CCAAT/enhancer binding protein regulates the promoter activity of the rat GLUT2 glucose transporter gene in liver cells

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The liver-specific expression of the GLUT2 glucose transporter gene is suppressed in cultured hepatoma cell lines as well as in hepatocytes in primary culture. To understand the underlying mechanism involved in this process, we analysed the rat GLUT2 promoter region. A DNase I footprinting assay with rat liver nuclear extract revealed eight protected regions within a −500 bp region of the GLUT2 promoter (sites A to H). Three of these sites (B, F and H) were occupied by transcription factors that are considerably enriched in liver cells compared with spleen or kidney. The proteins binding to these sites were investigated by a combination of DNase I footprinting assay and electrophoretic mobility-shift assay with the addition of specific oligonucleotide competitors and specific antibody against known transcription factors. As a result it was revealed that hepatocyte nuclear factor 3 binds to site B (−120 to −70), and CCAAT/enhancer binding protein α (C/EBPα) and C/EBPβ bind to site F (−375 to −356) and site H (−500 to −471). The binding of C/EBP to sites F and H was markedly decreased within 4 h when liver cells were subjected to primary culture, suggesting that C/EBP might be responsible for the decreased expression of GLUT2 in this process. In contrast, Western blot analysis revealed that C/EBPα began to decrease after 1 h of hepatocyte culture, and C/EBPβ was not changed significantly throughout the culture period, suggesting that C/EBP could be regulated at the transcriptional level as well as the post-translational level when hepatocytes were put in culture. To confirm the role of C/EBP in the regulation of GLUT2 promoter activity, sites F and H were ligated to a chloramphenicol acetyltransferase (CAT) reporter gene and co-transfected with a C/EBP expression vector into HepG2 cells. The co-expression of C/EBPα and C/EBPβ resulted in 9.1-fold and 3.8-fold increases of CAT activities in the site F-CAT and site H-CAT constructs respectively. These results indicate that C/EBPα and C/EBPβ regulate the promoter activity of the GLUT2 gene and might be responsible for the down-regulation of the GLUT2 gene when hepatocytes are subjected to primary culture.

INTRODUCTION

Cellular differentiation requires the precise and tightly controlled appearance and interplay of various transcription factors. The tissue-specific transcription factors as well as ubiquitous factors are involved in the initiation and maintenance of the transcription of a range of genes in a cell-specific manner. In recent years, several transcription factors that might be involved in the transcription of liver-specific genes, such as albumin, α-fetoprotein, α_1-antitrypsin, transthyretin and transferrin, have been discovered and characterized [1,2]. Hepatocyte nuclear factor 1 (HNF-1) [3,4], HNF-3 [5,6], HNF-4 [5,7] and the CCAAT/enhancer binding protein (C/EBP) [8–10] are the major liver-enriched *trans*-acting factors that have key roles in the transcriptional regulation of genes in liver cells. The possible roles of these transcription factors in the progression through different stages of hepatic cell commitment and differentiation are under discussion [11].

The role of C/EBP in tissue growth and differentiation has drawn special attention. C/EBP is expressed abundantly in liver and fat cells [12] and is considered to have a direct role in regulating the transcription of some enzymes involved in controlling the metabolic pathways of carbohydrates and lipids [13]. It has also been suggested that C/EBPα is a key factor in the differentiation of preadipocytes into fat cells [14]. A decrease in the level of C/EBPα mRNA species has been observed in primary cultured hepatocytes [15], suggesting a role for C/EBPα in the maintenance of the quiescent, mature state of adult hepatocytes [16].

One of the most important functions of the liver is the regulation of glucose metabolism. Hepatic glucose production and utilization involves the regulation of many kinds of gene, such as those for glucokinase, pyruvate kinase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and phosphoenolpyruvate kinase. The liver-enriched transcription factors also interact with *cis*-elements of these genes, which mediate the regulation of the genes' expression after hormonal or metabolic stimulation [17]. Hepatic glucose metabolism begins or ends with the movement of glucose into or out of the hepatocyte. This transport of glucose through the membrane of liver cells is accomplished by a specific facilitative transporter, GLUT2 [18,19]. This type of glucose transporter is expressed mainly in liver cells and has a higher K_m than other types [20]. Pancreatic β-cells also express GLUT2, although the regulation of this molecule differs in a manner related to the functions of two cell types [21,22].

Although many genes referred to as being liver-specific are transcribed exclusively or preferentially in liver, the hepatic expression of the GLUT2 gene shows some additional features not generally found in liver-specific genes. GLUT2 is expressed early in liver, before the appearance of glucokinase, increases

Abbreviations used: CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer binding protein; EMSA, electrophoretic mobility-shift assay; HNF, hepatocyte nuclear factor.

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throughout the developmental stages and becomes the main glucose transporter in fully differentiated liver cells [23]. However, GLUT2 fails to be transcribed in established hepatoma cells such as HepG2 [19,24] or in cultures of primary hepatocytes, in which the expression of other liver-specific genes is maintained [25–27]. This phenomenon suggests that the GLUT2 gene might be regulated not only in a tissue-specific manner but also by the differentiation status of liver cells. Therefore studies on the gene regulation of GLUT2 might provide an insight into the molecular mechanism of expression of the liver-specific gene. So far the interactions of transcription factors on many liver-specific genes have been studied to improve our understanding of tissue-specific expression; however, the molecular mechanism of transcriptional regulation of GLUT2 expression in liver cells is as yet largely unknown.

