Identification of an mRNA-binding Protein and the Specific Elements That May Mediate the pH-responsive Induction of Renal Glutaminase mRNA*

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Various segments of the 3′-nontranslated region of the renal glutaminase (GA) mRNA were tested for their ability to enhance turnover and pH responsiveness. The combined effects were retained in the 340-base R-2 segment. However, the combined R-1 and R-3 fragments also imparted a partial destabilization and pH responsiveness to a chimeric β-globin mRNA. RNA electrophoretic mobility shift assays indicated that cytosolic extracts of rat renal cortex contain a protein that binds to the R-2 and R-3 RNAs. The binding observed with the R-2 RNA was mapped to a direct repeat of an 8-base AU region of the GA mRNA and mediate its pH-responsive stabilization.

Increased catabolism of glutamine is an important component of the renal adaptation that occurs in response to onset of metabolic acidosis (1). In normal acid-base balance, the kidney metabolizes very little of the plasma glutamine (2). In contrast, during metabolic acidosis, approximately one-third of the arterial glutamine is extracted in a single pass through this organ. The increased renal catabolism of glutamine yields ammonium ions, which are largely excreted in the urine to facilitate the excretion of acids. The further catabolism of the carbon skeleton of glutamine generates bicarbonate ions, which are added to the blood to partially compensate the decrease in pH. The mitochondrial glutaminase catalyzes the initial reaction in the primary pathway for renal metabolism of glutamine (3). During chronic acidosis, the level of this activity is increased 7–20-fold within the rat renal proximal convoluted tubule (4, 5). The increased activity results from an increased rate of glutaminase synthesis (6), which correlates with an increase in the level of total and of translatable GA1 mRNA (7–9). However, the increased level of GA mRNA is not due to an increased rate of transcription (8, 9). These results suggested that increased expression of the GA gene may result from the selective stabilization of the GA mRNA.

Rat kidney contains two forms of GA mRNA, a 5.0-kilobase and a less abundant 3.4-kilobase mRNA (8, 9). The smaller GA mRNA is probably synthesized by use of an internal polyadenylation site that is located at position 2984 in the GA cDNA (10). The levels of both GA mRNAs are coordinately affected in response to onset and recovery from metabolic acidosis (8, 9). Recent studies have indicated that a 955-bp segment, which comprises the portion of the 3′-nontranslated region that is common to both GA mRNAs, contains a pH-responsive instability element (11). This was demonstrated by cloning this segment into the 3′-nontranslated region of a gene that encodes a β-globin/growth hormone (βG) mRNA. The parent βG construct produced high levels of a very stable βG mRNA when expressed in LLC-PK1-F cells, a pH-responsive line of pig renal proximal tubule-like cells (12, 13). The level of the βG mRNA was unaffected by transferring the cells to acidic medium (pH 6.9, 10 mM HCO3−). In contrast, the chimeric construct, βG-GA, which contains the additional GA cDNA sequence, was expressed at significantly lower levels in stable transfectants of LLC-PK1-F cells grown in normal medium (pH 7.4, 25 mM HCO3−). The decreased expression resulted from the more rapid turnover (t1⁄2 = 4.6 h) of the βG-GA mRNA. The transfer of the latter cells to acidic medium resulted in a pronounced stabilization and a gradual induction of the βG-GA mRNA.

In the current study, the apparent half-lives and the pH responsiveness of various deletions of the βG-GA construct were characterized. RNA gel shift analyses were also performed to further map the binding sites of a 48-kDa GA mRNA binding protein that is contained in cytosolic extracts of rat renal cortex. The apparent binding of this protein is decreased significantly in extracts prepared from acutely acidic rats. The results of the two approaches indicate that redundant AU-rich binding elements mediate the inherent instability and the pH-responsive stabilization of the GA mRNA.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dATP and [α-32P]UTP (specific activity 3000 Ci/mmole) were purchased from ICN Biochemicals or Amersham Corp. Restriction enzymes, T7 polymerase, and RNase T1 were acquired from Boehringer Mannheim and New England Biolabs. Oligolabeling kit was

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1 The abbreviations used are: GA, glutaminase; βG, β-globin; PCR, polymerase chain reaction; BSA, bovine serum albumin; bp, base pair(s).
from Pharmacia Biotechnology. GeneScreen Plus was purchased from NEN Life Science Products. Gel-blotting paper was purchased from Schleicher and Schuell. RNAsein was from Promega. Sequenase version 2.0 and agarose-LMP were obtained from U.S. Biochemical Corp. Dulbecco’s modified Eagle’s medium/F12 medium and Genetitin were produced by Life Technologies, Inc. Guanidinoacetate and sarcosyl-lauryl sarcosine were obtained from Fluka. Tissue culture plates were purchased from Dow Corning. All other biochemicals were purchased from Sigma.

