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Mechanism of Altered Renal Glutaminase Gene Expression in Response to Chronic Acidosis†

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Received November 2, 1990; Revised Manuscript Received March 22, 1991

ABSTRACT: Increased rat renal ammoniagenesis is sustained during chronic metabolic acidosis by the cell-specific induction of the regulatory enzymes of glutamine catabolism and of gluconeogenesis. A glutaminase-specific cDNA hybridizes to 6.0- and 3.4-kb mRNAs that are contained in total or poly(A)$^+$ RNA isolated from rat kidney. When translated in a rabbit reticulocyte lysate, each of the fractionated mRNAs produces the 72-kDa precursor of the mitochondrial glutaminase. The levels of both mRNAs are increased 5-fold within 1 day following onset of chronic acidosis and reach a maximum (8-fold) after 5 days. During recovery from chronic acidosis, the levels of the glutaminase mRNAs are returned to normal within 1 day. The observed changes in mRNA levels correlate with equivalent changes in the relative levels of translatable glutaminase mRNA. Nuclear run-on assays indicate that the rate of transcription of the renal phosphoenolpyruvate carboxykinase gene is increased and decreased in response to onset and recovery from chronic acidosis, respectively. In contrast, the rates of transcription of the glutaminase and β-actin genes are unaffected by alterations in acid-base balance. Thus, the increase in renal glutaminase activity during chronic acidosis results from an equivalent increase in the levels of total and translatable glutaminase mRNAs which apparently results from an increased stability of the glutaminase mRNA.

During normal acid-base balance, the rat kidney extracts very little, if any, of the plasma glutamine (Squires et al., 1976). Renal extraction and catabolism of plasma glutamine are increased rapidly following onset of metabolic acidosis (Hughey et al., 1980). The acute increase in renal ammoniagenesis results primarily from changes in the concentrations of metabolites and H^+ that regulate flux through the mitochondrial glutaminase, glutamate dehydrogenase, and α-ketoglutarate dehydrogenase (Tannen & Sastrasinh, 1984). During chronic acidosis, the initial changes in renal metabolites and plasma pH are largely compensated (Parry & Brosnan, 1978). The increased rat renal ammoniagenesis and gluconeogenesis are now sustained by the induction of glutaminase (Curthoys & Lowry, 1973), glutamate dehydrogenase (Wright & Kuepper, 1989), and phosphoenolpyruvate carboxykinase (Burch et al., 1978) that occur solely within the proximal convoluted segment of the nephron.

The increase in mitochondrial glutaminase activity is due to a gradual increase in the relative rate of glutaminase synthesis (Tong et al., 1986). The increased rate of synthesis reaches a plateau within 5 days that is 5-fold greater than normal. The apparent half-life of the glutaminase is unaltered during acidosis. As a result, the total renal glutaminase activity is increased approximately 5-fold after 7 days of chronic acidosis. The increase in the relative rate of glutaminase synthesis correlates with an increase in the relative level of translatable GA[1] mRNA (Tong et al., 1987). In vitro translation of rat renal poly(A)$^+$ RNA yields a 72-kDa peptide that is immunoprecipitated with anti-glutaminase IgG. Pulse-chase experiments with primary cultures of rat renal proximal tubular epithelial cells and in vitro processing studies (Perera et al., 1990) have established that the 72-kDa peptide is the cytosolic precursor of the 68- and 65-kDa peptides that constitute the mitochondrial glutaminase.

In this study, Northern blot analysis and nuclear transcription run-on assays were performed to further characterize the mechanism of glutaminase induction during chronic acidosis. The observed increase in the relative levels of translatable GA mRNA correlates with equivalent changes in the level of total GA mRNA. However, this increase occurs without increasing the rate of transcription of the GA gene. Thus, increased renal glutaminase activity that occurs in response to chronic acidosis apparently results from an increase in the stability of the GA mRNA.

Materials and Methods

[α-^{32}P]dCTP and [α-^{32}P]CTP (sp act. 3000 Ci/mmol), L-[^{35}S]methionine (sp act. 800 Ci/mmol), and Gene Screen were obtained from New England Nuclear Research. ^{14}C-Labeled protein molecular weight standards were purchased from Bethesda Research Laboratories. Glutamate dehydrogenase, restriction enzymes, and calf liver tRNA were products of Boehringer Mannheim. Oligolabeling kit and ACS solution were obtained from Pharmacia and Amersham, respectively. Oligo(dT)-cellulose type 2 was purchased from Collaborative Research, Inc. Low-melting-temperature aga-

† Supported in part by National Institutes of Health Grant DK37124.
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[1] Abbreviations: GA, glutaminase; PCK, phosphoenolpyruvate carboxykinase.

