Specificity and functional analysis of the pH-responsive element within renal glutaminase mRNA

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Laterza, Omar F., and Norman P. Curthoys. Specificity and functional analysis of the pH-responsive element within renal glutaminase mRNA. Am J Physiol Renal Physiol 278: F970–F977, 2000.—The specificity and the functional significance of the binding of a specific cytosolic protein to a direct repeat of an eight-baseAU sequence within the 3′-nontranslated region of the glutaminase (GA) mRNA were characterized. Competition experiments established that the protein that binds to this sequence is not an AUUUA binding protein. When expressed in LLC-PK1-F cells, the half-life of a β-globin reporter construct, βG-phosphoenolpyruvate carboxykinase, was only slightly affected (1.3-fold) by growth in acidic (pH 6.9, 10 mM HCO$_3$) vs. normal (pH 7.4, 25 mM HCO$_3$) medium. However, insertion of short segments of GA mRNA containing the direct repeat or a single eight-baseAU sequence was sufficient to impart a fivefold pH-responsive stabilization to the chimeric mRNA. Furthermore, site-directed mutagenesis of the direct repeat of the 8-base AU sequence was sufficient to impart a fivefold pH-responsive stabilization to the chimeric mRNA. Thus either the direct repeat or a single eight-base AU sequence is both sufficient and necessary to create a functional pH-response element.

LLC-PK1-F cells; proximal tubule; metabolic acidosis; renal ammoniagenesis

METABOLIC ACIDOSIS IS CHARACTERIZED by a decrease in blood pH and HCO$_3$ concentration. Mild forms of acidosis occur in response to a high-protein diet, prolonged exercise, or a sustained fast (14). More severe forms are associated with clinical disorders such as diabetic ketoacidosis, genetic acidurias, and acute renal failure. When metabolic acidosis is sustained and uncompensated, it becomes life threatening. Increased renal ammoniagenesis and gluconeogenesis from glutamine function as a compensatory response to the onset of acidosis (7). In normal acid-base balance, the kidney extracts and catabolizes very little, if any, of the plasma glutamine (27). However, during metabolic acidosis, as much as one-third of the plasma glutamine is extracted during a single pass through the kidney (16, 27). The initial reactions in the primary pathway of renal catabolism of glutamine are catalyzed by the mitochondrial glutaminase (GA) and glutamate dehydrogenase (7). The combined deamidation and deamination reactions yield two ammonium ions and α-ketoglutarate. The ammonium ions serve as expendable cations and are primarily excreted in the urine. This process facilitates the excretion of acids while conserving essential Na$^+$ and K$^+$ ions. The subsequent conversion of α-ketoglutarate to glucose generates HCO$_3$ ions, which are added to the blood and partially compensate the systemic acidosis (2).

During chronic metabolic acidosis, the activity of the rat renal mitochondrial GA is increased 7- to 20-fold (6, 31). This increase occurs solely within the proximal convoluted tubule. The cell-specific increase in activity results from an increased rate of GA synthesis (28) that correlates with an increased level of GA mRNA (17, 18, 29). However, the observed increases occur without increasing the rate of transcription of the GA gene (17, 18). The selective stabilization of the GA mRNA was initially demonstrated by stable transfection of LLC-PK1-F cells (12), a pH-responsive porcine proximal tubule-like cell line, with various β-globin (βG) constructs (15). The parent construct, βG, produced a very stable mRNA that was expressed at high levels in cells grown in normal medium (pH 7.4, 25 mM HCO$_3$). Neither the level of the βG mRNA nor its half-life was affected by transfer of the cells to an acidic medium (pH 6.9, 10 mM HCO$_3$). In contrast, a chimeric construct, βG-GA, which also encodes a 956-base segment of the 3′-nontranslated region of the GA mRNA, was expressed at significantly lower levels when stable transfectants of the LLC-PK1-F$^+$ cells were grown in normal medium. The decreased expression resulted from the more rapid turnover ($t_{1/2}$ = 4.6 h) of the βG-GA mRNA. Transfer of the latter cells to acidic medium resulted in a pronounced stabilization (6-fold) and a gradual induction of the βG-GA mRNA. These studies indicated that the 3′-nontranslated region of the GA mRNA contains a pH-response element (pH-RE).

