Published in final edited form as:

Mol Endocrinol. 1998 September ; 12(9): 1294–1309.

IRE-ABP (Insulin Response Element-A Binding Protein), an SRY-Like Protein, Inhibits C/EBP α (CCAAT/Enhancer-Binding Protein α)-Stimulated Expression of the Sex-Specific Cytochrome P450 2C12 Gene

Colleen Buggs,

Diabetes Unit and Medical Services Massachusetts General Hospital Harvard Medical School
Boston, Massachusetts 02114

Nargis Nasrin,

Diabetes Unit and Medical Services Massachusetts General Hospital Harvard Medical School
Boston, Massachusetts 02114

Agneta Mode,

Department of Medical Nutrition Karolinska Institute Novum, S-14186 Huddinge, Sweden

Petra Tollet,

Department of Medical Nutrition Karolinska Institute Novum, S-14186 Huddinge, Sweden

Hui-Fen Zhao,

National Research Council Biotechnology Research Institute Montreal, Quebec H4P 2R2,
Canada

Jan-Åke Gustafsson, and

Department of Medical Nutrition Karolinska Institute Novum, S-14186 Huddinge, Sweden

Maria Alexander-Bridges

Diabetes Unit and Medical Services Massachusetts General Hospital Harvard Medical School
Boston, Massachusetts 02114

Abstract

In primary hepatocytes, overexpression of an insulin response element-A binding protein (IRE-ABP), a member of the SRY family of high-mobility group (HMG) proteins, inhibits CCAAT/enhancer-binding protein α (C/EBP α)-mediated activation of the female-specific cytochrome P450 2C12 (*CYP2C12*) gene, but not the male-specific cytochrome P450 2C11 (*CYP2C11*) gene. IRE-ABP and C/EBP α have overlapping specificity for the C/EBP α target site in the *CYP2C12* promoter and compete for binding to *CYP2C12* DNA *in vitro*. In contrast, IRE-ABP and C/EBP α bind distinct sequences in the *CYP2C11* promoter. A single amino acid substitution in the HMG domain of IRE-ABP impairs its ability to bind DNA and to inhibit the effect of C/EBP α on *CYP2C12* gene expression. Therefore, the ability of IRE-ABP to inhibit C/EBP α -stimulated *CYP2C12* gene expression requires a functional DNA-binding domain. Taken together, our findings suggest that SRY-like proteins can bind to a subset of sequences recognized by the C/EBP family of DNA-binding proteins and modulate gene transcription in a context-specific manner.

INTRODUCTION

Insulin response element-A binding protein (IRE-ABP) is a member of the SRY-like family of transcriptional regulators that was isolated from a rat adipose tissue library by virtue of its ability to bind an insulin response element-A (IRE-A), located in the upstream region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (1, 2). The IRE-ABP cDNA encodes a high-mobility group (HMG) DNA-binding domain that is 67% identical in amino acid sequence to the mammalian testis-determining factor, mouse SRY (mSry) and 98% identical to an SRY-related HMG box protein that is expressed in mice during early embryogenesis, referred to as a4 or SOX4 (2–7). Thus IRE-ABP is likely to be a rat homolog of mouse a4. In a previous study, we showed that IRE-ABP and mSry have overlapping specificity for the DNA sequence 5'-PyCTTTG^A/_T^A/_T and that their HMG boxes contain highly conserved amino acids that are critical for binding to this site (2). Previous studies suggest that SRY-like proteins can activate transcription through multimers of these sites (8, 9), but little is known about how these proteins modulate the activity of natural promoters. We hypothesized that IRE-ABP, like other members of the SRY family of transcriptional regulators, might modulate the expression of sex-specific genes in its target tissues.

The cytochrome P450 2C12 gene (*CYP2C12*) encodes a steroid sulfate 15 β hydroxylase that is induced in the liver of female rats during puberty (10, 11). In contrast, the cytochrome P450 2C11 (*CYP2C11*) gene encodes a steroid 16 α - and 2 α -hydroxylase that is induced in the liver of male rats during puberty (12–14). Differential expression of these genes in male and female rats is set by sexually dimorphic patterns of GH secretion and is mediated at the level of transcription initiation (11, 13, 15). In male rats, GH is secreted in episodic bursts every 3 to 4 h with low or undetectable levels in between. GH pulses of lower amplitudes occur more frequently in females, and base line levels are higher than in males, resulting in a continuous pattern of GH secretion (16, 17).

The male pattern of GH secretion activates the transcription factor STAT5b (18, 19). Furthermore, in male mice disruption of the STAT5b gene results in loss of male-specific liver *CYP* gene expression (20). Thus, it is presumed that STAT5b regulates *CYP2C11* gene expression in rat liver (18, 19). Both the male and female pattern of GH secretion can induce expression of hepatic nuclear factor-6 (HNF-6), but the female pattern is somewhat more effective. HNF-6 can bind and enhance transcription from the *CYP2C12* promoter (21).

The female pattern of GH secretion leads to the marked induction of an unidentified liver nuclear protein complex referred to as the GH-regulated nuclear factor (GHNF) (22). In liver extracts isolated from female rats, GHNF binds several regions in the *CYP2C12* promoter and at least one region in the *CYP2C11* promoter. One of the GHNF-binding sites in the *CYP2C12* gene (–231 to –185) contains a putative binding site for the CCAAT/enhancer-binding protein α (C/EBP α).

C/EBP α is an important determinant of liver gene expression (23–30) and can activate the expression of both the *CYP2C12* and *CYP2C11* genes (14, 31). In primary hepatocytes, we have shown that C/EBP α stimulates expression of the *CYP2C12* gene through the sequence, 5'-TTATCAATGTT (–229 to –207) (31). However, regulation of C/EBP α alone does not appear to account for female-specific expression of the *CYP2C12* gene (22, 31).

We noticed that the C/EBP α site in the *CYP2C12* gene carries a recognition sequence for SRY-like HMG proteins, 5'-PyC^A/_TTTG^A/_T^A/_T, on the antisense strand (2, 32–34). In this report we examined whether IRE-ABP could modulate the effect of C/EBP α on expression of the *CYP2C12* gene expression. We show that, in primary hepatocytes, overexpression of IRE-ABP inhibits C/EBP α -mediated activation of the *CYP2C12* gene, but not the control

gene *CYP2C11*. This observation correlates with the presence of overlapping binding sites for C/EBP α and IRE-ABP in the *CYP2C12* promoter, but not the *CYP2C11* promoter. We propose that the overlap in DNA-binding specificity between IRE-ABP and activators, such as C/EBP α in certain target genes, is one mechanism by which SRY-like proteins may modulate the expression of sex-specific genes.

RESULTS

Transcriptional Activation of the *CYP2C12* and *CYP2C11* Genes by C/EBP α Is Differentially Inhibited by IRE-ABP

To determine whether IRE-ABP modulates the effect of C/EBP α on expression of the endogenous *CYP2C12* gene, we overexpressed C/EBP α in the presence and absence of IRE-ABP in primary rat hepatocytes. Overexpression of a C/EBP α expression vector in primary hepatocytes stimulated endogenous P450 2C12 mRNA levels 4.3-fold (Fig. 1). When IRE-ABP was cotransfected with C/EBP α , stimulation of P450 2C12 mRNA by C/EBP α was completely blocked.

Overexpression of C/EBP α also stimulated P450 2C11 mRNA levels 3.8-fold. In contrast to its effect on P450 2C12 mRNA levels, however, IRE-ABP did not block the effect of C/EBP α on P450 2C11 mRNA levels. Therefore, in primary hepatocytes, IRE-ABP differentially modulates C/EBP α -mediated activation of the endogenous *CYP2C12* and *CYP2C11* genes.

An IRE-ABP expression plasmid (0, 1.5, 3.0, and 6.0 μ g) was cotransfected with a C/EBP α expression plasmid (3.0 μ g) into primary hepatocytes isolated from female rats. Total nucleic acid was extracted from the cells and P450 2C12 and 2C11 mRNA levels were analyzed. Data are expressed as the fold-induction compared with the values in control cells transfected with empty vectors. All plates were transfected with the same amount of plasmid by substitution with empty vectors.

We next examined whether IRE-ABP and C/EBP α together could regulate the expression of chloramphenicol acetyl transferase (CAT) reporter genes driven by the *CYP2C12* and *CYP2C11* promoters (Fig. 2). In transiently transfected NIH-3T3 cells, IRE-ABP had no effect on basal expression of the *CYP2C12*- and *CYP2C11*-CAT constructs (Fig. 2A). In the absence of IRE-ABP, C/EBP α stimulated *CYP2C12*- and *CYP2C11*-CAT activities 11- and 24-fold, respectively (Fig. 2A). In the presence of IRE-ABP, however, C/EBP α -stimulated *CYP2C12*-CAT activity was inhibited 80% (Fig. 2A, *left*), while IRE-ABP had no effect on C/EBP α -stimulated *CYP2C11*-CAT activity (Fig. 2A, *right*).

Similar results were obtained in transfected Chinese hamster ovarian (CHO) cells (Fig. 2B). C/EBP α stimulated *CYP2C12*-CAT activity 4.0-fold, and this effect was blocked by IRE-ABP (Fig. 2B, *left*), whereas C/EBP α stimulated *CYP2C11*-CAT activity 12-fold, and this effect was not impaired by IRE-ABP (Fig. 2B, *right*). These results indicate that activation of the *CYP2C12* gene by C/EBP α and inhibition of this effect by IRE-ABP is mediated through sequences in the 5'-flanking region of the *CYP2C12* gene. In contrast, the control promoter *CYP2C11* provides a natural setting in which the functional C/EBP α sites are not subject to inhibition by IRE-ABP.

