HADHB, HuR, and CP1 Bind to the Distal 3'-Untranslated Region of Human Renin mRNA and Differentially Modulate Renin Expression*

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Production of renin is critically dependent on modulation of *REN* mRNA stability. Here we sought to elucidate the molecular mechanisms involved. Transfections of renin-expressing Calu-6 cells with reporter constructs showed that a cis-acting 34-nucleotide AU-rich “renin stability regulatory element” in the *REN* 3'-untranslated region (3'-UTR) contributes to basal *REN* mRNA instability. Yeast three-hybrid screening with the *REN* 3'-UTR as bait isolated HADHB (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein) β-subunit) as a novel *REN* mRNA-binding protein. Recombinant HADHB bound specifically to the 3'-UTR of *REN* mRNA, as did the known mRNA stabilizers HuR and CP1 (poly(C)-binding protein-1). This required the renin stability regulatory element. Forskolin, which augments *REN* mRNA stability in Calu-6 cells, increased binding of several proteins, including HuR and CP1, to the *REN* 3'-UTR, whereas 4-bromocrotonic acid, a specific thiolase inhibitor, decreased binding and elevated renin protein levels. Upon decreasing HADHB mRNA with RNA interference, renin protein and mRNA stability increased, whereas RNA interference against HuR caused these to decrease. Immunoprecipitation and reverse transcription-PCR of Calu-6 extracts confirmed that HADHB, HuR, and CP1 each associate with *REN* mRNA in vivo. Intracellular imaging revealed distinct localization of HADHB to mitochondria, HuR to nuclei, and CP1 throughout the cell. Immunohistochemistry demonstrated enrichment of HADHB in renin-producing renal juxtaglomerular cells. In conclusion, HADHB, HuR, and CP1 are novel *REN* mRNA-binding proteins that target a cis-element in the 3'-UTR of *REN* mRNA and regulate renin production. cAMP-mediated increased *REN* mRNA stability may involve stimulation of HuR and CP1, whereas *REN* mRNA decay may involve thiolase-dependent pathways.

Renin, secreted by renal juxtaglomerular cells, is rate-limiting in angiotensin II generation and regulated by perturbations in NaCl delivery to the distal nephron and renal perfusion pressure (1). Thus, the human renin gene (*REN*) requires tight control to ensure expression appropriate to need. A better understanding of this is necessary in view of the importance of the renin system as a major therapeutic target in hypertension. We have found that *REN* expression is subject to both transcriptional (2) and post-transcriptional (3) control. A role for the 3'-untranslated region (3'-UTR) of *REN* mRNA in cell- and tissue-specific expression has been demonstrated in transgenic studies (4). Via an action on an intermediate gene to stabilize *REN* mRNA, cAMP increases *REN* mRNA by 100-fold (3, 5), reminiscent of effects on other mRNAs (6, 7).

Stabilization of specific mRNAs is an important mode whereby a cell determines protein expression levels. The 3'-UTR is a common target for such control via structural domains (cis-elements) for mRNA-binding proteins (RBPs) (8). A major class of destabilizing elements comprises adenosine-uridine pentamers (AUAUA, AU-rich elements (AREs)) (9) present in mRNAs for c-fos, granulocyte/macrophage colony-stimulating factor, c-myc, junB, nur77, γ-interferon, various interleukins (interleukin-1, -2, and -3), and tumor necrosis factor (5, 9, 10). An ARE containing the nonamer UUAUUUA(U/A)(U/A) is more indicative of rapid destabilization (11, 12). The combination of functionally and structurally distinct sequence motifs, including AU pentamers and nonamers and other U-rich stretches, determines the ultimate destabilizing ability of each particular ARE. Other binding motifs include the C-rich motif targeted by ~42-kDa poly(C)-binding proteins (CPs) (13).

Proteins that bind to 3'-UTRs, in particular to AREs, include AUFI (14), 3-oxoacyl-CoA thiolase (15), glyceraldehyde-3-phosphate dehydrogenase (16), heterogeneous nuclear ribonucleoproteins A1 (17) and C (17), AUH (AU enoyl-CoA hydratase-binding protein) with enoyl-CoA hydratase activity (18), and

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the ELAV (embryonic lethal abnormal vision) family of RBPs such as HuR (8, 19). HuR (36 kDa) contains three RNA recognition motif RNA-binding domains (20, 21) and binds with high affinity to AREs to stabilize specific ARE-containing mRNAs for GLUT1, c-fos, granulocyte/macrophage colony-stimulating factor, plasminogen activator inhibitor-2, and p21WAF1 (20, 22–25). Some complex cis-elements are targeted by members of different RNA-binding protein families, e.g. in androgen receptor mRNA (26) and p21WAF1 mRNA (27), two sequences are tailed by HuR and CP1.