We previously reported the genomic organization and promoter sequence of the rat GLUT2 glucose transporter gene [28]. We also showed that the same transcriptional unit is used in the liver and in pancreatic β-cells, although the regulatory mechanism in response to a physiological signal in a living organism might be different. In the present study we identified several DNA–protein binding sites in this promoter and found that at least two sites were bound by liver-enriched transcription factor C/EBP. We also showed that C/EBP binding activity to GLUT2 promoter was decreased during primary hepatocyte culture; finally we showed that C/EBP could trans-activate the rat GLUT2 gene promoter in hepatoma cell line HepG2. This result implies a possible regulatory mechanism of GLUT2 promoter activity in relation to the hepatocyte differentiation status and the expression of C/EBP.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts from liver, spleen and kidney of male Sprague–Dawley rats were prepared as described in [29]. Protein concentration was determined by the method of Bradford [30]. The extracts were frozen in aliquots and stored at -70 °C.

DNase I footprinting assay

DNA fragments of approx. 300 bp were labelled in one strand and purified as follows. The promoter region was subjected to combined treatment with *Hae*III, *Hin*cII and *Alu*I to obtain overlapping promoter fragments; the resulting fragments were subcloned into pGEM-4Z vector. The promoter fragments were isolated by double digestion with *Eco*RI (or *Hin*dIII) and *Pst*I (or *Kpn*I) to obtain 5′-overhanging and 3′-overhanging ends. The fragments were labelled with Klenow fragment and [α-^{32}P]dATP, then purified by PAGE. DNA–protein binding reactions were performed with 50000 c.p.m. (approx. 1 ng) of probe per reaction in a solution containing 10 mM Hepes, pH 7.9, 60 mM KCl, 7% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly(dI-dC)·poly(dI-dC) and the indicated amount of nuclear extract. After 30 min of incubation on ice, 5 μl of DNase I, freshly diluted in a solution containing 10 mM Hepes, pH 7.9, 60 mM KCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$ and 7% (v/v) glycerol, was added to the reaction, which was then kept at room temperature for 2 min. Dilutions of DNase I ranged from 1:200 to 1:2000 of stock (10 units/μl), depending on the amount of protein in the reaction. Digestion reactions were stopped by the addition of 80 μl of a stop solution containing 20 mM Tris/HCl, pH 8.0, 20 mM EDTA, 250 mM NaCl, 0.5% SDS, 4 μg of yeast tRNA and 10 μg of proteinase K. The samples were incubated for 30 min at 45 °C, extracted with phenol/chloroform, precipitated with ethanol and resuspended in formamide dye. The samples were resolved in 6% (w/v) polyacrylamide/7 M urea sequencing gel. The protected regions were mapped with reference to the migration of Maxam–Gilbert A+G sequencing products. For the competition assay, 1.5 pmol of oligonucleotides for specific transcription factors were added to the binding reactions; 40 μg of nuclear extract was used per reaction. The oligonucleotides for HNF-1 [31], HNF-3 [5], HNF-4 [7], HNF-5 [32], C/EBP [9] and NF-Y [33] were synthesized; AP-1, Oct-1 and TFIID consensus oligonucleotides were purchased from Promega.

Electrophoretic mobility-shift assay (EMSA) and supershift assay

The oligonucleotides used in these assays were as follows: site F, 5′-GCCTCTACTCTTATCTGACTCAACAGG-3′; site H, 5′-AGTTAACAATCTTGATTTCCACATCACAAACGTGCA-3′. The formation of protein–DNA complexes was induced by the same procedure as for the DNase I footprinting assay. After 30 min on ice, the samples were resolved in 4% (w/v) non-denaturing polyacrylamide gel polymerized in 0.25 × TBE (where TBE is Tris/borate/EDTA). For competition assays, a 100–200-fold molar excess of various unlabelled competitor DNA species was added to the reaction mixture before the addition of nuclear extracts. For the supershift assay, 1 μg of anti-C/EBPα, anti-C/EBPβ or anti-C/EBPδ (SantaCruz Biotech) was added to the DNA–protein binding reaction. The dried gels were exposed to X-ray film at -70 °C with an intensifying screen.

Primary hepatocyte culture and nuclear extract preparation

Hepatocytes were isolated from male Sprague–Dawley rats (approx. 200 g) by the collagenase perfusion method [34]. Dissociation into individual hepatocytes was performed in Williams's E medium containing 5 mM glucose, 0.1 i.u/ml insulin, 10 nM dexamethasone, 10% (v/v) fetal calf serum, 26 mM sodium bicarbonate, 100 i.u./ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The cells were filtered through four layers of gauze and centrifuged at 50 ***g*** for 4 min. The pellets were washed twice with 40 ml of medium and subjected to Percoll density-gradient centrifugation. The pellets, which were composed of viable hepatocytes, were suspended in modified Williams's E medium described above. For each hepatocyte preparation the cell viability was estimated by the exclusion of Trypan Blue. Hepatocytes (10^8) were plated on 10 cm dishes in a final volume of 10 ml of medium and maintained at 37 °C in an air/CO_2 (19:1) atmosphere. Nuclear extracts were prepared by the method of Schreiber et al. [35] at the designated time point of hepatocyte maintenance.

Construction of plasmids

The region containing site A (+56 to +189) was obtained by digesting the promoter with *Acc*I and *Eco*RI, subcloned into pCAT3 basic vector and named pCAT(+56/+189). pCAT-F(+56/+189) or pCAT-H(+56/+189) was constructed as follows. Site F and site H double-stranded oligonucleotides were subcloned into pT7Blue(R) vector, and the resulting recombinant DNA species were treated with *Kpn*I and *Xba*I to obtain site F or site H. The fragments were inserted into the *Kpn*I and *Nhe*I site of pCAT(+56/+189). The constructs were confirmed by DNA sequencing in both directions.

