Cooperative Binding of NF-Y and Sp1 at the DNase I-hypersensitive Site, Fatty Acid Synthase Insulin-responsive Element 1, Located at \([-500\) in the Rat Fatty Acid Synthase Promoter\(*\)

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In vitro DNase I footprint analysis of the rat fatty acid synthase (FAS) promoter from \([-568\) to \([-468\) revealed four protein binding sites: A, B, and C boxes and the FAS insulin-responsive element 1 (FIRE1). As demonstrated by gel mobility shift analysis and supershift experiments, FIRE1, located between \([-516\) and \([-498\), is responsible for binding NF-Y. The C box located downstream of FIRE1 was shown by in vitro footprinting to be a Sp1 binding site, and furthermore, competition with Sp1 also abolished FIRE1 binding. Since the half-life of the Sp1-NF-Y-DNA complex is significantly longer than the half-lives of the Sp1-DNA or NF-Y-DNA complexes, the two transcription factors are deemed to bind cooperatively in the FAS promoter at \([-500\). It is unusual that NF-Y binds at this distance from the start site of transcription. NF-Y binding sites are found in the promoters of at least three other FAS genes, viz. goose, chicken, and man. A second NF-Y binding site is located in the FAS promoter at the more usual position of \([-103\) to \([-87\), and it too has a neighboring Sp1 site. CTF/NF-1 competes for proteins binding to the B box. The A box binds Sp1 and contains a 12/13 match of the inverted repeat sequence responsible for binding the nuclear factor EF-C/RFX-1 in the enhancer regions of hepatitis B virus and the major histocompatibility complex class II antigen promoter. The same relative positions of NF-Y and Sp1 binding sites in the promoters of FAS genes of goose, rat, chicken, and man emphasize the involvement of these transcription factors in the diet and hormonal regulation of FAS.

Fatty acid synthase (FAS; EC 2.3.1.85),† responsible for fatty acid synthesis \(\text{de novo}\), is one of the main lipogenic enzymes (1, 2). Not surprisingly, this enzyme, which converts dietary calories into a storage form of energy, reacts to diet, the cognate mRNA undergoing a severalfold induction when a previously starved animal is refed (3, 4). This refeeding phenomenon is not encountered in diabetic animals, suggesting that the peptide hormone insulin may have a direct or indirect effect on the nutritional response (5). To study the molecular basis of the nutritional response we embarked on a systematic investigation of the promoter of the FAS gene of \(Rattus norvegicus\) and have shown that its chromatin structure responds to a nutritional stimulus as demonstrated by its altered sensitivity to DNase I (6). The distribution of DNase I-hypersensitive sites in the rat FAS promoter changed and their number increased when hepatic chromatin from refed animals was compared with that of starved animals. In the region of the DNase I-hypersensitive site located at approximately \([-500\), we identified a tripartite element, FIRE1, with strong sequence homology to the insulin-responsive element of the human GAPDH gene (7). Using gel mobility shift assays we showed that the protein binding properties of FIRE1 were dependent on each of the three regions, viz. \(5^{'-GCCT}\), a 6-nucleotide spacer, and a \(3^{'}\)-palindrome. Transient transfection of the human hepatoma cell line HepG2 with successively deleted FAS promoter constructs fused to the chloramphenicol acetyltransferase gene has shown that the promoter construct retaining the DNase I-hypersensitive site mediates a 2.5-fold effect of insulin as measured by CAT activity (8). Based on these results we propose that the nutritional effect on the expression of the rat FAS gene may be insulin-mediated via protein(s) binding to the FIRE1 element. A second insulin-responsive element, FIRE2, located at \([-300\) base pairs on the promoter of the rat FAS gene, was revealed by these transfection studies. Yet a third element, FAS-IRS-A, has been postulated by Sul and co-workers (9), between nucleotides \([-68\) and \([-52\) and has been defined by gel mobility shift assay and \(\text{in vitro}\) footprint analysis. This location of FAS-IRS-A could well be the same as that of FIRE3, which we claimed to be responsible for the 2-fold insulin-mediated stimulation of the FAS promoter activity observed with the \([-179\) FAS/CAT promoter construct in H4IIE and HepG2 (8). Furthermore, three tandem repeats of FAS-IRS-A conferred insulin responsiveness on a heterologous promoter in \(3T3-L1\) adipocytes (9). However, the insulin response conferred by these tandem repeats of FAS-IRS-A could not be repeated in our hands. The ubiquitous basic helix-loop-helix leucine zipper-containing transcription factors USF1 and USF2 have been identified as major components of the protein complex(es) binding to FAS-IRS-A (10). USF has also been shown to bind to RFX-1, enhancer factor C/member of the RFX family.