0006-2960/91/0430-7522$02.50/0

rose and regular agarose were purchased from FMC. SDS and CH_3HgOH were purchased from BDH Chemicals Ltd. and Alfa, respectively. Guanidinium thiocyanate and sodium *N*-lauroylsarcosine were purchased from Fluka. All other biochemicals were purchased from either Sigma or Fisher. Rabbit reticulocyte lysate was prepared by Dr. Rosemary Jagus according to the method of Pelham and Jackson (1976). The pPCK10, which encodes rat phosphoenolpyruvate carboxykinase, was obtained from R. Hanson (Yoo-Warren et al., 1983). The pHFβA-1, which encodes human β-actin, was obtained from L. Kedes (Gunning et al., 1983).

Male Sprague-Dawley rats weighing 200–250 g were purchased from Zivic-Miller Laboratories and maintained on Purina Rat Chow. Chronic metabolic acidosis was induced by providing 1.5% NH_4Cl as the sole source of drinking water for up to 7 days. After drinking the NH_4Cl solution for 5 days, a set of acidotic rats was given water and allowed to recover from acidosis. Acute metabolic acidosis was induced by stomach loading rats with 20 mmol of NH_4Cl/kg body weight and then allowing them to drink 1.5% NH_4Cl. Acute recovery of 5-day chronic acidotic rats was induced by stomach loading with 20 mmol of $NaHCO_3$/kg body weight.

Rat phosphate-dependent glutaminase activity was measured as described (Curthoys & Weiss, 1974). Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Rabbit polyclonal anti-rat renal glutaminase IgGs were prepared as described (Curthoys et al., 1976a,b). The IgG fraction was purified from rabbit antiserum by chromatography on DEAE-Affi-Gel Blue (Tong et al., 1986). The purified IgG fraction had an antibody titer of 15 units/mL. Rat brain tissue was homogenized in a Potter–Elvehjem homogenizer and then diluted with $^1/_4$ volume of 5% Triton X-100, incubated for 1 h at 4 °C, and centrifuged for 30 min at 48 000 rpm in a 50Ti rotor. The glutaminase activity in the resulting supernatant was about 3 units/mL. This supernatant was added as carrier to coprecipitate the glutaminase precursor synthesized by in vitro translation.

pGA-12 and pGA-13 contain 2.7- and 2.1-kb GA cDNAs, respectively, which were isolated by screening a λgt10 rat kidney cDNA library with pGA-1 (Banner et al., 1988) and subcloned into the *Eco*RI site of pGEM-4Z. Transformation of *Escherichia coli* HB101, screening of transformants, plasmid DNA preparation, and restriction enzyme digests were performed according to standard procedures (Maniatis et al., 1982). The 0.7-kb 3′ *Eco*RI fragment of pGA-12, the 2.0-kb *Sac*I fragment of pGA-13, and the 2.1-kb *Bam*HI fragment of pHFβA-1 (Gunning et al., 1983) were isolated on a 1% low-melting-temperature agarose gel and used as probes for Northern blot analysis (Feinberg & Vogelstein, 1984).

Total RNA was isolated from the kidneys of control and experimental rats according to the procedure of Chirgwin et al. (1979). Aliquots of the isolated RNA were fractionated by electrophoresis on an 1% agarose gel containing 3% formaldehyde (Goldberg, 1980). The integrity of the RNA was analyzed by visualization of the 28S and 18S rRNA with ethidium bromide. Fractionated RNAs were transferred to Gene Screen, cross-linked by UV irradiation (Church & Gilbert, 1984), and subsequently hybridized (Amasino, 1986) with probes that were labeled with [α-^{32}P]dCTP by using the Pharmacia Oligolabeling Kit. The hybridized filters were exposed to Kodak X-omat film. Radioactivity was determined by liquid scintillation counting of the hybridized areas excised from the Gene Screen and corrected for background by subtraction of radioactivity associated with equivalent areas cut from blank regions of the same filter. All data were calculated as specific hybridization relative to that of β-actin.

