

DNase I hypersensitivity sites and nuclear protein binding on the fatty acid synthase gene: identification of an element with properties similar to known glucose-responsive elements

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We have shown previously that fatty acid synthase (FAS) gene expression is positively regulated by glucose in rat adipose tissue and liver. In the present study, we have identified in the first intron of the gene a sequence closely related to known glucose-responsive elements such as in the L-pyruvate kinase and S14 genes, including a putative upstream stimulatory factor/major late transcription factor (USF/MLTF) binding site (E-box) (+292 nt to +297 nt). Location of this sequence corresponds to a site of hypersensitivity to DNase I which is present in the liver but not in the spleen. Moreover, using this information from a preliminary report of the present work, others have shown that a +283 nt to +303 nt sequence of the FAS gene can confer glucose responsiveness to a heterologous promoter. The protein binding to this region has been investigated *in vitro* by a

combination of DNase I footprinting and gel-retardation experiments with synthetic oligonucleotides and known nuclear proteins. DNase I footprinting experiments using a +161 nt to +405 nt fragment of the FAS gene demonstrate that a region from +290 nt to +316 nt is protected by nuclear extracts from liver and spleen. This region binds two ubiquitous nuclear factors, USF/MLTF and the CAAT-binding transcription factor/nuclear factor 1 (CTF/NF1). Binding of these factors is similar in nuclear extracts from liver which does or does not express the FAS gene as observed for glucose-responsive elements in the L-pyruvate kinase and S14 genes. This suggests a post-translational modification of a factor of the complex after glucose stimulation.

INTRODUCTION

We have shown previously in cultured adipose tissue of suckling rats that the fatty acid synthase (FAS) and acetyl-CoA carboxylase genes were responsive to glucose alone in a concentration-dependent manner and that glucose 6-phosphate could be the metabolite involved in this effect [1]. Insulin had no effect *per se* but was potentiating the glucose effect, probably by enhancing glucose metabolism. We have confirmed these data in the liver, showing that glucose was absolutely necessary for the induction of the FAS gene in cultured hepatocytes of suckling rats and that the role of insulin was indirect, probably by allowing the transcription of the glucokinase gene, thus enhancing the glucose-phosphorylating capacity of the hepatocyte (C. Prip-Buus, D. Perdureau, F. Fougelle, P. Ferré and J. Girard, unpublished work). Actinomycin D totally blocks the glucose effect on the accumulation of FAS mRNA, showing that the glucose effect is transcriptional [2]. The same kind of glucose effect (through the generation of a glucose metabolite) has since been confirmed for the acetyl-CoA carboxylase and liver-type pyruvate kinase (L-PK) genes in a β -pancreatic cell line [3,4].

For some other glucose-responsive genes, namely the L-PK (liver) and the S14 (liver, adipose tissue) genes, the DNA elements which determine glucose responsiveness (GIRE for Glucose Responsive Element) have been characterized [5–9]. They involve

a core sequence containing (1) a canonical (S14) or (2) a degenerated (L-PK) 5'-CACGTG-3' E-box, located from –172 nt to –143 nt in the L-PK gene and from –1457 nt to –1428 nt in the S14 gene, which bind a protein of the upstream stimulatory factor/major late transcription factor (USF/MLTF) family. Moreover, it has been shown recently that the flanking bases of the 5'-CACGTG-3' core are determinant for the glucose effect in the S14 gene [8]. Since the original USF/MLTF sequence does not confer glucose responsiveness in the context of other promoters and since all available data suggest that the binding site for USF/MLTF family members is restricted to 5'-NNNC-ACGTGNNN-3', it has been suggested that protein–protein interaction between a USF/MLTF factor and an accessory factor may provide for specific regulation [8].

A search in the FAS gene promoter has not revealed such a USF/MLTF consensus sequence. However, in the first intron of the FAS gene a 5'-CACGTG-3' sequence does exist, located from +292 nt to +297 nt. Using the information from a preliminary report of the present work, indicating that this element corresponded to a hypersensitive DNase I site, Towle's group has shown that a sequence +283 nt to +303 nt of the FAS gene confers glucose responsiveness (20-fold stimulation) in the context of a heterologous promoter [8]. The aim of the present paper was to analyse the characteristics of this element in the context of the FAS gene, namely hypersensitivity to DNase

Abbreviations used: CTF, CAAT-binding transcription factor; FAS, fatty acid synthase; GIRE, glucose responsive element; L-PK, liver-type pyruvate kinase; MLTF, major late transcription factor; NF1, nuclear factor 1; tk, thymidine kinase; USF, upstream stimulatory factor.

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I, footprinting with hepatic nuclear extracts and binding of specific nuclear transcription factors.

EXPERIMENTAL

Analysis of DNase-I hypersensitive sites

Nuclei were isolated from fresh tissues according to [10]. Isolated nuclei were suspended in 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 0.1 mM EGTA, 0.2 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris/HCl, pH 7.5, 5% (v/v) glycerol, 0.5 mM dithiothreitol, 0.1 mM phenylmethanesulphonyl fluoride. They were stored at -80°C until required for use. Frozen nuclei were thawed and resuspended at a DNA concentration of 5 mg/ml, in the digestion buffer [60 mM KCl, 0.1 mM EGTA, 5% (v/v) glycerol, 15 mM Tris/HCl, pH 7.5, 0.5 mM dithiothreitol]. Nuclei were prewarmed (37°C for 10 min) and digestions were started by adding nuclei into tubes containing 5 mM MgCl_2 and DNase I over a 1–8 $\mu\text{g}/\text{ml}$ range. Control tubes contained MgCl_2 but no DNase I. After 3 min of digestion at 37°C , the reaction was stopped by addition of the same volume of a buffer containing 150 mM NaCl, 15 mM EDTA, 0.3% SDS and 50 mM Tris/HCl, pH 7.5.