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† The abbreviations used are: FAS, fatty acid synthase; ADD1, adipocyte differentiation-dependent factor 1; AP-1, AP-2, activating proteins 1 and 2; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer-binding protein; ChoxE, carbohydrate response element; CREB, cAMP-responsive element-binding protein; CTF/NF-1, CCAAT/binding transcription factor/nuclear factor-1; IRS-A, insulin-responsive sequence A; FIRE, FAS insulin-responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP-1, insulin-like growth factor-binding protein-1; IRE, insulin-responsive element; MLTF-1, major late transcription factor-1; mTF, mouse transferrin; SREBP, sterol regulatory element-binding protein; USF, upstream stimulatory factor; TPH, tryptophan hydroxylase; EF-C/RFX-1, enhancer factor C/member of the RFX family.
Identification of NF-Y/Sp1 Binding Sites in FAS Promoter

Carbohydrate-responsive elements (ChoRE) found in the promoters of several lipidogenic genes (11). In vitro footprint analysis and the ability to confer glucose responsiveness on a heterologous promoter, permitted the definition of a ChoRE at position −1448 and −1428 in the promoter of the SREBP1 gene (11) and at −171 and −124 in the liver-type pyruvate kinase gene (12). A similar sequence has been postulated to be present in the promoter of the rat FAS gene within the first intron and has been shown to confer glucose responsiveness on a heterologous promoter. This sequence is not liver-specific since a footprint was found at the same position when using extracts from spleen, and gel mobility shift studies have shown that it binds the ubiquitous USF/MLTF transcription factor and CTF/NF-1 (13).

Another member of the basic-helix-loop-helix leucine zipper transcription factor family, ADD1, was found by screening a rat adipocyte library with the EC oligonucleotide having the sequence 5′-GATCCAACTTGGCGATACAGG-3′ (14). The artificial EC oligonucleotide has a 9-nucleotide identity with part of the FIRE1 element we have defined by DNase I hypersensitivity between −518 and −495 of the promoter of the rat FAS gene. A CAT reporter gene containing multiple copies of the relevant region of the FAS promoter could be stimulated when the ADD1 protein was coexpressed in NIH 3T3-L1 fibroblasts. ADD1 is the murine counterpart of the human SREBP-1, which has been shown to bind to the promoter of the rat FAS gene (15). ADD1/SREBP-1 is involved in the sterol regulation of the FAS promoter, thus linking the regulation of lipogenesis with that of cholesterol (16).

In this study we investigated the protein binding characteristics of FIRE1, i.e. we undertook footprint analysis of the promoter region that contains a diet-induced DNase I-hypersensitive site and whose sequence contains both E- and CAAAT-box motifs (6). Using a series of gel mobility shift analyses, we searched for the FIRE1 binding factor by a process of elimination and identified the transcription factor binding thereon as one which has been conserved from yeast to mammals, namely NF-Y. The initial in vitro footprint analysis indicates the presence of a Sp1 binding site next to FIRE1. The cooperative binding has been demonstrated between NF-Y and Sp1 by measuring the half-lives of the relevant DNA-protein complexes. We also found a second NF-Y/Sp1 binding site, albeit with a different spacer, at −103/−82 of the FAS promoter and will comment on the remarkable conservation between the promoters of FAS genes of birds and mammals (17–20).

**EXPERIMENTAL PROCEDURES**

**Transfection**—The *Drosophila melanogaster* Schneider line 2 cells (21) were cultured at 27 °C in Schneider's *Drosophila* medium (Sigma) supplemented with 10% fetal calf serum, 120 μg/ml penicillin, 120 μg/ml streptomycin, and 25 μg/ml fungizone. Transfections were done by the calcium phosphate co-precipitation method (22) using 3 μg of the appropriate plasmids and 100 ng of the expression plasmid P$_{cat}$-Sp1. The transfected cells were incubated for 48 h, and extracts were prepared as described previously (8). H4IE cells were cultured and transfected exactly as described in O'Brien et al. (23). This protocol minimizes variability arising from differences in transfection efficiency. Where indicated 10 μM insulin was added for a period of 24 h. Luciferase activity was measured in a Lumat luminometer LB9501 (Berthold, UK) with luciferin as the substrate (Promega Biotech Inc.).

