Characterization of the human liver fructose-1,6-bisphosphatase gene promoter

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Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11), an important gluconeogenic enzyme, catalyses the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and P_i. Enzyme activity is mainly regulated by the allosteric inhibitors fructose 2,6-bisphosphate and AMP. Although some observations about hormonal regulation of the enzyme have been published, the FBPase promoter has not been studied in detail. Here we report an *in vitro* characterization of the FBPase promoter with respect to the elements that are required for basal promoter activity. Transient transfection of H4IIE rat hepatoma cells, combined with site-directed mutagenesis, demonstrated that an enhancer box, three GC-boxes and a nuclear factor κ B (NF- κ B)-binding

element are important for hepatic FBPase promoter activity. These elements are found in the region located between –405 to +25 bp relative to the transcription start site. Electrophoretic-mobility-shift assays and supershift analysis confirmed that upstream stimulatory factor 1 (USF1)/USF2, specificity protein 1 (Sp1)/Sp3 and NF- κ B respectively bind to these sites. The present study provides the basis for a more comprehensive screening for mutations in FBPase-deficient patients and for further studies of the transcriptional regulation of this gene.

Key words: FBPase, nuclear factor κ B, specificity protein, upstream stimulatory factor.

INTRODUCTION

Fructose-1,6-bisphosphatase (FBPase) is one of the seven enzymes that catalyse unidirectional reactions of hepatic glucose metabolism. Three substrate cycles, i.e. the glucose/glucose 6-phosphate, fructose 6-phosphate/fructose 1,6-bisphosphate and phosphoenolpyruvate/pyruvate cycles, encompass reactions catalysed by different enzymes in the glycolytic and gluconeogenic pathways [1]. FBPase catalyses the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and P_i, a rate-controlling reaction of hepatic and renal gluconeogenesis. Acute regulation of enzyme activity occurs primarily by the allosteric inhibitor fructose 2,6-bisphosphate. The bifunctional enzyme (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase) is responsible for synthesis and hydrolysis of this regulator. Glucagon, acting via cAMP, regulates the synthesis/hydrolysis ratio of this regulator and subsequently affects rates of gluconeogenesis/glycolysis via its target enzymes FBPase/phosphofructokinase (PFK). The hormonal regulation of the FBPase gene itself has not been studied in detail, although some observations have been published. For example, cAMP has a stimulatory effect on FBPase mRNA levels, and activation of the FBPase gene by 1,25-dihydroxyvitamin D₃ during monocytic differentiation has been reported [2]. Also, FBPase mRNA levels increase in diabetic rats and mice [3,4], which may be explained by the response of this gene to increased intracellular cAMP and to low plasma insulin levels. However, no evidence for FBPase as a candidate gene for diabetes was found in affected families using linkage analysis [5].

FBPase deficiency is an autosomal recessive condition, characterized principally by episodes of hypoglycaemia and metabolic acidosis (reviewed in [6,7]). There is a high mortality rate during

the neonatal period, and FBPase deficiency may be one of the inherited metabolic diseases responsible for up to 25 % of sudden-infant-death-syndrome ('SIDS') cases [8,9]. Point mutations in the coding sequence of the FBPase gene in patients with FBPase deficiency have been reported [10,11]. Splice defects and large deletions in the gene may also cause this defect (B. Herzog, unpublished work). El-Maghrabi et al. [12] proposed that mutations either in the promoter or in the fructose-2,6-bisphosphatase half of the bifunctional enzyme may also lead to this disease. We decided, therefore, to characterize the FBPase promoter so that regions of this gene that might contribute to FBPase deficiency could be identified.

Two isoforms of FBPase exist. We cloned and studied the human liver FBPase gene (*FBP1*) promoter. There is a second isoform of FBPase, referred to as muscle-FBPase or FBP2 [13,14] that is encoded by a separate gene [14a] for which the physiological function remains unclear. Here we report an *in vitro* characterization of transcription factors that are involved in the basal promoter activity of human liver *FBP1*. These data provide a basis for a more precise screening of the *FBP1* for disease-causing gene mutations and for further studies of the regulation of *FBP1*.

