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THE 5′ REGION OF THE RAT PHOSPHOENOLPYRUVATE CARBOXYKINASE GENE CONFERS pH SENSITIVITY TO CHIMERIC GENES EXPRESSED IN RENAL AND LIVER CELL LINES CAPABLE OF EXPRESSING PEPCK

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The 5′ flanking regions of the rat phosphoenolpyruvate carboxykinase gene were used to form chimeric gene constructs with the human growth hormone gene. These constructs were transfected into several renal and one liver cell line and the production of growth hormone (HGH) measured by immunoassay. Cyclic-AMP and glucocorticoid responsiveness of HGH production was observed in all cell lines. In two lines, the rat NRK52E renal epithelial line and the rat H4IIE hepatoma cell line, both capable of expressing PEPCK, lowering extracellular pH increased HGH production several fold. Comparison of hormone and pH effect on cells transfected with a thymidine kinase promoter-HGH chimera indicated that the PEPCK 5′ flanking region effects were specific. Thus, part of the pH responsiveness of the PEPCK gene in vivo may be attributed to properties of the 5′ flanking regions.

Introduction: The gene for cytoplasmic phosphoenolpyruvate carboxykinase(GTP) is expressed in a highly organ-specific manner, and has been the subject of a number of studies [2,5,9,13,18]. This major enzyme of gluconeogenesis is expressed in liver and kidney and in both organs, responds to fasting, glucocorticoids and cAMP. In vivo, differences exist between liver, where insulin is the major suppressive hormone and kidney where insulin is without effect. In addition, glucose loading is suppressive in both organs (Pollock, AS, Am. J. Physiol. in press). In the kidney, metabolic acidosis is a major inducer of transcription of this message, while in liver, this effect is less pronounced [8]. Much of the hormonal responsiveness of the PEPCK gene resides in the 5′ flanking region, where a cAMP-responsive sequence and several GRE-like sequences reside. Previously reported transfections of PEPCK-chimeric genes have been performed in liver cells, where hormone responsiveness has been the issue of study. In this report, we evaluate the pH responsiveness of the 5′ region in renal cells.

Methods: Cell Culture All cells described were maintained in DME, glucose, 5 mM; HCO_3, 24 mM; non-essential amino acids, 0.1 mM; penicillin and streptomycin, 100 μg/ml each; in 5% CO_2 air. The cell lines used for these studies were: the OK cell line, the LLC-PK1 cell line (a gift of Dr. J. Handler), the NRK52E cell line [6] (obtained from ATCC, and the H4IIE hepatoma line (obtained from Dr. C. Grunfeld).

Isolation of PEPCK enhancer/promoter Fragment and promoter/reporter construction. A rat lambda genomic library was screened with the 5′PstI fragment of plasmid pPCK10,

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containing 1.4 kb of the rat PEPCK cDNA as well as three 30 mer oligonucleotides complementary to the published PEPCK genomic sequence. Three separate clones were isolated after three rounds of screening and decreasing plaque density and plaque purified. One of them, λPC1, contained a 15 kb insert comprised of four EcoRI fragments containing the entire PEPCK gene as well as 5 kb of 5′ flanking DNA. The lambda fragments were subcloned into pGEM3Z and restriction mapping and Southern blotting with oligonucleotide probes, and double stranded dideoxy sequencing was used to identify plasmids bearing the first exon-bearing coding region (pλPC1.1) as well as the 5′ flanking region (pλPC2.2). A promoter/reporter construct, pPCP2.0GH was constructed as follows. A 527 bp EcoRI-BglII fragment, corresponding to nucleotides –464 to +62 [1] was isolated from pλPC1.1 and together with the HindIII-EcoRI polylinker fragment of pUC18 was ligated with the HindIII-BamHI fragment of p0GHXE bearing the gene for Human Growth Hormone (HGH). p0GHXE was prepared by removing the EcoRI site of p0GH with EcoRI, fill in with Klenow fragment and re-ligation. The identity of the PEPCK fragment in this construct was verified by double stranded sequencing from the M13 primer binding site contained in the p0GH plasmid host. Plasmid pPCP2.0GH was linearized with EcoRI and ligated with the 4.5 kb 5′ genomic fragment contained in pλPC2.2. Restriction mapping and southern blotting with oligonucleotides identified recombinants in which the 5′ fragment was inserted in the native orientation (pPCP3.0GH).

