Identification of an oxygen-responsive element in the 5'-flanking sequence of the rat cytosolic phosphoenolpyruvate carboxykinase-1 gene, modulating its glucagon-dependent activation

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The glucagon-stimulated transcription of the cytosolic phosphoenolpyruvate carboxykinase-1 (*PCK1*) gene is mediated by cAMP and positively modulated by oxygen in primary hepatocytes. Rat hepatocytes were transfected with constructs containing the first 2500, 493 or 281 bp of the *PCK1* 5'-flanking region in front of the chloramphenicol acetyltransferase (*CAT*) reporter gene. With all three constructs glucagon induced *CAT* activity with decreasing efficiency maximally under arterial pO_2 and to about 65 % under venous pO_2 . Rat hepatocytes were then transfected with constructs containing the first 493 bp of the *PCK1* 5'-flanking region in front of the luciferase (*LUC*) reporter gene, which were block-mutated at the CRE1 (cAMP-response element-1; -93/-86), putative CRE2 (-146/-139), promoter element (P) 1 (-118/-104), P2 (-193/-181) or P4 (-291/-273) sites. Glucagon induced *LUC* activity strongly when the P1 and P2 sites were mutated and weakly when the P4 site was mutated; induction of the P1, P2 and P4 mutants was positively modulated by the pO_2 . Glucagon also induced *LUC* activity strongly when the putative CRE2 site was altered; however, induction of the CRE2 mutant was not modulated by the pO_2 . Glucagon did not induce *LUC* activity when the CRE1 site was modified. These experiments suggested that the CRE1 but not the putative CRE2

was an essential site necessary for the cAMP-mediated *PCK1* gene activation by glucagon and that the putative CRE2 site was involved in the oxygen-dependent modulation of *PCK1* gene activation. To confirm these conclusions rat hepatocytes were transfected with simian virus 40 (SV40)-promoter-driven *LUC*-gene constructs containing three CRE1 sequences (-95/-84), three CRE2 sequences (-148/-137) or three CRE1 sequences plus two CRE2 sequences of the *PCK1* gene in front of the SV40 promoter. Glucagon induced *LUC* activity markedly when the CRE1, but not when the CRE2, sites were in front of the SV40-*LUC* gene; however, induction of the (CRE1)₃SV40-*LUC* constructs was not modulated by the pO_2 . Glucagon also induced *LUC* activity very strongly when the CRE1 and CRE2 sites were combined; induction of the (CRE1)₃(CRE2)₂SV40-*LUC* constructs was positively modulated by the pO_2 . These findings corroborated that sequences of the putative CRE2 site were responsible for the modulation by oxygen of the CRE1-dependent induction by glucagon of *PCK1* gene transcription.

Key words: cAMP-response element, gluconeogenesis, hypoxia, metabolic zonation, PCK.

INTRODUCTION

Phosphoenolpyruvate carboxykinase (PCK; EC 4.1.1.32) can be located in both the cytosol (PCK1) and the mitochondria (PCK2) [1,2]. The cytosolic [3,4] and mitochondrial [5] genes have been cloned. The cytosolic PCK1 is the key gluconeogenic enzyme; it is expressed primarily in liver and kidney cortex ([6,7], reviewed in [8]). In the liver, expression is stimulated mainly by glucagon via cAMP under the permissive action of glucocorticoids and thyroid hormones [9–11]; it is inhibited by insulin [12,13]. In kidney, expression is enhanced mainly by acidosis [14,15] via decreases in pH or bicarbonate levels, which act independently from each other [16].

In liver parenchyma, most key enzymes are distributed heterogeneously so that the periportal or upstream and perivenous or downstream zones have different catalytic potentials. This is the basis of the model of metabolic zonation (reviewed in [17–19]). PCK1 is preferentially localized in the periportal zone, as are the other glucose-forming enzymes fructose-1,6-bisphosphatase and glucose-6-phosphatase, whereas the glucose-utilizing enzymes glucokinase and liver pyruvate kinase are localized in the

perivenous area. These zonal expression patterns of the genes of carbohydrate-metabolizing enzymes may be caused by an oxygen gradient and a decreasing glucagon/insulin ratio established during the passage of blood through the liver sinusoids [17–19]. It was shown previously in primary rat hepatocyte cultures that glucagon induced higher transcription rates and mRNA amounts of the *PCK1* gene, as well as increased protein and activity levels, under arterial compared with venous oxygen tensions [20–22]. Conversely, in the same system, glucokinase mRNA was induced by insulin to a higher extent under venous than under arterial oxygen tensions [23].

