7A1*). Insulin down-regulates *CYP7A1* transcription. The aim of our study was to characterize the sequences of hamster *CYP7A1* promoter, mediating the response to insulin. We therefore performed transient transfection assays with *CYP7A1* promoter/luciferase chimaeras mutated at putative response elements and studied protein–DNA interactions by means of gel electrophoresis mobility-shift assay. Here we show that two sequences confer insulin responsiveness on hamster *CYP7A1* promoter: a canonical insulin response sequence TGTTTTG overlapping a binding site for hepatocyte nuclear factor 3 (HNF-3) (at nt −235 to −224) and a binding site for HNF-4 at nt −203 to −191. In particular we show that the hamster *CYP7A1* insulin response sequence is part of a complex unit involved in specific interactions with multiple transcription factors such as members of the HNF-3 family; this region does not bind very strongly to HNF-3 and as a consequence partly contributes to the transactivation of the gene. Another sequence located at nt −138 to −128 binds to HNF-3 and is involved in the tissue-specific regulation of hamster *CYP7A1*. The sequence at nt −203 to −191 is not only essential for insulin effect but also has a major role in the liver-specific expression of *CYP7A1*; it is the target of HNF-4. Therefore the binding sites for liver-enriched factors, present in the hamster *CYP7A1* proximal promoter in close vicinity and conserved between species, constitute a regulatory unit important for basal hepatic expression and tissue restriction of the action of hormones such as insulin.

Key words: bile acids, cholesterol metabolism, hepatocyte nuclear factors, liver-specific genes.

INTRODUCTION

Insulin performs a central role in homoeostasis and in most cases regulates the levels of specific proteins by acting at the level of gene transcription [1].

The binding of insulin to its receptors and the consequent autophosphorylation of tyrosine residues on the β subunit are the initial events in the hormone action. Distal to receptor activation, insulin activates cytoplasmic protein serine/threonine kinases by using multiple signal-transduction pathways, such as the MAPK (mitogen-activated protein kinase) cascade [2] and the PI-3K (phosphoinositide 3-kinase) pathway [3]. The effects of insulin on gene expression seem to be very complex and have not been fully elucidated. It has been reported that insulin can act as a modulator of the hepatocyte nuclear factor (HNF) network [4]. However, the mechanisms by which insulin can induce or repress liver-restricted genes still need to be unravelled. Several genes involved in carbohydrate metabolism are negatively regulated by insulin through members of the C/EBP (CAAT-enhancer-binding protein) and HNF-3 families.

The metabolic effects of insulin are highly compromised in the diabetic state, in particular in non-insulin-dependent diabetes mellitus and the associated insulin resistance. In addition to lipid and lipoprotein abnormalities ([5], and references therein), an underestimated trait of diabetes is a complex and still not well elucidated pattern of disorders in bile acid metabolism. For example, an increase in bile acid pool size and excretion is found in humans with untreated diabetes [6] and in experimental animals with insulin deficiency [7–10].

The hepatic conversion of cholesterol into bile acids is physiologically relevant in that it represents one of the main methods of cholesterol elimination from the body. Bile acid biosynthesis occurs primarily through a 'classic' pathway initiated by the 7α-hydroxylation of cholesterol, although the role of an 'alternative' route has since emerged [11]. Cholesterol 7α-hydroxylase (encoded by *CYP7A1*) catalyses the initial, rate-limiting, step in the classic pathway [12]. The enzyme is expressed selectively in the liver and is regulated, primarily at the level of gene transcription, by bile acids returning to the liver via enterohepatic circulation [13,14], cholesterol [15] and hormones such as insulin [16–18] and glucocorticoids [17–19].

Evidence from studies performed on transgenic mice carrying a disrupted *CYP7A1* locus implies that the gene is down-regulated in the foetal period and then switched on immediately after birth [20]. In this regard, spatial and temporal regulation of *CYP7A1* expression during rat liver development has been confirmed recently [21]. Hormonal changes occurring after birth, namely a decrease in insulin levels and an increase in the concentrations of glucagon and glucocorticoids, have been invoked as a major cause of the onset of another liver-specific gene encoding tyrosine

Abbreviations used: AP-1, activator protein 1; *CYP7A1*, cholesterol 7α-hydroxylase gene; HNF, hepatocyte nuclear factor; IRS, insulin response sequence; PEPCK, phosphoenolpyruvate carboxykinase; TTR, transthyretin.

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aminotransferase [22]. These events might also be critical for the onset of expression of other genes, for example *CYP7A1*, which has been demonstrated to be required for normal postnatal development [20].

In previous studies we reported that insulin and glucocorticoids regulate *CYP7A1* transcription in opposite directions by interacting with the proximal promoter of the rat [17], human [18] and hamster [23] genes, although putative responsive sequences mediating hormone action seem to be located differently in the three promoters [23].

Insulin-mediated down-regulation of *CYP7A1* transcription activity would explain the increase in the bile acid pool and excretion in humans with untreated type I diabetes mellitus [6] and in experimental animals with insulin deficiency [7–10].

The aim of the present study was the functional characterization of sequences located in the hamster *CYP7A1* promoter in response to insulin. Here we show that two sequences can mediate insulin action on the hamster *CYP7A1* promoter.

EXPERIMENTAL

Construction of plasmids

The wild-type plasmid p-445 spanning the region −445 to −10 nt upstream of the first ATG codon was made by PCR [24] with the upstream primer 5′-AGGGTACCTAACAACACAAACA-ACC-3′ bearing a *Kpn*I restriction site (underlined) and the downstream primer 5′-AGATGACTCGAGCAGGAAAACT-CCCAAG-3′ bearing a *Xho*I restriction site and cloned upstream of the luciferase gene in pGL2 Basic (Promega, Madison, WI, U.S.A.) cut with *Kpn*I and *Xho*I.

