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Repression and activation of transcription of phosphoenolpyruvate carboxykinase gene during liver development

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Abstract Transcriptional activation of the hepatic phosphoenolpyruvate carboxykinase (PEPCK) gene at birth is critical since PEPCK appearance initiates hepatic gluconeogenesis. A delayed appearance results in hypoglycemia, while a premature appearance results in neonatal diabetes, both are incompatible with sustaining life. Experiments using transgenic mice and transfected hepatoma cells suggest that both repression and activation underlie the correct onset of hepatic PEPCK gene transcription. In transgenic mice, transgenes driven by the proximal PEPCK promoter are prematurely expressed in the fetal liver and over-expressed in the neonatal liver, indicating that sequences upstream of the proximal promoter restrain perinatal expression. In Hepa1c1c7 cells, which mimic the fetal liver, the proximal PEPCK promoter (597 bp) exhibited a 3.5–10-fold higher activity than longer promoters. Repression of the longer promoter (2000 bp) was diminished upon deletion of the sequence spanning positions −840 to −1116 which contains a PPAR/RXR recognition element. The intact 2000 bp PEPCK promoter could be markedly activated by co-transfecting the transcription factor HNF-1 together with C/EBP. It could be repressed by co-transfection with RXRα and adding PPARα relieved this inhibition.

Key words: Phosphoenolpyruvate carboxykinase; Liver; Development; Transcription; Modulation; Peroxisome proliferator-activated receptor; Retinoid X receptor; HNF-1; C/EBP

1. Introduction

The liver plays a major role in mammalian glucose homeostasis by providing glucose to the blood via gluconeogenesis. This function becomes critical after the detachment of the newborn from the maternal supply of glucose at birth. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the limiting step of gluconeogenesis. In fact, it regulates gluconeogenesis since its activity readily changes in response to a variety of hormonal and dietary conditions. These changes are the consequences of modulations of PEPCK gene transcription (for reviews see [1,2]).

As a result of the activation of hepatic PEPCK gene transcription [3], the appearance of hepatic PEPCK at birth initiates gluconeogenesis. The timing of this appearance is critical, since delaying it results in hypoglycemia, while its premature appearance results in neonatal diabetes, both of which are incompatible with sustaining life (for review see [4]). Thus, the activation of hepatic PEPCK gene transcription at birth and the prevention of its premature activation prior to birth are equally important.

The PEPCK gene is expressed in three tissues, in the gluconeogenic liver and kidney and in the glyceroneogenic adipose tissue. Of these, hepatic expression of PEPCK is most directly associated with glucose homeostasis in the blood.

The activation of PEPCK gene transcription at birth correlates the late appearing transcription factors from the C/EBP family. Studies by us and by others, in cultured hepatoma cells and in transgenic mice, have suggested that the PEPCK gene is a target of members of the C/EBP family for transcription factors [5–8]. Its activation at birth coincides with the abruptly increasing hepatic levels of the C/EBP family of proteins at this time [9,10]. Knockout of the gene for C/EBPα results in neonatal lethality with hypoglycemia, associated with the lack of appearance of hepatic PEPCK and glucose 6-phosphatase at birth [11]. Furthermore, we have recently demonstrated, using mice homozygous for disruption of C/EBPβ, that this activator is also associated with the developmental activation of hepatic PEPCK gene transcription at birth [12].

Although hepatic PEPCK gene transcription initiates at birth [3], it can be activated in rat fetuses by in utero injecting them with either cAMP [13] or agents that reduce insulin, such as streptozotocin or anti-insulin serum [14,15]. More recent experiments have established that these two treatments differentially induce the expression of genes encoding C/EBPα and C/EBPβ, along with activating PEPCK gene transcription in the fetal liver [12]. Furthermore, we have shown that the premature activation of hepatic PEPCK gene by in utero injection of Bt_2cAMP is considerably reduced in mice homozygous for the disruption of the C/EBPα gene [12]. These data document the involvement of C/EBP transcription factors in the activation of hepatic PEPCK gene transcription. However, in addition to activation, other data imply that the hepatic gene transcription might be repressed prior to birth: (a) PEPCK is expressed in several hepatoma cells that are poor in C/EBPα [16,17]; (b) this factor is already expressed in the fetal liver albeit at a low level [9,18]; (c) using PEPCK antibody for immunohistochemical analyses revealed that, in addition to the main three PEPCK-expressing tissues, a minimal level of expression of the gene can be detected in many tissues (such as the salivary glands), except the fetal liver [19]. Since the PEPCK gene is probably transcribed at low levels in many tissues the failure to detect any expression in the fetal liver is surprising, suggesting that even a minimal PEPCK expression in the fetal liver is inhibited. In this study we examine this issue, using transgenic mice and hepatoma cell lines.