More recent studies have shown that multiple segments of the GA mRNA function as pH-responsive elements (pH-REs) (20). Experiments using additional chimeric βG constructs indicated that a 340-base segment of the GA mRNA, termed R-2, retained most of the functional characteristics of the 3′-nontranslated region. However, the remainder of the 3′-nontranslated region also served as a weak pH-RE. RNA gel shift analyses were used to identify a 48-kDa protein, which binds with high affinity to the R-2 RNA. Mapping
studies demonstrated that the high-affinity binding site within the R-2 RNA consisted of a direct repeat of an eight-base AU sequence. In the present study, the function of the eight-base AU sequence was further analyzed. The resulting data suggest that either the direct repeat or a single copy of the eight-base AU sequence is necessary and sufficient to function as a pH-RE.

MATERIALS AND METHODS

Materials. [α-{³²P}]dCTP and [α-{³²P}]UTP (specific activity 3,000 Ci/mmol) were purchased from ICN Biochemicals or Amersham. Restriction enzymes, T7 RNA polymerase, and yeast tRNA were acquired from Boehringer Mannheim and New England Biolabs. The oligolabeling kit was from Pharmacia Biotechnology. GeneScreen Plus was purchased from New England Nuclear. GENECLEAN was manufactured by Bio101. Guanidine thiocyanate and sodium-3,000 Ci/mmol) were purchased from ICN Biochemicals or Amersham. Restriction enzymes, T7 RNA polymerase, and yeast tRNA were acquired from Boehringer Mannheim and New England Biolabs. The oligolabeling kit was from Pharmacia Biotechnology. GeneScreen Plus was purchased from New England Nuclear. GENECLEAN was manufactured by Bio101. Guanidine thiocyanate and sodium-

Construction of plasmids. The specificity of the RNA binding was characterized by using transcripts produced from two plasmids, pBS-GA(R-2I) (20) encodes a 29-base segment of the 3'-nontranslated region and polyadenylation site of the GA mRNA. pmβ-G-A was synthesized by using the Quick-Change Site Directed Mutagenesis kit from Stratagene to mutate the two 8-base pH-REs within pβ-G-G. The inserted fragment includes nucleotides 2010–2965 of the GA cDNA that encode the 3'-nontranslated region of the 3.4-kb rat GA mRNA. pmβ-G-G was synthesized by using the Quick-Change Site Directed Mutagenesis kit from Stratagene to mutate the two 8-base pH-REs within pβ-G-G. The inserted fragment includes nucleotides 2010–2965 of the GA cDNA that encode the 3'-nontranslated region of the 3.4-kb rat GA mRNA. pmβ-G-G was synthesized by using the Quick-Change Site Directed Mutagenesis kit from Stratagene to mutate the two 8-base pH-REs within pβ-G-G. The inserted fragment includes nucleotides 2010–2965 of the GA cDNA that encode the 3'-nontranslated region of the 3.4-kb rat GA mRNA. pmβ-G-G was synthesized by using the Quick-Change Site Directed Mutagenesis kit from Stratagene to mutate the two 8-base pH-REs within pβ-G-G. The inserted fragment includes nucleotides 2010–2965 of the GA cDNA that encode the 3'-nontranslated region of the 3.4-kb rat GA mRNA.

In vitro transcription. The templates used to transcribe the R-2I and (AUUU)₈ RNAs were obtained by restricting the pH-RE-BP to the R-2I RNA (20). The PCR reaction was performed by using annealing and elongation temperatures of 50 and 68°C, respectively. The resulting plasmids were initially screened by restricting with Ssp I (a site that is mutated in mβ-G-G) and confirmed by the dideoxyribonucleotide sequencing.

