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Original articles

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PEPCK mRNA LOCALIZATION IN PROXIMAL TUBULE AND GENE REGULATION DURING METABOLIC ACIDOSIS

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To identify the nephron segments expressing PEPCK in control and acidotic conditions, PEPCK mRNA was localized in rat kidney using the technique of reverse transcription and polymerase chain reaction (RT-PCR) in individual microdissected S_1, S_2, and S_3 segments of the rat proximal tubule. In controls, the number of tubules expressing PEPCK mRNA was greatest in the S_3 segment, moderate in the S_2 segment, and least in the S_1 segment of the proximal tubule. After NH_4Cl feeding, strong signals for PEPCK mRNA were detected in all three proximal tubule segments. *In situ* hybridization demonstrated expression of PEPCK mRNA only in the medullary rays in controls. After NH_4Cl, PEPCK mRNA was expressed throughout the cortex, confirming the RT-PCR results. These data demonstrate the ability of the rat kidney cortex to modulate the expression of PEPCK mRNA during metabolic acidosis by recruitment of additional cells in the proximal nephrons. Studies with cultured LLC-PK_1-F^+ cells indicated that increased PEPCK gene transcription at acid pH required a *cis*-acting element (enhancer) in the more distal 5' flanking region of the promoter.

Key words: Phosphoenolpyruvate carboxykinase; pH; RT-PCR; LLC-PK_1-F^+ cells; transfections

INTRODUCTION

The kidney, like the liver, is capable of synthesizing glucose from its precursors including lactate, pyruvate, glycerol and several amino acids. The ability of the kidney to synthesize glucose exceeds that of the liver on a per weight basis, and increases dramatically during several pathophysiologic conditions such as potassium depletion,

acidosis, starvation, and a low carbohydrate diet (see 1). The gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first committed step of gluconeogenesis, *viz.*, the conversion of oxaloacetate to phosphoenolpyruvate.

Metabolic acidosis markedly stimulates renal ammonium synthesis and excretion from glutamine, the principal substrate for ammoniagenesis (2,3). As part of this process, the activities of the enzymes phosphate dependent glutaminase (PDG), glutamate dehydrogenase (GDH), and PEPCK increase dramatically (2,3). In metabolic acidosis, PEPCK acts to stimulate glutamine metabolism by converting α-ketoglutarate, the end product of the deamination and deamidation of glutamine, to glucose. Temporally, PEPCK is the first enzyme with measurable stimulation in activity after a metabolic acidosis induced by NH_4Cl administration. This stimulation is due to increased synthesis of the enzyme (4-6). Curthoys and Lowry were the first to demonstrate that the activity of PEPCK increased only in the proximal tubule after NH_4Cl feeding (7).

In additional experiments, Curthoys and colleagues reported the results of nuclear run-on experiments demonstrating that the acute induction of PEPCK mRNA was due to an increased rate of transcription (8). PEPCK has also been characterized in LLC-PK_1-F^+ cells, a mutant strain of cells derived from pig kidney (9,10). LLC-PK_1-F^+ cells have the enzymes necessary for glucose synthesis and are capable of growing in the absence of glucose in the medium (9). The enzyme is located in both cytoplasm and mitochondria of these cells in a ratio of 2:1, but only cytoplasmic PEPCK activity increases in acidic medium (10). Kaiser and Curthoys (11) demonstrated an increased rate of PEPCK transcription in LLC-PK_1-F^+ cells incubated in acidic medium.

Recently, Schoolwerth *et al.* showed that after NH_4Cl gavage feeding PEPCK mRNA content increased in kidney cortex within 4 hours, with maximal levels of mRNA achieved at 10 hours (12). In addition, using *in situ* hybridization, they demonstrated that PEPCK mRNA was localized to the medullary rays of kidneys from control rats. Ten hours after NH_4Cl administration, the expression of PEPCK mRNA spread throughout the entire cortex. This study was the first to demonstrate a change in distribution pattern of PEPCK mRNA in the kidney cortex. However, because cDNA rather than cRNA probes were used, this *in situ* hybridization study did not provide sufficient resolution to determine precisely which portions of the tubule expressed mRNA. To further reduce background and to enhance sensitivity of the signal, *in situ* hybridization was repeated in the present study using a riboprobe complementary to PEPCK mRNA (13). The present study also utilized the technique of reverse transcription-polymerase chain reaction (RT-PCR) carried out on microdissected tubule segments to provide a more precise topographical localization of PEPCK mRNA along the proximal tubule.

Finally, the present studies evaluated the transcriptional regulation of PEPCK in acidosis. Previous studies have provided conflicting data as to whether sequences in the PEPCK promoter could account for the accelerated rate of transcription observed at acid pH. We tested the hypothesis that an acid response element serves as an enhancer

to accelerate renal PEPCK expression. The activation of PEPCK at acid pH *in vitro*, simulating *in vivo* metabolic acidosis, was evaluated by transfecting 5' flanking sequence elements of the PEPCK gene ligated to a reporter system into cultured LLC-PK_1-F^+ renal cells. Our studies indicate that an acid response element resides in an 1,850 bp fragment in this upstream region of the PEPCK promoter.

MATERIALS AND METHODS

Materials

PCR primers were synthesized by Operon Technologies, Alameda, California. All chemicals and materials were molecular biology grade and were either purchased as, or treated to become, Ribonuclease (RNAse) free.

Experimental animals

Male rats weighing 150-200g were obtained from either Harlan Sprague Dawley, Indianapolis, Indiana, the National Cancer Institute, Frederick, Maryland, or Harlan Sprague Dawley, Zeist, the Netherlands. The animals were housed in the Animal Resource facility of Virginia Commonwealth University or in the Animalium of the Academic Medical Center, Amsterdam. The Animal Care and Use Committee approved all animal protocols. Animals were allowed free access to commercial rat chow and water. Acute metabolic acidosis was induced by a single gavage feeding of 20 mmol NH_4Cl/ kg body weight. Acidotic animals were subsequently sacrificed ten hours after the gavage. Control animals were housed in similar cages but were not manipulated. The ten-hour time point was chosen as Schoolwerth *et al.* had previously demonstrated that this was the peak time of PEPCK mRNA expression after gavage feeding of NH_4Cl (12).

Arterial blood samples were taken from a small group of NH_4Cl-fed and control rats to verify induction of acidosis with this protocol. Following anesthesia with a single intramuscular injection of xylazine and ketamine, the arterial blood samples were obtained from the abdominal aorta. Blood pH and [HCO_3^-] were determined using an automated blood gas analyzer. The pH was 7.22 ± 0.10 and plasma HCO_3^- 17.3±6.2 compared to 7.36 and 26.65, in acidotic rats and control rats, respectively.

