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RT-PCR Localization of Phosphoenolpyruvate Carboxykinase (PEPCK) mRNA in Rat Proximal Tubule Segments During Ammonium Chloride Acidosis

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RT-PCR localization of phosphoenolpyruvate carboxykinase (PEPCK) mRNA in rat proximal tubule segments during ammonium chloride acidosis,

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RT-PCR localization of phosphoenolpyruvate carboxykinase (PEPCK) mRNA in rat proximal tubule segments during ammonium chloride acidosis

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at the Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

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Dedication

I would like to dedicate this thesis to Lady MacBeth, who has provided unconditional canine love and support despite my long absences between walks while attending graduate school and medical school. Her enthusiastic, vocal greetings, no matter what the hour and no matter what my mood was, provided a remarkable release from the many difficulties that are graduate studies.

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Abstract

RT-PCR LOCALIZATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) mRNA IN RAT PROXIMAL TUBULE SEGMENTS DURING AMMONIUM CHLORIDE ACIDOSIS

By Matthew R. Craig, Bachelor of Arts, University of Virginia, 1992

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at the Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, December 1998

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In metabolic acidosis, the early increase in PEPCK mRNA and enzyme protein content contributes to the accelerated rates of ammonium and glucose formation. *In situ* hybridization demonstrated that expression of PEPCK was confined to medullary rays of rat kidney cortex in controls and spread throughout the cortex 10 hours following NH_4Cl feeding (*Am.J.Physiol.*, 267: F400, 1994). To identify the specific nephron segments expressing PEPCK in control and acidotic conditions, the mRNA for PEPCK along the nephron of the rat kidney was localized 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. Two-millimeter segments of tubule were permeabilized, the mRNA reverse transcribed using oligo-dT as a downstream primer and the cDNA

product amplified by PCR (35 cycles). Primers specific for β-actin were used to confirm transfer of tubule, and only tubules positive for β-actin were amplified with primers specific for PEPCK. Both primers were designed to span at least one intron to avoid amplification of genomic DNA. The PCR products were detected using agarose gel electrophoresis and ethidium bromide staining. Verification of PCR product was performed by restriction enzyme digestion.

Under control conditions, the number of tubules expressing PEPCK mRNA as detected by RT-PCR was greatest in the S_3 segment, moderate in the S_2 segment, and least in the S_1 segment of the proximal tubule. Ten hours after gavage feeding of 20-mmol/kg bodyweight NH_4Cl, strong signals for PEPCK were detected in all three proximal tubule segments. These data demonstrate the ability of the rat kidney cortex to modulate the expression of PEPCK mRNA along the proximal tubule under physiological conditions, and to increase expression of PEPCK mRNA during metabolic acidosis by the recruitment of additional cells in the proximal nephron.

Introduction

The kidney is the only organ other than the liver capable of significant glucose synthesis. The formation of glucose from non-carbohydrate precursors is known as gluconeogenesis. These non-carbohydrate precursors include lactate, pyruvate, glycerol and some amino acids.[23] The gluconeogenic amino acids glutamine and alanine are quantitatively the most important gluconeogenic amino acids *in vivo*.[14] Apart from the liver, the kidney is the only organ which readily synthesizes glucose from lactate, pyruvate and amino acids.[47] Elucidation of the gluconeogenic nature of the kidney cortex was first described by Benoy and Elliott in 1937[10] and was corroborated by H. A. Krebs, *et al* in 1963.[48]

The ability of the kidney to synthesize glucose exceeds that of the liver on a per weight basis[46], and increases dramatically during metabolic perturbations such as potassium depletion,[26] acidosis,[2,41,52] starvation[8,33] and a low carbohydrate diet.[48] During normal homeostasis, the kidney can provide up to twenty percent of the total body glucose;[23] and under certain conditions the contribution by the kidney to total body glucose can increase to as much as forty-five to fifty percent.[42,75] The ultimate end product of renal gluconeogenesis is thought to be glucose instead of glycogen, as very little glycogen is stored by the kidney.[48]

The gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first committed step of gluconeogenesis, and is described as the rate-limiting enzyme of gluconeogenesis.[33, 79] PEPCK, first described in 1954 by Utter and Kurahashi, catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (PEP) [Equation 1].[80] The high-energy intermediate PEP can then form either glucose through gluconeogenesis, or be transformed into pyruvate for oxidation in the mitochondria. The latter pathway most likely occurs when glucose availability is high and free fatty acid levels are low.[17]

Oxaloacetate + GTP $\Leftrightarrow$ Phosphoenolpyruvate + GDP + CO_2
Equation 1

The enhancement of renal gluconeogenesis was shown to occur between oxaloacetate and phosphoenolpyruvate by measuring intermediates of gluconeogenesis during metabolic acidosis in the rat.[1] G.A.O Alleyne concluded in 1968 that the activity of PEPCK increased in the kidneys of acidotic rats, and served to remove α-ketoglutarate, a by-product of glutamine and glutamate breakdown. The conversion of PEP to glucose from the metabolic precursor α-ketoglutarate is considered the principal end product of renal α-ketoglutarate metabolism, as the oxygen uptake by the kidney is much less than anticipated if complete oxidation of PEP via pyruvate were to occur.[26] α-Ketoglutarate is produced by the kidney from the metabolism of glutamine when glutamine is deaminated and then deamidated.[76] The ammonium produced is excreted by the kidney as ammonium ions, resulting in the excretion of excess protons. This process is known as ammoniagenesis, and increases during metabolic acidosis. It was once

thought that PEPCK acted to control ammoniagenesis by modulating the level of α-ketoglutarate via oxaloacetate, but this theory has since been abandoned. Ammoniagenesis has been shown to occur in the absence of gluconeogenesis, using a specific inhibitor of PEPCK.[9,11,18] PEPCK, therefore, is not regulatory for ammonium formation by the kidney, but influences glutamine metabolism by affecting oxaloacetate levels. Renal gluconeogenesis is not rate-limiting for the production of ammonium by the kidney, and ammonium production occurs before any increase in renal glucose production.[65] The increase in renal gluconeogenesis has been suggested to be a consequence rather than a cause of glutamine hydrolysis, and serves as a means to dispose of the carbon skeleton of glutamine.[76] Renal gluconeogenesis is therefore considered to be a "salvage reaction" for the carbon skeleton of glutamine.[47]

The kidney acts to maintain acid-base homeostasis by filtering and reabsorbing bicarbonate. When bicarbonate is low, the kidney synthesizes “new” bicarbonate by metabolizing glutamine with the concomitant production of ammonium, which is excreted in the urine. During acidosis, plasma bicarbonate concentration is low, and glutamine utilization by the kidney is increased.[77] Glutamine serves as a "sink" for the disposal of excess hydrogen ions, providing both a source of urinary ammonium and a carbon source for subsequent metabolism of the carboxylate anions. The NH_4^+ produced is excreted in the urine to avoid the bicarbonate consuming process of urea formation by the liver. The carboxylate anions produced during the metabolism of glutamine are converted to the neutral end products glucose or CO_2, with concomitant production of

bicarbonate.[30] The net effect of these mechanisms serves to increase plasma [HCO_3^-] and restore blood pH.

PEPCK has been demonstrated to be present with high specific activity in liver, kidney cortex, and white adipose tissue, with lower activities in lung, jejunum and brain.[32] The intracellular location of PEPCK exhibits a wide variation among species. PEPCK is present in both a mitochondrial and cytosolic form, but differs in its subcellular distribution. In almost every species studied, with the exception of the chicken, the subcellular distribution is consistent throughout all tissues.[34] In the hamster, rat and mouse, hepatic PEPCK enzyme activity exists mainly in the cytosol (90% cytosolic/10% mitochondrial).[31] In human, guinea pig, sheep and cow, subcellular location is almost equivalent (~40% cytosolic/~60% mitochondrial), and in rabbit and pigeon, the enzyme is almost entirely mitochondrial (~5% cytosolic/~95% mitochondrial).[31,32] In the chicken, hepatic PEPCK is predominantly mitochondrial (~5% cytosolic/~95% mitochondrial). Renal PEPCK abundance in the chicken is also mostly mitochondrial, but to a lesser degree than that of the liver (~20% cytosolic/~80% mitochondrial).[81,20]

Hepatic PEPCK enzymes from the cytosol and mitochondria of the rat have been demonstrated to exist as immunologically distinct enzymes, in that the antibody to one enzyme does not cross react with the counterpart enzyme.[34] However, the PEPCK enzymes present in the cytosol of rat adipocytes and in the cytosol of rat hepatocytes have been established to be immunologically identical.[6] Additionally, using the antibody for cytosolic rat liver PEPCK, cytosolic rat renal PEPCK was found to be

immunologically indistinguishable.[53] The differences in the subcellular localization of the enzyme and its response to metabolic perturbations in different organisms have led to the generalization that the mitochondrial enzyme is constitutively expressed, while the cytosolic form is inducible and is regulated according to the metabolic needs of the tissue.[32]