The *PCK1* gene could contain 5'-flanking sequences that are involved in the modulation by oxygen of its glucagon-dependent transcription. This sequence could represent a known DNA element, such as a cAMP-response element (CRE), or an independent so-far-unknown oxygen-regulatory element. It was the aim of the present study to test whether 5'-flanking sequences are responsible for the modulation by oxygen of the glucagon-dependent induction of the *PCK1* gene in hepatocytes. Transient transfection of primary rat hepatocytes with serially deleted *PCK1* promoter-chloramphenicol acetyltransferase (*CAT*) gene

Abbreviations used: AP1, activator protein 1; CAT, chloramphenicol acetyltransferase; CRE, cAMP-response element; DOTAP, 1,2-dioleoyloxy-3-(trimethylamino)propanesulphate; EPO, erythropoietin; GRE, glucocorticoid-response element; HRE, hypoxia regulatory element; IRE, insulin-response element; LUC, luciferase; NF- κ B, nuclear factor κ B; NRE, normoxia regulatory element; P1–P4, promoter elements 1–4; PCK, phosphoenolpyruvate carboxykinase; SV40, simian virus 40.

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constructs showed that the first 281 bp of the 5'-flanking region were sufficient to mediate the modulation by oxygen of the glucagon-dependent induction of the *PCK1* gene. Transfection with constructs containing the first 493 bp of the *PCK1* 5'-flanking region mutated at the CRE1, putative CRE2, promoter element (P) 1, P2 or P4 sites [24,25] (see Figure 2, below) in front of the luciferase (*LUC*) gene indicated that sequences of the putative CRE2 site, -148/-137, were involved in the modulation of *PCK1* gene activity by oxygen. Transfection with constructs consisting of three CRE1 or three CRE2 elements of the *PCK1* gene in front of the simian virus 40 (SV40)-promoter-driven *LUC* gene showed that the CRE1 sequence, but not the putative CRE2 sequence, was responsible for cAMP-mediated induction of *LUC* activity independent of the pO_2 . Yet three CRE1 sequences and two putative CRE2 sites together in front of the SV40-promoter-driven *LUC* gene permitted the modulation by oxygen of the glucagon-dependent induction of *LUC* activity.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and purchased from commercial suppliers. Collagenase, 1,2-dioleoyloxy-3-(trimethylamino)propanesulphate (DOTAP) and fetal calf serum were from Boehringer Mannheim (Mannheim, Germany). Medium M199 was from Gibco BRL (Eggenstein, Germany). Hormones were delivered from Serva (Heidelberg, Germany). Hyperfilm and [^{14}C]chloramphenicol were supplied by Amersham Buchler (Braunschweig, Germany). Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium) or NAPS (Göttingen, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Gene constructs

The plasmids pPCKSCAT-2500, pPCKSCAT-281, pPCKSCAT-177, pPCKSCAT-137 and pPCKSCAT-71 were provided kindly by Dr. E. Schmidt (Department of Anatomy, University of Amsterdam, Amsterdam, The Netherlands). Construction of the promoter fragments was described by Short et al. [9] (The nomenclature of the PCK constructs is according to Beale et al. [3], which is 3 bp different from the nomenclature used by Short et al. [9].) Plasmid pPCKSCAT-493 was constructed by excising the 2 kb *XbaI* fragment from pPCKSCAT-2500 and religation of the remaining vector. The plasmid PCK-493-LUC and the block mutants were constructed by performing PCR with wild-type and block-mutated PCK-CAT constructs as templates, provided kindly by Dr. R. W. Hanson (School of Medicine, Case Western Reserve University, Cleveland, OH, U.S.A.). The PCR products (-493/+33) were cloned into the *SmaI* site of pUC18, excised by *KpnI* and *BglII* and ligated into the *KpnI* and *BglII* site of the luciferase vector pGL3 Basic (Promega, Mannheim, Germany). All constructs were sequenced to analyse the mutations (Table 1).

The plasmids (CRE1)₃SV40-LUC, (CRE2)₃SV40-LUC and (CRE1)₃(CRE2)₂SV40-LUC were constructed as follows. The 43mer single-stranded sense and antisense oligonucleotides for (CRE1)₃ (sense, 5'-gatcCCTTACGTCAGACCTTACGTCAG-AattCCTTACGTCAGA-3'; the original *PCK1* sequence is shown in upper case, the CRE1 sequence is underlined, non-*PCK* nucleotides are shown in lower case), which had a *BglII* overhang (gatc) at the 5' end and an *EcoRI* site (GAattC) between the second and the third CRE1 motifs (TTACGTCA) for analytical reasons, were phosphorylated by T4 polynucleotide kinase

(Gibco BRL) and annealed. The 19mer sense and antisense oligonucleotides for CRE2 (sense, 5'-gatcTGTTAGGTCAGT-TCC-3'; style as above, with the CRE2 sequence underlined), which had a *BglII* overhang at the 5' end, were also phosphorylated by T4 polynucleotide kinase and annealed. Additionally, the annealed double-stranded CRE2 oligonucleotide was oligomerized by incubation with T4 ligase, leading to an oligomerized random product. Another oligomerization was carried out with a mix of annealed double-stranded (CRE1)₃ and CRE2 oligonucleotides. The double-stranded (CRE1)₃, the random-oligomerized CRE2 and the random-oligomerized (CRE1)₃/CRE2 oligonucleotides were cloned into the previously *BglII*-digested and dephosphorylated pGL3prom (Promega) in front of the SV40 promoter driving the *LUC* gene. Afterwards, all constructs were sequenced; they contained the oligonucleotides in 5'→3' direction and were named according to their structure: (CRE1)₃SV40-LUC (5'-gatcCC-TTACGTCAGACCTTACGTCAGAattCCTTACGTCAGA-3'); (CRE2)₃SV40-LUC (5'-gatcTGTTAGGTCAGTTC-CgatcTGTTAGGTCAGTTCgatcTGTTAGGTCAGTTC-3'); and (CRE1)₃(CRE2)₂SV40-LUC (5'-gatcCCTTACGTCAGACCTTACGTCAGAattCCTTACGTCAGAgatcTGTTAGGTCAGTTCgatcTGTTAGGTCAGTTC-3').

