Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene

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A functionally important cis-acting element termed P2 was identified in the liver promoter of the glucokinase gene. Element P2 was delineated by footprinting in vitro with nuclear proteins from rat liver and spleen. Its core sequence in the rat gene is a canonical CACGTG E-box. In the electrophoretic mobility-shift assay with nuclear proteins from rat liver, hepatocytes and hepatoma cells, an oligonucleotide with P2 in the context of the glucokinase promoter sequence gave rise to a DNA–protein complex shown to contain the upstream stimulatory factor (USF) by specific competition experiments and by reactivity with anti-USF antibodies. Transient transfection of hepatoma HepG2 cells, combined with site-directed mutagenesis, demonstrated that the P2 element was important for liver glucokinase promoter activity. Co-transfection of an expression plasmid coding for USF1 activated reporter gene expression in a manner dependent on an intact P2 element, whereas an expression plasmid for c-Myc was ineffective. Expression of a truncated form of USF1 lacking the transcription activation domain and the basic region decreased reporter activity by a dominant-negative effect. The functional significance of the P2 element was also demonstrated in transient transfection of primary hepatocytes.

INTRODUCTION

Glucokinase (ATP:hexose 6-phosphotransferase; EC 2.7.1.1), a member of the hexokinase family (hexokinase type IV or D), exhibits a narrow tissue distribution [1,2]. The expression of glucokinase is restricted to cell types that are known or suspected to have a primary role in sensing hyperglycaemia and defending glucose homoeostasis [3]. The major site of expression, both in the total amount of glucokinase per organ and in enzyme specific activity, is the liver [4]. The liver is intrinsically capable of adapting its rate of glucose uptake or release in response to changes in the concentration of perfused glucose, even in the absence of hormonal or neural stimuli [5]. This ability is crucial for glucose homoeostasis and is conferred on liver cells primarily by glucokinase [6].

Transcription of the glucokinase gene in the liver is initiated at a liver-specific promoter used uniquely in this tissue, whereas transcription in all extrahepatic cells expressing glucokinase is initiated at another promoter localized far upstream in the locus [7]. In contrast with the upstream promoter, which seems to function constitutively throughout postnatal life, the liver promoter first becomes active at weaning and remains acutely regulatable in adult life by insulin and glucagon, which turn transcription on and off respectively [8]. The mechanisms responsible for the transcriptional regulation of the glucokinase gene in the hepatocytes remain largely unknown.

The delineation of regulatory DNA sequence elements that could control transcriptional initiation at the liver promoter is still at an early stage. Transgenic mice expressing a glucokinase transgene in liver in a copy-number-dependent and nutritionally regulated manner have been generated by Magnuson and co-workers [9], but this required the use of an 80 kbp DNA transgene; putative regulatory regions within this long stretch of DNA have not been localized in transgenic animals. By using transient transfection of reporter plasmids driven by the liver glucokinase promoter and flanking region, we have identified a liver-specific enhancer between nt —1000 and —700 upstream of the transcription start site in the rat gene, conserved at a similar position in the human gene [10]. This enhancer exhibited very stringent cell specificity, being active in primary hepatocytes but not in hepatoma or insulinoma cells. In addition to the enhancer, the proximal promoter region between —123 and the start site was shown to support the expression of the reporter gene in primary hepatocytes and hepatoma cells, but not in insulinoma cells [10]. The present study was undertaken to characterize the promoter proximal region in more detail. The delineation of a functionally important cis-acting element of the promoter and the identification of the cognate transcriptional activator are reported.

EXPERIMENTAL

Plasmids

The reporter plasmids GK-180Luc, P2m1GK-180Luc and P2m2GK-180Luc were constructed with liver glucokinase promoter fragments generated by PCR. The template for PCR amplification was the previously described GK-400Luc [10]. The following 5’ primers were used for GK-180Luc, P2m1GK-180Luc and for P2m2GK-180Luc respectively: 5’-tcccccggGTCAAAACCGAACCCACG-3’, 5’-tcccccggGTCAAAACCGAACCCACG-3’, 5’-tcccccggGTCAAAACCGAACCCACG-3’ and 5’-tcccccggGTCAAAACCGAACCCACG-3’. Capital letters designate the liver promoter sequence of the rat glucokinase gene [11] starting at —101 with respect to the start site of transcription. Bold letters indicate mutated nucleotides. Lower-case letters indicate 5’ extensions for digestion with

Abbreviation used: USF, upstream stimulatory factor.