IRE-ABP and C/EBP α Bind to Overlapping Sites in the Promoter of the *CYP2C12*, but Not the *CYP2C11* Gene

To determine why IRE-ABP inhibits C/EBP α -mediated activation of *CYP2C12* gene expression, but not *CYP2C11* gene expression, we used deoxyribonuclease I (DNase I) footprint analysis to identify the IRE-ABP- and C/EBP α -binding sites in these genes. C/

EBP α and IRE-ABP were expressed as glutathione-S-transferase (GST) fusion proteins, affinity purified on glutathione Sepharose beads, and eluted with glutathione or cleaved with thrombin to remove GST.

As shown in Fig. 3A, GST-C/EBP α (0.15–0.9 μ g) bound to two regions in the *CYP2C12* promoter, as compared with GST alone (Fig. 3A, compare lanes 2–5 to lane 1; see *stippled circles*). Thrombin cleaved IRE-ABP (Fig. 3A, lanes 7–10), and GST-IRE-ABP (Fig. 3A, lanes 11–12) bound to four regions in the *CYP2C12* promoter of which only IRE-ABP sites 2–4 are shown (Fig. 3A, compare lanes 10 and 12 to lane 6). While IRE-ABP binding to sites 2 and 3 is clearly demonstrated by DNase I footprinting, binding to site 4 was better demonstrated by electrophoretic mobility shift assay (EMSA) (see Fig. 4A, lane 1). In the *CYP2C12* promoter, the two C/EBP α -binding sites overlap with IRE-ABP binding sites 2 and 4. In contrast, in the *CYP2C11* promoter, GST-C/EBP α (0.06–0.6 μ g) bound to three sites (Fig. 3B, compare lanes 1 and 2 to lanes 3–5; see *stippled circles*), each of which was distinct from the one region bound by GST-IRE-ABP (0.06–0.6 μ g), referred to as Site A (compare lanes 1 and 2 to lanes 6–8; see *solid rectangles*).

The sequence and location of the IRE-ABP- and C/EBP α -binding sites in the *CYP2C* promoters are presented in Tables 1 and 2, respectively; a schematic representation of the binding sites is shown in Fig. 3C. In Table 1, the IRE-ABP binding sites are designated sites 1–4, with binding site 1 being most proximal to the TATA box. Binding sites 2 and 3 contain the sequence motif 5'-ACAAAGT, and binding sites 1, 2, and 4 contain the sequence motif 5'-^A/_TCAAT^A/_G^A/_T which has a CAAT box. Therefore, the IRE-ABP-binding sites in the *CYP2C12* promoter, 5'-^A/_TCAAT^T/_A^G/_A^A/_T, conform to previously reported HMG consensus sequences, 5'-PyCTTTG^A/_T^A/_T and 5'-AACAAATA (2, 32, 33, 35–38).

In the *CYP2C12* promoter, C/EBP α protein (0.3 μ g) bound nucleotides (nts) –222 to –204 (Fig. 3A, lane 3), a previously described functional C/EBP α target site (31). This C/EBP α binding site overlaps IRE-ABP DNA-binding site 4. At higher concentrations of C/EBP α (0.9 μ g), a second C/EBP α -binding site located between nts –121 and –105 becomes apparent (Fig. 3A, lane 5); this site overlaps IRE-ABP-binding site 2 (Fig. 3A, lanes 10 and 12). Examination of the IRE-ABP- and C/EBP α -binding sites 4 and 2 in the *CYP2C12* promoter indicates that they carry the sequence 5'-TCAAT (see Tables 1 and 2).

In the *CYP2C11* gene promoter, there is one IRE-ABP-binding site located between nt –113 to –101, referred to as site A (see Fig. 3C). Site A contains the sequence 5'-TTCTTTGTG (see Table 1) and does not bind C/EBP α . Three C/EBP α -binding sites were detected in the *CYP2C11* promoter; two upstream (–194 to –153 and –148 to –132) and one downstream (–88 to –69) of IRE-ABP-binding site A (Table 2). These C/EBP α sites do not carry an HMG consensus sequence and do not bind IRE-ABP (Fig. 3B, lanes 6–8). Therefore, although both IRE-ABP and C/EBP α bind the 5'-flanking sequences of the *CYP2C11* gene, their binding sites do not overlap.

A schematic diagram depicting the location of the binding sites for IRE-ABP and C/EBP α in the *CYP2C12* and *CYP2C11* promoters and their relationship to previously described sex-specific and GH-dependent sites is shown in Fig. 3C. IRE-ABP-binding site 4 in the *CYP2C12* promoter as well as IRE-ABP-binding site A in the *CYP2C11* promoter overlap sequences bound by the GH-regulated nuclear factor GHNF, previously described by Waxman *et al.* (22). Sex-specific and GH-dependent DNase I hypersensitivity sites have also been identified in the *CYP2C11* and *CYP2C12* promoters using liver nuclear extracts from male and female rats (14, 15, 22). In the *CYP2C12* gene, the sex-specific and GH-responsive DNase I hypersensitivity sites are located at nt –99 and –140, respectively (15).

A female-specific hypersensitive region also exists between nts –112 and –159 (15). *C/EBPα* and IRE-ABP cause hypersensitivity to DNase I in these regions. In particular, binding of IRE-ABP to *CYP2C12* increased sensitivity to DNase I near nt –120, which is part of binding site 2 (Fig. 3A, lanes 7–12).

IRE-ABP Is a Component of the Liver Nuclear Protein Complexes That Bind *CYP2C11* and *CYP2C12* DNA

We have previously reported that *C/EBPα* is a component of the rat liver protein complex that binds site 4 in the *CYP2C12* DNA (31). To determine whether IRE-ABP also binds site 4, we used specific antibodies raised against IRE-ABP and *C/EBPα* to detect these proteins in EMSA gels (Fig. 4A). The results shown below were obtained in three independent liver nuclear extracts isolated from male rats in which we expect the female *CYP2C12* gene to be repressed. Under the conditions of this assay, no significant difference in binding pattern was seen in extracts prepared from male and female rats (data not shown).

Purified IRE-ABP protein was preincubated with partially purified preimmune (PI) chicken IgY (Fig. 4A, lane 1) or immune (I) chicken IgY raised against the full-length IRE-ABP protein (Fig. 4A, lane 2) before addition of *CYP2C12* site 4 DNA. In the presence of the IRE-ABP antibody, purified IRE-ABP was completely supershifted (Fig. 4A, compare lane 1 to lane 2).

Next we examined the binding of liver nuclear extract to wild-type site 4 DNA and a mutant of site 4 with the wild-type HMG consensus sequence 5'-ACAATGT changed to 5'-CGCCCTG (Fig. 4A, lanes 3–6, as compared with lanes 7–9). Several complexes were detected with wild-type site 4 DNA (Fig. 4A, lane 3, complexes I–V). In the presence of IRE-ABP antibody, two of the complexes (I and II) were diminished in intensity (Fig. 4A, lane 4) as compared with the PI control (Fig. 4A, lane 3), and two new supershifted (ss) bands appeared (Fig. 4A, lane 4). Complex I comigrates with bacterial IRE-ABP. Thus, the IRE-ABP antibodies shift bacterial IRE-ABP as well as an activity that comigrates with IRE-ABP in liver nuclear extracts (complex I on the *CYP2C12* probe). In the presence of antibody to IRE-ABP, the intensity of complexes II, IV, and V was diminished as well. Thus, there are IRE-ABP-like activities that migrate with lower mobility than pure IRE-ABP in liver nuclear extract. These differences in migration may be the result of posttranslational modifications, interaction of IRE-ABP with other proteins such as *C/EBPα*, or binding of other IRE-ABP-like gene products.

When the binding was performed using the mutant probe, complex I was not detected (compare lanes 3 and 7). The mutant probe also failed to show the two supershifted IRE-ABP bands (Fig. 4A, lane 8). Furthermore, although the mutant probe bound to new complexes that migrated similarly to complexes II–V, these complexes were markedly less sensitive to the IRE-ABP antibody (Fig. 4A, compare lanes 3 and 4 to lanes 7 and 8). Thus, IRE-ABP antibodies detect an IRE-ABP-like binding activity in the complex that binds site 4.

The EMSA gel conditions used to detect IRE-ABP do not favor binding of *C/EBPα* complexes. Nevertheless, preincubation of a *C/EBPα* antibody with liver nuclear extract supershifted a minor band, complex III, that bound to the wild-type but not the mutant site 4 DNA probe (Fig. 4A, compare lanes 3 and 5 to lanes 7 and 9). Therefore, although the mutant probe detects a band that comigrates with complex III, the new complex does not contain *C/EBPα*. The supershift complex was more complicated in the presence of both the *C/EBPα* and IRE-ABP antibodies than with either antibody alone, but multiple attempts failed to resolve these complexes (Fig. 4A, lane 6).

