Identification of a cAMP response element within the glucose-6-phosphatase hydrolytic subunit gene promoter which is involved in the transcriptional regulation by cAMP and glucocorticoids in H4IIE hepatoma cells

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The expression of a luciferase reporter gene under the control of the human glucose 6-phosphatase gene promoter was stimulated by both dexamethasone and dibutyryl cAMP in H4IIE hepatoma cells. A *cis*-active element located between nucleotides –161 and –152 in the glucose 6-phosphatase gene promoter was identified and found to be necessary for both basal reporter-gene expression and induction of expression by both dibutyryl cAMP and dexamethasone. Nucleotides –161 to –152 were functionally replaced by the consensus sequence for a cAMP response element. An antibody against the cAMP response element-binding protein caused a supershift in gel-electrophoretic-mobility-shift assays

using an oligonucleotide probe representing the glucose 6-phosphatase gene promoter from nucleotides –161 to –152. These results strongly indicate that in H4IIE cells the glucose 6-phosphatase gene-promoter sequence from –161 to –152 is a cAMP response element which is important for the regulation of transcription of the glucose 6-phosphatase gene by both cAMP and glucocorticoids.

Key words: gluconeogenesis, insulin, liver, phosphoenolpyruvate carboxykinase.

INTRODUCTION

The glucose 6-phosphatase hydrolytic subunit (G6Pase), a key gluconeogenic enzyme, catalyses the hydrolysis of glucose 6-phosphate to glucose, which is important for the maintenance of blood glucose levels [1–3]. Glucagon (acting via cAMP) and glucocorticoids increase G6Pase activity by inducing G6Pase gene expression in the liver during starvation [4–7]. The recent cloning of the promoter regions of the human [8], rat [9] and mouse [10] G6Pase genes has made it possible to study the molecular basis of control of G6Pase gene expression. An insulin response element has recently been described in the G6Pase promoter and shown to be highly similar to one in the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter [10]. In addition, the binding of the hepatic nuclear factors HNF 1 α and HNF 3 γ to three activation elements between nt –234 and –66 has been shown to be essential for basal G6Pase gene-promoter expression [11]. In the same report a cAMP response element (CRE) was identified between nt –136 and –129 [11]. CREs mediate the stimulation of the transcription of many genes after activation of the adenylate cyclase/cAMP pathway (for review see [12]). The most critical protein binding to a CRE is the so-called CRE-binding protein (CREB). An increase in the level of cAMP induces the phosphorylation of CREB by protein kinase A, enabling CREB to act as a docking protein for the CREB-binding protein.

In this paper we have identified a second CRE within the G6Pase promoter which is involved in the induction of G6Pase gene transcription by both cAMP and glucocorticoids.

EXPERIMENTAL

Materials

Restriction nucleases, modifying enzymes, pGL3 basic vector, pSV- β -galactosidase control vector and the luciferase-detection reagent were purchased from Promega (Southampton, U.K.). The site-directed mutagenesis and sequencing kits were purchased from Amersham (Braunschweig, Germany). Plasmid-isolation kits were from Qiagen (Hilden, Germany). Insulin, dexamethasone, β -oestradiol, retinoic acid and tri-iodothyronine were purchased from Sigma (Deisenhofen, Germany); N⁶,2'-O-dibutyryl cAMP (db₂cAMP) was from Boehringer Mannheim (Mannheim, Germany) and Calbiochem (Nottingham, U.K.).

Cell culture and transfections

H4IIE and HepG2 hepatoma cells were cultivated in Dulbecco's modified Eagle's medium (DMEM)/10 % fetal calf serum. H4IIE cells were transfected using 10 μ g of reporter-gene construct and 2 μ g of pSV- β -galactosidase control vector per dish as described elsewhere [8]. Following transfection (18 h) the cells were shocked by 15 % glycerol for 2 min and subsequently incubated for 4 h in DMEM. The medium was then exchanged to DMEM with or without dexamethasone (1 μ M), db₂cAMP (500 μ M), oestradiol (1 μ M), retinoic acid (2 μ M) or tri-iodothyronine (100 nM). Following a subsequent incubation of 24 h the cells were harvested and the luciferase activities measured. HepG2 cells were transfected in similar way, except that only 5 μ g of reporter-

Abbreviations used: G6Pase, glucose 6-phosphatase hydrolytic subunit; CRE, cAMP response element; CREB, CRE-binding protein; db₂cAMP, N⁶,2'-O-dibutyryl cAMP; PEPCK, phosphoenolpyruvate carboxykinase; IBMX, 3-isobutyl-1-methylxanthine; RLA, relative luciferase activity; DMEM, Dulbecco's modified Eagle's medium.