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In all reactions the 3’ primer was the commercial GL primer 2 (Promega). The DNA fragments after PCR were digested with SalI and BglII, inserted into the corresponding sites of the Gl2 Basic luciferase vector (Promega) and verified by manual dideoxy sequencing with Sequenase. The other glucokinase/luciferase plasmids have been described previously [10]. Plasmid P490Luc was constructed by inserting a KpnI-BglII promoter fragment of the rat phosphoenolpyruvate carboxykinase gene (from plasmid P490CAT [12], a gift from Dr. Richard Hanson) into GL2 Basic.

Expression plasmids for full-length human USF1 (in which USF stands for upstream stimulatory factor) and a truncated form lacking the first 207 N-terminal amino acids (transcriptional activation and basic domains, Δ-tbT-D-U1) were provided by Dr. Michel Raymondjean and Dr. Axel Kahn [13]. A luciferase reporter plasmid with a binding site for c-Myc from the prothymosin α gene (36E-tkLUC [15]) were gifts from Dr. Laurie Desbarats and Dr. Martin Eilers. The expression plasmids for a hybrid protein containing the complete mouse USF2 sequence fused to the C-terminal activation domain of VP16 (USF2/VP16) was made available by Dr. Elisabeth Kaytor and Dr. Howard Towle [16].

Cell culture and transient transfection
The human hepatoma cells HepG2 were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and nutrient mix F12, supplemented with 2 mM glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Cells were seeded in six-well tissue culture dishes 40 h before transfection and were transfected by a standard calcium phosphate/DNA co-precipitation method, with 4 μg of luciferase reporter plasmid and 0.5 μg of the plasmid CMV-β (Invitrogen) encoding β-galactosidase for normalization purposes. Effector plasmids encoding transcription regulatory proteins were added in amounts of 1 μg or as specified in the table legends, and the total amounts of plasmid DNA in control samples without effector plasmids were equalized by the addition of empty vector. The cells were generally cultured for 21 h before being harvested for assays of the reporter enzyme activities as described previously [10].

Hepatocytes from rats were isolated, transfected by electroporation and maintained in primary culture as described previously [10], except that donor animals were fasted for 24 h instead of 48 h before the experiments.

Nuclear extracts from tissues and cells
Nuclear proteins from liver or spleen were incubated with labelled probe in a total of 15 μl of a reaction mixture containing (final concentrations): 10 mM Hepes, 10 mM Tris/HCl, pH 8.0, 60 mM KC1, 3 mM MgCl2, 0.5 mM EDTA, 0.4 mM dithiothreitol, 2 μg of poly(dI-dC) as non-specific competitor and 4% (v/v) glycerol. When specified, 0.5 μg of sonicated single-stranded salmon testes DNA was present as an additional non-specific competitor. In reaction mixtures with nuclear extracts from cultured cells, KC1 was replaced by 80 mM NaCl. The labelled probe (7.5 to 15 fmol) was added last to the reaction mixtures. Unlabelled competitor oligonucleotides were added 8 min before the labelled probe in amounts indicated in the Figure legend. When antibodies to USF (Santa Cruz Biotechnology) were used, they were allowed to react with the nuclear proteins for 40 min at room temperature before the addition of the labelled probe. Reactions were incubated at room temperature for 15 min after the addition of the probe and terminated by addition of 2.5 μl of a 15% (w/v) Ficoll. Samples (8 μl) were loaded on a 4% (w/v) polyacrylamide gel made in a low-ionic-strength buffer containing 36 mM Tris, 36 mM boric acid and 0.8 mM EDTA. The gel was subjected to electrophoresis at 130 V for 100 min with recirculation of the running buffer and cooling at 13 °C. The dried gels were exposed to X-ray films with one intensifying screen.