In situ Hybridization

In situ hybridization was performed using standard techniques, previously published (13), except that ^{35}S-labeled RNA probes instead of DNA probes were used.

Microdissection of Individual Proximal Tubule Segments

The method of tubule dissection was modified from the technique of Moriyama et al. (14) and has been previously described by Ecelbarger et al. (15). Individual 1-2 mm proximal tubule segments were dissected in the VRC dissection solution on a Wild M8 dissection microscope. Tubular segments were identified using established criteria (16,17) . Any attached blood vessels, tissue, or glomeruli

were carefully removed before transfer of the tubule. A total of 2 mm of tubule(s) were washed and transferred to a PCR tube containing a hypotonic solution of ribonuclease inhibitor and dithiothreitol and frozen on dry ice until reverse transcription. The harvest of tubules was completed within one hour. Two hundred tubule segments were harvested from 16 rats, yielding 73 S_1, 61 S_2, and 66 S_3 segments for RT-PCR analysis.

Reverse Transcription-Polymerase Chain Reaction

The permeabilized tubules were reverse transcribed in a 30 ml reaction volume containing the following reactants (final concentration in mM): 50 Tris-HCl (pH 8.3), 75 KCl, 3.0 $MgCl_2$, 10 DTT, 0.25 each dNTP, plus 0.5 µM Oligo-dT and 200 units of MMLV-RT). A control sample without the addition of reverse transcriptase was also run in parallel with the experimental samples to rule out genomic amplification. The reaction tubes were incubated at 42°C for 60 minutes. The samples were then maintained at 4°C (if PCR was done the same day) or stored frozen at -20°C until PCR analysis.

PEPCK mRNA was amplified in 20 µl of the RT mixture in the same tube using primers specific for PEPCK. The primer sequences used for PEPCK were as follows: sense (1493-1513): 5' GCAGCATGGGGTGTTTGTAGG 3' and antisense (2139-2159): 5'TCCCTAGCCTGTTC-TCTGTGC 3'. The expected PCR product was 667 base pairs in length. The PCR was performed in a final volume of 60 µl and the final concentrations of the reactants were (in mM): 50 KCl, 10 Tris-HCl, 1.5 $MgCl_2$, 2.0 each dNTP, plus 0.5 µM of each PEPCK primer and 1.5 units of *Taq* polymerase. The contents of each reaction tube were amplified in a TempCycler (Coy Labs, Ann Arbor, Michigan) in the following sequence: 3 minutes at 94°C, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds with a final extension of 7 minutes at 72°C. All the tubes were kept at 4°C until analysis by agarose gel electrophoresis. A sample without template (water blank) was performed in parallel with each PCR reaction to monitor for contamination.

To verify transfer and permeabilization of the tubule, and the integrity and reverse transcription of the mRNA population, one-third of the prepared cDNA (10 µl) was amplified with primers specific for β-actin cDNA according to the protocol of Nudel, *et al.* (18). The predicted PCR product was 698 base pairs in length. This „control" reaction was performed first. PCR for PEPCK was performed subsequently only on those samples that were positive for β-actin. Of the total, 95 samples were not found to be positive for β-actin and therefore felt to contain degraded mRNA. These were not further analyzed.

Analysis of PCR Products

For analysis of PCR products, the entire volume was electrophoresed in a 1.5% agarose gel in TBE buffer (45 mM Tris-borate and 1 mM EDTA). The DNA was stained with ethidium bromide and visualized with UV light. Each microdissected, reverse transcribed and amplified tubule segment was scored as positive or negative for either β-actin or PEPCK based on the presence or absence of the predicted PCR product of proper size. The water blank was negative in each reaction indicating the absence of contamination. No attempt was made to quantitate the PCR products.

The identity of the PCR products was confirmed by restriction enzyme digestion analysis. The PCR product for β-actin was cut with PvuII producing two fragments of 235 and 463 base pairs as predicted (data not shown). The PEPCK PCR product was cut with EcoRI producing two fragments of 398 and 269 base pairs as predicted (data not shown).

Cells

LLC-PK_1-F^+ cells were grown in large culture flasks in Dulbecco's Modified Eagle Medium (D-MEM: 5.5 mM glucose; pH 7.4; osmolarity 300 mOsm) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 mg/ml streptomycin. Cells were grown in 5% CO_2 /air-balanced incubator at 37°C for 10-14 days**.** Cells were collected using 0.25% trypsin and 0.02% EDTA in PBS. They were washed twice with serum-free medium, and 1 ml of splitting solution/flask was added. Cells, resuspended in 3-5 ml of medium containing 10% FBS, were placed in a 10ml tube and centrifuged at 500 rpm at 4°C for 10 min. After removal of the supernatant, medium containing serum was added, and this cell suspension was used for transient transfections. Periodically, cells cultured for several days in the absence of antibiotics were tested for mycoplasma contamination using PCR technique (Stratagene instruction manual).

Isolation of DNA fragments of the PEPCK gene

Two fragments from genomic DNA coding for the PEPCK gene were isolated and cloned into the expression vector pCT_1 containing the chloramphenicol acetyltransferase (CAT) gene as a reporter system. These two fragments are subclones of the genomic DNA clones that were described earlier (19). The constructs of pCT_1 were made as follows. A 550 bp genomic DNA fragment was isolated from PAX plasmid by digestion with XbaI and BglII, and was subsequently ligated into pCT_1 between