The regulation of PEPCK varies with modulator and tissue type. The activity of PEPCK can be altered *in vivo* by administration of glucagon, norepinephrine, ACTH glucocorticoids, thyroxine and insulin. The response to these mediators is tissue-specific, as glucocorticoids increase the amount of the enzyme in the kidney, and decrease the amount of the enzyme in adipose tissue.[24] Glucocorticoid administration and starvation both lead to an increase in the activity of renal PEPCK.[33,66] Insulin increases the activity of the enzyme in the liver, but has no effect on the renal enzyme.[79] Rats fed high protein diets demonstrated higher renal PEPCK activity than rats fed low protein diets, and this increase was attenuated by sodium bicarbonate administration.[73] PEPCK in the liver and kidney of the rat also differ in their response to metabolic acidosis. Following NH_4Cl feeding, renal PEPCK increased while hepatic PEPCK was unchanged,[4,74] or even decreased.[40] The stimulation of renal PEPCK after NH_4Cl feeding occurs in thyroidectomized, parathyroidectomized, hypophysectomized and adrenalectomized animals.[37] Additionally, adrenalectomy does not effect the renal increase of PEPCK due to fasting.[24,27]

The cytosolic PEPCK enzyme is induced in rat renal proximal tubule cells in response to metabolic acidosis.[16,37] Under similar conditions, however, the mitochondrial

enzyme is constitutively expressed.[51,75] Increased synthesis of PEPCK enzyme during metabolic acidosis is the primary factor determining the increased renal level of the enzyme.[63] During starvation, which results in metabolic acidosis, the high rate of PEPCK synthesis was reduced after sodium bicarbonate feeding.[37]

In the isolated perfused kidney, Bowman demonstrated that more glucose was produced from lactate at a perfusate pH of 7.23 than from lactate at a perfusate pH of 7.45.[12] Glucose production from gluconeogenic precursors, pyruvate, glutamine and other amino acids, was greater at an acidic perfusate pH than at a basic perfusate pH. These data demonstrate the dependence of perfusate pH on renal gluconeogenesis by the rat tubule, and that glucose production by the kidney is increased by an acidic tubular pH. Renal gluconeogenesis itself is exclusive to the cortex, and is limited to the proximal tubule of the nephron.[16,28] Using microdissected nephrons, PEPCK enzyme activity is highest in the proximal tubule.[28,29]

In a study by Schoolwerth, *et al*, *in situ* hybridization was used to determine that PEPCK mRNA was localized to the medullary rays of control kidneys. Ten hours after gavage feeding of 20 mmol NH_4Cl/kg bodyweight, the expression of PEPCK mRNA spread throughout the entire cortex. Using a dot blot assay, Schoolwerth and colleagues demonstrated that PEPCK mRNA expression peaked 16-fold after ammonium chloride ingestion, and returned to control levels after thirty hours. This study by Schoolwerth, *et al* was the first to demonstrate a change in the distribution pattern of PEPCK mRNA in the kidney cortex.[74] Using the techniques of microdissection, reverse transcription and the polymerase chain reaction, the present study will attempt to provide an enhanced

illustration of the localization of PEPCK mRNA expression along the rat proximal tubule.

Polymerase Chain Reaction

The polymerase chain reaction, or PCR, is a powerful *in vitro* technique for the enzymatic amplification of a region of DNA between two portions of a known DNA sequence. The PCR process was conceived by Kary Mullis during a moonlit drive up the coast of California[58] and first appeared in print in December of 1985.[69] In 1989, PCR was heralded by *Science* as a "major scientific development," and Taq DNA polymerase was selected as the molecule of the year.[44] Dr. Mullis received the Nobel Prize for Chemistry in 1993.

A complete PCR reaction contains all the necessary components for DNA amplification: the DNA target template of interest, a molar excess of each adenine, cytosine, guanine and thymine deoxynucleotide base, a quantitatively large amount of the specific oligonucleotide primers, and a thermostable DNA polymerase. Most commonly, the thermostable enzyme *Thermus aquaticus*, or *Taq* DNA polymerase, is used for amplification, although other DNA polymerases are available. Because *Taq* DNA polymerase is generally insensitive to the 95°C denaturation step, it does not need to be replenished with each cycle.[68]

Taq DNA polymerase is stable at 95°C for up to 40 minutes and has an optimal extension rate at a reaction temperature of 70-80°C, which makes it an ideal enzyme for PCR amplification. *Taq* can add mononucleotides onto the end of a growing DNA chain at the rate of 50 to 60 nucleotides per second. Additionally, it has an inherent 5'→3'

exonuclease activity, which removes nucleotides from the template strand ahead of the growing strand. These combined factors make *Taq* DNA polymerase a very versatile and effective enzyme for use in PCR.[61]

The PCR process is initiated when the double-stranded DNA target is first denatured or "unzipped" by heating to 90-95°C. The specific oligonucleotide primers hybridize to the complementary regions of the DNA target sequence after cooling to 55-60°C. The primer-dependent DNA polymerase then catalyzes the elongation of the oligonucleotide primer at 72° C, using the complementary DNA strand as a template and the free deoxynucleotides as building blocks. The single-stranded primers are generally 20 to 30 nucleotides in length, and flank a region of DNA anywhere from 100 to 5000 or more bases in length. The amplification reaction proceeds through many cycles of template denaturation, oligonucleotide primer annealing and extension by the DNA polymerase. At the end of each cycle, a new double-stranded DNA molecule exists, which can serve as a target for the next round of amplification. The amount of DNA target template has theoretically doubled, and accumulates exponentially with each cycle. Twenty cycles of PCR can yield a million-fold amplification of target DNA within two hours (10^{20} = 1,048,576).[22]

The first round of denaturation and extension of the DNA template results in double stranded DNA of indeterminate length, but contains the DNA region of interest. The DNA polymerase will continue to add bases onto the growing strands until it falls off or reaches the end of the template. The second cycle of amplification also leads to products of various lengths. By the third cycle, however, the amplification products,

which are defined by the distance between the two primers, begin to accumulate, and are amplified exponentially. The longer products are also produced, but are only amplified linearly.

The simple beauty of PCR is that it allows for the amplification and detection of a small amount of nucleic acids. This small amount of nucleic acids could be due to a low number of target copies within a large population of nucleic acid molecules, or a small amount of cellular starting material.[59] With the addition of reverse transcription, single stranded cDNA copies of cellular mRNA can be amplified. Reverse transcription PCR (RT-PCR) provides extraordinary sensitivity in detecting rare copies of nucleic acid sequences in large samples or in samples with very small amounts of starting material. This latter trait is exploited in the current study, as microdissected rat kidney tubules were examined for β-actin and PEPCK mRNA. To attempt to preserve as much cellular mRNA as possible, the tubule cells were permeabilized and the mRNA examined directly, without extraction or purification. This avoided having to extract mRNA from very small amounts of tissue, where product loss would be most probable. This technique of microdissection, isolation and direct lysis followed by RT-PCR was introduced by Moriyama *et al* in 1990 and has been used successfully for many renal tubular and vascular studies.[21,45,57,78]

Rationale for Thesis

The kidney synthesizes glucose and increases glucose production during metabolic acidosis. This gluconeogenic increase occurs in kidney cortex and is concomitant with an increase in PEPCK mRNA throughout the entire cortex. *In situ* hybridization data by Schoolwerth, *et al* suggest that PEPCK mRNA is localized to the S_3 segment of proximal tubule under control conditions,[74] but are insufficient to identify the precise segment in which PEPCK mRNA is expressed. The same study by Schoolwerth, *et al* described PEPCK mRNA expression as having expanded to include the entire cortex 10 hours following NH_4Cl administration, which suggests that all segments of the proximal tubule are expressing PEPCK mRNA. This proposition needs further refinement by microdissection to augment its validity. The present studies were therefore performed to resolve the localization of PEPCK mRNA in the rat proximal tubule during control and acidotic conditions. Rats were studied ten hours after gavage feeding of 20 mmol NH_4Cl/kg bodyweight; this causes acute metabolic acidosis and results in a 16-fold increase in PEPCK mRNA in the rat kidney cortex.[74] The S_1, S_2 and S_3 segments of the rat proximal tubule were isolated and examined for PEPCK mRNA expression during acidosis and physiological control conditions. Using RT-PCR, microdissected rat nephron segments were analyzed for the presence or absence of β-actin mRNA, the gene for a constitutive structural protein. A positive result with β-actin should confirm proper

transfer and permeabilization of tubule, in addition to successful reverse transcription of the mRNA population present in the sample. Only β-actin positive tubules were analyzed for the presence or absence of PEPCK mRNA by RT-PCR

Under control conditions it was predicted that PEPCK mRNA expression would be highest in the S_3 segment of the proximal tubule, as suggested by the *in situ* hybridization data of Schoolwerth, *et al.*[74] Following metabolic acidosis, PEPCK mRNA expression was present throughout the cortex, and it was anticipated to be detected by RT-PCR in all segments of the proximal nephron. These studies attempt to demonstrate the localization in the proximal tubule of PEPCK mRNA under normal conditions and following an acid insult. By examining the PEPCK mRNA expression in sub-segments of the proximal tubule, these studies endeavor to substantiate that during acute metabolic acidosis there is a recruitment of proximal tubule cells which were not previously expressing PEPCK mRNA.