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gene vector and, in some experiments, 1 μ g of an expression vector for the glucocorticoid receptor were transfected with the pSV- β -galactosidase control vector per dish. The luciferase activities were normalized by measuring the β -galactosidase activities in the extracts [8]. Each series of experiments was performed at least three times with at least two different plasmid preparations, and each transfection was performed in triplicate. In each series of experiments three plates were co-transfected with 10 μ g of pGL3 basic vector and 2 μ g of pSV- β -galactosidase vector. The normalized luciferase expression in these cell extracts was set as 1, unless otherwise indicated. Data are presented as means $\pm$ S.E.M. ($n = 3$) unless otherwise indicated. Statistical analysis (t -test) was performed using the InStat program.

Reporter-gene constructs

The 5'-flanking region of the human G6Pase gene up to nt -3920 relative to the transcription start site was obtained from a previously described cosmid clone [8] and sequenced in both directions by a combination of automated fluorescence sequencing and manual sequencing. The -3920/+57 fragment was cloned into the *SacI/XhoI* sites of the reporter-gene plasmid pGL3 basic. The construction of the reporter-gene construct containing the -1227/+57 fragment has been described previously [8]. Deletion mutants were created by exonuclease III (erase-a-base, Promega) or, in the case of the constructs containing the promoter fragments -180/+4, -161/+4, -150/+4 and -63/+4, by PCR using primers with nested *XhoI* and *SacI* restriction sites for subsequent cloning into pGL3 basic. The plasmid CRE2mut, in which the palindromic sequence from nt -161 to -152 in the -180/+4 construct was mutated at positions -157, -154 and -153 from 5'-TTTACGTAAA-3' to 5'-TTTAGGTCCA-3' (mutated bases are underlined), was generated using the site-directed mutagenesis kit from Stratagene (Heidelberg, Germany). For the plasmids CRE2mut/CRE2 and -63/+4/CRE2 a double-stranded oligonucleotide containing the sequence 5'-TTTACGTAAA-3' was cloned into the *KpnI/SacI* sites of each plasmid. The construct CRE2mut/-3kb-CRE2 was generated by cloning an oligonucleotide with the sequence 5'-TTTACGTAAA-3' into the *BamHI* site of CRE2mut, approximately 3 kb upstream of the TATA box of the G6Pase gene promoter. The plasmid CRE2mut/CREcons was created by cloning a double-stranded oligonucleotide with the consensus sequence of a CRE (5'-TGACGTCA-3') into the *KpnI/SacI* site of the plasmid CRE2 mut. The plasmid TK-pGL3 was generated by cloning the promoter fragment from nt -109 to +56 of the human herpes virus thymidine kinase gene into the *XhoI/HindIII* sites of pGL3. The plasmid TK-pGL3/CRE2 contained a double-stranded oligonucleotide with the sequence 5'-TTTACGTAAA-3' within the *KpnI/SacI* sites of TK-pGL3. All plasmids were verified by restriction mapping and sequencing of the flanking regions (deletion mutants) or the entire inserts (PCR constructs and site-directed mutated plasmids).

Isolation of nuclear extracts and gel-electrophoretic-mobility-shift assays

Nuclear extracts were isolated according to the method of Schreiber et al. [13]. For gel-electrophoretic-mobility-shift assays nuclear extracts (9 μ g of protein) were incubated in a total of 20 μ l with a 32 P-labelled double-stranded oligonucleotide (40000 c.p.m.) and 1 μ g of a mixture of double-stranded poly(dI-dC) and poly(dA-dT) in 20 mM Tris/HCl (pH 7.9)/100 mM NaCl/20% (v/v) glycerol/200 μ M dithiothreitol for 20 min at room temperature. Where indicated nuclear extracts were pre-incubated at room temperature for 10 min with 1 μ l of