The mutants of p-445 used in the present study were generated by PCR-based mutagenesis [25] and cloned into pGL2 Basic vector containing the luciferase gene. Schematic representations of the mutated promoters are shown in Figures 2 and 5 and Tables 1 and 3, in which bold type indicates responsive elements and lower case the mutated nucleotides.

Plasmids were verified by sequencing with the Sequenase kit (Amersham Italia Srl) and purified with Nucleobond (Macherey-Nagel, Düren, Germany) columns in accordance with the manufacturer's instructions.

The HNF-3α expression vector, pCMV-HNF-3α (in which CMV stands for cytomegalovirus), was made by restriction digestions of pL-H3α (a gift from Dr William Chen, The Rockefeller University, New York, NY, U.S.A.) with *Bam*HI and subcloning the resulting 1.6 kb HNF-3α cDNA into pcDNA3 cut with *Bam*HI. pCMV-HNF-4 was made as described previously [26].

Cell culture and transient transfection assays

HepG2 were purchased from A. T. C. C. (Manassas, VA, U.S.A.) and grown in 12-well cluster plates as described previously [23,27].

Confluent cells were transfected by the calcium phosphate precipitation technique [17,23,28]. pCMVβ expression vector for bacterial β-galactosidase (Clontech, Palo Alto, CA, U.S.A.) was used to normalize for transfection efficiency. When indicated, cells were co-transfected with the expression vector for HNF-3α and HNF-4. Luciferase and β-galactosidase activities were assayed as described [29].

Electrophoretic mobility-shift assay

Nuclear extracts were prepared from HepG2 cells by the method described by Dent and Latchman [30]. HNF-3α was synthesized *in vitro* by using the TNT® Coupled Reticulocyte Lysate System (Promega) in accordance with the manufacturer's instructions. Unprogrammed reactions were performed with pcDNA3 empty vector. The oligonucleotides used in the assays are shown in Figures 3 (top panel), 4 (top panel) and 6 (upper panel). They bore either GATC or CTAG overhangs. Double-stranded wild-type oligonucleotides were labelled by filling in 5′ extensions with the Klenow fragment of *Escherichia coli* DNA polymerase I with [α-^{32}P]dCTP (Amersham Italia Srl). Protein binding was performed in binding buffer containing 12 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 15% (v/v) glycerol and 2 μg of poly(dI-dC)·(dI-dC), as described [26]. Labelled double-stranded oligonucleotides (100000 c.p.m.) were added and incubated for 15 min at room temperature in a final volume of 20 μl.

Competition experiments were performed with a 10–100-fold molar excess of the unlabelled double-stranded oligonucleotides shown in Figures 3 (top panel), 4 (top panel) and 6 (upper panel), which were added together with the labelled probes.

The reactions products were fractionated on a 4% or 5% (w/v) non-denaturing polyacrylamide gel in 0.5 × TBE (45 mM Tris/borate/1 mM EDTA) at 120 V for 2 h at room temperature. Gels were dried and exposed to X-ray films for an appropriate duration.

Statistical analysis

Results are presented as means ± S. D. for experiments performed in triplicate. Statistical analysis was performed with an unpaired Student's *t* test using Excel 97 (Microsoft Corporation, Redmonton, WA, U.S.A.).

RESULTS

Functional characterization of a putative insulin response sequence (IRS)

The functional activity of a putative IRS (TGTTTTG) [31] located between nt −228 and nt −222 in hamster *CYP7A1* promoter (Figure 1) was investigated by means of transient transfection assays. Site-directed mutagenesis of the *CYP7A1* promoter was performed either in the putative IRS (p-445hm1) or downstream of it (p-445hm3) (Table 1). As expected, when the luciferase gene was driven by the wild-type *CYP7A1* promoter, the transcription activity was significantly inhibited in the pres-

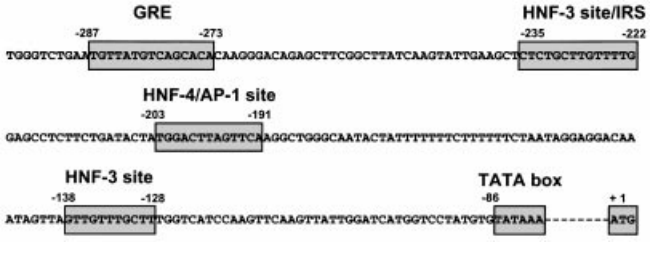

Figure 1 Organization of the hamster *CYP7A1* proximal promoter: schematic representation of *CYP7A1* proximal promoter

Putative responsive sequences are boxed. Numbering refers to the first ATG codon. Abbreviation: GRE, glucocorticoid response element. See also Table 2.

Table 1 Mapping of a negative IRS in the hamster CYP7A promoter at nt −228/−222 by site-directed mutagenesis

Cells grown in 12-well plates were co-transfected with plasmids carrying the wild-type or mutated promoter fused to the luciferase gene (2.5 μg/well) and pCMVβ expression vector (100 ng/well). Transfected cells were treated with 1 μM insulin in serum-free medium for 40 h and then harvested for enzyme activities determination. Results are representative of at least three independent experiments. **Bold face** indicates CYP7A1 responsive elements and lower-case indicates mutated nucleotides. 'Normalized transcriptional activity' represents the luciferase activity corrected for the β-galactosidase activity and is expressed as 'relative light units'/A_{420}. A single asterisk (*) indicates statistical significance at $P < 0.05$ versus wild-type promoter.