Cell Culture—LCL-PK,-F" cells were obtained from Gerard Getraunthaler (12). Cells were cultured as described previously (13) using 10% FCS. Dulbecco’s modified Eagle’s and Ham’s F12 media containing 5 mM glucose and 10% fetal bovine serum at 37 °C in a 5% CO2 atmosphere. Cell lines expressing the various β-GA chimeric mRNAs were produced by transfection with calcium phosphate-precipitated DNA (14) and selection with Geneticin. Total RNA was isolated from LCL-PK,-F" cells according to the procedure of Chomczynski and Sacchi (15), and mRNA half-life determinations and Northern analyses were performed as described previously (11).

Synthesis of pβG-GA Constructs—The initial β-globin expression vector (pβG) was created by ligating a Pou/Ub/III fragment of the rabbit β-globin gene into the HindIII site of pRe/RSV (Invitrogen). Thus, pβG contains the strong viral promoter derived from the long terminal repeat of the Rous sarcoma virus followed by the transcription start site, the 5′-nontranslated region, the full coding sequence, and two introns of the rabbit β-globin gene; a multicloning site containing four unique restriction sites; and the 3′-nontranslated region and polyadenylation site of the bovine growth hormone gene (11). pβG-GA was created by cloning a 955-bp AccI/DrdI restriction fragment from pGA-12 (10) into the multicloning site of pβG. The insert GA cDNA fragment contains 72 bp of coding sequence and extends to a site 18 bp 5′ of the internal polyadenylation signal (Fig. 1). The initial set of deletion constructs (pβG-GA9, pβG-GA2684, and pβG-GA) was generated by PCR amplification of the desired sequences within pGA-12 (10). The 5′ primer (primer 2175, 5′-CAGCTAGATT-TACAACTATTGCTCTCCGG-3′) and the 3′ primer (primer 2985, 5′-ACGTCTTAGTTGTTGAACTGAGACCC-3′) were used to synthesize pβG-GA9, which contains bases 2175–2985 of the 3′-nontranslated region of the GA cDNA (Fig. 1A). The two primers contained SpeI and XhoI restriction sites, respectively (in boldface type). Similarly, the cDNA insert for pβG-GA2684, which extends from base 2017 to 2866 was synthesized using primer 2015 (5′-AGAGCAGAATGG-ACTAGT-3′) and primer 2884 (5′-CAGCTAGATTGTTGAACTGAGACCC-3′). Finally, pβG-GA3, which extends from base 2175 to 2866 was synthesized using primer 2175 (see above) and primer 2884 (5′-CAGCTAGATTGTTGAACTGAGACCC-3′). In each case, the primers were designed to have similar Tm values. For the PCR amplification, the primers were diluted into a final concentration of 0.2 μM, and the pGA-12 cDNA template was used at a final concentration of 0.27 ng/ml. The fragments produced from the PCR reaction were purified from a 1% low melting temperature agarose gel, digested with BsaII and PvuII, and reamplified by PCR amplification with BsaII and the internal restriction enzyme, and ligated into the respective sites in the multicloning region of pβG. The remaining constructs were produced by deletion of sequences from pβG-GA9, which contained GA sequence from bases 2344–2883. pβG-GA9 contained the same sequence as pβG-GA3 but was inserted in the reverse orientation. The validity of all constructs was verified by restriction mapping andideoxy nucleotide sequencing.