RESULTS

Activity of liver glucokinase promoter in HepG2 cells

Reporter plasmids with the firefly luciferase gene driven by the liver glucokinase promoter and 5' flanking DNA were transiently transfected in HepG2 cells, a human hepatoblastoma cell line commonly used as model for the hepatocyte cell type. Luciferase activity was readily detectable with constructs containing 1 kb of flanking sequence (GK-1,000Luc) and was maintained at about the same level with progressive deletion down to nt 102 with respect to the start of transcription (GK-180Luc) (Figure 1). Further deletion of the next 67 nt to position 35, just upstream of the putative TATA box, caused a drastic decrease in promoter activity. Thus the DNA sequence between 101 and 35 is essential for activity of the liver glucokinase promoter in HepG2 cells. This DNA segment was previously shown to support liver glucokinase promoter activity in primary rat hepatocytes and the rat hepatoma FTO-2B, but to be virtually inactive in insulinoma cells [10]. In addition, results in Figure 1 show that the DNA segment between 1000 and 700, previously characterized as a liver-specific enhancer in experiments with primary rat hepatocytes [10], is ineffective in HepG2 cells. Thus the proximal promoter region is active both in primary hepatocytes and
Upstream stimulatory factor and liver glucokinase

Figure 1 Liver glucokinase promoter drives reporter gene expression in transiently transfected HepG2 hepatoma cells

Rat glucokinase genomic fragments were subcloned upstream of the firefly luciferase gene of the promoterless plasmid GL2 basic. Endpoints of the glucokinase genomic sequence relative to the start site of transcription in liver are given below the scheme of each construct. The plasmids were introduced into HepG2 cells by the calcium phosphate/DNA co-precipitation method. Luciferase activity was measured in cell extracts collected 21 h after transfection. Normalization of the data for transfection efficiency was done by measuring β-galactosidase activity elicited by a CMV–β-galactosidase plasmid co-transfected with the test plasmids. The results are expressed as relative luciferase activity, a value of 100 being assigned to the activity of GK-1,000Luc in each experiment. The values are means ± S.E.M. for four independent transfections.

Footprinting of the liver glucokinase promoter in vitro

To identify potential binding sites for transcriptional activators in the proximal promoter region, a DNA fragment extending from nt −310 to +16, end-labelled at the +16 position, was subjected to DNase I protection assay in vitro with nuclear protein prepared from rat liver. Two protected sequence elements were clearly mapped in this experiment (Figure 2A). The first element, termed P1, was between −54 and −35 and is not examined further in the present paper. The second element, termed P2, was between −89 and −81. Both elements were footprinted with spleen nuclear protein as well as liver nuclear protein, indicating that the putative proteins binding to these DNA elements are not restricted to liver. The sequence of the P2 element, 5′-CCCACGTGG-3′, contains the core motif CACGTG, which serves as a binding site for transcriptional activators of the basic helix–loop–helix (bHLH) class such as c-Myc and its heterodimerization partners, and the upstream stimulatory factors USF1 and USF2.

Trans-activation of glucokinase promoter by co-transfection of USF1 in HepG2 cells

The ability of c-Myc and USF1 to activate the liver glucokinase promoter in HepG2 cells was tested by transient transfection of effector plasmids encoding these activators, together with re-
Figure 3  Testing for trans-activation of liver glucokinase promoter by co-expressed c-Myc and USF1

The reporter luciferase plasmids designated below the plots were transfected into HepG2 cells together with expression plasmids for human c-Myc (left panel) or human USF1 (right panel), or with a control empty vector. Luciferase activity was measured 21 h after transfection and was normalized for β-galactosidase activity from plasmid CMV-β-galactosidase used as a control for transfection efficiency. For a given luciferase reporter, results are expressed as the ratio of activity measured in the presence of the co-transfected expression plasmid to the activity measured with the control empty vector. Values are means ± S.E.M. for six independent transfections. Luciferase activities (means ± S.E.M.) elicited by the various reporter plasmids in the presence of control empty vector, expressed in relative luminometer units after normalization for β-galactosidase activity, were: GK-180Luc, 453 ± 51.6; P2m1GK-180Luc, 244 ± 34.7; 36EtkLuc, 752 ± 91.8; P490Luc, 352 ± 35.5.

180Luc). Two control reporter plasmids were also used. Plasmid 36EtkLuc contains a target binding site for c-Myc from the prothymosin-α gene [15]. Plasmid P490Luc expresses luciferase from the phosphoenolpyruvate carboxykinase promoter, a promoter not known to harbour functional binding sites for bHLH proteins.