Plasmid			Normalized transcriptional activity		
			Control	1 μM insulin	Statistical significance
Wild-type p-445	**p-445wt**	-235 **CTCTGCTTGTTTTG**GAGCC -217 TATA luciferase	305·9±15·63	205·5±10·52	$P<0·005$
p-445hm1	**p-445hm1**	**CTCTGCTTt**gggg**t**GAGCC TATA luciferase	136·1±8·46*	125·4±11·82	n.s.
p-445hm3	**p-445hm3**	**CTCTGCTTGTTTTG**tctaa TATA luciferase	200·2±5·84*	125·4±13·27	$P<0·005$

Table 2 Consensus sequences of negative IRS and binding sites of known transcription factors

Response element	Consensus sequence	Reference
IRS	T(G/A)TTTTG	[31]
HNF-3-binding site	A(A/T)TRTT(G/T)RYRY	[33]
HNF-4-binding site	AGGTCA N AGGTCA	[35]
AP-1-binding site	TGA N TCA	[36]

ence of 1 μM insulin (33% inhibition) (Table 1). This concentration was chosen in accordance with previous studies [17,18] to achieve a maximal inhibition of promoter activity. HepG2 cultures transfected with a plasmid carrying the mutated IRS (p-445hm1) lost their responsiveness to insulin, indicating that this sequence can mediate the effect of the hormone. In contrast, mutation of the 3′ flanking nucleotides (p-445hm3) did not affect the hormone-mediated down-regulation of transcription (37% inhibition). Moreover, the comparison of normalized activities in control transfected cells showed that disruption of the IRS also affected the constitutive promoter activity, because the basal transcription rate of p-445hm1 mutant was 44% of that of the wild-type promoter. This finding therefore suggested that nuclear factors expressed by HepG2 cells in the absence of exogenous stimuli transactivate the promoter by interacting with this sequence. It should be mentioned that p-445hm3 mutant also displayed a lower activity (65%) than the native promoter. This could be due to several factors, for example to a lower affinity of this mutant for another transcription factor that contributes to the basal activity. It has been demonstrated that the distal IRS present in the promoter of phosphoenolpyruvate carboxykinase (PEPCK) can interact with transcription factors such as members of the CCAAT-enhancer-binding protein and HNF-3 families [32]. The region surrounding the core IRS of hamster *CYP7A1* promoter (nt −235 to −224, shown in Figure 1) displays a high similarity to the consensus sequence of the binding site for HNF-3 (8/11 [33] or 9/12 [32]) (Table 2). This site is extensively disrupted in mutant p-445hm1, but not in mutant p-445hm3. To assess whether HNF-3 can transactivate the hamster *CYP7A1* gene through this sequence, co-transfection experiments were performed, overexpressing HNF-3α in HepG2 cultures. The

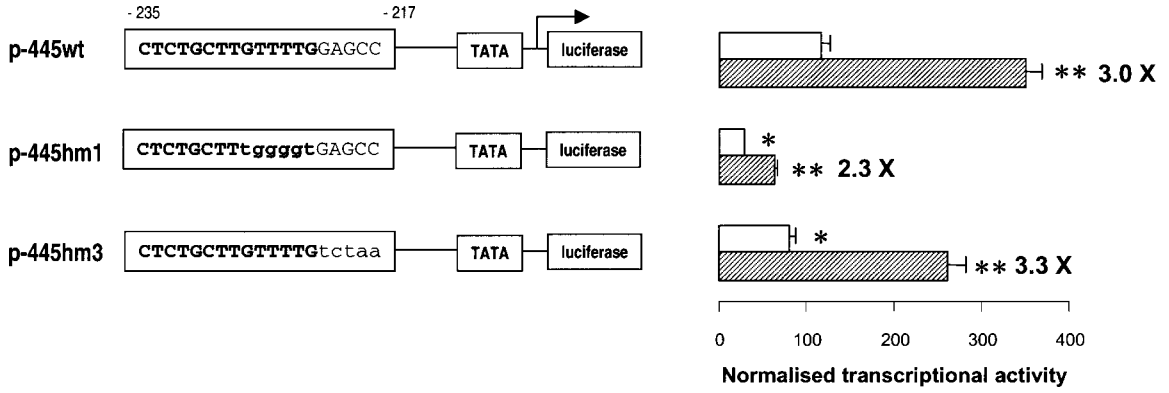

Figure 2 Functional analysis of hamster *CYP7A* IRS

Transcriptional activity of the wild-type (wt) and mutated promoters in HepG2 cells co-transfected with pcDNA3 empty vector (350 ng) (open bars) and pcDNA3 HNF-3α expression vector (350 ng) (hatched bars). Luciferase activities were normalized to β-galactosidase activity. Results are representative of at least three independent experiments. *$P < 0.05$ compared with wild-type promoter; **$P < 0.005$ compared with pcDNA3 co-transfected cells.