Transfections, experimental design and analysis. Cells were transfected by electroporation by the method of Chu [4]. Approximately 10^8 cells were transfected with 100 μg of the indicated plasmid and plated in 20-30 100 mm dishes. In each experiment, a parallel group of cells was transfected with pTKGH (the HSV thymidine kinase promoter driving HGH gene) and treated identically. The medium was changed and experimental medium was applied on the fifth day after transfection. After ≈14 hours in the experimental medium, the medium was aspirated and HGH was measured by double antibody I^{125} immunoassay for HGH (Nichols). Experimental media was buffered with 25 mM HEPES and an air incubator was used. The remaining cell monolayer was rinsed with iced saline and total RNA was prepared. Each experimental data point consists of the mean HGH production of three identically plates. To correct for generalized effects on HGH production, a ratio of the PEPCK-driven HGH production to the TK-driven HGH production (of identically treated plates) was formed. Previous studies indicated that with a common pool of electroporated cells, HGH expression between identically treated plates was characterized by a coefficient of variation of <10%. Therefore, no discreet correction for transfection efficiency was made so long as the only comparisons made were among plates from the same transfection. Northern blots or dot-blots of total or polyA$^+$-selected RNA were done where indicated. Probes were labeled by the random-hexamer method to > 10^9 cpm/ μg DNA. General molecular biological techniques were as described in Maniatis [11].

Results: The relationship between the PEPCK-HGH chimeras and the PEPCK gene are outlined in figure 1. The 527 bp EcoRI-BglII fragment contained in pPCP2.0GH, is known to contain a cAMP-responsive region and a GRE [20,21]. The changes reported; the ratio between PEPCK- and Herpes Simplex Virus TK- promoter driven HGH expression was primarily due to changes in expression of the PEPCK-construct. The TK-driven expression usually varied by <15%. Figure 2 summarizes transfection studies of the PEPCK-HGH chimeras in the OK (opossum) and LLC-PK1 (porcine) cell lines. These lines were chosen because of several proximal-tubule like features they have display and the existence of an extensive literature dealing with them [10,15,17]. When pPCP2.0GH and pPCP3.0GH were transfected into the OK and LLC-PK1 cell lines, cAMP responsiveness was observed as previously reported in liver. Initially, no glucocorticoid responsiveness was noted until exogenous glucocorticoid receptor was provided by co-transfection with p6RGR (a glucocorticoid receptor expression vector [14]), at which time glucocorticoid responsiveness was restored (figure 2). However, no effect of lowering pH on PEPCK-HGH chimera expression was noted. Previous studies with the OK cell line suggest that lowering the extracellular pH to 7.0 reduces intracellular pH in the OK cell line to < 6.8 [17], therefore,