Animals

Male Wistar rats (200–260 g, from Winkelmann, Borcheln, Germany) were kept on a 12 h:12 h day/night rhythm (light from 7 am to 7 pm) with free access to water and food. Rats were anaesthetized with pentobarbital (60 mg/kg of body weight) prior to preparation of hepatocytes between 8 and 9 am.

Cell culture and transfection experiments

Liver cells were isolated by collagenase perfusion. Cells (5×10^6) in 60-mm-diameter culture dishes were maintained under standard conditions in an atmosphere of 16% O₂/79% N₂/5% CO₂ (by vol.) in 2.5 ml of medium M199 containing 1 nM insulin added as a growth factor for culture maintenance, 100 nM dexamethasone required as a permissive hormone and, until the first change of medium, 4% newborn calf serum. For determination of PCK activity, medium was changed after 4 h and culture was continued for another 43 h with changing medium once after 22 h. Induction of PCK was started at 47 h by adding fresh medium M199 with 10 nM glucagon/100 nM dexamethasone/0.5 nM insulin at 16% O₂/79% N₂/5% CO₂ (by vol.) mimicking arterial oxygen tensions, and at 8% O₂/87% N₂/5% CO₂ (by vol.) mimicking venous oxygen tensions. These values take into account the oxygen-diffusion gradient from the media surface to the cells [17]. PCK activity was determined in duplicate 4, 6 and 8 h after induction, as described [26].

Transfection experiments were carried out in 1.5 ml of medium M199 containing 100 nM dexamethasone, 1 nM insulin and 4% newborn calf serum. Freshly isolated cells were transfected with the PCK1-CAT constructs using a cationic liposome technique by adding 84 µl of HEPES-buffered saline, containing 2.5 µg of construct DNA and 12.5 µg of DOTAP, to a 1.5 ml suspension of 5×10^5 cells in Petri dishes [27]. After 7 h, 1 ml of medium M199 containing 100 nM dexamethasone and 0.5 nM insulin was added to the cells and transfection proceeded for another 13 h. Then the medium was changed and cells were further cultured under standard conditions for 27 h. CAT activity was induced at 47 h by adding fresh media with 10 nM glucagon, 100 nM

Table 1 Wild-type and mutated regulatory elements of the *PCK1* promoter

The mutated bases are shown in bold.

Position	Site	Wild-type sequence	Block mutation (BM)	
			Sequence	Construct
—93/—86	CRE1	TTACGTCA	TGCATGCA	CRE1-BM LUC
—146/—139	CRE2	TTAGGTCA	TGCATGCA	CRE2-BM LUC
—118/—106	P1	TGGCTATGATCCA	TCTCGAGGTTACC	P1-BM LUC
—193/—181	P2	CATTAAACACAGC	CTCGAGGTTACCT	P2-BM LUC
—291/—273	P4	GTTTGCATCAGCAACAGTC	CTTGGTTACCAGATCTCGC	P4-BM LUC

dexamethasone and 0.5 nM insulin under arterial or venous pO_2 . CAT activity was determined in duplicate 4, 6, 8, 12 and 16 h after induction as described [28]. PCK-LUC constructs were transfected by calcium phosphate precipitation [29]: 2.5 μ g of construct DNA in 150 μ l of transfection buffer, composed of 7.5 μ l of 2.5 M $CaCl_2$, 75 μ l of 2 $\times$ Hepes (pH 7.05) and 67.5 μ l of H_2O , was added to 5×10^5 freshly isolated hepatocytes in 1.5 ml of medium M199. The cells were cultured for 5 h under standard conditions. Then medium was changed and culture was continued for 27–31 h under standard conditions. Luciferase activity was determined in duplicate after stimulation of the cells with 10 nM glucagon for 8–12 h under arterial or venous oxygen tensions following the Promega protocol for the Luciferase Assay System.

RESULTS

Primary rat hepatocytes were transiently transfected with serially deleted PCK1 promoter-CAT-gene constructs, block-mutated

PCK1 promoter-LUC-gene constructs and PCK1-CRE SV40-promoter-LUC-gene constructs in order to characterize DNA sequences responsible for the modulation by oxygen of the induction of the PCK by glucagon.

Modulation by oxygen of the glucagon-induced activation of serially deleted PCK1 promoter-CAT-gene constructs: localization of the oxygen responsiveness within the first 281 bp of the promoter

In rat hepatocyte cultures, glucagon elevated endogenous PCK activity from basal levels by 6.5-fold to a transient maximum after 6–7 h under arterial pO_2 . Under venous pO_2 , glucagon induced PCK to only about 65% of the value reached under arterial pO_2 (Figure 1).