Materials

Male Sprague Dawley rats were procured either from Harlan Sprague Dawley, Indianapolis, Indiana or from the National Cancer Institute, Bethesda, Maryland. Xylazine, Ketamine and Sodium Pentobarbital were obtained from the Animal Resource Facility of the Medical College of Virginia, Richmond, Virginia. Vanadyl Ribonucleoside Inhibitor (VRC) and Dithiothreotol (DTT) were obtained from Gibco BRL, Gaithersburg, Maryland. Collagenase, Pronase, Bovine Serum Albumin (BSA), glycerol, agarose (Standard and Low Melting point) and mineral oil were acquired from Sigma Chemical Company, St. Louis, Missouri. Hyaluronidase was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Triton X-100 was obtained from Boehringer-Mannheim, Indianapolis, Indiana. Wild M8 stereomicroscope was manufactured by Wild Heerbrugg, Ltd., Heerbrugg, Switzerland. Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT), Recombinant Ribonucleoside Inhibitor (RNasin), *Thermus aquaticus* DNA Polymerase (*Taq* DNA Polymerase), Oligo-$(dT)_{15}$, 100bp DNA ladder and deoxynucleotide triphosphates {dATP, dCTP, dGTP, and dTTP (dNTPs)} were obtained from Promega, Madison, Wisconsin. PCR primers were synthesized by Operon Technologies, Alameda, California. Xylene cyanol FF and bromophenol blue were obtained from Bio-Rad, Hercules, California. QIAquick Gel Extraction kit was obtained from Qiagen, Inc. Chatsworth, California. Restriction

enzymes were obtained from New England Biolabs, Beverly, Massachusetts. PCR Thermocycler, TempCycler®, supplied by Coy Labs, Ann Arbor, Michigan. The DNA analysis computer software used was the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin. All chemicals and materials were molecular biology grade and were either purchased as, or treated to become, RNase free.

Methods

Animals

Male Sprague Dawley rats, 150-200g, were housed in the Animal Resource facility of the Medical College of Virginia, Virginia Commonwealth University after obtaining them from the vendor. Control and acidotic animals were allowed free access to commercial rat food and water. Acute metabolic acidosis was induced by a single gavage feeding of 20 mmol NH_4Cl/kg bodyweight.[74] Acidotic animals were subsequently sacrificed ten hours following feeding NH_4Cl. Control animals resided in similar cages without manipulation. The peak of PEPCK mRNA expression was previously demonstrated by Schoolwerth, *et al* to occur ten hours subsequent to gavage feeding of 20 mmol NH_4Cl/kg bodyweight.[74] This outcome provided the ten hour focal point for the present investigation.

Blood analysis

To investigate the extent of acidosis ten hours after ammonium chloride feeding, arterial blood samples (0.3mL) were taken from a small group of ammonium chloride fed and control rats. Ten hours after ammonium chloride feeding, rats were anesthetized according to bodyweight with an intramuscular injection of xylazine and ketamine. The abdominal aorta was exposed and approximately 0.3mL of arterial blood was collected in

a heparinized, air-tight syringe. The syringe was sealed with a cork and kept on ice until analysis. Blood pH and [HCO_3^-] was determined using an automated blood gas analyzer.

Preparation of rat kidney cortex

The method of tubule dissection and preparation was similar to previous reports, with some modification.[21] Rats were anesthetized according to bodyweight with an intramuscular injection of xylazine and ketamine, followed by an intraperitoneal injection of sodium pentobarbital. After the animal was tranquilized, an abdominal incision was made exposing the internal organs. The employed method of tissue preparation was adapted from previous reports.[21,57] The aorta was ligated above the bifurcation of the aorta and additionally between the left and right renal arteries. The aorta was cannulated with polyethylene tubing (PE-90) below the left kidney and secured with 3-0 suture silk. The left kidney was selectively perfused in vivo initially with ice-cold perfusion solution (1ml/10g bodyweight), followed by a second perfusion of 1ml/10g bodyweight of the same solution containing collagenase (1mg/mL), hyaluronidase (1000U/mL) and bovine serum albumin (1mg/mL) warmed to 37°C. The pH 7.4 perfusion solution contained 135mM NaCl, 5.0mM KCl, 1.0mM Na_2HPO_4, 3.0mM sodium acetate, 1.2mM Na_2SO_4, 5.5mM glucose, 2.5 $CaCl_2$, 1.2mM $MgSO_4$ and 5.0mM Hepes, and was suffused with 100% oxygen.

After perfusion, the kidney was removed, decapsulated and demedullated. Portions of the cortex and outer stripe of the outer medulla were cut into 1-2mm cubes. These pieces were then transferred into separate 25mL Erlenmeyer flasks containing 8-12mL of the 37°C buffered enzyme solution. The pieces were incubated for 20 minutes

at 37° C in a shaking water bath. The digestion solution was bubbled with 100% oxygen prior to perfusion and during incubation. Following incubation, the tissue was washed once with enzyme-free perfusion solution before being placed in an ice-cold perfusion medium containing 10mM Vanadyl Ribonucleoside Complex (VRC), an effective RNase inhibitor, and kept on ice until dissection. To remove any particulate matter, the VRC solution was refined by low speed centrifugation followed by filtration through a 0.22 μm syringe filter.

Dissection of tubules

Individual 1-2mm proximal tubular segments were isolated using polished, sterile No. 5 Dumont forceps and a Wild M8 dissecting microscope in a dissecting dish cooled to 4°C with a Peltier cooling element. The dissecting dish was directly illuminated from below. Tubular segments were identified using established criteria[25,45,49,71,82] and measured with a calibrated ocular micrometer. S_1 and S_2 segments of proximal tubule were obtained from the cortical pieces and the S_3 segments were obtained from the medullary rays of the outer stripe of the outer medulla. Any attached blood vessels or glomeruli were carefully removed before transfer of tubule. One or two nephron segments for a total length of 2mm were transferred with a Drummond pipette to a separate dish containing 10mL VRC-free dissection medium. After rinsing the tubule(s) in the VRC-free medium, the segment(s) were transferred with a second Drummond pipette along with 2μl of dissection solution to a thin-wall RT-PCR microcentrifuge tube containing 8μl of lysis buffer (2.5% Triton X-100, 1 U/ml RNase inhibitor, 5mM DTT).

The samples were kept frozen on dry ice until reverse transcription. The harvest of tubules was completed one hour after the end of the digestion of the rat kidney cortex.

First strand cDNA synthesis (Reverse transcription [RT])

To produce a stable cDNA copy of the mRNA present, each sample was heated for 10 minutes at 65°C to reduce mRNA secondary structure, quick-chilled on ice and centrifuged at 12,000 rpm to collect any condensate. An Eppendorf benchtop microcentrifuge was used for this and all subsequent microtube centrifugations. Treatment of the samples with DNase to remove genomic DNA proved to be detrimental to the survival of the mRNA and was not performed. The permeabilized tubules were reverse transcribed using of Moloney Murine Leukemia virus Reverse Transcriptase (MMLV-RT) and Oligo-dT as a downstream primer. Each total RNA sample was incubated with a molar excess of oligo-dT primer at 65°C for ten minutes before transcription to improve the efficiency of the MMLV-RT. Heat denaturing total RNA in the presence of RT primer prior to reverse transcription decreases secondary structure of the mRNA and enhances specific primer annealing.[13] The final concentration of reactants was 0.67% Triton X-100, 50mM Tris-HCl (pH 8.3), 75mM KCl, 3.0mM $MgCl_2$, 10mM DTT, 0.25mM each of dNTP's, 0.5μM Oligo-dT and contained 200 units of MMLV-RT. The total volume of the reaction mixture was 30μl. Controls without addition of reverse transcriptase were performed to rule out any possible genomic DNA amplification. A water blank was reverse transcribed with each reaction to rule out any incidental mRNA contamination. The reaction tubes were incubated for 10 minutes at 23°C, 60 minutes at 42°C and then 10 minutes at 95°C to stop the reaction by inactivating

the enzyme. The samples were maintained at 4°C or stored frozen at -20°C until PCR analysis.

Polymerase Chain Reaction (PCR)

To confirm transfer and permeabilization of tubule, and reverse transcription of the mRNA population, one-third of the prepared cDNA (10μl) was amplified with primers specific for β-actin cDNA. β-actin is a constitutive structural protein whose expression is unaffected by ammonium chloride feeding.[35,36,56] β-actin has been previously demonstrated to be present in all nephron segments, and has served as a positive control in other published RT-PCR reports.[78] The β-actin primer sequence was acquired from a previous report.[45] The primer pair spanned at least one intron and the predominant PCR product was predicted to be 698 base pairs in length. β-actin primer sequences and base definitions are listed in Table 1.

β-actin cDNA amplification was performed in a 25 μl final volume in 600 μl Gene-Amp PCR tubes. The amplification protocol was optimized for magnesium and dimethyl sulfoxide (DMSO) concentration by the method of Ausubel, *et al.*[5] The final optimized concentration of reactants was 50mM KCl, 10mM Tris-HCl, 0.1% Triton X-100, 2.0mM $MgCl_2$, 2.0mM each of dNTPs, 0.9% DMSO, 0.5μM each of β-actin specific primers and contained 0.625 units of *Thermus aquaticus* (*Taq*) DNA polymerase. For each reaction, a "master mix" was made containing all of the PCR ingredients except for the cDNA sample, as advocated in the literature.[50] The contents of each reaction tube were overlaid with one drop of mineral oil to prevent the evaporation of reactants. A water blank (no cDNA added) was utilized to detect any nonspecific contamination

present in the reagents or pipettes. The tubes were placed in an automated thermocycler and incubated for 3 minutes at 94° before cycling 35 times through a reaction profile of 94° for 30 seconds (template denaturation), 58° for 30 seconds (primer annealing) and 72° for 45 seconds (primer extension). The tubes were then incubated for 7 minutes at 72° for a final extension and chilled at 4° C until analysis by agarose gel electrophoresis.