an anti-CREB antibody (New England Biolabs, Schwalbach, Germany). The reaction mixtures were resolved on 6% non-denaturing polyacrylamide gels, which were then dried and autoradiographed. The labelled double-stranded oligonucleotides had the sequences: 5'-AGACTGCTTTACGTAAAGAGAGA-3' (the sequence corresponding to the promoter sequence between nt -161 and -152 is underlined); 5'-AGACTGCTTTAGGTCCAGAGAGA-3' (mutated nt corresponding to positions -157, -154 and -153 of the promoter sequence are shown in bold); and 5'-AGATTGCCTGACGTCAAGAGCT-3' (the consensus sequence of a CRE is underlined).

RESULTS

Dexamethasone and cAMP synergistically stimulate the G6Pase gene promoter in H4IIE cells

We studied the expression of the reporter gene luciferase under control of the human G6Pase gene promoter in the rat hepatoma cell line H4IIE (Table 1). In accordance with our previous study [8], the expression of the 1.2 kb promoter-reporter-gene construct could be induced approximately 10-fold by the artificial glucocorticoid dexamethasone (Table 1). Here we demonstrate that the induction of gene transcription by dexamethasone was specific and could not be mimicked by other hormones binding to nuclear receptors, for example oestradiol, retinoic acid and triiodothyronine (Table 1). Reporter-gene expression was stimulated approximately two-fold by db_2 cAMP (Table 1). This effect was confirmed by application of the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Table 1). Dexamethasone and db_2 cAMP had a synergistic effect on the reporter-gene expression, resulting in an approximately 20-fold induction (Table 1). db_2 cAMP had no effect in our previous report [8], due to the use of a db_2 cAMP preparation with lower purity.

Reporter-gene activities of constructs containing either the -3920/+57 or the -1227/+57 fragments of the G6Pase promoter are induced by db_2 cAMP and dexamethasone to a similar extent in H4IIE cells

To study whether *cis*-active sequences in the G6Pase gene promoter located further upstream than 1.2 kb from the transcription start site could enhance the basal transcription rate of

Table 1 Regulation of the luciferase reporter-gene construct under the control of the -1257/+57 G6Pase gene promoter fragment

H4IIE hepatoma cells were co-transfected with 10 μ g of reporter-gene construct containing the G6Pase promoter fragment -1227/+57 and 2 μ g of pSV- β -galactosidase vector. Following glycerol shock the cells were incubated with the indicated hormones and hormone mimetics. The reporter-gene activities were measured and normalized. The background expression of the promoter-less vector pGL3 basic was set as 1. Data are means $\pm$ S.E.M. ($n = 6$). *, $P < 0.05$ versus no mediator; †, $P < 0.05$ versus dexamethasone or db_2 cAMP.

Mediator	Relative luciferase expression
None	2.7 $\pm$ 0.6
db_2 cAMP (500 μ M)	5.5 $\pm$ 1.4*
Dexamethasone (1 μ M)	26.2 $\pm$ 4.2*
Dexamethasone (1 μ M) + db_2 cAMP (500 μ M)	43.9 $\pm$ 4.6†
Retinoic acid (2 μ M)	2.6 $\pm$ 0.7
β -Oestradiol (1 μ M)	2.9 $\pm$ 0.4
Tri-iodothyronine (100 nM)	2.9 $\pm$ 0.5
Forskolin (19 μ M) + IBMX (500 μ M)	9.2 $\pm$ 1.6*

Figure 1 Effect of deletions in the flanking sequence of the G6Pase gene on the basal promoter activity and its induction by dexamethasone and db_2cAMP in H4IIE cells

The indicated fragments of the G6Pase 5'-flanking region were cloned into the promoter-less and enhancer-less luciferase reporter vector pGL3 basic. H4IIE cells were co-transfected with these plasmids and with pSV- β -galactosidase control vector. Solid bars indicate basal expression in the absence of hormone mimetics, open bars indicate the expression in the presence of 1 μM dexamethasone and 500 μM db_2cAMP . At least three independent experiments were carried out with two preparations of each construct. Data are expressed as means $\pm$ S.E.M. ($n = 3$) relative to the background expression of a promoter-less vector pGL3 basic, which was set as 1.

the luciferase reporter gene, we cloned and sequenced the 5'-flanking region of the human G6Pase gene up to nt -3920 from the transcription start site. The expression of the luciferase reporter gene in response to db_2cAMP and dexamethasone was similar when under the control of either the promoter fragment -3920/+57 or the -1227/+57 fragment (Figure 1). This indicated that no additional *cis*-active elements were present in the longer fragment that significantly influenced the G6Pase promoter activity under these experimental conditions.