Post-transcriptional stabilization of REN mRNA could explain how, despite a weak dideoxy sequencing (Australian Genome Research Facility, Brisbane, 1 transfected with 1 factor, plasminogen activator inhibitor-2, and p21WAF1 in androgen receptor). The Calu-6 cell line was maintained as described previously (30).

The major role in the regulation of human renin expression is the ELAV (embryonic lethal abnormal vision) family of RBPs (31). pGEX-6P2-HuR (a gift from Dr. Henry Furneaux, University of Connecticut Health Center) was generated from pGEX-2T-HuR as described (31). pGEX-2T-HuR was linearized with a specific thiolase inhibitor, 4-bromocrotonic acid (4-BCA; 100 μM), for 8 h. Cytoplasmic extracts were prepared as described (30, 39). Cells were scraped from the culture dishes with chilled phosphate-buffered saline (PBS) and centrifuged at 450 × g (Jouan C3-12 centrifuge) at 4 °C, and the supernatant was discarded with unla- beled PBS, and recentrifuged. Cell pellets were incubated with cyto- plasmic extract buffer (10 mM HEPES, 3 mM MgCl2, 40 mM KC1, 5% glycerol, 0.2% Nonidet P-40, and 1 mM dithiothreitol) with freshly added protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.2% Nonidet P-40) and then into pCARel or anti-CP1 polyclonal antibody (1:1000; from Dr. Arnold Strauss, Vanderbilt University), mouse anti-HuR monoclonal antibody 19F12 (1:2000; from Dr. Maria Czyzyk-Krzeska, University of Cincinnati) for 1 h, ade2, LYS2::LexA op4-HIS3, ura3::LexA op5-17-2Luc, and pLexMRS2/Zeocin) were supplied with the RNA-Protein Hybrid Hunter kit. Yeast cells were maintained on plates of 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar at 30 °C and transformed with pH3-UTR, pH3-MUT, or pH3-IRF by the lithium acetate method (33). Transformants were selected on minimal essential me- dium YC plates (DOBA™ and CSM™, Bio 101, Inc.) deficient in uracil (YC-Ura medium) to ensure maintenance of their pH3 plasmid. Those containing pH3-UTR were amplified in YC-Ura medium and transformed with 250 μg of pYEStrep2 plasmids from a randomly primed human kidney cDNA library (Invitrogen). The transformants were plated onto YC plates containing uracil, boar- d, and false positives were eliminated based on ability to grow in the presence of 5 mM 5-fluorouracil acid (Sigma) (34) and 0–25 mM 3-ami- notetrazolium (Sigma) and to metabolize X-gal (35, 36). The library prey plasmid was rescued from the yeast as described (37, 38).

Preparation of Cytoplasmic Extracts—Calu-6 cells were grown to 90% confluence in T-75 culture flasks. The medium was replenished; and 12–24 h later, the cells were treated with 10 μM forskolin for 24 h or a specific thiolase inhibitor, 4-bromocrotonic acid (4-BCA; 100 μM), for 8 h. Cytoplasmic extracts were prepared as described (30, 39). Cells were scraped from the culture dishes with chilled phosphate-buffered saline (PBS) and centrifuged at 450 × g (Jouan C3-12 centrifuge) at 4 °C, and the supernatant was discarded with unla- beled PBS, and recentrifuged. Cell pellets were incubated with cyto- plasmic extract buffer (10 mM HEPES, 3 mM MgCl2, 40 mM KC1, 5% glycerol, 0.2% Nonidet P-40, and 1 mM dithiothreitol) with freshly added protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.2% Nonidet P-40) and then into pCARel or anti-CP1 polyclonal antibody (1:1000; from Dr. Arnold Strauss, Vanderbilt University), mouse anti-HuR monoclonal antibody 19F12 (1:2000; from Dr. Maria Czyzyk-Krzeska, University of Cincinnati) for 1 h, ade2, LYS2::LexA op4-HIS3, ura3::LexA op5-17-2Luc, and pLexMRS2/Zeocin) were supplied with the RNA-Protein Hybrid Hunter™ kit. Yeast cells were maintained on plates of 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar at 30 °C and transformed with pH3-UTR, pH3-MUT, or pH3-IRF by the lithium acetate method (33). Transformants were selected on minimal essential me- dium YC plates (DOBA™ and CSM™, Bio 101, Inc.) deficient in uracil (YC-Ura medium) to ensure maintenance of their pH3 plasmid. Those containing pH3-UTR were amplified in YC-Ura medium and transformed with 250 μg of pYEStrep2 plasmids from a randomly primed human kidney cDNA library (Invitrogen). The transformants were plated onto YC plates containing uracil, boar-