To amplify PEPCK, the remaining reverse transcribed tubule cDNA (20μl) was amplified in the same tube as the reverse transcription reaction using primers specific for PEPCK. Only those tubule segments shown to contain β-actin cDNA were amplified with primers specific for PEPCK. The primers for PEPCK were previously designed in the laboratory. The primer pair spanned at least one intron and the predominant PCR product was predicted to be 667 base pairs in length. PEPCK primer sequences and base definitions are listed in Table 1. The amplification protocol was optimized for magnesium and dimethyl sulfoxide (DMSO) concentration by the method of Ausubel, *et al.*[4] The PCR was performed in a final volume of 60 μl and was optimized to contain 50mM KCl, 10mM Tris-HCl, 0.1% Triton X-100, 1.5mM $MgCl_2$, 2.0mM each of dNTPs, 0.5μM each of PEPCK specific primers and contained 1.5 units of *Taq* polymerase. For each reaction, a "master mix" was made containing all of the PCR ingredients except for the cDNA sample. The contents of each reaction tube were overlaid with one drop of mineral oil to prevent the evaporation of reactants. A water blank (no cDNA added) was utilized to detect any nonspecific contamination present in the reagents or pipettes. The PEPCK PCR amplification profile was identical to the β-actin PCR time and temperature profile.

Oligonucleotides for amplification

The details of the oligonucleotide primers used for β-actin and PEPCK PCR amplification are given in Table 1. The sequence of the β-actin primers was chosen from a previous report,[45] and the PEPCK primer pair sequence was previously determined in the laboratory. All primers were synthesized by Operon Technologies, Alameda California. To confirm the specificity of the primers, each sequence was compared to 240,449 known, published sequences in the GenEMBL data bank and were validated for the absence of any coincidental homology to other sequences.

Agarose Gel Electrophoresis

For analysis of PCR products, 5 μl of Gel loading buffer Type III[70] (0.25% xylene cyanol FF, 0.25% bromophenol blue, 30% glycerol) was added to the reaction mixture, and the entire volume fractionated in a 1.5% agarose gel prepared with 45mM Tris-borate and 1mM EDTA at 20 Vhr/cm. A DNA molecular weight standard was electrophoresed in the outermost lanes of the gel to resolve the size of the DNA product. The DNA was visualized by ethidium bromide staining using a variable intensity ultraviolet illuminator and photographed with a Polaroid camera. Ethidium bromide is a fluorescent dye that intercalates between stacked bases of DNA, and causes the DNA to fluoresce when exposed to ultraviolet light of 265 nanometer wavelength.[39] 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 visible on an agarose gel.

PCR product enzyme digestion

To confirm that the predominant PCR amplification products were actually the proper products, the PCR results were analyzed by restriction digestion analysis to demonstrate the precision of the PCR primers. The sequence of the predicted PCR amplification products for β-actin and PEPCK was created using the sequence editor function of the Wisconsin Package (v9.0) by the Genetics Computer Group (GCG). The expected PCR products were analyzed for the presence or absence of cleavage sites for restriction enzymes available in the laboratory using the restriction site-mapping program of GCG. The expected PCR products were also analyzed with the restriction enzyme site map-sorting program of GCG, to ascertain the predicted size of the cleavage products. Enzymes were selected to cut or not cut the expected PCR product into pieces easily identifiable on an agarose gel. Total mRNA extracted from rat kidney cortex (previously extracted and available in the laboratory) was reverse transcribed and amplified by PCR according to the conditions described earlier in this report. The PCR product was then electrophoresed on a low melting point agarose gel. A DNA molecular weight standard was electrophoresed in the outermost lanes of the gel to resolve the size of the PCR product. The DNA was visualized by ethidium bromide staining using a variable intensity ultraviolet illuminator, and the DNA band of expected size was removed from the gel using a sterile scalpel. The excised band was separated from the agarose using a QIAquick Gel Extraction kit according to the manufacturer's instructions. The purified DNA product was then incubated with a restriction enzyme and buffer supplied by the enzyme manufacturer. DNA, enzyme and enzyme buffer were incubated together

according to the manufacturer's directions. After digestion, 5μl of Gel loading buffer Type III was added to the reaction mixture, and the entire volume fractionated in a 2% agarose gel prepared with 45mM Tris-borate and 1mM EDTA and electrophoresed at 20 Vhr/cm to analyze the digestion products. A DNA molecular weight standard was electrophoresed in the gel to resolve the size of the DNA product. The DNA was visualized by ethidium bromide staining using a variable intensity ultraviolet illuminator and photographed with a Polaroid camera.

Statistics

The number of observed β-actin positive tubules expressing or not expressing PEPCK mRNA in each identified proximal tubule segment from control and acidotic kidneys was compared using Chi-square analysis. The null hypothesis that the variables of a contingency table are independent of each other was rejected at the alpha level of $P < 0.05$. When the null hypothesis is rejected ($P < 0.05$), then both variables are considered to be statistically dependent, and an experimental influence between the two variables can be contemplated. When the null hypothesis is not rejected, then both conditions can be considered independent of each other, and there is no indication of a relationship between the two categories. Both variables are then described as being statistically independent, and that one variable does not influence the other to any appreciable degree.

Data manipulation and statistical analysis was performed using Microsoft Excel (v7.0). No attempt was made to quantitate the RT-PCR products, as the amount of starting material (total RNA) varied from tubule to tubule and was neither extracted nor quantitated, and the amount of mRNA degradation prior to reverse transcription was not

accounted for by an exogenous cRNA standard. Each examination of an individually dissected tubule was evaluated on a dichotomous positive/negative basis, depending on whether an ethidium bromide stained band of DNA was visible on an agarose gel.

Results

Blood gas values from control and acidotic rats

Blood gas data from control and acidotic Sprague Dawley rats are shown in Table 2. Arterial blood was taken from a small sample of control and acidotic rats to determine acid-base status. Blood was drawn into a heparinized syringe from the abdominal aorta and analyzed in an automatic clinical blood gas analyzer. The rats used for blood gas analysis were not used for tubule dissection, and vice versa. The blood gas data for control and acidotic rats published in three reports is also shown in Table 2 for comparison, along with the method and duration used to establish acidotic conditions. The blood gas data for the present studies correlates well with the previously published data, and demonstrates that a significant acidosis was induced by gavage feeding of 20 mmol/kg NH_4Cl. No statistical analysis was performed with the present blood gas data, as the sample size is too small to make any statistical inferences

PEPCK mRNA expression in tubules of control rats

The number of segments expressing PEPCK mRNA as detected by RT-PCR in the proximal tubule of control rats is shown in Table 3. In these experiments, 2 millimeter segments of the S_1, S_2 and S_3 proximal tubule from control rats were identified, isolated and collected. The tubule cells were permeabilized, and the mRNA population reverse transcribed using the enzyme MMLV and oligo-dT as a downstream

primer. A freeze-thaw procedure in the presence of an RNase inhibitor, as used in this report, has been shown to produce a proper RT-PCR product with as little as four cells, and was quite applicable for the successful reverse transcription of the tubular cell mRNA population.[43] Transfer and successful reverse transcription to produce cDNA was confirmed first by obtaining a 698 base pair PCR amplification product visible on an ethidium bromide stained agarose gel using primers specific for β-actin. When the PEPCK or β-actin amplifications were carried out in the absence of target cDNA (water blank), no products were visible on the gel.

In control rats, the number of tubules expressing PEPCK mRNA in S_1, S_2 and S_3 segments of the rat proximal tubule was found to be statistically dependent by Chi-square contingency table analysis ($P < 0.05$). All statistical calculations for tubules from control rats can be found in Table 5. The null hypothesis that the expression of PEPCK mRNA is independent of proximal tubular location was rejected with a χ^2 test statistic of 7.73 and two degrees of freedom. The calculations in Table 5 (part A) indicate that there is a dependence on tubular location for PEPCK mRNA expression under control conditions. This statistical dependence suggested that there is a strong indication for a relationship between sub-segment location and PEPCK mRNA expression under physiological conditions.

To expose the relationship between tubule sub-segment location and expression of PEPCK mRNA during control conditions, the number of tubules expressing PEPCK mRNA in each sub-segment was weighed against the number of tubules expressing PEPCK mRNA in each of the other two proximal tubule sub-segments. These

calculations for control tubules are shown in Table 5 (parts B, C and D). These calculations demonstrate that the expression of PEPCK mRNA is more likely to occur in the S_3 segment of the proximal tubule under control conditions, and less likely to occur in the S_1 segment of the proximal tubule under control conditions. The S_3 segment expresses PEPCK mRNA more readily than S_1 or S_2 segments under control conditions, and the S_1 segment tends to express PEPCK mRNA less readily than S_2 or S_3 proximal tubule segments ($P < 0.05$). A statistical significance between the number of S_2 tubules expressing PEPCK mRNA and S_1 and S_3 tubules expressing PEPCK mRNA cannot be demonstrated, and therefore does not show any indication of a relationship ($P > 0.5$).

PEPCK mRNA expression in tubules of acidotic rats

The number of β-actin positive segments expressing PEPCK mRNA as detected by RT-PCR in the proximal tubule of rats ten hours after gavage feeding of 20 mmol/kg bodyweight is shown in Table 4. Acidotic proximal tubule isolation, collection, permeabilization and RT-PCR analysis was performed in the same manner as control proximal tubules.