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2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), F12 and fetal calf serum were purchased from Biological Industries, Kibutz Beit Haemek, Israel. Nytran membrane (Schleicher and Schuell) was used for blot hybridization. Radioactive signals were quantified by phosphorimaging (Fujix BAS 1000, Fuji, Japan).

2.2. Animals

Lines of transgenic mice [20], containing the entire rat PEPCK gene, in which a 465 bp fragment in the last exon was replaced by a non-homologous counterpart 485 bp fragment from the chicken PEPCK cDNA [21], were used in these experiments. The independent lines of transgenic mice used, F8, M12, M9 and M16, contain the chimera gene with 540 bp of the 5′ flanking region of the PEPCK gene (designated CRC540). Transgenic mice were detected by PCR (polymerase chain reaction) [22] of tail DNA using the 5′ and 3′ primers GGGAAAGCCTCTGCCAAGTCA and CACAGTTTT-GAAGACTGTGTGCTC of the 485 bp chicken insert, spanning positions +4935 to +5420 of the chimera PEPCK gene. A unique *Hpa*I restriction site in the amplified insert was used for verification. Nineteen day old fetuses and 1 day old newborn mice were used in these experiments.

2.3. Molecular probes and RNA analysis

PEPCK cDNA probe was a 1.6 kb *Pst*I fragment [23]. The 485 bp fragment of the chicken PEPCK cDNA (c485) and its counterpart rat cDNA were as described previously [20]. The rpL7 cDNA for the rat ribosomal protein L7 [24] was used to normalize RNA amounts in the Northern blots. The analysis was as previously described [20], using 50 μg total hepatic RNA prepared by the method of Chirgwin et al. [25] as described [20].

2.4. Cell culture, transfection conditions and CAT assays

Hepa1c1c7 and HepG2 hepatoma cell lines were grown in the growth medium containing a 1:1 mixture of DMEM and F12, and 10% fetal calf serum. Cells were transfected by calcium phosphate precipitation, essentially according to Chen and Okayama [26], as previously described [17], using 1 μg of supercoiled plasmid and additional carrier pBS DNA (Stratagene), to make a total of 20 μg. The transfection efficiency was monitored by including 0.1 μg of pS16-GH as an internal standard [27], 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 radioimmunoassay [27], using a commercial kit (St. Nichols, San Diego, CA, USA) according to the supplier's instructions. Where indicated, 1 μg each of HNF-1α, C/EBPα and HNF-4 or peroxisome proliferator-activated receptor (PPAR) α and retinoid X receptor (RXR) α expression vectors were added to the transfection mix. 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.

2.5. Plasmids used in transfection

The previously described plasmids 597-pck-CAT and 4800-pck-CAT contain 597 bp and 4800 bp respectively of the rat PEPCK promoter region fused to the CAT reporter gene [28]. The derived plasmid 2000-pck-CAT contains 2000 bp of the PEPCK promoter and plasmid Δ(840–1116) contains an internal deletion in the 2000 bp PEPCK promoter, spanning positions −840 to −1116 of the transcription start site. The deleted sequence contains PPARE (a PPAR/RXR motif) at positions −897 to −997 of the transcription start site [29]. DNA amounts of the constructs containing longer PEPCK promoters than 597-pck-CAT were corrected to achieve the same number of molecules as that of the proximal promoter (597-pck-CAT). Expression vectors encoding the liver enriched transcription factors used in this work included: HNF-1α (also termed HNF-1) [30], C/EBPα [16], HNF-4 [31], PPARα [32] and RXRα [33].

3. Results

In the transgenic mice used in this work the transgenes driven by the proximal rat PEPCK promoter (540 bp) prematurely expressed in the fetal liver, albeit at low levels (Fig. 1). This was in contrast to the lack of expression of the endogenous PEPCK gene (Fig. 1) or to previous findings showing a lack of expression in the fetal liver of both the endogenous PEPCK gene and transgenes driven by 2000 bp of the rat PEPCK promoter [20]. Furthermore, even in 1 day old newborn mice, when both the endogenous gene and transgenes were activated, the hepatic expression of the transgenes markedly exceeded that of the endogenous gene (Fig. 1). Again, this was in contrast to previous findings that expression of transgenes driven by the 2000 bp PEPCK promoter did not exceed that of the endogenous gene [20]. These results suggested that sequences upstream of position −540 of the PEPCK gene exerted an inhibitory effect on its transcription during the perinatal period.