Rat Renal Cortical Cytoplasmic Extracts—Rats were made acutely acidotic by stomach-loading them with 20 mmol of NH4Cl/kg body weight. Normal and 16-h acutely acidotic rats were anesthetized with 1 mg/kg body weight of pentobarbital and opened with a midline incision. The kidneys were perfused in situ with a Krebs-Henseleit solution. They were removed immediately, decapsulated, sliced longitudinally, and placed in ice-cold Krebs-Henseleit solution. The cortex was dissected from the papilla and the medulla, cut into small pieces, weighed, and placed in an equal volume of 40 mM Hepes, pH 7.4, containing 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 10 μg/ml antipain, 2 μg/ml trypsin, and 1 mM phenylmethylsulfonyl fluoride. The tissue was homogenized using a Dounce homogenizer. An aliquot of homogenate was examined microscopically for released nuclei and then centrifuged for 10 min at 1,000 × g to pellet intact cells and nuclei. The supernatant was centrifuged at 10,000 × g for 10 min to pellet the mitochondria and then centrifuged for 2 h at 100,000 × g to pellet membrane bound organelles and polyribosomes. The final supernatant

pH-responsive Instability Element of GA mRNA

**FIG. 1. Schematic representation of the 955-bp AccI/DrdI fragment of the GA cDNA.** A, the PCR 5′- and 3′ (-) primers and the indicated restriction sites that were used to generate the various deletion constructs of pβG-GA are positioned according to scale. All of the 5′ primers encoded an SpeI site, while the 3′ primers contained an XhoI site. TAA, stop codon; pA, internal polyadenylation site. B, various fragments of GA cDNA were cloned into pBlueScript. The indicated restriction sites were used to terminate the transcription of the three non-overlapping segments of RNA, (R-1, R-2, and R-3) that were used as probes for the initial RNA gel shift assays. The lengths of GA sequences in each RNA are indicated in bases (b).
RESULTS

A pH-responsive stabilization of the βG-GA chimeric mRNA, which contains 955 bases of the 3'-nontranslated region of the GA mRNA, was observed in LLC-PK1-F-"F" cells (11). Six deletion constructs were synthesized and tested to further delineate the region that mediates this response (Fig. 2). The separate cell lines, when grown in normal medium (pH 7.4, 25 mM HCO₃⁻), expressed levels of the chimeric mRNAs that were similar to that of the full-length βG-GA mRNA. In addition, the levels of all six mRNAs were increased 1.5–2-fold when the cells were maintained in acidic medium (pH 6.9, 10 mM HCO₃⁻) for 18 h (data not shown). The apparent half-lives of the chimeric mRNAs were measured by selectively inhibiting polymerase II transcription with 65 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (21). The parent βG mRNA was very stable, and its rate of turnover was unaffected when the LLC-PK1-F-"F" cells were transferred to acidic medium (11). In contrast, the βG-GA mRNA, which contains the original 955-base insert, decayed with a half-life of 4.6 h in cells grown in normal medium. Following transfer to acidic medium, the apparent half-life of the βG-GA mRNA was increased approximately 6-fold (t₁/₂ = 29 h). The chimeric mRNAs produced from the βG-GA-G, βG-GA₂, and βG-GA₃ constructs contain deletions of either or both of the putative stem-loop structures previously identified in the GA mRNA (10). All three mRNAs exhibited a rapid turnover in cells grown in normal medium (t₁/₂ values range from 5.2 to 6.7 h) and a significant stabilization when transferred to acidic medium (t₁/₂ values range from 15 to 29 h). Thus, neither of the two putative stem-loop structures is the primary determinant of the rapid turnover or the pH-responsive stabilization of the GA mRNA.

βG-GA₄ contains two segments of GA mRNA sequence that extend from base 2175 to 2343 and base 2684 to 2894. This mRNA was destabilized in cells grown in normal medium (t₁/₂ = 8 h) and exhibited a slight pH response (1.9-fold). In contrast, βG-GA₅, mRNA which contains only the bases 2344–2683, i.e. the bases deleted in the βG-GA₄ construct, was also significantly destabilized (t₁/₂ = 8 h) and exhibited a strong pH response (3.8-fold). Interestingly, when this sequence was inserted into βG in the reverse orientation, the resulting βG-GA₅ mRNA was less effectively destabilized (t₁/₂ = 15 h) but was still slightly pH-responsive (1.8-fold). These results suggest that the 3'-nontranslated region of the GA mRNA contains multiple functional elements that contribute to its inherent lability and pH-responsive stabilization.

