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

Patrick B. IYNEDJIAN¹

Division of Clinical Biochemistry and Diabetes Research, University of Geneva School of Medicine, 1, rue Michel-Servet, CH-1211 Geneva 4, Switzerland

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 homeostasis [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 homeostasis 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'-tccccggGTCAAACCCGACCCACG-3', 5'-tccccggGTCAAACCCGACCCCTGTACAGTTCTTTGTCCTGGC-3' and 5'-tccccggGTCAAACCCGACCCCATGCGGTTCTTTGTCCTGG-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.

¹ E-mail iynedjian@medecine.unige.ch

*Sma*I. In all reactions the 3' primer was the commercial GLprimer 2 (Promega). The DNA fragments after PCR were digested with *Sma*I and *Bgl*II, 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 *Kpn*I-*Bgl*II 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 -bTD-U1) were provided by Dr. Michel Raymondjean and Dr. Axel Kahn [13]. An expression plasmid encoding human c-Myc [14] and 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 μ g/ml streptomycin and 10 % (v/v) 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 μ g of luciferase reporter plasmid and 0.5 μ g of the plasmid CMV- β (Invitrogen) encoding β -galactosidase for normalization purposes. Effector plasmids encoding transcription regulatory proteins were added in amounts of 1 μ g 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 rat livers and spleens were prepared as described previously [10]. Nuclear extracts from cultured HepG2 cells or primary hepatocytes were prepared by the method of Schreiber et al. [17], with buffers modified by the addition of 0.5 mM Na orthovanadate, 2 mM β -glycerophosphate and protease inhibitors (antipain, aprotinin, chymostatin, leupeptin, pepstatin, each at 2.5 μ g/ml). Nuclear proteins were stored at -75°C .

DNAse I protection assay *in vitro*

Methods were exactly as described previously [10].

Electrophoretic mobility-shift assay

An oligonucleotide probe containing the P2 element was obtained by annealing the following oligonucleotides: 5'-CAAGCCCG-ACCCACGTGGTCTTTGTC-3' and 5'-CAGGACAAAG-AACCACGTGGGGTCGGG-3'. Bold letters designate the P2 element identified by footprinting. After annealing and end-

filling with the Klenow fragment of DNA polymerase, the double-stranded oligonucleotide reproduces the liver glucokinase promoter sequence from nt -99 to -69 , except that the lower-case g at the fourth position of the first oligonucleotide is an A $\rightarrow$ G transition introduced to permit end-labelling with the Klenow fragment and [α - ^{32}P]dCTP. The foregoing probe is termed the rat probe. A probe (called competitor 2 in the legend to Figure 4) with a point mutation in the P2 element (bold lower-case) was obtained by annealing the following oligonucleotides: 5'-CAAGCCCGACCCCA**a**GTGGTCTTTGTC-3' and 5'-CAGGACAAAGAACCAC**t**GGGGTCGGG-3'.

A probe with the human promoter sequence from -93 to -64 [18] was obtained by annealing the following oligonucleotides: 5'-CAGCTGTACCCACATGGGCCTACCCT-3' and 5'-CAGAGGGTAGGCCCATGTGGGGTAACAG-3'. The above double-stranded oligonucleotide is called the human probe.

Nuclear proteins from liver or spleen were incubated with labelled probe in a total of 15 μ l of a reaction mixture containing (final concentrations): 10 mM Hepes, 10 mM Tris/HCl, pH 8.0, 60 mM KCl, 3 mM MgCl_2 , 0.5 mM EDTA, 0.4 mM dithiothreitol, 2 μ g of poly(dI·dC) as non-specific competitor and 4 % (v/v) glycerol. When specified, 0.5 μ g of sonicated single-stranded salmon testes DNA was present as an additional non-specific competitor. In reaction mixtures with nuclear extracts from cultured cells, KCl 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 μ l of 15 % (w/v) Ficoll. Samples (8 μ l) 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

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 $\pm$ S.E.M. for four independent transfections.

hepatoma cells, whereas the remote enhancer is uniquely active in primary hepatocytes.

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 zip (bHLHZ) 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 2 Footprinting of liver glucokinase promoter *in vitro*

(A) Gel electrophoresis of products obtained in the DNase I protection assay *in vitro* with a -310 to +11 liver glucokinase promoter fragment. The fragment was end-labelled at position +11 with polynucleotide kinase and [γ - 32 P]ATP. The fragment was subjected to limited digestion with DNase I in the presence of the indicated amounts of nuclear protein from the livers or spleens of fasted, glucose-refed (FR) rats. The four left-hand lanes show the products of Maxam-Gilbert sequencing of the fragment. (B) Diagram of footprints P1 and P2 revealed in (A). The liver promoter sequence readable from the Maxam-Gilbert products (from nt -124 to -7 in [11]) is shown. The boxes delineate the protected elements. The putative TATA box is also highlighted. Arrows denote positions with hypersensitive cleavage.

porter plasmids containing either the wild-type liver glucokinase promoter from -101 to +79 (GK-180Luc) or a similar sequence except for a block mutation replacing the wild-type CACGTG motif in the P2 element with a TGTACA sequence (P2m1GK-

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 $\pm$ S.E.M. for six independent transfections. Luciferase activities (means $\pm$ 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 transcription activators [19].