**Plasmids and Oligonucleotides**—The FAS minimal promoter was created by using an oligonucleotide corresponding to the rat FAS sequence from −610 to −12 relative to the transcriptional start site of the FAS mRNA (17). The double-stranded NheI and BglII sites was cloned into pGL2 basic (Promega). Two copies of the A oligonucleotide representing the A box were inserted in the NheI site upstream of the FAS minimal promoter to create plasmid A. Plasmid C with two copies of the C box was constructed in a similar manner. The promoter fragment for footprint analysis was derived from the FAS/CAT construct −565/+65 (8). pGM-NF-YA was constructed by inserting the entire NF-YA-coding sequence contained on an EcoRI/XhoI fragment of pYA-PB9 (24) into pGM-Z3Z (+) (Promega). The *SalI/XhoI* herpes simplex virus thymidine kinase promoter fragment of CAT5 (25) was inserted into the XhoI site of pGL2 basic to create Luc5. LucTK-IGGBP-1 contains the IRE of the promoter of the IGBP-1 gene (26) upstream of the luciferase promoter. pGL2(−816) contains the FAS promoter from −816 to +67 upstream of the luciferase gene of pGL2 basic. In the pGL2(−816) derivative pGL2(−816)AFIRE1 the FIRE1 core sequence 5′-TGTCGAATTCTGTC-3′ has been replaced by a PstI restriction site. The mutation was created by the Chameleon double-stranded, site-directed mutagenesis kit (Stratagen). Similarly, pGL2(−816)AC box contains a HindIII site instead of the core sequence 5′-CCAGC CCCCC-′3′.

Single-stranded oligonucleotides were synthesized using the Applied Biosystem model 392 with appropriate restriction endonuclease sites *BamHI* and *XhoI* (not shown) at their termini. AP-1, AP-2, C/EBP, CREBP, MLTF-1, NF-1, and Sp1 (27) are consensus binding sites for the corresponding transcription factors. FIRE1, FIRE2 (6), FAS-IRS-A (9), CAAAT, and A, B, and C oligonucleotides (17) represent sequences in the FAS promoter. mTF/C/EBP (28) and IRE-AGAPDH (7) are derived from the mouse transferrin and human GAPDH promoters, respectively. The EC box is a synthetic sequence containing a binding site for C/EBP and an overlapping E-box motif (14). The CAAAT(−91) is derived from the human tryptophan hydroxylase promoter (29). IGFBP-1 is an IRE in the promoter of the Igfbp-1 gene (30). The core sequence of each oligonucleotide is underlined. The sequences of oligonucleotides are as follows (coding strand): AP-1: 5′-CCGTTTATGTCCATAGCCGGAAA-′3′; AP-2: 5′-GTCAGGAGTCTCAGCCCGCCGCGC-′3′; CAAAT(−91): 5′-GCCTTGTCGCCGGAAGG-′3′; CAAAT(−157): 5′-TGTCAGTCTGGCAGGCAAGG-′3′; CAAAT(−216): 5′-GAGATTCCAGTCCCAAGGCGGCGG-′3′; FIRE1: 5′-TGTCGAATTCTGTC-′3′; CCAAT(−91): 5′-GTCAGGAGTCTCAGCCCGCCGCGC-′3′; CREB: 5′-AGAGGATCCGTCCCATCCAGGAGGCAAGGAGG-′3′; CCAGC CCCCGCTCGGCT-′3′; FIRE1 C box: 5′-TGTCGAATTCTGTC-′3′; FAS A box: 5′-TGCCACTAGAGCAGCCACCCGCGGCGACACTGGGGCC-′3′; FAS B box: 5′-TGCCACTAGAGCAGCCACCCGCGGCGACACTGGGGCC-′3′; CAAAT(−91): 5′-GTCAGGAGTCTCAGCCCGCCGCGC-′3′; CAAAT(−157): 5′-GCCTTGTCGCCGGAAGG-′3′; CAAAT(−216): 5′-GAGATTCCAGTCCCAAGGCGGCGG-′3′; IGFBP-1-IRE: 5′-CCGTTTATGTCCATAGCCGGAAA-′3′; Sp1: 5′-ATTTCCTCACCTCCCCTTCACTTGGTGAAGG-′3′; Sp1: 5′-ATTTCCTCACCTCCCCTTCACTTGGTGAAGG-′3′.
Identification of NF-Y/Sp1 Binding Sites in FAS Promoter

FIG. 1. DNase I footprint analysis of the rat FAS promoter from −451 to −566 with FAS-induced rat liver extract reveals four protein binding sites. The sense (A) and the antisense strands (B) were labeled at either end and used with (+) or without (−) rat liver extract in footprint reactions. The competing oligonucleotides FIRE1 and Sp1 were included at a 100-fold molar excess where indicated. The regions protected from DNase I digestion are indicated by open rectangles. Maxam-Gilbert A + G and C + T sequence reactions of the probes are shown in lanes 7 and 8 (A) and lanes 9 and 10 (B). Asterisks identify DNase I-hypersensitive sites. The reaction products appearing in B above the C box correspond to vector sequences.