To confirm that IRE-ABP-binding site A in the *CYP2C11* gene promoter binds IRE-ABP but not C/EBP α , we examined the binding of purified IRE-ABP and C/EBP α proteins to this site (Fig. 4B, lanes 1 and 2, respectively). As expected from the DNase I protection assay, IRE-ABP bound site A but C/EBP α did not. Several liver nuclear complexes were detected with wild-type site A DNA (Fig. 4B, lanes 3–6). Preincubation of male liver nuclear extract with an antibody to IRE-ABP led to a marked decrease in intensity of a prominent complex (Fig. 4B, lane 4), as compared with preincubation of this extract with PI serum (lane 3). We presume that the complex was supershifted and that the complex did not enter the well (Fig. 4A, lane 4). When compared with nonimmune (NI) IgG (Fig. 4B, lane 5) a specific antibody to C/EBP α (IgG) did not alter binding of liver nuclear complexes to site A (Fig. 4B, lane 6). The major IRE-ABP-like activity detected with the *CYP2C11* probe (see Fig. 4B, lane 4) migrates similarly to an IRE-ABP activity (complex II) detected with the *CYP2C12* probe (see Fig. 4A, lane 4). While the *CYP2C11* site A probe detects only one major band, the *CYP2C12* site 4 probe detects several bands that are additional bands that are inhibited by the IRE-ABP antibody. We suspect these additional complexes represent IRE-ABP in complex with other proteins (such as C/EBP α).

Combined, these observations demonstrate that the *CYP2C12* site 4 binds both C/EBP α and IRE-ABP (or an IRE-ABP-like activity), while the *CYP2C11* site A binds IRE-ABP and not C/EBP α .

IRE-ABP and C/EBP α Modulate Gene Expression via Overlapping Target Sites Examined on a Heterologous Promoter

The functional effect of C/EBP α on the *CYP2C12* gene maps to sequences located between nts –220 and –207 (31). Having shown that both IRE-ABP and C/EBP α are components of the nuclear protein complex that binds site 4 in the *CYP2C12* promoter, we examined whether the inhibitory effect of IRE-ABP on C/EBP α could be mapped to this region. The IRE-ABP binding sites at site 4 extend upstream and downstream of the C/EBP α footprint, from nts –231 to –184. To capture the full effect of IRE-ABP, we cloned nts –231 to –184 upstream of a heterologous promoter. Having shown that IRE-ABP does not inhibit C/EBP α -stimulated *CYP2C11*-CAT activity (Fig. 2), we chose to use nt –65 to +26 of this promoter, referred to as the minimal *CYP2C11* promoter (min-CAT), to drive expression of the CAT reporter gene (site 4-min-CAT).

In CHO cells, C/EBP α alone did not stimulate min-CAT activity. Although IRE-ABP alone inhibited the basal activity of min-CAT by 50%, there was no effect of IRE-ABP alone on basal expression of the site 4-min-CAT construct (Fig. 5, panels A and B). In the absence of IRE-ABP, C/EBP α stimulated site 4-min-CAT activity 4-fold, whereas in the presence of IRE-ABP, the ability of C/EBP α to stimulate site 4-min-CAT activity was completely blocked (Fig. 5B). Therefore, sequences located between nt –231 to –183 in the *CYP2C12* gene promoter confer activation of gene transcription by C/EBP α and inhibition of this effect by IRE-ABP.

IRE-ABP and C/EBP α Bind with Overlapping Specificity to Site 4 in the *CYP2C12* Gene Promoter

To understand the molecular mechanism by which IRE-ABP inhibits the effect of C/EBP α on the *CYP2C12* gene, we examined whether IRE-ABP and C/EBP α compete for binding to the functional C/EBP α target site using nts –222 to –195 in site 4 DNA. IRE-ABP alone (Fig. 6, lane 1) and C/EBP α alone (lane 3) formed distinct complexes on the ³²P-labeled site 4 DNA. In the presence of a 2-fold molar excess of IRE-ABP over C/EBP α , binding of C/EBP α to the limiting amount of probe was markedly diminished (Fig. 6, lane 4 as compared with lane 3), indicating that IRE-ABP displaces C/EBP α from site 4 DNA. To determine

whether the HMG consensus sequence 5'-ATCAATGT was the target of both IRE-ABP and C/EBP α , this site was changed to 5'-GGCCCTGG and the EMSA repeated. Binding of purified IRE-ABP and C/EBP α protein to site 4 DNA was inhibited by mutation of the HMG site (Fig. 6, compare lanes 1 and 3 to lanes 2 and 5). Thus, IRE-ABP and C/EBP α can bind the same sequence in site 4 DNA.

A Single Amino Acid Substitution in the HMG Box of IRE-ABP Impairs Its Ability to Bind DNA and Interact with C/EBP α *in Vitro*

We were unable to identify mutations with the *CYP2C12*-binding site 4 that eliminated IRE-ABP binding while retaining C/EBP α -mediated transcriptional activation of *CYP2C12* gene expression. Therefore, we determined whether binding of IRE-ABP to site 4 DNA was critical to its inhibitory effect on C/EBP α by correlating the ability of mutant IRE-ABP proteins to compete with C/EBP α for binding to site 4 DNA with their ability to inhibit C/EBP α -mediated activation of the *CYP2C12* gene.

We engineered two point mutations in the HMG domain of IRE-ABP, one of which inhibits and the other of which enhances binding to DNA in the setting of the human SRY protein (C. Buggs and M. Alexander-Bridges, unpublished observations). In the binding-deficient mutant, a glycine is substituted for tryptophan at position 12 in the HMG box (IRE-ABP·Gly), an invariant residue among all HMG box proteins; whereas in the binding-enhanced mutant, a threonine is substituted for proline at position 25 (IRE-ABP·Thr), a substitution that is tolerated in certain HMG-box proteins (40) such as LEF-1 (41). In the setting of SRY, both mutations are associated with sex reversal in humans.

Wild-type IRE-ABP alone (Fig. 7, lane 1), when added at concentrations one third of those used in Fig. 6, forms two complexes on site 4 DNA, with the monomer as the major complex. In contrast, the larger quantity of IRE-ABP added in Fig. 6 resulted in the predominant appearance of the IRE-ABP dimer complex (Fig. 6, lane 1). The IRE-ABP·Gly mutant does not bind to site 4 DNA (Fig. 7, lane 4), whereas the IRE-ABP·Thr mutant shows enhanced binding to site 4; in particular, more of the dimer form is recovered (Fig. 7, lane 6).

When IRE-ABP (0.15 μ g) and C/EBP α (0.15 μ g) were added in equal concentrations to site 4 DNA (Fig. 7, lane 3), the intensity of the IRE-ABP was slightly diminished, and a new minor complex with mobility slower than that of the IRE-ABP dimer was observed (Fig. 7, lane 3) that was not present when either protein was added alone (Fig. 7, lanes 1 and 2). The apparent abundance of this new complex was strikingly increased, however, when the high-affinity IRE-ABP·Thr DNA-binding mutant and C/EBP α bound to DNA (Fig. 7, compare lane 3 to lane 7; see C/EBP α ·IRE-ABP). In contrast, the IRE-ABP·Gly mutant had no effect on the ability of C/EBP α to bind DNA (Fig. 7, lane 5) nor was this new complex evident in the presence of C/EBP α . Together, these observations indicate that IRE-ABP and C/EBP α co-occupy site 4 DNA.

To determine whether the inhibitory effect of IRE-ABP might be mediated, in part, by an interaction with C/EBP α , we used an *in vitro* protein interaction assay (42). *In vitro* translated C/EBP α was incubated with equivalent amounts of GST or GST·IRE-ABP bound to Sepharose beads. The beads were washed and the eluate subjected to electrophoresis as described in *Materials and Methods*. *In vitro* translated C/EBP α bound to GST·IRE-ABP, but not to GST alone (Fig. 8, compare lane 3 to lane 2). Furthermore, C/EBP α interacted with the HMG DNA-binding domain of IRE-ABP (data not shown), which led us to determine whether mutations within the HMG domain might affect the ability of IRE-ABP to interact with C/EBP α as well as to bind DNA. In comparison to wild-type GST·IRE-ABP, which bound more than 25% of the input C/EBP α (Fig. 8, lane 3 as compared with

lane 1), the GST/IRE-ABP-Gly mutant bound very little C/EBP α (Fig. 8, lane 4). In contrast, the GST/IRE-ABP-Thr mutant bound amounts comparable to wild-type IRE-ABP (Fig. 8, lane 5 as compared with lane 3). Therefore, a single point mutation in the HMG domain of IRE-ABP blocks its ability to bind DNA and interact with C/EBP α *in vitro*. Ethidium bromide intercalates DNA and inhibits the interaction of DNA with proteins. In the presence of ethidium bromide (200 μ g/ml), the wild-type and mutant IRE-ABP proteins bind C/EBP α to a comparable extent (lanes 7–9). Thus, we conclude that the direct interaction of C/EBP α and IRE-ABP is not affected by mutations in the HMG-binding domain of IRE-ABP.

The Inhibitory Effect of IRE-ABP Requires a Functional DNA-Binding Domain

To determine whether the mutation in IRE-ABP that inhibits binding of IRE-ABP to DNA would also block its inhibitory effect on activation of *CYP2C12* gene expression, CHO cells were transfected with C/EBP α and wild-type or mutant IRE-ABPs. C/EBP α alone stimulated *CYP2C12*-CAT activity 3.0-fold (Fig. 9). In the presence of wild-type IRE-ABP and IRE-ABP-Thr, *CYP2C12*-CAT activity was stimulated 1.2- and 0.3-fold, respectively, by C/EBP α (Fig. 9). In contrast, in the presence of IRE-ABP-Gly, C/EBP α stimulated *CYP2C12*-CAT activity 5.3-fold (Fig. 9). This finding indicates that a single amino acid mutation in the HMG domain of IRE-ABP, which disrupts binding to DNA, can also interfere with the inhibitory effect of IRE-ABP on gene transcription, whereas a mutation that increases its binding retains the ability to repress transcription.

