Involvement of HNF-1 in the regulation of phosphoenolpyruvate carboxykinase gene expression in the kidney

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Abstract The cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (PEPCK) gene is differentially expressed in several tissues. A specific set of regulatory elements in the promoter are responsible for the control of PEPCK gene transcription and, in turn, determine its distinct metabolic role in each tissue. DNase I footprinting analysis of the PEPCK promoter, using nuclear proteins from tissues which express the gene for PEPCK, and transient expression assays in renal cell lines have demonstrated that the HNF-1 recognition motif (P2) in the PEPCK promoter characterizes kidney-specific expression. This site is required also for the response to acidosis. Since the P2 site is not involved in the expression of the PEPCK gene in the liver, we propose that its critical role in the kidney stems from a combination of abundance of HNF-1 together with low concentrations of members of the C/EBP family in this tissue.

Key words: PEPCK; Kidney; Transcription; Basal; Modulation; HNF-1

1. Introduction

Gluconeogenesis in the liver and kidney plays an essential role in controlling glucose homeostasis in mammals. In humans hepatic gluconeogenesis is the predominant process which provides glucose to the blood while renal gluconeogenesis becomes an important factor only during prolonged starvation and during acidosis [1]. Thus, because it is differentially regulated, the same gluconeogenic pathway seems to play different roles in the liver and kidney.

Of the three gluconeogenic enzymes, PEPCK catalyzes the key and limiting step of gluconeogenesis. The activity of the cytosolic form of PEPCK readily changes in response to a variety of hormonal and dietary stimuli, as a result of alterations of the transcriptional rate of the PEPCK gene (for a recent review, see [2]). Experimental metabolic acidosis in the rat selectively induces the renal, and not hepatic, expression of the PEPCK gene [3]. Furthermore, while fasting induces PEPCK gene transcription in both liver and kidney, this stimulation can be differentially abolished in the two tissues i.e. by glucose in the liver and by alkalosis in the kidney [4]. Thus, the differential modulation of PEPCK gene expression in the liver and kidney is transcriptionally regulated.

The transcriptional regulation of the rat PEPCK gene has been intensively studied (reviewed in ref. [5]). This single copy gene [6], is selectively expressed in several tissues arising from different embryonic origins: the liver (endoderm), kidney (intermediate mesoderm), and adipose tissue (dorsal mesoderm). Using cell lines and transgenic mice, the *cis*-regulatory regions involved in this selective pattern of expression was analyzed [7–11]. These analyses have shown that each of the PEPCK-expressing tissues utilizes distinct regulatory sequences to direct expression of the gene.

Transcription of the PEPCK gene is modulated by a number of hormones and other factors in a tissue specific manner. Detailed studies in hepatoma cells have led to the identification of distinct hormone response elements in the PEPCK promoter [12–14]. It is conceivable that the *cis*-elements which determine basal transcription in the liver and kidney also contribute to the tissue-specific modulation of the gene in the two tissues.

Here we show by transient transfection experiments with cultured kidney cell lines that the HNF-1 recognition motif in the PEPCK promoter (P2) plays a dominant role in the kidney in both the basal expression of the gene and in its response to acidic pH.

2. Materials and methods

2.1. Materials

Dulbecco Modified Eagle's medium (DMEM), F12 and fetal calf serum were purchased from Biological Industries, Kibutz Beit Haemek. Quantification of radioactive signals was performed using a phosphorimager (Fujix BAS 1000, Fuji, Japan).

2.2. Cell culture studies

The proximal tubule cell line from porcine kidney LLC-PK1 (PK1) cells (passage 163 obtained from Dr. A. Moran) was grown as previously described [9], except that the growth medium contained a 1:1 mixture of DMEM and F12, and 10% fetal calf serum. The same medium, but free of glucose, was used for the selection of the PK22 sub-line of cells enriched in PEPCK activity. The glucose-free selection medium contained 10 mM pyruvate and 10% dialyzed fetal calf serum (containing less than 30 μM glucose) [15]. $PK1F^{+}$ was a PEPCK-enriched sub-line selected by Dr. G. Gstraunthaler, from parental PK1 cells of passage 280, that was kindly provided to us by Dr. Norman P. Curthoys.