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

Figure 4 Electrophoretic mobility-shift assay with P2 oligonucleotide probes

(A) The labelled oligonucleotide was the rat probe defined in the Experimental section. Nuclear proteins were from the livers of fasted, glucose-refed rats. The input of protein in the binding reactions was $0.5 \mu\text{g}$. The crescendo symbol at the top of the figure indicates the presence of increasing amounts of unlabelled competitor oligonucleotide numbered as follows: 1, unlabelled rat oligonucleotide in molar excesses of 6-fold and 18-fold; 2, unlabelled competitor 2 defined in the Experimental section in molar excesses of 6-fold and 18-fold; 3, unlabelled human oligonucleotide defined in the Experimental section in molar excesses of 54-fold and 162-fold. (B) The labelled oligonucleotide was the human probe. Nuclear extract was from cultured HepG2 cells. The protein input in the binding reactions was $5 \mu\text{g}$. Sonicated single-stranded salmon testes DNA was present in addition to poly(dI-dC) as a non-specific competitor. The crescendo indicates 3-fold and 9-fold excesses of unlabelled rat oligonucleotide as specific competitor. In both panels the arrows designate the specific retarded complex and asterisks designate non-specific bands.

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₊₁GTgC (A₊₁ = 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 $\rightarrow$ A transversion converting the core CACGTG motif to CAAGTG, 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).

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 μ g of protein), nuclear extracts from cultured primary hepatocytes from rats (hepato) (2 μ g of protein), and nuclear extracts from HepG2 cells (2 μ g 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.

These results indicated that the specific retarded band reflects binding to element P2 and suggested that the binding 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 the human HepG2 hepatoma cells, a retarded complex was clearly seen (Figure 4B). The specific nature of the complex was shown by competition with an excess of unlabelled rat oligonucleotide (Figure 4B) or human oligonucleotide (results not shown).

Antibodies recognizing USF1 and USF2 were used in supershift assays with nuclear extracts from rat liver, cultured rat hepatocytes and HepG2 cells (Figure 5). This analysis included nuclear proteins both from the livers of fasted rats, in which the glucokinase gene is turned off, and from the livers of glucose-refed rats, in which the gene is induced. Proteins from both types of animal gave rise to the specific complex described above. In the presence of anti-USF antibodies the complex was replaced by two bands of markedly slower mobility, strongly suggesting that the complex reflects the binding of USF proteins to the oligonucleotide probe. The weaker intensity of retarded bands with proteins from fasted rats in Figure 5 was not a reproducible observation and was probably due to a lower input of proteins in this particular experiment. Nuclear extracts from cultured rat

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

Reporter plasmids with wild-type sequence (GK-180Luc) or a block mutation of the P2 element (P2m1GK-180Luc) were co-transfected into HepG2 cells with empty vectors (no effector), or expression vectors for the artificial trans-activator USF2/VP16 (50 ng of plasmid) alone or together with the dominant-negative variant Δ -bTD-U1 (1 μ g of plasmid). Cell extracts were prepared for assay of luciferase activity 20 h after transfection. Luciferase activity was normalized for β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid and was expressed relative to the activity of the reporter in the absence of co-expressed protein. Results are means $\pm$ S.E.M. for four separate experiments.

	Luciferase activity (%)		
	No effector	+ USF2/VP16	+ USF2/VP16 + Δ -bTD-U1
Reporter			
GK-180Luc	100	1860 $\pm$ 268	130 $\pm$ 15
P2m1GK-180Luc	100	76 $\pm$ 4	95 $\pm$ 12

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 trans-activator would then test for the role of endogenous USF proteins in the transcription of reporter genes (Table 2). Luciferase activity driven by GK-180Luc was modestly (20% inhibition), but significantly ($P < 0.022$) decreased by co-transfection of plasmid Δ -bTD-U1. Luciferase activity from P2m1GK-180Luc was unchanged, indicating that the negative action of the deleted USF1 protein occurred via a factor binding to the P2 element, presumably endogenous USF.