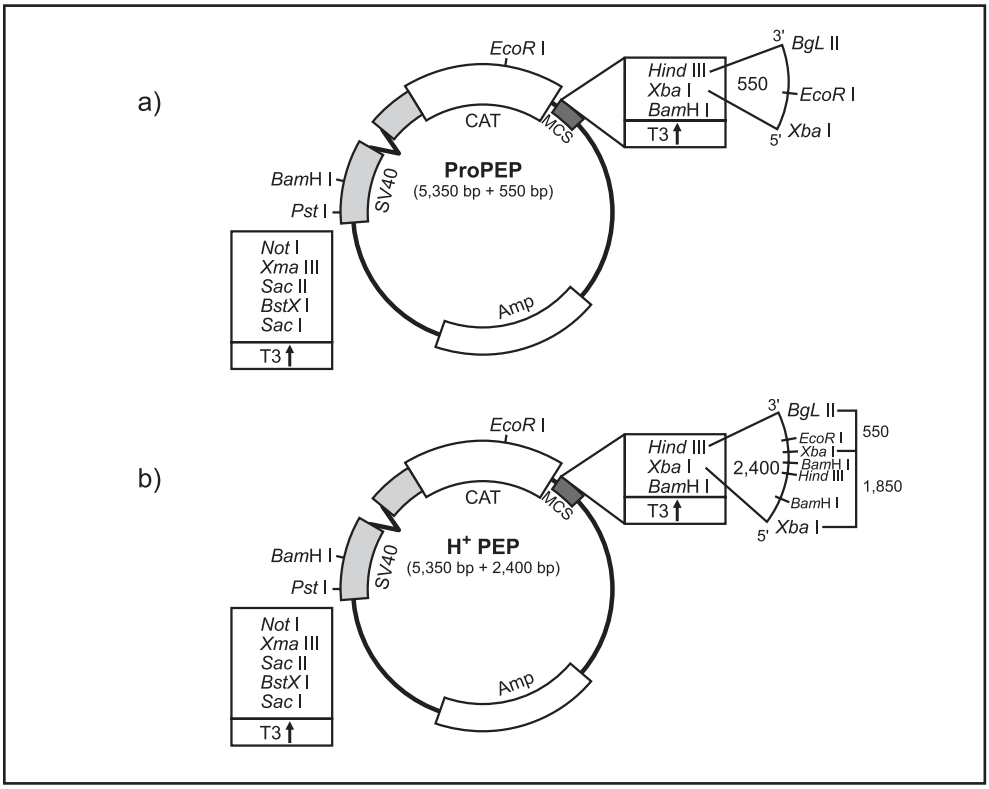

Fig. 1. ProPEP (*Fig. 1a*) and H^+PEP (*Fig. 1b*) constructs

the HindIII and XbaI sites. This 550 bp segment, corresponding to –480 to +72 bp in the PEPCK promoter region, was named ProPEP (*Fig. 1*, top panel). An 1,850 bp genomic DNA fragment was isolated from PAX using XbaI and was subsequently cloned into the „550" with XbaI, to yield a 2,400 bp fragment of the PEPCK gene, named H^+PEP (*Fig. 1*, bottom panel). To confirm that the fragment was ligated in the right orientation, plasmid minipreps were digested with HindIII and KpnI, and placed on an agarose gel. The sequence of the entire 2,400 bp fragment was compared against the Genbank database to confirm that it represented native rat PEPCK genomic sequence (see below).

Sequencing of the promoter and adjacent 5' fragment of the PEPCK gene

The 550 bp and 2,400 bp DNA segments were sequenced using an ABI Prism 377 XL Automated DNA Sequencer (Perkin Elmer/Applied Biosystems, employing FS-DNA sequencing kit/dye terminator chemistry). The GenBank accession number for rat PEPCK is K03243.

Transient transfections of LLC-PK_1-F^+ cells with DNA fragments from the 5' flanking region of the PEPCK gene

Cells were transfected with the following expression vectors using liposomes to deliver the DNA fragment, ProPEP or H^+PEP. 24-48 hrs prior to transfection, confluent cells were split and plated in 6-well tissue culture dishes (1-2 x 10^6 cells/well). For each transfection 5-10 mg of DNA diluted in 100 ml in OPTI-MEM I reduced-serum medium and 10-15 ml of lipofectamine diluted in 100 ml of the above medium were combined and incubated for 15-30 min at room temperature. After incubation, 800 ml of OPTI-MEM I was added to the DNA/liposome complex and 1 ml of this mixture was overlaid onto cells pre-washed with 2 ml of OPTI-MEM I. Cells were incubated for 24 hrs at 37°C in a CO_2 incubator. The next day, the DNA-containing medium was replaced with fresh medium containing 10% FBS; pH was adjusted to 7.40±0.01 or 6.99±0.01 (at 37°C). The cells were harvested 48 hrs later (i.e., 72 hrs after adding DNA/lipid complex). Several experiments were performed with both LLC-PK_1-F^+ cells grown to subconfluence and confluence. We observed no difference under these two conditions; thus the results were pooled. Co-transfections were performed with control vector pCAT3, a positive control vector, to monitor transfection efficiencies in LLC-PK_1-F^+ cells and to normalize CAT expression.

Harvesting/lysing transfected cells

After the third day of culture following transfection, cells were washed with PBS and then exposed to ice-cold PBS plus 2 mM EDTA for 5 min. After centrifugation, cells were lysed with 0.1% Triton X-100. A sample of each lysate (5-10 ml) was taken for determinations of protein concentration (207±14 mg/lysate, n=49). The cell lysates were frozen rapidly by placing each tube at -70°C for 15 min and stored at -20°C for further analysis.

Statistics

The number of β-actin positive tubules expressing PEPCK mRNA in each proximal tubule segment from control and acidotic rats were compared using Chi-square analysis with a $p < 0.05$ indicating a statistical difference. CAT activities from transfected cells incubated at pH 7.4 and 7.0 were analyzed using the Student t-test for unpaired data. Data manipulation and statistical analysis was performed using Microsoft Excel (v. 7.0) software.

RESULTS

Animal studies. In situ hybridization.

Fig. 2 is a representative photograph of *in situ* hybridization using an RNA probe complementary to PEPCK mRNA in control rat kidney and in the kidney of a rat 10 hours after NH_4Cl. The control rats have expression predominantly in the medullary rays and into the outer stripe of the outer medulla, the location of S_3 proximal tubules. After an acid load, expression was clearly seen throughout the cortex. These data confirm the previous *in situ* hybridization performed with standard DNA probes (12) and suggest that PEPCK is expressed in baseline conditions in the S_3 proximal tubule segment. In metabolic acidosis additional cells are recruited from the S_1 and S_2 proximal tubule segments to synthesize glucose via the PEPCK pathway.