To verify that the IRE-ABP wild-type and mutant proteins were equivalently expressed in cells, we overexpressed the IRE-ABP wild-type and mutant constructs in cells and subjected cellular extracts to Western analysis. We found that twice as much of the IRE-ABP-Gly mutant protein was expressed than wild-type IRE-ABP protein, while expression of the IRE-ABP-Thr mutant and wild-type IRE-ABP was equivalent (data not shown). Therefore, the inability of IRE-ABP-Gly to inhibit gene transcription is, most likely, due to a loss of DNA-binding activity, as opposed to a decrease in expression of the mutant protein in cells.

DISCUSSION

C/EBP α acts in concert with other transcriptional regulators to dictate liver-specific gene expression (27). In this report we show that the HMG transcriptional regulator, IRE-ABP, binds the C/EBP α target site in the female-specific *CYP2C12* gene, 5'-TTATCAATGTGTT, and prevents activation of this gene by C/EBP α . A mutation in the HMG DNA-binding domain of IRE-ABP, which inhibits binding of IRE-ABP to DNA, also blocks transcriptional repression by IRE-ABP. In contrast, in the male-specific *CYP2C11*, the binding sites for IRE-ABP and C/EBP α do not overlap, and IRE-ABP binding does not prevent activation by C/EBP α . Thus, we conclude that IRE-ABP is a context-specific repressor of gene expression.

Previous reports suggest that the ability of SRY-like proteins to activate transcription may be a prominent feature of this family of genes (7–9, 43, 44). For example, SRY and related proteins, such as SOX4, have been shown to bind and activate transcription through multimerized 5'-ACAAT sequences driving a heterologous promoter (7, 8). In the setting of a native promoter, however, we find that IRE-ABP binds the sequence 5'-ACAAT in the *CYP2C12* gene and acts as a context-specific repressor of C/EBP α -activated gene transcription. IRE-ABP inhibited C/EBP α -stimulated *CYP2C12* expression in three separate assay formats, using: 1) the endogenous *CYP2C12* gene (Fig. 1), 2) the native *CYP2C12* promoter to drive expression of a CAT reporter gene (Fig. 2), and 3) the monomeric site 4 from the 5'-flanking region of the *CYP2C12* gene to drive expression of a heterologous promoter (Fig. 5). In contrast, IRE-ABP did not footprint the C/EBP α sites that mediate

activation of the *CYP2C11* gene by C/EBP α , nor did C/EBP α footprint the IRE-ABP site in the *CYP2C11* gene (see Fig. 3C). Thus, the male-specific *CYP2C11* gene, wherein the IRE-ABP site does not overlap with C/EBP α sites, serves as a control gene whose expression can be examined in the setting of a native promoter. The observation that IRE-ABP has no effect on C/EBP α activation of *CYP2C11* suggests that the ability of IRE-ABP to inhibit C/EBP α -activated transcription of *CYP2C12* requires that the two proteins bind to adjacent or overlapping DNA sequences. These findings provide new insights into the molecular mechanisms that underlie the ability of SRY-like architectural proteins to regulate gene transcription during differentiation and in response to hormonal stimuli.

C/EBP α and IRE-ABP can interact directly, independent of their ability to bind DNA. Nevertheless, several observations indicate that the ability of IRE-ABP to inhibit C/EBP α -activated *CYP2C12* gene expression depends primarily on binding of IRE-ABP to *CYP2C12* DNA rather than to C/EBP α . The direct protein-protein interaction between IRE-ABP and C/EBP α can be visualized in the presence of ethidium, which intercalates DNA and prevents DNA-dependent protein interactions. Under these conditions, the interaction of C/EBP α with IRE-ABP is equivalent for wild-type IRE-ABP, the IRE-ABP-Gly mutant that is deficient in DNA binding, and the IRE-ABP-Thr mutant that enhances binding to DNA (Fig. 8, lanes 6–8). Thus, the IRE-ABP-Gly mutant that inhibits DNA-dependent protein interactions between IRE-ABP and C/EBP α does not inhibit the direct interaction of these proteins (Fig. 8, compare lanes 3 and 4). Nevertheless, the IRE-ABP-Gly mutant lacks the ability to inhibit C/EBP α -activated *CYP2C12* gene expression. Moreover, the C/EBP α -activated expression of *CYP2C11* is not inhibited by wild-type IRE-ABP, indicating that the protein-protein interaction is not sufficient to cause inhibition of C/EBP α action at this gene. Thus, although we cannot conclusively distinguish whether the ability of IRE-ABP to inhibit C/EBP α action on the *CYP2C12* gene results from simple competition for the activator site or from a protein-protein interaction that occurs once these proteins are bound to adjacent sites on DNA, our data indicate that repression of C/EBP α by IRE-ABP requires IRE-ABP binding to DNA.

We propose that once bound to DNA, IRE-ABP modulates C/EBP α -mediated transcriptional activation via one or more mechanisms: 1) IRE-ABP could simply displace C/EBP α from DNA, 2) IRE-ABP could interact with C/EBP α so as to mask the interaction of C/EBP α with the transcriptional machinery, or 3) IRE-ABP could bend *CYP2C12* DNA so as to prevent C/EBP α from interacting with components of the transcriptional machinery. In support of the third mechanism, sequence-specific HMG box proteins are thought to activate transcription by bending DNA and facilitating the assembly of nucleoprotein complexes (40, 45–47). For example, studies indicate that the T cell-specific HMG box protein LEF-1 stimulates gene expression by binding and bending DNA, which, in turn, facilitates interactions between transcription factors bound to sequences flanking the HMG site (41). Inasmuch as similar results have been obtained with SRY (41), it seems plausible that IRE-ABP could inhibit activation of *CYP2C12* gene expression by inducing a bend in DNA that inhibits interactions between C/EBP α and neighboring proteins. Although this mechanism does not require that IRE-ABP interact with C/EBP α , it does not exclude a contribution from such a direct interaction.

In this study we found that IRE-ABP can differentially modulate the expression of two sexually dimorphic cytochrome P450 genes, *CYP2C11* and *CYP2C12*, which are differentially regulated at the level of transcription initiation in the liver of adult male and female rats (11, 13, 15). This regulation in rodents has been shown to be attributable to a sexually dimorphic pattern of GH secretion: the male rat secretes GH in a pulsatile pattern, whereas the female rat secretes GH in a more continuous pattern (16, 17). These two patterns of GH secretion cause differential activation/induction of transcription factors. The

transcription factor STAT5b is activated by pulsatile GH secretion (18, 19) and activates expression of male-specific *CYP* genes in mice (20). In contrast, the HNF-6 transcription factor, whose expression is dependent on GH, is more efficiently induced by the relatively continuous pattern of GH secretion that occurs in female rats, and HNF-6 can increase transcription from the female *CYP2C12* promoter (21).

In addition to HNF-6, continuous GH secretion induces binding of an as yet unidentified protein complex, GHNF, which binds to sequences in both the *CYP2C12* and the *CYP2C11* promoters (21, 22). Thus, three or more transcription factors (STAT5b, HNF-6, and GHNF) have been implicated as mediators of the differential effect of GH signaling on the expression of the *CYP2C12* and *CYP2C11* genes.

In this study we show that IRE-ABP binds to two distinct motifs, 5'-TCAAT^G/_A^T/_A in the *CYP2C12* gene and 5'-TCTTTGT in the *CYP2C11* gene, both of which conform to the consensus sequence for SRY-like proteins, 5'-PyC^A/_TTTG^A/_T^A/_T. In the *CYP2C12* gene, IRE-ABP binds to four sites, most tightly to sites 2 and 4. Site 1 overlaps the HNF-6 site, 5'-AAATCAATAT, located between nts -38 to -47 (see Table 1) (21). As shown in Fig. 3C, IRE-ABP binding Site 4 (-231 to -183), which is also bound by C/EBP α (-222 to -204) overlaps a GHNF-binding site (-231 to -185) (22). In the *CYP2C11* gene, the single high-affinity IRE-ABP binding site A (-115 to -105) also overlaps the GHNF binding site (-135 to -85) (see Fig. 3C), but this site, 5'-TCTTTGT, is not a target for C/EBP α (22). Thus, the binding of IRE-ABP overlaps with that of GHNF in both the *CYP2C12* and *CYP2C11* genes, despite the fact that the structure of the binding sites is different in each gene. Therefore, IRE-ABP (or an HMG protein with similar specificity) may modify the binding or activity of the putative transactivator GHNF.

IRE-ABP is itself unlikely to be GHNF, because we did not detect the marked female-specific differences in IRE-ABP-binding activity that have been observed for GHNF. Furthermore we have not seen differences in IRE-ABP mRNA levels in the liver of male and female rats (our unpublished observations). We speculate that IRE-ABP, in addition to its inhibition of C/EBP α action on the *CYP2C12* gene, also acts as a constitutive repressor of both the *CYP2C11* and *CYP2C12* genes by modifying the activity of GHNF and possibly HNF-6.

Our results are consistent with the following model for differential regulation of the sex-specific *CYP2C12* and *CYP2C11* genes by a repressor, such as IRE-ABP in female and male rats. Each gene contains a site (site A in *CYP2C11* and site 4 in *CYP2C12*) that can bind both an activator (C/EBP α) and a repressor of transcription (IRE-ABP). Depending on which proteins are bound, expression of one gene is activated while the other is markedly inhibited. In this study, we have used C/EBP α and IRE-ABP to illustrate that this effect can occur; however, we can only speculate as to how HNF-6 or GHNF may function in this context.