EXPERIMENTAL

Cloning and sequencing

The *FBP1* gene promoter was cloned using the Genome Walker System (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions. Subcloning for sequence analysis was performed in the pGEM-T vector (Promega, Madison, WI,

Abbreviations used: AP2, activator protein; E-box, enhancer box; EMSA, electrophoretic mobility-shift assay; FBPase, fructose-1,6-bisphosphatase; *FBP1* and *Fbp1*, human and rodent liver FBPase genes respectively; *FBP2* and *Fbp2*, human and rodent muscle FBPase genes; HNF, hepatocyte nuclear factor; NF- κ B, nuclear factor κ B; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; Sp, specificity protein; USF, upstream stimulatory factor.

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Table 1 Oligonucleotides used in the present study

Oligonucleotides used for generating the 5' deletion series or used for the Quick Change site-directed mutagenesis protocol (Stratagene) are listed. Oligonucleotides used in EMSA and competition studies are also listed and are named according to their positions in the promoter or according to the affected binding element. Abbreviations: comp., competitor; Mut, mutant; WT, wild-type.

Name	Sequence
Second 5' deletion series	
—410	CGGGGTACCCTGCAGAGCTAAAGCCAGGC
—360	CGGGGTACCAGCTGGTGGCATTGAGCAAG
—310	CGGGGTACCAGATTTTGGAGGAAACAGTCA
—260	CGGGGTACCCGAGTCCCGCTCCACAGTGC
—207	CGGGGTACCGGGGCGACAGCCAGGAAGT
—158	CGGGGTACCGGGTTGGGAGACCGCAGGGA
—111	CGGGGTACCAACAGGCGGAGCCGCGGTCT
—77	CGGGGTACCGCGGGGCGCAAGAGTGTTC
Re1	CATGGTGGCTTTACCAACAG
Site-directed mutagenesis (sense strand)	
MEB1/2	CAGAGCTAAAGCCAGGCTGCAGTGACTGGCTGCGGGTTTCG
M1/2	GCAGGGCGCGTGGGAAAGGGGGTTGGGAGA
SpMut1/2	GTCTGGTCCGCGGGGTGCAGTGCAGGCAAGAGTGTCCCG
M3/4	AGAGTGTTCGCGGAAAGGGGGCCGACCC
M22/23	GGGTGGGAGACCGCAGCTCGGATCCCCGACCTTGC
Oligonucleotides used in gel-shift arrays (sense strand)	
415/385	AGGAGCTGCAGAGCTAAAGCCAGGCCATGT
405/375	GAGCTAAAGCCAGGCCATGTGGACTGGCTG
395/365	CAGGCCATGTGGACTGGCTGCGGGTTTCGC
385/355	GGACTGGCTGCGGGTTTCGCTGCCTAGCTG
375/345	CGGGTTTCGCTGCCTAGCTGGTGGCATTGA
110/80	ACAGGCGGAGCCGCGGTCTGGTCCGCGGGG
100/70	CCGCGGTCTGGTCCGCGGGGTAGGCGGGGC
90/60	GTCCGCGGGGTAGGCGGGGCGCAAGAGTGT
80/50	TAGGCGGGGCGCAAGAGTGTTCGCGGGGC
70/40	GCAAGAGTGTTCGCGGGGCGGGGGCCGA
60/30	TCCCGGGGCGGGGGCCGACCCGCTCTA
200/170	CAGCCAGGAAGTCTAGGGGCGGCGAGGCG
190/160	AGTCTAGGGGCGGCGAGGCGCGTGGGGGC
180/150	CGGCGAGGCGCGTGGGGCGGGGTTGGG
170/140	GCGTGGGGCGGGGTTGGGAGACCGCAGG
160/130	GGGGTTGGGAGACCGCAGGAGGATCCCC
EMSA/supershifts (sense strand)	
WT E-box	396/365
Mut E-box	TGCAGTGCAGGACTGGCTGCGGGTTTCGC
Consensus USF	GATCTCGGTACGTGACCGGA
WT NF-κB	CGCAGGGAGGATCCCCGACCTT
Mut NF-κB	CGCAGCTCGGATCCCCGACCTT
Consensus NF-κB	TCAGAGGGGACTTTCGAGAGG
WT first GC-box	GCAGGGCGCGTGGGGCGGGGGTTGGGAGA
Mut first GC-box	GCAGGGCGCGTGGGAAAGGGGTTGGGAGA
WT second GC-box	90/60
Mut second GC-box	GTCTGGTCCGCGGGGTGCAGTGCAGGCAAGAGTGTCCCG
WT third GC-box	AGAGTGTTCGCGGGGCGGGGGCCGACCC
Mut third GC-box	AGAGTGTTCGCGGAAAGGGGGCCGACCC
Comp. Sp	ATTCGATCGGGGCGGGGCGAGC
Mut. comp. Sp	ATTCGATCGGTTCCGGGCGAGC

U.S.A.). The sequence of approx. 4 kb upstream of the transcription start site was determined using an A. L. F. DNA Sequencer (Amersham Pharmacia Biotech, Freiburg, Germany) and a 377 DNA Sequencer (PE Biosystems, Foster City, CA, U.S.A.) respectively, and the appropriate sequencing kits for cycle sequencing.