RNA gel shift analyses were performed to determine if cytosolic extracts of rat kidney cortex contain a protein that exhibits specific binding to the 3'-nontranslated region of the GA mRNA. Initial experiments were performed using three RNAs, R-1, R-2, and R-3, which correspond to bases 2009–2343, 2344–2683, and 2684–2963 of the GA mRNA, respectively (Fig. 1B). Due to the length of the RNA probes, it was necessary to digest the free RNA and the RNA-protein complexes with RNase T1 before separating them by electrophoresis on a native polyacrylamide gel. When R-1 was used as a labeled probe, a minor band was detected, which exhibited a greater electrophoretic mobility than the undigested probe. However, this band is nonspecific because it was effectively competed by an excess of unlabeled RNA. The half-lives of the chimeric mRNAs expressed in LLC-PK1-F-"F" cells, when grown in normal medium, were similar to the half-life of the chimeric mRNAs that were incubated in acidic medium (t₁/₂ values range from 5.2 to 6.7 h) and a significant stabilization when transferred to acidic medium (t₁/₂ values range from 15 to 29 h). Thus, neither of the two putative stem-loop structures is the primary determinant of the rapid turnover or the pH-responsive stabilization of the GA mRNA.

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binding of the R-2 RNA. Furthermore, the amount of the bound R-2 probe was increased with increasing amounts of the cytosolic extract (data not shown). When labeled R-3 was incubated with the renal cortical extract, two discrete bands were observed (Fig. 3B). These bands were also specific. They exhibited a mobility different from the undigested probe and they were competed by a 500-fold excess of unlabeled R-3. They were not competed by unlabeled R-1 or the nonrelated RNA obtained from pBlueScript. However, they were effectively competed by R-2. Competition with increasing amounts of R-2 and R-3 indicated that the observed bands were more effectively competed with lower amounts of R-2 than with itself (data not shown). Thus, this segment apparently contains two specific binding sites that have lower affinity for the same protein that binds to R-2.

Cytoplasmic extracts were also prepared from the renal cortex of rats that were made acutely acidotic. The apparent binding of the labeled R-2 and R-3 probes to cytosolic proteins from normal and acutely acidotic rats was then compared (Fig. 4). The labeled R-2 probe produced a significant shift when incubated with 1.5 μg of the normal cytosolic extract. The intensity of this shift was nearly doubled when 3.0 μg of extract was used. In contrast, no specific binding was evident when 1.5 μg of the extract from an acutely acidotic rat was used. A slight binding was detectable with 3.0 μg of the latter extract. In contrast, with the labeled R-3 probe, the apparent binding responsible for the larger of the two shifted bands was reduced 2-fold in acidotic versus normal extracts, whereas the binding responsible for formation of the smaller band was apparently unchanged. Thus, the onset of acidosis causes a large decrease in the apparent binding to the R-2 RNA but only a slight decrease in the intensity of only one of the two bands observed with the R-3 RNA.

Additional RNA probes, R-2A through R-2I, were generated to further map the site of protein binding to the R-2 segment of the GA mRNA (Fig. 5). The full banding pattern was retained in the first three constructs, which deleted increasing lengths of sequence from the 5’-end of R-2. The R-2D probe, which contained the sequence deleted in the R-2A through R-2C probes, and the R-2E probe, which lacked only the 82-base GA sequence from the 3’-end of R-2, failed to exhibit any protein binding. Based upon these data, it was anticipated that the specific binding was limited to the region containing the final 82 bases of the R-2 sequence. However, when this sequence (R-2F) was tested, only a less intense band that has a slower mobility was produced. Thus, three additional probes (R-2G through R-2I) were tested. All of the latter constructs including R-2I, which contains a 29-base sequence from GA, retained the initial banding pattern. Probes R-2F and R-2I were effectively competed with a 250- or 500-fold excess of unlabeled R-2F and R-2I RNAs, respectively, but only slightly competed with the same excess of unlabeled R-3A RNA. They were not competed with an unrelated 96-base RNA obtained from pBlueScript (data not shown). These results indicate that the high affinity and specific binding characteristic of the R-2 probe occurs within the 29-base GA sequence of the R-2 RNA.