The -161 to -150 portion of the G6Pase promoter is required for regulation by both db_2cAMP and dexamethasone in H4IIE cells

In order to define *cis*-active elements between nt -1227 and the TATA box, reporter-gene constructs with sequential deletions of the promoter were transfected. Truncation of the 5'-flanking promoter sequence to nt -161 had no significant influence on the reporter-gene expression in H4IIE cells in the presence or absence of dexamethasone and db_2cAMP (Figure 1). The reporter-gene activities following transfection of constructs -180/+57 or -180/+4 were identical, indicating that the truncation of the 3' end of the G6Pase gene promoter from nt +57 to +4 did not affect reporter-gene expression. Shortening of the 5' end of the promoter fragment from nt -161 to -150 significantly decreased basal expression to 0.4 ± 0.1 relative luciferase activity (RLA; -150/+57 construct) or 0.6 ± 0.2 RLA (-150/+4 construct) compared with the -180/+4 construct (1.3 ± 0.2 RLA; $P < 0.05$) and the -161/+4 construct (2.2 ± 0.3 RLA; $P < 0.05$). Reporter-gene expression driven by the G6Pase gene-promoter fragments -150/+57 or -150/+4 could still be induced by dexamethasone and db_2cAMP (1.4 ± 0.2 RLA or 1.7 ± 0.3 RLA, respectively, $P < 0.05$ versus respective basal expression), although the induction was less than with the longer constructs. From these results it was clear that the promoter sequence between nt -161 and -150 was important

Figure 2 Similarity of the nucleotide sequence of CRE2 and its location relative to the insulin response element in the human, mouse and rat G6Pase genes

Sequences similar to CRE2, in the human sequence located between nt -161 and -152, are represented by shaded boxes. Two of the motifs of the insulin response sequence (IRS, [10]) are shown by open boxes. For comparison, the sites of the mutations introduced into the vector CRE2mut are also shown, with the mutated bases underlined. Bold letters indicate differences between the human, mouse and rat gene sequences.

for the reporter-gene expression and the extent of its induction by dexamethasone and db_2cAMP in H4IIE cells. This region contains the 10 bp perfect palindromic sequence 5'-TTTACG-TAAA-3'. The sequence and position of this motif relative to the previously described insulin response element [10] are conserved between the human, rat and mouse G6Pase genes (Figure 2).

The CRE consensus sequence compensates the effect of mutations within the sequence from nt -161 to -152 on the regulation of the G6Pase promoter in H4IIE cells

To investigate the significance of the palindromic sequence between nt -161 and -152 for G6Pase expression, this region was mutated within the construct -180/+4, generating construct CRE2mut (Figure 2). Due to the similarity of the sequence -161/-152 to the consensus sequence of a CRE, the mutations were introduced in such a way as to prevent potential CREB binding [14], while not interfering with the overlapping motif of the insulin response element located between nt -164 and -158 [10] (Figure 2). The mutagenesis of nt -157, -154 and -153 within construct -180/+4 decreased the reporter-gene expression by a similar degree to that resulting from deletion of the sequence -161 to -150, as can be seen by comparing CRE2mut with the construct -150/+4 (Figure 3). In the case of the construct CRE2mut, both basal luciferase expression (0.4 ± 0.1 RLA) and the ability of db_2cAMP and dexamethasone to induce reporter-gene expression (0.8 ± 0.2 RLA) were reduced compared with the expression of the construct -180/+4 (basal expression, 1.3 ± 0.2 RLA; in the presence of db_2cAMP and dexamethasone, 34.0 ± 4.0 RLA, $P < 0.05$). The plasmid CRE2mut/CRE2 further demonstrated the significance of the sequence -161/-152. In order to generate this plasmid, an oligonucleotide with the sequence of nt -161 to -152 was cloned into the multi-cloning site of CRE2mut, approximately 40 bp from the 5' end of the promoter insert. The introduction of this sequence restored the responsiveness of reporter-gene expression to dexamethasone and db_2cAMP and increased basal reporter-gene expression. Basal reporter-gene expression (1.3 ± 0.2 RLA) and its induction in the presence of db_2cAMP (3.7 ± 1.0 RLA) were within the same range for both the CRE2mut/CRE2 and the wild-type construct -180/+4. How-