using Klenow fragment in the presence of [α-32P]dATP, and the antisense strand released from pBS(−566/−451) as a SalI/SacI fragment was end-labeled at the SalI site. Labeled DNA (~20,000 cpm) was incubated for 30 min with 2 μg of poly(dI-dC)/poly(dI-dC) and 60 μg of nuclear protein extract of induced rat liver (6) in a final volume of 40 μl (20 mM HEPES, pH 7.9, 25 mM NaCl, 1.25 mM MgCl₂, 0.62 mM CaCl₂, 0.2 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol). For competition experiments oligonucleotide competitors (100-fold molar excess) were included in the binding reaction. To determine the optimal conditions a titration was performed for each probe using increasing concentrations of DNase I in the same amount of nuclear extract. 5–10 milliunits of DNase I in DNase I buffer (1 mM MgCl₂, 1 mM dithiothreitol, 20 mM KCl) were added, and following a 2-min incubation at room temperature the reaction was terminated by the addition of 160 μl of 10% poly(dI-dC) and 60 μg of proteinase K (25 μg/μl). After a 30-min incubation at 42 °C reactions were extracted twice with phenol/chloroform/isoamylalcohol and ethanol-precipitated before analysis on a 5% polyacrylamide, 7M urea sequencing gel.

In Vitro Transcription/Translation—After linearization at the XhoI site 3′ to the inserted cDNA pGEM-NF-YA (NF-YA) and pCiteCBF-C (CBF-C) or at the BglII site in pYB-EM38 (NF-YB) the plasmids were transcribed and translated in vitro as described in Roder et al. (32).

RESULTS

In Vitro DNase I Footprinting Analysis of an Insulin-responsive Region of the FAS Promoter—To examine the protein binding properties around the diet-induced DNase I-hypersensitive site at −500 of the FAS promoter, a restriction fragment extending from −451 to −566 was subjected to in vitro DNase I footprint analysis. Suitably labeled probes were incubated with a nuclear extract prepared from the livers of rats fed a FAS-induction diet (4). As shown in Fig. 1A, there are four protected areas on the sense strand designated A, B, C, and FIRE1. The FIRE1 footprint on the sense strand extends from −498 to −514 and on the antisense strand from −501 to −516. Competition studies with FIRE1 confirm the footprint (Fig. 1B). Of the remaining three protected regions B and C flank FIRE1 and the A box is located upstream of the B box. The protected region in A extends from −536 to −564 and in B from −520 to −534 on the sense strand. Identical footprints were obtained when the analysis was performed using nuclear extracts prepared from the livers of rats fed a normal diet or starved (data not shown).

Since the FAS promoter has been shown to have a high GC content (17), 67% in this region, the presence of binding sites for the general transcription factor Sp1 (33) are to be expected. The C box extending from −470 to −489 on the antisense and −488 to −468 on the sense strand is indeed the result of Sp1 binding (Fig. 1B), since the footprint is abolished when an oligonucleotide representing the Sp1 consensus sequence is included in the assay. Furthermore, the addition of the Sp1 oligonucleotide to the binding assay also removes the footprint, suggesting cooperativity between Sp1 bound to the C box and any protein(s) binding to FIRE1.

A and C Bind Sp1 whereas B Binds CTF/NF-1 or a Related Protein—The proteins binding to the A, B, and C boxes have been identified by gel mobility shift assays. Enhancement of protein binding to the A and C boxes was observed following the addition of Zn²⁺ to the assay (data not shown). With recombinant Sp1 we have confirmed that Sp1 binds to the C box and does so strongly, since a 30-fold molar excess of an oligonucleotide corresponding to the C box abolishes binding. On the other hand, the affinity of the A box for Sp1 is lower, since a
Identification of NF-Y/Sp1 Binding Sites in FAS Promoter

300-fold molar excess of an oligonucleotide corresponding to the A region is required to abolish binding (data not shown). These differences in binding to the Sp1 consensus sequence of the A and C boxes can probably be explained by the number of mismatches to the Sp1 consensus sequence; viz. 1/9 for the C box and 2/9 for the A box.

From preliminary studies we know that the B box does not bind Sp1. Therefore, we tested cognate oligonucleotides for C/EBP, CREB, Sp1, AP-1, AP-2, and MTLF-1 (27) DNA binding sites as competitors for B box binding in gel mobility shift assays. Upon incubation with nuclear extracts from H4IIE, the B box represented by the oligonucleotide extending from −536 to −504 are DNA-protein complexes, U is an unspecific DNA-protein complex, F is the free DNA.

First evidence that the FIRE1-binding protein is found in several different cell types and is independent of dietary status (Fig. 4A). A signal with the same mobility could be detected using nuclear extract from differentiated mouse 3T3-L1 adipocytes (data not shown).