In male rats, expression of the male gene *CYP2C11* is most likely determined by activated STAT5b (20), which we presume overrides an inhibitory effect of IRE-ABP on this gene. As regards expression of the female *CYP2C12* gene in the male, the relatively low levels of the activator HNF-6 and of GHNF seen with pulsatile GH secretion are insufficient to overcome the repressive effect of IRE-ABP at site 4; and as seen in Figs. 1, 2 and 5, IRE-ABP can effectively inhibit the ability of C/EBP α to activate *CYP2C12* gene expression at this site. These conditions result in the repressed state of the female gene *CYP2C12* in the male.

In the female rat, we surmise that the robust induction of GHNF and HNF-6 overrides the negative effect of IRE-ABP and activates the "female" *CYP2C12* gene. However, *CYP2C11*

also carries a GHNF-binding site. Why then is this gene repressed in the liver of female rats and not induced by GHNF? Our data indicate that IRE-ABP has a higher affinity for the GHNF-binding site in the *CYP2C11* gene, binding site A (see Fig. 3B), than it does for binding site 4 in the *CYP2C12* gene (Fig. 3A). Therefore, IRE-ABP should compete more effectively with GHNF at *CYP2C11* (site A) than it does at *CYP2C12* (site 4). As a result, the male gene, *CYP2C11*, remains repressed in the female, despite induction of GHNF and activation of *CYP2C12*.

This model does not require marked changes in expression or DNA-binding activity of IRE-ABP, but instead relies on observed differences in apparent affinity and specificity of IRE-ABP for its sites in the *CYP2C12* and *CYP2C11* genes (Fig. 3, A and B). However, it is clear that additional studies are required to establish whether this model can explain the sex-differentiated expression of *CYP2C* genes and to elucidate the relationship of IRE-ABP and GHNF.

In summary, we find that IRE-ABP, an SRY-related HMG box protein, can bind C/EBP α sites that contain a CAAT box and inhibit C/EBP α -activated gene transcription. Our findings also indicate that SRY-like proteins can inhibit gene expression by binding to DNA so as to interfere with the activating function of other transcription factors; in the case of the *CYP2C12* gene, C/EBP α and IRE-ABP bind the same site. These observations illustrate a previously unappreciated mechanism by which SRY-like proteins can modulate gene expression during cellular differentiation and in response to hormonal stimuli.

MATERIALS AND METHODS

Plasmids

A pCDNA-C/EBP α eukaryotic expression vector, containing the cDNA sequence for rat C/EBP α , was used for transfection experiments in NIH-3T3 and CHO cells (48). A CMV-C/EBP α expression vector, containing the cDNA sequence for mouse C/EBP α was used for transfection experiments in primary hepatocytes (49).

The CDM8-IRE-ABP expression plasmid was constructed by cloning full-length IRE-ABP cDNA into an *EcoRI* cut CDM8 vector (a kind gift from B. Seed, Massachusetts General Hospital). The mutant CDM8-IRE-ABP constructs were made by PCR-directed mutagenesis as follows: the reverse primer used to synthesize IRE-ABP·Gly was 5' - GCCGCTTGGAGATCTCGGCGTTGTGCATGTCTGGGCGACTGCTCCATGATCTTGC GCCGCTCGATCTGCACCCCACCATAAAG-3' (the single nt substitution is underlined); the reverse primer used to synthesize IRE-ABP·Thr has the sequence 5' - GCCGCTTGGAGATCTCGGCGTTGTGCATGTCTGGTCGACTGCTCCA-3' (the nt substitution is underlined); and the forward primer used to generate the mutant IRE-ABP proteins was 5' -GACTCACTATAGGGAGACCGGAAGCTTAAT-3'. PCR reactions consisted of wild-type CDM8-IRE-ABP plasmid (0.200 μ g) as the template, 200 μ M deoxynucleoside triphosphate, 0.2–1.0 μ M primer, 2 mM MgSO₄, 10% dimethylsulfoxide, 100 μ g/ml BSA, and *Pfu* DNA Polymerase (1–2 U) in 1 $\times$ *Pfu* DNA polymerase reaction buffer (Stratagene, La Jolla, CA). The reaction cycle included 1.0 min at 94 C, 1.0 min ramp to 52 C, 1.0 min at 52 C, 1.0 min ramp to 72 C, 1.0 min at 72 C, 1.0 min ramp to 94 C, and was repeated 30 times. PCR products were extracted, digested with *NcoI* and *BglII*, and then cloned into *NcoI*- and *BglII*-cut wild-type CDM8-IRE-ABP from which the corresponding wild-type *NcoI/BglII* IRE-ABP fragment had been removed.

IRE-ABP cDNA was cloned into *NcoI*- and *HindIII*-cut PGEX-GT vector (a kind gift of J. Dixon) to make GST-IRE-ABP (2). The mutant GST-IRE-ABP plasmids were made by digesting the mutant CDM8-IRE-ABP plasmids with *NcoI* and *BglII* and subcloning each

IRE-ABP fragment with the single nt substitution, into the GST-IRE-ABP plasmid from which the corresponding wild-type *NcoI/BglII* fragment had been removed. The sequence of the mutant IRE-ABP constructs was confirmed.

The *CYP2C12*-CAT reporter construct containing *CYP2C12* sequences –500 to +23 and the *CYP2C11*-CAT construct containing *CYP2C11* sequences –200 to +26 have been previously described and kindly provided by Dr. C. Legraverend and Dr. A. Ström (13, 14). The min-CAT construct was made by generating a *CYP2C11* promoter fragment (nt –65 to +26) by use of PCR with the forward primer 5'-gaattcaagcttCAGAAGCTCATGTTGAATTG and the reverse primer 5'-CATTTTAGCTTCCTTAGCTCCTGAAAATCTCGCCAAGCTCctcgagatcc (*lowercase letters* indicate restriction enzyme sites), and the *CYP2C11*-CAT construct (described above) was used as the template. The site 4-min-CAT construct was made by generating a promoter fragment containing *CYP2C12* sequences (–231 to –183) fused to *CYP2C11* sequences (–65 to +26) in a PCR reaction using the forward primer 5'-gaattcaagcttGAATGAGTGTATTTATCAATGTTACATGAAATAACTCAATAAACAGAGCTCATGTTGAATTG (*lowercase letters* indicate restriction enzyme sites), the same reverse primer used to make the min-CAT construct and the *CYP2C11*-CAT construct as the template. Both fragments were cut with *HindIII/XhoI* and used to replace the minimal promoter fragment in the *CYP2C11*-CAT construct described (14).

The *CYP2C12*-CAT construct used in Fig. 9 was made by generating a *CYP2C12* fragment (–231 to +23) by PCR with the forward primer 5'-gaattcaagcttTTAAAGATTTGAATGAGTGT and the reverse primer, 5'-CATTTTAGCTTCCTTAGCTCCTGAAAATCTCGCCAAGCTCctcgagatcc-3' (*lowercase letters* indicate restriction enzyme sites) using the *CYP2C12*-CAT construct as the template. The PCR product was extracted, digested with *HindIII* and *XhoI*, and then cloned into *HindIII/XhoI* cut pBLCAT3 that had been digested with *NdeI* and *HindIII*, blunted, and religated.

The PCR reactions for all reporter constructs contained the following: DNA template (200 ng), 200 μ M deoxynucleoside triphosphate, 0.2–1.0 μ M primer, 100 μ g/ml BSA and Vent DNA polymerase (1.0–2.0 U) in 1 $\times$ reaction buffer from New England BioLabs (Beverly, MA). Reactions were cycled for 1.0 min at 94 C, 1.0 min ramp to 55 C, 1.0 min at 55 C, 1.0 min ramp to 72 C, 1.0 min at 72 C, 1.0 min ramp to 94 C, for 30 cycles. All of the reporter constructs were sequenced by dideoxy-mediated chain termination method with a DNA sequencing kit (United States Biochemical, Cleveland, OH) to verify that promoter sequences were correct. Plasmids used as cotransfection controls were Rous sarcoma virus (RSV)- β -galactosidase (RSV- β GAL) and RSV-GH (RSV-GH).

Cell Culture

Hepatocytes were isolated from 8-week-old Sprague-Dawley female rats (B&K Universal AB, Stockholm, Sweden) that had been maintained under standardized conditions of light and temperature, with free access to chow and water (11, 31, 50). Cells were cultured on plastic at a density of 5×10^6 cells per 100-mm dish in 10 ml DMEM-Ham's F12 medium (1:1), supplemented with vitamins (GIBCO BRL, Gaithersburg, MD), Na_2SeO_3 (0.1 μ M), insulin (0.1 μ g/ml), streptomycin (50 U/ml), and FCS (5%). The serum concentration was reduced to 2% and 0% after 18 h and 42 h, respectively. NIH-3T3 cells were cultured in DMEM medium supplemented with 10% FBS and 1% glutamine. CHO cells were cultured in Ham's F12 medium supplemented with 10% FBS, 1% glutamine, and 0.1% gentamicin (G418).

Transfections, Solution Hybridization, and Reporter Gene Assays

Hepatocytes were transfected by DOTAP (Boehringer Mannheim, Indianapolis, IN) after 50 h in culture and harvested 15 h later. C/EBP α expression plasmid (3.0 μ g) was cotransfected with increasing amounts of IRE-ABP (1.5–6.0 μ g) or a control plasmid (6.0 μ g) to maintain total DNA concentration at 6.0 μ g. Solution hybridization of total nucleic acid was performed from pooled samples of three transfected dishes, and P450 2C12 and P450 2C11 mRNAs were analyzed using specific [³⁵S]UTP-labeled *CYP2C11* and *CYP2C12* cRNA probes (triplicate determination) as previously described (11, 31, 50).