Chimeric βG mRNAs

![Chimeric βG mRNAs](image-url)
described procedure (1). Cytosolic extracts of rat renal cortex were prepared as described previously (20). An aliquot of extract containing 3 µg of protein was preincubated for 10 min at room temperature with 0.5 µg of yeast tRNA in 10 µl of a reaction mixture containing 10 mM HEPES, pH 7.4, 25 mM potassium acetate, 2.5 mM magnesium acetate, 0.5% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and 10 U RNAsin. Then, [32P]-labeled RNA and specified amounts of unlabeled RNAs were added as indicated. The reaction mixture was incubated at room temperature for 20 min, and the samples were then loaded onto a 5% polyacrylamide gel and subjected to electrophoresis at 170 V by using a 90 mM Tris, 110 mM boric acid, 2 mM EDTA running buffer. Gels were dried and exposed to either a film or a PhosphorImager screen. The addition of RNAsin was necessary to maintain the integrity of the probe during the initial incubation with the cytosolic extracts.

Isolation of stable cell lines. LLC-PK₁-F⁺ cells (12) were obtained from Gerhard Gstraunthaler and cultured in a 50:50 mixture of Dulbecco's modified Eagle's and Ham's F₁-12 media containing 5 mM glucose and 10% fetal bovine serum at 37°C in a 5% CO₂-95% air atmosphere. Cell lines expressing the various chimeric mRNAs were produced by transfection with calcium phosphate-precipitated DNA (3). A confluent 10-cm plate of cells was split 1:4 and grown for 24 h. The medium was replaced 1 h before the addition of 20 µg of calcium phosphate-precipitated DNA. The precipitated DNA was allowed to interact with the cells for 18 h, and then the cells were washed twice with 5 ml of phosphate-buffered saline. After washing, 10 ml of selection medium containing 0.5 mg/ml G-418 were added to the growing cells. The medium was changed every 2 days. About 10–14 days later, the G-418-resistant colonies were treated with trypsin and grown in medium containing 0.2 mg/ml G-418.

mRNA half-life analysis. The various transfected LLC-PK₁-F⁺ cell lines were grown for 10–14 days in medium containing 0.2 mg/ml G-418. They were then maintained in medium without G-418 for 24 h and subsequently treated for 8 h in normal or acidic media. At time 0, 65 µM 5–6-dichloro-1-β-ribofuranosyl benzimidazole (DRB), a specific inhibitor of RNA polymerase II transcription (10), was added to each plate. At 3, 6, or 9 h post-DRB treatment, total cellular RNA was isolated as described previously (5). RNA concentrations were determined by measuring the absorbance at 260 nm.

Northern analysis. A 507-bp fragment of rabbit β-globin cDNA was excised from pRSV-β-g (11) with Hind III and Bgl II. A 2.0-kb fragment of the 18S ribosomal RNA cDNA from Acanthamoeba castellanii was excised from pA2 with Hind III and EcoR I (8). The fragments were separated on 1% agarose gels, excised, and purified by using GENECLEAN. The synthesis of oligolabeled cDNA probes and Northern analysis was performed as described previously (15).

RESULTS

Previous experiments have demonstrated that a direct repeat of an eight-base AU-rich sequence within the rat GA mRNA functions as a high-affinity protein binding site (20). Competition studies were conducted to test whether the protein that binds to this sequence is a previously identified AU-rich element binding protein (4). The initial identification of a protein that binds to an AU-rich element was performed by using four contiguous AUUUUA motifs (26). Such motifs function as instability elements in mRNAs that encode various cytokines and immediate early-response proteins (23). At least nine other proteins were subsequently identified to bind to related sequences (4). There are no AUUUA motifs present within the 29-base sequence (R-2I) from the GA mRNA that contains the direct repeat of the 8-base AU-rich elements. However, this segment is very AU rich and may associate with an AUUUUA binding protein. The pBS-AUUUA plasmid was specifically constructed to test this hypothesis. It contains the sequence (ATTT)₅A and thus encodes five contiguous copies of the AUUUUA motif or three overlapping copies of the UUUUUUAAU motif (32). The specificity of the binding was tested by comparing the ability of cold R-2I or (AUUU)₅A RNA to compete the interaction observed with [32P]-labeled R-2I probe (Fig. 2). A 25-fold excess of cold R-2I RNA was sufficient to effectively compete the shifted band. However, no apparent competition was evident even when a 150-fold excess of cold (AUUU)₅A RNA was added. Therefore, the protein that binds to the R-2I RNA apparently is not a previously characterized AU-rich element binding protein that recognizes the various AUUUUA destabilizing motifs.