In cultured rat hepatocytes transfected with pPCKSCAT-2500, a construct that contained the PCK1 promoter sequence

Figure 1 Modulation by oxygen of the glucagon-dependent activation of the endogenous *PCK1* gene and transfected PCK1 promoter-CAT-gene constructs in primary rat hepatocyte cultures: time course of induction

Hepatocytes were transfected with the promoter-CAT constructs pPCKSCAT-2500, -493 and -281, using the cationic liposome method [27] and cultured under standard conditions. After 47 h, cells were induced with 10 nM glucagon (Ggn) for 4–16 h under arterial (16% O_2 , by vol., ■) or venous (8% O_2 , by vol., □) pO_2 . The highest endogenous PCK activity and transfected CAT activity for each plasmid was set equal to 100%. Values are means $\pm$ S.E.M. of 5–8 independent experiments. Statistics: Student's *t* test for paired values (16% O_2 versus 8% O_2), **P* < 0.01.

Figure 2 Modulation by oxygen of the glucagon-dependent induction of PCK1 promoter-CAT- or LUC-gene constructs transfected into primary rat hepatocyte cultures: abolition of the oxygen effect by mutation of the putative CRE2 site

(a) The 5'-flanking region of the rat cytosolic PCK1 gene with its *cis*-acting regulatory elements [6,8,24,25]. (b) Hepatocytes were transfected in parallel by the cationic liposome method [27], with serially deleted PCK promoter fragments fused to the CAT reporter gene (pPCKSCAT). Cells transfected with pPCKSCAT-2500, -493, -281 and -71 were cultured under standard conditions and after 47 h induced with 10 nM glucagon each under arterial (16% O₂, by vol.) or venous (8% O₂, by vol.) pO₂ for 12 h. Basal, non-glucagon-induced CAT activity is shown on the left of the histogram and the glucagon-induced activity is shown on the right side of the zero line. Values are means $\pm$ S.E.M. of 4 independent experiments. Statistics: Student's *t* test for paired values (16% O₂ versus 8% O₂), **P* < 0.05. (c) Hepatocytes were transfected in parallel by the calcium phosphate-precipitation method [29] with LUC gene constructs driven by a 493-bp PCK1 promoter in its native form (pPCKLUC-493) or block-mutated (BM) at either the CRE1, putative CRE2, P1, P2 or P4 sites (CRE1-BM LUC, etc.). The symbol on each construct indicates the site of mutation. Transfected cells were cultured under standard conditions and after 27 h induced with 10 nM glucagon each under arterial (16% O₂, by vol.) and venous (8% O₂, by vol.) pO₂ for 12 h. Basal, non-glucagon-induced LUC activity is shown on the left of the histogram and glucagon-induced activity is shown on the right of the zero line. Values are means $\pm$ S.E.M. of the number of independent experiments given in parentheses. Statistics were as for (b). GRU, glucocorticoid-response unit; ARE, accessory-factor regulatory element; HNF3, hepatic nuclear factor 3; RLU, relative light unit; PR, protein.

from -2500 to +69, with the most important regulatory elements such as insulin-response element (IRE; -416/-402), glucocorticoid-response element (GRE) 1 (-388/-374), GRE2 (-367/-353), P4 (-291/-273), P3 (-267/-234), P2 (-193/-181), putative CRE2 (-146/-139), P1 (-118/-104) and CRE1 (-93/-86) (Figure 2a and Table 1) as well as the CAAT (-110/-104) and TATA (-31/-25) boxes [6,7,24,25], induction of CAT activity by glucagon was modulated by oxygen in the same way as endogenous PCK activity. Glucagon induced CAT activity within 8 h from basal levels of 18% to the maximum of 100%, or by 5.5-fold, under arterial pO₂ and to about 70%, or by 3.8-fold, under venous pO₂ (Figure 1). After transfection with pPCKSCAT-493, a construct that provided essentially all liver-specific *cis*-acting elements [8] (Figure 2a), glucagon elevated CAT activity within 16 h from basal 25% to maximal levels of 100% or by 4-fold under arterial pO₂ and to about 60% or by 2.4-fold under venous pO₂ (Figure 1). After transfection with pPCKSCAT-281, a construct which lacked several important *cis*-acting elements, such as the IRE [12] and the GREs and thus the glucocorticoid-response unit [30] and, in addition, parts of P4 (Figure 2a), glucagon exerted only a weak induction. The basal CAT activity of about 35% was increased within 12 h to the maximum of 100% or by 3-fold under arterial pO₂ and to about 70% or by 2-fold under venous pO₂ (Figure 1).