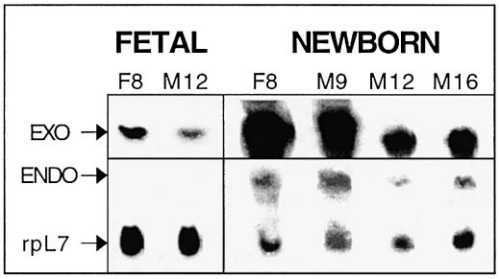

Fig. 1. Hepatic perinatal expression of PEPCK gene and transgene. Northern blot hybridization of 50 μg/lane of total hepatic RNA from independent lines of transgenic mice (F8, M12, M9, M16, see Section 2) prepared from 19 day old fetuses (Fetal) and 1 day old neonates (Newborn). The c485 probe reveals the transgene expression (EXO) and its counterpart rat probe reveals the endogenous PEPCK gene expression (ENDO). The ribosomal protein cDNA L7 (rpL7) monitors RNA loading.

To further investigate this phenomenon, we utilized the mouse hepatoma Hepa1c1c7 cell line [34] (Hepa), which mimics the fetal liver [17]. Transient transfection experiments with the CAT reporter gene driven by various sizes of the rat PEPCK promoter showed (Fig. 2A) that the promoter activities decreased about 3.5- and 10-fold when their sequence length increased from 597 bp to 2000 and 4800 bp respectively. In contrast, similar analyses using the PEPCK expressing HepG2 human hepatoma cells (Fig. 2B) or H4IIEC3 rat hepatoma cells (results not shown) showed that all promoters shared similar activities regardless of size.

The existence of inhibitory sequences upstream of position −2000 has been previously documented [35]. The region between positions −2000 and −597 of the transcription start site has not been similarly analyzed. This region contains a PPARE recognition site for the PPAR, which bind PPARE as heterodimers with the RXR [29]. Internal deletion of this sequence (within positions −1116 to −840 of the PEPCK gene) diminished the inhibition of the 2000 bp promoter activity in Hepa cells (Fig. 2A), but had no effect on the activity of this promoter in HepG2 cells (Fig. 2B).

Since the deletion mutant has implicated PPARE in the repression of PEPCK promoter activity, experiments were designed to assess the role of its cognate factors in this modulation. Members of the PPAR family are nuclear receptors that form heterodimers with RXR and have been shown to bind PPARE of the rat PEPCK promoter and stimulate its activity in NIH3T3 cells [29]. In contrast, RXR can form

homodimers which, in turn, can repress the activities of various target promoters via appropriate recognition sites [36–38]. To investigate whether RXRα could inhibit the PEPCK promoter activity we co-transfected its expression vector either alone or with PPARα expression vector, together with the CAT gene driven by the 2000 bp PEPCK promoter. Although this promoter activity was already inhibited (Fig. 2), RXRα by itself further inhibited its activity (Fig. 3). This inhibition disappeared by adding PPARα expression vector, causing even a stimulation (×2.5) of the promoter activity. Thus, the distal PPARE in the PEPCK promoter was capable of mediating either repression or a slight activation of the promoter activity, depending on the cognate transcription factors available.

Besides repression, it was evident (Fig. 1) that in the newborn liver not only transgenes driven by the proximal promoter were markedly activated, but the endogenous PEPCK gene was activated as well. Previous evidence documented that this activation was linked to the rise in the hepatic level of C/EBP family of proteins because it failed to occur upon the disruption of the C/EBP genes leading to neonatal lethality [11,12]. Hepa cells lack liver-enriched transcription factors such as C/EBPα and HNF-1α. We have previously shown that these factors synergistically stimulated the proximal promoter activity in these cells [17]. To investigate whether these factors could stimulate the longer promoter activity as well we co-transfected their expression vectors together with PEPCK-CAT constructs driven by the longer promoters (4800 bp, results not shown and 2000 bp, Fig. 3). The results showed that these factors markedly stimulated the activities of the longer promoters.

4. Discussion

Our present results demonstrate that transgenes driven by the proximal PEPCK promoter (540 bp) exhibit temporal unrestrained hepatic expression in the perinatal period, resulting in premature onset of expression in the fetal liver and overexpression in the neonatal liver. Complementary transfection experiments in hepatoma cells have assisted us in: (a) reconstructing the repressed activities of longer PEPCK promoters (containing sequences upstream of position −597 of the PEPCK gene); (b) disclosing the role of the distal PPAR/RXR recognition sequence which, depending on the cognate factors, confers either repression or stimulation of the promoter activity; (c) showing that the repression disappears in the presence of specific liver-enriched transcription activators.