Figure 3 Mutation and deletion of the CRE2 sequence decreased basal promoter activity and responsiveness to hormone mimetics in H4IIE cells, an effect which could be abolished by the introduction of the consensus sequence for CRE

CRE2mut was based on the construct $-180/+4$, but contained mutations in the sequence from nt -161 to -152 . CRE2mut/CRE2 and CRE2mut/CREcons contained the wild-type sequence $-161/-152$ and the consensus sequence for CRE respectively, within 200 bp of the TATA box of the promoter. In CRE2mut/-3kb-CRE2 the sequence $-161/-152$ was cloned into the reporter-gene construct approximately 3 kb upstream from the TATA box. H4IIE cells were co-transfected with the indicated plasmids and with the pSV- β -galactosidase control vector. Black bars indicate basal expression. The presence of db_2cAMP ($500 \mu\text{M}$) is indicated by grey bars (second from top in each group), the presence of dexamethasone ($1 \mu\text{M}$) by striped bars and of both dexamethasone and db_2cAMP by open bars. At least three independent experiments were carried out with two preparations of each construct. Data are expressed as means $\pm$ S.E.M. ($n = 3$) relative to the background expression of the promoter-less vector pGL3 basic, which was set as 1.

ever, the extent of reporter-gene induction by dexamethasone alone or in combination with db_2cAMP was significantly higher with the construct CRE2mut/CRE2 than with the construct $-180/+4$ (98.3 ± 21.0 RLA compared with 34.0 ± 4.0 RLA in the presence of both hormone mimetics, $P < 0.05$). Furthermore, the introduction of the wild-type sequence into the CRE2mut had no significant effect on reporter-gene expression when the oligonucleotide was cloned approximately 3 kb from the transcription start site (construct CRE2mut/-3kb-CRE2). These experiments demonstrated that the nt from -161 to -152 were sensitive to mutagenesis and must be located in relative proximity to the transcription start site in order to mediate the induction by cAMP and dexamethasone. The sequence between nt -161 and -152 possesses a similarity with the CRE consensus sequence, as mentioned above and in [14]. We investigated whether the CRE consensus sequence could functionally replace the respective G6Pase promoter sequence by generating the reporter construct CRE2mut/CREcons. As can be seen in Figure 3, the CRE consensus sequence was able to overcome the effect of the mutations between nt -161 and -152 . The induction of reporter-gene expression in the presence of dexamethasone was approximately four-fold higher with the construct CRE2mut/CREcons than with the construct CRE2mut/CRE2. This difference was not observed for basal expression or the induction by db_2cAMP alone.

The sequence from nt -161 to -152 confers db_2cAMP and dexamethasone responsiveness to a minimal G6Pase promoter, but only db_2cAMP responsiveness to a thymidine kinase promoter

Recently, binding sites for CREB, HNF 1α and HNF 3γ have been identified within the promoter region between nt -226 and

Figure 4 The sequence from nt -161 to -152 confers db_2cAMP and dexamethasone responsiveness to a G6Pase promoter fragment beginning at nt -63 , but only db_2cAMP responsiveness to a thymidine kinase promoter fragment

H4IIE hepatoma cells were transfected with (A) plasmids $-63/+4$ and $-63/+4/\text{CRE2}$ and with (B) the thymidine kinase gene-promoter constructs TK-pGL3 and TK-pGL3/CRE2. Black bars indicate basal expression. The presence of db_2cAMP ($500 \mu\text{M}$) is indicated by grey bars (second from top in each group), the presence of forskolin ($10 \mu\text{M}$) and IBMX ($500 \mu\text{M}$, in A only) by vertical-striped bars, the presence of dexamethasone ($1 \mu\text{M}$) by diagonal-striped bars and of both dexamethasone and db_2cAMP by open bars. At least three independent experiments were carried out with two preparations of each construct. Data are expressed as means $\pm$ S.E.M. ($n = 3$) relative to the background expression of a promoter-less vector pGL3 basic (A) or relative to the luciferase expression in the absence of hormone mimetics (B).