To study the role of C/EBP on GLUT2 promoter activity, pMSV-C/EBPα or pMSV-C/EBPβ (kindly donated by Dr. S. McKnight), which are the overexpression vectors of C/EBPα or C/EBPβ, was used for transfection.

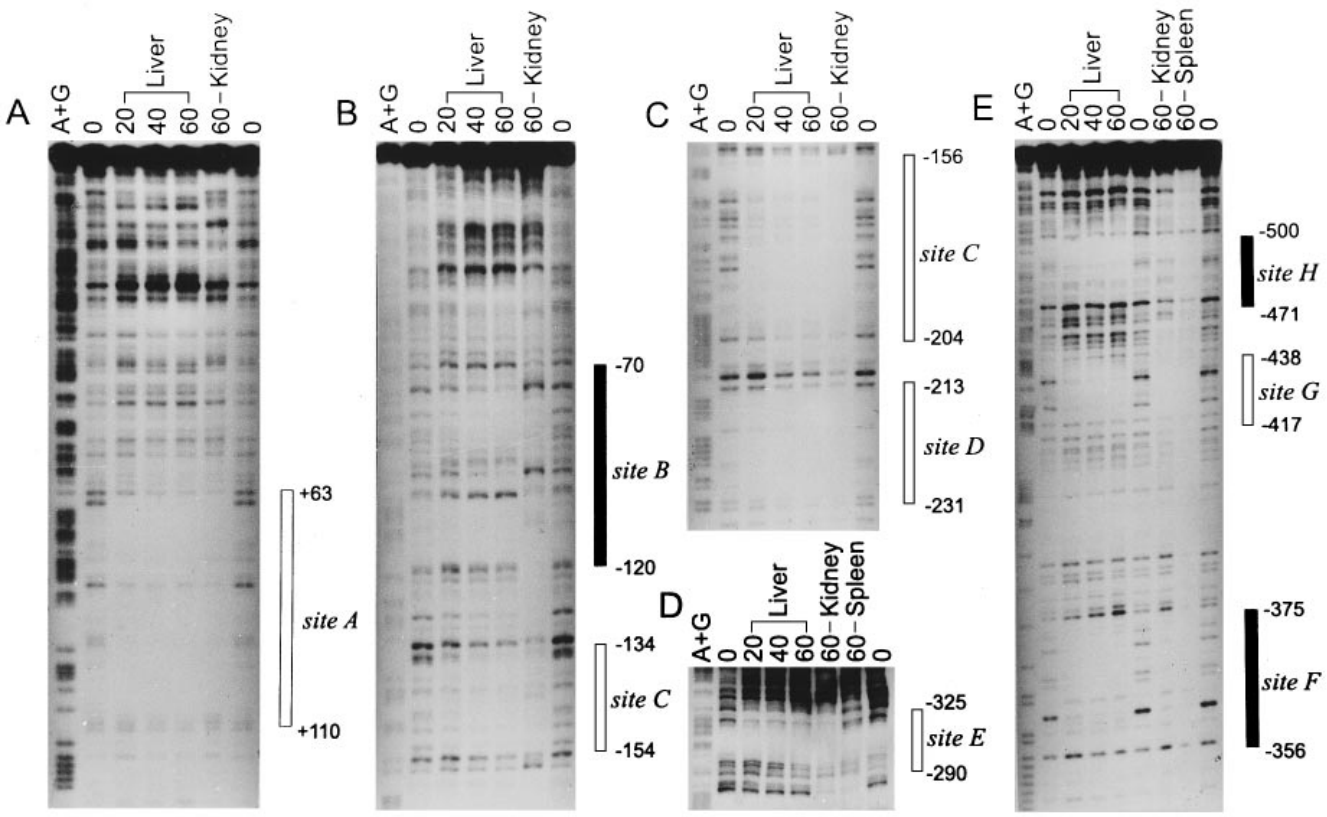

Figure 1 DNase I footprinting assay of the rat GLUT2 promoter region

DNase I footprinting assay was performed as described in the Materials and methods section with the indicated amounts (in μg) of nuclear proteins from liver, kidney and/or spleen. The probes used were ^{32}P-end-labelled fragments of: +190 to −149, coding strand (**A**); +2 to −275, non-coding strand (**B**); −150 to −444, non-coding strand (**C**); −150 to −444, coding strand (**D**); and −328 to −552, coding strand (**E**). The lane headed A + G contained Maxam–Gilbert sequencing products. Nucleotide numbers refer to the positions with respect to the previously reported promoter region [28]; position −1 represents the nucleotide preceding the start of transcription. The protected regions are indicated by boxes with their names and positions (sites A to H); liver-specific protected regions are marked by black boxes. The experiments were also performed with labelling of the opposite strand of each probe to confirm the protected regions (results not shown).