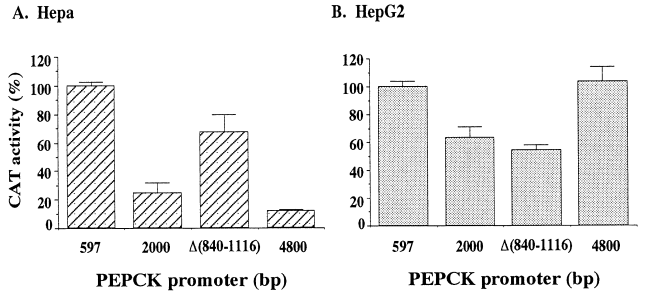

Fig. 2. Effect of the size of the PEPCK promoter activity in hepatoma cell lines. Transient transfection experiments in Hepa cells (A) and HepG2 cells (B) with CAT reporter gene driven by various sizes of the PEPCK promoter (597, 2000 and 4800 bp) and with a 2000 bp promoter containing an internal deletion of positions −840 to −1116 of the transcription start site (Δ(840–1116)). The histograms represent the means ± S.E.M. (indicated by the vertical lines) of CAT activity from 3–5 independent transfection experiments for each construct. CAT activity is expressed as percent of the activity measured of 597-pck-CAT.

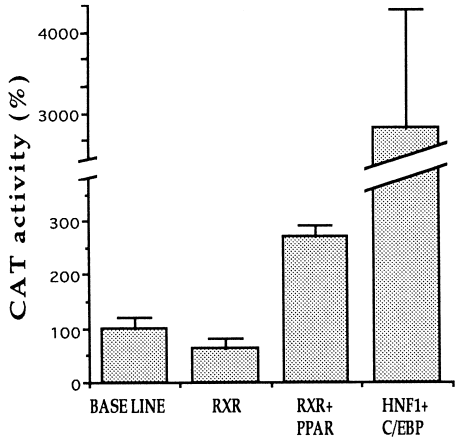

Fig. 3. Modulation of the 2000 bp PEPCK promoter in Hepa cells by RXR, PPAR/RXR, and by HNF-1α and C/EBPα. 2000-pck-CAT was transfected without or co-transfected with the expression vector for RXRα alone or together with PPARα or with those for HNF-1α together with C/EBPα. Note the larger scale (×10) for the activation by HNF-1α and C/EBPα. For details of CAT activity see legend to Fig. 2.

Taken together, our findings strongly suggest that two mechanisms regulate the correct onset of hepatic PEPCK gene expression after birth: (a) activation of the gene expression by distinct liver-enriched transcription factors which, in turn, appear relatively late in development; (b) prevention of a premature onset of expression, via specific regulatory sequences and their cognate transcription factors. The latter is partially mediated via PPARE, which can confer repression or activation depending on the availability of its cognate transcription factors. As previously reported [29], PPARE binds either a heterodimer comprising PPAR and RXR or a homodimer of RXRα. It has been shown that when RXRα dimerizes to form a homodimer it inhibits, rather than activates, a number of promoters [36–,38]. As is now evident, RXRα also inhibits the PEPCK promoter activity when supplemented alone. However, the co-supplemented PPARα abolishes this inhibition and even moderately stimulates the longer promoter activity. The significance in vivo of this versatile behavior of PPARE lends support from the relatively late perinatal appearance of PPARα [39]. Furthermore, intensive studies in recent years have shown that nuclear receptors can mediate either repression or activation of target gene expression, depending on their binding to adapter molecules such as NCoR a corepressor or NCoA a coactivator (for recent reviews see [40,41]). In the case of the hepatic PEPCK promoter, although the repression disappears in the presence of the heterodimer PPAR/RXR the stimulation by these receptors is moderate.

In contrast, C/EBP together with HNF-1 markedly stimulate all PEPCK promoters reaching similar high levels of activities in their presence regardless of the promoter size. This might occur either by erasing the inhibition or, alternatively, by excessively stimulating the longer promoters to overshadow the inhibition. A synergistic stimulation by these two transcription factors has been shown for the human albumin pro-

moter as well [42]. However, the significance of these findings in vivo is still open; the albumin gene is not dependent in vivo on HNF-1 or on C/EBPα since it begins to be expressed in the fetal liver preceding the hepatic expression of C/EBPα or HNF-1α [43–45]; it is expressed in Hepa cells which fail to express either of these transcription factors [17]; and it is expressed in neonatal mice homozygous for the disruption of the C/EBPα [11] or HNF-1 genes [46]. In contrast, hepatic PEPCK gene expression is critically dependent on C/EBPα (although not on HNF-1 [46]) since it is not expressed in the fetal liver or in Hepa cells and the onset of its hepatic expression in neonates is blocked in mice homozygous for the disruption of C/EBPα gene [3,11,17]. The present findings that transgenes driven by the proximal PEPCK promoter prematurely express in the fetal liver, albeit at a low level, correlate the absence of PPARE in these transgenes. It is attractive to propose that, in the absence of PPARE, early activation of the transgenes by the existence of low levels of C/EBPα and HNF-1α in the fetal liver is facilitated. A burst of the transgene expression in the neonatal liver correlates the rise in the level of C/EBPα at this age [12].

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