Nuclei were incubated with RNase A (50 $\mu\text{g}/\text{ml}$) for 1 h at 37°C and then incubated overnight at 37°C with proteinase K (100 $\mu\text{g}/\text{ml}$). DNA was extracted twice with phenol/chloroform (1:1, v/v) and precipitated with 4 vol. of 100% ethanol for 1 h at -20°C . After centrifugation and washing with 70% ethanol, pellets were dissolved in 10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and the DNA was quantified spectrophotometrically at 260 nm and stored at -20°C .

The samples were electrophoresed on a 1% agarose gel. ^{35}S -DNA mass markers were run in parallel. Blots were hybridized with a +361 to +656 probe labelled with [α - ^{32}P]dCTP using a random hexamer priming kit (Multiprime DNA labelling system, Amersham).

DNase I footprinting

Nuclear extracts were prepared according to Gorski et al. [10] from isolated liver nuclei. The 245 bp fragment containing the FAS sequence from +161 to +405 was prepared in the following way. A double-stranded DNA fragment was synthesized by PCR from genomic liver DNA using as primers: 5'-TCCTCAACAGCCCTGCGCTC-3' from +161 nt to +180 nt and 5'-ACCTCAAGCCACGGGGAAGC-3' from +880 nt to +861 nt. The PCR product was cleaved by *Sma*I which cuts 3' to +405 to generate the 245 bp fragment. This fragment was then inserted at the *SrF1* site of the plasmid Bluescript SK(+) (kit PCR-script SK(+), Stratagene), amplified and sequenced for control. The coding strand was labelled by cleaving with *Not*I which cuts in the plasmid polylinker 23 bp upstream of the 5' end of the FAS fragment, filling in the overhanging end with [α - ^{32}P]dCTP, and digesting with *Eco*RI to cleave 14 bp downstream of the 3' end of the FAS fragment. This generates a 282 bp fragment containing the 245 bp FAS fragment (+161 to +405). Labelling of the non-coding strand was obtained cleaving first by *Eco*RI, filling in the overhanging end with [α - ^{32}P]dATP and then cleaving with *Not*I.

All the end-labelled fragments were isolated by acrylamide gel electrophoresis. DNA-binding reactions were carried out in 20 μl containing 25 mM Hepes, pH 7.6, 5 mM MgCl_2 , 34 mM KCl, and 1 μg of poly(dI:dC). A typical reaction contained 2–3 ng of end-labelled fragment (15000 c.p.m.) with increasing amounts of nuclear extract (20–40 μg) and was incubated at $+4^{\circ}\text{C}$ for 10 min. DNase I digestions were performed by adding 1 μl of a

solution containing DNase I (100–400 ng) in 10 mM Hepes, pH 7.6, 25 mM CaCl_2 for 5 min at 4°C . The digestions were terminated by adding 80 μl of a stop buffer containing 20 mM Tris/HCl, pH 8.0, 20 mM EDTA, 250 mM NaCl, 0.5% SDS, 1 μg of herring sperm DNA and 10 μg of proteinase K. Following 60 min of incubation at 45°C , samples were extracted twice with phenol/chloroform and precipitated with 300 μl of 100% ethanol and 5 μl of 5 M NaCl. After centrifugation, the pellet was resuspended in 3 μl of 90% formamide, 0.1% xylene cyanol and 0.1% Bromophenol Blue. Equal counts were loaded on a 6% polyacrylamide/7 M urea sequencing gel. DNase I cleavage sites protected from digestion were identified using cleavage sequencing reactions of the same labelled fragments [11].

Oligonucleotides

The following oligonucleotides were synthesized by Genset (Paris, France). In these sequences, the E-box, which corresponds to the consensus binding site of USF/MLTF, is indicated in bold characters and the nuclear factor 1 (NF1) consensus binding site(s) is underlined.

FAS 1 (+285/+316):

5'-CCGCTGT**CACGTGGGG**CCCGCGCCAGCCGGGT-3'
3'-GGCGACAG**TGCAC**CCCGCGCGGTCGGCCCA-5'

FAS 1 M (+285/+316): The NF1 site GCCAG is mutated to AAAAG

5'-CCGCTGT**CACGTGGGG**CCCGCAAAAACCCGGGT-3'
3'-GGCGACAG**TGCAC**CCCGCGCGTTTTCGGCCCA-5'

mFAS 1 (+285/+316): The USF/MLTF site CACGTG is mutated to AAAGTG

5'-CCGCTGT**AAAGTGGGG**CCCGCGCCAGCCGGGT-3'
3'-GGCGACAT**TTTAC**CCCGCGCGGTCGGCCCA-5'

FAS 2 (+285/+303):

5'-CCGCTGT**CACGTGGGG**CC-3'
3'-GGCGACAG**TGCAC**CCCGCGG-5'

S14 (-1446/-1428):