Cell culture, DNA transfection and chloramphenicol acetyltransferase (CAT) assay

Cells (10^5) of the human hepatoma cell line HepG2 were spread in 6 cm plates and cultured at 37 °C in Earle's minimal essential medium (MEM) with 10 % (v/v) fetal calf serum. After 1–2 days of culture, various CAT constructs were transfected with lipofectin reagent. In brief, 3 μg of CAT constructs, 1.5 μg of pMSV-C/EBPα and/or pMSV-C/EBPβ, 1 μg of pCMV-β-galactosidase and 15 μl of lipofectin in 2 ml of OPTI-MEM I medium lacking serum were mixed and added to the HepG2 cells. To transfect constant amounts of DNA, sample DNA species were supplemented with an appropriate control vector. After a 15 h incubation period the remaining liposome–DNA complexes were removed and the medium was replaced with serum-containing medium; 72 h after transfection, cells were scraped and resuspended in 100 μl of 0.25 M Tris/HCl, pH 7.8, and disrupted by freezing and thawing. The reactions were centrifuged at 10000 ***g*** for 30 s, after which the extracts were collected. Aliquots of 30 μl were used for β-galactosidase assay, whereas aliquots of 2 μl were used for measurement of protein concentration. The remaining aliquots were heat-treated at 65 °C for 10 min. For the measurement of CAT activities, the amounts of cell extract to be loaded on a TLC plate were normalized with respect to β-galactosidase activities and protein concentrations. The CAT activities were expressed as percentage conversion into acetylated chloramphenicol from chloramphenicol.

Western blot analysis

Nuclear extracts (30 μg) prepared from indicated time points of primary hepatocyte culture were subjected to SDS/PAGE [12 % (w/v) gel], transferred to nitrocellulose membranes and detected by enhanced chemiluminescence (ECL, Amersham) [36] with anti-C/EBPα and anti-C/EBPβ as primary antibodies (SantaCruz Biotech).

Statistical analysis

All transfection studies were performed in three separate experiments in which triplicate dishes were transfected. The results are given as means ± S.E.M. Statistical analysis was performed with SigmaStat software (Jandel Scientific).

RESULTS

Identification of the protein-binding sites in the rat GLUT2 gene promoter

Previously we cloned the promoter of the rat GLUT2 gene [28]. In the present study we performed DNase I footprinting experi-

Figure 2 Summary of protein-binding regions in the rat GLUT2 promoter

The transcription initiation site is designated +1 [28]. The boxed areas represent the protected regions of rat GLUT2 promoter identified by DNase I footprinting assay with rat liver nuclear extract. The known consensus sequences are underlined and their names are given underneath.

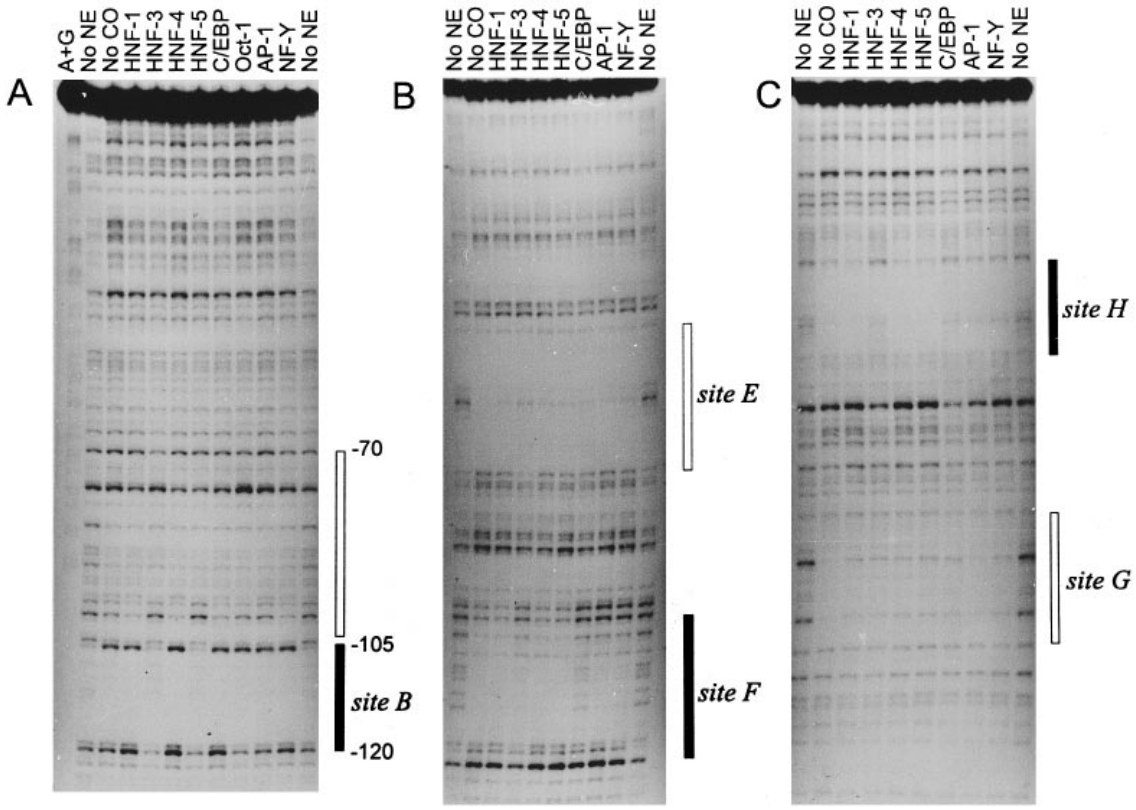

Figure 3 DNase I footprinting competition experiments

DNase I footprinting assay with liver nuclear extracts (NE, 40 μg of protein) in the absence or presence of a 200-fold molar excess of competing oligonucleotides (CO) for known transcription factors, as indicated above each lane. The probes used were ^{32}P-end-labelled fragments of +2 to −275 (**A**) and −328 to −552 (**B** and **C**). The lane marked A+G was the Maxam–Gilbert sequence ladder. The protected regions are indicated by open boxes with their names and positions, and liver-specific protected regions are marked by filled boxes.