Studies were performed to establish the functionality of the identified binding site. Either one or two of the eight-base AU-rich sequences were introduced into a chimeric mRNA that has a moderate rate of turnover and is minimally responsive to changes in extracellular pH. The chimeric mRNA chosen for these studies contained the coding region of βG mRNA and the 3'-nontranslated region of PCK mRNA (15). LLC-PK₁-F⁺ cells were stably transfected with pBG-PCK, and the apparent half-life of the βG-PCK mRNA was measured...
The apparent half-life of the βG-PCK mRNA was determined to be 8.5 and 11.3 h in cells grown in normal and acidic media, respectively (Fig. 3). This difference is not a significant pH-responsive stabilization. Thus the βG-PCK mRNA constitutes an appropriate control mRNA for the functional studies.

The R-2H segment of the pGA cDNA was inserted just upstream of the PCK sequence in the βG-PCK plasmid (Fig. 1). This 76-bp fragment extends from position 2574 to position 2649 of the GA cDNA and contains both 8-base AU sequences. The βG-GA(R-2H)-PCK plasmid was stably transfected into LLC-PK1-F+ cells, and the half-life of the chimeric mRNA was measured by using normal and acidic media (Fig. 4). The apparent half-life of the βG-GA(R-2H)-PCK mRNA was 6.0 h in cells grown in pH 7.4 medium, but the mRNA was significantly stabilized when the cells were grown in pH 6.9 medium. Because no significant decrease in the level of the the βG-GA(R-2H)-PCK mRNA was observed after 9 h, it was not possible to calculate an accurate half-life. However, the half-life in pH 6.9 medium must be at least 30 h, which would produce a 20% decrease in 9 h. Thus the insertion of the 76-base segment is sufficient to impart pH-responsiveness to the βG-PCK mRNA.

The R-2F segment of the pGA cDNA was also inserted just upstream of the PCK sequence in the βG-PCK plasmid (Fig. 1). This segment contains 82-bp and extends from the Ssp I site at position 2602 to the BstE II site at position 2683 of the GA cDNA. It contains only the second of the two 8-base AU-rich sequences of the pH-RE. Again, the half-life of the βG-GA(R-2F)-PCK mRNA in cells grown in normal medium was 5.7 h and was increased to >30 h by transfer of the cells to acidic medium (Fig. 5). Thus a single eight-base AU sequence is also sufficient to act as an effective pH-RE.

The two 8-base AU-rich elements encoded within the βG-GA cDNA were mutated to further assess their
role in mediating the increased stability of the β-GA mRNA that occurs in response to a decrease in extracellular pH. Both the wild-type and the mutated β-GA constructs were transfected into LLC-PK₁F₁ cells that were derived from the same split. The half-life of the β-GA mRNA in cells grown in pH 7.4 media was 5.8 h, whereas the half-life increased to 15 h when the cells were transferred to acidic medium (Fig. 6). Thus a 2.6-fold pH-responsive stabilization of the half-life of the β-GA mRNA was observed. The mutated β-GA mRNA had a half-life of 7.0 h in LLC-PK₁F₁ cells grown in normal medium, and it remained unchanged in cells grown in acidic media (Fig. 7). Thus the mutation of the two 8-base AU sequences completely abolished the stabilization of the β-GA mRNA. This result indicates that the pH-RE is necessary for the
Table 1. Comparison of the apparent half-life values of the various chimeric \( \beta \)-globin mRNAs