Poly(A)$^+$ RNA was prepared by oligo(dT)–cellulose affinity chromatography (Aviv & Leder, 1972) and fractionated by electrophoresis on a 1% agarose gel containing 5 mM CH_3HgOH (Weislander, 1979). Lanes containing total RNA were stained with ethidium bromide, and the 28S and 18S rRNAs were used as guides to slice the gel. The lane containing the rat renal poly(A)$^+$ RNA was cut into 3-mm slices starting 12 mm above the 28S rRNA and extending to the 18S rRNA. The gel slices were melted in 0.5 M ammonium acetate and extracted with phenol. The RNA was precipitated with ethanol at −20 °C following the addition of 10 μg of calf liver tRNA. The concentration of the poly(A)$^+$ RNA solutions was determined by using the [^{3}H]poly(U) hybridization assay of Milcarek et al. (1974). In vitro translation and immunoprecipitation of the glutaminase precursor were carried as described by Perera et al. (1990).

The 2.1-kb *Bam*HI fragment of pHFβA-1 (Gunning et al., 1983), a 2.8-kb *Eco*RI fragment of λGA-104 (isolated by screening a random primed λgt11 rat brain cDNA library with pGA-2), and the 1.6-kb *Bgl*II fragment of pPCK10 (Yoo-Warren et al., 1983) were subcloned into pBluescriptII-SK(−), and the resulting plasmids were used to synthesize antisense RNA (Krieg & Melton, 1987). Rat renal nuclei were isolated by methods adapted from Morris et al. (1987) and Meisner et al. (1985). The recovery was approximately 5×10^7 nuclei/g wet weight of kidney. Nuclei were diluted to 2×10^5 nuclei/μL and dispensed into 100-μL aliquots for immediate use or were frozen on dry ice and stored at −75 °C for up to 1 month.

The transcription reaction mixture (200 μL) contained 0.21 μM [α-^{32}P]CTP (3000 Ci/mmol), 25% glycerol, 75 mM sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (pH 7.5), 100 mM KCl, 5 mM magnesium acetate, 1 mM $MnCl_2$, 50 μM EDTA, 1 mM ATP, 0.5 mM GTP and UTP, 4 mM dithiothreitol, 0.1 mg/mL heparin, 8.8 mM phosphocreatine, 8 μg of creatine phosphokinase, 40 units of RNasin, 20 μg of nucleoside 5′-diphosphate kinase, and approximately 2×10^7 nuclei. After a 10-min incubation at 25 °C, nuclei were digested with ribonuclease-free DNase I. Yeast tRNA was added and nuclear RNA was isolated by adding a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium lauroylsarcosinate, 0.1 M 2-mercaptoethanol, 2 M sodium acetate (pH 4.0), water-saturated phenol, and chloroform/isoamyl alcohol (24:1). The labeled nuclear RNA was precipitated with 2-propanol, washed in 70% ethanol, dissolved, and centrifuged through a Sephadex G-50 spin column. In a typical reaction, $(2–5) \times 10^7$ cpm of [α-^{32}P]CTP were incorporated into RNA. Samples containing 10 μg of the antisense RNAs were immobilized on a Gene Screen Plus membrane by using a Schleicher and Schuell Minifold II slot-blot apparatus. Hybridizations were carried out in 50% formamide, 0.25 M sodium phosphate (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 100 μg/mL denatured salmon sperm DNA, 10 μg/mL poly(A)$^+$ RNA, 7% sodium dodecyl sulfate, and 10^7 cpm/mL RNAs at 53 °C for 3 days. After treating with RNase and washing, the hybridized nuclear RNAs were quantitated by autoradiography and densitometry using a computerized Microscan 2000 image analyzer (Technology Resources, Inc., Nashville TN). Hybridization efficiency (~30%) was determined simultaneously by quantitating the extent of binding of ^{3}H-labeled sense PCK RNA. All data were calculated as specific hybridization relative to that of β-actin.

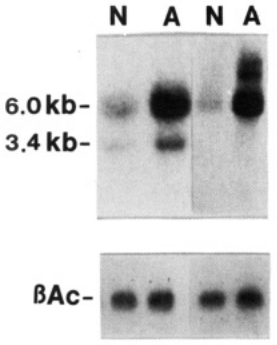

FIGURE 1: Effect of chronic metabolic acidosis on the relative abundance of GA mRNA. Aliquots containing 15 μg of total RNA isolated from the kidneys of normal rats (N) and of rats that were made 7-day chronically acidotic (A) were characterized by Northern blot analysis. The samples were hybridized with GA cDNAs that correspond to coding sequence, pGA-13 (left panel), or to the 3′-terminal end of pGA-12 (right panel). The blots were stripped and rehybridized for β-actin mRNA (βAc).