ments to localize the DNA sequences able to interact with *trans*-acting factors within the region +190 to −732 nt. The experiment with rat liver nuclear extracts showed eight protected regions, which we designated as follows: site A (+63 to +110), site B (−120 to −70), site C (−204 to −134), site D (−231 to −213), site E (−325 to −290), site F (−375 to −356), site G (−438 to −417) and site H (−500 to −471) (Figures 1 and 2). DNase I footprinting experiments with rat kidney or spleen nuclear extracts showed different patterns of protection in three regions (Figure 1). Therefore sites B, F and H were regarded as liver-enriched *trans*-acting protein-binding sites. Computer analysis of these sites revealed that site B was compatible with

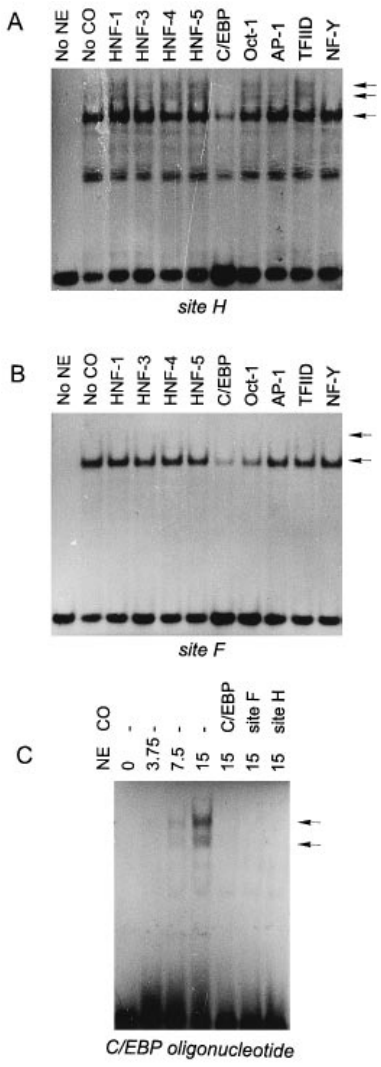

Figure 4 EMSA of sites H and F

(**A**, **B**) EMSA with site H (**A**) or site F (**B**) oligonucleotide as a probe with liver nuclear extract (NE; 10 μg of protein per reaction) was performed in the absence or presence of 100-fold molar excess of competing oligonucleotides (CO) for known transcription factors, as indicated at the top of each lane. (**C**) EMSA with C/EBP consensus [9] as a probe. ^{32}P-labelled C/EBP oligonucleotide was incubated with the indicated amounts (in μg) of liver nuclear extract (NE) in the absence or presence of a 100-fold molar excess of unlabelled C/EBP, site F or site H oligonucleotide (CO), as indicated at the top of the lanes.

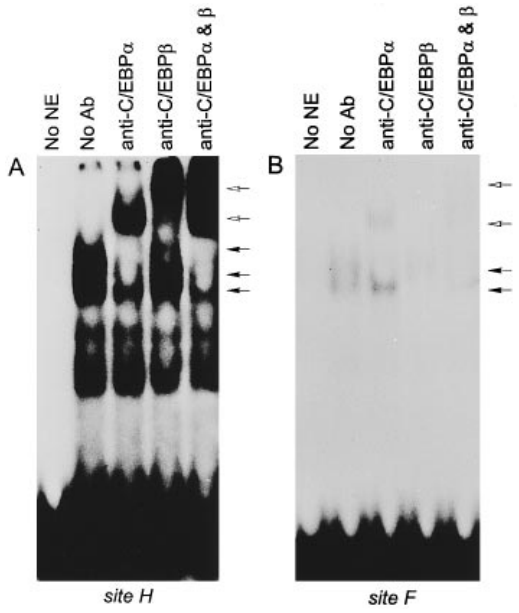

Figure 5 Supershift assay of sites H and F with anti-C/EBP antibodies

Labelled site H (**A**) or site F (**B**) was incubated with rat liver nuclear extract (NE, 10 μg) in the absence or presence of anti-C/EBPα and/or anti-C/EBPβ antibody (Ab), as indicated above the lanes, and the resulting complexes were subjected to electrophoresis in 4% (w/v) non-denaturing gel. The DNA–protein complexes are indicated by filled arrows; the positions of supershifted DNA–protein–antibody complexes are indicated by open arrows. No supershift was observed in the experiment with the addition of anti-C/EBPδ antibody to both sites (results not shown).

binding sites for the HNF-3, site F had a perfect matched sequence for AP-1, and site H had the consensus sequences for both C/EBP and Oct-1 (Figure 2). Although site A did not contain a TATA box, it contained an initiator (Inr) sequence [37] that is known to be a binding site for general transcription factors including RNA polymerase. The protein binding sites are summarized in Figure 2.