As shown in Fig. 6A, R-2I RNA contains a direct repeat of an 8-base AU sequence in which 7 of the 8 bases are identical. To determine if the AU sequences constitute the binding element, three additional 29-base RNA sequences, which contain GC substitutions of 5 bases in the center of the first, second, or both AU regions, respectively, were synthesized. Mutation of either AU sequence greatly reduced the specific binding of the rat cytosolic protein (Fig. 6B). When both sites were mutated, no specific binding was detectable. Thus, the two 8-base AU sequences may act synergistically to create the cis-acting element

### A. R-2 Probe

| BSA   | Extract | Competitor |
|-------|---------|------------|
|       | - + + + + |
| -     | - + + + + |
| -     | - R-1 R-2 R-3 NS |

### B. R-3 Probe

| BSA   | Extract | Competitor |
|-------|---------|------------|
|       | - + + + + |
| -     | - + + + + |
| -     | - R-1 R-2 R-3 NS |

**Fig. 3.** RNA gel shift analysis of GA mRNA binding proteins. A. 32P-labeled R-2 RNA was synthesized by in vitro transcription and incubated in the presence (+) of 3 μg of BSA or 3 μg of a cytosolic extract of rat renal cortex. The incubations were performed in the absence or presence of a 500-fold excess of unlabeled competitor RNA. The competitor RNAs included the R-1, R-2, and R-3 segments of the 3’-nontranslated region of GA mRNA and a nonspecific (NS) RNA synthesized from pBlueScript. All samples were treated with RNase T1, separated by electrophoresis on a nondenaturing polyacrylamide gel. The gel was then dried and exposed either to x-ray film or a PhosphorImager screen. B, analysis was performed as described in A, except R-3 RNA was used as the 32P-labeled probe.

undigested probe and represented specific binding. The band was effectively competed with a 500-fold excess of unlabeled R-2 but was not competed by the same concentration of R-1 or the nonrelated RNA obtained from pBlueScript. The addition of a 500-fold excess of unlabeled R-3 RNA slightly reduced the
for the binding of a specific rat renal cytosolic protein.

To identify the protein that binds to the R-2 segment, UV cross-linking experiments were performed (Fig. 7). Five of the 32P-labeled R-2 probes that exhibit specific binding (Fig. 5) were incubated with 3 μg of a rat renal cortical cytosolic extract or with 3 μg of BSA, digested with RNase T1, and exposed to UV light. The complexes were then subjected to SDS-polyacrylamide gel electrophoresis. Four of the probes, including the 76-base R2-H, produced a single 48-kDa protein-RNA complex. In contrast, the R2-F probe produced a complex, which has a slightly greater mobility. No band was observed when the 32P-labeled R-2B probe was incubated with BSA. Thus, a single protein is responsible for producing the complex banding pattern characteristic of the R-2 probes.

The probes R3-A through R3-D were synthesized to further map the specific binding that occurs within the R-3 segment of the GA mRNA (Fig. 8). The R-3A probe, which contains the initial 108 bases of the R-3 segment, produced a single sharp band that has a mobility that is different from either of the bands observed with the R-3 segment. Despite this difference, the binding to this segment was still specific. It was competed with a 250- or 500-fold excess of the R-2H and R-3A probes, but it was not competed by the unrelated 96-base RNA obtained in contrast, the R2-F probe produced a complex, which has a slightly greater mobility. No band was observed when the 32P-labeled R-2B probe was incubated with BSA. Thus, a single protein is responsible for producing the complex banding pattern characteristic of the R-2 probes.

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from pBlueScript (data not shown). The binding pattern that is characteristic of the R-3 segment was retained in the R-3B probe. When this segment was further subdivided, the characteristic binding pattern was still retained in the R3-D probe, whereas the R3-C probe failed to exhibit any binding. The binding observed with the R-3D RNA was also specific. It was competed effectively by either the R-2H or R-3D RNAs, but it was not competed by the unrelated RNA from pBlueScript (data not shown). Thus, the final 84-base GA sequence of the R-3 probe contains the site that is responsible for the protein binding characteristic of this segment. Attempts to UV-cross-link any of the various 32P-labeled R-3 probes failed to label any of the proteins contained in a rat renal cortical extract (data not shown).