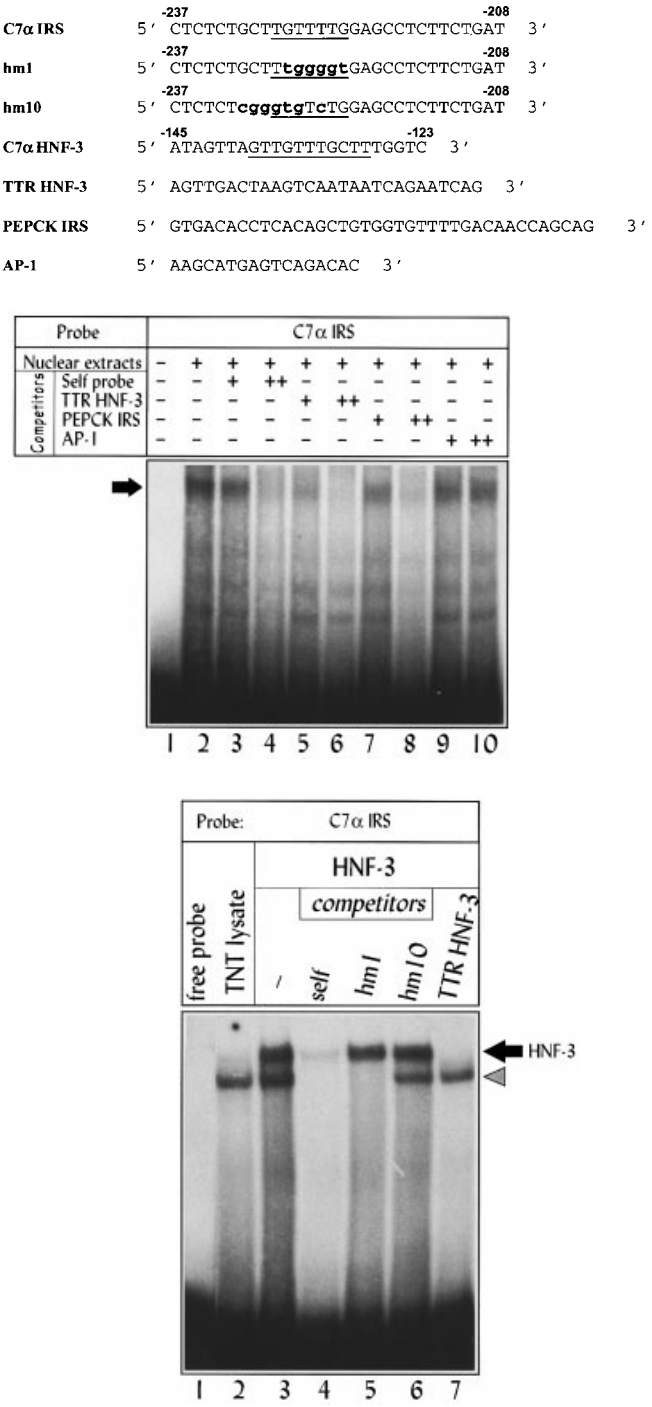

Figure 3 Analysis of nuclear protein binding to hamster *CYP7A1* IRS

Top panel: sequences of the competitors used: C7α IRS, wild-type *CYP7A1* promoter sequence; hm1, mutant hm1 of *CYP7A1* promoter sequence; hm10, mutant hm10 of *CYP7A1* promoter sequence; C7α HNF-3, wild-type *CYP7A1* promoter sequence; PEPCK IRS, IRS of the PEPCK promoter; TTR HNF-3, HNF-3-binding site of the TTR promoter; AP-1, AP-1-binding site of the collagenase promoter. Putative consensus sequences in hamster *CYP7A1* are underlined. Bold lower case indicates mutated nucleotides. Middle panel: competition band-shift assays with ^{32}P-labelled probe corresponding to hamster *CYP7A1* IRS at nt −235 to −222 incubated with nuclear extracts from HepG2 cells. Unlabelled competitors were in 10-fold (+) and 100-fold (++) molar excess. Bottom panel: competition band-shift assays with ^{32}P-labelled probe corresponding to hamster *CYP7A1* IRS at nt −235 to −222 incubated with HNF-3α synthesized *in vitro* (lanes 3–7). Lane 2, unprogrammed reticulocyte lysate. Unlabelled competitors were in 100-fold molar excess. The black arrow indicates the complex with HNF-3; the grey arrowhead indicates an unidentified complex.

wild-type promoter and p-445hm3 mutant were stimulated by approx. 3-fold, whereas the mutated promoter p-445hm1 was stimulated to a smaller extent (2.3-fold) (Figure 2). The modest loss of responsiveness can be ascribed to the presence of additional binding sites for HNF-3, in particular to the sequence located more proximally at nt −139 to −128 (Figure 1) that is similar to that characterized in the human *CYP7A1* promoter [34]. Disruption of this element negatively affected basal promoter activity, which accounted for 38.95±6.42% of the wild-type promoter (results not shown).

Interactions between the putative IRS/HNF-3-binding site (nt −235 to −222) and nuclear proteins were investigated by means of gel retardation assays. Nuclear extracts from HepG2 cells were incubated with the radiolabelled wild-type probe and competition experiments were performed with the competitors shown in Figure 3 (top panel). In particular, the radiolabelled probe gave rise to a protein–DNA complex (Figure 3, middle panel, lane 2) that was specifically competed for by excess unlabelled self probe (Figure 3, middle panel, lanes 3 and 4). The complex was also competed for by an excess of double-stranded oligonucleotide containing the distal IRS of the gene for PEPCK (lanes 7 and 8) or the HNF-3-binding site of the transthyretin (TTR) promoter (lanes 5 and 6). No competition was observed with the activator protein 1 (AP-1)-binding site of the collagenase promoter, used as a non-specific competitor (lanes 9 and 10). To investigate further the identity of the protein interacting with the IRS at nt −235 to −222, we performed gel mobility-shift assays with HNF-3α synthesized *in vitro*. As shown in Figure 3 (bottom panel), the sequence under investigation bound specifically to HNF-3α (lane 3), but also to an unknown factor present in reticulocyte lysates (compare lanes 3 and 2). The complex with HNF-3α was competed for by 100-fold excess of unlabelled probes bearing the self sequence (lane 4) and the HNF-3-binding site of the TTR promoter (lane 7), but not by the mutated probes (lanes 5 and 6). In contrast, the unknown band could have been due to interactions between an unidentified factor and the region flanking the sequence TGTTTTG, because the oligonucleotide hm1 (lane 5), but not the oligonucleotide hm10 (lane 6), competed out this complex.