As shown in Figure 3 (left panel), co-expression of c-Myc with the 36EtkLuc reporter resulted in a 2.2-fold increase in luciferase activity, showing that exogenous c-Myc was capable of stimulating the expression of a reporter with a bona fide target element for c-Myc. In contrast, both the wild-type and mutated glucokinase promoter/luciferase reporters, as well as the P490Luc plasmid, displayed a slight decrease in luciferase activity after transfection with the c-Myc plasmid. Thus the P2 E box of the glucokinase promoter seems to be unable to serve as a c-Myc target in the present system.

Co-transfection of the USF1 expression plasmid (Figure 3, right panel) stimulated by 7-fold the luciferase activity driven by the wild-type glucokinase promoter. Luciferase driven by the mutated promoter was stimulated 4-fold. The stimulation by USF1 specifically dependent on the integrity of the CACGTG motif was therefore 1.7-fold. Co-transfection of USF1 also resulted in stimulation of luciferase activity from the two control plasmids, a 3.7-fold stimulation with 36EtkLuc and a 2.7-fold stimulation with P490Luc. These effects are comparable in magnitude to the 4-fold stimulation of the P2m1GK-180-Luc and can be considered non-specific in the present analysis. Non-specific stimulation might result from the presence of cryptic E boxes in the reporter plasmids. Also, E-box-independent stimulation of transcription by USF proteins, mediated by so-called initiator elements near the transcription start site, have been reported in several systems [20,21]. Interestingly, the sequence surrounding the liver transcriptional start site in the rat glucokinase gene, TTA¬TTATGC (A¬_ = start), conforms to the consensus initiator element [22] at six of seven positions (indicated by capital letters). Whether USF or its newly cloned interacting partner TFII-I [23] binds to this element remains to be investigated.

Binding of USF in nuclear extracts of liver-derived cells to glucokinase gene P2 element

An oligonucleotide probe with the sequence of the liver glucokinase promoter from nt -99 to -69 of the rat gene was subjected to electrophoretic mobility-shift assay with nuclear protein from rat liver. As shown in Figure 4A (arrow), a major retarded complex was discernible. The formation of this complex was inhibited by the inclusion in the binding reaction of excess unlabelled oligonucleotide probe (designated competitor 1 in Figure 4). Two other bands (Figure 4, asterisks) with intermediate migration between the major complex and free oligonucleotide were considered non-specific because they were little affected by excess unlabelled oligonucleotide. An oligonucleotide identical in sequence to the labelled probe except for a C → A transversion converting the core CACGTG motif to CaGTC, a change known to cause a drastic decrease in the binding affinity of oligonucleotides for USF [24], was completely unable to prevent the formation of the specific complex (Figure 4A, competitor 2).
Overall this sequence was 50 nt identical with the rat sequence, indicating that the negative action of the deleted USF1 protein might be USF.

A third competitor was synthesized to reproduce the sequence of the liver promoter of the human glucokinase gene from nt −93 to −64 with respect to the transcriptional start site. Overall this sequence was 50 % identical with the rat sequence, with 8/9 identity in the P2 element, the core CACGTG motif being changed to CACATG. The human oligonucleotide could act as specific competitor of the rat probe, but the molar excess of oligonucleotide necessary was approx. 30-fold larger than with the rat oligonucleotide (Figure 4A). Thus the human sequence can serve as binding site for USF proteins, although the binding affinity seems to be substantially decreased compared with that of the rat gene. In a mobility-shift assay with the human oligonucleotide as labelled probe and nuclear extracts from liver nuclei, a retarded complex with the nuclear extract of animal gave rise to the specific complex described above. In refeeding rats, in which the gene is induced. Proteins from both types of hepatocytes and HepG2 cells produced the same specific complex and supershifted bands as proteins from liver nuclei. Fainter bands below the major retarded complex with the nuclear extract from cultured hepatocytes were also displaced by anti-USF antibodies and therefore seem to represent truncated USF proteins, perhaps reflecting limited proteolysis during the preparation of the extracts. The supershifting of the retarded complex from HepG2 cells was somewhat less complete than with nuclear proteins from rat origin. This might have been due to a weaker affinity of the antibodies for human than for rodent USFs.