We know from sequence comparisons of FIRE1, FIRE2 (6), and FIRE3 (8) (FAS-IRS-A (9)) in the promoter of the rat FAS and IRE-A in the promoter of the human GAPDH gene (7) that they have in common a sequence-independent, tripartite structure consisting of a GCCN motif, a spacer, and a 3′-palindrome. The 3′-palindrome of FIRE1 harbors the E-box hexanucleotide CANNTG (35) capable of binding proteins containing the basic helix-loop-helix leucine zipper motif. This hexanucleotide overlaps with four nucleotides of the pentanucleotide CCAAT found in several promoters and enhancers (36). We therefore looked to see if FIRE1 binds any of the proteins binding to other E- and CCAAT-box-containing promoter sequences. The experiment was performed using nuclear extracts of H4IIE and labeled FIRE1 oligonucleotide. None of the oligonucleotides tested corresponded to the sequences, FAS-IRS-A (9), FAS-Chore (11, 13), EC box (15), FIRE2 (6), and IRE-A GAPDH (7) challenged the protein(s) binding to FIRE1 (Fig. 4B). This lack of competition between FIRE1 and each of the oligonucleotides was confirmed in a “reverse” experiment, i.e. using labeled FIRE2, FAS-IRS-A, FAS-Chore, IRE-A GAPDH, and EC box as the probes and FIRE1 as the competitor (data not shown). Further consensus binding sites for well characterized DNA-binding factors, such as CREB, Sp1, AP-1, AP-2, CREF/Sp1, and MTLF-1 (27) were not capable of competing out the complex with FIRE1 detected in H4IIE nuclear extract (Fig. 4C). These experiments eliminated known E-box-binding proteins as candidates for FIRE1-binding protein(s).

**Involvement of C/EBP in FIRE1 Binding**—We now turn our attention to the CCAAT motif. In gel mobility shift assay with nuclear extracts from H4IIE the DNA-protein complex(es) bound to FIRE1 can be abolished by an oligonucleotide corresponding to a C/EBP (37) binding site from the mouse transferrin promoter mTF C/EBP (28) and a sequence of the FAS promoter itself between −103 and −87 (CCAAT)FAS (Fig. 4D). FIRE1 and the FAS promoter region between −103 and −87...
contain a common CCAAT motif that might be responsible for the same protein (FIRE1-binding protein) binding to both sequences. Indeed, a labeled probe corresponding to the FAS promoter region between nt −103 and −87 showed the same retarded complex in gel shifts as FIRE1 and could be competed out by unlabeled FIRE1 (32). In a FAS-induced hepatic nuclear extract the gel mobility shift pattern with mTF C/EBP is complex due to the binding of different C/EBP isoforms. This is in contrast to the single band obtained with FIRE1 (Fig. 5). Since C/EBP isoforms are resistant to thermal or chemical denaturation (38) the FAS-induced liver nuclear extract was incubated for 5 min at 80 °C prior to gel mobility shift assay with FIRE1 and mTF C/EBP (Fig. 5). The mTF C/EBP shows binding of several heat stable proteins, whereas the FIRE1-binding protein (BFIRE1) disappears. This experiment shows the heat instability of the FIRE1-binding protein and therefore its significant difference from the other C/EBP isoforms. The thermally unstable complex obtained with mTF C/EBP migrates at the same position as the FIRE1-binding protein and can be competed out by FIRE1 (data not shown). The common core sequence CAAT of FIRE1 and mTF C/EBP is probably responsible for the formation of this heat-labile DNA-protein complex.

**Immunological Characterization of FIRE1-binding Protein(s)**—To further clarify that FIRE1-binding protein is not identical to one of the C/EBP isoforms, antibodies against rat C/EBPa and C/EBPβ were used in supershift assays (Fig. 6). FAS-induced liver nuclear extract was exposed to antibodies raised against rat C/EBPa and C/EBPβ prior to incubation with labeled mTF C/EBP oligonucleotide or FIRE1. Despite the complex gel mobility shift pattern, the use of isoform-specific antibodies made it possible to identify DNA-protein complexes containing C/EBPa and C/EBPβ because they are shifted to a higher position in the gel. No such supershift was detectable with the FIRE1 oligonucleotide. Further evidence for a common factor binding to mTF C/EBP and FIRE1 is that a complex of apparently the same size as the FIRE1 complex is revealed in the two supershifted samples (Fig. 6, lanes 2 and 3). Antiserum against rat C/EBPβ was also incapable of supershifting the FIRE1 complex (data not shown). The experimental results underline clearly that some of the members of the C/EBP family involved in the binding to the mTF C/EBP are not binding to FIRE1 but do not rule out that other members of the C/EBP family (37, 38) bind to FIRE1.