Plasmid constructs

A fragment of ≈ 2.2 kb of the FBP1 gene promoter was cloned into the *SacI* and *Eco47III/SmaI* sites of the pGL3 luciferase reporter vector (Promega). A series of reporter constructs containing 5' deletion mutations were generated using restriction

digestion, blunting and re-ligation at positions -1.9 kb (*KpnI*), -1.1 kb (*BalI*), -405 bp (*PstI*), -126 bp (*Tth111I*) and -56 bp (*XmaI*). A more defined deletion series of the promoter region up to -410 bp was generated by PCR (see Table 1 for oligonucleotide sequences). The primer code corresponds to the length of the generated promoter construct upstream of the transcription start site. Primer Re1 was used as a reverse primer. All 5' primers contain a *KpnI* site. PCR products were cloned into the pGL3 reporter vector by using *KpnI* (5' site) and *HindIII* (3' site) digestion. The mutation of the -405 construct to make the constructs mutE-box, mutNF- κ B site (where NF- κ B is nuclear factor κ B), mut1stGC-box, mut2ndGC-box and mut3rdGC-box were performed using the Quick Change site-directed mutagenesis

Table 2 Deletions of the *FBP1* promoter allow identification of specific regions necessary for promoter activity

No significant changes in promoter activity were observed when the promoter was deleted from -2200 to -405. More detailed deletions revealed that the regions from -405 to -360, -207 to -158, -158 to -126, and -111 to -77 bp contribute to promoter activity. The luminescence data are expressed as the ratio of relative light units (firefly luciferase activity/*Renilla* luciferase activity) of each promoter construct relative to the activity of the empty reporter vector (pGL3). Results are averages $\pm$ S.E.M. for three to six experiments.

Reporter construct	Luminescence
-2200	39.74 $\pm$ 6.11
-405	40.77 $\pm$ 2.08
-360	11.68 $\pm$ 1.59
-260	12.62 $\pm$ 2.90
-207	19.96 $\pm$ 3.38
-158	7.43 $\pm$ 3.21
-126	17.34 $\pm$ 4.01
-111	12.61 $\pm$ 1.86
-77	1.88 $\pm$ 0.27
pGL3	(1)

kit (Stratagene, La Jolla, CA, U.S.A.) and the following primer pairs: MEB1 and MEB2 (for mutE-box); M1 and M2 (for mut1stGC-box), SpMut1 and SpMut2 (for mut2ndGC-box);

M3 and M4 (for mut3rdGC-box); M22 and M23 (for mutNF- κ B site) (Table 1). The DNA sequence of each mutant was verified.

Cell culture and transient transfections

The maintenance and transfection of H4IIE cells has been described previously [15]. Dual-luciferase reporter assays (Promega) were performed according to the manufacturer's instructions.

Nuclear extracts and electrophoretic mobility-shift assays (EMSAs)

EMSAs were performed as described previously [16]. H4IIE cells were incubated in serum-free media for 4 h. For NF- κ B EMSAs, cells were treated with 1 μ M PMA to stimulate nuclear localization of NF- κ B. Subsequently, cells were washed twice in 5 ml of ice-cold PBS and were harvested by centrifugation. The cell pellet was resuspended in buffer A [20 mM Hepes (pH 7.9)/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/0.5 mM PMSF/20 mM NaF/1 mM Na₃VO₄/1 mM Na₄P₂O₇/0.4 mM Na₂MoO₄/0.125 μ M okadaic acid/1 μ g/ml aprotinin/1 μ g/ml leupeptin]. After homogenization in a tight-fitting Dounce homogenizer, cell lysates were centrifuged at 2000 *g* for 10 min, and nuclear pellets were resuspended in buffer B [buffer A containing 420 mM NaCl and 20% (v/v) glycerol]. The resus-