**DISCUSSION**

The rate of mRNA turnover is an important factor in the regulation of gene expression (22). The time required to adjust the level of an mRNA to a new steady state is primarily dependent upon the rate of mRNA turnover (23). Thus, the rapid induction of an mRNA is feasible only if the mRNA has a relatively short half-life. The mRNAs that encode immediate early response proteins have half-lives of less than 1 h (24). This rapid degradation is due to AUUUA sequences that are located in U-rich areas of the 3' nontranslated regions (25) and to specific instability elements contained in the coding regions (26, 27) of the mRNAs. In addition, various sequence elements have been identified that participate in altering the stability of specific mRNAs in response to physiological stimuli. For example, the iron response element is a stem-loop structure that is repeated five times within the 3' nontranslated region of the transferrin receptor mRNA (28). During iron sufficiency, the iron response element-binding protein binds iron in an iron-sulfur complex (29). The resulting change in conformation reduces its ability to bind to the iron response elements and thus permits the rapid degradation of the transferrin receptor mRNA. Deletion analysis identified a 250-base segment as the minimal functional element that retained the ability to impart both rapid degradation and iron-responsive regulation to a truncated transferrin receptor mRNA (30). This segment contained three iron-response elements and four additional stem-loop structures, which may serve as instability elements. The cAMP- and differentiation-dependent induction of the Na⁺/glucose transporter in epithelial cells is also due to stabilization of its mRNA (31). However, this response is mediated by a U-rich element which is contained in the 3' nontranslated region of the Na⁺-coupled glucose transporter 1 mRNA (32). The apparent affinity of a protein that exhibits specific binding to this sequence is increased in response to a cAMP-dependent protein phosphorylation.

The rat renal glutaminase is expressed in various cells located in the proximal, distal, and collecting duct segments of the nephron (4, 5). However, in response to acidosis, the enzyme is induced solely in the S1 and S2 segments of the proximal convoluted tubule. Recent studies suggest that this cell-specific induction is due to a pH-responsive instability element
that is contained in the 955-base segment that constitutes the 3’-nontranslated region of the smaller GA mRNA (11). The introduction of this sequence into a β-globin reporter gene construct produced a chimeric βG-GA mRNA, which exhibits a pH-responsive stabilization and induction when expressed in LLC-PK1-F⁺ cells, a pH-responsive pig renal proximal tubule-like cell line (13, 33). In contrast, the level of βG-GA mRNA expressed in COS-7 renal fibroblasts was unaffected by changes in medium pH (11). Furthermore, the expression of a βG-phosphoenolpyruvate carboxykinase construct, which contains the 3’-nontranslated region of the phosphoenolpyruvate carboxykinase mRNA, in LLC-PK1-F⁺ cells was also unaffected by transfer to acidic medium. The latter experiment is an important control, since the pH-responsive induction of the endogenous phosphoenolpyruvate carboxykinase mRNA is due primarily to an increased rate of transcription (8, 9, 34).

Six deletions of the pβG-GA construct were designed to further map the pH-responsive region of the GA mRNA. The first three constructs established that neither of the previously identified stem-loop structures of the GA mRNA (10) contributes significantly to its basal instability or pH-responsive stabilization. The βG-GA₅ mRNA also retained properties that are nearly identical to those observed with the βG-GA mRNA. This observation suggests that the primary cis-acting element that mediates these effects is contained within the 340-base sequence between positions 2344 and 2683 of the GA mRNA. This segment contains a single AUUUA sequence and two separate AU-rich regions (10). The AUUUA sequence was unlikely to contribute to the basal instability of the GA mRNA, since it lacks a U-rich flanking region, which is characteristic of functional AUUUA elements (26). The pβG-GA₅ construct contains a GA sequence, which is the inverted complement of that contained in pβG-GA₆. The resulting βG-GA₅ mRNA exhibits a 2-fold lower basal instability and a reduced pH-responsive stabilization when compared with the βG-GA₆ mRNA. However, neither effect is completely lost in the antisense construct. These observations suggest that both specific sequences and unique secondary structures may contribute to the binding determinants of the respective cis-acting element. The pβG-GA₄ construct contains the sequence from pβG-GA₅ that is deleted in the pβG-GA₆ and pαG-GA₆ constructs. Surprisingly, the βG-GA₄ mRNA also exhibited a basal instability (t₁/₂ = 8.1 h) that is comparable with βG-GA₅ and a slight pH responsiveness. Thus, the 3’-nontranslated region of the GA mRNA must contain redundant instability elements, one of which is contained within the 340-base segment of the βG-GA₆ mRNA, and a second element that is located in one of the two segments of βG-GA₄.