In parallel, we characterized the second putative HNF-3-binding site located at nt −138 to −128 in the hamster *CYP7A1* promoter. As shown in Figure 4 (middle panel), this sequence interacted specifically with HNF-3α synthesized *in vitro* (Figure 4, middle panel, lane 4). A complex with a similar electrophoretic mobility was observed after incubation with nuclear extracts (Figure 4, middle panel, lane 2). Moreover, we compared the ability of several unlabelled double-stranded oligonucleotides to compete for this complex. The oligonucleotide containing hamster *CYP7A1* IRS (nt −235 to −222) competed less efficiently than the self unlabelled probe containing the proximal HNF-3-binding site at nt −138 to −128 (Figure 4, middle panel, compare lanes 6 and 5). The oligonucleotide containing the HNF-3 site of the TTR promoter was the most efficient competitor (Figure 4, middle panel, compare lane 7 with lanes 5 and 6). As expected, the double-stranded oligonucleotide bearing the mutated proximal binding site for HNF-3 (hm9) did not compete out the proximal promoter (Figure 4, middle panel, lane 8). These results suggest a hierarchy between HNF-3-binding sites present in hamster *CYP7A1* promoter, the proximal sequence (nt −138 to −128) being stronger than the distal one (nt −235 to −224). These observations were confirmed in a reciprocal experiment with the HNF-3 site of the TTR promoter as labelled probe and the two *CYP7A1* HNF3 sites as unlabelled competitors. The *CYP7A1* HNF3 oligonucleotide (nt −138 to −128) competed out the complex (Figure 4, bottom panel, lane 5) more

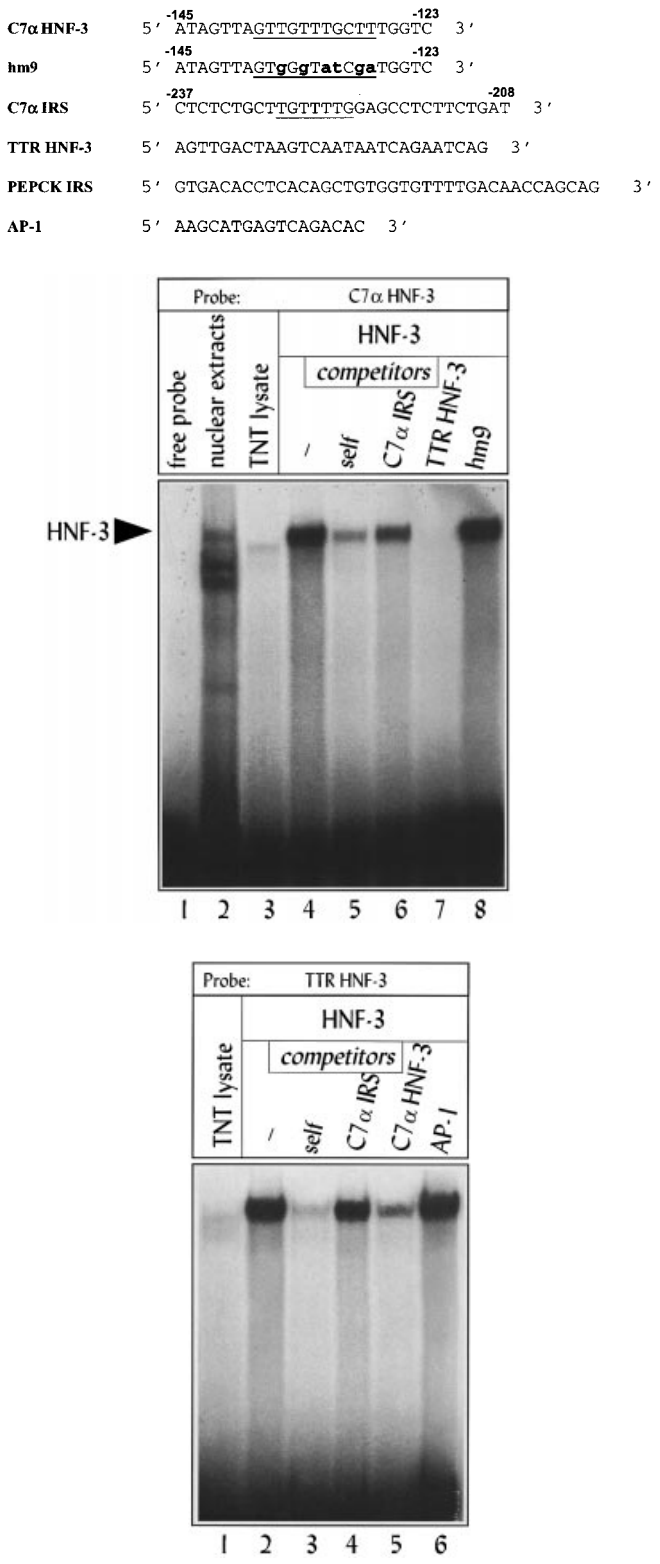

Figure 4 Analysis of nuclear protein binding to hamster *CYP7A1* proximal HNF-3 site