PEPCK mRNA expression across β-actin positive tubules collected from acidotic rats was not found to be dependent on tubular location by Chi-square analysis ($P > 0.3$). All statistical calculations for tubules from acidotic rats can be found in Table 6. The null hypothesis that the expression of PEPCK mRNA is independent of proximal tubular location was accepted with a χ^2 test statistic of 2.23 and two degrees of freedom. The calculations in Table 6 (part A) indicate that there is no dependence on tubular location for PEPCK mRNA expression under acidotic conditions ($P > 0.3$). These data reveal that

PEPCK mRNA expression appears balanced along the acidotic nephron. Parts B, C and D of Table 6 illustrate that when sub-segments were compared individually, no relationship can be elucidated. These data demonstrate that during metabolic acidosis, expression of PEPCK mRNA is independent of tubular location, and that there is no indication of a relationship between proximal tubular location and PEPCK mRNA expression during metabolic acidosis.

PEPCK mRNA expression in proximal tubules of acidotic versus control rats

The number of tubules expressing PEPCK mRNA in acidotic S_1 proximal tubules was significantly greater than those expressing PEPCK mRNA in the S_1 segments of control rats ($P < 0.05$). Statistical calculations for PEPCK mRNA expression in tubules of acidotic versus control rats can be found in Table 7. These calculations demonstrate that the increase in the number of S_1 tubules expressing PEPCK mRNA is dependent on acid-base status, and increases significantly during metabolic acidosis ($P < 0.05$). These calculations also demonstrate that the increase in the number of S_2 tubules expressing PEPCK mRNA is dependent on acid-base status, and increases significantly during metabolic acidosis ($P < 0.05$). The data do not indicate any relationship between the number of control and acidotic S_3 tubules expressing PEPCK mRNA. The number of tubules in acidotic S_3 proximal tubules was not significantly greater or lesser than those expressing PEPCK mRNA in the S_3 segments of control rats ($P > 0.4$). Taken together, these data demonstrate that following ten hours of ammonium chloride induced acidosis, there is a significant increase in the number of S_1 and S_2 tubules expressing PEPCK mRNA.

PCR product analysis

In order to verify that the primers specific for β-actin had amplified the proper DNA sequence, the anticipated β-actin PCR product sequence was analyzed for restriction enzyme cleavage sites of restriction enzymes present in the laboratory using the computer site-mapping program of GCG. The results from the GCG restriction enzyme site analysis of the β-actin PCR product are shown in Figures 1 and 2. The PCR amplification product was incubated with each identified restriction enzyme according to the manufacturer's instructions and the digest fractionated on an ethidium bromide stained agarose gel. The results from the restriction enzyme digestion analysis of the β-actin PCR product are shown in Figure 3. The enzyme PvuII cut at one site, producing the predicted DNA fragments of 235 and 463 base pairs in length. FokI had six cleavage sites and was predicted to produce seven DNA fragments. However, incomplete cleavage of some and/or preferential cleavage of other certain sites produced digestion products of unanticipated size. BglII and EcoRI did not have enzyme recognition sites and did not cut, as expected.

In order to verify that the primers specific for PEPCK had amplified the proper DNA sequence, the expected PEPCK PCR product sequence was analyzed for restriction enzyme cleavage sites of restriction enzymes present in the laboratory using the computer site-mapping program of GCG. The results from the GCG restriction site analysis of the PEPCK PCR amplification product are shown in Figures 4 and 5. The PCR amplification product was incubated with each identified restriction enzyme according to the manufacturer's instructions and the digest fractionated on an ethidium bromide stained

agarose gel. The results of the restriction digest of the PEPCK RT-PCR product are shown in Figure 6. The enzyme EcoRI cut at one site and produced the predicted DNA fragments of 398 and 269 base pairs in length. Neither HindIII nor PvuII had enzyme recognition sites therefore and did not cut, as anticipated.

Discussion

The observations presented in this study further extend the details on the renal localization of PEPCK mRNA expression in the proximal tubule of the rat under control conditions and acute NH_4Cl induced 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 message 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 PEPCK mRNA expression under normal physiological conditions is localized to the S_3 portion is consistent with data from Schoolwerth, *et al.*, who demonstrated by *in situ* hybridization that PEPCK mRNA message was confined primarily to the medullary rays of control kidneys.[74] The investigations by Schoolwerth, *et al* suggested that the S_3 segment of the proximal tubule predominantly expressed PEPCK mRNA under control conditions, as the present data substantiate.

Ten hours following a single gavage feeding of 20 mmol NH_4Cl/kg bodyweight, a significant increase in the number of S_1 and S_2 segments of the proximal tubule of the rat expressing PEPCK mRNA was detected ($P < 0.05$). There was no significant detectable change in the number of S_3 tubules expressing PEPCK mRNA in response to metabolic

acidosis ($P > 0.4$). The number of S_1 and S_2 tubules expressing PEPCK mRNA in response to acidosis was almost twice that of control levels. These changes in PEPCK mRNA expression are also consistent with data of Schoolwerth, *et al* who demonstrated that PEPCK mRNA message reached a maximum distribution throughout the entire cortex of the rat kidney ten hours after NH_4Cl administration.[74] The results of Schoolwerth, *et al* suggest that the S_1 and S_2 segments of the proximal tubule increase expression of PEPCK mRNA after ten hours of ammonium chloride induced acidosis, as the present data also demonstrate.

The results presented here suggest that under physiological conditions, the S_3 segment of the rat nephron 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. In response to an acid insult, however, cells of the S_1 and S_2 proximal rat nephron not previously expressing PEPCK mRNA are recruited to express PEPCK mRNA. This provides evidence of a finely controlled mechanism along the proximal tubule of the rat that is sensitive to perturbations in acid-base balance.

The present data also coincide with those of Burch, Brehe *et al* and Schmidt, Dubach and Guder, who demonstrated that PEPCK enzyme activity was localized to the proximal tubule of the rat and increased significantly in the cortex of acidotic rats when compared to control.[15, 72] This increase was described as greatest in the proximal convoluted tubule and less in the proximal straight tubule, with no increase in the late 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 portion of the proximal straight tubule" are extrapolated to S_1, S_2 and S_3 portions of the proximal tubule, respectively. The current studies demonstrate a significant increase in PEPCK mRNA in the "proximal convoluted tubule" (S_1) and in the "proximal straight tubule" (S_2), with no significant increase in PEPCK mRNA in the "late portion of the proximal straight tubule" (S_3) during acidosis when compared to control. This extrapolation may be a bit tenuous, as data by these investigators was contributed a decade or more before standardization of kidney ultrastructure.[49]

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 producing PEPCK enzyme protein, were induced to express PEPCK messenger RNA in response to metabolic acidosis. Other investigators have demonstrated an increase in PEPCK mRNA along a similarly acute timeline and with a relatively similar increase in expression. Pollock demonstrated by quantitative Northern analysis a three fold increase in renal PEPCK mRNA four hours after NH_4Cl (10 mmol/kg) feeding.[67] 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.[38] 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.[19] 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.[35] Iynedjian, Ballard and Hanson showed that

the rate of synthesis of renal PEPCK enzyme more than tripled between eight and thirteen hours after NH_4Cl feeding.[37] The increase in the renal PEPCK response has been demonstrated to be due to an increase mediated at the level of PEPCK mRNA transcription.[35,36,55] When present data are considered with data of these previous investigations, it can be surmised 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. There probably exists, therefore, some sort of signaling pathway 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.

The renal increase in PEPCK is primarily due to a disturbance of acid-base homeostasis. Renal PEPCK increases following an acid load, and this increase can be reversed with administration of sodium bicarbonate.[36, 37] Although fasting has been demonstrated to cause an increase in renal PEPCK,[37,55,67] this increase can also be reversed by sodium bicarbonate administration.[37,55,60] Metabolic acidosis is considered to be the major factor which brings about an increase in renal gluconeogenesis during fasting. The increase in renal PEPCK during fasting is believed to be due purely to an acid-base disturbance (ketoacidosis) resulting from starvation.[37,55] To this end, it was not deemed necessary to account for the possibility that the experimental animals may avoid food for the ten hours after NH_4Cl administration. The effect of possible fasting would ultimately manifest itself as metabolic acidosis, the goal of the NH_4Cl treatment. Administration of anesthetic can result in hypoventilation and possibly a concomitant

respiratory acidosis by experimental animals. Fourteen hours of respiratory acidosis has been shown to cause an increase in renal PEPCK activity in cortical slices.[3] However, no difference in PEPCK mRNA expression in the kidneys of anesthetized rats compared to the kidneys of rats sacrificed by decapitation has been demonstrated.[67]

In these studies, dissected tubules were assayed first for the presence of β-actin mRNA, which is the gene for the constitutive structural protein. β-actin was chosen as a "housekeeping" gene because its sequence in the rat has been well characterized, cloned and sequenced.[62] The possibility exists that β-actin mRNA expression could be altered by NH_4Cl administration and could therefore lead to erroneous results. However, other investigators have demonstrated that β-actin mRNA expression is not affected by administration of NH_4Cl.[35,36,56] Using oligo-dT as a downstream primer for reverse transcription, β-actin served as an excellent control marker to confirm transfer and permeabilization of tubule and successful reverse transcription of the poly$(A)^+$-RNA population. The use of oligo-dT as a downstream primer created a cDNA copy of the entire mRNA population of the dissected tubule. This process was highly effective in determining which tubules could be used for PEPCK mRNA analysis. Without first determining that permeabilization and reverse transcription was successful, PEPCK mRNA analysis would have led to many false negatives, or scoring tubules as not expressing PEPCK mRNA, when in fact the mRNA had been degraded prior to RT-PCR analysis. This method of dissection and RT-PCR analysis could be expanded to assay many different mRNA populations along the rat proximal tubule. The only limitation for

this procedure would be the knowledge of the gene of interest, so that PCR primers could be properly designed.