In the pPCKSCAT-transfected hepatocytes, basal and glucagon-induced CAT activity decreased with increasing deletions of the PCK1 promoter. The direct comparison of pPCKSCAT-2500, -493 and -281, when transfected *in parallel*, showed a stepwise reduction in basal and glucagon-induced CAT activity from pPCKSCAT-2500 with the highest values to pPCKSCAT-281 with the lowest; but with all three constructs the modulation by oxygen of the induction was maintained (Figure 2b). After transfection of the serially deleted pPCKSCAT-177 and pPCKSCAT-137 (results not shown), in which further important DNA elements, such as P4, P3 and P2 and the putative CRE2, respectively, were missing (Figure 2a), glucagon no longer enhanced CAT activity and thus modulation by oxygen of induction could not occur. In hepatocytes transfected with pPCKSCAT-71, which in addition to the so-far-deleted DNA elements also lacked P1, including the CAAT box, and CRE1 (Figure 2a), neither basal nor glucagon-induced CAT activity could be measured (Figure 2b).

Thus, serial deletion of sequences from -2500 to -281 resulted in a stepwise decrease in both basal and glucagon-, i.e. cAMP-, induced PCK1 promoter activity, but it did not abrogate the modulation by oxygen of the glucagon-elicited induction. This suggested that the oxygen responsiveness was located predominantly within the first 281 bp of the PCK1 promoter.

Figure 3 Modulation by oxygen of the glucagon-dependent induction of PCK1-CRE SV40-LUC-gene constructs transfected into primary rat hepatocyte cultures: conferring of oxygen responsiveness by CRE2 sequences

Hepatocytes were transfected in parallel using the calcium phosphate-precipitation method [29] with triplicates of CRE1 [(CRE1)₃SV40-LUC] or CRE2 [(CRE2)₃SV40-LUC] or a combination of a CRE1 triplicate and a CRE2 duplicate [(CRE1)₃(CRE2)₂SV40-LUC] in front of the SV40-promoter-driven LUC-gene constructs. The CRE-consensus sequences are underlined; the changed base between CRE1 and CRE2 is boxed. Transfected cells were cultured under standard conditions and after 31 h induced with 10 nM glucagon each under arterial (16% O₂, by vol.) or venous (8% O₂, by vol.) pO₂ for 12 h. Fold induction is the ratio of glucagon-induced to basal LUC activity. Values are means ± S.E.M. of 3 independent experiments. Statistics were as for Figure 2.

Modulation by oxygen of the glucagon-induced activation of block-mutated PCK1 promoter-LUC-gene constructs: abrogation of oxygen responsiveness by mutation of the putative CRE2 promoter element

PCK1 promoter-LUC-gene constructs containing the first 493 bp of the PCK 5'-flanking region were transfected into primary rat hepatocytes; they were mutated, when indicated, in the CRE1, putative CRE2, P1, P2 or P4 sites (Table 1 and Figure 2a). As expected, a mutation in the CRE1 site had no effect on the basal LUC activity but completely abolished its inducibility by glucagon (Figure 2c). Mutations in the P1 or P2 sites reduced both the basal and the glucagon-induced LUC activity but had no influence on the modulation by oxygen of the induction (Figure 2c). A mutation in the P4 site also reduced the basal LUC activity, but nearly abolished the inducibility by glucagon; yet the oxygen responsiveness was maintained (Figure 2c). A mutation in the putative CRE2 again caused a reduction of both the basal and glucagon-enhanced LUC activities; in addition, it abolished the modulation by oxygen of the induction (Figure 2c). These findings suggested that sequences of the putative CRE2 site mediated the modulation by oxygen of the glucagon-dependent activation of the PCK gene.

Modulation by oxygen of the glucagon-induced activation of PCK1-CRE SV40-LUC-gene constructs: conferring of oxygen responsiveness by sequences of the putative CRE2 promoter element

The results obtained thus far clearly indicate that the CRE1 but not the CRE2 was essential for the cAMP-mediated glucagon-

dependent activation of the PCK1 gene and that the CRE2 had a key role in the modulation by oxygen of this activation. In order to verify these conclusions directly, rat hepatocytes were transfected with SV40-promoter-driven LUC-gene constructs, which contained several PCK1-CRE1 or PCK1-CRE2 sites, or both, as enhancers in front of the SV40 promoter.

After transfection of hepatocytes with a construct containing three CRE1 elements, LUC activity could be induced by glucagon; however, the level of induction was the same under arterial and venous pO₂ (Figure 3). Thus the three CRE1 sites conferred responsiveness to glucagon, but not to oxygen. Three putative CRE2 sites, instead of three CRE1 sites, were unable to mediate glucagon responsiveness (Figure 3). However, three CRE1 and two CRE2 sites together conferred inducibility by glucagon and its modulation by oxygen. This confirmed that the CRE1 site established glucagon, i.e. cAMP responsiveness, and that the putative CRE2 site or parts thereof effected the modulation by oxygen of the glucagon responsiveness. Thus the combination of these two regulatory DNA elements of the PCK1 promoter was required for the oxygen effect on the glucagon-dependent activation of PCK1 transcription.