A

B

-415 E-box (-390/-385) -345
AGGAGCTGCAGAGCTAAAGCCAGGCCATGTGACTGGCTGCGGGTTTCGCTGCCTAGCTGGTGGCATTGA

-200 1st GC-box (-164/-159) NF- κ B (-142/-132)
CAGCCAGGAAGTCTAGGGGGCGGCGGCGCGTGGGGCGGGTTGGGAGACCGCAGGAGGATCC

-100 2nd GC-box (-79/-74) 3rd GC-box (-54/-49) -30
CCGCGGTCTGTCGCCGGGTAGCGGGGCGCAAGAGTGTTCGGGGCGGGGGCCGACCCGCTCTAA

C

Figure 1 EMSA reveals DNA-binding activity from different regions of the *FBP1* gene promoter

(A) DNA binding was examined over regions of the *FBP1* gene promoter that extend from -415 to -345 (left panel), -200 to -130 (middle panel) and -110 to -30 bp (right panel). Overlapping sets of oligonucleotides were incubated with H4IIE nuclear extracts and analysed by EMSA, as described in the Experimental section. (B) Several DNA-protein complexes were identified by EMSA in the experiment illustrated in (A). Examination of the *FBP1* gene promoter sequence allowed us to identify several putative transcription-factor-binding sites. The sequence of the *FBP1* gene promoter is illustrated, and the location of each of these putative sites is underlined. (C) Data from Table 2 is compared with the location of the putative DNA-binding elements. The location of the E-box and GC-box motifs correlates with the loss of activity by 5' deletions, whereas deletion of the putative NF- κ B motif leads to increased promoter activity.

pendent nuclei were exposed to consistent agitation for 30 min and centrifuged at 13 000 *g* for 20 min. The supernatant was used for DNA-binding reactions. Protein amounts were determined using the Bio-Rad (Hercules, CA, U.S.A.) Protein Assay reagent. Oligonucleotides used in arrays covering promoter regions and corresponding oligonucleotides to the DNA-binding motifs [enhancer box (E-box), GC-boxes, NF- κ B site; see Table 1] were annealed and end-labelled using polynucleotide kinase in the presence of [γ - 32 P]dATP. DNA-binding reactions were performed in a buffer containing 10 mM Tris/HCl (pH 7.6), 50 mM NaCl, 0.5 mM dithiothreitol, 10 % glycerol, 0.2 % Nonidet P40 and 3 μ g of poly(dI-dC) using 5 μ g of nuclear protein and the labelled probe (approx. 60 000–80 000 c.p.m./reaction). For supershift assays, reactions were performed in the presence or absence of 1 μ l of polyclonal antibodies specific for NF- κ B subunit p65 (sc-109x), NF- κ B subunit p50 (sc-114x), specificity protein 3 (Sp3) (sc-644x), activator protein 2 α (AP2 α) (sc-184x), upstream stimulatory factor 1 (USF1) (sc-229x), USF2 (sc-861x) and monoclonal antibody against Sp1 (sc-420x) (Santa Cruz, La Jolla, CA, U.S.A.). Samples were incubated at room temperature for 25 min and analysed by electrophoresis on a non-denaturing 6 % (w/v)-polyacrylamide gel in TAE buffer [7 mM Tris (pH 7.6)/3 mM sodium acetate/1 mM EDTA]. For supershift analysis, reaction mixtures were preincubated with the antibody for 20 min before adding the probe.

RESULTS

We cloned and sequenced approx. 4 kb of the FBP1 gene promoter from human liver in order to determine what regions

of the FBP1 gene promoter are important for transcriptional activity. Various regions of the promoter from –2200 to +25 bp were subcloned into the pGL3 luciferase reporter vector for transfection studies in H4IIE rat hepatoma cells. The promoter region extending from –2200 to +25 bp relative to the transcription start site [12] showed activity. No significant changes in promoter activity were observed when the promoter was deleted from –2200 to –405 bp (Table 2).

The nucleotide sequence extending from –405 to +25 bp relative to the transcription start site was characterized in the present study, as discussed below. Sequence analysis revealed only minor differences to the GenBank[®] published sequence [12]. A more defined 5' deletion series (in steps of about 50 bp) revealed that three regions were important for transcriptional activity (Table 2). Loss of activity was observed when the regions between –405 and –360 bp and between –111 and –77 bp were deleted from the promoter. Increasing and decreasing activities were observed when regions between –260 and –126 bp were deleted. These promoter regions, therefore, were subjected to a more detailed analysis.