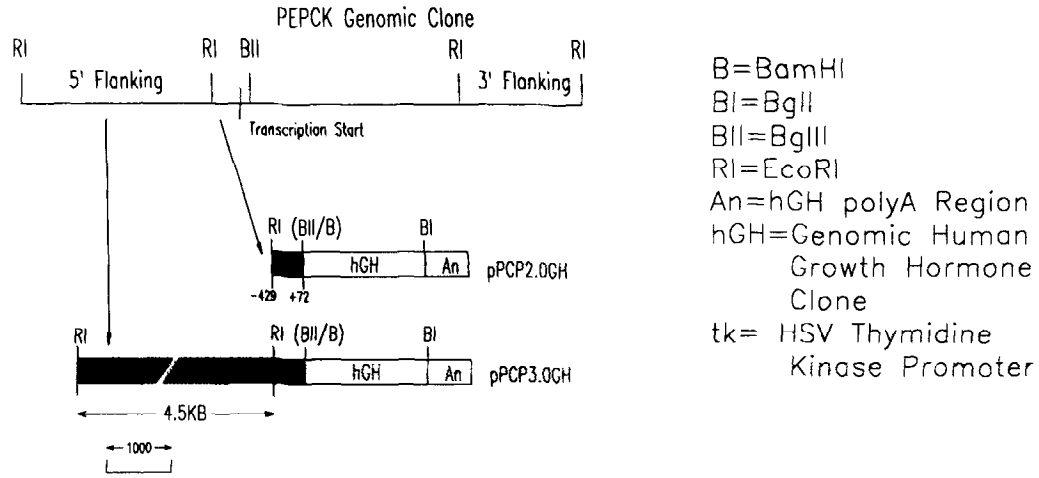

Figure 1. Origin and construction of enhancer/reporter constructs used in these studies. Bases are numbered relative to the PEPCK mRNA transcription start site [1]. The TK-GH plasmid uses the HSV-tk promoter driving HGH production.

appreciable intracellular pH decrease occurred. It is of note that the both the 527 bp 5' region and the longer 5 kb 5' region responded identically in the two renal cell lines evaluated.

Although retaining proximal tubule-like features, the OK and LLC-PK1 cell lines are not of rat/murine origin and, in our hands are not gluconeogenic and express no hybridizable PEPCK message or PEPCK enzyme activity. Additional studies were performed in both the NRK52E renal and the H4IIE hepatoma cell lines. The former is a rat renal cell line subclone of the well known NRK line, chosen to represent epithelial components in the original mixed culture. Recent reports indicate it is both gluconeogenic and ammoniagenic; both proximal tubule-like metabolic features [16]. The H4IIE is a rat hepatoma line which expresses PEPCK in a hormonally-responsive manner [3]. Northern blots of NRK52E cell RNA revealed very low levels of PEPCK mRNA expression which does increase with lowered pH and cAMP. Figure 3 demonstrates the results of transfection experiments in the NRK52E line. Decreasing extracellular pH to 7.0 increased HGH expression 3 fold over control. The cell line was also cAMP responsive and dexamethasone responsive (without addition of glucocorticoid-receptor expression plasmid).

In vivo, hepatic expression of the PEPCK gene is subject to positive modulation by conditions such as fasting and by specific mediators such as cAMP and glucocorticoids [2,3,5,19]. Metabolic acidosis, which induces renal PEPCK mRNA, has an early suppressive effect, and a later slight stimulatory effect of hepatic PEPCK mRNA. When transfected liver cells were exposed to low pH, HGH secretion increased 3 fold. This effect was additive to the effect of glucocorticoids; 10^{-6} M dexamethasone produced a larger effect at pH 7.0 that 7.4. Simultaneous slot-blots on A^+ RNA from the transfected hepatocytes probed with a PEPCK probe indicated that the expression of the native H4IIE cell PEPCK gene paralleled that of the chimera expression (see figure 4). It should be noted that the cDNA probe used for the slot blots did not include sequences expected in the PEPCK chimera transcript.