In experiments involving the effect of acidic pH, the cells were grown in 25 cm^2 Falcon T flasks, which were sealed with a screw cap after adjusting the pH by CO_2 aeration. The standardized timing of the aeration enabled us to achieve either pH 7.4 or pH 6.9, which

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Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); CRE, cAMP response element; HNF-1, hepatic nuclear factor-1; C/EBP, CCAAT/enhancer binding protein; kbp, kilobase pair(s); bp, base pair(s); rp, ribosomal proteins; hGH, human growth hormone; bGH, bovine growth hormone; SV40, simian virus 40; RSV, Rous Sarcoma virus; DMEM, Dulbecco Modified Eagle's medium; CAT, chloramphenicol acetyltransferase; PPAR, peroxisome proliferator-activated receptor

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was kept constant for the entire 16 h period of the experiment. The cultured medium used in these experiments contained 29 mM HCO_3^-, previously shown to be optimal for the pH effect [16].

2.3. Transfection conditions and CAT assays

Cells were transfected by calcium phosphate precipitation, essentially according to Chen and Okayama [17], as previously described [18], using 5 μg of each supercoiled plasmid and additional carrier pBS DNA (Stratagene), to make a total of 20 μg. The transfection efficiency was monitored by including as an internal standard 0.1 μg of pS16-GH [19], containing the human somatotropin (hGH) gene driven by the ribosomal protein S16 (rpS16) promoter. The levels of hGH secreted into the medium were determined by radio-immune assay [19], using a commercial kit (St. Nichols, San Diego, CA) according to the supplier's instructions. For experiments involving the effect of acidosis, the hGH reporter gene was driven by the Rous Sarcoma virus (RSV) promoter/enhancer, rather than by the rpS16 promoter. Where indicated, 5 μg of either HNF-1α or HNF-1β expression vectors were added to the transfection mix. The calcium-phosphate-DNA precipitates were left on the cells for 5 h. After their removal, the cells were rinsed and shocked for 1.5 min. with 20% glycerol. The transfected cells were harvested after two days and the activity of the chloramphenicol acetyltransferase (CAT) reporter was determined as previously described [18]. The percent of acetylated ^{14}C labeled chloramphenicol, from the sum of acetylated and non-acetylated spots identified by autoradiography of the TLC plates, was quantified using a phosphorimager apparatus. CAT activity was normalized to the amount of hGH secreted into the medium as previously described [18].

2.4. PEPCK activity assay

Activity was determined in the post-mitochondrial supernatant of the cells, using a $^{14}CO_2$ fixation assay [20]. One unit of enzyme activity catalyzes the fixation of 1 μmol of $NaH^{14}CO_3$ per minute at 37°C.

2.5. Plasmids used in transfection

pSV2-cat is a plasmid containing the structural gene encoding the bacterial chloramphenicol acetyltransferase (CAT) gene fused to the simian virus 40 (SV40) early promoter-enhancer region in pBR322 [21]. The previously described plasmid 597-pck-CAT, has 597 bp of the PEPCK promoter region fused to the CAT reporter gene [7]. The derived plasmid Δ362-205-pck-CAT (ΔP3,P4) contains an internal deletion in the PEPCK promoter, spanning positions −362 to −205 of the transcription start site [7], which has two C/EBP recognition sequences [22,23]. Plasmid Δ487-417-pck-CAT (ΔP6) also derives from 597-pck-CAT and contains an internal deletion between positions −487 to −417 of the transcription start site [9] which has an AF1 site, the HNF-4 recognition sequence [22,24]. Plasmid mh-597-pck-CAT (mP2), where the P2 binding site (an HNF-1 motif [25]) in the sequence spanning positions −155 to −205 of plasmid 597-pck-CAT was replaced by 50 bp of a polylinker sequence from the commercial plasmid pBlueScript (Stratagene), has been previously described [18]. PEPCK-CAT containing plasmids, where the P1 and CRE sites in the PEPCK promoter were mutated (mP1 and mCRE, respectively), and the plasmid containing the unmodified PEPCK promoter (−460 to +73) were described previously [13]. Expression vectors encoding hepatic enriched transcription factors used in this work included: HNF-1α (also termed HNF-1) [26] and HNF-1β (also termed vHNF-1) [27].