NIH-3T3 and CHO cells were plated at a density of approximately 5×10^5 cells per 35-mm dish 24 h before transfection and transfected with a calcium phosphate method (51). Amounts of DNA used for each experiment are indicated in the corresponding figure legends. NIH-3T3 cells were incubated with DNA calcium phosphate precipitates overnight, washed, and incubated in fresh medium. CHO cells were exposed to DNA/calcium phosphate precipitate for 4 h and then shocked with 10% glycerol in $1 \times$ PBS for 3 min before the addition of fresh medium. Cells were harvested 48 h after addition of the DNA and CAT assays were performed as described in Ref. 51.

In NIH cells, RSV-GH was used as a cotransfection control, and GH levels were measured using a RIA kit (Nichols Institute Diagnostic, San Juan, CA). In CHO cells, RSV- β GAL was used as a cotransfection control, and β -galactosidase levels were measured by a luminescence assay (Galactolight, Tropix, Bedford, MA).

DNase I Footprinting Analysis

The *CYP2C12*-CAT construct used in Fig. 2 was digested with *Xho*I, labeled with [³²P]dCTP, and then digested with *Hind*III to release the *CYP2C12* DNA fragment. The *CYP2C11*-CAT construct used in Fig. 2 was digested with *Xho*I, labeled with [³²P]dCTP, and digested with *Nde*I to release the *CYP2C11* DNA fragment. Purified GST fusion proteins were prepared as described and cleaved with thrombin to remove GST (2, 51). The proteins were incubated with 30×10^3 cpm of the labeled *CYP2C12* DNA and *CYP2C11* DNA probes for 1 h in binding buffer: 25 mM HEPES, pH 7.9, 20% glycerol, 78 mM NaCl, 7 mM MgCl₂, and 10 mM dithiothreitol (DTT), and 0.02 mg/ml BSA. DNase was added to the reaction for 1 min, and the reaction was stopped by the addition of phenol. DNA was purified by phenol/chloroform extraction, precipitated, and resuspended in dH₂O. Equivalent counts ($\sim 2 \times 10^3$ cpm) of recovered DNA were loaded on a 6% sequencing gel.

EMSA

Gel shift mobility assays were performed with purified GST proteins prepared as previously described (2). The proteins were cleaved with thrombin to remove GST (51). C/EBP α and IRE-ABP were preincubated with antibody for 25 min at room temperature and then added to the DNA probe (25×10^3 cpm) for 25 min at 4°C in the following buffer: 20 mM HEPES, pH 7.8, 0.1 mM EDTA, 20% glycerol, 50 mM KCl, 50 mM NaCl, 6.5 mM MgCl₂, 5 mM DTT, 0.1 μ g deoxyinosinic-deoxycytidylic acid (dIdC), and 0.1 mg/ml BSA. Reactions were electrophoresed on a 4.5% or 5% nondenaturing polyacrylamide gel in $0.5 \times$ TBE (45 mM Tris borate, 1 mM EDTA, pH 8.0) at 170 V for 1–1.5 h or up to 90 min at 4°C, as indicated in the figure.

Liver nuclear extracts (10 μ g) from male rats were prepared as previously described (13). The extracts were preincubated with or without serum (PI or I as indicated in the figures) in a reaction buffer (18 μ l) containing 20 mM HEPES, pH 7.8, 0.1 mM EDTA, 20% glycerol, 50 mM KCl, 50 mM NaCl, 5 mM DTT for 25 min at room temperature and then added to a probe mix containing 6.5 mM MgCl₂, 0.05 μ g dIdC, 5 μ g BSA, and DNA probe ($20\text{--}30 \times 10^3$ cpm).

and further incubated on ice for 25 min. Reactions were subjected to electrophoresis on a 4.5% nondenaturing polyacrylamide gel in TBE at 170 V for 1.5–2.0 h at 4 C.

Oligonucleotides

The following oligonucleotides were synthesized by Massachusetts General Hospital Core Facility and used in the EMSAs:

Wild-type *CYP2C12* site 4, nts –222 to –196

5'-agcttTATTTATCAATGTTACATGAAATAACTCg

Mutant *CYP2C12* site 4, nts –222 to –196

5'-agcttGCCGGCGCCCTGTTACATGCGGCCGCTCg

Wild-type *CYP2C11* site A (–129 to –96)

5'-agcttATTTTAACAGGGTCAAGGTCCACAAAGAAGAAATA.

Lowercase letters indicate restriction enzyme sites added for end labeling.

In Vitro Translations and Protein-Protein Interactions

The pCDNA-C/EBP α plasmid used in transfection studies was also used for *in vitro* synthesis of proteins in rabbit reticulocyte lysate using protocols of the supplier (Promega, Madison, WI). GST pull-down assays were performed using a method described by Lai *et al.* (42) with the following modifications. Glutathione agarose beads (Pharmacia, Piscataway, NJ) were incubated with GST or GST-fusion proteins. Using one half of the bound beads, the amount of GST-fusion protein bound to the beads was quantitated by subjecting proteins released from the beads to SDS/PAGE and staining the gel with Coomassie blue. The other half of the bound beads (bearing ~0.5–1.0 μ g of GST-fusion proteins) were incubated with or without 200 μ g/ml ethidium bromide and 35 S-labeled *in vitro* translated C/EBP α (10 μ l) in a solution containing 100 mM NaCl, 1.0 mM EDTA, 20 mM Tris, and 0.5% NP-40 (NETN) at 4 C for 2 h with constant rotation. The beads were washed four times with 1 ml of the NETN solution. Bound proteins were resuspended in SDS sample buffer (51), resolved by electrophoresis on a 12% SDS polyacrylamide gel, and visualized by autoradiography.

IRE-ABP Antibody

A chicken polyclonal antibody (1:125,000 by ELISA) was raised against the full-length GST-IRE-ABP fusion protein. Chicken IgY was precipitated from egg yolk proteins using polyethylene glycol. The precipitate was resuspended in PBS and filtered by a 0.2- μ m filter (40 μ g of PI and I sera was used in each reaction). A commercial antibody to C/EBP α was obtained (Santa Cruz Biochemicals, Santa Cruz, CA) and 3.0 μ g of antibody were used in each reaction.

Acknowledgments

We thank Dr. D. D. Moore, Dr. R. Kingston, Dr. J. Avruch, and Dr. H. Kronenberg for discussion and critical reading of the manuscript; Dr. D. Page for unpublished information about SRY sex-reversal mutations; Leia Harris for assistance with graphics; and Harriet Silverman for secretarial assistance.

This study was supported by grants from Howard Hughes Medical Institute, the National Institute of Diabetes and Digestive and Kidney Diseases (Grant F31-DK-08729) as well as grants from the Swedish Medical Research Council (Grant 03X-06807), the Karolinska Institute, and the American Scandinavian Foundation.

REFERENCES

- Nasrin N, Ercolani L, Denaro M, Kong XF, Kang I, Alexander M. An insulin response element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein by insulin in cultured cells by nutritional manipulations. *Proc Natl Acad Sci USA*. 1990; 87:5273–5277. [PubMed: 2164673]
- Nasrin N, Buggs C, Kong XF, Carnazza J, Goebel M, Alexander-Bridges M. DNA-binding properties of the product of the testis-determining gene and a related protein. *Nature*. 1991; 354:317–320. [PubMed: 1956382]
- Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature*. 1990; 346:245–250. [PubMed: 2374589]
- Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischau A-M, Lovell-Badge R, Goodfellow PN. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*. 1990; 346:240–244. [PubMed: 1695712]
- Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature*. 1990; 348:450–452. [PubMed: 2247150]
- Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R. Male development of chromosomally female mice transgenic for Sry. *Nature*. 1991; 351:117–121. [PubMed: 2030730]
- van de Wetering M, Oosterwegel M, van Norren K, Clevers H. Sox-4, an Sry-like HMG box protein, is a transcriptional activator in lymphocytes. *EMBO J*. 1993; 12:3847–3854. [PubMed: 8404853]
- Dubin RA, Ostrer H. Sry is a transcriptional activator. *Mol Endocrinol*. 1994; 8:1182–1192. [PubMed: 7838151]
- Cohen DR, Sinclair AH, McGovern JD. SRY protein enhances transcription of Fos-related antigen 1 promoter constructs. *Proc Natl Acad Sci USA*. 1994; 91:4372–4376. [PubMed: 8183916]
- Gustafsson J-Å, Ingelman-Sundberg M. Regulation and properties of a sex-specific hydroxylase system in female rat liver microsomes active on steroid sulfates. *J Biol Chem*. 1974; 249:1940–1945. [PubMed: 4817969]
- Mode A, Wiersma-Larsson E, Gustafsson J-Å. Transcriptional and posttranscriptional regulation of sexually differentiated rat liver cytochrome P-450 by growth hormone. *Mol Endocrinol*. 1989; 3:1142–1147. [PubMed: 2797005]
- Strom A, Mode A, Zaphiropoulos P, Nilsson AG, Morgan E, Gustafsson J-Å. Cloning and pretranslational hormonal regulation of testosterone 16 alpha-hydroxylase (P-45016 alpha) in male rat liver. *Acta Endocrinol (Copenh)*. 1988; 118:314–320. [PubMed: 2455430]
- Legraverend C, Mode A, Wells T, Robinson I, Gustafsson J-Å. Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. *FASEB J*. 1992; 6:711–718. [PubMed: 1537461]
- Strom A, Eguchi H, Mode A, Legraverend C, Tolle tP, Stromstedt PE, Gustafsson J-Å. Characterization of the proximal promoter and two silencer elements in the CYP2C11 gene expressed in rat liver. *DNA Cell Biol*. 1994; 13:805–819. [PubMed: 8068205]
- Sundseth SS, Alberta JA, Waxman DJ. Sex-specific, growth hormone-regulated transcription of the cytochrome P450 2C11 and 2C12 genes. *J Biol Chem*. 1992; 267:3907–3914. [PubMed: 1740438]
- Tannenbaum GS, Martin JB. Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology*. 1976; 98:562–570. [PubMed: 1261487]
- Eden S. Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology*. 1979; 105:555–560. [PubMed: 572295]
- Waxman DJ, Ram PA, Park SH, Choi HK. Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, Stat 5-related DNA binding protein. *J Biol Chem*. 1995; 270:13262–13270. [PubMed: 7768925]