NF-Y, also known as CBF, CP1, or YEBP, was originally identified as binding to the conserved Y-box element in the
human or mouse major histocompatibility complex class II Ea
promoter (39–42). This Y box contains an inverted CCAAT
sequence. As seen in Fig. 7 preincubation of NF-YA and NF-YB
antisera with HepG2 extracts results in a supershift of the
FIRE1 complex. The CCAAT box-binding protein NF-Y is
involved in a number of systems, among them the mouse tryptophan
hydroxylase (TPH) promoter (43). We therefore tested the
CCAAT$_{TPH}$ as well as the CCAAT$_{FAS}$ oligonucleotides for com-
petition of the FIRE1 complex with NF-YA. The successful
competition with both oligonucleotides strengthens the results of
the supershift with anti-NF-Y antisera.

Proof that NF-Y also binds to CCAAT$_{FAS}$ was obtained by
supershifts with anti-NF-YA and NF-YB antisera and com-
petition with appropriate oligonucleotides. The results of this
experiment are shown in Fig. 7B.

Next we demonstrated the direct interaction of NF-Y with
FIRE1 using in vitro synthesized NF-YA and NF-YB (Fig. 8).
When unprogrammed rabbit reticulocyte lysate was incubated
with FIRE1, a weak band could be observed indicating the
presence of NF-Y in the lysate. There was no difference in the
band intensity with in vitro translated NF-YA or NF-YB show-
ing that neither alone binds to labeled FIRE1. However, the
binding signal was enhanced approximately 10-fold when both
in vitro translated NF-YA and NF-YB were included in the
assay. The retarded complex obtained migrates at the same
position as the FIRE1 complex obtained with HepG2 extract and
can be competed out with FIRE1 but not with AP-1 (27).
Furthermore, incubation with NF-YB antisera supershifted the
complex.

**Neither FIRE1 nor C Box Plays a Role in the Insulin Re-
response of FAS in H4IIE—**Transient transfection of hepatoma
cell lines with successively deleted FAS/CAT promoter fusion
plasmids (8) suggested the presence of three IREs in the FAS
promoter (FIRE1, 2, and 3). To test whether FIRE1 or its
neighboring C box is involved in the insulin response of the
FAS promoter in H4IIE cells, we constructed several versions of
the FAS/luciferase promoter fusion plasmid of pGL$_{-816}$.
As a control we used the previously identified IRE in the
promoter of the human Igf1 gene (26). This element has been
shown to mediate an inhibitory effect of insulin on a
heterologous promoter in human and rat hepatoma cell lines
(23, 26). The plasmids listed in Table I were transfected into
H4IIE cells, and their ability to confer insulin-modulated lucif-
erase expression was assayed. LucTK-IGFBP-1 showed a 40% inhi-
bition of activity after insulin treatment for 24 h. Under the
same conditions, the FAS wild type promoter construct
pGL$_{-816}$ showed a 2.3-fold stimulation of luciferase activity,
which was not altered upon deletion of either FIRE1 or C box.

**NF-Y and Sp1 Bind Cooperatively to FIRE1 and C Box—**Gel
mobility shift assays were performed with rat liver nuclear
extract and an oligonucleotide (FIRE1/C box) containing FIRE1
and the C box (Fig. 9A). Four distinct complexes were formed
with the FIRE1/C-box oligonucleotide when both NF-Y and Sp1
were allowed to bind (lane 1). One complex (NF-Y/Sp1) corre-
sponds to NF-Y as it is almost completely eliminated by FIRE1
competition (lane 3) and supershifted by NF-YB antibodies
(lane 5). The other two complexes are not influenced by FIRE1
competition and are also found when nuclear extract is incu-
bated with labeled C box or Sp1 oligonucleotide (cf. lanes 3 and
7 and data not shown). Two complexes (NF-Y/Sp1 and Sp1)
correspond to Sp1 as they are competed out by Sp1 (C box; lane
2) and supershifted by Sp1 antibodies (lane 4). The super-
shifted Sp1 \(BS(Sp1)\) complex migrates to the same position as the NF-Y\(z\) Sp1 complex (cf. lanes 1 and 4 and data not shown). The GC band is caused by an immunologically unrelated GC box-binding protein, despite competition by the C-box oligonucleotide and an Sp1 consensus oligonucleotide (data not shown). Interestingly, competition with the C box creates a new complex \(BNF-Y\) not observed previously. In this case NF-Y binds on its own to FIRE1/C box since no free Sp1 is available. This complex is identical to the NF-Y/FIRE complex (cf. lanes 2 and 6). Since the upper band \((NF-Y/Sp1)\) is eliminated by both NF-Y and Sp1 competitors and supershifted by both anti-NF-YB and Sp1 antisera, it must be caused by binding of both NF-Y and Sp1. As expected from the footprint results, the formation of the NF-Y/Sp1 complex sequesters the NF-Y and precludes the formation of the DNA-NF-Y complex (lane 2), a further indication of cooperative binding.