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The renin-expressing human pulmonary adenocarci-

**Plasmids**—All pLuc plasmids (see Fig. 1A) were generated on a pBlues backbone (Invitrogen). pLucSV was created by transferring nucleotides 245–347 of human CP1 (32) into pGEM-Zf (Promega) as a HindIII/H9262 BamHI fragment into the HindIII/BamHI sites of pBlues, pLucUTR was made by transferring the REN 3'-UTR from IMAGE clone 2161619 (GenBank™/EBI accession number A4803344) as a HindIII/EcoRI fragment into pGEM-Zf (Promega) and then into pBlues (BamHI/ XbaI fragment (see Fig. 1A)). pLucSV and pH3-MUT were generated by site-directed mutagenesis. Briefly, (Promega) was linearized with HindIII and lacking classical AREs. Here we sought to identify novel 

**PREPARATION OF RNA**—The plasmid constructs used in the yeast three-hybrid system included pH3 (‘bait’), pYEStrp2 (‘prey’), and pH3-IRE/pYEStrep2-IRP (positive control ‘bait/prey’ interactors) (RNA-Protein Hybrid Hunter™ kit, Invitrogen). The REN 3'-UTR (nucleotides 1310–1457 of GenBank™/EBI accession number E01074) was cloned into plasmid pH3 from IMAGE clone 2161619 as a blunt-ended Bsu361/HindIII fragment and termed pH3-UTR. For riboprobe generation, specific REN 3'-UTR fragments were PCR cloned and then cDNA from pCARel into the EcoRv site of pBluescript (Stratagene) (see Fig. 1C). GST-HADHB was cloned from DH5α as specified by the manufacturer (Amersham Biosciences). GST-HuR (a gift from Dr. Henry Furneaux, University of Connecticut Health Center) was generated from pGEX-2T-HuR as described (31). pGEX-6P2-CP1 (a gift from Dr. Mike Kiledjian, Rutgers University) generated a fusion protein (GST-CP1) that contained amino acids 13–347 of human CP1 (32). All constructs were confirmed by digestion sequencing. Australian Genome Research Facility, Australia, and the Department of Immunology, Royal Perth Hospital.

**UV Cross-linking of RNA-Protein Complexes**—UV cross-linking was performed as described above using 20 μg of Calu-6 cytoplasmic extract, 200 ng of GST-HADHB, 200 ng of GST-HuR, or 200 ng of cleaved CP1 and 100 rpm cDNA (10–20 pg). Briefly, following incubation at 22 °C for 30 min, 0.3 units of RNase T1 (Roche Diagnostics) was added for 10 min, followed by 50 μg of Bioorbitase (Novagen) for 10 min. A 10× DMSO acrylamide gel, dried, and analyzed using a PhosphorImager (Amer- sham Biosciences). In RNA competition assays, a 100-fold excess of unlabeled sense RNA transcript (e.g. REN or pBluescript) was preincubated with the extract for 30 min at 22 °C prior to incubation with the labeled probe as described above. In some assays, antibodies to specific RNA-binding proteins (HADHB, HuR, and CP1) were added as de- scribed (25, 27) in an effort to supershift RNA-protein complexes.