19. Gerbert CA, Park SH, Waxman DJ. Regulation of signal transducer and activator of transcription (STAT)5b activation by the temporal pattern of growth hormone stimulation. *Mol Endocrinol*. 1997; 11:400–414. [PubMed: 9092792]
20. Udy GB, Towers RP, Snell RG, Wilkins R, Park SH, Ram PA, Waxman D, Davey HW. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci USA*. 1997; 94:7239–7244. [PubMed: 9207075]
21. Lahuna O, Fernandez L, Karlsson H, Maiter D, Lemaigre FP, Rousseau GG, Gustafsson J-Å, Mode A. Expression of hepatocyte nuclear factor 6 in rat liver is sex-dependent and regulated by growth hormone. *Proc Natl Acad Sci USA*. 1997; 94:12309–12313. [PubMed: 9356445]
22. Waxman DJ, Zhao S, Choi HK. Interaction of a novel sex-dependent, growth hormone-regulated liver nuclear factor with CYP2C12 promoter. *J Biol Chem*. 1996; 271:29978–29987. [PubMed: 8939943]
23. Graves BJ, Johnson PF, McKnight SL. Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell*. 1986; 44:565–576. [PubMed: 3004739]
24. Johnson PF, Landschulz WH, Graves BJ, McKnight SL. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev*. 1987; 1:133–146. [PubMed: 2824279]
25. Friedman AD, Landschulz WH, McKnight SL. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev*. 1989; 3:1314–1322. [PubMed: 2558052]
26. Christy RJ, Yang VW, Ntambi JM, Geiman DE, Landschulz WH, Friedman AD. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev*. 1989; 3:1323–1335. [PubMed: 2606350]
27. McKnight SL, Lane MD, Gluecksohn-Waelsch S. Is CCAAT/enhancer-binding protein a central regulator of energy metabolism? *Genes Dev*. 1989; 3:2021–2024. [PubMed: 2697636]
28. Park EA, Roesler WJ, Liu J, Klemm DJ, Gurney AL, Thatcher D, Shuman J, Friedman A, Hanson RW. The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol Cell Biol*. 1990; 10:6264–6272. [PubMed: 2147222]
29. Liu J, Park EA, Gurney AL, Roesler WJ, Hanson RW. Cyclic AMP induction of phosphoenolpyruvate carboxykinase (GTP) gene transcription is mediated by multiple promoter elements. *J Biol Chem*. 1991; 266:19095–19102. [PubMed: 1655770]
30. Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR, Darlington GJ. Impaired energy homeostasis in C/EBP α knockout mice. *Science*. 1995; 269:1108–1112. [PubMed: 7652557]
31. Tollet P, Lahuna O, Ahlgren R, Mode A, Gustafsson J-Å. CCAAT/enhancer-binding protein- α -dependent transactivation of CYP2C12 in rat hepatocytes. *Mol Endocrinol*. 1995; 9:1771–1781. [PubMed: 8614413]
32. Giese K, Cox J, Grosschedl R. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell*. 1992; 69:1–20. [PubMed: 1555234]
33. Haqq CM, King C-Y, Donahoe PK, Weiss MA. SRY recognizes conserved DNA sites in sex-specific promoters. *Proc Natl Acad Sci USA*. 1993; 90:1097–1101. [PubMed: 8430080]
34. Giese K, Pagel J, Grosschedl R. Distinct DNA-binding properties of the high mobility group domain of murine and human SRY sex-determining factors. *Proc Natl Acad Sci USA*. 1994; 91:3368–3372. [PubMed: 8159753]
35. Waterman ML, Jones KA. Purification of TCF-1 1991 DNA-binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1. *Genes Dev*. 1990; 5:2567–2578.
37. Denny P, Swift S, Connor F, Ashworth A. An *SRY*-related gene expressed during spermatogenesis in the mouse encodes a sequence-specific DNA-binding protein. *EMBO J*. 1992; 11:3705–3712. [PubMed: 1396566]
38. Harley VR, Lovell-Badge R, Goodfellow PN. Definition of a consensus DNA binding site for SRY. *Nucleic Acids Res*. 1994; 22:1500–1501. [PubMed: 8190643]

39. Tollet P, Hamberg M, Gustafsson J-Å, Mode A. Growth hormone signalling leading to CYP2C12 gene expression in rat hepatocytes involves phospholipase A2. *J Biol Chem.* 1995; 270:12569–12577. [PubMed: 7759504]
40. Grosschedl R, Giese K, Pagel J. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet.* 1994; 10:64–100.
41. Giese K, Kingsley C, Kirshner JR, Grosschedl R. Assembly and function of a TCR alpha enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. *Genes Dev.* 1995; 9:995–1008. [PubMed: 7774816]
42. Lai JS, Herr W. Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. *Proc Natl Acad Sci USA.* 1992; 89:6958–6962. [PubMed: 1495986]
43. Haqq CM, King C, Ukiyama E, Falsafi S, Haqq TN, Donahoe PK, Weiss MA. Molecular basis of mammalian sexual determination: activation of Mullerian inhibiting substance gene expression by SRY. *Science.* 1994; 266:1494–1500. [PubMed: 7985018]
44. Hosking BM, Muscat GE, Koopman PA, Dowhan DH, Dunn TL. Trans-activation and DNA-binding properties of the transcription factor, Sox-18. *Nucleic Acids Res.* 1995; 23:2626–2628. [PubMed: 7651823]
45. Bianchi ME. The HMG-box domain. In: Lilley, DMJ., editor. *DNA-Protein: Structural Interactions.* Oxford University Press; New York: 1995. p. 177-200.
46. Lilley DM. HMG has DNA wrapped up. *Nature.* 1992; 357:282–283. [PubMed: 1589033]
47. Wolffe AP. Architectural transcription factors. *Science.* 1994; 264:1100–1101. [PubMed: 8178167]
48. Ron D, Habener JF. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* 1992; 6:439–453. [PubMed: 1547942]
49. Legraverend C, Antonson P, Flodby P, Xanthopoulos KG. High level activity of the mouse CCAAT/enhancer binding protein (C/EBP α) gene promoter involves auto-regulation and several ubiquitous transcription factors. *Nucleic Acids Res.* 1993; 21:1735–1742. [PubMed: 8493090]
50. Tollet P, Enberg BAM. Growth hormone (GH) regulation of cytochrome P-450IIC12, insulin-like growth factor-I (IGF-I), and GH receptor messenger RNA expression in primary rat hepatocytes: a hormonal interplay with insulin, IGF-I, and thyroid hormone. *Mol Endocrinol.* 1990; 4:1934–1942. [PubMed: 2082191]
51. Ausubel, FA.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K., editors. *Current Protocols in Molecular Biology.* Vol. vol 1. John Wiley; New York: 1997.

Fig. 1.
IRE-ABP and C/EBP α Differentially Regulate Expression of *CYP2C12* and *CYP2C11*
Genes in Primary Hepatocytes

Fig. 2.

Regulation of *CYP2C12*-CAT and *CYP2C11*-CAT Constructs by *C/EBP α* and IRE-ABP in Cultured Cell Lines

A, NIH-3T3 cells were transfected with a *CYP2C12* promoter-CAT construct (8.0 μ g) or a *CYP2C11* promoter-CAT construct (8.0 μ g) in the presence of a *C/EBP α* expression plasmid (0.3 μ g) or its control expression plasmid (0.3 μ g) and an IRE-ABP expression plasmid (0.9 μ g) or its control plasmid (0.9 μ g), as indicated in the figure. An RSV-GH expression plasmid (4.0 μ g) was included to control for transfection efficiency. The fold induction of CAT activity by *C/EBP α* in the absence (*striped bar*) or presence (*solid bar*) of IRE-ABP was normalized to CAT expression in the presence of control plasmids. Results from the average ($\pm$ SEM) of three experiments with triplicate samples are shown. The fold induction of GH is shown *below* the graphs. B, CHO cells were transfected with the same amount of *CYP2C12*- and *CYP2C11*-CAT constructs as in panel A and a control or *C/EBP α* expression plasmid (0.1 μ g) as well as a control or IRE-ABP expression plasmid (0.2 μ g). CAT activity was normalized to basal CAT activity of cells transfected with control plasmids. The fold induction of CAT activity by *C/EBP α* in the absence (*striped bar*) or presence (*solid bar*) of IRE-ABP is shown. Results from a representative experiment of triplicate samples (the average $\pm$ SEM) are shown. The fold induction of β -galactosidase (β gal) activities is shown *below* the graphs.