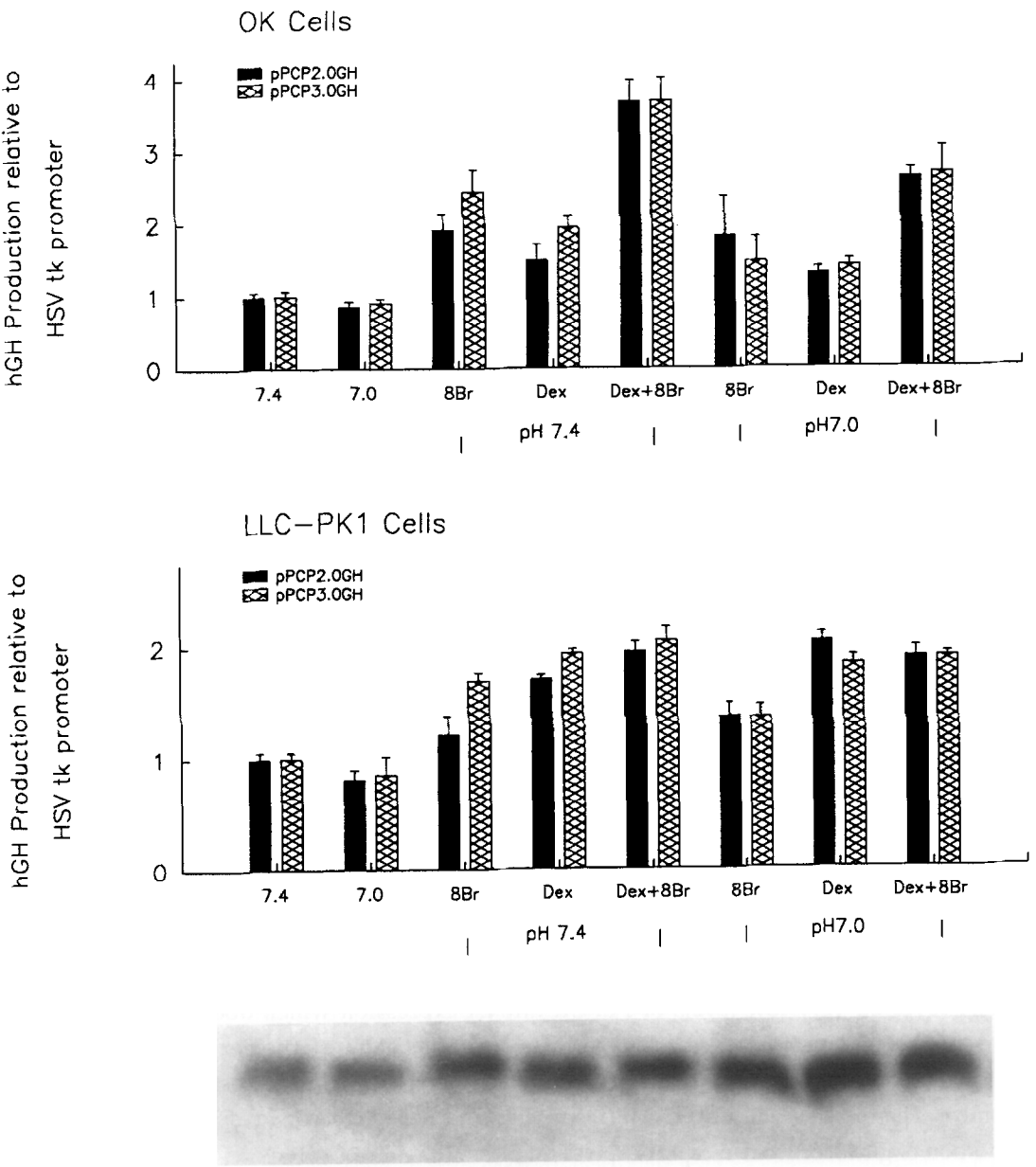

Figure 2. Response of the PEPCK 5′ enhance regions in two renal epithelial cell lines. The two upper panels indicate the relative HGH production in the indicated conditions in the OK and LLC-PK1 cell lines transfected with pPCP2.0GH and pPCP3.0GH. The lower panel is a northern blot of OK cell RNA from an experiment depicted in the upper graph probed for HGH RNA. The quantities expressed are the ratio of the PEPCK-driven to the TK-driven expression of HGH in parallel cultures transfected at the same time and exposed to the same experimental media.

Discussion: These studies were undertaken to determine whether the 5′ flanking regions of the rat PEPCK gene conferred pH-sensitivity on chimeric gene transcription in renal cells. In vivo, systemic pH changes rapidly and specifically affect renal, and to a lesser extent hepatic PEPCK mRNA expression. The results indicate that in renal and hepatocyte cell

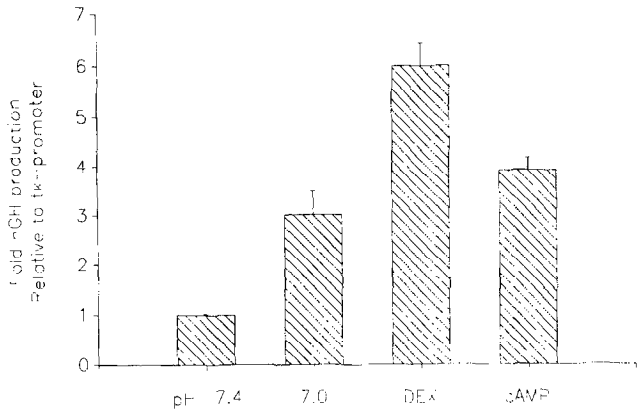

Figure 3. HGH production from pPCP2.0GH transiently expressed in the rat NRK52E cell line , after overnight exposure to the indicated agents. Dexamethasone was 10^{-6}M and cAMP was 8Br-cAMP, 0.1 mM. The ratio expressed is calculated from parallel TK-driven constructs as described above.

lines which are capable of expressing PEPCK at all, lowering extracellular pH induces increase in chimeric gene expression, relative to that seen with the viral HSV-tk promoter.