DISCUSSION

The present study has shown (i) that the first 281 bp of the 5'-flanking region of the rat cytosolic PCK1 gene were sufficient for the modulation by oxygen of the glucagon-dependent activation of the gene (Figures 1 and 2b), (ii) that within this region the putative CRE2 site (−148/−137; 5'-TGTTAGGTCAGT-3') in contrast to the CRE1 site (−95/−84; 5'-CCTTACGTCAGA-3'; where italics indicate the CRE core sequences) did not act as the key regulatory element for the activation of the gene by glucagon and (iii) that the putative CRE2 site was responsible, or contained an important regulatory element, for the modulation by oxygen of the activation (Figures 2c and 3).

Oxygen as regulator of constitutive and modulator of induced gene expression

Oxygen plays a role as an effector of gene expression in bacteria, yeast and mammals (reviewed in [31]). Oxygen may act as an inhibitory or stimulatory regulator of constitutive gene expression or as a negative or positive modulator of hormone- or substrate-activated gene expression. In mammals many oxygen-regulated genes are known.

In constitutive gene expression, hypoxia (1% O₂ ≈ 0.93 kPa ≈ 7 mmHg) was found to activate the genes for erythropoietin (EPO) in kidney and fetal liver [32–34], for vascular endothelial growth factor [35,36] and platelet derived growth factor β [37] in endothelial cells, for endothelin-1 in lung and right atrium [38] and for angiotensin-converting enzyme in pulmonary endothelial cells [39] as well as for tyrosine hydroxylase, a key enzyme of catecholamine synthesis, in the carotid body and in pheochromocytoma cells [40]. Hypoxia also induced the genes coding for the glycolytic enzymes lactate dehydrogenase A, phosphoglycerate kinase 1, aldolase A and pyruvate kinase M in HepG2, Hep3B or HeLa cells [41,42]. Hyperoxia, not only hypoxia, can also influence gene expression. Strong hyperoxia (95% O₂ ≈ 89.3 kPa ≈ 670 mmHg) activated the cytochrome P4501A1 and P4501A2 genes in lung and liver [43]. Slight hyperoxia (20% O₂ ≈ 18.6 kPa ≈ 140 mmHg) stimulated the genes for glutathione peroxidase in heart muscle cells [44] and for surfactant protein A in lung pneumocytes [45].

In hormone-induced gene expression, oxygen acted as a positive modulator of the induction by cAMP of the surfactant

protein A gene in lung pneumocytes [45]. In primary rat hepatocytes the glucagon-dependent expression of the *PCK* gene was positively modulated by arterial pO_2 [20–22] and, reciprocally, the insulin-dependent expression of the glucokinase gene was negatively modulated by arterial pO_2 [23].

Oxygen-regulatory elements and transcription factors

So far, only a few genes have been found to be positively modulated by normoxia. Little is known about possible normoxia regulatory elements (NREs) and the corresponding transcription factors mediating the modulation. The majority of genes modulated by oxygen have been shown to be induced by hypoxia. The hypoxia regulatory elements (HREs) and the transcription factors involved in the induction by hypoxia have been defined (see below).

NREs

In the human glutathione peroxidase gene, two similar 13-bp oxygen-responsive elements CCTCAAAGAAAGT and CC-TCTGAGAAAAA (differences are underlined) were found to bind disparate proteins and to confer oxygen sensitivity of gene expression [44]. In the present study, an 8-bp oxygen-responsive element, TTAGGTCA, was identified in the rat *PCK1* promoter at positions –146/–139 (Figures 2c and 3). It was previously named CRE2, on the basis of a 75% similarity (6 out of 8 bp) with the palindromic CRE consensus sequence TGACGTCA and of a footprint with liver nuclear extracts [24]. However, functional evidence had not been presented. In the present study the putative CRE2 site was shown not to confer inducibility by cAMP, but to confer modulation by oxygen of the CRE1-dependent gene activation (Figure 3). Thus the CRE2 site can be described as an NRE. The CRE1 site at position –93/–86, with a 7-out-of-8-bp similarity to the CRE consensus sequence, was found to confer inducibility by cAMP in line with previous studies [9] (Figure 3). The CRE2 alone, in contrast with the CRE1, did not bind nuclear protein from primary hepatocytes; yet the CRE1 and CRE2 linked by the intervening nuclear-factor-1 element did so [46]. This result is consistent with the present study, in which only the combination of CRE1 and CRE2 permitted the modulation by oxygen of the glucagon-dependent activation of the transfected LUC construct in primary hepatocytes (Figure 3). Other genes, the expression patterns of which are also periportal/zonated (reviewed in [17–19]), such as tyrosine aminotransferase and serine dehydratase, contain promoter elements with a 7-out-of-8-bp similarity with the CRE2 or, more correctly, the NRE, of the rat *PCK1* gene. It remains to be established whether these sequences can function as a NRE.

Nuclear factor κ B (NF- κ B)-binding element

NF- κ B is activated by H_2O_2 (reviewed in [47]), which could be formed by the oxygen sensor as the second messenger [22,48,49]. However, the 5'-flanking region up to –2500 of the rat *PCK1* gene does not contain the binding site for NF- κ B, (subunits p65/p50) 5'-GGGAATTCCC-3'. Therefore, it is unlikely that NF- κ B is involved in the oxygen-dependent zonated expression of the *PCK1* gene.