Determination of the transcription factors bound to liver-specific DNA elements in rat GLUT2 promoter

To investigate the nature of liver-specific proteins binding to sites B, F and H, we first performed a competition assay with oligonucleotides corresponding to binding sites for known transcription factors. As shown in Figure 3(A), the addition of HNF-3 or HNF-5 competitors markedly inhibited the protection of the -105 to -120 region in site B. In this regard, the HNF-3 family recognizes two apparently unrelated DNA sequence motifs, and one of them is included in the binding site consensus sequence for HNF-5 [32]. Roux et al. [38] reported that the sequences for HNF-3 displayed a common feature and moreover that the DNA-binding specificities of HNF-3 and HNF-5 were identical. Our results also showed that HNF-3 and HNF-5 bound to the same sequence of the rat GLUT2 promoter. Another liver-specific protected region, site F, competed with C/EBP consensus oligonucleotide (Figure 3B). This was somewhat unexpected because computer analysis showed that site F matched with the AP-1 binding site (Figure 2). The addition of AP-1-binding site, however, failed to compete with protein binding for site F (Figure 3B). Site H had the sequences for binding C/EBP or Oct-1, but only C/EBP oligonucleotide outcompeted the protection (Figure 3C). These results were further confirmed by EMSA experiments: EMSA of site H revealed three shifted bands (Figure 4A), and slowly migrating bands were displaced not by Oct-1 competitor but by C/EBP competitor. Site F also showed C/EBP binding, but the binding was less strong than that exerted by site H (Figure 4B). This result was confirmed by the experiment with ^{32}P-labelled C/EBP oligonucleotide as a probe. The unlabelled form of site F or site H, as well as C/EBP oligonucleotide itself, could outcompete the binding of nuclear proteins to C/EBP probe (Figure 4C).

Binding of C/EBPα and C/EBPβ to sites H and F

The binding of C/EBP to the GLUT2 promoter was further investigated by a supershift experiment with antibodies against C/EBPα, C/EBPβ and C/EBPδ. As shown in Figure 5, super-

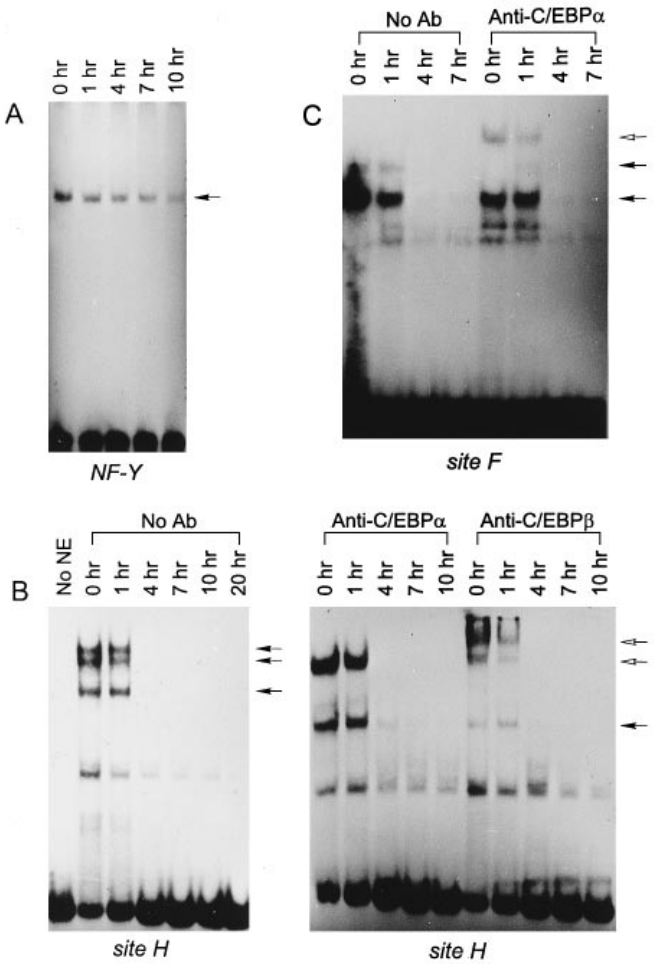

Figure 6 Binding of C/EBP to sites H and F decreased during primary hepatocyte culture

Hepatocytes were cultured for 10 h and nuclear extracts were prepared at the indicated time points as described in the Materials and methods section. For EMSA, 10 μg of nuclear extracts (NE), prepared at each time point of culture as indicated, was incubated with ^{32}P-labelled NF-Y [33] consensus double-stranded oligonucleotide (**A**), site H (**B**) or site F (**C**) as a probe. For supershift assay, the nuclear extracts were incubated with anti-C/EBPα or anti-C/EBPβ antibody (Ab) for 10 min before the addition of the probe. The reaction mixtures were subjected to non-denaturing PAGE (4% gel). The DNA–protein complexes are indicated by filled arrows; the positions of supershifted DNA–protein–antibody complexes are indicated by open arrows.

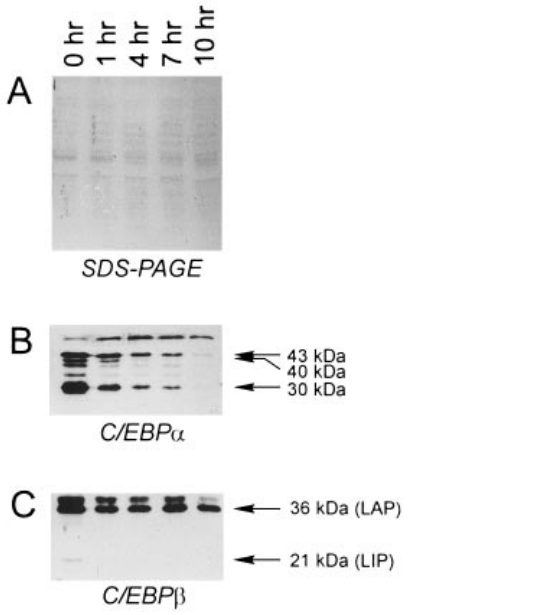

Figure 7 Western blot analysis of C/EBP proteins during primary hepatocyte culture

Primary hepatocyte nuclear extracts (30 μg) prepared from the indicated time points of culture were resolved by SDS/PAGE (12% gel) and either stained with Coomassie Brilliant Blue (**A**) or transferred to a nylon membrane. The membrane was blocked with 5% (w/v) non-fat dried milk, and incubated with 1:2000 diluted anti-C/EBPα (**B**) or anti-C/EBPβ (**C**) (SantaCruz Biotech) as a primary antibody. Horseradish peroxidase-conjugated anti-(rabbit IgG) horse radish peroxidase conjugated antibody was used as a secondary antibody (diluted 5000-fold), and detected with an enhanced chemilumineiscence (ECL) system. Arrows indicate the protein components of C/EBPα or C/EBPβ. Abbreviations: LAP, liver-activating protein; LIP, liver-inhibitory protein.

shifted bands appeared when anti-C/EBPα or anti-C/EBPβ antibody was added to the binding reaction with site H or site F. The supershifted bands were more evident in site H.