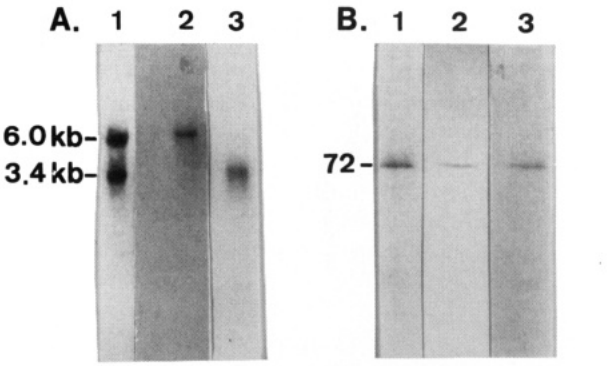

FIGURE 2: Identification and in vitro translation of the separated forms of glutaminase mRNAs. Aliquots containing total poly(A)$^+$ RNA (lane 1) and the separated 6.0-kb (lane 2) and 3.4-kb (lane 3) glutaminase mRNAs were either subjected to Northern blot analysis (panel A) or translated in a rabbit reticulocyte lysate (panel B). The resulting proteins were immunoprecipitated with anti-glutaminase IgG and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography. The sizes of the precipitated proteins are indicated in kDa.

RESULTS

The pGA-13 cDNA hybridized to two distinct mRNAs contained in total RNA isolated from rat kidney (Figure 1). The larger and more abundant mRNA was approximately 6 kb in length, whereas the smaller mRNA was approximately 3.4 kb. Both forms of GA mRNA were increased 8-fold following adaptation to chronic metabolic acidosis. In contrast, the level of β-actin mRNA was unaffected by changes in acid–base balance. pGA-1, the original glutaminase cDNA (Banner et al., 1988), hybridized to the same species of mRNA and demonstrated the same level of induction. In contrast, probes obtained from pGA-12 exhibit a different pattern of hybridization. pGA-12 contains 0.2 kb of sequence that encodes the C-terminal segment of the glutaminase and 2.5 kb of 3′-noncoding sequence. Probes restricted from the coding sequence and from the initial portion of the 3′-nontranslated sequence hybridize to both forms of GA mRNA. However, fragments isolated from the distal portion of the 3′-nontranslated sequence hybridize solely with the 6.0-kb form of GA mRNA (Figure 1).

In order to determine whether both forms of GA mRNA encode the 72-kDa cytosolic precursor of the mitochondrial glutaminase, rat renal poly(A)+ RNA was fractionated on a CH_3HgOH gel. The mRNAs eluted from individual slices of the gel produced discrete sets of proteins when translated in a rabbit reticulocyte lysate. Northern blot analysis was carried out to identify fractions that contained the two separated mRNAs (Figure 2A). In vitro translation of the two RNA fractions containing the separated 6.0- or 3.4-kb GA mRNAs both produced a 72-kDa peptide that was immunoprecipitated with anti-glutaminase IgG (Figure 2B). Therefore, the two hybridizable RNAs represented translatable forms of GA mRNA. Thus, in the remainder of the experiments, the level of GA mRNA was determined as the sum of the intensity of the 6.0- and 3.4-kb mRNAs.

The glutaminase activity measured in a crude homogenate of rat kidney is increased gradually following onset of chronic acidosis (Figure 3). This increase reaches a maximum after 7 days that is 5-fold greater than normal. The decrease in glutaminase activity during recovery from chronic acidosis also occurred slowly, requiring 11 days to return to normal (Tong et al., 1986). In contrast, the changes in the relative level of GA mRNA during onset and recovery occurred rapidly (Figure 3). Within 1 day, the level of GA mRNA increased 5-fold, and after 5 days it plateaued at a level 8-fold greater than normal. Similarly, 1 day of recovery was sufficient for