To measure their relative stabilities the half-lives, i.e., the time at which 50% of the complex remains were determined for the complexes NF-Y\(z\) Sp1, NF-Y, and Sp1. As shown in Fig. 9B, NF-Y binding to FIRE1 has a half-life of 5.5 min. However, when Sp1 is given the opportunity to bind simultaneously with NF-Y the half-life of the NF-Y/Sp1 complex increases 4.4-fold. In the opposite experiment examination of Sp1 binding to the C box also revealed a stabilization of binding when in an NF-
Y-Sp1 complex (Fig. 9C). The half-life of the Sp1 complex alone was 8.0 min. In the presence of NF-Y, the half-life of Sp1 increased approximately 2.5-fold. These findings confirm that the adjacent binding sites for NF-Y and Sp1 stabilize the NF-Y-Sp1 complex. Thus we have demonstrated that in the FAS promoter at ~500 NF-Y and Sp1 bind cooperatively.

**DISCUSSION**

A prerequisite for the positioning and functioning of transcription factors modulating a gene’s transcriptional activity is rearrangement of the chromatin structure in its promoter (44). We observed that the chromatin structure of the promoter of the rat FAS gene is sensitive to dietary signals, not unexpected for a lipogenic gene. The dietary signal was sent out upon refeeding a starved animal and was detectable as a 30-fold increase in the amount of FAS mRNA (4). Since this transcriptional reaction to refeeding was not observed in a diabetic animal (5), the amount of circulating insulin could be the humoral factor triggering the transcriptional response.

The discovery that the position of the diet-induced DNase I-hypersensitive site at ~500 coincided with a sequence of the rat FAS promoter (17) having similarity to the postulated IRE of the human GAPDH gene suggested that this may be a region of the FAS promoter receiving insulin-induced signals. This is the first indication that the region of the FAS promoter designated FIRE1 (6) might be the end point of an insulin cascade. However, transfection studies in H4IIE using FAS/Luc constructs suggest that neither FIRE1 nor its neighboring C box is directly involved in insulin regulation, since no difference in response to insulin could be detected between constructs carrying the wild type promoter or the same promoter deleted for FIRE1 or C box. This correlates with the results obtained with successively deleted versions of the FAS promoter transiently transfected in H4IE and examined for their insulin response (6).

The results of the in vitro footprint analysis suggest that that part of the FAS promoter surrounding the DNase I-hypersensitive site can be subdivided into at least four discrete protein binding regions. From the results of the gel mobility shift analysis we know that the A and C boxes bind the general transcription factor Sp1, the B box binds NF-1, and that FIRE1 binds NF-Y. A computer search has shown that in the region between ~900 and ~300 there are no less than 26 sequence elements with 89% homology to the Sp1 consensus sequence and therefore no lack of opportunity for bound Sp1 molecules to interact with each other and other transcription factors causing looping of the DNA (45) with all its implications for the activation of the transcription machinery of the FAS promoter. Using Schneider cells we showed that two tandem copies of the C box upstream of the FAS minimal promoter were twice as effective as two copies of the A box in the same plasmid. Interestingly, the sequence of the A box contains a 12/13 match of the inverted repeat sequence of the EF-C/RFX-1 binding site 5′-GTTGC(T/C)NG(G/A)CAAC-3′ located in the enhancer regions of hepatitis B virus, polyomavirus, and the major histocompatibility complex class II antigen promoter (46), suggesting that other factors may also bind to the A box. The Sp1 binding capacity of the C box appears to be important for proteins binding to FIRE1, since the inclusion of Sp1 as a competitor in the footprint analysis closes not only the C box “window” but also the FIRE1 “window.” Indeed, measurements of the half-lives of Sp1, NF-Y, Sp1/NF-Y, complexed with DNA showed significant differences. In the NF-Y-Sp1-DNA complex, the interaction of both proteins with their target sites is stabilized. Cooperative binding allows the formation of NF-Y-Sp1-DNA complexes rather than NF-Y-DNA or Sp1-DNA complexes. Sp1 is involved in the regulation of many housekeeping genes, either alone or acting synergistically with another transcription factor (15, 47). The juxtaposition of putative Sp1 and IREs in the promoters of genes whose expression is regulated by insulin may be coincidental but is nevertheless remarkable (48). For instance, potential Sp1 binding sites are found in close proximity to postulated insulin-responsive elements in the promoters of several genes including FAS (8, 9), the human GAPDH gene (7), and rat gene 33 (49). Synergy of Sp1 and SREBP-1 is found in the promoters of the low density lipoprotein receptor and FAS genes (15), suggesting a common regulatory mechanism in lipid and cholesterol metabolism.