Sets of overlapping 30 bp oligonucleotides corresponding to different regions of the FBP1 gene promoter extending from –415 to –345 bp, –200 to –130 bp, and –110 to –30 bp, were next constructed (Table 1). These oligonucleotides were employed in an EMSA using nuclear extracts isolated from H4IIE cells (Figure 1A). DNA binding activity was detected throughout the –415 to –345 bp region, although the most prominent activity was found using the oligonucleotides corresponding to –405/–375 bp and –395/–365 bp. Examination of the sequence encompassing this region of the promoter

Figure 2 USF1 and USF2 bind to the E-box element at –390/–385

The E-box oligonucleotide (–395/–365; Table 1) was used in all EMSA reactions, except for lanes 2 and 5. In lanes 2 and 5, a mutated E-box oligonucleotide (Table 1) was incubated with nuclear extracts. Oligonucleotide competitors and antibodies were added to EMSA reactions as follows: lanes 1 and 4, no antibody or competitor was added; lane 3, a 50-fold excess of a consensus USF element oligonucleotide (see Table 1) was used as a competitor; lane 6, an antibody specific for USF1 was added to the reaction mixtures; lane 7, an antibody specific for USF2 was added to the reaction mixtures.

Figure 3 NF-κB binds to an element at -142/-133 bp

Nuclear extracts from PMA-stimulated H4IIE cells were incubated with either an oligonucleotide (oligo) containing the putative *FBP1* NF-κB element (Table 1; lanes 2, 4, 5 and 6), a mutated *FBP1* NF-κB element (Table 1; lane 3) or a consensus NF-κB element (Table 1; lane 7), and analysed by EMSA. Antibodies against the p50 (lane 4) and the p65 (lane 5) subunit of NF-κB, or a consensus NF-κB element as a competitor (60-fold molar excess in lane 6), were used to identify NF-κB-binding activity.

revealed a putative E-box motif at -390/-385 bp (Figure 1B). Inspection of the region extending from -200 to -130 bp revealed DNA-binding activity from the -180/-150 bp and -170/-140 bp oligonucleotides, and a putative GC-box was identified at -164/-159 bp (Figure 1B). DNA binding activity was also observed in the -90 to -30 region of the proximal promoter. Examination of this sequence revealed the existence of two more GC-boxes at -79/-74 bp and -54/-49 bp (Figure 1B). We also identified a putative NF-κB DNA-binding element at -142/-133 bp (Figure 1B).

As indicated in Figure 1(C), the location of these putative binding elements correlates with the activity of the truncated promoter constructs observed in Table 2. The promoter activity decreased from -405 to -360 bp, from -207 to -158 bp, and from -126 to -77 bp. The putative E-box, and two GC-boxes, are located between these positions and may be targets for transcriptional activators. Deletion of the region from -158 to -126 bp of the promoter, which encodes the putative NF-κB

site, led to an increase in activity, suggesting that NF-κB may repress the *FBP1* gene promoter.

Supershift analysis and the use of competitor oligonucleotides allowed us to identify the transcription factors that bind to these promoter elements. USF1 and USF2 both bind to the E-box, since antibodies directed against both factors prevent formation of a protein complex with the corresponding oligonucleotide. Additionally, a consensus E-box is a competitor for DNA binding (Figure 2).

Nuclear extracts from H4IIE hepatoma cells treated with PMA (a phorbol ester that stimulates nuclear localization of NF-κB) shifted the NF-κB oligonucleotide in EMSAs. NF-κB DNA binding activity was decreased in the presence of antibodies specific for either the p50 or p65 subunits of NF-κB. Also, a competitor oligonucleotide encoding an NF-κB consensus site decreased DNA binding (Figure 3). Although there are other known subunits of NF-κB, we chose to examine only the p50 and p65 subunits, since these subunits constitute the majority of