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 Δ -bTD-U1 plasmid was transfected without exogenous USF trans-activator. Luciferase activities were measured 44 h after transfection. Results are means $\pm$ S.E.M. for five separate experiments. * Difference from 'no effector' condition statistically significant, with $P < 0.022$.

Reporter	Luciferase activity (%)	
	No effector	+ Δ -bTD-U1
GK-180Luc	100	82 $\pm$ 4.9*
P2m1GK-180Luc	100	97 $\pm$ 11.5

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 $\pm$ S.E.M. for seven independent transfections in HepG2 cells and eight transfections in primary hepatocytes.

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 CATGcG. 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.

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 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. Co-transfection 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 in the electrophoretic mobility-shift assay, albeit with decreased affinity compared with the rat sequence.

Co-expression of an artificial variant of USF1 lacking trans-activation and basic domains significantly inhibited glucokinase promoter activity in HepG2 cells by a dominant-negative effect mediated via the P2 element. The inhibition of reporter activity amounted to only 20 % with the dominant-negative variant, compared with a 50 % inhibition caused by a full block mutation of the core P2 element. The rather limited efficacy of the dominant-negative variant might be explained by an incomplete ability of newly synthesized truncated USF1 molecules to disrupt

performed 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 factors 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/USF2a 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 CATGTG *cis*-acting element that seems to confer insulin inducibility on the gene [35]. Sul and Wang [36,37] showed that the CATGTG 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-refed 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 vivo* 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 periportal 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 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.

Thanks go to Karen Nazaryan for help with the preparation of nuclear extracts from cultured cells, to Florence Bourgoin for help with the construction of plasmid P2m2GK-180Luc, and to Asllan Gjinovci for dedicated assistance in the isolation of hepatocytes. I also thank Laurie Desbarats, Elisabeth Kaytor, Michel Cole, Martin Eilers, Axel Kahn, Michel Raymondjean and Howard Towle for the gifts of plasmids. This work was supported by grant 32-42207.94 from the Swiss National Science Foundation.

REFERENCES

- 1 Iynedjian, P. B., Mobius, G., Seitz, H. J., Wollheim, C. B. and Renold, A. E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1998–2001
- 2 Iynedjian, P. B., Pilot, P.-R., Nussipikel, T., Milburn, J. L., Quaade, C., Hughes, S., Ucla, C. and Newgard, C. B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7838–7842
- 3 Jetton, T. L., Liang, Y., Pettepher, C. C., Zimmerman, E. C., Cox, F. G., Horvath, K., Matschinsky, F. M. and Magnuson, M. A. (1994) *J. Biol. Chem.* **269**, 3641–3654
- 4 Weinhouse, S. (1976) *Curr. Top. Cell. Regul.* **11**, 1–50
- 5 Glinesman, W. H., Hern, E. P. and Lynch, A. (1969) *Am. J. Physiol.* **216**, 698–703
- 6 Rossetti, L., Giaccari, A., Barzilai, N., Howard, K., Sebel, G. and Hu, M. (1993) *J. Clin. Invest.* **92**, 1126–1134
- 7 Magnuson, M. A. and Shelton, K. D. (1989) *J. Biol. Chem.* **264**, 15936–15942
- 8 Iynedjian, P. B. (1993) *Biochem. J.* **293**, 1–13
- 9 Niswender, K. D., Postic, C., Jetton, T. L., Bennett, B. D., Piston, D. W., Efrat, S. and Magnuson, M. A. (1997) *J. Biol. Chem.* **272**, 22564–22569
- 10 Iynedjian, P. B., Marie, S., Wang, H., Gjinovci, A. and Nazaryan, K. (1996) *J. Biol. Chem.* **271**, 29113–29120
- 11 Magnuson, M. A., Andreone, T. L., Printz, R. L., Koch, S. and Granner, D. K. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4838–4842
- 12 Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J., Friedman, A. and Hanson, R. W. (1990) *Mol. Cell. Biol.* **10**, 6264–6272
- 13 Lefrançois Martinez, A. M., Martinez, A., Antoine, B., Raymondjean, M. and Kahn, A. (1995) *J. Biol. Chem.* **270**, 2640–2643
- 14 Philipp, A., Schneider, A., Vasrik, I., Xiong, Y., Beach, D., Alitalo, K. and Eilers, M. (1994) *Mol. Cell Biol.* **14**, 4032–4043
- 15 Gaubatz, S., Imhof, A., Dosch, R., Werner, O., Mitchell, P., Buettner, R. and Eilers, M. (1995) *EMBO J.* **14**, 1508–1519
- 16 Kaytor, E. N., Shih, H. and Towle, H. C. (1997) *J. Biol. Chem.* **272**, 7525–7531
- 17 Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
- 18 Tanizawa, Y., Matsutani, A., Chiu, K. C. and Permutt, M. A. (1992) *Mol. Endocrinol.* **6**, 1070–1081
- 19 Hanson, R. W. and Reshef, L. (1997) *Annu. Rev. Biochem.* **66**, 581–611
- 20 Du, H., Roy, A. L. and Roeder, R. G. (1993) *EMBO J.* **12**, 501–511
- 21 Luo, X. and Sawadogo, M. (1996) *Mol. Cell Biol.* **16**, 1367–1375
- 22 Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B. and Smale, S. T. (1994) *Mol. Cell Biol.* **14**, 116–127
- 23 Roy, A. L., Du, H., Gregor, P. D., Novina, C. D., Martinez, E. and Roeder, R. G. (1997) *EMBO J.* **16**, 7091–7104