5'-TCGACAGTTCT**CACGTG**GGTGGCC-3'
3'-GTCAAGAG**TGCAC**ACCACCGGAGCT-5'

Ad-MLTF from the major late adenovirus promoter (-70/-43):

5'-AGGTGTAGGCC**CACGTG**ACCGGGTGTTC-3'
3'-TCCACATCCG**TGCAC**TGGCCCAAGG-5'

L4 element from the L-PK promoter (-172/-143):

5'-ATGGGCG**CACGGG**GCACT**CCCGT**GTTTCT-3'
3'-TACCCGCG**TGCC**CCGTGAG**GGC**ACCAAGGA-5'

NF1 from the adenovirus 2 (+20/+49):

5'-TATTTGGATTGAAGCCAATATGATAATGA-3'
3'-ATAAA**ACCTA**ACT**TCGGT**TATACTATTACT-5'

Mobility-shift assays

Double-stranded oligonucleotides were prepared by combining equivalent amounts of the complementary single-stranded DNA in a solution containing 67 mM Tris/HCl, pH 7.5, 13 mM MgCl_2 ,

1.3 mM EDTA, 1.3 mM spermidine and 6.7 mM dithiothreitol, heating to 90 °C for 5 min and then cooling to room temperature. The annealed oligonucleotides were 5'-labelled with polynucleotide kinase and [γ - 32 P]ATP. Binding reactions were carried out in 20 μ l mixtures containing 10 mM Hepes, pH 8.0, 50 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 4 mM spermidine, 2 mM dithiothreitol, 2 μ g of albumin, 17.5% glycerol and 1 μ g of poly(dI:dC). A typical reaction mix contained 1000 c.p.m. (1 ng) of end-labelled DNA with 5 μ g of nuclear extract. Following incubation at 4 °C for 10 min, samples were electrophoresed on a 6% polyacrylamide gel containing 45 mM Tris, 45 mM boric acid and 1.25 mM EDTA, pH 8.0. For competition experiments, the conditions were the same as above except that competitor DNAs were included in the binding mixture before adding the nuclear extract.

The full-length cDNA clone encoding the 43 kDa human MLTF [12] (kindly provided by Drs. Pognonec and Roeder) was subcloned into pBLKS+ and transcribed with T7 RNA polymerase. RNA was then translated in rabbit reticulocyte lysate (Promega) and the translated product was used for gel-mobility-shift assays.

Purified CAAT-binding transcription factor 1 (CTF 1) [13] was a gift from Dr. Mermod, Institut de Biologie Animale, Lausanne, Switzerland.

RESULTS

A DNase I hypersensitive site is localized in the region of the putative GIRE

In order to localize the hypersensitive sites associated with the FAS gene, Southern blots of *Pst*I-digested genomic DNA from control and DNase I-treated nuclei isolated from suckling and weaned rat liver and weaned rat spleen have been probed with a 32 P-labelled probe (+361, +656). In the absence of DNase I, it yielded a single 2.4–2.7 kb DNA fragment (Figure 1), which fits with the known *Pst*I sites located at –1607 nt and +815 nt.

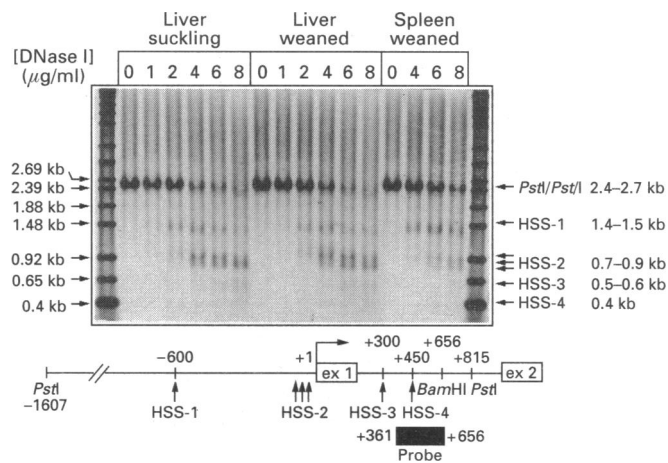


Figure 1 Localization of DNase I hypersensitive sites associated with FAS gene

Rat liver or spleen nuclei were digested with increasing concentrations of DNase I as indicated. DNA isolated from undigested and DNase I-digested nuclei was digested with *Pst*I, electrophoresed and transferred as described in the Experimental section. The blot was hybridized with a +361 to +656 probe. The size of the undigested or digested fragments is indicated on the right-hand side of the blot. Map: position of the four hypersensitive sites (HSS), probe, exons (ex) and known *Bam*HI and *Pst*I restriction sites on the *Pst*I/*Pst*I fragment.