HREs

The prototype of hypoxia-inducible genes is the EPO gene. The oxygen sensitivity is located in an element 3' to the poly-A-addition site of the gene [33,50,51]. An 8-bp sequence,

TACGTGCT [31,52], was found to bind a nuclear factor named hypoxia-inducible factor 1 [53], present in many EPO- and non-EPO-producing cell types only under hypoxic conditions [50,54,55]. Also the genes of the glycolytic enzymes lactate dehydrogenase A, phosphoglycerate kinase 1 and aldolase A [41,42] were shown to contain a functional HRE. The gluconeogenic *PCK1* gene does not possess a complete 8-bp HRE in its 5'-flanking region, but only a 6-out-of-8-bp element, CGTGCT, at positions –129/–124, which probably did not bind hypoxia-inducible factor 1 [56]. Thus it is unlikely that an HRE is involved in the oxygen-dependent zonated expression of the rat *PCK1* gene; if it were involved, it would have to exert an inhibitory effect which has not been described so far.

Activator protein 1 (AP1)-binding element

Jun, and Fos which form AP1, are known to be induced by reduced pO_2 [57]. In the *PCK* promoter, several putative AP1-binding sites are known, such as the CRE1, P3 and P4 sites [58]. Since Fos inhibits the expression of the *PCK1* gene [58], which is dependent on the CRE1 and P4 sites (Figure 2), AP1 cannot be involved in the positive modulation by oxygen of the cAMP-elicited induction of the gene via these two sites. However, AP1 might play a role via the P3 site, inhibiting *PCK1* expression at perivenous pO_2 . This possibility remains to be studied.

Conclusion

The findings of this study represent strong evidence for the proposal that the interaction of proteins bound to the CRE1 and NRE ($\cong$ CRE2) elements was responsible for the modulation by oxygen of the glucagon-dependent *PCK* transcription, with CRE1 being the gene-activating element and NRE ($\cong$ CRE2) being the modulating element.

REFERENCES

- Utter, M. F. and Kurahashi, K. (1954) *J. Biol. Chem.* **207**, 787–802
- Nordlie, R. C. and Lardy, M. A. (1963) *J. Biol. Chem.* **238**, 2259–2265
- Beale, E. G., Chrapkiewicz, N. B., Scoble, H. A., Metz, R. J., Quick, D. P., Noble, R. L., Donelson, J. E., Biemann, K. and Granner, D. K. (1985) *J. Biol. Chem.* **260**, 10748–10760
- Ting, C. N., Burgess, D. L., Chamberlain, J. D., Keith, T. P., Falls, K. and Meister, M. H. (1993) *Genomics* **16**, 698–706
- Modaressi, S., Brechtel, K., Christ, B. and Jungermann, K. (1998) *Biochem. J.* **333**, 359–366
- McGrane, M. M., Yun, J. S., Moorman, A. F., Lamers, W. H., Hendrick, G. K., Arafah, B. M., Park, E. A., Wagner, T. E. and Hanson, R. W. (1990) *J. Biol. Chem.* **265**, 22371–22379
- Beale, E. G., Clouthier, D. E. and Hammer, R. E. (1992) *FASEB J.* **6**, 3330–3337
- Pilkis, S. J. and Granner, D. K. (1992) *Annu. Rev. Physiol.* **54**, 885–909
- Short, J. M., Wynshaw-Boris, A., Short, H. P. and Hanson, R. W. (1986) *J. Biol. Chem.* **261**, 9721–9726
- Wynshaw-Boris, A., Short, J. M., Loose, D. S. and Hanson, R. W. (1986) *J. Biol. Chem.* **261**, 9714–9720
- Runge, D., Schmidt, H., Christ, B. and Jungermann, K. (1991) *Eur. J. Biochem.* **198**, 641–649
- O'Brien, R. M., Lucas, P. C., Forest, C. D., Magnuson, M. A. and Granner, D. K. (1990) *Science* **249**, 533–537
- Christ, B., Nath, A. and Jungermann, K. (1990) *Biol. Chem. Hoppe-Seyler* **371**, 395–402
- Watford, M. and Mapes, R. E. (1990) *Arch. Biochem. Biophys.* **282**, 399–403
- Hwang, J. J. and Curthoys, N. P. (1991) *J. Biol. Chem.* **266**, 9392–9396
- Kaiser, S. and Curthoys, N. P. (1991) *J. Biol. Chem.* **266**, 9397–9402
- Jungermann, K. and Katz, N. (1989) *Physiol. Rev.* **69**, 708–764
- Gebhardt, R. (1992) *Pharmacol. Ther.* **53**, 275–354
- Jungermann, K. and Kietzmann, T. (1996) *Annu. Rev. Nutr.* **16**, 179–203
- Nauck, M., Wölflle, D., Katz, N. and Jungermann, K. (1981) *Eur. J. Biochem.* **119**, 657–661