2.6. DNase I footprinting assays

Nuclear extracts from rat liver and kidney were prepared by a modification [22] of the method of Gorski et al. [28]. Nuclear extracts from cultured adipocytes were prepared according to Dignam et al. [29], with a modification adapted from Gorski et al., where $MgCl_2$ was replaced by spermine spermidine [28]. DNase I footprinting assays were performed essentially as previously described [22] using the ^{32}P-end labeled probe containing the 610 bp *Bam*HI-*Bgl*II fragment of the rat PEPCK promoter, spanning positions −540 to +69 of the gene.

3. Results

3.1. Analysis of distinct sequences in the PEPCK promoter

Previous DNase I footprint analysis of the PEPCK promoter compared the binding by nuclear extracts from tissues that express PEPCK (liver and kidney) to extracts from non-expressing tissues (spleen and brain) [25]. This comparative analysis readily identified sequences that specifically bind hepatic nuclear proteins, later identified as C/EBP binding sites [22,23]. In contrast, the pattern of binding to the PEPCK promoter of proteins extracted from renal nuclei did not differ from footprints obtained with nuclear extracts from tissues which do not express the gene for PEPCK. To verify the sequences within the PEPCK promoter which differentially bind renal nuclear proteins, footprint analysis of nuclear extracts from the three PEPCK-expressing tissues, kidney, liver and adipocytes was performed (Fig. 1). Hepatic nuclear proteins footprinted all sites. These included the tissue-non-specific CRE site (cAMP response element) which binds CREB, AP1 and C/EBP, the P1 site which binds nuclear factor 1 (NF1), and the tissue-specific sites including P2, which binds hepatic nuclear factor 1 (HNF-1), and P3 and P4 which bind members of the C/EBP family (for reviews, see [2,30]).

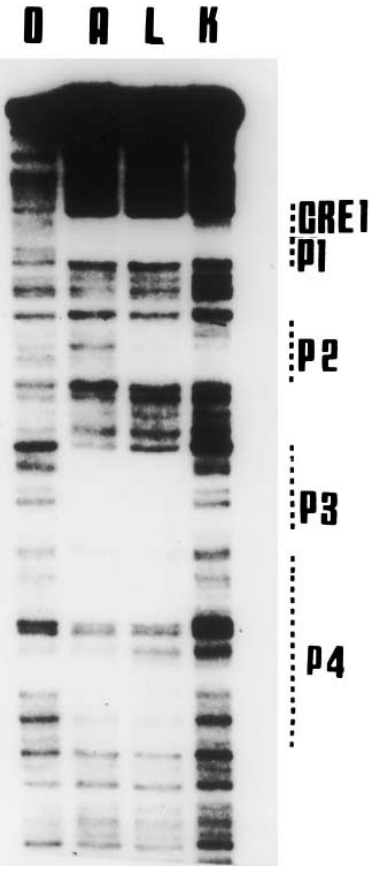

Fig. 1. Footprinting of the PEPCK promoter region with nuclear extracts from the kidney, liver and adipocytes. Ten fentomols of ^{32}P-end labeled 610 bp of *Bam*HI-*Bgl*II fragment encompassing the entire rat PEPCK promoter up to position −540 of the gene were incubated either with 20 μg of nuclear proteins extracted from the kidney (K), liver (L) and adipocytes (A) or in the absence of proteins (O). After digestion with DNase I the DNA was separated on a sequencing gels. The protected sequences are indicated on the right. Analysis of sequences upstream of position −362 is not shown.