Moriyama, *et al* employed the dissection/RT-PCR amplification technique to examine aldose reductase mRNA expression in segments of the rat nephron. They reasoned that when this procedure did not identify aldose reductase mRNA in a dissected tubule, the gene was not expressed in this segment. They concluded that the RT-PCR of microdissected nephron segments provides a practical and sensitive means for determining gene expression in nephron segments.[57]

There exists intrinsic drawbacks of the reverse transcription and amplification systems that restricts 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 and inconsistencies in the amplification of the cDNA product produced. A variable number of enzyme inhibitors can also be present, and can vary from tube to tube even among replicate samples. To this end, the amount of product obtained from a given sample following reverse transcription and a given number of PCR cycles cannot be assumed to be an accurate reflection of the amount of starting material.[64] 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. Other investigators have demonstrated that the "practical detection threshold" for DNA on an agarose gel was 20 nanograms, although faint bands could be visualized at 10 nanograms.[21] 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.[39] These visible quantities represent a tremendous amount of amplified DNA, and 35 cycles of PCR was chosen to produce this result. This resulted in confirming that samples were truly positive or negative for PEPCK mRNA expression, without having to make comparisons between faint and strong bands on an agarose gel.

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Tables

Table 1

PCR primer oligonucleotide sequences, cDNA targets and base definitions for rat β-actin and rat phosphoenolpyruvate carboxykinase.

PCR primer oligonucleotide, cDNA	5'—3' Sequence
Rat β-actin 3 (sense), bases[62] 2168-2193	5'-CTGATCCACATCTGCTGGAAGTGG-3'
Rat β-actin 4 (antisense), bases[62] 3078-3053	5'-ACCTTCAACACCCCAGCCATGTACG-3'
PEPCK-1 (antisense), bases[7] 2139-2159	5'-TCCCTAGCCTGTTCTCTGTGC-3'
PEPCK-4 (sense), bases[7] 1493-1413	5'-GCAGCATGGGGTGTTTGTAGG-3'

Table 2

Plasma bicarbonate and plasma pH values for control and acidotic rats from the present study and previous reports. Values are means ± SE of (n) rats, or when only two values are demonstrated, mean [actual values]. No statistical analysis was performed on these data.

Data Source and feeding protocol	Experimental condition	Plasma [HCO_3^-]	Plasma pH	n
Current studies	Control	26.65 [24.6, 28.7]	7.36 [7.31, 7.4]	2
10 hours post 20mmol/kg NH_4Cl feeding	Acidotic	17.3 ± 6.2 mmol/L	7.22 ± 0.10	3
GAO Alleyne[2]	Control	24.2±0.4 mmol/L	7.39±0.01	19
10 mmol/kg NH_4Cl by stomach tube; measured after 6 hours	Acidotic	16.5±0.3 mmol/L	7.23±0.01	22
Bennett and Alleyne[9]	Control	23.9±0.5 mmol/L	7.37±0.01	13
1mmol/kg NH_4Cl at 0800, 2000 and 0800; sac'ed at 1000.	Acidotic	12.3±1.7 mmol/L	7.09±0.2	7
Mapes and Watford[54]	Control	26.6±0.6 mmol/L	7.31±0.02	4
1.5% NH_4Cl as drinking H_2O for 6 days	Acidotic	17.7±2.3 mmol/L	7.21±0.05	4

Table 3

The number of segments expressing PEPCK mRNA as detected by RT-PCR in the proximal tubule of control rats. Each positive value represents a tubule that produced a PEPCK RT-PCR amplification product of predicted size visible on an agarose gel, after first producing a β-actin RT-PCR amplification product of predicted size visible on an agarose gel. Each negative value represents a tubule that did not produce visible PEPCK product, after first producing a visible β-actin amplification product.

Control Tubules	Proximal Tubule Segment		
	S_1	S_2	S_3
# of PEPCK positive samples	6	6	14
# of PEPCK negative samples	14	8	5
Total # of samples	20	14	19

Table 4

The number of segments expressing PEPCK mRNA as detected by RT-PCR in the proximal tubule of acidotic rats, ten hours following administration of 20 mmol NH_4Cl/kg bodyweight. Each positive value represents a tubule that produced a PEPCK RT-PCR amplification product of predicted size visible on an agarose gel, after first producing a β-actin RT-PCR amplification product of predicted size visible on an agarose gel. Each negative value represents a tubule that did not produce visible PEPCK product, after first producing a visible β-actin amplification product.

Acidotic Tubules	Proximal Tubule Segment		
	S_1	S_2	S_3
# of PEPCK positive samples	15	12	12
# of PEPCK negative samples	8	3	2
Total # of samples	23	15	14

Table 5

Chi-Square statistical test for independence. PEPCK mRNA expression within the proximal tubule of control rats. PEPCK mRNA expression within the proximal tubule of control rats was examined by comparing observed and expected values for S_1, S_2 and S_3 proximal tubules. "P value" is the calculated probability associated with the chi-squared distribution when comparing the observed and expected values. " Calc χ^2" is the value of the chi-squared distribution for the statistic based on the calculated probability and degrees of freedom {df = (r-1)(c-1)}. Tubules are categorized as "Yes" if PEPCK mRNA positive, and "No" if PEPCK mRNA negative. See Table 3 for further explanation.

A

Observed Values

Tubules Express PEPCK?	Yes	No	
S_1	6	14	20
S_2	6	8	14
S_3	14	5	19
	26	27	53

P Value: **0.021**
Calc χ^2: 7.733

Expected Values

Tubules Express PEPCK?	Yes	No	
S_1	9.811	10.189	20
S_2	6.868	7.132	14
S_3	9.321	9.679	19
	26	27	53

B

Observed Values

Tubules Express PEPCK?	Yes	No	
S_1	6	14	20
$S_2 + S_3$	20	13	33
	26	27	53

P Value: **0.031**
Calc χ^2: 6.965

Expected Values

Tubules Express PEPCK?	Yes	No	
S_1	9.811	10.189	20
$S_2 + S_3$	16.189	16.811	33
	26	27	53

C

Observed Values

Tubules Express PEPCK?	Yes	No	
S_2	6	8	14
$S_1 + S_3$	20	19	39
	26	27	53

P Value: 0.589
Calc χ^2: 1.060

Expected Values

Tubules Express PEPCK?	Yes	No	
S_2	6.868	7.132	14
$S_1 + S_3$	19.132	19.868	39
	26	27	53

D

Observed Values

Tubules Express PEPCK?	Yes	No	
S_3	14	5	19
$S_1 + S_2$	12	22	34
	26	27	53

P Value: **0.007**
Calc χ^2: 9.829

Expected Values

Tubules Express PEPCK?	Yes	No	
S_3	9.321	9.679	19
$S_1 + S_2$	16.679	17.321	34
	26	27	53

Table 6

Chi-Square statistical test for independence. PEPCK mRNA expression within the proximal tubule of acidotic rats. PEPCK mRNA expression within the proximal tubule of acidotic rats was examined by comparing observed and expected values for S_1, S_2 and S_3 proximal tubules. "P value" is the calculated probability associated with the chi-squared distribution when comparing the observed and expected values. "Calc χ^2" is the value of the chi-squared distribution for the statistic based on the calculated probability and degrees of freedom {df = (r-1)(c-1)}. Tubules are categorized as "Yes" if PEPCK mRNA positive, and "No" if PEPCK mRNA negative. See Table 4 for further explanation.

A

Observed Values

Tubules Express PEPCK?

	Yes	No	
S_1	15	8	23
S_2	12	3	15
S_3	12	2	14
	39	13	52

P Value: 0.328
Calc χ^2: 2.231

Expected Values

Tubules Express PEPCK?

	Yes	No	
S_1	17.25	5.75	23
S_2	11.25	3.75	15
S_3	10.5	3.5	14
	39	13	52

B

Observed Values

Tubules Express PEPCK?

	Yes	No	
S_1	15	8	23
$S_2 + S_3$	24	5	29
	39	13	52

P Value: 0.147
Calc χ^2: 3.837

Expected Values

Tubules Express PEPCK?

	Yes	No	
S_1	17.25	5.75	23
$S_2 + S_3$	21.75	7.25	29
	39	13	52

C

Observed Values

Tubules Express PEPCK?

	Yes	No	
S_2	12	3	15
$S_1 + S_3$	27	10	37
	39	13	52

P Value: 0.596
Calc χ^2: 1.035

Expected Values

Tubules Express PEPCK?

	Yes	No	
S_2	11.25	3.75	15
$S_1 + S_3$	27.75	9.25	37
	39	13	52

D

Observed Values

Tubules Express PEPCK?

	Yes	No	
S_3	12	2	14
$S_1 + S_2$	27	11	38
	39	13	52

P Value: 0.279
Calc χ^2: 2.555

Expected Values

Tubules Express PEPCK?

	Yes	No	
S_3	10.5	3.5	14
$S_1 + S_2$	28.5	9.5	38
	39	13	52

Table 7

Chi-Square statistical test for independence. PEPCK mRNA expression in control versus acidotic tubules. PEPCK mRNA expression in control versus acidotic tubules was examined by comparing observed and expected values for S_1, S_2 and S_3 proximal tubules from control and acidotic rats. The "P value" is the calculated probability associated with the chi-squared distribution when comparing the observed and expected values. " Calc χ^2" is the value of the chi-squared distribution for the statistic based on the calculated probability and one degree of freedom {(r-1)(c-1) = 1}. Tubules are categorized as "Yes" if PEPCK mRNA positive, and "No" if PEPCK mRNA negative. See Tables 3 and 4 for further explanation.