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>pH 7.4</th>
<th>pH 6.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta )-G-PCK</td>
<td>8.5</td>
<td>11.3</td>
</tr>
<tr>
<td>( \beta )-G-GA(R-2H)-PCK</td>
<td>6.0</td>
<td>30</td>
</tr>
<tr>
<td>( \beta )-G-GA(R-2F)-PCK</td>
<td>5.7</td>
<td>30</td>
</tr>
<tr>
<td>( \beta )-GA</td>
<td>5.8</td>
<td>15</td>
</tr>
<tr>
<td>m( \beta )-GA</td>
<td>7.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

\( \beta \)-G, \( \beta \)-globin; PCK, phosphoenolpyruvate carboxykinase; GA, glutaminase.

The data for the experiments that test the function of the pH-RE are summarized in Table 1.

### DISCUSSION

In previous studies (15, 20), a \( \beta \)G reporter construct, which encodes a very stable mRNA, was used to demonstrate that the 3'-nontranslated region of the GA mRNA contains both an instability element and a pH-RE. To determine whether shorter segments of the GA mRNA function as the pH-RE, it was necessary to utilize a reporter construct that encodes a mRNA that has a moderate half-life. Thus the \( \beta \)G-G-PCK expression vector (15) was used to determine whether the eight-base AU sequences from the GA mRNA can function as a pH-RE. When stably transfected into LLC-PK1-F \(^{+} \) cells, this vector expresses a high level of \( \beta \)-G-PCK mRNA, a chimera that contains the coding region of \( \beta \)-globin mRNA and the 3'-nontranslated region of the PCK mRNA. The level of the transgenic mRNA can be readily quantified by using a \( \beta \)G cDNA probe without interference from the endogenous PCK mRNA. Furthermore, the \( \beta \)-G-PCK mRNA has a relatively rapid half-life that is only slightly stabilized when the transfected cells are transferred to acidic medium (15). In the present study, only a 30% increase in the stability of the \( \beta \)-G-PCK mRNA was observed. Thus the \( \beta \)-G-PCK mRNA exhibits the properties necessary to study the function of a pH-RE.

Surprisingly, the insertion of either the direct repeat of the eight-base AU sequence or only the second of the two elements produced an identical pH-responsive stabilization. The \( \beta \)-G-GA(R-2H)-PCK and the \( \beta \)-G-GA(R-2F)-PCK mRNAs were both degraded with a half-life of \( \sim 6 \) h in cells maintained in pH 7.4 medium. When the cells were transferred to pH 6.9 medium, both half-lives were increased to \( > 30 \) h. Thus the insertion of only a single eight-base AU sequence is sufficient to function as an effective pH-RE. Mutation of either eight-base AU sequence to introduce five G and C bases greatly reduced the binding activity of the pH-REBP in RNA gel shift experiments (20). However, binding to the R-2F RNA, which contained only the second of the eight-base AU sequences, was only slightly reduced compared with the binding observed with the R-2H RNA. The results of the functional assay are consistent with the latter observation. Therefore, the context of the eight-base AU sequence may significantly affect the binding properties and function of the pH-RE.

In a second set of experiments, the identical mutations used in the previous binding studies (20) were introduced into the direct repeat of the pH-RE encoded in the p\( \beta \)G-GA plasmid. In total, 10 of the 16 A and U residues were converted to G and C residues. Because the size of the encoded mRNA was not altered by the mutagenesis, any observed changes in the pH responsiveness could not be due to alterations in the length or relative positioning of elements within the chimeric mRNA. In the present study, the half-life of the \( \beta \)-GA mRNA was 5.8 h in cells grown in normal medium, and it increased to 15 h in cells grown in acidic medium. This reflects a 2.6-fold increase in stability, which is slightly less than previously observed (15). However, the observed stabilization was both significant and sufficient to study the effects of the mutation. The difference in the observed half-lives of the \( \beta \)-GA mRNA in the two studies could be due to the use of cells with a different split number or to slight changes in growth conditions. To control such variables, the wild-type and the mutated p\( \beta \)G-GA plasmids were transfected into cells that were split the same number of times in culture and that were grown under identical conditions.