All except one of these sites were bound by nuclear proteins from adipocytes. The only site in the PEPCK promoter which failed to bind nuclear proteins from adipocytes was the P2

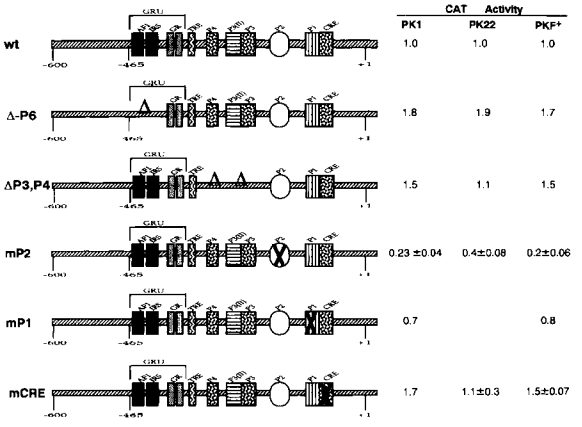

Fig. 2. Analysis of sequences in the PEPCK promoter region which regulate its transcription in several lines of kidney cells. The figures represent CAT activities driven by the native PEPCK promoter (wt) and by the following mutants: Δ487-417-pck-CAT (ΔP6) [9] which contains an internal deletion of the HNF-4 recognition site; Δ362-205-pck-CAT (ΔP3,P4) which contains an internal deletion of two C/EBP recognition sequences [7]; mh-597-pck-CAT (mP2), where the HNF-1 recognition sequence has been replaced by a non-relevant polylinker [18]; mP1, where the P1 element was mutated and mCRE, where the CRE element was mutated [13]. CAT activity was determined using phosphorimager, calibrated according to the amount of hGH secreted, and expressed as percent of the unmodified PEPCK promoter activity. Most values are the average of two independent transfection experiments except for the P2 mutant (mP2) and the CRE mutant (mCRE) which are the averages ± S.E. of eight and three independent transfection experiments, respectively.

site, which is known to bind HNF-1. Because this site was efficiently footprinted by nuclear proteins from the PEPCK-non expressing spleen [22,25], and considering the expression of vHNF-1 (HNF-1β) in many tissues including the spleen [27], the adipocytes appear to lack HNF-1.

In contrast to the liver and adipocytes, the kidney nuclear proteins footprinted only two of the sites, P1 and P2. Strikingly, the kidney proteins not only failed to bind the bona fide C/EBP recognition P3 and P4 sites but, also failed to bind the CRE site, which is not involved in determining the tissue specific expression of the PEPCK gene. Binding of kidney nuclear proteins was limited to P1, which contains the ubiquitous NF-1 motif and P2, containing the HNF-1 motif (Fig. 1). We also observed binding of renal proteins to the P6 site of the PEPCK promoter (data not shown), which is known to bind members of the super family of nuclear receptors [31,32]. Thus, the binding of renal nuclear proteins was considerably restricted compared to those obtained by nuclear proteins from the other two PEPCK-expressing tissues and notwithstanding the comparable levels of PEPCK gene expression in the three tissues.

3.2. Basal PEPCK promoter activity in kidney cell lines

Transient transfection experiments were employed to analyze the role of the distinct recognition sites of the PEPCK promoter in conferring its renal expression. To this end, we used LLC-PK1 porcine kidney cells (PK1) which weakly express the PEPCK gene (17 ± 6 milliunits per mg protein) and two derivative sub-cell lines, enriched for this expression, that have been obtained by selecting PK1 cells in glucose-free medium in the presence of 10 mM pyruvate. These included PK22 cells which express the gene 3 times higher than PK1 (52 ± 6 milliunits/mg protein) and PK1F$^+$, containing 15 times higher PEPCK activity (260 ± 15). These enzymatic activities were in accord with the activities of the PEPCK promoter in these cells determined by transient transfection of the cells with 597-pck-CAT plasmid containing the PEPCK promoter driving the reporter CAT gene [7]. Thus, relative to the activity of the SV40 early promoter/enhancer (pSV2-cat [21]), PEPCK promoter activity was 1.4 ± 0.20 in PK1 cells; 4.1 ± 1.4 in PK22 cells and 9.0 ± 2.3 in PK1F$^+$ cells.

The functional roles of the recognition sequences of the PEPCK promoter have been assessed using distinct mutants including: (a) deletion of the sequence containing the HNF-4 recognition sequence (P6) in the plasmid Δ487-417-pck-CAT (ΔP6), and deletion of the C/EBP recognition sequences P3 and P4 in the plasmid Δ362-205-pck-CAT (ΔP3,P4) [7,9]; (b) replacement of the HNF-1 recognition sequence P2 by a non-relevant sequence in the plasmid mh-597-pck-CAT (mP2) [18]; and (c) mutations of the P1 and CRE sites in the plasmids mP1 and mCRE [13].