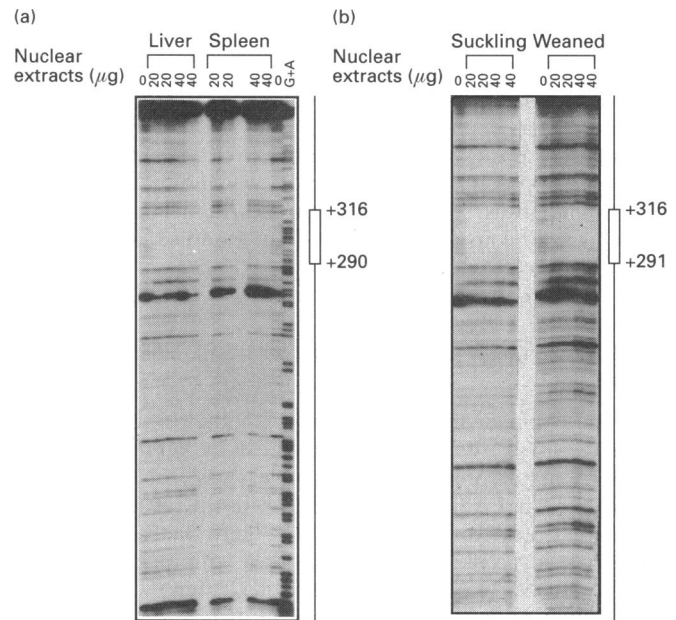


Figure 2 Nuclear factors from liver and spleen bind to the FAS (+161 to +405) fragment in the region of the USF/MLTF-like domain

The 245 bp fragment from the FAS gene (+161 to +405) including the HSS-3 site was subjected to DNase I footprinting. Various amounts of nuclear extracts from liver or spleen of suckling and weaned rats (a) and of nuclear extracts from liver of suckling and weaned rats (b) were incubated with the labelled non-coding strand. The end-points of the protected region shown in the box were identified by chemical sequencing. The protected region is +290 5'-GTCACGTGGGCGCCGCCACCGGGT-3' +316.

Three fragments of 1.4–1.5 kb, 0.7–0.9 kb, 0.55–0.65 kb were clearly generated by increasing amounts of DNase I in liver nuclei (Figure 1). A fourth one of 0.4 kb, although weak, was consistently found. These four fragments correspond to hypersensitive DNase I sites labelled HSS-1, HSS-2, HSS-3 and HSS-4 respectively. HSS-1 is located approximately at –0.6 kb and is found with the same intensity in nuclei of weaned rat liver, a tissue which expresses the FAS gene, and in the nuclei of the liver of suckling rats or of the spleen, tissues which do not express FAS. HSS-2 encompasses a larger protected region around the transcription start site, a region which in addition to the TATA box contains an insulin response element [14]. The sensitivity of this site is higher in the nuclei of liver than in the nuclei of spleen. HSS-3 is located at +0.25–0.35 kb, a region corresponding to the putative GIRE (+292) and is readily apparent only in the liver nuclei. The site is present in the liver of both suckling and weaned rats with a similar hypersensitivity. Similar characteristics are found for HSS-4 located at +0.4 kb.

DNase I hypersensitivity experiments were also conducted using a *Bam*HI/*Bam*HI (–2729, +656) fragment probed with the same 32 P-labelled probe (+361, +656). The same hypersensitive sites were detected (results not shown).

The GIRE DNA region is protected from DNase I digestion by nuclear extracts

Using a probe spanning nucleotides +160 to +405 (coding strand and non-coding strand) of the FAS gene, one region was protected against the nucleolytic attack of DNase I by liver nuclear extracts. This region corresponds to nucleotides +290 to +316 on the non-coding strand (Figure 2) and +291 to +315 on

the coding strand (results not shown). The protected sequence 5'-GTCACGTGGGCGCCGCGCCAGCCGGGT-3' includes part of the sequence (underlined) which has been shown to confer glucose responsiveness to a heterologous promoter (S14) [8] and contains a canonical USF/MLTF E-box (bold characters). A similar footprint was observed in the liver and spleen (Figure 2a), suggesting that the nuclear factors which bind to DNA are of the ubiquitous type. Finally, the nutritional status of the animal, suckling or weaned rats, did not modified the protected region (Figure 2b).

The protected region including the putative GIRE binds at least two proteins

The interaction of nuclear proteins with the protected region +290 to +316 was further investigated by gel-mobility-shift assays. A major protein-DNA complex was formed with the oligonucleotide FAS 1 (+285 to +316) in the presence of liver nuclear extracts from weaned rats (Figure 3a, lane 2). This complex was specific since it disappeared with an excess of the same unlabelled FAS 1 oligonucleotide (Figure 3a, lane 3). Competition with a 30-fold excess of the Ad-MLTF oligonucleotide led to the partial, but not total, disappearance of the complex (Figure 3a, lane 4). Competition was already maximal with a 5-fold excess of the Ad-MLTF oligonucleotide and did not increase further (results not shown). This suggests (1) that a factor of the USF/MLTF family is indeed part of the complex and (2) that another factor is binding in this region.

Concerning the second factor, examination of the sequence +296 to +310, 5'-TgGGC-N₅-GCCAg-3' showed a similarity (capital letters) with the known consensus sequence for the ubiquitous factor CTF/NF1, TT/CGGA/C-N₅-GCCAA [15-17]. We thus tried a competition experiment between FAS 1 oligonucleotide and a canonical adenovirus NF1 oligonucleotide in a 30-fold excess. The complex partially disappeared (Figure 3a, lane 5). Competition was already maximal with a 2.5-fold excess of the NF1 oligonucleotide and did not increase further (results not shown). A competition experiment with both Ad-MLTF and NF1 oligonucleotides led to the total disappearance of the complex (Figure 3a, lane 6), indicating that the second factor binding to the protected region was probably of the CTF/NF1 family.