Testing dominant-negative forms of USF

Variant USF proteins with mutations or deletions in the trans-activation domain or basic region necessary for DNA binding can in principle inhibit the transcription of USF-activated genes by forming non-functional dimers with wild-type subunits [25]. To implement this approach, an incomplete USF1 protein with truncation of the N-terminal trans-activation domain and contiguous basic region (Δ bTD-U1) was used [13]. The truncated protein was first co-expressed in HepG2 cells with a USF2/VP16 fusion protein, which was shown by Towle and co-workers [16] to act as a very strong transcriptional activator of presumed USF target genes. The results in Table 1 show that, by itself, the USF2/VP16 activator stimulated luciferase expression from the GK-180Luc reporter plasmid approx. 20-fold. The effect of the superactivator was entirely mediated via the P2 element because P2m1GK-180Luc (with the block mutation in P2) did not display any stimulation at all. Furthermore the stimulation of the wild-type promoter was suppressed (93 % inhibition) on co-transfection with the plasmid encoding the truncated USF1 protein. These results showed that the truncated USF1 was able to act in the present system as a dominant-negative form of USF.

Transfecting the expression plasmid for truncated USF1 in the absence of plasmid for exogenous USF-activator would then test the role of endogenous USF proteins in the transcription of reporter genes (Table 2). Luciferase activity driven by GK-180Luc was modestly (20 % inhibition) on co-transfection with the plasmid encoding the truncated USF1 protein. These results showed that the truncated USF1 was able to act in the present system as a dominant-negative form of USF.

Table 1 Dominant-negative variant of USF inhibits activation of liver glucokinase promoter by a USF2/VP16 fusion protein

<table>
<thead>
<tr>
<th>Reporter</th>
<th>No effector</th>
<th>+USF2/VP16</th>
<th>+USF2/VP16 + Δ bTD-U1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK-180Luc</td>
<td>100</td>
<td>1860±268</td>
<td>130±15</td>
</tr>
<tr>
<td>P2m1GK-180Luc</td>
<td>100</td>
<td>76±4</td>
<td>95±12</td>
</tr>
</tbody>
</table>

Figure 5 Electrophoretic mobility-shift assay with anti-USF antibodies

The labelled probe was the rat oligonucleotide, as in Figure 4(A). Conditions for the binding reactions without (-) or with (+) antibodies recognizing USF1 and USF2 are given in the Experimental section. Nuclear proteins were from: the livers of rats after glucose refeeding (FR) or in the fasting state (F) (0.5 µg of protein), nuclear extracts from cultured primary hepatocytes from rats (hepato) (2 µg of protein), and nuclear extracts from HepG2 cells (2 µg of protein). The filled arrow shows the specific retarded complex without antibodies; the open arrow shows the complex supershifted by antibodies. Asterisks designate non-specific bands.
The plasmid GK-180Luc contains the wild-type promoter sequence. The P2m1 and P2m2 mutations are described in the Experimental and Results sections. The plasmid GK-120Luc has the liver glucokinase promoter sequence from −34, and GL2 basic is the promoterless vector. Transfection methods for the two cell types and the luciferase assay are described in the Experimental section. Luciferase activities were normalized to the β-galactosidase activity driven by the co-transfected CMV-β-galactosidase plasmid. In each experiment a value of 100 was given to the luciferase activity elicited by GK-180Luc; other values are expressed relative to this value. Results are means ± S.E.M. for seven independent transfections in HepG2 cells and eight transfections in primary hepatocytes.

**Figure 6 Site-directed mutations in P2 element decrease liver glucokinase promoter activity in HepG2 cells and primary hepatocytes**

The plasmid GK-180Luc contains the wild-type promoter sequence. The P2m1 and P2m2 mutations are described in the Experimental and Results sections. The plasmid GK-120Luc has the liver glucokinase promoter sequence from −34, and GL2 basic is the promoterless vector. Transfection methods for the two cell types and the luciferase assay are described in the Experimental section. Luciferase activities were normalized to the β-galactosidase activity driven by the co-transfected CMV-β-galactosidase plasmid. In each experiment a value of 100 was given to the luciferase activity elicited by GK-180Luc; other values are expressed relative to this value. Results are means ± S.E.M. for seven independent transfections in HepG2 cells and eight transfections in primary hepatocytes.

**Table 2 Effect of dominant-negative variant of USF on activity of liver glucokinase promoter in HepG2 cells**

Experiments were similar to those in Table 1, except that the ΔoTD-U1 plasmid was transfected without exogenous USF trans-activator. Luciferase activities were measured 44 h after transfection. Results are means ± S.E.M. for five separate experiments. * Difference from ‘no effector’ condition statistically significant, with \( P < 0.022 \).