-66 [11]. In accordance with these results the basal reporter-gene expression of a construct containing a promoter fragment at nt $-63/+4$ was very low (0.3 ± 0.1 RLA) and could not be induced significantly by dexamethasone or db_2cAMP (Figure 4A). However, the reporter-gene expression of plasmid $-63/+4/\text{CRE2}$, which contained the nt -161 to -152 could be induced by dexamethasone (Figure 4A). In addition, db_2cAMP and a combination of forskolin and IBMX elevated reporter-gene expression, demonstrating that reporter-gene expression of the construct $-63/+4/\text{CRE2}$ could be regulated by cAMP. Basal expression of the construct $-63/+4/\text{CRE2}$ was not significantly elevated compared with construct $-63/+4$ and the extent of its expression in the presence of the hormone mimetics was approximately 20% of that observed for the CRE2mut/CRE2 construct (compare Figure 3 with Figure 4A). This was probably caused by the loss of *cis*-active elements located between nt -180 and -63 , which contribute to basal expression [11].

Figure 5 The sequence from nt -161 to -152 influences dexamethasone responsiveness to the G6Pase promoter fragment in human HepG2 cells

HepG2 cells were transfected with the indicated plasmids together with an expression vector for the glucocorticoid receptor. CRE2mut was based on the construct $-180/+4$, but contained mutated sequence from nt -161 to -152 . CRE2mut/CRE2 and CRE2mut/CREcons contained the wild-type sequence $-161/-152$ and the consensus sequence for CRE within 200 bp of the TATA box of the promoter. Basal expression is indicated by black bars, the presence of db₂cAMP (500 μ M) by grey bars (second from top in each group), the presence of dexamethasone (1 μ M) by striped bars and of both dexamethasone and db₂cAMP by open bars. At least three independent experiments were carried out with two preparations of each construct. Data are expressed as means $\pm$ S.E.M. ($n = 3$) relative to the background expression of the promoter-less vector pGL3 basic, which was set as 1.

These experiments indicated that the sequence from nt -161 to -152 was sufficient to mediate the induction of reporter-gene expression by dexamethasone and db₂cAMP where the G6Pase gene-promoter fragment lacked all previously described *cis*-active elements, including the CRE between nt -136 and -129 [11]. Similar results to the expression of construct $-63/+4$ /CRE2 were obtained with a construct $-105/+4$ /CRE2 (results not shown). The luciferase expression of the plasmid TK-pGL3, under control of the thymidine kinase gene promoter, was slightly induced by db₂cAMP (1.3 ± 0.1 RLA, basal luciferase activity was set as 1, Figure 4B, $P < 0.05$). The luciferase expression of plasmid TK-pGL3/CRE2, which contained the sequence of the G6Pase promoter from nt -161 to -152 , was more highly induced by db₂cAMP (2.1 ± 0.2 RLA, basal luciferase activity was set as 1, $P < 0.05$). This data confirmed earlier results [14], demonstrating that the sequence motif 5'-TTTACGTAAA-3' possessed transactivation activity. The luciferase expression of plasmids TK-pGL3/CRE2 and TK-pGL3 in response to dexamethasone were not significantly different, indicating that the sequence from nt -161 to -152 is not in itself sufficient to mediate the regulation by glucocorticoids.

The sequence between nt -161 and -152 is involved in the activation of the G6Pase promoter by dexamethasone in HepG2 cells

In order to determine whether the sequence of the human G6Pase promoter between nt -161 to -152 had an effect on reporter-gene expression in a human cell line as well as in the rat hepatoma cell line, human HepG2 hepatoma cells were transfected with plasmids $-180/+4$, CRE2mut, CRE2mut/CRE2 and CRE2mut/CRE2cons (Figure 5). Following transfection of the construct $-180/+4$, the reporter-gene expression was stimulated by dexamethasone and db₂cAMP to a much lower extent in HepG2 cells than in H4IIE cells (compare Figures 3 and 5). This