Mutation of the recognition consensus sites for USF/MLTF and CTF/NF1

In order to confirm the respective roles of each nuclear factor in the formation of the complex, mobility-shift experiments were performed using mFAS 1 and FAS 1M oligonucleotides mutated respectively on the USF/MLTF and CTF/NF1 recognition sites. When a gel-mobility-shift assay was performed using liver nuclear extracts and the FAS 1 oligonucleotide mutated on the E-box (mFAS 1), the complex formed was not competed with by an excess of Ad-MLTF oligonucleotide but was totally abolished by competition with an excess of NF1 oligonucleotide (Figure 3b). Conversely, when a gel-mobility-shift assay was performed using liver nuclear extracts and the FAS 1 oligonucleotide in which the NF1 recognition sequence 5'-GCCA-3' is mutated to 5'-AAAA-3' (FAS 1M), the complex formed was not competed with by an excess of NF1 oligonucleotide but was totally abolished by competition with an excess of Ad-MLTF oligonucleotide (Figure 3c). These experiments confirm the respective roles of factors of the USF/MLTF and CTF/NF1 family in the formation of the complex.

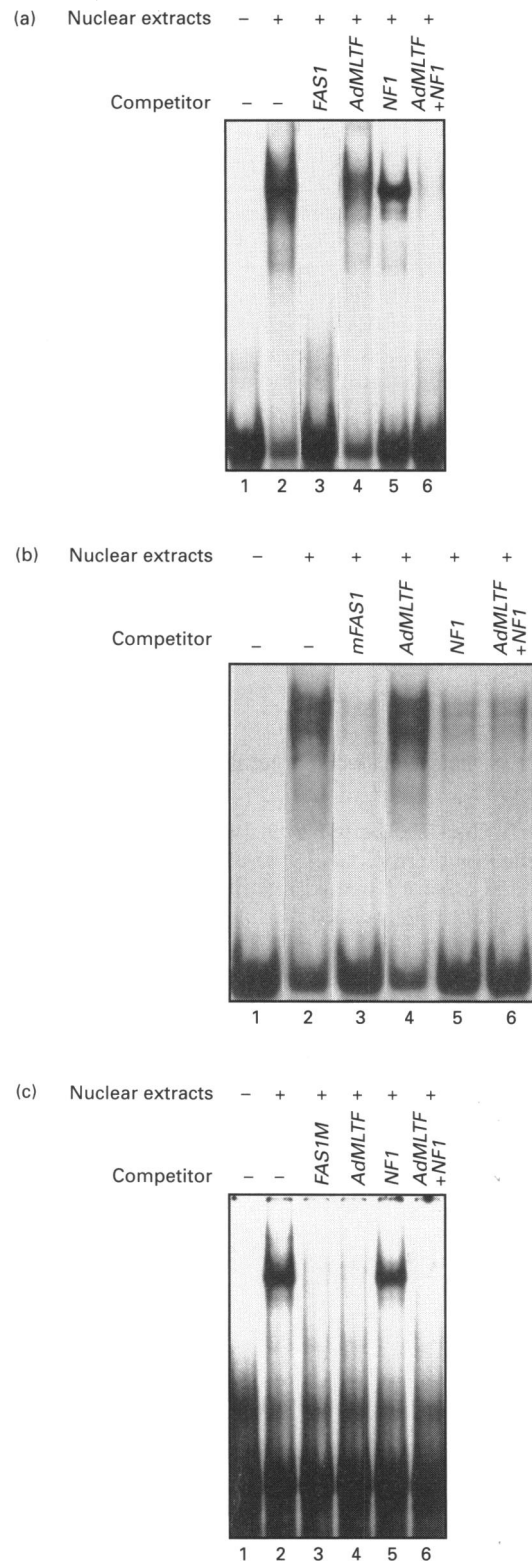


Figure 3 Binding of nuclear extracts to wild-type or mutated FAS 1 (+285 to +316) oligonucleotides in the absence or presence of various competitors

Hepatic nuclear extracts (5 μ g) of weaned rats were incubated with ³²P-labelled FAS 1 (a), mFAS 1 (b) and FAS 1M (c) oligonucleotide and subjected to mobility-shift assay in the absence or presence of 30 ng of competitor oligonucleotides. For mFAS 1, the mutation consists of the replacement of the E-box +292 5'-CACGTG-3' +297 by a 5'-AAAGTG-3' sequence. For FAS 1M, the mutation consists of the replacement of +306 5'-GCCA-3' +310 sequence by a 5'-AAAA-3' sequence.

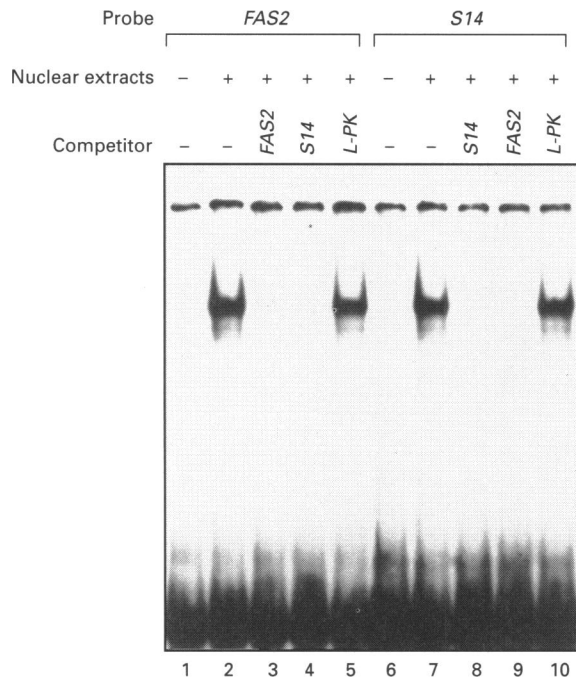


Figure 4 Comparison of the binding of nuclear extracts to FAS 2 (+285 to +303) and S14 (-1446 to -1428) oligonucleotides

Hepatic nuclear extracts (5 μ g) of weaned rats were incubated with 32 P-labelled FAS 2 and S14 oligonucleotides and subjected to mobility-shift assay in the absence or presence of 30 ng of competitor oligonucleotides.