- 24 Bendall, A. J. and Molloy, P. L. (1994) *Nucleic Acids Res.* **22**, 2801–2810
- 25 Krylov, D., Kasai, K., Echlin, D. R., Taparowsky, E. J., Arnheiter, H. and Vinson, C. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12274–12279
- 26 Blackwell, T. K., Huang, J., Ma, A., Kretzner, L., Alt, F. W., Eisenman, R. N. and Weintraub, H. (1993) *Mol. Cell. Biol.* **13**, 5216–5224
- 27 Sawadogo, M. and Roeder, R. G. (1985) *Cell* **43**, 165–175
- 28 Sirito, M., Lin, Q., Maity, T. and Sawadogo, M. (1994) *Nucleic Acids Res.* **22**, 427–433
- 29 Aperlo, C., Boulukos, K. E., Sage, J., Cuzin, F. and Pognonec, P. (1996) *Genomics* **37**, 337–344
- 30 Viollet, B., Lefrançois-Martinez, A. M., Henrion, A., Kahn, A., Raymondjean, M. and Martinez, A. (1996) *J. Biol. Chem.* **271**, 1405–1415
- 31 Diaz-Guerra, M.-J. M., Bergot, M.-O., Martinez, A., Cuif, M.-H., Kahn, A. and Raymondjean, M. (1993) *Mol. Cell. Biol.* **13**, 7725–7733
- 32 Shih, H. and Towle, H. C. (1994) *J. Biol. Chem.* **269**, 9380–9387
- 33 Kennedy, H. J., Viollet, B., Rafiq, I., Kahn, A. and Rutter, G. A. (1997) *J. Biol. Chem.* **272**, 20636–20640
- 34 Vallet, V. S., Henrion, A. A., Bucchini, D., Casado, M., Raymondjean, M., Kahn, A. and Vaulont, S. (1997) *J. Biol. Chem.* **272**, 21944–21949
- 35 Moustaid, N., Beyer, R. S. and Sul, H. S. (1994) *J. Biol. Chem.* **269**, 5629–5634
- 36 Wang, D. and Sul, H. S. (1995) *J. Biol. Chem.* **270**, 28716–28722
- 37 Wang, D. and Sul, H. S. (1997) *J. Biol. Chem.* **272**, 26367–26374
- 38 Iynedjian, P. B., Gjinovci, A. and Renold, A. E. (1988) *J. Biol. Chem.* **263**, 740–744
- 39 Iynedjian, P. B., Jotterand, D., Nospikel, T., Asfari, M. and Pilot, P.-R. (1989) *J. Biol. Chem.* **264**, 21824–21829
- 40 Iynedjian, P. B., Marie, S., Gjinovci, A., Genin, B., Deng, S.-P., Buhler, L., Morel, P. and Mentha, G. (1995) *J. Clin. Invest.* **95**, 1966–1973
- 41 Katz, N., Teutsch, H. F., Jungermann, K. and Sasse, D. (1977) *FEBS Lett.* **83**, 272–276
- 42 Moorman, A. F. M., De Boer, P. A. J., Charles, R. and Lamers, W. H. (1991) *FEBS Lett.* **287**, 47–52
- 43 Kietzmann, T., Roth, U., Freimann, S. and Jungermann, K. (1997) *Biochem. J.* **321**, 17–20
- 44 Pognonec, P., Kato, H. and Roeder, R. G. (1992) *J. Biol. Chem.* **267**, 24563–24567
- 45 Boyd, K. E. and Farnham, P. J. (1997) *Mol. Cell. Biol.* **17**, 2529–2537
- 46 Desbarats, L., Gaubatz, S. and Eilers, M. (1996) *Genes Dev.* **10**, 447–460
- 47 Valera, A., Pujol, A., Gregori, X., Riu, E., Visa, J. and Bosch, F. (1995) *FASEB J.* **9**, 1067–1078