The OK and LLC-PK1 cell lines are well studied renal epithelial-like lines, however, they are not gluconeogenic nor, in our hands, do they express PEPCK message. In contrast, the renal NRK52E, and hepatoma H4IIE lines, both derived from the rat, do express the PEPCK message. In these cells, the 527 bp 5′ flanking region of the rat PEPCK gene confers pH sensitivity.

The expression of the PEPCK gene has been well studied in liver and hepatocytes. In addition to being uniquely sensitive to dietary state, the regulation of PEPCK gene expression serves as a superb example of multi-hormonal regulation; insulin, glucocorticoids and cAMP all affecting its transcription and message stability. Recent studies of nuclease-hypersensitivity [7] indicate that expression of the PEPCK gene in liver is associated with at least 4 hypersensitive regions spread over > 10 kb. Therefore,

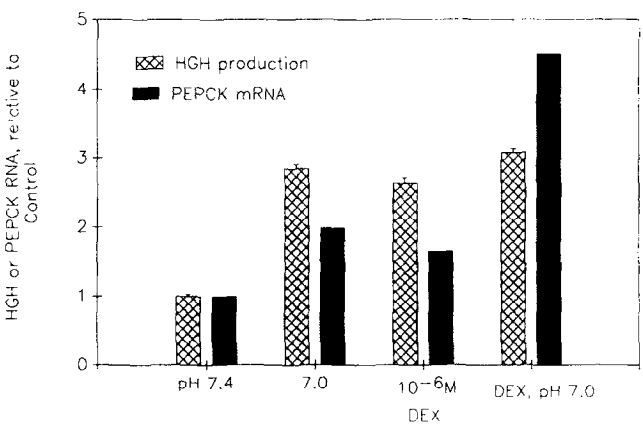

Figure 4. HGH production (relative to the TK promoter) from pPCP2.0GH transiently expressed in the rat H4IIE hepatoma cell line. The hatched bars indicate the relative HGH production and the filled bars represent hybridization intensity on simultaneous dot-blots of A^+ RNA probed with a PEPCK probe.

regulation of PEPCK expression is likely to be considerably more involved than demonstrated by this relatively short flanking region segment. The kidney, which also expresses high levels of PEPCK mRNA and enzyme, has been less well studied. It is clear from several studies that in kidney *in vivo*, physiological states in which renal acid excretion is increased are associated with significant induction of the renal PEPCK message. It appears that *in vivo* systemic pH changes might be the most potent inducers of the PEPCK message in kidney, although recent evidence suggests that it may also increase in liver in the setting- although with a different time course.

Several published studies on this same enhancer/promoter fragment indicate that it confers cAMP and glucocorticoid sensitivity by virtue of consensus GRE and cAMP-responsive regions located within 200 bp of the transcription start site. Although published studies of this flanking region in liver cells have not clearly demonstrated insulin sensitivity, these same regions confer dietary-sensitivity when expressed in the transgenic mouse [12]. Dietary manipulations which have been reported to induce this gene, such as fasting and high protein diets, are both associated with mild *in vivo* systemic acidosis. Therefore it is possible that many of the effects previously reported in both liver and kidney *in vivo* are a reflection of systemic acid-base alterations. In that sense, the observation that this hormonally-responsive enhancer/promoter fragment conferred pH sensitivity in both liver and kidney cell lines (which are able to express PEPCK mRNA) suggests that intracellular pH sensitivity in the appropriate cell type may be a transcriptional property of this gene region. Further study of genetic elements contained in this region may be germane to *in vivo* mechanisms of systemic pH homeostasis.

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