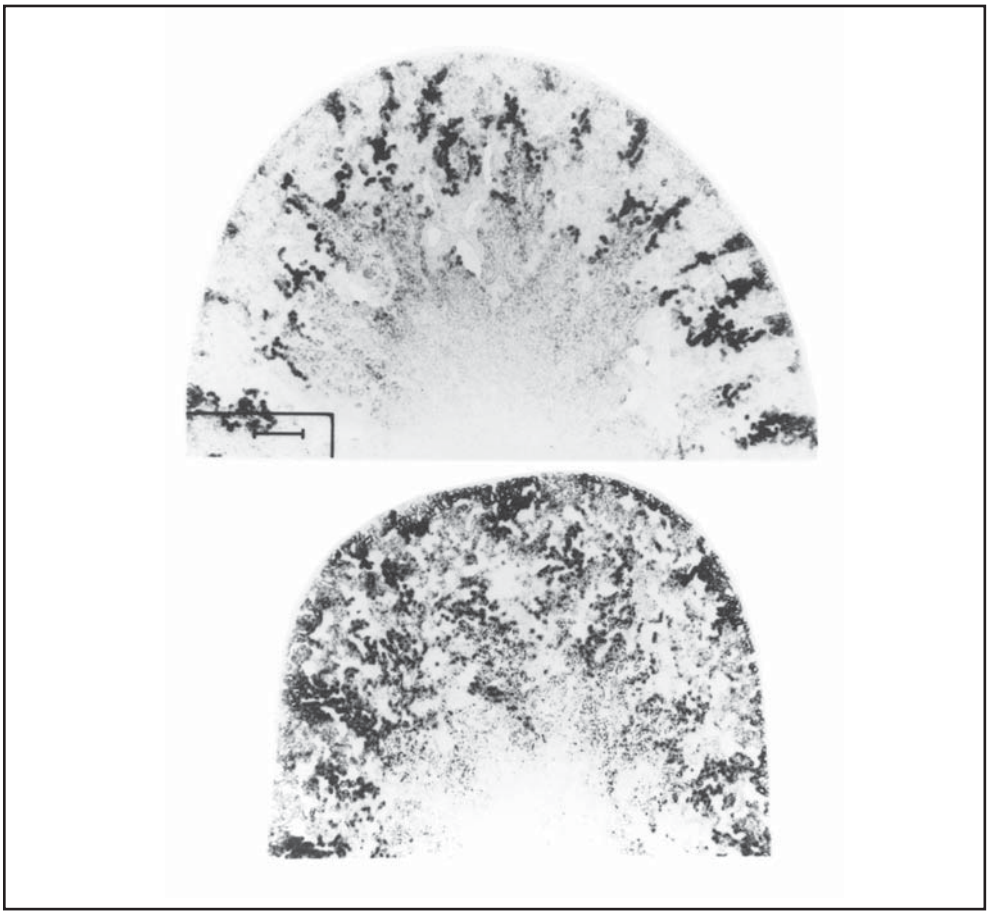

Fig. 2. In situ hybridization using RNA probes. PEPCK mRNA is shown in rat kidney cortex at 0 hrs (top panel) and 10 hrs (bottom panel) after NH_4Cl.

RT-PCR

Expression of β-actin mRNA in individual proximal tubule segments was determined by RT-PCR from control and acidotic rats. We interpreted the absence of β-actin mRNA expression to represent either failure of proper transfer of the tubule or degradation of the mRNA prior to reverse transcription. Therefore, only tubules expressing β-actin were analyzed for expression of PEPCK mRNA.

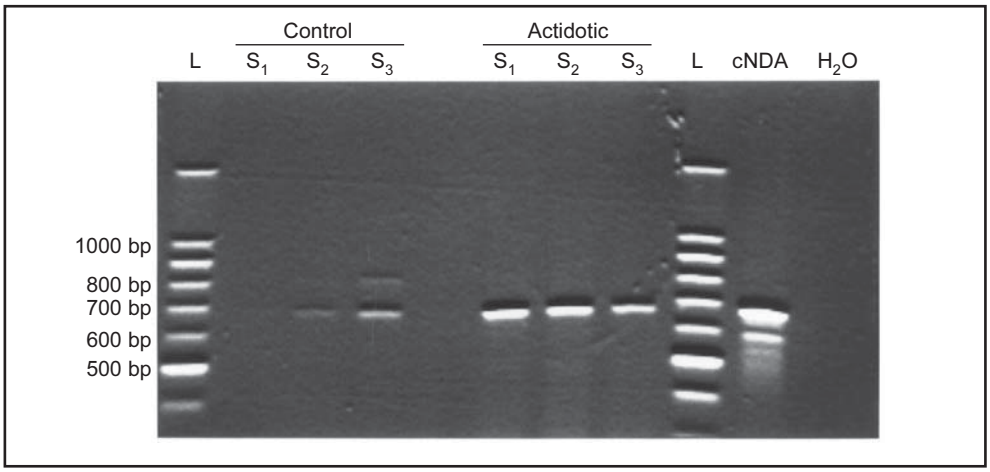

Fig. 3. PEPCK PCR products from one control and one acidotic rat. L, ladder

In *Fig. 3* a representative agarose gel demonstrates expression of PEPCK mRNA from control and acidotic rats in isolated proximal tubule segments. The bands correspond to the predicted size of 667 base pairs and to an amplicon derived from rat PEPCK cDNA. *Fig. 4* shows the number of segments expressing PEPCK mRNA as detected by RT-PCR in the proximal tubule of control and acidotic rats. Under control conditions, PEPCK expression was predominantly in the S_3 segment of the proximal tubule. Although some S_1 and S_2 segments expressed PEPCK mRNA under control conditions, less than half of them did compared to almost three-fourths (74%) of the S_3 segments. Ten hours after gavage feeding with NH_4Cl, a marked increase in expression of PEPCK mRNA in the S_1 and S_2 segments of the proximal tubule was found. In the S_1 segment, PEPCK mRNA was expressed in 65% of the samples from acidotic rats compared to only 30% from control rats. In the S_2 segment, PEPCK mRNA expression went from 43% of the tubules in control rats to 80% in acidotic rats. The difference in PEPCK mRNA expression between control and acidosis in both the S_1 and S_2 segments of the proximal tubule was significant ($p< 0.05$). Expression of PEPCK mRNA in the S_3 proximal tubules under acidotic conditions was 86% compared to 74% in controls, but this difference did not reach statistical significance ($p>0.4$). Tubule samples dissected from control rats sometimes produced

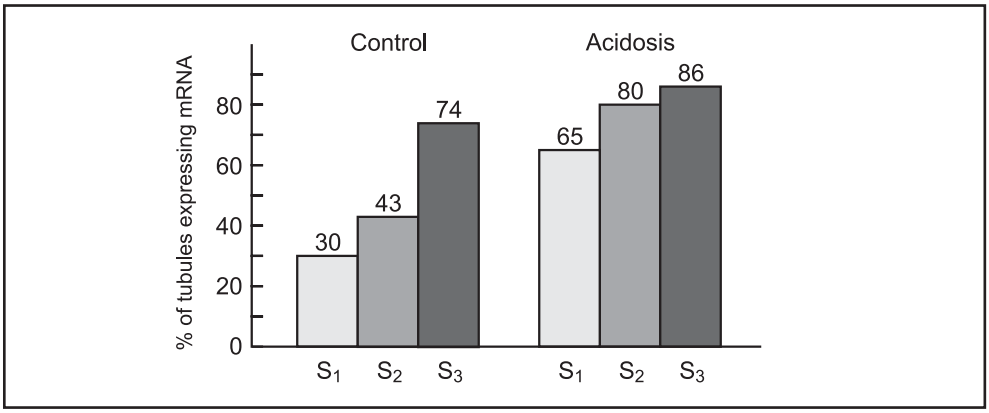

Fig. 4. Summary of PEPCK expression in S_1, S_2, and S_3 tubule segments in control and acidotic rats. The number of segments expressing PEPCK mRNA as detected by RT-PCR in the S_1, S_2, and S_3 segments of proximal tubule in control and acidotic rats, ten hours after administration of 20 mmol/l NH_4Cl/kg. The data were tested from 6-14 control tubule segments and 12-15 segments from acidotic rats. *, $p<0.05$, acidosis vs. control.

a second band (approximately 1000bp), representing amplification of genomic DNA. This second band also was present in the absence of the reverse transcription step, and when samples were pretreated with DNase B this band no longer appeared (studies not shown), thus confirming its identity as genomic DNA. This band was not present in tubules dissected from acidotic rats. We speculate that significant genomic amplification occurs only when the PEPCK mRNA in the tubule is not abundant.