Figure 6 CREB bound to a 32 P-labelled oligonucleotide with the sequence of the G6Pase promoter from nt -161 to -152 , but not after mutagenesis of positions -157 , -154 and -153

Gel-electrophoretic-mobility-shift assays were carried out with nuclear proteins of H4IIE cells using double-stranded oligonucleotide probes with the sequence of the G6Pase promoter from nt -161 to -152 either unmutated (lanes 1 and 2) or with mutations at nt corresponding to positions -157 , -154 and -153 (lanes 3 and 4). A third oligonucleotide comprising the consensus sequence of a CRE was also used (lanes 5 and 6). Supershift experiments were carried out with an antibody against CREB (lanes 2, 4 and 6). The arrow indicates the supershifted band; a and b symbolize protein complexes and c indicates free probe.

was also the case when the glucocorticoid receptor was co-expressed. The induction of reporter-gene expression by dexamethasone either alone or in combination with db₂cAMP was significantly lower with construct CRE2mut than with construct $-180/+4$, indicating an involvement of the promoter sequence between nt -161 and -152 in the glucocorticoid response in HepG2 cells. In contrast to H4IIE cells, the mutations had no significant influence on either basal activity, or the induction by db₂cAMP alone. However, as was the case in H4IIE cells, the induction of reporter-gene expression by dexamethasone either alone or in combination with db₂cAMP was increased following transfection with constructs CRE2mut/CRE2 and CRE2mut/CREcons. The data presented (Figure 5) were obtained in the presence of co-transfected glucocorticoid receptor protein, as controversial data are available in the literature about the extent to which HepG2 cells express functional glucocorticoid receptor

protein [15–17]. The omission of the co-transfected receptor plasmid had in our hands no significant effect on the results described above (results not shown).

CREB binds to an oligonucleotide with the sequence corresponding to nt –161 to –152 of the G6Pase promoter

In order to characterize the binding of nuclear proteins to the sequence between nt –161 and –152 we performed gel-electrophoretic-mobility-shift assays using nuclear protein extracts from H4IIE cells and double-stranded synthetic oligonucleotides corresponding to either the wild-type or mutated G6Pase promoter sequence from nt –161 to –152, or a third oligonucleotide with the consensus sequence of a CRE. When using the wild-type oligonucleotide the formation of two protein complexes (a and b) was observed (Figure 6, lane 1). Pre-incubation of the extracts with an antiserum against CREB led to a shift of complex a (Figure 6, arrow, lane 2). When an oligonucleotide with the consensus sequence of a CRE was used as a probe, a major protein complex a was formed (Figure 6, lane 5). Pre-incubation with the anti-CREB antibody resulted in a supershift similar to that observed with the wild-type oligonucleotide (Figure 6, lane 6). Parallel gel-shift assays were performed using an oligonucleotide with the promoter sequence from nt –161 to –152, containing the mutations of CRE2mut within the palindromic sequence as described above. The major difference between the binding pattern seen with this oligonucleotide and those seen with the wild-type and consensus sequences was the lack of the protein complex a, which could be supershifted with the anti-CREB antibody (Figure 6, lanes 3 and 4). These experiments indicated that the wild-type, but not the mutant sequence, between nt –161 and –152 was capable of binding CREB. Identical results were obtained using nuclear extracts of human HepG2 hepatoma cells (results not shown).

DISCUSSION

The results of the present study strongly indicate that nt –161 to –152 act as a CRE within the G6Pase gene promoter. This sequence mediated responsiveness to elevated cAMP levels, was able to bind CREB, and could be functionally replaced by a consensus sequence for CRE. Mutations within this sequence known to suppress CREB binding [14] abolished *trans*-activation activity, as did deletion of the sequence. In addition, this sequence was active only when cloned in relative proximity to the transcription start site. CREs located more than 500 bp distal to the TATA box are less active [12]. We named this element CRE2, to distinguish it from the CRE recently identified in the G6Pase promoter between nucleotides –136 and –129 [11], which we will subsequently refer to as CRE1. The presence of CRE1 is most likely to be responsible for the low induction of reporter-gene expression by cAMP observed with the constructs –150/+4 and CRE2mut, both of which lacked the CRE2 motif. This effect was not observed with the constructs –63/+4 (Figure 4), which lacked CRE1.