Top panel: sequences of the competitors used: C7α HNF-3, wild-type *CYP7A1* promoter sequence; hm9, mutant hm9 of *CYP7A1* promoter sequence; C7α IRS, wild-type *CYP7A1* promoter sequence; TTR HNF-3, HNF-3-binding site of the TTR promoter; AP-1, AP-1-binding site of the collagenase promoter. Putative consensus sequences in hamster *CYP7A1* are underlined. Bold lower case indicates mutated nucleotides. Middle panel: competition band-shift assays with ^{32}P-labelled probe corresponding to hamster *CYP7A1* IRS at nt −138 to −128 incubated with nuclear extracts from HepG2 cells (lane 2) or HNF-3α synthesized *in vitro* (lanes 4–8). Lane 3, unprogrammed reticulocyte lysate. Unlabelled competitors were in 100-fold molar excess. Bottom panel: competition band-shift assays with ^{32}P-labelled probe corresponding to the HNF-3-binding site of the TTR promoter incubated with HNF-3α synthesized *in vitro* (lanes 2–6). Lane 1, unprogrammed reticulocyte lysate. Unlabelled competitors were in 100-fold molar excess.

efficiently than the distal sequence at nt −235 to −224 (Figure 4, bottom panel, lane 4), which displaced the radioactive probe only slightly. This was probably due to the strong binding of HNF-3 to the TTR promoter, because 100-fold excess of the unlabelled self probe could not compete out the complex completely (Figure 4, bottom panel, lane 3). As expected, a double-stranded oligonucleotide containing an unrelated sequence (AP-1) did not displace the labelled probe (Figure 4, bottom panel, lane 6).

Taken together, our results show that insulin might down-regulate hamster *CYP7A1* through an IRS; this element is part of a complex unit involved in specific interactions with multiple transcription factors, among which are members of the HNF-3 family. A second HNF-3-binding site is present at nt −138 to −128 and contributes to the basal promoter activity.

Sequence from nt −203 to nt −191 is involved in insulin responsiveness

In the screening of hamster *CYP7A1* promoter, another region was tested for insulin responsiveness. As shown in Figure 1, the sequence between nt −203 and nt −191 is highly similar to the consensus of the binding site for HNF-4 [35]. The mutation of this promoter sequence of the hamster *CYP7A1* gene led to a complete loss of inhibition by insulin in mutant hm4, or even a slight stimulation with mutant hm5 (Table 3). Moreover, lower levels of basal transcription activity were observed in comparison with the wild-type promoter (less than 10%). This finding suggests that the region under investigation is the target of endogenous transcription factors expressed by HepG2 cells, which positively affect gene transcription. The relevance of this sequence in HNF-4-dependent transactivation was also confirmed in co-transfection experiments. As shown in Figure 5, wild-type hamster *CYP7A1* promoter was stimulated by increased levels of HNF-4 protein (3.3-fold stimulation). In contrast, disruption of the putative binding site not only prevented HNF-4-dependent transactivation, but also caused a slight, but statistically significant, decrease in transcription activity in response to HNF-4. In particular, the normalized activity dropped from 9.76 ± 1.19 to 3.73 ± 0.29, amounting to 60% inhibition, for p-445hm4, and from 16.32 ± 1.97 to 11.44 ± 0.2, amounting to 30% inhibition, for p-445hm5. This paradoxical observation might be due to a phenomenon of competition for factors (i.e. common mediators to the transcription initiation complex) present at limiting concentration, in comparison with excess unbound HNF-4. In addition, the mutants hm4 and hm5 retained their ability to respond to glucocorticoid hormones (results not shown), thus indicating that, despite the mutation of this site, the promoter was still able to respond to other extracellular stimuli. As a control, mutation of *CYP7A1* promoter in a different region (p-445hm3) did not impair the stimulatory effect of HNF-4.

A further analysis of the sequence under investigation revealed that the element ACT(T)AGT at position −200 to −194 might resemble a binding site for AP-1 [36]. This hypothesis was also supported by the observation that this putative AP-1-binding site is essential for the inhibitory effect exerted by phorbol esters on

Table 3 Effect of insulin on mutants in the region at nt -203/-191 of the hamster CYP7A promoter description (see Table 1)

Cells grown in 12-well plates were co-transfected with plasmids carrying the wild-type or mutated promoter fused to the luciferase gene (2.5 μg/well) and pCMVβ expression vector (100 ng/well). Transfected cells were treated with 1 μM insulin in serum-free medium for 40 h and then harvested for enzyme activities determination. Results are representative of at least three independent experiments. **Bold face** indicates CYP7A1 responsive elements and lower-case indicates mutated nucleotides. 'Normalized transcriptional activity' represents the luciferase activity corrected for the β-galactosidase activity and is expressed as 'relative light units/A_{420}. A single asterisk (*) indicates statistical significance at $P < 0.05$ versus wild-type promoter.