Nucleotide sequences of the promoter and adjacent 5' DNA fragment of the PEPCK gene

The nucleotide sequence of ProPEP and H^+PEP were determined for two reasons. One, GenBank data were available only for the minimal PEPCK promoter and adjacent 5' flanking sequences to –1,000 bp. We wished to determine the sequence of the entire 2,400 bp construct. Second, it was important to show that the sequences of H^+PEP were adjacent to and contiguous with ProPEP. The 550 bp and 2,400 bp DNA segment sequences are shown on *Fig. 5*; the 2,400 bp DNA fragment (H^+PEP) contains the minimal PEPCK promoter (ProPEP) and immediately adjacent 5' DNA sequences (nucleotides –2330 to +72 relative to the transcription start site). Based on GenBank comparison, this segment shows 99.9% nucleotide sequence homology with the rat PEPCK gene sequences, including the minimal promoter region and the 1,000 bp fragment immediately upstream from this minimal promoter region (20). A detailed restriction map of the 2,400 bp clone is also virtually identical with that reported earlier (19), and confirms the location of sites identified in both constructs used in this study (data not shown).

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5' 13     8      5
TCTAGAAATC CTTGACTAAT ATAACAGTAT AATTTTGAAC CTGTTTCTGA GTGCCTGGTC TTCCTCTCTT

CCTCTCCAAA GCGGCTTCAC ATTCATAAAC AACTGTTTAC AGGCTTGACA GAAAGCCTCC CTGACCTAAG
                   9
GGAACATGAG CCCAGTAGCC CCTGAGAGTC TAGGGGGTCT CCCTATGTCT GGGTGAGAAA CCATGTGGGA
           9                                 11
AACTCCCACT CGGTGGGAAT CCCTGGCCCT CTGCTTGCCC TGTACGAGCG CTGAACATCA CACAAGGTGG

GCTGGGAGCT CTTTGGGGAG TCCTAAGAGG GCAGCTGGCA ATGGACACCT AGCAGTCCCT TTGAGACTTA
                                 10
TTTCAGATGG AGCTGTAGAA AGATGCCATG GCTCACAGTG CCTCCCTGGG AAGGGGGCAG AGGGCTGCCC

AGTGAGGCCT CTTGCGAGCA GGAAATCACC AGAGACAAGG AAAGACCAGA CCCCAGGATG ACCTCAGTTA

GGCCTTGCCC GACTGTCCTC AGAGTCCCAT TCTCTGTGTC CTGGTTCTTT TAGAAGATCA TGGACCTCCA

GGTCATTTCG TAACCGGAAT CTGCCTTGCG GGGGGTTTTG ACAAGCTATG GTATAGTGTA TGTGGGGGTA

CTGACGAATT GGAAGATCAT GGAGACCCCT TCTCCTCCTC CATCATTGGT CTGCCACATC CCTCCCAGGA

GACTCACAGC AGAGAGACCT TGGATGTATG TAGGGTGCTT TAAAACTCCA GCTGAGTTAC AGTCTCTCCT
                                 10
TTCTGTTTTC ACCTTAACCT TCCAGGGATG CAAACCCACG ACAGGTTTAG CAGCAGAGTG GAGGCTGGCC

ATGAATCTCA GAGAAAGTGC TCACTGGAAA GGCTGGTTTA GCCCAGGCCT GATGTGGAGG CACTGAGCTG
                  10
GACGTTCTAG CGGGGTTGAC ACCCAACAGT TTACATAGGG GGAGGCCACC CCTCCTGAGC AGTCTCGGTG

ACTTGAAGAG GAAGCCGCTT CTTCTGTACC AACACAGAAG CTCCAGCGAA CCCCCAGAAT GCTGGCAGTG
                                 4
TGGGTGCTAT GTAAAAGTAT TTACATAGCT TTGTAGAGTG AGCCAAGCCC AGTCTGTTTG GGATGACTCT

TCACAGTGCC TCGAATCTGT CACACGTCTT AGTAAGCAGA GTCACAGAGT TTCTGTCACA TCATCCTCCT
                        10
GCCTACAGGG AAGTAGGCCA TGTCCCTGCC CCCTACTCTG AGCCCAGCTG TGGGAGCCAG CCCTGCCCAA

TGGGCTCTCT CTGATTGACT TCTCACTCAC TTCTAAACTC CAGTGAGCAA CTTCTCTCGG CTCGTTCAAT

TGGCGTGAAG GTCTGTGTCT TGCAGAGAAG GTTCTTCACA ACTGGGATAA AGGTCTCGCT GCTCAAGTGT

AGCCCAGTAG AACTGCCAAG CCCCTTCCCC TCCTCTCCCT AGACTCTTGG ATGCAAGAAG AATCCAGGCA

GCTCCAAGGG TGATTGTGTC CAACCTAGAA TGTCTTGAAA AAGACATTAA GGGGACTAGA GAAGACAGGG

GATCCAACGG TTCTCTGCAG CCCAGCCTGA CTGACATGTA ACTCTTCTGG TTCTCACCAG CCAGCTGGAC

CTGCTTAGTA TTCTTTCTGC CTCAGTTTCC CAGCCTGTAC CCAGGGCTGT CATAGTTCCA TTTCAGGCAG

TAGTAATGAA TGAGCTGACA TAAAACATTT AGAGCAGGGG TCAGTATGTA TATAGAGTGA TTATTCTATA
                 7                10
TCAGGCATTG CCTCCTCGGA ATGAAGCTT ACAATCACCCC TCCCTCTGCA GTTCATCTTG GGGTGGCCAG
                                12
AGGATCCAGC AGACACCTAG TGGGGTAACA CACCCCAGCC AACTCGGCTG TTGCAGACTT TGTCTAGAAG