NF-Y, also known as CBF or CP1, consists of three subunits, A, B, and C, and the yeast homologues are heme activator proteins 3, 2, and 5, respectively (50, 51). CBF-C can in fact complex with CBF-A and -B. The yeast homologue of NF-Y appears to have a general role in energy metabolism since several genes involved in mitochondrial function, gluconeogenesis, the glyoxalate cycle, and even fatty acid metabolism are regulated by it (52). The cross-species nature of the protein(s) binding to FIRE1 was vindicated by positive competition with mTF C/EBP. This transcription factor has been shown to bind specifically to the pentanucleotide 5′-GCAAT-3′ (28). This was the first experimental evidence that the transcription factor binding to FIRE1 could be a C/EBP-binding protein. Comparing the protein complex of FIRE1 with that of mTF C/EBP using heat-treated nuclear extracts from the livers of rats induced for FAS showed that one of the proteins in the smear obtained with mTF C/EBP and the FIRE1 complex migrate at the same position and both are heat-labile. However, following this up with supershift analysis using antiserum directed against C/EBPα, C/EBPβ, and C/EBPδ relegated these factors to those previously tested and found not to bind to FIRE1.

Our analysis of the FAS promoter provides further evidence for the interaction of the general transcription factors Sp1 and NF-Y. Cooperativity between NF-Y and Sp1 has been demonstrated in the regulation of the major histocompatibility complex class II-associated invariant chain gene expression (53) and in the transcriptional regulation of the farnesyl diphosphate synthase gene which is regulated by cholesterol status. The authors of this study pose the question whether or not the rodent equivalent of SREBP-1, ADD1, requires NF-Y for normal function (54). While we cannot answer this question, our results illustrate binding of NF-Y in HepG2 cells to the corresponding region of the FAS promoter that binds ADD1 in NIH 3T3 fibroblasts. However, some other factor must also be involved because the EC oligonucleotide which pulled out ADD1 (14) does not compete for FIRE1 binding in rat hepatocytes. It could be that in differentiated adipocytes ADD1 rather than NF-Y, or the mouse equivalent thereof, binds to the DNA. Interestingly, the juxtaposition of NF-Y and Sp1 binding sites found at ~500 in the FAS promoter is repeated at ~103–~87 FAS fragment, albeit with different spacing. On the basis of competition studies the CAATNF-VAS (~103/~87) element competes for FIRE1 binding. There is also a supershift when this oligonucleotide is incubated with HepG2 nuclear extract pre-treated with anti-NF-YB antiserum. Using in vitro translated NF-YA and NF-YB we have been able to show that both NF-YA and NF-YB are required to form the FIRE1 complex. This is in agreement with the hypothesis put forward by Sinha et al. 1995 (51) that the three components of NF-Y are necessary for binding to DNA. The position of FIRE1 is unusual for a NF-Y binding site and is to our knowledge the first time that NF-Y has been shown to bind so far upstream of the transcription start site. Furthermore, it is also the first example of a repeat of an NF-Y/Sp1 combination within the same promoter.

Inspection of the sequence of the promoter of the goose FAS
gene (18) reveals that it has a high degree of sequence similarity with the rat FAS promoter (17). For instance, in the goose FAS promoter there is a reverse CCAAT box and two nucleotides downstream a Sp1 binding site between −513 and −508 with only two mismatches to the consensus sequence. Continuing the analogy, A and B boxes are also found upstream of the postulated FIRE1 in the goose FAS promoter between −577 and −545. Furthermore, the goose A box contains an inverted repeat of the EF-C/RFX-1 binding site (46) The goose equivalent of the rat CCAAT\textsubscript{FAS} motif is located at −94 and −88. Although we have no functional data for the goose (18), chicken (19), and human (20) FAS promoters, the similarity at the nucleotide level and the conserved relative positions of the cis-elements functionally tested in the rat FAS promoter is compelling.

It is known that NF-Y, like Sp1, introduces distortions in the double helix (55) and creates an environment for the recruitment of transcription factors which more than likely exert their effects via protein/protein interactions rather than DNA/protein interactions. This could mean that the NF-Y/Sp1 interactions set the stage for the transcriptional activation of the FAS gene. Our experiments show that the region of the diet-induced NDAse I-hypersensitive site at −500 is occupied by a set of general transcription factors whose orchestrated performance may be the result of glucose/insulin-induced effect on the activation of one or more of them.

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