Plasmid			Normalized transcriptional activity		
			Control	1 μM insulin	Statistical significance
Wild-type p-445	**p-445wt**	-203 -191 TGGACTTAGTTCA — TATA — luciferase	305·9±15·63	205·5±10·52	P<0·005
p-445hm4	**p-445hm4**	ccaACTTAGTTCA — TATA — luciferase	20·36±0·39	18·15±4·47	n.s.
p-445hm5	**p-445hm5**	TGGcagcgaaTCA — TATA — luciferase	20·08±1·22	26·86±0·61	P<0·005

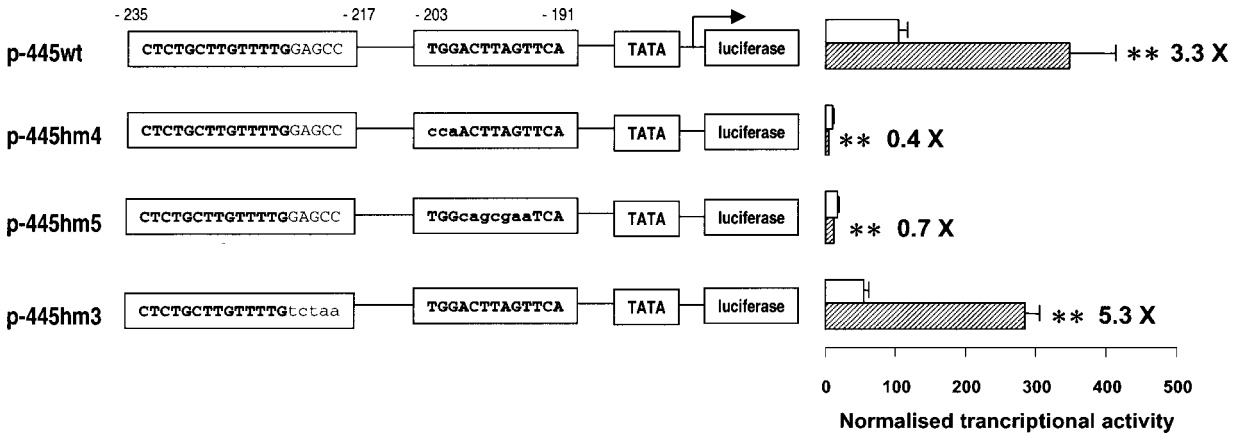

Figure 5 Functional analysis of hamster *CYP7A1* HNF-4/AP-1-binding site

Experimental conditions were as described in the legend to Figure 2. Transcription activity of the wild-type (wt) and mutated promoters in HepG2 cells co-transfected with pcDNA3 empty vector (350 ng) (open bars) and pcDNA3 HNF-4 expression vector (350 ng) (hatched bars). Luciferase activities were normalized to β-galactosidase activity. Results are representative of at least three independent experiments. $^{**}P < 0.005$ compared with pcDNA3 co-transfected cells.

the rat [27] and hamster promoters (E. De Fabiani, M. Crestani, M. Marrapodi, A. Pinelli, V. Golfieri and G. Galli, unpublished work). Therefore, to understand the molecular interactions involving the potential HNF-4/AP-1-binding site with nuclear proteins, gel retardation assays were performed with a radiolabelled oligonucleotide containing the wild-type sequence and the non-radioactive competitors reported in Figure 6 (upper panel). The radioactive probe containing the native sequence interacted with nuclear proteins, giving rise to several bands, but only the complex characterized by lower electrophoretic mobility was efficiently competed for by excess unlabelled self probe and not by the mutated one (Figure 6, lower panel, lanes 3 and 4, and 5 and 6, respectively). A similar competition was observed in the presence of excess oligonucleotide containing the HNF-4-binding site present in the promoter of the HNF-1 gene (Figure 6, lower panel, lanes 7 and 8). A competitor containing the AP-1-binding site of the collagenase promoter was almost ineffective, because only very modest competition was observed with a 100-fold molar excess of the unlabelled oligonucleotide (Figure 6, lower panel, lanes 9 and 10). This result clearly excludes the possibility that this sequence is a functional binding site for the AP-1 complex.

In summary, the functional study by means of co-transfection experiments and gel retardation assays demonstrated that the sequence from nt -203 to nt -191 is essential for insulin effect, has a major role in the liver-specific expression of *CYP7A1* and is the target of HNF-4.

DISCUSSION

It has been shown that insulin inhibits cholesterol 7α-hydroxylase expression by acting at the transcriptional level [16]. In previous studies we demonstrated that the responsiveness of hamster *CYP7A1* to insulin is due to one or more elements present in the proximal promoter [23]. To define these elements, we performed a functional study of the hamster *CYP7A1* promoter, focusing on putative response sequences represented in Figure 1.

Here we have reported the existence of two insulin response elements in hamster *CYP7A1* proximal promoter: a canonical IRS (TGTTTTG) (nt -228 to -222) and a direct repeat 1 (DR-1)-like element TGGACTTAGTTCA located at position -203 to -191 (where the spacer nucleotide is underlined). The first element is a weak binding site for HNF-3α; the second sequence is an HNF-4-binding site. In this regard it is important to

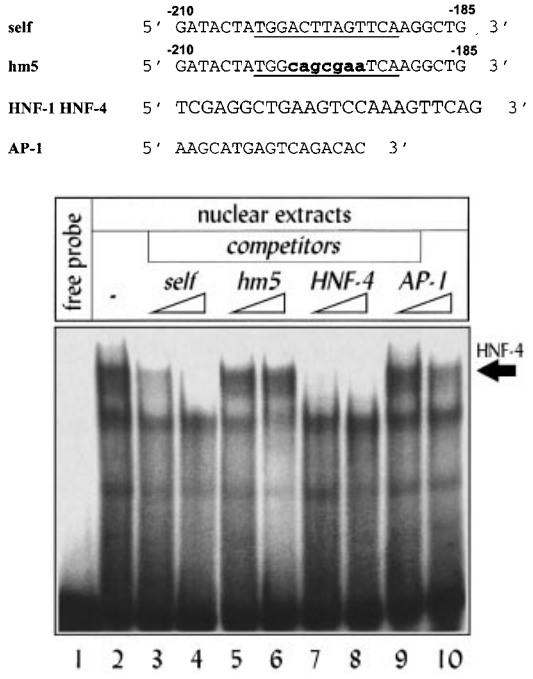

Figure 6 Analysis of nuclear proteins binding to hamster *CYP7A1* HNF-4/AP-1-binding site