Observed Values — **S_1 tubules** — *Expected Values*

	Tubules Express PEPCK? Yes	No				Tubules Express PEPCK? Yes	No	
Control	6	14	20	P Value:	**0.021**	9.767	10.23	20
Acid	15	8	23	Calc χ^2:	5.31	11.23	11.77	23
	21	22	43			21	22	43

Observed Values — **S_2 tubules** — *Expected Values*

	Tubules Express PEPCK? Yes	No				Tubules Express PEPCK? Yes	No	
Control	6	8	14	P Value:	**0.039**	8.69	5.31	14
Acid	12	3	15	Calc χ^2:	4.24	9.31	5.69	15
	18	11	29			18	11	29

Observed Values — **S_3 tubules** — *Expected Values*

	Tubules Express PEPCK? Yes	No				Tubules Express PEPCK? Yes	No	
Control	14	5	19	P Value:	0.403	14.97	4.03	19
Acid	12	2	14	Calc χ^2:	0.698	11.03	2.97	14
	26	7	33			26	7	33

Figures

Figure 1

Restriction enzyme site analysis (MAP) of the predicted β-actin PCR amplification product, 698 base pairs in length. The sequence of the PCR product was analyzed using the computer site-mapping program of GCG for restriction enzymes BglII, EcoRI, PvuII and FokI, enzymes that were readily available in the laboratory. Restriction enzymes FokI and PvuII have recognition sequences within the β-actin PCR product, and their predicted cleavage locations are indicated. The restriction enzymes BglII and EcoRI do not have recognition cleavage sites.


(Linear) MAP of: bactinpcr.seq check: 6231 from: 1 to: 698
LOCUS 698 bp DNA ROD 04-AUG-1986
DEFINITION Rat cytoplasmic beta-actin PCR product
ACCESSION J00691
NID g202653
KEYWORDS actin; beta-actin; beta-cytoplasmic actin.
SOURCE Rat gene library, clones pAc[-R1,-18.1,-4.1] from bacteriophage Act
With 4 enzymes: BGLII ECORI PVUII FOKI
June 16, 1996 16:39 ..
FokI
ACCTTCAACACCCCAGCCATGTACGTAGCCATCCAGGCTGTGTTGTCCCTGTATGCCTCT
1
60
TGGAAGTTGTGGGGTCGGTACATGCATCGGTAGGTCCGACACAACAGGGACATACGGAGA
GGTCGTACCACTGGCATTGTGATGGACTCCGGAGACGGGGTCACCCACACTGTGCCCATC
61
120
CCAGCATGGTGACCGTAACACTACCTGAGGCCTCTGCCCCAGTGGGTGTGACACGGGTAG
FokI
TATGAGGGTTACGCGCTCCCTCATGCCATCCTGCGTCTGGACCTGGCTGGCCGGGACCTG
121
180
ATACTCCCAATGCGCGAGGGAGTACGGTAGGACGCAGACCTGGACCGACCGGCCCTGGAC
PvuII
ACAGACTACCTCATGAAGATCCTGACCGAGCGTGGCTACAGCTTCACCACCACAGCTGAG
181
240
TGTCTGATGGAGTACTTCTAGGACTGGCTCGCACCGATGTCGAAGTGGTGGTGTCGACTC
AGGGAAATCGTGCGTGACATTAAAGAAGAAGCTGTGCTATGTTGCCCTAGACTTCGAGCAA
241
300
TCCCTTTAGCACGCACTGTAATTTCTCTTCGACACGATACAACGGGATCTGAAGCTCGTT
FokI
GAGATGGCCACTGCCGCATCCTCTTCCTCCCTGGAGAAGAGCTATGAGCTGCCTGACGGT
301
360
CTCTACCGGTGACGGCGTAGGAGAAGGAGGGACCTCTTCTCGATACTCGACGGACTGCCA
CAGGTCATCACTATCGGCAATGAGCGGTTCCGATGCCCCGAGGCTCTCTTCCAGCCTTCC
361
420
GTCCAGTAGTGATAGCCGTTACTCGCCAAGGCTACGGGGCTCCGAGAGAAGGTCGGAAGG
FokI
FokI
TTCCTGGGTATGGAATCCTGTGGCATCCATGAAACTACATTCAATTCCATCATGAAGTGT
421
480
AAGGACCCATACCTTAGGACACCGTAGGTACTTTGATGTAAGTTAAGGTAGTACTTCACA
GACGTTGACATCCGTAAAGACCTCTATGCCAACACAGTGCTGTCTGGTGGCACCACCATG
481
540
CTGCAACTGTAGGCATTTCTGGAGATACGGTTGTGTCACGACAGACCACCGTGGTGGTAC
FokI
TACCCAGGCATCGCTGACAGGATGCAGAAGGAGATTACTGCCCTGGCTCCTAGCACCATG
541
600
ATGGGTCCGTAGCGACTGTCCTACGTCTTCCTCTAATGACGGGACCGAGGATCGTGGTAC
AAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCTGTGTGGATTGGTGGCTCTATC
601
660
TTCTAGTTCTAGTAACGAGGAGGACTCGCGTTCATGAGACACACCTAACCACCGAGATAG
CTGGCCTCACTGTCCACCTTCCAGCAGATGTGGATCAG
661
698
GACCGGAGTGACAGGTGGAAGGTCGTCTACACCTAGTC
Enzymes that do cut:
FokI PvuII
Enzymes that do not cut:
BglII EcoRI

Figure 1

```
(Linear) MAPSORT of: bactinpcr.seq  Check: 6231  from: 1  to: 698

LOCUS        698 bp     DNA              ROD       04-AUG-1986
DEFINITION   Rat cytoplasmic beta-actin PCR product
ACCESSION    J00691
NID          g202653
KEYWORDS     actin; beta-actin; beta-cytoplasmic actin.
SOURCE             Rat  gene  library,  clones  pAc[-R1,-18.1,-4.1]  from
bacteriophage Act

 With 4 enzymes: FOKI PVUII BGLII ECORI

                        July 2, 1996 14:54  ..

FokI GGATGnnnnnnnnn'nnnn_

Cuts at:       0*      16      133      303      430      475      573      698
   Size:          16      117      170      127       45       98      125

  Fragments arranged by size:

                 170      127      125      117       98       45       16

PvuII CAG'CTG

Cuts at:       0*     235      698
   Size:         235      463

 Enzymes that do cut:
     FokI     PvuII
 Enzymes that do not cut:
    BglII     EcoRI
```

Figure 2

Restriction enzyme product prediction (MAPSORT) for β-actin PCR product digested with restriction enzymes BglII, EcoRI, PvuII and FokI. FokI enzyme digestion is predicted to produce seven fragments of various lengths between 16 and 170 base pairs. PvuII enzyme digestion is predicted to produce two DNA digestion products, 235 and 463 base pairs in length. Restriction enzymes BglII and EcoRI do not have recognition sequences within the β-actin PCR product and are not predicted to cut.

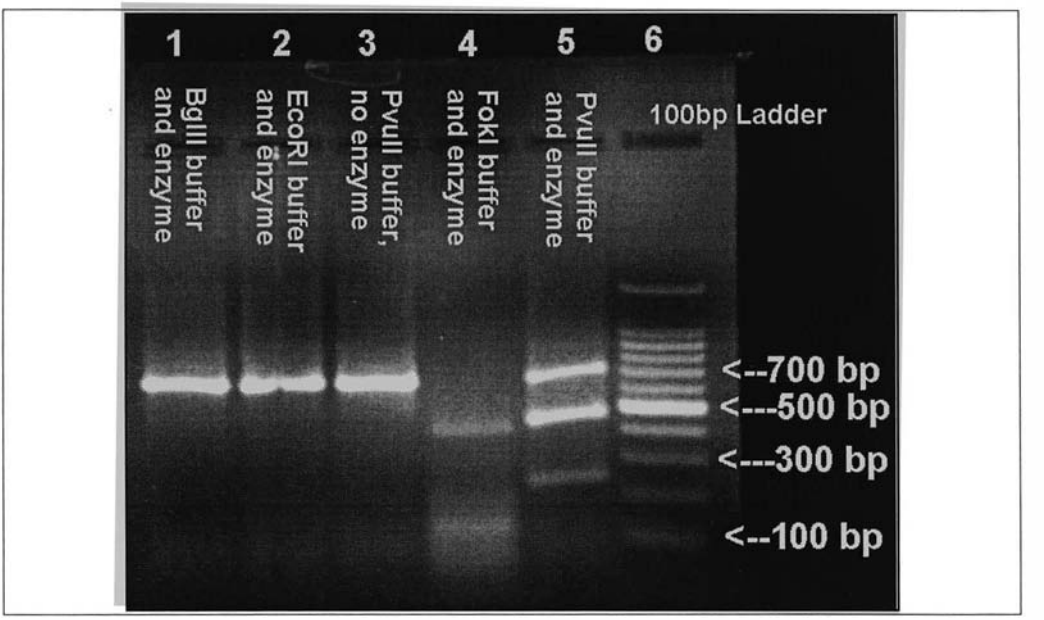

Figure 3

Ethidium bromide stained agarose gel of the restriction enzyme digest of the β-actin PCR product with restriction enzymes BglII, EcoRI, PvuII and FokI. Lane 1: BglII buffer and enzyme; no digestion product produced. Lane 2: EcoRI enzyme and buffer; no digestion product produced. Lane 3: PvuII enzyme buffer with no enzyme; no digestion product produced. Lane 4: FokI buffer and enzyme; incomplete digestion of β-actin PCR product visible. Lane 5: PvuII buffer and enzyme; 698 (uncut), 235 and 463 (cut) base pair DNA enzyme digestion products visible. Lane 6: 100 base pair DNA standard.