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Luciferase activity (%)</th>
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<tbody>
<tr>
<td>GK-180Luc</td>
<td>100</td>
</tr>
<tr>
<td>P2m1GK-180Luc</td>
<td>100</td>
</tr>
<tr>
<td>No effector</td>
<td>82 ± 4.9*</td>
</tr>
<tr>
<td>+ ΔoTD-U1</td>
<td>97 ± 11.5</td>
</tr>
</tbody>
</table>

**DISCUSSION**

A functional cis-acting element of the E-box type has been identified in the liver promoter of the glucokinase gene. This element termed P2 is localized from nt −89 to −81 with respect to the transcriptional start site in the rat gene and contains a core CACGTG motif, known to serve as consensus binding site for factors of the bHLH/HOM class of transcriptional activators such as USF and c-Myc [24]. In the context of the glucokinase promoter, the CACGTG motif was protected against digestion with DNase I by nuclear protein from liver and spleen, as shown by the footprinting assay in vitro. An oligonucleotide with the P2 element and flanking glucokinase gene sequence produced a specific retarded complex with liver nuclear protein in the electrophoretic mobility-shift assay. Competition experiments suggested that the formation of the complex depended on USF-specific target sequence. The complex was supershifted by antibodies recognizing USF1 and USF2. By transient transfection experiments combined with site-directed mutagenesis, the P2 element was shown to be functionally important for promoter activity in hepatoma cells and primary hepatocytes. Cointransfection of an expression plasmid encoding USF1 into HepG2 hepatoma cells resulted in the activation of the glucokinase promoter, dependent on the integrity of the P2 element. Activation was noted in spite of the presence of endogenous USF proteins in HepG2 cells (Figure 5), perhaps explaining the relatively small effect of exogenous USF1. In the same assay, a c-Myc expression plasmid did not stimulate glucokinase promoter activity, although the activity of a promoter with a well-defined c-Myc target site was stimulated. Collectively, these multiple lines of evidence provide a strong indication of a role for USF proteins as activators of transcription at the liver glucokinase promoter. It is noteworthy that the P2 core sequence CACGTG in the rat gene is replaced at an equivalent position by CACATG in the human gene. An oligonucleotide with the human sequence was shown to form a shifted complex with USF and c-Myc [24]. In the context of the glucokinase promoter, the CACGTG motif was protected against digestion with DNase I by nuclear protein from liver and spleen, as shown by the footprinting assay in vitro.

Mutations of P2 element impair glucokinase promoter activity in hepatoma cells and normal hepatocytes

The previous experiments made use of hepatoma cells as surrogates for hepatocytes. To verify the relevance of the findings to the normal liver cell, transient transfection experiments with P2-mutated GK-180Luc constructs were done in rat hepatocytes in primary culture. In primary hepatocytes as well as in HepG2 cells (Figure 6), plasmid P2m1GK-180Luc, bearing the block mutation of the P2 element, gave rise to approx. 50 % luciferase activity compared with wild-type GK-180Luc. A similar decrease in reporter activity in both cellular systems was noted with plasmid P2m2GK-180Luc, which harbours a more selective double point mutation of the P2 element changing the CACGTG core motif to CAcGcG. The latter mutation was previously shown to cause a marked decrease in sequence-specific binding of USF to oligonucleotides [16,26]. These results demonstrate the functional role of the P2 element for liver glucokinase promoter activity in normal hepatocytes and support the identification of USF as the transcriptional activator acting via this element. It should be pointed out that the same double point mutation as in plasmid P2m2GK-180Luc, when introduced into E boxes of the metabolic response units of the L-type pyruvate kinase or S14 genes, did not abolish the glucose inducibility of plasmids transiently transfected into hepatocytes [16]. On this basis, Kaytor et al. [16] proposed that an unidentified factor different from USF might be responsible for trans-activation of the L-type pyruvate kinase and S14 genes. The present results strongly argue against the possibility that this hypothetical trans-activator is the P2-binding factor involved in the regulation of the liver glucokinase promoter.
preformed endogenous USF dimers [25], and/or by the presence of other activators acting along with USF via the P2 element.