- 21 Hellkamp, J., Christ, B., Bastian, H. and Jungermann, K. (1991) *Eur. J. Biochem.* **198**, 635–639
- 22 Kietzmann, T., Freimann, S., Bratke, J. and Jungermann, K. (1996) *FEBS Lett.* **338**, 228–232
- 23 Kietzmann, T., Roth, U., Freimann, S. and Jungermann, K. (1997) *Biochem. J.* **321**, 17–20
- 24 Roesler, W. J., Vandenbark, G. R. and Hanson, R. W. (1989) *J. Biol. Chem.* **264**, 9657–9664
- 25 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
- 26 Seubert, W. and Huth, W. (1965) *Biochem. Z.* **343**, 176–191
- 27 Modaressi, S., Christ, B., Bratke, J., Zahn, S., Heise, T. and Jungermann, K. (1996) *Biochem. J.* **315**, 807–814
- 28 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051
- 29 Immenschuh, S., Hinke, V., Ohlmann, A., Giffhorn-Katz, S., Katz, N., Jungermann, K. and Kietzmann, T. (1998) *Biochem. J.* **334**, 141–146
- 30 Imai, E., Stromstedt, P. E., Quinn, P. G., Carlstedt-Duke, J., Gustafsson, J. A. and Granner, D. K. (1990) *Mol. Cell. Biol.* **10**, 4712–4719
- 31 Bunn, H. F. and Poyton, R. O. (1996) *Physiol. Rev.* **76**, 839–885
- 32 Imagawa, S., Goldberg, M. A., Doweiko, J. and Bunn, H. F. (1991) *Blood* **77**, 278–285
- 33 Semenza, G. L., Nejfelt, M. K., Chi, S. M. and Antonarakis, S. E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5680–5684
- 34 Eckardt, K. U., Pugh, C. W., Meier, M., Tan, C. C., Ratcliffe, P. J. and Kurtz, A. (1994) *Ann. N. Y. Acad. Sci.* **718**, 50–63
- 35 Shweiki, D., Itin, A., Soffer, D. and Keshet, E. (1992) *Nature (London)* **359**, 843–845
- 36 Goldberg, M. A. and Schneider, T. J. (1994) *J. Biol. Chem.* **269**, 4355–4359
- 37 Kourembanas, S., Hannan, R. L. and Faller, D. V. (1990) *J. Clin. Invest.* **86**, 670–674
- 38 Kourembanas, S., Marsden, P. A., McQuillan, L. P. and Faller, D. V. (1991) *J. Clin. Invest.* **88**, 1054–1057
- 39 King, S. J., Booyse, F. M., Lin, P. H., Traylor, M., Narkates, A. J. and Oparil, S. (1989) *Am. J. Physiol.* **256**, C1231–C1238
- 40 Czyzyk-Krzeska, M. F., Furnari, A. B., Lawson, E. E. and Millhorn, D. E. (1994) *J. Biol. Chem.* **269**, 760–764
- 41 Firth, J. D., Ebert, B. L., Pugh, C. W. and Ratcliffe, P. J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6496–6500
- 42 Semenza, G. L., Roth, P. H., Fang, H. M. and Wang, G. L. (1994) *J. Biol. Chem.* **269**, 23757–23763
- 43 Okamoto, T., Mitsuhashi, M., Fujita, I., Sindhu, K. R. and Kikkawa, Y. (1993) *Biochem. Biophys. Res. Commun.* **197**, 878–885
- 44 Cowan, D. B., Weisel, R. D., Williams, W. G. and Mickle, D. A. (1993) *J. Biol. Chem.* **268**, 26904–26910
- 45 Acarregui, J. M., Snyder, M. J. and Mendelson, R. C. (1993) *Am. J. Physiol.* **264**, L465–L474
- 46 Christ, B., Nath, A. and Jungermann, K. (1991) *Biochem. Biophys. Res. Commun.* **181**, 367–374
- 47 Pahl, H. L. and Baeuerle, P. A. (1994) *BioEssays* **16**, 497–502
- 48 Fandrey, J., Frede, S. and Jelkmann, W. (1994) *Biochem. J.* **303**, 507–510
- 49 Kietzmann, T., Porwoll, T., Zierold, K., Jungermann, K. and Acker, H. (1998) *Biochem. J.* **335**, 425–432
- 50 Beck, I., Ramirez, S., Weinmann, R. and Caro, J. (1991) *J. Biol. Chem.* **266**, 15563–15566
- 51 Pugh, C. W., Tan, C. C., Jones, R. W. and Ratcliffe, P. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10553–10557
- 52 Wenger, R. H. and Gassmann, M. (1997) *Biol. Chem.* **378**, 609–616
- 53 Semenza, G. L. and Wang, G. L. (1992) *Mol. Cell. Biol.* **12**, 5447–5454
- 54 Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2423–2427
- 55 Wang, G. L. and Semenza, G. L. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4304–4308
- 56 Kietzmann, T., Bratke, J. and Jungermann, K. (1997) *Kidney Int.* **51**, 542–547
- 57 Webster, K. A., Discher, D. J. and Bishopric, N. H. (1993) *J. Biol. Chem.* **268**, 16852–16858
- 58 Gurney, A. L., Park, E. A., Giralt, M., Liu, J. and Hanson, R. W. (1992) *J. Biol. Chem.* **267**, 18133–18139

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