Fig. 3.
Identification of Binding Sites for IRE-ABP and C/EBPα in the *CYP2C12* and *CYP2C11* Promoters

A, DNase I footprinting analysis was performed on the coding strand of the *CYP2C12* gene (–500 to +23) with GST alone (0.9 μg, lane 1) and increasing amounts of GST·C/EBPα (0.15–0.9 μg, lanes 2–5) or GST alone (0.60 μg, lane 6) and increasing amounts of thrombin-cleaved IRE-ABP (0.05–0.6 μg, lanes 7–10) or GST·IRE-ABP (0.15 μg and 0.6 μg, lanes 11 and 12). The *CYP2C12* G ladder was obtained by Maxim-Gilbert sequencing of the probe; the location of nts –69 to –285 is indicated on the left. C/EBPα-binding sites are indicated with *stippled circles*. IRE-ABP binding sites 2, 3, and 4 in the *CYP2C12* gene are indicated with *solid rectangles*. The region of footprint 1 was not retained on this gel. B, The coding strand of the *CYP2C11* gene (–200 to +26) was incubated with no protein (lane 1), GST alone (0.6 μg, lane 2), increasing amounts of GST·C/EBPα (0.06–0.60 μg, lanes 3–5), or increasing amounts of GST·IRE-ABP (0.06–0.60 μg, lanes 6–8). The *CYP2C11* G ladder obtained by Maxim-Gilbert sequencing of the probe is shown in lane 9. C, A schematic drawing summarizing binding sites for IRE-ABP and C/EBPα in the *CYP2C12* and *CYP2C11* promoters is shown. IRE-ABP binding sites 1–4 are indicated with *solid rectangles*, the C/EBPα-binding sites are indicated with *stippled circles*. Previously reported DNase I hypersensitivity sites are indicated with an *asterisk* (*) (15). Binding sites for a previously described GH-regulated nuclear factor (GHNH) are indicated by *brackets* (22). IRE-ABP binding site 4 overlaps the GHNH binding site at nts –231 to –185. IRE-ABP binding site A overlaps the GHNH site located between nts –135 to –85. Site 1 overlaps the binding site for the GH-sensitive transcription factor HNF-6 (not shown), located between nts –38 to –47 (21).

Fig. 4.

IRE-ABP is a Component of the Liver Nuclear Complexes That Bind *CYP2C12* Site 4 and *CYP2C11* Site A

A, IRE-ABP binding to *CYP2C12* site 4. EMSA was performed with 32 P-labeled wild-type (lanes 1–6) or mutant (lanes 7–9) *CYP2C12* site 4 DNA (nts –222 to –195). Pure IRE-ABP protein was preincubated with PI IgY (lane 1) or I IgY raised against IRE-ABP (lane 2). Nuclear extract isolated from rat liver (10 μ g) was preincubated with PI IgY (lanes 3, 5, and 7), I IgY raised against IRE-ABP (lanes 4, 6, and 8), a commercial antibody raised against C/EBP α (lanes 5 and 9), or antibodies to IRE-ABP and C/EBP α together (lane 6) for 25 min at room temperature, after which site 4 DNA probe was added and the incubation continued for 25 min at 4 C. The protein-DNA complexes were separated on a 4.5% TBE gel as described in *Materials and Methods*. The radioautograph is shown. Five nuclear protein complexes are indicated with *Roman numerals*; complexes that represent supershifted (ss) bands are indicated with an *arrow*. B, IRE-ABP binding to *CYP2C11* site A. EMSA was performed with 32 P-labeled wild-type site A DNA containing *CYP2C11* sequences –129 to –96. Purified IRE-ABP protein (lane 1) or C/EBP α protein (lane 2) was preincubated with binding buffer on ice for 25 min. Nuclear extract isolated from rat liver (10 μ g) was preincubated with PI IgY (lane 3), I IgY raised against IRE-ABP (lane 4), or normal (NI) rabbit IgG (lane 5) or I IgG raised against C/EBP α (lane 6) as described in Fig. 4A. A radioautograph of the EMSA gel is shown.

Fig. 5.

Binding Site 4 Confers C/EBP α -Mediated Activation and IRE-ABP- Mediated Inhibition to a Heterologous Promoter

CHO cells were transiently transfected with a CAT construct driven by the minimal *CYP2C11* promoter sequences (nt -65 to +26), min-CAT, or a construct that contains Site 4 (nt -231 to -183) in the *CYP2C12* gene driving the minimal *CYP2C11* promoter sequences, site 4-min-CAT. A, The min-CAT plasmid (8.0 μ g) was cotransfected with a C/EBP α expression plasmid (0.1 μ g) in the presence of a control plasmid (0.2 μ g) or an IRE-ABP expression plasmid (0.2 μ g). B, The site 4-min-CAT plasmid (8.0 μ g) was cotransfected with a C/EBP α expression plasmid (0.1 μ g) in the presence of control plasmid (0.2 μ g) or an IRE-ABP expression plasmid (0.2 μ g). CAT activity was normalized to the basal CAT activity of cells transfected with control vectors, and the fold induction of CAT activity by C/EBP α in the absence (*striped bars*) or presence (*solid bars*) of IRE-ABP is shown. Results from the average ($\pm$ SEM) of three experiments with triplicate samples are shown.

Fig. 6.

IRE-ABP and C/EBP α Compete for Binding to DNA Sequences in *CYP2C12* Site 4 DNA
 EMSA was performed with 32 P-labeled site 4 DNA containing *CYP2C12* sequences nt -222 to -196. IRE-ABP (0.7 μ g) alone was incubated with wild-type (lane 1) or mutant (lane 2) site 4 DNA. C/EBP α (0.35 μ g) alone or in the presence of IRE-ABP (0.7 μ g) was incubated with wild-type (lanes 3 and 4, respectively) or mutant site 4 DNA (lanes 5 and 6, respectively). The proteins were subjected to electrophoresis on a 5% TBE gel. The radioautograph of the EMSA gel is shown.

Fig. 7.

Effect of HMG Box Mutations in IRE-ABP on the Binding of IRE-ABP and C/EBP α to *CYP2C12* Site 4 DNA

EMSA was performed with an end-labeled site 4 DNA probe containing *CYP2C12* sequences -222 to -196. IRE-ABP wild-type or mutant protein (0.15 μ g) was preincubated in the absence or presence of C/EBP α (0.15 μ g) before the addition of the *CYP2C12* DNA probe. The proteins were loaded onto a 4.5% TBE gel as follows: wild-type IRE-ABP (Wt), lane 1; C/EBP α , lane 2; wild-type IRE-ABP (Wt) and C/EBP α , lane 3; glycine mutant IRE-ABP (Gly), lane 4; glycine mutant IRE-ABP (Gly) and C/EBP α , lane 5; threonine mutant IRE-ABP (Thr), lane 6; and threonine mutant IRE-ABP (Thr) and C/EBP α , lane 7. The probe was run off the gel to improve the resolution of the complexes.

IRE-ABP and C/EBP α Interact *in Vitro*

Mol Endocrinol. Author manuscript; available in PMC 2012 October 04.

Fig. 9.

An HMG Box Mutation Blocks the Inhibitory Effect of IRE-ABP on C/EBP α -Mediated Activation of a *CYP2C12*-CAT Construct

CHO cells were transiently transfected with a *CYP2C12*-CAT construct containing sequences -231 to +23 fused to the CAT gene. The *CYP2C12*-CAT plasmid (8.0 μ g) was cotransfected with a C/EBP α expression plasmid (0.1 μ g) in the presence of a control plasmid (0.2 μ g) or wild-type and mutant IRE-ABP expression plasmid (0.2 μ g). CAT activity was normalized to basal CAT activity of cells transfected with control vectors, and the fold induction of CAT activity by C/EBP α in the absence (*striped bar*) or presence of IRE-ABP wild-type (*solid bar*) or mutant IRE-ABP-Gly (*open bar*) or mutant IRE-ABP-Thr (*speckled bar*) was determined. Results from two experiments with triplicate samples are shown.

Table 1
Summary of IRE-ABP Binding Sites in the *CYP2C12* and *CYP2C11* Gene Promoters

Nucleotides	Binding Site	Sequence	Recognition Motif
2C12 Promoter			
-231 to -205	4	TGAAATGAGTGTATTATTCAATGTTACA	TCAATGT
-204 to -183	4	TGAAATAAAGCTCAATAAAATTAAAG	TCAATAA
-151 to -136	3	GACAAAGTTAAAGGAG CAATGAACTTGTCTGATGATGAC	ACAAAGT
-100 to -127	2		TCAATGA
-21 to -47	1	TTTATATAACACCATATAAAAAATCAATAT	TCAATAT
2C11 Promoter			
-113 to -101	A	GTCCACAAAGAAAG	ACAAAGA
IRE-ABP Consensus Sequence in the <i>CYP2C12</i> and <i>CYP2C11</i> genes			A ₁ /TCAA ^T _A /G _A A _T

IRE-ABP-binding sites 1–4 in the *CYP2C12* gene promoter and IRE-ABP-binding site A in the *CYP2C11* gene promoter are summarized above. The HMG core motif is shown in *bold*.

Table 2
Summary of C/EBP α -Binding Sites in the *CYP2C12* and *CYP2C11* Gene Promoters

Nucleotides	Binding Site	Sequence	Recognition Motif
2C12 Promoter			
-222 to -204	4	TATTTATCAATGTTACATG	TCAAT
-105 to -125	2	GAACTTTGTCTCAATGATAAG	TCAAT
2C11 Promoter			
-189 to -172		CCCTGGTTAGTTACACAG	TTAGTTA
-173 to -148		ATTGTGGTAGTAGAAAGAGGAAG	TTGTGGTAG GTAGAAAG
-143 to -127		GTTTTCACTTGTGTATT	TTGTGTATT
-83 to -64		TAGTTGATTGGTCACCTTAGG	GTTGATTG
C/EBP α Consensus Sequence in the <i>CYP2C12</i> and <i>CYP2C11</i> genes			T ^T ₁ _G NNGNAA ^T ₁ _G