**UVC Linking RNA-Protein Complexes**—RNA-protein binding reactions were carried out as described above using 20 μg of Calu-6 cytoplasmic extract or 200 ng of cleaved CP1 fusion protein and 2 × 10^7 cpm RNA (15–30 pg) 32P-labeled riboprobe (39, 41, 44). After adding labeled riboprobe, samples were placed in a microplate reader and incubated for 30 min, incubated with 20 μg of RNase A (Roche Diagnostics) at 37 °C for 15 min, and boiled for 3 min in SDS sample buffer. RNA- protein complexes were separated by 10% SDS-PAGE and analyzed with a PhosphorImager using 13C-labeled Rainbow molecular mass markers (Amersham Biosciences). The RNA-Protein Hybrid Facilitated Assay for HADHB, HuR, and CP1—A portion of the UV-crosslinked gel (see above) was transferred to polyvinylidine difluoride (PVDF) membrane (Osmonics, Inc., Minnetonka, MA) at 35 V overnight in buffer containing 20 mMtir, 150 mM glycine, 20% meth- anol, and 0.2% SDS. After blocking with 10% nonfat dried milk in TBS-T (20 mM Tris-Cl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) at room temperature, membranes were incubated with a monoclonal antibody to HuR (1:2000; from Dr. Arnold Strauss, Vanderbilt University), mouse anti-HuR monoclonal antibody 19F12 (1:2000; from Dr. Henry Furneaux), or anti-CP1 polyclonal antibody (1:1000; from Dr. Maria Czyzyk-Krzeska, University of Cincinnati) for 1–2 h at 22 °C,
followed by secondary antibody (Amersham Biosciences); and the proteins were visualized by ECL. Plus reagents on ECL Hyperfilm (both from Amersham Biosciences).

Thiolase Inhibitor Studies—Freshly seeded Calu-6 cells (4 × 10^6) were treated with either 100 μM 4-BCA (45 or vehicle (Eagle's minimal essential medium) for 0–8 h. At the time points indicated, cells were collected and subjected to 12% SDS-PAGE, and proteins were electroblotted onto PVDF membranes and then blocked overnight in 5% (w/v) skim milk. Membranes were incubated sequentially with rabbit anti-renin polyclonal antibody (1:350; a gift from Roche Diagnostics) and goat anti-rabbit IgG antibody (1:30,000; Sigma) for 2.5 h each. Immune complexes were visualized by incubation of the membranes with 5-bromo-4-chloro-3-indoly phosphate/nitro blue tetrazolium substrate (Promega) for 2–5 min. Blots were dried, scanned and quantified using Molecular Analyst software (Bio-Rad).

Immunoprecipitation and Reverse Transcription (RT)-PCR Assay—Calu-6 cells grown to ~70% confluence in 10-cm dishes were washed with PBS, trypsinized, washed again with PBS, and then lysed in 1 ml of cytoplasmic extract buffer (see REMSA procedures described above) on ice for 20 min. Lysates were centrifuged at 11,500 rpm for 10 min, and the supernatant was removed to fresh tubes. Anti-HADHB antibody (10 μl), anti-HuR antibody (10 μg), anti-CP1 antibody (10 μg), anti-p21 WAF1 monoclonal antibody 15091A (BD Biosciences) or no antibody was added to lysates and incubated for 60 min at 4 °C on a rotating wheel. Fifty μl of a 50% slurry of a mixture of protein A beads (Amersham Biosciences) and protein G beads (Sigma) (pre-washed and equilibrated in cytoplasmic extract buffer) was added to each sample and incubated for a further 60 min. After centrifugation at 4500 rpm for 2 min, the supernatants were removed; the pelleted beads were washed five times with cold cytoplasmic extract buffer; and RNA was extracted using TRIzol reagent (Invitrogen). RNA was treated with RNase-free DNase I (Promega) to eliminate any genomic DNA, and RT was performed using random hexamers (Promega) and Superscript II (Invitrogen) following standard procedures. PCR was performed for 40 cycles, with annealing at 55 °C, using primers REN-F (5’-gtg gtc ctc acc tgt ctc-3’)/REN-R (5’-cct gaa cat aag cgg acc aag t-3’), flanking an intron site within REN cDNA to avoid any genomic DNA effect. PCR products (~225 bp) were resolved on ethidium bromide-stained 1.5% agarose gels. RNA Interference (RNAi) Experiments—Small inhibitory RNA (siRNA) oligonucleotides were designed 261 and 88 nucleotides 3’ of the first ATG codon in HADHB and HuR mRNAs, respectively. A 21-mer of each was generated with an 8-mer (cct gct tca) targeted to the 3’-end for T7 priming. Selected sequences were subjected to a BLAST search to determine specificity for the HADHB and HuR genes: HADHB target mRNA, 5’-aa gac cgg aua cca gaa gag u-3’; antisense HADHB siRNA oligonucleotide template (with U residues replaced by T residues), 5’-aa gac ctt gtc aca gat cat gac cgg aag a-3’; sense HADHB siRNA oligonucleotide template, 5’-aa aat cct ggt gca tca ggt g cct gtc tcc-3’; HADHB mRNA stability assay, 5’-aa cct gac cca gca gga gga g-3’; antisense Hsu siRNA oligonucleotide template, 5’-aa taa ctc atc gtt cgt gct gct gtc tcc-3’; sense Hsu siRNA oligonucleotide template, 5’-aa taa ctc atc gtt cgt gct gct gtc tcc-3’; antisense nonsense siRNA oligonucleotide template, 5’-aa tgg cag gac att aca cca ctc gtc tcc-3’; and sense nonsense siRNA oligonucleotide template, 5’-aa atg gta tga tct ggt gac a cct gtc tcc-3’.