TTTCACGTCT CAGAGCTGAA TTCCCTTCTC ATGACCTTTG GCCGTGGGAG TGACACCTCA CAGCTGTGGT

GTTTTGACAA CCAGCAGCCA CTGGCACACA AAATGTGCAG CCAGCAGCAT ATGAAGTCCA AGAGGCGTCC
  10
CGGCCAGCCC TGTCCTTGAC CCCCACCTGA CAATTAAGGC AAGAGCCTAT AGTTTGCATC AGCAACAGTC
                   3
ACGGTCAAAG TTTAGTCAAT CAAACGTTGT GTAAGGACTC AACTATGGCT GACACGGGGG CCTGAGGCCT
                   7
CCCAACATTC ATTAACAACA GCAAGTTCAA TCATTATCTC CCCAAAGTTT ATTGTGTTAG GTCAGTTCCA
                     6
AACCGTGCTG ACCATGGCTA TGATCCAAAG GCCGGCCCCT TACGTCAGAG GCGAGCCTCC AGGTCCAGCT
                   2                1
GAGGGGCAGG GCTGTCCTCC CTTCTGTATA CTATTTAAAG CGAGGAGGGC TAGCTACCAA GCACGGTTGG
                                 3'
CCTTCCCTCT GGGAACACAC CCTTGGCCAA CAGGGGAAAT CCGGCGAGAC GCTCTGAGAT
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Fig. 5. Sequence of PEPCK ProPEP and H^+PEP DNA fragment sequences. The numbers above the residues enclosed in boxes refer to the following: 1, Start site (+1); 2, “TATA box” 1; 3, “CAAT box”; 4, GCRE; 5, “TATA box” 2; 6, CREB-PEPCK; 7, NF-E_1; 8, AP-1; 9, NF-kB; 10, AP-2; 11, Sp1; 12, end of 550 bp ProPEP fragment (Xba I); 13, 5’ end of 2,400 bp H^+PEP fragment (Xba I). The sequence of ProPEP is identical to the first 550 bp of the 2,400 H^+PEP fragment.

PEPCK gene expression in LLC-PK$_1$-F$^+$ cells; effect of low pH

CAT activities expressed in LLC-PK$_1$- F$^+$ cells transfected with ProPEP and H$^+$PEP were, as expected, 1% and 3%, respectively, of the expression level of CAT observed in cells transfected with pCAT3-control (data not shown). Transfection of the ProPEP into LLC-PK$_1$-F$^+$ cells resulted in an expression level that was 2-times higher than the background level determined in cells transfected with pCT$_2$ at both pH values (data not shown). However, incubation of the transfected cells in acidic medium (pH 7.0) for 48 hrs did not enhance expression of ProPEP *(Fig. 6)*. As the minimal PEPCK promoter alone was insufficient to mediate the response to acidic pH, LLC-PK$_1$-F$^+$ cells were transfected with the 2,400 bp DNA fragment (H$^+$PEP, including the 550 bp minimal PEPCK promoter and adjacent upstream 1,850 bp fragment) in the pCT$_1$ vector. Expression activity of this H$^+$PEP-containing plasmid was also evaluated relative to pCT$_1$ containing only ProPEP. In medium at pH 7.0, CAT activity in extracts from H$^+$PEP transfected cells was found to be 2.8-fold higher ($p<0.007$) than at pH 7.4 and 4-fold higher than that demonstrated for ProPEP-transfected into cells at medium pH 7.0 ($p<0.001$). These findings suggest that a pH response element is contained in H$^+$PEP. This was confirmed by results obtained in additional experiments that evaluated and were normalized for transfection efficiency. Normalization of CAT activities determined in lysates obtained from LLC-PK$_1$-F$^+$ cells co-transfected with ProPEP and pSV-bGAL revealed no effect of decreased medium pH on the level of PEPCK expression. However, in acidic medium, PEPCK gene expression was increased 2.7-fold in cells to which H$^+$PEP was introduced together with pSV-bGAL, compared to the response measured at pH 7.4. A more pronounced effect of low medium pH on the expression level induced by H$^+$PEP was apparent when compared to ProPEP-generated CAT activities in the co-transfected cells at pH 7.0; PEPCK expression was 9.6-fold higher. Comparison of normalized CAT activity levels in

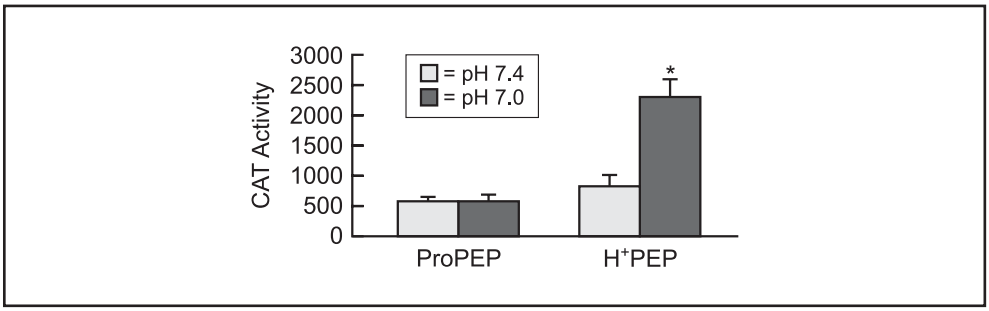

Fig. 6. Comparison of CAT activities expressed in LLC-PK$_1$-F$^+$ cells transfected with plasmid DNA. Data are presented as cpm/100 mg protein. The data represent the mean ± SE of 15, 12, 11, and 6 transfection experiments with ProPEP (pH 7.4 and pH 7.0) and H$^+$PEP (pH 7.4 and pH 7.0), respectively. ProPEP, pCT$_1$ containing the 550 bp minimal promoter sequence; H$^+$PEP, pCT$_1$ containing the PEPCK 550 bp minimal promoter and 1,850 bp immediately upstream (5') from the minimal promoter. *, significantly different from H$^+$PEP at pH 7.4, $p=0.007$.

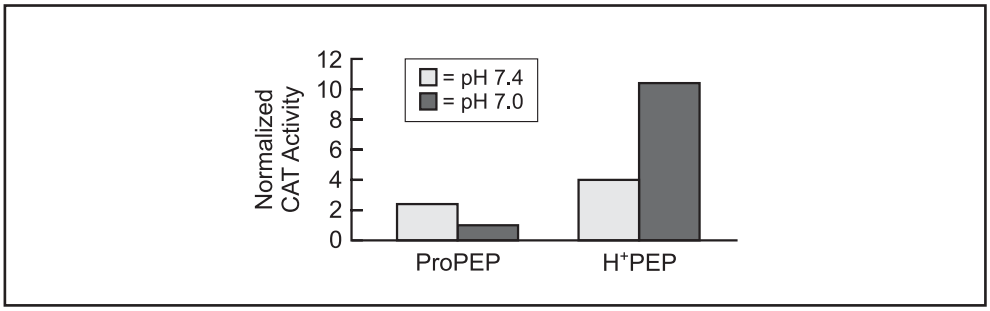

Fig. 7. Normalized CAT activities expressed in LLC-PK1-F+ cells co-transfected with pSV-βGAL. Data were normalized by dividing CAT activity (cpm/100 mg protein) by β-galactosidase activity (OD/100 mg protein) in 2-3 experiments. The ratio of normalized CAT activity with H^+PEP at pH 7.0 was 2.7-times that at pH 7.4.

cells cotransfected with pCAT3-control and pSV-bGAL at two pH values confirmed that transfection efficiency was unchanged by medium H^+ content.