Binding of C/EBP to rat GLUT2 promoter decreased during culture of primary hepatocytes

During primary culturing of hepatocytes, the cells were harvested for nuclear extract preparation at the indicated time points. First, EMSA experiments were performed with an NF-Y oligonucleotide, which binds the ubiquitous NF-Y proteins as an internal control of protein [33,39]. The binding of DNA by this transcription factor was not changed significantly during a 10 h culture period (Figure 6A). In contrast, C/EBP binding to sites H and F showed a marked change between 1 and 4 h after the hepatocytes were put in culture (Figures 6B and 6C). These results suggested that decreased C/EBP expression might be partly responsible for the GLUT2 gene expression known to be decreased when hepatocytes were subjected to primary culture.

To correlate the change of protein amount in C/EBP with its binding to site H or site F during primary culture, the nuclear extracts used for EMSA at the indicated incubation times were subjected to Western blot analysis. During this period, total protein concentrations remained unchanged (Figure 7A). As reported previously, immunoblotting with anti-C/EBPα antibody showed three major protein bands of 43, 40 and 30 kDa [40]. These proteins decreased markedly after 1 h of culture, and showed a gradual decrease after that (Figure 7B). Liver-activating protein (36 kDa) and liver-inhibitory protein (21 kDa), which constitute C/EBPβ, did not change notably (Figure 7C).

Overexpression of C/EBPα and C/EBPβ resulted in an increase of promoter activity in HepG2 cells

The GLUT2 gene has been reported to be suppressed in hepatoma cell lines such as HepG2 cells as well as in primary cultured hepatocytes. From the experiments described above, the decreased binding of C/EBPs to GLUT2 promoter in these cells was thought to be responsible for decreased GLUT2 gene expression; if this were so the overexpression of C/EBP should restore the GLUT2 promoter activity. To examine this possibility we constructed the plasmid containing site H or site F in front of a minimal promoter of the GLUT2 gene in pCAT3-basic vector, and examined their activity when C/EBPα and/or C/EBPβ was overexpressed in HepG2 cells. The CAT reporter assay showed definite trans-activation by C/EBP on sites H and F, and the

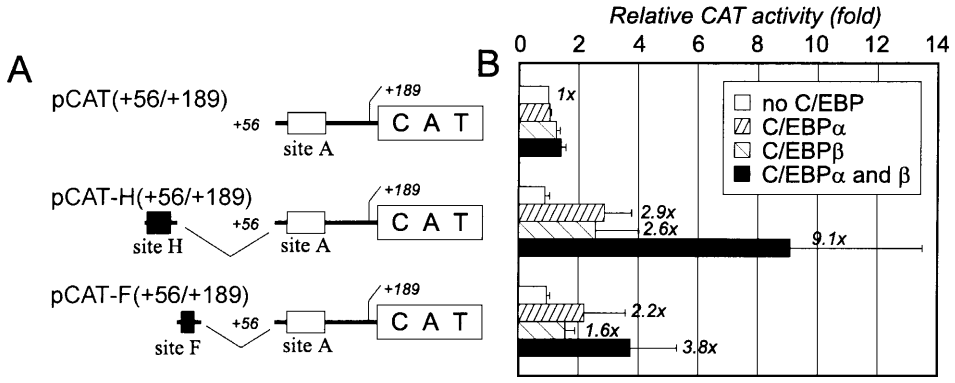

Figure 8 Effect of C/EBP overexpression on the GLUT2 promoter activity

(**A**) Plasmid constructs used in CAT assay. pCAT(+56/+189) was used for the minimal promoter element, and pCAT-H(+56/+189) and pCAT-F(+56/+189) were constructed as described in the Materials and methods section. (**B**) Effects of C/EBP overexpression on the promoter activity of the GLUT2 promoter. HepG2 cells (10^5 cells) were co-transfected with each CAT construct, with or without pMSV-C/EBPα and/or pMSV-C/EBPβ, as indicated; 72 h after transfection, cells were scraped and the CAT activities were measured by TLC. All results were normalized with respect to β-galactosidase activities and protein concentrations, and were calculated as percentage conversions into acetylated chloramphenicol from chloramphenicol. The result is shown as relative CAT activities compared to that of pCAT(+56/+189) without C/EBP overexpression.

interaction of C/EBPα and C/EBPβ was found to be synergistic in this promoter (Figure 8). The activities of the site H-CAT and site F-CAT constructs were increased 9.1-fold and 3.8-fold respectively when pMSV-C/EBPα and pMSV-C/EBPβ were co-transfected in HepG2 cells, suggesting that C/EBP is one of the main regulators of liver-specific expression of the GLUT2 gene.