The R-2 RNA, which contains the 340-base GA sequence found in the βG-GA₅ mRNA, exhibits a high affinity and specific binding to a unique rat renal cortical cytosolic protein. Because of the size of the R-2 RNA, the detection of specific binding required the prior digestion of the RNA-protein complex with RNase T1. Thus, the broad binding pattern may result from the retention of various lengths of RNA. However, the observed pattern is highly reproducible using either the same extract or extracts prepared from eight different rats. In contrast, the preceding R-1 region, which contains 335 bases, does not exhibit specific binding to any protein contained in the cytosolic extract.

The RNA gel shift patterns produced by the R-3 RNA, which contains the 280 bases that are 3’ to the R-2 sequence, are also very interesting. This sequence produced two well-resolved bands. Given this resolution, these bands are likely to represent complexes that are formed with different proteins or with the same protein binding to different sites. However, both bands are competed effectively with an excess of unlabeled R-2 or R-3 RNAs. The finding that R-2 competes more effectively suggests that the elements within R-3 bind the same protein as R-2 but with a lower affinity. Thus, the R-2 and R-3 sequences contain redundant but not identical elements.

The binding element within the R-2 RNA was mapped to two direct repeats of an 8-base AU sequence. The partial loss of the protein binding observed with the R-2F RNA initially suggested the presence of a protein binding site at or near the SspI restriction site. Only the first of the two 8-base AU-sequences is disrupted by SspI digestion (Fig. 6A). The partial loss of binding to the R-2 site would occur if both of the AU sequences were required for maximal binding. This hypothesis was confirmed by the mutation analysis, which established that disruption of either AU sequence greatly reduced the specific binding, whereas total binding was lost by disruption of both sequences. R-2E RNA failed to exhibit specific binding apparently because it contains only a portion of one 8-base AU-sequence.
Again, the SapI restriction enzyme used to produce the R-2E RNA cuts within the initial AU sequence.

UV cross-linking studies suggest that a single protein binds to the R-2 region. Probes R-2B, R-2C, R-2G, and R-2H, which produced the same binding pattern in a native acrylamide gel, also formed the same 48-kDa RNA-protein complex following UV cross-linking and separation on an SDS gel (Fig. 7). The same complex was also formed with the 29-base R-2I RNA (data not shown). The R-2F RNA again produced a slightly different pattern. Following UV cross-linking, it formed a single RNA-protein complex, which had a slightly greater electrophoretic mobility. This difference could reflect a slight decrease in the number of nucleotides that are retained in the digested complex. RNase T1 digestion would cause a greater stability or an increase in the half-life of the GA mRNA that decreases binding would cause a greater stability or an increase in the half-life of the GA mRNA. This suggests that the specific sequences that have an 88% identity to either of the two AU-rich regions contains an extensive AU-rich region that has four 8-base sequences that have an 88% identity to either of the two AU-rich regions.

Mapping studies indicate that the R-3 region contains multiple protein binding sites. The R-3A fragment apparently binds a single protein, while the R-3D fragment apparently binds two distinct proteins. In all cases, the full-length R-2 RNA or the shorter R-2H fragment was a more effective competitor than either of the R-3 RNA segments. This suggests that the protein that binds to the 8-base AU sequence may serve as a binding element for a protein proposed by Shyu and co-workers (35) to explain how an AUUUA sequence may serve as a binding element for a protein that recruits an RNAse that causes the rapid degradation of the poly(A) tail and primes the RNA for further degradation. Future studies will be directed at identifying the specific GA mRNA-binding protein and characterizing its mechanism of action.

The GA mRNA-binding protein may regulate the stability of other mRNAs that are also induced in response to onset of metabolic acidosis. For example, rat renal glutamate dehydrogenase activity is also increased in the proximal tubule in response to metabolic acidosis (36). The increase in the level of glutamate dehydrogenase mRNA occurs with kinetics similar to that observed for the GA mRNA (37). The 3′-nontranslated region of the glutamate dehydrogenase mRNA (38) also contains an extensive AU-rich region that has four 8-base sequences that have an 88% identity to either of the two AU-rich direct repeats found in the primary GA mRNA binding element. Thus, it will be interesting to determine if any of the sequences from the glutamate dehydrogenase mRNA can also function as a pH-responsive instability element.

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