Deletion of the recognition consensus site for CTF/NF1 and comparison with the S14 GIRE

A mobility-shift assay was performed with the oligonucleotide FAS 2 (+285, +303) centred on the 5'-CACGTG-3' motif and in which the CTF/NF1 site has been deleted. A complex was formed in the presence of liver nuclear extracts (Figure 4, lane 2). A competition with the S14 GIRE oligonucleotide, which in gel-shift experiments binds exclusively a member of the USF/MLTF family [7,8], led to the total disappearance of the complex (Figure 4, lane 4). Conversely in a gel-mobility-shift assay with the S14 oligonucleotide, competition with an excess of the FAS 2 oligonucleotide led to the disappearance of the complex (Figure 4, lane 9). This shows that the S14 GIRE and FAS 2 oligonucleotide bind similar factors. Competition with the L4 oligonucleotide (glucose-responsive element) from the L-pyruvate kinase promoter at a 30-fold excess gave no competition with the FAS or S14 oligonucleotide (Figure 4, lanes 5 and 10), confirming that the affinity of the degenerated E-box of the L-PK gene for USF/MLTF factors is lower than a canonical one [9].

Concerning the binding sites for CTF/NF1, since the oligonucleotide FAS 2 contains the sequence 5'-TGGGC-3' but not the sequence 5'-GCCAG-3' it can also be concluded that the former sequence is not sufficient for CTF/NF1 binding whereas the latter is absolutely necessary. It can be pointed out that in the HSV thymidine kinase (tk) promoter a single sequence similar to the one present in the FAS gene, 5'-CGCCAG-3', is sufficient to bind NF1 with a good affinity [15].

Purified USF/MLTF and CTF1 bind to FAS 1 oligonucleotide

We performed an experiment with 43 kDa USF/MLTF prepared by *in vitro* translation of its cDNA clone. A gel-mobility-shift

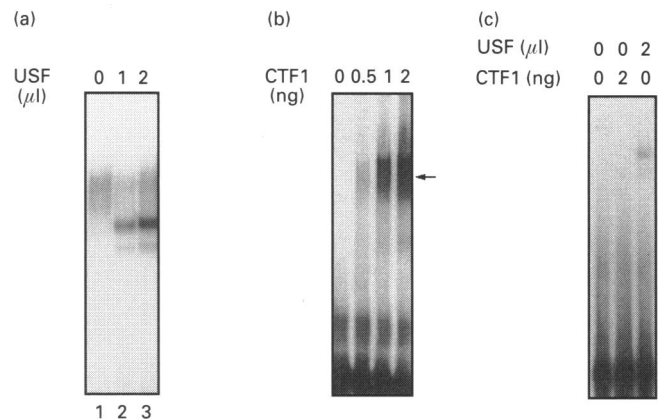


Figure 5 USF/MLTF and CTF1 binding on wild-type and mutated FAS 1 (+285 to +316) oligonucleotide

(a) The 32 P-labelled FAS 1 oligonucleotide was incubated with 1 or 2 μ l of the *in vitro* translated USF/MLTF lysate (lanes 2 and 3) or the unprogrammed lysate (lane 1). (b) The 32 P-labelled FAS 1 oligonucleotide was incubated with increasing amounts (0.5, 1 and 2 ng) of purified CTF1. The CTF1/FAS 1 complex is indicated by an arrow. (c) The 32 P-labelled FAS 1M oligonucleotide (mutated on the CTF/NF1) binding site was incubated with 2 ng of purified CTF1 or with 2 μ l of the *in vitro* translated USF/MLTF lysate.

assay using the FAS 1 oligonucleotide gave rise to the appearance of a complex in the presence of a translated USF lysate but not with the unprogrammed lysate (Figure 5a). Moreover, a similar experiment performed with the oligonucleotide mFAS 1, mutated on the USF/MLTF recognition site, did not give rise to a complex (results not shown). We also performed an experiment with purified CTF1. Purified CTF1 gave rise to the appearance of a complex in a gel-mobility-shift assay when using the FAS 1 oligonucleotide (Figure 5b) but not when using the oligonucleotide FAS 1M mutated on the CTF/NF1 recognition sequence (Figure 5c). FAS 1M was, however, still able to bind USF/MLTF (Figure 5c).

Since the consensus binding sites of the USF/MLTF and CTF/NF1 factors could be partially overlapping on the FAS 1 sequence, we investigated whether binding of the factor of one family precludes the binding of the factor of the other family or whether they can bind on the same oligonucleotide. We performed a gel-mobility-shift assay using USF/MLTF prepared by *in vitro* translation and purified CTF1. Clearly, when both USF and CTF1 were added together, a heavier complex appeared (Figure 6, lanes 6 and 7) when compared with USF alone (lanes 2 and 3) or CTF1 alone (lanes 4 and 5). This strongly suggests that the two factors are able to bind at the same time on the DNA sequence.