The analyses revealed (see Fig. 2) that replacement of the HNF-1 recognition motif (mP2) markedly reduced PEPCK promoter activity in all three cell lines tested. The importance of this sequence for basal promoter activity was further accentuated by the lack of significant effects of deleting or mutating other sites in the PEPCK promoter. Deletion of the P3, P4 and P6 sites and mutation of the CRE site resulted in a slightly elevated activity, while mutation of the P1 site resulted in a slightly reduced activity of the PEPCK promoter (Fig. 2). We have previously reported that deletion of the sequence spanning positions −205 to −98 of the PEPCK promoter, which contained both the P2 and P1 sites, completely abolished its activity in PK1 cells [9]. Our present data suggests that the P2 element may play a more significant role than P1

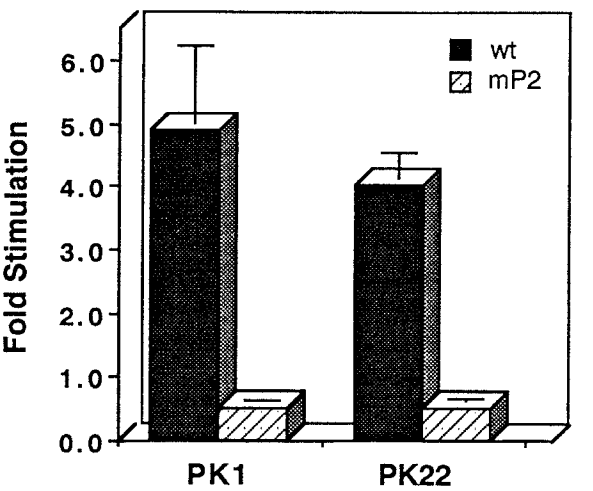

Fig. 3. Transactivation of the PEPCK promoter in sub-lines of kidney cultured cells by HNF-1α. Cultured kidney cells were transfected with 597-pck-CAT (wt) or with the P2 mutant (mP2) alone, or co-transfected with HNF-1α expression vector. CAT activities were quantified using the phosphorimager and calibrated according to the amount of hGH secreted. The activity of the unmodified PEPCK promoter in each cell line, when transfected alone, was used to normalize the activity of the mutant (mP2) and the fold stimulation of the wild type and mutant PEPCK promoter by the co-transfected expression vector coding for HNF-1α. The histogram represents the average fold stimulation in the presence of the transcription factor, for 6 independent experiments. The S.E. of the mean is indicated by the vertical bars.