Figure 4 Sp1 and Sp3, but not AP2 α , bind to three GC-box elements

Oligonucleotides encoding the three GC-boxes were incubated with nuclear extracts from H4IIE cells and analysed by EMSA and supershift analysis. **(A)** EMSAs were performed using oligonucleotides encoding either the first, second or third GC-box from the FBP1 gene promoter (see Table 1), as indicated at the top of the Figure. Lane 1, no competitor added; lane 2, a consensus Sp competitor oligonucleotide was included in reaction mixtures; lane 3, a mutated consensus oligonucleotide (Table 1) was included in the reaction mixture. **(B)** A supershift analysis with each GC-box was performed. Lane 1, free probe; lane 2, no antibody added; lane 3, an antibody specific for Sp1 was added to the reaction mixture; lane 4, an antibody specific for Sp3 was added to the reaction mixture; lane 5, an antibody specific for AP2 α was included in the reaction mixture. Antibodies against SP1 and SP3, but not against AP2 α , resulted in a supershift or decreased DNA binding of each GC-box, as indicated by the arrow.

NF- κ B activity in H4IIE cells ([17]; M. Waltner-Law, M. C. Daniels and D. K. Granner, unpublished work).

Sp-family members bind to all three identified GC-boxes, since DNA binding was competed for by a consensus Sp-oligonucleotide, but not by a mutated Sp-oligonucleotide (Figure 4A). Antibodies directed against both Sp1 and Sp3 changed the band-shift pattern on all three GC-boxes, whereas an AP2 α antibody, as a control, showed no effect (Figure 4B).

These binding sites were mutated in the context of the -405 to +25 bp FBP1 gene promoter construct to investigate their

functional relevance. The mutations that were introduced into the GC-boxes, E-box and NF- κ B site led to a loss of binding of the proteins, as shown in Figures 2 and 3. Transient transfection experiments revealed that the mutation of each of these sites affected promoter activity when compared with the wild-type promoter. Mutation of the E-box and three GC-boxes led to a significant decrease in activity, whereas mutation of the NF- κ B site led to an increase in promoter activity (Table 3). These findings are in agreement with the results obtained in the 5'-promoter-deletion experiments (Figure 1C).

Table 3 Mutation of each identified element affects promoter activity

The E-box, three GC-boxes or NF- κ B element were mutated within the context of the $-405/+25$ bp promoter construct and transfected into H4IIE cells. The data are presented as in Table 2. Results are averages $\pm$ S.E.M. for four to ten experiments. Abbreviation: Mut, mutant.

Reporter construct	Luminescence
—405	31.47 $\pm$ 3.71
MutE-box	19.98 $\pm$ 4.03
Mut first GC-box	10.86 $\pm$ 2.91
Mut second GC-box	8.45 $\pm$ 1.25
Mut third GC-box	18.21 $\pm$ 1.61
MutNF- κ B	52.57 $\pm$ 13.87
pGL3	(1)

DISCUSSION

Deletion analysis of the FBP1 gene promoter demonstrated that the region between -405 and $+25$ bp relative to the transcription start site is important for promoter activity. Several putative DNA-binding elements within this region were identified when the promoter sequence was first published [12]. In the present study we show that at least five functionally important sites exist within this area. Four of these sites bind to factors that activate *FBP1* transcription (Sp1, Sp3, USF1 and USF2), whereas one site binds to a factor (NF- κ B) that can repress *FBP1* transcription.

The region of the FBP1 gene promoter from -405 to $+25$ bp has a relatively high GC content (about 65%) and it contains three GC-box motifs. EMSA experiments indicate that members of the Sp family of transcription factors, which are involved in the regulation of a great variety of housekeeping genes, bind to these sites [18]. Sp1 and Sp3 bind to these GC-boxes in the FBP1 gene promoter and mutation of these sites in the context of an otherwise wild-type promoter decreases transcriptional activity. Sp-family members recognize the Sp-binding site with identical affinity [19], and they may form large homo- or hetero-meric complexes [20], observations that may explain the incomplete antibody supershifts seen in the EMSA experiments. The interactions of these transcription factors are complicated. In some cases Sp3 inhibits Sp1-activated transcription [21], whereas the effects of Sp1 and Sp3 are additive in other cases [22]. Changes in the ratio of Sp1/Sp3 in the nucleus may therefore result in a wide range of transcriptional activity [23]. The relative contributions of these transcription factors to the regulation of *FBP1* remain to be established.