siRNA transcription templates (double-stranded DNA) were generated using an Ambion siRNA kit according to the manufacturer’s instructions. After annealing of T7 promoter sequence to siRNA oligonucleotides, double-stranded RNA was generated by in vitro transcription with T7 RNA polymerase. Leader sequences were removed by RNAse T1 and Dnase I. siRNAs were purified by phenol/chloroform extraction and quantified by absorbance at 260 nm, and integrity was determined by PAGE.

Calu-6 cells were plated at 3 × 10^5 cells/well in 2-cm 6-well plates and transfected the next day with siRNA (100 μM/well) using OligofectAMINE (Invitrogen) and Opti-MEM serum-free medium (Invitrogen) as recommended. After 4 h, the medium was replaced by Dulbecco’s modified Eagle’s medium and 5% (w/v) FBS serum; and then 1 μCi [3H]leucine, 1 μM phenyl-methylsulfonyl fluoride, 1 μM dithiothreitol, and 1 μg/ml pepstatin, and the Bradford assay (Bio-Rad) for total protein was performed. For Western blotting, 10 μg of total protein was separated on 10% Bis-Tris polyacrylamide gels (Invitrogen) in 1X MOPS running buffer (Invitrogen) and transferred to PVDF membranes in 20 mM Tris, 150 mM glycine, 20% methanol, and 0.02% SDS at 35 V overnight. Membranes were blocked with 10% skim milk in TBS-T at 22 °C for 1 h prior to addition of rabbit anti-renin antibody (1:200), mouse anti-HuR monoclonal antibody 19F12 (1:4000), rabbit anti-HADHB polyclonal antibody (1:2000), or goat anti-actin monoclonal antibody (1:1000; 1:19, sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 10% skim milk in TBS-T for >1 h at 22 °C. After washing with 10% skim milk in TBS-T, membranes were incubated with peroxidase-conjugated secondary antibody for 1 h (1:10,000, mouse or rabbit, Amersham Biosciences; 1:5000, sheep or goat, Silenus Laboratories Pty., Ltd., Victoria, Australia) and washed again with TBS-T prior to detection with ECL. Plus reagents on ECL Hyperfilm.

For RT-PCR studies, a 105–106-time RT-PCR, ActD (7.5 μg/ml; Sigma) was added to inhibit transcription. Total RNA was extracted from cells using TRIzol at 0, 2, 4, and 8 h after ActD addition. RT was performed using oligo(dT) (Promega) and Superscript III (Invitrogen) following standard procedures. Real-time PCR for REN and β-actin mRNAs (REN-PCR F, 5’-agaggtggagaccatcttgag-3’; REN-PCR R, 5’-gtcgataagctggattattc-3’; β-actin sense, 5’-gcaacaccagttgctctg-3’; and β-actin antisense, 5’-tactctgtttgtctgtcaca-3’) was performed for 35 cycles, with annealing at 60 °C (REN) or 58 °C (β-actin) (Corbett RotorGene, Sydney, Australia). Data were normalized to β-actin and expressed as a ratio relative to REN mRNA at the zero time point.