The transcriptional activator USF was initially identified as a trans-activator of the adenovirus major late promoter in HeLa cells [27]. A large number of cellular genes have subsequently been reported to be targets for USF factors, which were shown to have a wide tissue distribution [28]. Two separate genes encode the highly similar USF1 and USF2 proteins in mammals [28,29]. Further diversity is afforded by the alternative splicing of transcripts from the USF2 gene [13,28]. The USF proteins belong to the class of bHLH proteins and act as sequence-specific trans-activators in the form of homodimers or heterodimers, binding DNA in a sequence-specific manner at E-box recognition elements [24]. In the liver, USF1/USF2 heterodimers seem to be the favoured DNA-binding species [30].

Cellular genes of metabolic interest, including the genes for the glycolytic enzyme L-type pyruvate kinase gene and the S14 protein, are known to contain cis-acting elements of the E-box type [31,32]. These elements are part of the metabolic response units that confer transcriptional inducibility in response to a high glucose level. Evidence suggesting that USF proteins are the E-box binding activators implicated in the response to high glucose levels is strong [33,34] but not undisputed [16]. The promoter of the rat gene for fatty acid synthase contains a CATGTTG cis-acting element that seems to confer insulin inducibility on the gene [35]. Sul and Wang [36,37] showed that the CATGTTG element in the fatty acid synthase promoter could serve as a binding site for USF factors and suggested that USF might have a role in insulin signalling to the fatty acid synthase gene. The foregoing observations raise the question of whether the P2 element and USF factors might be involved in the positive regulation of liver glucokinase gene transcription by insulin [38–40]. The footprint at the P2 element in vitro was similar with liver nuclear proteins from fasted rats (gene repressed) and glucose-reared rats (gene transcribed). Also, no consistent difference was noted in the electrophoretic mobility-shift assay patterns obtained with nuclear proteins from the two types of animal or with nuclear extracts from CAMP-treated hepatocytes (gene repressed) or insulin-treated hepatocytes (gene transcribed) (P. B. Iynedjian, unpublished work). Thus there is at present no evidence for the regulated binding of USF to the P2 element. In addition, no specific, P2-element-mediated, effect of insulin on the expression of glucokinase promoter luciferase reporter plasmids could be obtained in transient transfections of either HepG2 cells or primary hepatocytes. In spite of these negative results, a more detailed analysis of the occupancy of the P2 element in diverse metabolic situations in animals and cells using footprinting experiments in vitro seems to be warranted.

Glucokinase and its mRNA are present in higher concentrations in the perivenous area than in the periportal area of the liver lobule [41,42]. Kietzmann et al. [43] reported recently that primary rat hepatocytes cultured under a reduced partial pressure of oxygen (that is, mimicking the perivenous situation) exhibit increased insulin induction of glucokinase mRNA compared with hepatocytes cultured under an oxygen partial pressure mimicking the perivenous situation. It is interesting that the sequence-specific binding activity of USF and its ability to stimulate transcription in a system in vitro have been shown to be positively regulated under reducing conditions by a mechanism involving cysteine thiol groups [44]. This raises the intriguing hypothesis that USF might have a role in causing or maintaining the metabolic zonation of glucokinase.

The DNA-binding sites of the various members of the bHLH proteins class of transcriptional regulators display considerable sequence overlap, making the formal identification of specific factors implicated in the regulation of given genes problematic [45,46]. The evidence shown here that the P2 cis-acting element of the liver glucokinase promoter is a target for USF proteins acting as transcriptional regulators of the gene is strong but does not exclude the possibility that, under special circumstances, other bHLH proteins might be involved. Interestingly, Valera et al. [47] have reported that transgenic mice overexpressing c-Myc in the liver under control of the phosphoenolpyruvate carboxykinase promoter have increased levels of glucokinase mRNA and enzyme activity in the liver. It is tempting to speculate that transcription of the glucokinase gene in the livers of these animals might have been stimulated by promiscuous binding of c-Myc (presumably as c-Myc/max dimers) to the P2 element. Mice with targeted disruption of genes encoding bHLH proteins will provide interesting animal models for analysing the role of particular factors of this class, although functional redundancy between diverse members of this class might be expected.

Finally, it should be stressed that the P2 cis-acting element accounted for only part (50 %) of the liver glucokinase promoter activity. The functional role of P1, the other DNA element revealed by footprinting assay in vitro, and the identity of the factor(s) binding to it, remain to be investigated.

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