USF transcription factors, members of the basic helix-loop-helix leucine zipper ('bHLH-zip') family [24], were first discovered in HeLa cells [25]. These factors, which are encoded by the ubiquitously expressed USF1 and USF2 genes [26], form homo- and hetero-dimers; however the USF1·USF2 heterodimer is the predominant DNA-binding form [27]. These factors play an essential role during embryonic development [28], possibly in conjunction with co-activators [29]. The USF factors are also involved in the regulation of several genes that are involved in glucose and fatty acid metabolism, such as those encoding fatty acid synthase [30,31], pdx-1 [32], pyruvate kinase [33], glucokinase [34] and spot 14 [35]. The USF factors may also be involved in the transcriptional response of the L-type pyruvate kinase and S14 genes to glucose [36,37]. *FBP1* can now be added to the list of genes regulated by USF factors.

Members of the NF- κ B/Rel family regulate various physiological functions through the formation of homo- and heterodimers [38,39]. The p50/p65 heterodimer binds to the FBP1 gene

promoter and may have a repressive effect on *FBP1* activity (Table 3). This is somewhat surprising, since NF- κ B is typically a transcriptional activator. Repression of transcription is not without precedent, however, since NF- κ B represses the PEPCK gene (M. Waltner-Law, M. C. Daniels and D. K. Granner, unpublished work).

Other putative DNA-binding elements, including those for hepatocyte nuclear factor 4 (HNF4), Sp1 and AP2, have been identified in the FBP1 gene promoter in the region located between -273 and -201 bp [12]. The 5' deletion experiments reported in this paper showed no change in activity when these sites were deleted, so this region was not subjected to further analysis. However, deletion of the promoter region from -260 to -207 bp resulted in an increase in activity, so it is conceivable that a repressor binds to this area. Also, a putative CCAAT-box, identical with the CAAT element from the rat liver PEPCK gene promoter, is located at $-116/-110$ bp [40,18]. Although binding of nuclear proteins to an oligonucleotide encoding this putative CCAAT-box was observed, we did not further investigate this putative element since no significant loss of activity was observed when this site was deleted. Finally, the liver-enriched factors HNF1, HNF3 and HNF4 play a role in the expression of other gluconeogenic genes such as those encoding PEPCK and glucose-6-phosphatase [16,41–43]. Although we only observed the binding of USF, Sp and NF- κ B to the FBPase promoter, we cannot exclude the possibility that HNF also binds to this promoter.

Mutation of the E-box or GC-boxes alone did not completely abolish promoter activity, a finding that suggests redundancy at this level of gene expression. Small sequence alterations within these sites are therefore unlikely to cause a complete loss of gene transcription and the subsequent loss of enzyme expression in liver. By contrast, certain point mutations in the coding sequence completely abolish FBPase enzyme activity [10,11]. Larger deletions in the promoter region could abolish transcription, and it is conceivable that a resultant low level of gene expression could contribute to FBP1 deficiency in the critical, postnatal period of development.

The human, rat and mouse liver promoters have similar structures [12,14a,44] (mouse *Fbp1*: EMBL accession number AJ245391). All have a relatively high GC content in the region located between -250 to -1 bp, and the rat and mouse promoters also have the putative binding sites for USF, Sp and NF- κ B elements shown here to be involved in the transcriptional regulation of the human gene. On the basis of this information it is possible that these promoters are regulated by similar mechanisms.

Insulin and cAMP co-ordinately regulate the transcription of glycolytic and gluconeogenic genes. The key enzymes of gluconeogenesis, PEPCK, FBPase and glucose-6-phosphatase, are inhibited by insulin and stimulated by cAMP, whereas their glycolytic counterparts, glucokinase, PFK and pyruvate kinase, are stimulated by insulin and inhibited by cAMP [1]. PEPCK is regulated exclusively at the level of transcription, whereas FBP1 is regulated both allosterically, by the inhibitors fructose 2,6-bisphosphate and AMP, and hormonally [3,44]. Although the mRNAs that encode the two FBPase isoforms are induced after treatment of HL-60 cells with vitamin D₃ [2] or the mouse embryonal carcinoma F9 cells with retinoic acid [13], the low level of FBP1 expressed by H4IIE cells is not significantly affected by hormonal treatment (results not shown). Mechanisms by which the promoter is regulated, the influence of this regulation on gluconeogenic flux under various physiological conditions, and the contribution of hormonal regulation of FBP1 to disease remain enigmatic [3–5]. The present study provides the basis for a more comprehensive screening for mutations in the FBP1 gene

and for further analysis of the regulation of the human FBPI gene.

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