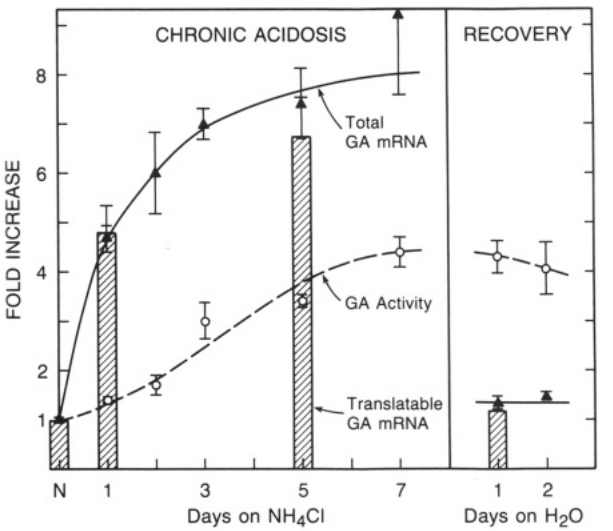

FIGURE 3: Effect of onset and recovery from chronic acidosis on glutaminase activity, relative abundance of GA mRNA, and relative level of translatable GA mRNA. Glutaminase-specific activity (O---O) was measured in crude homogenates of kidney tissue. The relative abundance of GA mRNA (▲—▲) was quantitated relative to that of β-actin mRNA, and translatable GA mRNA (shaded bars) was determined by using poly(A)$^+$ RNA. All values are the mean ± SD of at least three determinations.

the total GA mRNA to return to normal. The changes in total GA mRNA abundance correlate with equivalent changes in the relative level of translatable GA mRNA. Thus, the changes in the relative rate of glutaminase synthesis that occur in response to alterations in acid–base balance (Tong et al., 1987) are due primarily to changes in levels of both total and translatable GA mRNA.

To identify the possible mechanism responsible for the changes in GA mRNA, transcription run-on assays were performed with rat renal nuclei isolated at different times following onset and recovery from acidosis (Figure 4). The relative rates of phosphoenolpyruvate carboxykinase and β-actin transcription were also determined. The observed hybridization was specific for antisense RNA and was inhibited greater than 90% by the presence of 2 μg/mL α-amanitin. Therefore, the labeled RNAs that hybridized were specific products of RNA polymerase II transcription. The rate of β-actin mRNA synthesis calculated as ppm was unaffected by alterations in acid–base balance. The relative rate of

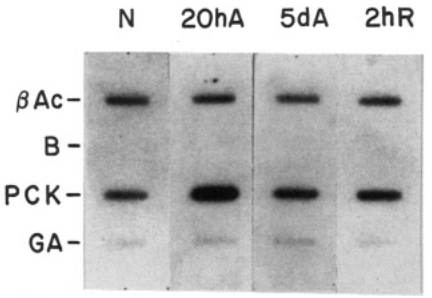

FIGURE 4: Effect of acidosis on the rates of glutaminase, phosphoenolpyruvate carboxykinase, and β-actin transcription. Renal nuclei from normal (N), acute and chronic acidotic rats (20hA and 5dA), and rats following 2-h acute recovery from 5-day chronic acidosis (2hR) were incubated with [α-^{32}P]CTP. The [^{32}P]CTP-labeled nuclear RNAs were hybridized to a filter containing 10 μg of β-actin (βAc), glutaminase (GA), and phosphoenolpyruvate carboxykinase (PCK) antisense RNAs. The blank lane (B) contained no RNA.

Table I: Effect of Onset and Recovery from Acidosis on the Relative Rates of Synthesis of GA and PCK mRNAs by Isolated Rat Renal Nuclei[a]

condition	PCK mRNA	GA mRNA
normal	1.0 ± 0.13	1.0 ± 0.14
20-h acute acidosis	3.2 ± 0.53	1.1 ± 0.37
1-day chronic acidosis	2.2 ± 0.43	0.9 ± 0.23
5-day chronic acidosis	1.8 ± 0.60	1.2 ± 0.12
2-h recovery from chronic acidosis	1.2 ± 0.42	1.0 ± 0.02

[a] Nuclei were isolated from normal rats, from rats that were made acutely or chronically acidotic, and from rats that were made acidotic for 5 days and then acutely treated with $NaHCO_3$ to correct the acidosis. [^{32}P]RNA synthesized in the transcription run-on assays was hybridized simultaneously to PCK, GA, and β-actin antisense RNAs. Specific hybridization was measured relative to that of the β-actin mRNA. The values for the relative rates of PCK and GA transcription were then normalized by dividing by the respective value measured in nuclei isolated from normal rats. Values are the mean ± SD of the data obtained from at least three rats.

transcription of PCK gene was increased approximately 3-fold at 20 h following onset of acute acidosis (Table I). However, the development of chronic acidosis resulted in only a 2-fold increase in the relative rate of transcription of PCK. The increased rate of transcription returned to normal within 2 h following acute recovery from chronic acidosis. In contrast, the relative rate of GA mRNA transcription was unaffected by onset or recovery from chronic acidosis.