DISCUSSION

The observations presented in this study further extend information on the renal localization of PEPCK mRNA expression in the proximal tubule of the rat under control conditions and acute metabolic acidosis. Under physiological conditions, PEPCK mRNA expression along the nephron was found to be statistically dependent on segment location and was confined predominantly to the S_3 segment of the proximal tubule of the rat nephron. PEPCK mRNA could be detected in some S_1 segments of β-actin positive proximal tubules, but the number of S_1 segments expressing PEPCK mRNA was found to be significantly less than expected when compared to S_2 and S_3. The conclusion that under normal physiological conditions PEPCK mRNA expression is localized to the S_3 portion is consistent with *in situ* hybridization studies presented here, and with our previous data, demonstrating that PEPCK mRNA is confined primarily to the medullary rays of control kidneys (12).

Ten hours following gavage feeding of NH_4Cl, a significant increase in the number of S_1 and S_2 segments expressing PEPCK mRNA was detected but no significant change in the number of S_3 tubules expressing PEPCK mRNA was observed. The number of S_1 and S_2 proximal tubules expressing PEPCK mRNA in response to acidosis was almost twice that of control levels. Schoolwerth *et al.* demonstrated that PEPCK mRNA message reached a maximum distribution throughout the entire cortex of the rat kidney ten hours after NH_4Cl administration (12). The present data together with the previous results suggest that the increase in expression of PEPCK mRNA in the S_1 and S_2 segments of the proximal tubule is maximal after ten hours of acidosis (12).

These results indicate that under physiological conditions, the S_3 segment of the

rat proximal tubule maintains a basal level of expression of PEPCK mRNA greater than that of the S_1 and S_2 segments. The S_1 and S_2 segments of the proximal tubule may contribute some, if only very little, of the PEPCK mRNA for enzyme synthesis. However, in response to an acid insult, cells of the S_1 and S_2 proximal rat nephron, not previously expressing PEPCK mRNA, are recruited to express PEPCK mRNA. These findings provide evidence of a proximal tubule mechanism that is finely controlled and sensitive to perturbations in acid-base balance. These findings suggest that if pH is the governing principle in PEPCK expression in the kidney, under normal conditions the intracellular pH in the S_3 segment is lower than in S_1 and S_2. However, to our knowledge there are no data to indicate a difference in intracellular pH between these nephron segments. While the transcription rate of renal PEPCK is known to be accelerated by glucocorticoids and cAMP, we are unaware of data indicating altered levels of these substances between renal proximal tubule segments (21,5,22) .

The present data also coincide with previous data demonstrating that PEPCK enzyme activity is localized to the rat proximal tubule and increases significantly in the cortex of acidotic rats when compared to control (23,24) . This increase was described as greatest in the proximal convoluted tubule and less in the proximal straight tubule, with no increase in the last portion of the proximal straight tubule. These findings correlate well with those of the current study when "proximal convoluted tubule", "proximal straight tubule" and "late proximal straight tubule" are extrapolated to the S_1, S_2 and S_3 portions of the proximal tubule, respectively. Such an extrapolation may be tenuous as data by these investigators was contributed a decade or more before standardization of kidney ultrastructure (16). Although *in situ* hybridization may not provide sufficient resolution to determine precisely which portions of the tubule are expressing the mRNA, we have obtained similar findings using different PEPCK probes. In contrast, the technique of RT-PCR in microdissected tubules, in which confirmation of the identity of the tubules was determined by light microscopic appearance and location within the tissue slice, is precise in defining the localization of mRNA expression.

The significant increase in PEPCK mRNA expression in S_1 and S_2 proximal tubules suggests that cells not previously expressing PEPCK mRNA, and subsequently not producing PEPCK enzyme protein, were induced to express PEPCK mRNA in response to metabolic acidosis. Other investigators have demonstrated an increase in PEPCK mRNA along a similarly acute time line and with relatively similar increases in expression. Pollock demonstrated a three-fold increase in renal PEPCK mRNA by quantitative Northern analysis four hours after NH_4Cl (10 mmol/kg) feeding (25). Iynedjian and Hanson utilized a cell-free wheat germ translational assay to determine that PEPCK mRNA increased three-fold six hours after gavage feeding of 10 mmol/kg NH_4Cl (6). Cimbala *et al.* revealed by Northern blotting with poly $(A)^+$ RNA that NH_4Cl administration resulted in a four-fold increase in PEPCK mRNA within six hours (20). Hwang and Curthoys employed nuclear run-on experiments to demonstrate

that the mRNA for cytosolic PEPCK increased six-fold relative to β-actin approximately eight hours after feeding of 20 mmol/kg NH_4Cl, thus demonstrating that the increase in PEPCK mRNA is due to synthesis of new mRNA rather than increased stability of mRNA (8). As mentioned, Schoolwerth *et al.* showed a 16-fold increase in renal cortex PEPCK mRNA by dot-blot analysis ten hours following NH_4Cl (20 mmol/kg) (12). These data plus the data of the present investigation indicate that the large increase in PEPCK mRNA detected in the kidney cortex is due to an increased amount of PEPCK mRNA transcribed by cells of the S_1 and S_2 proximal nephron. Thus, some sort of signaling pathway probably exists for cells not previously expressing PEPCK to begin transcribing PEPCK mRNA. The nature of the signal that mediates this increase is not known and remains to be elucidated. We hypothesize that the situation is analogous to pericentral and periportal enzyme gradients in hepatocytes (26). As with those hepatic enzymes that increase in cellular concentration from the pericentral toward the periportal regions under certain physiological conditions, renal PEPCK gradients likely exist with an increase in enzyme content proceeding from S_3 toward S_1 and S_2 nephron segments in acidosis. Christoffels *et al.* proposed that inducing factors are synthesized by the upstream cells (26). With regard to renal PEPCK, this would posit that unidentified inducing factors (possibly transcription factors) are synthesized in S_1 and/or S_2 segments "upstream" from S_3 cells.