Upper panel: sequences of the competitors used: self, wild-type *CYP7A1* promoter sequence; hm5, mutant hm5 of *CYP7A1* promoter sequence; HNF-4, HNF-4-binding site of the HNF-1 promoter; AP-1, AP-1-binding site of the collagenase promoter. Putative consensus sequences in hamster *CYP7A1* are underlined. Bold lower case indicates mutated nucleotides. Lower panel: competition band-shift assays with ^{32}P-labelled probe corresponding to hamster *CYP7A1* HNF-4/AP-1-binding site at nt −204 to −193 incubated with nuclear extracts from HepG2 cells. Unlabelled competitors were in 10-fold and 100-fold molar excess. The arrow indicates the band specifically competed for by unlabelled probes.

mention that corresponding sequences in the rat *CYP7A1* promoter have been reported to bind HNF-3α and HNF-4 translated *in vitro* and to be involved in both basal and retinoic acid-regulated transcription [26]. Moreover, in a preliminary report it has been shown that the rat promoter contains three IRSs and that two of them bind HNF-3 translated *in vitro* [37], although there is evidence that the regulation of *CYP7A1* by insulin in the hamster might be different from that in the rat.

CYP7A1 is a typical gene that is selectively expressed in liver cells [12]. Here we report that *CYP7A1* promoter can be transactivated to a similar extent by the increased availability of HNF-3 and HNF-4 (Figures 2 and 5). However, the region encompassing the IRS, although important for insulin responsiveness, seems to contribute only partly to HNF-3-activated transcription, as demonstrated by mutational analysis (Figure 2). Another HNF-3-binding site located more proximally at nt −138 to −128 (Figure 1 and Table 2), and similar to that characterized in the human *CYP7A1* promoter [34], is a stronger binding site for HNF-3. The role of this element in insulin-mediated regulation is still under investigation. However, preliminary results suggest that this element might not have a major role in the responsiveness to insulin (E. De Fabiani, M. Crestani, M. Marrapodi, A. Pinelli, V. Golfieri and G. Galli, unpublished work). It is conceivable that the distal HNF-3-binding site of the hamster *CYP7A1* promoter, which interacts more weakly with HNF-3, might be more susceptible than the distal one to regulation by extracellular signals such as insulin. Moreover, the surrounding sequence might bind other accessory factors that are critical for insulin responsiveness.

The direct repeat 1 (DR-1)-like element was critical for HNF-4 mediated transactivation. In spite of low levels of transcription activity in comparison with the wild-type promoter, the mutants at the HNF-4 site retained responsiveness to specific stimuli such as dexamethasone (results not shown) and excess HNF-4 levels (Figure 5).

Taken together, our results clearly indicate that insulin signalling converges on promoter regions that are critical for tissue-specific expression. In this regard, coincidence of insulin target sequences with HNF-3-binding sites has been reported for several liver-specific genes (PEPCK, insulin-like growth factor-binding protein, tyrosine aminotransferase) [32,38,39]. Moreover, convergence of multiple signals on complex regulatory units, for example the glucocorticoid response unit ('GRU'), has been evoked to explain the dominant effects exerted by insulin and phorbol esters over glucocorticoid-mediated transactivation [40]. In particular, the presence of an IRS partly overlapping a binding site for HNF3 identified as accessory factor 2, and necessary for the full response of the PEPCK promoter to dexamethasone, explains the phenomenon of insulin dominance over glucocorticoid-mediated transactivation [41]. A similar mechanism could also be applied to the hamster *CYP7A1* gene, because the negative effect of insulin is dominant over dexamethasone-induced transactivation, as reported previously [23]. However, it should be emphasized that despite the similarities of the response to insulin and glucocorticoids, the promoter architecture of *CYP7A1* gene is different from that of PEPCK and this necessarily implies that the mechanisms of regulation by different extracellular stimuli are probably different in the two genes.

It has been shown that HNF-4 mutations are associated with maturity onset of diabetes in the young (type 1), an autosomal dominant subtype of non-insulin-dependent diabetes mellitus [42]. Nevertheless, so far the interference of insulin with HNF-4-mediated transcription has not been fully investigated. It still remains to be demonstrated whether the insulin-mediated down-regulation of hamster *CYP7A1* promoter is due to changes in protein levels, post-translational modifications (e.g. phosphorylation) and, consequently, an altered binding affinity for transcription factors. Protein kinase B/Akt, which is downstream of phosphoinositide 3-kinase, can be translocated to the nucleus and has been reported to mediate the effect of insulin through a conserved IRS [43]. In addition, it has been shown that phosphorylation has important effects on the binding of HNF-4 DNA and transactivation activities, depending on the residues, tyrosine or serine, that undergo covalent modifications [44,45]. It is therefore conceivable that insulin might also down-regulate *CYP7A1* promoter by promoting the inactivation of HNF-4 through phosphorylation.

From our results we conclude that the binding sites for liver-enriched factors, present in the hamster *CYP7A1* proximal promoter in the close vicinity and conserved between species, constitute a regulatory unit important for basal hepatic expression and tissue restriction of hormone action, such as insulin.

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