Intracellular Localization Studies—For HuR, Calu-6 cells were cultured on Lab-Tek II chamber slides (Nalge Nunc International) and fixed with 100% ice-cold methanol. After blocking for 1 h with 5% bovine serum albumin, cells were incubated with anti-HuR antibody for 1 h and then with Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Inc.) before staining with 300 nM 4,6-diamidino-2-phenylindole (Molecular Probes, Inc.) and mounted with glycerol/gelatin/PBS (Sigma). Two-channel fluorescent images were acquired on a Zeiss Axioplan 2 imaging microscope. For colocalization of HADHB and mitochondrial proteins, cells were cultured, fixed, and blocked as described above and then incubated for 1 h with antibodies to HADHB and mitochondrial proteins (1:1000; mouse MAB1273, Chemicon International, Inc.). The secondary antibodies used were Alexa Flour 594-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). Finally, the cells were stained and mounted as described above before acquiring two- and three-channel fluorescent images. CP1 localization was as described for HADHB, but with anti-CP1 antibody and mouse antibody to SR proteins (1:50, SR1H4, Santa Cruz Biotechnology). All localizations were also tested 24 h after 10 μM forskolin addition to cells.

RESULTS

The REN 3′-UTR Contains a cis-Element—We made a reporter construct containing the REN 3′-UTR immediately 3′ of luciferase (pLucUTR) (Fig. 1A). Although the REN 3′-UTR does not contain classical AU-rich elements and nonamers or extended U-rich sequences, it is AU-rich; and we found a 34-nucleotide region at the 3′-end with homology (Fig. 1E) to a region of sarcotxin IIA mRNA (from Sarcophaga peregrina) that can bind a bifunctional RBP, 3-oxoacyl-CoA thiolase (15). Modeling using the mfold server2 showed stable stem-loop formation in the REN 3′-UTR (Fig. 1F), with the most distal involving the REN-SRE. REN-SRE deletion altered the overall structure, but preserved the 5′-end region (Fig. 1F). We thus made an REN-SRE mutant (pLucMUT) lacking this AU-rich region. Calu-6 cells were cotransfected with pPKX, pCMV-SEAP, and one of the luciferase constructs and then induced with ponasterone A for 24 h. The REN-SRE deletion led to a 3-fold increase in luciferase (Fig. 2A), suggesting that it may be a destabilizing element. When placed immediately 3′ of luciferase and 5′ of the SV40 poly(A) sequence (pLucSV-SRE), the activity decreased by ~20-fold (Fig. 2B). The REN-SRE had no effect on mRNA translation in rabbit reticulocyte lysates (1.3 ± 0.2)×10^6 relative light units for wild-type REN (REN-WT) versus (0.9 ± 0.1)×10^6 relative light units for mutant REN (REN-MUT) (n = 3; p = 0.7). Thus, the REN 3′-UTR can...
act in cis to regulate the activity of a heterologous reporter, and
the REN-SRE is a destabilizer.

Yeast Three-hybrid Screen

To find REN 3'-UTR protein interactors, we used the yeast three-hybrid system to screen a human kidney cDNA expression library. Multiple pRH3'-UTR/pYESTrp2 transformants grew on YC-Trp/H11002/Ura/H11002/His/H11002 plates. The pYESTrp2 library plasmid was rescued from these transformants and sequenced. One clone contained the 3' 30% (residues 285–410) of HADHB (GenBank®/EBI accession number BAA22061) (Fig. 1B). Yeast cells transformed with both the pYESTrp2-HADHB (prey) and pRH3'-UTR (bait) plasmids activated the reporter genes HIS3, indicated by survival on minimal essential medium plates in the absence of histidine and the presence of 20 mM 3-aminotetrazolium (a histidine antagonist), and lacZ, indicated by production of a blue color in the presence of X-gal (Fig. 3A). Their growth in the presence of 5-fluoroorotic acid (data not shown) indicated that the pRH3'-UTR plasmid was amplified in these yeast cells, thus validating the interaction between the RNA and the pYESTrp2 library plasmid (34). In contrast, yeast cells transformed with both the pYESTrp2-HADHB and pRH3' (containing no insert) plasmids were unable to grow on histidine-deficient plates and could not