Intrinsic drawbacks of the reverse transcription and amplification systems exist that restrict the methods from being reliably quantitative. These drawbacks include, but are not limited to, degradation of mRNA template during the reverse transcription step, incomplete reverse transcription of the mRNA population, probably related to accessibility of mRNA in the tubules, and inconsistencies in the amplification of the cDNA produced. A variable number of enzyme inhibitors can also be present, and can vary from tube to tube even among replicate samples. However, the amount of product obtained from a given sample following reverse transcription and a given number of PCR cycles can be assumed to reflect the presence of starting material (15) even though it cannot provide a precise quantitation of this amount. Because of these drawbacks, each examination of individual dissected tubules in this report was scored on a dichotomous positive/negative basis, describing whether or not a band could be visualized on an agarose gel after 35 cycles, which should be sufficient amplification for PEPCK mRNA species present in meaningful concentrations in the tubule segments. The use of ethidium bromide in an agarose gel as a detection method has also been described as being limited to 5 nanograms or more of DNA. For a PCR reaction of 35 cycles, this represents only a few copies of starting material. Thus, we can be reasonably sure that the samples were truly positive or negative for PEPCK mRNA expression. The use of the β-actin as a control eliminated many false negative samples where failure to visualize a band was due to factors other than the presence or absence of PEPCK mRNA in the dissected tubule segments.

The *in situ* hybridization data demonstrate that, in response to NH_4Cl-induced metabolic acidosis, expression of PEPCK expands from the medullary rays throughout the cortex, confirming our previous finding (12). Regional differences in optical density within a section represent equivalent differences in local mRNA concentration under the conditions used (27). The microdissection and *in situ* hybridization data therefore complement each other: The microdissection data are precise with respect to the tubular segments that are analyzed, but suffer from a number of inherent weaknesses with respect to mRNA quantitation (see above). The *in situ* hybridization data are precise with respect to topography and mRNA quantification but, due to the protease and detergent treatments, do not leave sufficient morphological details to identify individual tubule segments for correlation with the number of silver grains.

Our results also provide information to indicate that the sequences in the proximal 550 bp of the PEPCK promoter region are insufficient to explain the accelerated rate of gene transcription at acid pH. Rather, the data support the hypothesis that a *cis*-acting element in the more distal 5' flanking region of the PEPCK promoter serves as an enhancer. The relevant sequences are contained in an 1,850 bp segment in this flanking region.

Fig. 8 depicts the results of sequence analysis of the PEPCK –2400 promoter using the TRANSFAC database of transcription factor consensus binding sites. Those represented in cartoon format represent those sequences identified with high degree of stringent homology. While the presence of these putative binding sites does not indicate that these nuclear factors participate in the functional regulation of the PEPCK promoter in either basal or acid-responsive conditions, it suggests potential cis- and trans-interacting factors that may be responsible for regulation of the PEPCK gene. It is of interest that binding sites for several early-acting nuclear proteins, such as CEBP, NF-kB, NF-E2, AP-1, AP-3, AP-4 and the serum response factor are seen. Additionally, several consensus sites for interferon, steroid and glucocorticoid-responsive elements, which are known to interact with AP-1, are also present in the 2,400bp construct.

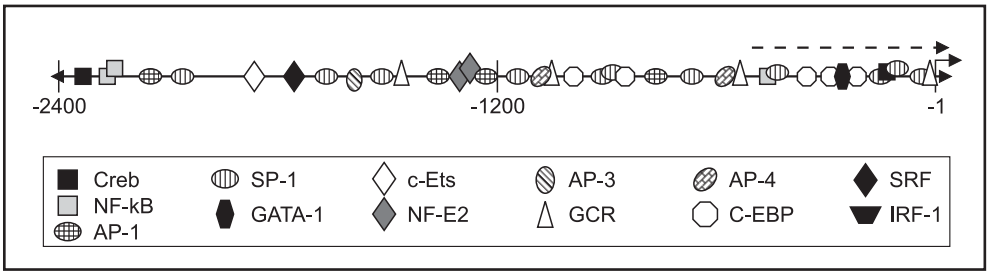

Fig. 8. Cartoon representation of the PEPCK 5' flanking sequence. Highly homologous putative consensus transcription factor binding sequences are demonstrated by symbols identified in the legend box. Angled arrow indicates transcription initiation site, and dashed arrow delineates the-550 minimal promoter construct described in the text. Abbreviations: Creb, cAMP response element binding protein; NF-kB, nuclear factor kappaB; GCR, glucocorticoid receptor; SRF, serum response factor; IRF-1, interferon response factor.

Demonstration of nuclear protein binding to these sequences awaits DNAse I footprinting and electrophoretic mobility shift assays. Confirmation of functional relevance of the cis- and trans interacting factors thus identified will require functional analysis in reporter gene assays in which these putative binding sites are specifically mutated.

Divergent results have been published on the site of transcriptional regulation of renal PEPCK. Holcomb, *et al* (28) showed reproducible but low initial levels of CAT activity in LLC-PK_1-F^+ cells transfected with PEPCK promoter (-460 to +73 bp); CAT activity was elevated 3.5-fold after incubation in acidic medium for 24 hours. More recently, these workers have not been able to duplicate these findings (personal communication). Cassuto, *et al.* (29) utilized several different cells, including parental LLC-PK_1 cells, PK22 cells, and LLC-PK_1-F^+ cells. They reported a 2.5-fold increase in CAT activity in PK22 cells transfected with a PEPCK promoter construct (597 bp, from the 5' flanking sequences proximal to the coding sequences) at pH 6.9 compared to pH 7.4. They did not report results obtained in LLC-PK_1-F^+ cells. We do not know if the additional 45 bp of their construct (our ProPEP construct contains nucleotides –480 to +72, or 552 bp) contain information which could resolve the different results from those we obtained.

More recently, Curthoys and Gstraunthaler (30) have postulated that the induction of PEPCK occurs via activation of the p38 stress-activated protein kinase and subsequent phosphorylation of transcription factor ATF-2. They hypothesize that ATF-2 binds to cAMP-response element 1 within the PEPCK promoter and may increase PEPCK transcription during metabolic acidosis (31).

In summary, we have demonstrated that metabolic acidosis induced by NH_4Cl results in recruitment of cells from the S_1 and S_2 proximal tubule to express PEPCK mRNA. A mechanism apparently exists for some signal of acidosis to be sensed by these cells to initiate synthesis of PEPCK mRNA. The identity and mechanism of this signal to turn on transcription in response to acidosis remain to be elucidated. We hypothesize that inducing factors are synthesized in S_1 and S_2 nephron segments in response to a reduction in cell pH.

The data obtained in this study also indicate that a *cis*-acting element likely is pivotal in the enhanced rate of PEPCK gene transcription that occurs at acid pH. They provide a base for future investigations on the molecular mechanisms responsible for basal and inducible tissue-specific PEPCK gene regulation *in vitro* and *in* vivo. The future identification of functionally relevant *cis*-acting sequences will provide information necessary to define the *trans*-acting factors that contribute to the pH-inducible regulation of the PEPCK gene.

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