Discussion

Northern analysis using either total or poly(A)$^+$ RNA indicates that rat kidney contains two forms of GA mRNA, a more abundant 6.0-kb mRNA and less abundant 3.4-kb mRNA. The relative levels of the two mRNAs are increased or decreased to the same extent in response to onset and recovery from chronic acidosis. Furthermore, when translated in vitro, both mRNAs yield the 72-kDa precursor of the mitochondrial glutaminase. The two forms of GA mRNA could be derived from a single gene by the use of different promoters, the presence of multiple polyadenylation signals, the selection of alternative exons, or a combination of these processes. Alternatively, the two GA mRNAs may be the products of different genes. However, the observation that the 3′-terminal *Eco*RI fragment of pGA-12 hybridizes solely with the 6.0-kb form of GA mRNA suggests that the two mRNAs result from the use of alternative polyadenylation signals.

The fold increases in the levels of total and translatable GA mRNA were greater than the corresponding induction of glutaminase activity. The RNAs used for this analysis were isolated from whole tissue. Thus, the transport of fully processed GA mRNA from the nuclei to the cytoplasm may be rate limiting during chronic acidosis. The accumulation of a nuclear pool of GA mRNA that in vivo is translationally inactive could explain the observed difference between the change in glutaminase activity and the levels of GA mRNA. Alternatively, this difference could be due to an inefficiency of the mitochondrial translocation and processing reactions. The de novo synthesis of a nuclear encoded mitochondrial protein requires the sequential binding of the cytoplasmic precursor to a receptor on the outer membrane of the mitochondria, membrane translocation, removal of the targeting signal by proteolysis, refolding to produce the active conformation, and localization of the mature enzyme to the appropriate compartment within the mitochondria (Schatz & Butow, 1983; Hartl & Neupert, 1990). Degradation of the newly synthesized glutaminase at any of these steps would result in a lower increase in glutaminase activity compared to the increase in GA mRNA.

The initial increase in PCK mRNA that occurs following acute onset of acidosis is due to increased transcription (Hwang & Curthoys, 1991). However, the level of PCK mRNA in rat kidney is increased 6-fold during chronic acidosis. Thus, the observed increase in transcription of the PCK gene can only partially account for the increase in PCK mRNA. Both cAMP and glucocorticoids cause a rapid increase in the levels of hepatic PCK mRNA by stimulating transcription (Lamers et al., 1982). However, both effectors also increase the stability of the PCK mRNA (Hod & Hanson, 1988; Peterson et al., 1989). Thus, it is likely that the mechanism responsible for the initial induction of PCK mRNA during acute acidosis may differ from the one which sustains the increased steady-state level during chronic acidosis.

In contrast to PCK gene expression, the observed rate of GA gene transcription was not affected by alterations in acid–base balance. The rate of renal GA transcription was significantly lower than that of PCK. As a result, the level of radioactivity that hybridized to the GA antisense RNA was too low to quantitate by direct scintillation counting. However, the bands were easily quantitated by densitometry. When the scintillation counting and densitometry methods were used to calculate the increase in the transcription rate of the PCK gene, they yielded identical results. In addition, the synthesis of transcripts that hybridized to the β-actin, glutaminase, and phosphoenolpyruvate carboxykinase antisense RNAs was inhibited by greater than 90% by the addition of a level of α-amanitin that specifically blocks RNA polymerase II activity. These observations support the conclusion that transcription of the GA gene is not increased during metabolic acidosis. The observed difference suggests that the induction of the GA and PCK genes by acidosis may be regulated by different processes. This hypothesis is supported by the observation that following acute onset of acidosis the induction of PCK mRNA and the induction of GA mRNAs occur with different kinetics (Hwang & Curthoys, 1991). The increase in PCK mRNA began immediately and was nearly complete before the pronounced increase in GA mRNA was initiated.

The 3′-nontranslated region of PCK mRNA contains AU-rich regions (Beale et al., 1985). AU-rich sequences have been shown to participate in the selective degradation of a number of transiently expressed mRNAs (Meijlink et al., 1985; Reeves et al., 1987; Shaw & Kamen, 1986; Wilson & Treisman, 1988). The 3′-noncoding region of the GA mRNA also contains varius AU-rich motives and a 32 base pair sequence of alternating AC residues. Thus, the large 3′-nontranslated

region may contain sequences which interact with specific factors that affect the stability of the GA mRNA. The binding of the same factor to the PCK mRNA could coordinate the induction of the two mRNAs during chronic acidosis.

Registry No. GA, 9001-47-2.

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