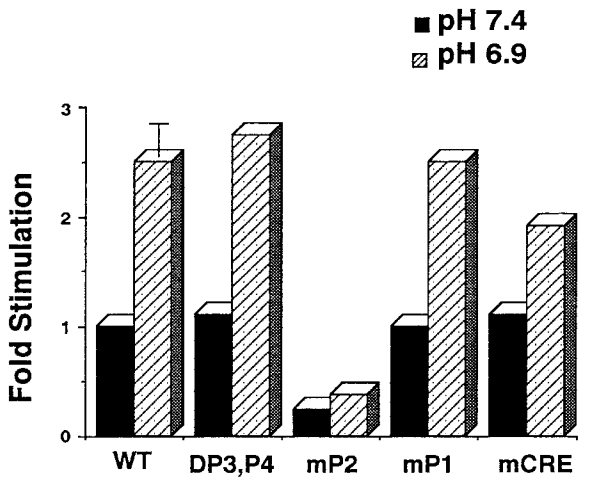

Fig. 4. Effect of acidic pH on transcription from the PEPCK promoter in PK22 cells: PK22 cells were transfected with 597-pck-CAT (wt) or with various derivative mutants indicated in the *x*-axis and described in the legend to Fig. 2. The transfection medium included RSV-hGH plasmid as an internal control. Twenty-four hours after transfection, the medium was replaced with either a medium at pH 7.4 or pH 6.9 for 16 h. CAT activity was determined using the phosphorimager, calibrated according to the amount of hGH secreted, and expressed relative to the unmodified PEPCK promoter activity at pH 7.4. The bargraphs represent the mean fold stimulation by acidic pH of CAT activity resulting from the wild type construct (2.5 ± 0.3 [S.E.M.] of 3 independent transfection experiments) and from the various mutant constructs (the means of two transfection experiments). However, the liver-specific P6 and P3 elements may also play a role in the expression of the PEPCK gene in the kidney, since their removal increases transcription from the PEPCK promoter in kidney cells (as can be seen here in Fig. 2) and in transgenic mice, where deletion of the P6 domain and mutation of the C/EBP recognition site (P3) result in an increased renal expression of the transgenes [11,34]. Recently, the disruption of the gene for C/EBPα has been shown to result in postnatal lethality with hypoglycemia and absence of hepatic PEPCK in the homozygous mutant mice [35], thus, corroborating the important role of this transcription factor for hepatic expression of the PEPCK gene. In contrast, the renal expression of the PEPCK gene in these mice has increased several fold over the normal level (Croniger, Darlington and Hanson, unpublished results). Thus, despite its low level in the kidney, C/EBPα might interact via the P3 element with some other factor to restrain the renal transcription of the PEPCK gene. We have recently shown in hepatoma cells, that members of the C/EBP family interact with HNF-1α via their respective recognition motifs in the PEPCK promoter to stimulate transcription from this promoter [18]. It is tempting to speculate that such interaction in kidney cells will restrain, rather than stimulate, the renal transcription of the PEPCK gene.

in the PEPCK promoter activity in kidney cells (Fig. 2). The dominant role of the P2 observed here in all three kidney cell lines, is in striking contrast to its failure to affect the promoter activity in hepatoma cells [13]. We have previously shown, however, that P2 could become important in hepatoma cells when the promoter activity was stimulated by co-transfection of HNF-1α together with members from the C/EBP family [18].

In view of the dominant role played by the P2 site in all three kidney cell lines it was conceivable that members of the HNF-1 family by themselves could stimulate the promoter activity in these cells. Indeed, when HNF-1α was co-transfected with the 597-pck-CAT plasmid the PEPCK promoter activity was stimulated 5 to 4 fold in PK1 and PK22 cells (Fig. 3). The trans-activation of the PEPCK promoter by HNF-1α was dependent on the presence of its cognate recognition motif in the promoter. Likewise, HNF-1β stimulated the promoter activity when co-transfected together with 597-pck-CAT (data not shown). It should be noted that the liver expresses only HNF-1α while the kidney expresses both HNF-1α and HNF-1β [27,33].

3.3. Modulation of the PEPCK promoter activity by acidic pH

As mentioned above the activity of the PEPCK gene can be modulated by external conditions such as pH. To determine whether the presence of an intact P2 was also required for the modulation of the PEPCK promoter activity by acidic pH, PK22 cells were transfected with 597-pck-CAT and 24 h later the cell culture medium was replaced by a medium containing 29 mM HCO_3^- at either pH 7.4 or pH 6.9 for 16 h (see Methods in Section 2). These experimental conditions have been used previously to demonstrate a maximal response of the endogenous PEPCK gene to acidic pH in $PK1F^+$ cells [16]. We noted that the acidic pH stimulated the PEPCK promoter by 2.5 ± 0.3 fold (the mean ± S.E. of three independent transfection experiments). The stimulation of CAT activity by acidic pH was also observed with mutated promoters, including mutations in the CRE, P1, P2 or P3/P4 sites. However, the extent of the stimulation was considerably lower when the P2 element (the HNF-1 motif) was mutated (Fig. 4) albeit, the basal activity with the mutated P2 was low.

4. Discussion

In this report we demonstrate that the renal expression of the gene for PEPCK specifically depends on a single regulatory element in the promoter that contains the HNF-1 motif (P2). This element appears to play a dominant role in both the basal and acidosis-stimulated activity of the promoter.

Previous studies with transgenic mice have shown that different elements are required for PEPCK gene expression in the liver, kidney and adipose tissue [8,10,11]. For example, the CRC362 transgene, which lacks the P6 element, is not expressed in the liver while it is fully expressed in the kidney [11] and fully responds to metabolic acidosis (Olswang and Reshef, unpublished results). The P6 element is important in cultured H4IIEC3 hepatoma cells [7,9] but not in cultured LLC-PK1 kidney cells ([9] and present work).