The bulk of these results thus demonstrates that as for the S14 and L-PK GIRE, a factor of the USF/MLTF family is involved in the complex formed with nuclear proteins of the FAS GIRE and that a second factor of the CTF/NF1 family is present.

DISCUSSION

The FAS gene is formed from 43 exons, of which the first and the last are non-coding, and 42 introns with a long (1200 bp) first intron. The promoter region has been sequenced up to -4860 bp [18,19]. It comprises an inverted CAAT box (-98 to -92), a sequence similar to a TATA box (-33 to -26) and a number of sequences similar to known elements (Sp1, AP-2, oestrogen/thyroid hormone). Recently, an element (-71 to -50) has

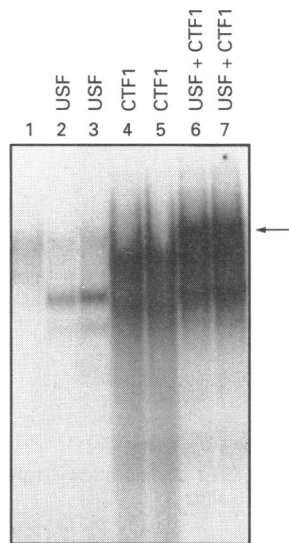


Figure 6 USF/MLTF and CTF1 are able to bind concomitantly to FAS 1 (+289 to +316) oligonucleotide

The 32 P-labelled FAS 1 oligonucleotide was incubated with the *in vitro* translated USF/MLTF lysate (lanes 2 and 3) or the unprogrammed lysate (lane 1), 0.5 ng or 1 ng of purified CTF1 (lanes 4 and 5) or *in vitro* translated USF/MLTF lysate and 0.5 or 1 ng of purified CTF1 (lanes 6 and 7). The heavier complex formed in lanes 6 and 7 is indicated by an arrow.

been found which interacts with specific nuclear protein(s) from liver and adipocytes and confers insulin responsiveness (2–3-fold stimulation) in an adipocyte cell-line [14]. It must be pointed out that in adipose tissue or in the liver, we have found no direct effect of insulin on FAS gene expression, suggesting that this insulin responsiveness could be linked to a differentiation rather than a regulatory process. In the first intron, in addition to the putative glucose response element (+292), consensus sequences for a glucocorticoid (+611), progesterone (+908) and fat-specific element (FSE) (+1005) have been detected [18].

We have shown in the present work that two DNase I hypersensitive sites are present when using the nuclei of liver, an organ which expresses the FAS gene, with a greater sensitivity, than when using nuclei of the spleen, an organ which does not express the FAS gene. One (HSS-2) is located in the proximal region of the promoter, near the transcription start site, and the other one (HSS-3) is in the first intron, near the putative GIRE region. None of them is sensitive to the nutritional conditions. These results differ from those of Roder et al. [20] since they do not find any hypersensitive site in the first intron nor around the transcription start site. The reasons for these discrepancies are unclear at the present time. HSS-1 found in the present study, a site with similar DNase I hypersensitivity in liver and spleen, could, however, correspond to the HSi-1 site in their study at –0.5 kb.

We have performed further studies in the region of the HSS-3 site and we have shown that nuclear proteins bind to a sequence which includes an element conferring glucose responsiveness to a heterologous promoter (S14) [8]. Indeed, a region protected by nuclear extracts from DNase I in footprint experiments (+290 to +316) is able to bind concomitantly USF/MLTF and CTF/NF1 factors. These factors are ubiquitous and it explains that similar footprints are found with nuclear extracts from liver and spleen.

We have now obtained evidence to think that we have indeed

identified the GIRE of the FAS gene. First, this element is present in a region hypersensitive to DNase I in a FAS-expressing tissue but not in a tissue which does not express FAS (spleen). Secondly, when inserted in an heterologous promoter, it confers glucose responsiveness [8]. Thirdly, it binds a factor of the USF/MLTF family, a striking similarity with the L-PK and S14 GIRE. Final evidence should stem from transfection experiments using the FAS gene itself.

As described for the L-PK and S14 GIRE [7,9,21], there was no qualitative or quantitative differences in the DNA–protein complex formed with nuclear extracts from suckling or weaned rats (present study) or from fasted and refed adult rats (results not shown), suggesting a post-translational modification of one factor of the complex after glucose stimulation. A recent study [22] suggests that a phosphorylation/dephosphorylation mechanism could be involved in the glucose signalling pathway of the S14 gene. Nevertheless, it is also possible that a protein which is part of the complex *in vivo* cannot be detected in the *in vitro* conditions used and it has been suggested for the GIRE that protein–protein interaction between a USF/MLTF factor and an accessory factor may provide for specific regulation [8].