Figure 4

Restriction enzyme site analysis (MAP) of the predicted PEPCK PCR amplification product, 667 base pairs in length. The sequence of the PCR product was analyzed using the computer site-mapping program of GCG for restriction enzymes EcoRI, HindIII and PvuII, enzymes, which were readily available in the laboratory. Restriction enzyme EcoRI contains a recognition sequence within the PEPCK PCR product, while restriction enzymes HindIII and PvuII do not. The cleavage site for EcoRI is illustrated.

```
(Linear) MAP of: pepckpcr.seq  check: 8851  from: 1  to: 667
LOCUS       667 bp    DNA             ROD       04-AUG-1986
DEFINITION  Rat phosphoenolpyruvate carboxykinase PCR product.
ACCESSION   K03248
NID         g206065
KEYWORDS    phosphoenolpyruvate carboxykinase.
 With 4 enzymes: ECORI HINDIII PVUII
                              April 19, 1996 15:17  ..

       GCAGCATGGGGTGTTTGTAGGAGCCGCCATGAGATCAGAGGCCACCGCTGCTGCAGAGCA
     1 ---------+---------+---------+---------+---------+---------+ 60
       CGTCGTACCCCACAAACATCCTCGGCGGTACTCTAGTCTCCGGTGGCGACGACGTCTCGT

       TAAGGGCAAGGTCATCATGCACGACCCCTTCGCTATGCGGCCCTTCTTTGGCTACAACTT
    61 ---------+---------+---------+---------+---------+---------+ 120
       ATTCCCGTTCCAGTAGTACGTGCTGGGGAAGCGATACGCCGGGAAGAAACCGATGTTGAA

       CGGCAAGTACCTGGCGCACTGGCTGAGCATGGCCCACCGCCCAGCAGCCAAGTTGCCCAA
   121 ---------+---------+---------+---------+---------+---------+ 180
       GCCGTTCATGGACCGCGTGACCGACTCGTACCGGGTGGCGGGTCGTCGGTTCAACGGGTT

       GATCTTCCACGTCAACTGGTTCCGGAAAGACAAAAACGGCAAGTTCCTCTGGCCCGGATT
   181 ---------+---------+---------+---------+---------+---------+ 240
       CTAGAAGGTGCAGTTGACCAAGGCCTTTCTGTTTTTGCCGTTCAAGGAGACCGGGCCTAA

        TGGTGAGAACTCCCGCGTGCTGGAGTGGATGTTCGGACGCATCGAAGGGGAAGACAGCGC
   241 ---------+---------+---------+---------+---------+---------+ 300
       ACCACTCTTGAGGGCGCACGACCTCACCTACAAGCCTGCGTAGCTTCCCCTTCTGTCGCG

       CAAGCTCACTCCCATTGGCTACGTCCCTAAGGAAGACGCCCTGAACTTGAAAGGCCTGGG
   301 ---------+---------+---------+---------+---------+---------+ 360
       GTTCGAGTGAGGGTAACCGATGCAGGGATTCCTTCTGCGGGACTTGAACTTTCCGGACCC

                                      EcoRI
                                        |
       GGACGTCAACGTGGAGGAGCTGTTCGGAATCTCTAAGGAATTCTGGGAGAAGGAGGTGGA
   361 ---------+---------+---------+---------+---------+---------+ 420
       CCTGCAGTTGCACCTCCTCGACAAGCCTTAGAGATTCCTTAAGACCCTCTTCCTCCACCT

       GGAGATCGACAAGTATCTGGAGGACCAGGTCAACGCCGACCTCCCTTACGAAATAGAGAG
   421 ---------+---------+---------+---------+---------+---------+ 480
       CCTCTAGCTGTTCATAGACCTCCTGGTCCAGTTGCGGCTGGAGGGAATGCTTTATCTCTC

       GGAGCTCCGAGCCCTGAAACAGAGAATCAGCCAGATGTAATCCCGATGGGGGGTGTCCTT
   481 ---------+---------+---------+---------+---------+---------+ 540
       CCTCGAGGCTCGGGACTTTGTCTCTTAGTCGGTCTACATTAGGGCTACCCCCCACAGGAA

       GAGAGTCGCCCCTTCCCGCTCACGGCACACGTTGGGAGCTAGGAGCAAACCAGCAAGCAC
   541 ---------+---------+---------+---------+---------+---------+ 600
       CTCTCAGCGGGGAAGGGCGAGTGCCGTGTGCAACCCTCGATCCTCGTTTGGTCGTTCGTG

       AATGCTGAGTAGATCAGAAAAGCACCTTTTAATAGTCAGTTGAGTAGCACAGAGAACAGG
   601 ---------+---------+---------+---------+---------+---------+ 660
       TTACGACTCATCTAGTCTTTTCGTGGAAAATTATCAGTCAACTCATCGTGTCTCTTGTCC

       CTAGGGA
   661 ------- 667
       GATCCCT
 Enzymes that do cut:
    EcoRI
 Enzymes that do not cut:
  HindIII     PvuII
```

Figure 4

```
(Linear) MAPSORT of: pepckpcr.seq  Check: 8851  from: 1  to: 667

LOCUS       667 bp    DNA          ROD       04-AUG-1986
DEFINITION  Rat phosphoenolpyruvate carboxykinase PCR product.
ACCESSION   K03248
NID         g206065
KEYWORDS    phosphoenolpyruvate carboxykinase.
SEGMENT     6 of 6 . . .

 With 3 enzymes: ECORI HINDIII PVUII

                      July 2, 1996 14:53  ..

EcoRI G'AATT_C

Cuts at:      0*    398     667
   Size:        398     269

 Enzymes that do cut:
    EcoRI
 Enzymes that do not cut:
  HindIII    PvuII
```

Figure 5

Restriction enzyme product prediction (MAPSORT) for PEPCK PCR product digested with restriction enzymes EcoRI, HindIII and PvuII. EcoRI enzyme digestion is predicted to produce two DNA digestion products, 269 and 398 base pairs in length. HindIII and PvuII restriction enzymes do not have recognition sequences within the PEPCK PCR product and are not predicted to cut.

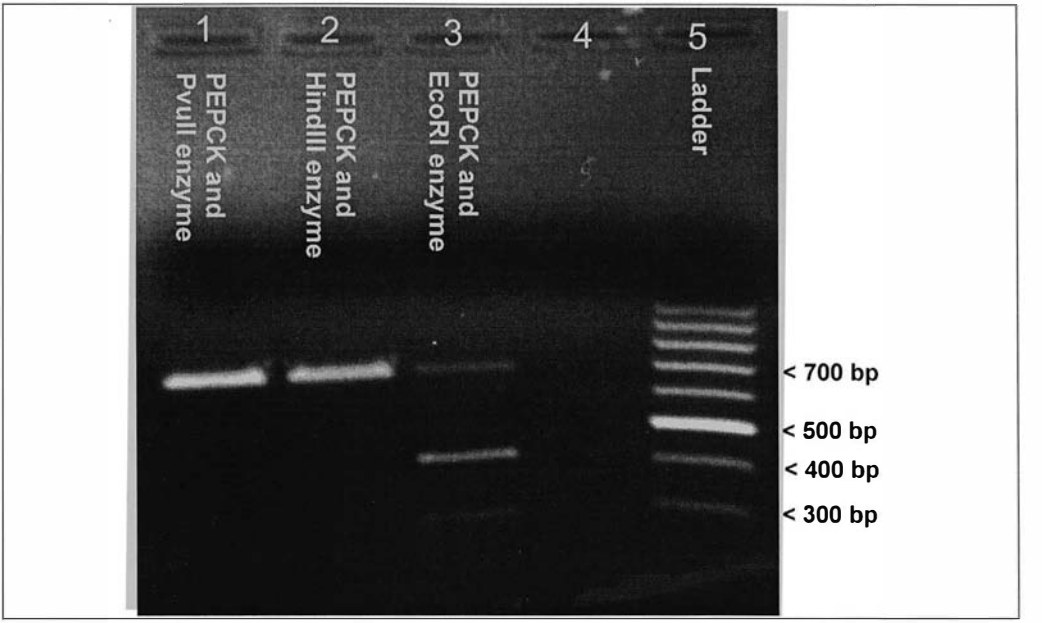

Figure 6

Ethidium bromide stained agarose gel of the restriction enzyme digest of the PEPCK PCR product with restriction enzymes EcoRI, HindIII and PvuII. Lane 1: PEPCK PCR product and PvuI enzyme; no digestion product produced. BglII buffer and enzyme with buffer; no digestion product produced. Lane 2: PEPCK PCR product and HindIII enzyme with buffer; no digestion product produced. Lane 3: PEPCK PCR product and EcoRI enzyme with buffer; 667 (uncut), 269 and 398 (cut) base pair DNA enzyme digestion products visible. Lane 4: blank. Lane 5: 100 base pair DNA standard.

Vita