In a previous study [14] using synthetic oligonucleotides with variations of the CRE consensus sequence, it was shown that an oligonucleotide with the 8 bp core sequence of CRE2, 5'-TTACGTAA-3', was able to bind recombinant CREB and activate transcription if cloned upstream of the thymidine kinase promoter. The binding region of CREB consists of an 8 bp core sequence, where the flanking nucleotides strongly influence the binding affinity [14]. Due to the perfect symmetry of the 10 bp sequence in the G6Pase gene promoter, we postulate that the two flanking nucleotides are part of CRE2, although we did not show

that they influenced transcriptional activity in this particular case. To our knowledge the sequence of CRE2 differs from all other CREs described so far.

The introduction of the consensus sequence for CRE did not augment the response to db₂cAMP (Figure 3). This may suggest that other transcription factors are necessary to mediate the full cAMP response and that in this case their absence limited the effect of the increased binding of CREB to the G6Pase promoter. From studies of the PEPCK promoter it is known that the so-called cAMP response unit consists of several *cis*-acting elements, besides CRE [18–20]. The same may also be true for the G6Pase promoter.

Our results indicate that not only does CRE2 mediate responsiveness of the G6Pase promoter to cAMP, but that intactness of CRE2 is also necessary for the induction of gene transcription by the synthetic glucocorticoid dexamethasone. This suggests an interaction between cAMP signalling and the glucocorticoid response at CRE2. The involvement of CRE2 in the glucocorticoid response may also explain the augmented induction of reporter-gene expression by dexamethasone using the construct CRE2mut/CREcons compared with the construct CRE2mut/CRE2 (Figure 4). Recombinant CREB binds with higher affinity to an oligonucleotide with the consensus sequence of CRE than to one with the core sequence of CRE2 [14] (Figure 6), and results in approximately 3.5-fold higher transcriptional activation [14]. Obviously, the higher binding affinity of CREB for CREcons over CRE2 influences the regulation of the G6Pase promoter by glucocorticoids. The increased expression of the construct CRE2mut/CRE2 compared with that of construct –180/+4 (Figure 3) could be explained by the greater accessibility of CREB to CRE2 in the CRE2mut/CRE2 construct, due to the lack of overlapping binding sites. The CRE2 sequence overlaps partly with a motif of the insulin response element [10]. The participation of a CRE in the glucocorticoid response has been reported for the regulation of the PEPCK [21,22] and somatostatin [23] genes. In the case of the PEPCK promoter, experimental data indicate a direct physical interaction between the glucocorticoid receptor and CREB [21]. The CRE of the PEPCK promoter was also found to enhance the glucocorticoid response by a stimulation of basal transcription [22]. The molecular basis of the proposed interaction between the glucocorticoid and cAMP responses at CRE2 is unclear. At least one additional *cis*-active sequence must be involved in the glucocorticoid response of the G6Pase promoter, because the CRE2 sequence does not transfer glucocorticoid responsiveness to the heterologous thymidine kinase gene promoter. In addition, this sequence must be localized between nt –63 and +4. Evidence for this assumption was provided by the cloning of CRE2 into the promoter fragment –63/+4, which led to the regulation of this construct by both cAMP and dexamethasone. A binding site for the glucocorticoid receptor within the gene promoter has not yet been identified. The data indicate a similar role of CRE2 in the regulation of the human promoter by glucocorticoids in both rat H4IIE and human HepG2 hepatoma cells. We performed most of our experiments with H4IIE cells, as the reporter gene was induced much more strongly by the hormone mimetics in these cells than in HepG2 cells. In addition, H4IIE cells have been established for the characterization of a species-heterologous G6Pase promoter [10]. The lack of a significant difference between the inducibility of the plasmid CRE2mut and the unmutated construct by db₂cAMP in HepG2 cells might indicate that in these cells CRE2 contributes less to the overall regulation by cAMP than is the case in H4IIE cells. From the study of the PEPCK promoter it is known that the role of *cis*-active elements can differ between these cell lines [18,22,24]. Further studies are

now needed to establish the extent to which CRE2 is involved in the expression of the G6Pase gene *in vivo*.

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