Further studies using transgenic mice [34] have recently shown that the C/EBP binding element, P3, is required for full expression of the PEPCK gene in the liver but not in the kidney. Likewise, this element is not required for PEPCK promoter activity in cultured kidney cells as shown in the present work. In contrast, the HNF-1 recognition domain is required for renal expression of the PEPCK gene. Thus, mutation of this recognition motif (P2) markedly reduces the transgene expression in the kidney but not in the liver [34]. Similarly, as shown here, mutation of the HNF-1 recognition motif (P2) in the PEPCK promoter markedly reduces its activity in cultured kidney cells regardless of whether the promoter activity is low (PK1 cells) or high ($PK1F^+$ cells). Taken together, these studies establish the differential roles of distinct sequences of the PEPCK gene in the regulation of its specific transcription in the liver and kidney. While at least two elements (the P6 and P3 sites) are required for full hepatic expression, a dominant single element, the HNF-1 recognition

motif (P2), appears to be critical for the renal-specific expression of the PEPCK gene.

In view of the high level of HNF-1α in both the liver and kidney it is, however, not clear why the P2 binding site of the PEPCK promoter is critically required for the renal expression of the PEPCK gene, while it is not required for expression in the liver. This issue is further emphasized by the disruption of the gene for HNF-1α in mice, which leads to lethality around the time of weaning. These mice develop renal Fanconi syndrome and fail to tolerate overnight fasting [33]. In contrast, hepatic PEPCK gene expression is normal in these mutant mice indicating its independence of HNF-1α. This differs from the expression of several other liver-specific genes that was affected by this mutation in the liver. In particular, the absence of expression of phenylalanine hydroxylase (4-monooxygenase), resulting in phenylketonuria [33].

Unlike the hepatic expression of the PEPCK gene, which is induced by birth, the renal expression is notably negligible during the first post-natal days [11] and attains adult levels only toward weaning (Olswang and Reshef, unpublished results). It would be interesting to find out whether renal PEPCK gene expression is affected in the HNF-1α mutant mice at weaning, when the mutation becomes lethal.

In addition to basal PEPCK gene transcription, we have now shown that the P2 sequence is required in cultured kidney cells for a full response of the PEPCK promoter activity to acidic pH. However, P2 itself is unlikely an acidosis response element because of the partial response of the P2 mutant to this treatment. Moreover, P2 seems to be necessary for modulation of the promoter activity by other treatments as well. Thus, in preliminary experiments we have recently observed a marked stimulation of the promoter activity in PK1 cells by the peroxisome proliferator-activated receptor (PPARα), whose recognition site in the promoter was previously assigned to P6 [32]. This trans-activation was considerably reduced when using the mP2 plasmid, containing a mutated P2 site (Heineman, Olswang and Reshef, to be published elsewhere). Since P2 does not bind PPAR, it most conceivably cooperates with sites which bind PPAR [32]. Cooperation between a bona-fide cell-specific element with elements mediating the gene response to various stimuli has been repeatedly shown. Thus, the response of the PEPCK gene to glucocorticoids requires cooperation between several elements, including P6 which is not a glucocorticoid receptor binding site [12], and its response to cAMP requires cooperation of the CRE with the cell-specific P3 element (a C/EBP motif) [13]. Such cooperation has been shown also in the cases of the tyrosine amino-transferase [36] and phenylalanine hydroxylase genes [37]. The involvement of P2 in more than one type of modulation of the PEPCK gene in the kidney cells is striking. This is even more emphasized in view of its dispensable role in the liver. It is tempting to speculate that the favored dominant role of P2 in the kidney is attained because the kidney nuclei are poor in proteins that bind to P3 and CRE sites, as evident by the lack of their footprinting by kidney nuclear proteins. Thus, we propose that the dominant role of P2 in the kidney not only reflects high levels of HNF-1α, but also the low levels of C/EBP in the kidney. This hypothesis, which is currently under investigation, also predicts that P2 will not play such an important role in adipocytes not only because adipocytes are poor in proteins that bind the P2 site but also because they abundantly express members from the C/EBP family.

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