The half-life of the m\( \beta \)-GA mRNA (7.0 h) in cells grown in normal medium is only slightly greater than the half-life of the wild-type \( \beta \)G-GA mRNA (6.0 h). This observation suggests that the pH-RE contributes very little to the inherent instability of the GA mRNA. This hypothesis is consistent with the effect of insertion of different segments of the 3'-nontranslated region of the GA mRNA on the stability of various reporter mRNAs. The insertion of large segments of GA mRNA sequences caused a reduction of the half-life of \( \beta \)G mRNA from \( \sim 30 \) to 4–7 h (15). In contrast, insertion of the R-2H and R-2F segments into the \( \beta \)-G-PCK mRNA only reduced stability from 8.5 to 6.0 h. However, mutation of the two 8-base AU sequences caused the complete loss of a pH-responsive stabilization of the \( \beta \)G-GA mRNA. The half-life of m\( \beta \)-GA mRNA was 7.0 h in cells grown in either normal or acidic medium. Thus the two 8-base AU sequences are necessary to impart a pH-responsive stabilization to the \( \beta \)-GA mRNA.

All of the functional studies were performed by using LLC-PK1-F \(^{+} \) cells, a pH-responsive line of porcine proximal tubule-like cells (12). These cells express two distinct GA mRNAs, which contain different 3'-nontranslated regions (22). Recent studies indicate that the levels of only the 4.5-kb porcine GA mRNA are increased when the LLC-PK1-F \(^{+} \) cells are transferred to acidic medium and that this increase results from a stabilization of the mRNA (13). Thus one would predict that the 3'-nontranslated region of the 4.5-kb porcine GA mRNA contains a pH-RE. Unfortunately, this segment of the 4.5-kb GA mRNA has not, as yet, been cloned and sequenced.

Two GA mRNAs are expressed in rat kidney (17), a 3.4-kb mRNA and a more abundant 4.7-kb mRNA. The previous studies to identify the pH-RE (15, 20) have focused solely on the 3'-nontranslated region of the 3.4-kb GA mRNA. A search for the direct repeat of the pH-RE (UUUAAAAAUUUAAAAUA) within the remain-
order of the 3'-nontranslated region that is unique to the 4.7-kb rat GA mRNA revealed no homologous sequences. However, two separate eight-base pH-REs were found within this sequence. Both of the putative pH-REs contained a single mismatch from the identified eight-base pH-RE. The levels of both the 3.4- and 4.7-kb GA mRNAs are coordinately induced and repressed during onset or recovery from acidosis, respectively (17, 18). Thus the direct repeat of the pH-REs that is located within the portion of the 3'-nontranslated region that is common to both forms of the GA mRNA probably acts as the primary cis-acting element. The individual pH-REs within the 3'-nontranslated region of the rat GA mRNA probably act as redundant sites that enhance the pH-responsive stabilization.

The pH-RE may mediate the stability of other mRNAs that are also induced in response to onset of metabolic acidosis. For example, rat renal glutamate dehydrogenase (GDH) activity is also increased in the proximal convoluted tubule in response to metabolic acidosis (30). The increase in the level of GDH mRNA occurs with kinetics similar to that observed for the GA mRNA (19). The 3'-nontranslated region of the GDH mRNA (9) contains four AU-rich 8-base sequences that have an 88% identity to either of the two pH-REs that constitute the direct repeat within the GA mRNA. Preliminary experiments indicate that the pH-RE binding protein partially purified from cytosolic extracts of rat renal cortex binds with high affinity to two of the four AU-rich elements within the 3'-nontranslated region of the GDH mRNA (J. Schroeder, A. Tang, and N. P. Curthoys, unpublished observations). Thus it will be interesting to determine whether either or both of these sequences can also function as a pH-RE.

This work was supported by Public Health Service Grant DK-37124 from the National Institute of Diabetes and Digestive and Kidney Diseases.

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Received 16 September 1999; accepted in final form 17 January 2000.

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