Could NF1 have a role in this context? The S14 oligonucleotide which confers glucose responsiveness to a heterologous promoter does not bind other factors than USF/MLTF in gel-shift assays [7,8]. However, comparison of the DNase I-protected GIRE regions of the S14 and FAS genes which contain only one E-box, shows that whereas it extends 5' of the 5'-CACGTG-3' core for S14, it extends on the 3' end in the FAS gene [7]. This suggests that in addition to the binding of an MLTF nuclear factor, binding of another factor might also be involved in the protection of this region in the S14 gene. It has been indeed shown in footprint experiments that for the S14 gene, competition with Ad-MLTF oligonucleotide does maintain a footprint on the –1440 to –1457 sequence. Interestingly enough, at the 5' end of the S14 GIRE, a sequence from –1449 to –1444, 5'-TGCCAG-3' (coding strand) is present in a position corresponding to a footprint which was not competed with by Ad-MLTF [8]. When this sequence is mutated, the glucose effect on a shortened S14 promoter is decreased [8]. The same sequence binds a factor of the CTF/NF1 family in the promoter of the human β -globin [15]. Whether it also corresponds to a true NF1-binding site not detected in gel-shift experiments remains to be determined.

The FAS oligonucleotide +283 5'-GGCCGCTGTCAG-TGGGCGCC-3' +303 which confers the glucose responsiveness to a heterologous promoter [8] does not contain the recognition site +306 5'-GCCAG-3' +310 for NF1. This would tend to suggest that a member of the NF1 family is not necessary for the glucose effect. However, the construction used to test the FAS sequence [8] contains a putative NF1 binding site (5'-TGCCAA-3') situated 20 nt downstream of the FAS oligonucleotide and a site 5'-TTGGCGTCCTGTCAAT-3' 230 nt downstream of the FAS oligonucleotide for which the binding of a factor of the CTF/NF1 family has been shown [23]. Thus, a possible role for NF1 in the context of the FAS promoter still remains a matter of debate.

Association of USF/MLTF in a GIRE with another nuclear factor is reminiscent of the situation seen for the L-PK GIRE in which an association between USF/MLTF and HNF4 is necessary in order to obtain the full responsiveness to glucose [5].

We are indebted to Dr. Pognonec and Dr. Roeder for the kind gift of the USF/MLTF cDNA clone, to Dr. Mermod for the kind gift of purified CTF 1 and to R. Parsa for helpful discussions in the setting of the DNase I experiments. This work was supported by a grant (92 G 0499 01) from the 'Ministère de l'Enseignement Supérieur et de la Recherche'. F. F. is a recipient of a grant from the Institut Français pour la Nutrition.

REFERENCES

- 1 Foufelle, F., Gouhot, B., Pégurier, J. P., Perdereau, D., Girard, J. and Ferré, P. (1992) *J. Biol. Chem.* **267**, 20543–20546
- 2 Foufelle, F., Gouhot, B., Perdereau, D., Girard, J. and Ferré, P. (1994) *Eur. J. Biochem.* **223**, 893–900
- 3 Brun, T., Roche, E., Kim, K.-H. and Prentki, M. (1993) *J. Biol. Chem.* **268**, 18905–18911
- 4 Marie, S., Diaz-Guerra, M.-J., Miquerol, L., Kahn, A. and Iynedjian, P. B. (1993) *J. Biol. Chem.* **268**, 23881–23890
- 5 Bergot, M. O., Diaz-Guerra, M. J. H., Puzenat, N., Raymondjean, M. and Kahn, A. (1992) *Nucleic Acids Res.* **20**, 1871–1878
- 6 Liu, Z., Thompson, K. S. and Towle, H. C. (1993) *J. Biol. Chem.* **268**, 12787–12795
- 7 Shih, H. and Towle, H. C. (1992) *J. Biol. Chem.* **267**, 13222–13228
- 8 Shih, H. and Towle, H. C. (1994) *J. Biol. Chem.* **269**, 9380–9387
- 9 Vaulont, S. and Kahn, A. (1994) *FASEB J.* **8**, 28–35
- 10 Gorski, K., Carneiro, M. and Schibler, U. (1986) *Cell* **47**, 767–776
- 11 Maxam, A. M. and Gilbert, W. (1980) *Methods Enzymol.* **65**, 503–509
- 12 Gregor, P. D., Sawadogo, M. and Roeder, R. G. (1990) *Genes Dev.* **4**, 1730–1740
- 13 Armentero, M. T., Horwitz, M. and Mermod, N. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11537–11541
- 14 Moustaid, N., Sakamoto, K., Clarke, S., Beyer, R. S. and Sul, H. S. (1993) *Biochem. J.* **292**, 767–772
- 15 Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J. and Tjian, R. (1987) *Cell* **48**, 79–89
- 16 Raymondjean, M., Cereghini, S. and Yaniv, M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 757–761
- 17 Faisst, S. and Meyer, S. (1992) *Nucleic Acids Res.* **20**, 3–26
- 18 Amy, C. M., Williams-Ahlf, B., Naggert, J. and Smith, S. (1990) *Biochem. J.* **271**, 675–679
- 19 Beck, K. F., Schreglmann, R., Stathopoulos, I., Klein, H., Hoch, J. and Schweizer, M. (1992) *DNA Sequence* **2**, 359–386
- 20 Roder, K., Klein, H., Kranz, H., Beck, K. F. and Schweizer, M. (1994) *Gene* **144**, 189–195
- 21 Vaulont, S., Puzenat, N., Levrat, F., Cognet, M., Kahn, A. and Raymondjean, M. (1989) *Mol. Biol.* **209**, 205–219
- 22 Sudo, Y. and Mariash, C. N. (1994) *Endocrinology* **134**, 2532–2540
- 23 MacDougald, O. A. and Jump, D. B. (1991) *Biochem. J.* **280**, 761–767