DISCUSSION

When hepatocytes were cultured *in vitro*, GLUT1 mRNA increased markedly within a few hours with a significant decrease in GLUT2 during the initial 24 h culture period, whereas albumin mRNA remained unchanged during this short-term culture [27]. In this cellular adaptation process, specific elements responsible for the down-regulation of GLUT2 mRNA in cultured or de-differentiated cells were unknown. The interest in the present study was to investigate the interplay between the DNA-binding protein and the GLUT2 promoter, which is involved in the suppression of GLUT2 gene expression in this phenomenon. The pattern of decrease in GLUT2 expression in the time course of the culture can vary with the conditions of culture, so we maintained the same culture environment and same nuclear extracts throughout the study. The GLUT2 promoter has two binding sites for the liver-enriched transcription factor C/EBP, and the binding of C/EBP to the GLUT2 promoter was significantly decreased during short-term culture of the hepatocyte, suggesting that this decrease in interaction is responsible for the decreased expression of GLUT2 in this process.

The fact that C/EBP regulates GLUT2 promoter activity provides a significant insight into hepatocyte differentiation. In liver, C/EBPα mRNA has been observed to decrease when hepatocytes are grown in culture or during liver regeneration [15], as well in as a hepatoma-derived cell line such as HepG2 [41]. Taking this together with the fact that GLUT2 mRNA was down-regulated during primary hepatocyte culture [27], we suggest a special role for C/EBPα in the appearance of GLUT2 in the hepatocyte differentiation programme as well as in the regulation of GLUT2 gene transcription in response to physiological stimuli in fully differentiated hepatocytes. It was also reported that C/EBPβ controls the down-regulation of albumin gene transcription during liver regeneration [42]. It was suggested that C/EBPβ, while enhancing the transcription of cell-cycle-related genes and controlling the G_1/S checkpoint, down-regulates albumin synthesis to prepare the hepatocyte for entry into the cell cycle during liver regeneration. In the present study, the maximal transcriptional activity of the GLUT2 gene promoter was observed when pMSV-C/EBPα and pMSV-C/EBPβ were co-transfected. Unlike the albumin gene, increasing the amount of pMSV-C/EBPβ did not yield a significant difference between experimental groups (results not shown), suggesting that C/EBPα and C/EBPβ trans-activate the GLUT2 promoter in a synergic manner.

The present study indicates the possible role of C/EBP in the decreased expression of the GLUT2 gene in primary culture. C/EBP binding activity to the GLUT2 promoter was strikingly decreased when the liver cell was subjected to primary culture. The binding of C/EBPα or C/EBPβ to sites F and H was decreased after 4 h of culture, whereas the amount of C/EBPα protein itself began to decrease after 1 h of culture. Moreover, little change in C/EBPβ protein content was observed during the culture period. This discrepancy could be explained in part by post-translational modification. It was reported that the phosphorylation status and the DNA-binding activity of the transcription factor could be regulated on terminal differentiation of the liver [43]. This implies the possibility that many transcription factors might be regulated by changes of the protein modification in the differentiation process. In fact, C/EBPβ is activated by phosphorylation, leading to the inactivation of an inhibitory domain [11,44], which also affects the DNA-binding activity [45]. The role of phosphorylation of C/EBPα in the transcriptional control of genes is more obscure, although the phosphorylation of C/EBPα by protein kinase C or MAP kinase resulted in an attenuation of site-selective DNA binding *in vitro* [46]. Our result indicates that the regulation of C/EBP could be achieved by its protein expression as well as its modification in controlling GLUT2 promoter activity when hepatocytes were put in culture, and possibly in the differentiation process. In other words, the phosphorylation status of C/EBP might be altered in response to extracellular signals during primary hepatocyte culture, leading to a decreased binding of C/EBP to its cognate sequence.

Another possible mechanism that affects the DNA-binding

activity of C/EBP to the GLUT2 gene is the interaction with other transcription factors. C/EBP belongs to the basic leucine zipper (bZip) family and forms a homodimer or heterodimer for DNA binding [47]. Analysis of the C/EBP sites within the diverse population of genes shows a significant degree of variability between DNA sequences [11]. Regulation of the C/EBP-DNA binding activity can be affected by the ability of each C/EBP protein to dimerize with other members of the C/EBP family and also with other nuclear factors [40]. In the present study, even though both sites F and H can bind C/EBP, their DNA sequences are quite different (Figure 2). Moreover, their C/EBP-binding affinities (Figures 4 and 5) and transactivation potentials (Figure 8) were also different, suggesting that the two sites (F and H) could be regulated differently by C/EBP depending on the cellular status.

We demonstrated the presence of C/EBP-binding sites in the rat GLUT2 promoter. In addition, we showed that the expression of C/EBPα and C/EBPβ restored the transcriptional activity of the GLUT2 gene, which was suppressed in HepG2 cells. In conclusion, lower levels of C/EBP in cultured hepatocytes or established cell lines might be responsible for decreased GLUT2 gene expression.

Previous studies of the physiological expression of GLUT2 in liver cells have been limited because the expression is not maintained in cultured hepatocytes or liver-derived cell lines. Recently it was reported that a cell line established by other researchers could express GLUT2 with the maintenance of the glucose responsibility [48]. Although the cell line was not physiological in terms of insulin response [49], it will nevertheless be interesting to determine the role of C/EBP in such a cell line. A study of whether C/EBP can affect the pattern of expression of the endogenous GLUT2 gene will also be interesting if the roles of other transcription factors, including HNF-3, are clarified as a whole. Our results will provide an insight into the molecular mechanisms involved in the regulation of GLUT2 expression during the development and differentiation of the liver cell.

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