Fig. 1. Plasmid constructs. A, constructs for transfections. P_{HSP}, heat shock protein promoter; Luc, luciferase. B, constructs for yeast three-hybrid screening. nt, nucleotides; DBD, DNA-binding domain. C, pBluescript constructs used in REMSA. D, GST/pGEX constructs for REMSA/UV cross-linking. E, 3'-UTRs of REN and sarcotoxin IIA mRNAs, showing alignment of the 3-oxoacyl-CoA thiolase-binding domain of the latter with the 34-nucleotide region (REN-SRE) in the distal REN 3'-UTR. Conserved residues are shaded. F, secondary structure predictions for the REN mRNA 3'-UTR, showing stem-loop plots of REN-WT and, in italics, the REN-SRE deletion mutant. ΔE values for each were −44.7 and −30.5 kJ/mol, respectively. The C-rich region is underlined.
metabolize X-gal (Fig. 3B). The positive control was the strong interaction of the iron-responsive element protein with the iron-responsive element RNA (Fig. 3C). The REN 3′-UTR/HADHB interaction was thus specific.

Interaction of Calu-6 Proteins with the REN 3′-UTR—Calu-6 cell cytoplasmic extracts and the REN 3′-UTR riboprobe (REN-WT) gave a distinct RNA-binding protein complex (RPC) in REMSA (Fig. 4A, lane 1) that could be competed with excess unlabeled REN-WT probe (lane 2), but not with excess unlabeled Bluescript (lane 3). Deletion of the REN-SRE to give the REN-MUT probe substantially reduced binding (Fig. 4A, lane 1 versus lane 6). Because binding by the ladder was efficiently competed with excess unlabeled REN-WT probe (Fig. 4A, lane 7), binding motifs 5′ of the REN-SRE may be present.

UV cross-linking revealed that multiple proteins targeted the REN 3′-UTR (Fig. 4B, lane 1). RPCs at ~47 and ~36 kDa corresponded to bands on Western blots with molecular masses of HADHB and HuR, respectively (Fig. 4B, *). Deletion of the REN-SRE decreased binding of the band at ~43 kDa and increased binding of the band at ~36 kDa (Fig. 4B, lane 6). The actin control confirmed equal loading on each lane (data not shown).

Regulation of Protein Binding to the REN 3′-UTR—Forskolin increased RPC binding to the REN-WT probe (Fig. 4B, lane 2), but REN-MUT binding was reduced substantially (compare lanes 6 and 7). The increase with forskolin was shown by UV cross-linking to be 2-fold or greater (by ImageQuant) for bands at ~97, ~55, and ~47 kDa (HADHB); ~43 kDa (CP1); ~36 kDa (HuR), and ~29 and ~25 kDa (Fig. 4B, compare lanes 1 and 2). Such increases were abolished by deletion of the REN-SRE (Fig. 4B, compare lanes 6 and 7). The thiol inhibitor 4-BCA reduced binding to each probe (Fig. 4B, compare lanes 3 and 8, respectively) of the ~43-, ~39-, ~36-, and ~28-kDa bands (compare lanes 1 and 3). An actin immunoblot confirmed equal loading on each lane (data not shown).

Binding of HADHB, HuR, and CP1 to the REN mRNA 3′-UTR—Recombinant GST-HADHB bound to the REN-WT 3′-UTR probe specifically in REMSA (Fig. 5A, lane 2), and the RPC was competed with excess (~100-fold unlabeled mRNA (REN-WT 3′-UTR) (lane 3), but not with this excess of pBluescript RNA (lane 4). Results for GST-HuR were similar (Fig. 5B, compare lanes 2 and 3). Anti-HuR monoclonal antibody decreased GST-HuR binding to the REN-WT probe and gave a supershift (Fig. 5B, compare lanes 4 and 5). Binding of GST-HuR to REN-MUT mRNA was less than to the REN-WT probe, consistent with the involvement of the REN-SRE in binding (data not shown). These data demonstrate direct and specific binding of HADHB and HuR to the REN 3′-UTR.

The sequence CCCUUCCC not far from the 3′-end of the REN 3′-UTR is homologous to a consensus CP-binding sequence (CCCUCUC (8, 26, 27). Because CPs are ~42 kDa, we tested whether the ~43-kDa RPC obtained upon UV cross-linking was CP1. Antibody to CP1 induced a supershift with a reduction in RPC intensity (Fig. 5C, lane 2). We made GST-CP1, cleaved it with PreScission™ protease (Amersham Biosciences), and showed in REMSA that it bound avidly to the REN-WT probe (Fig. 5C, lane 4). A supershift was produced by anti-CP1 antibody (Fig. 5C, lane 5). Cleaved CP1 bound, but less avidly, and could still be supershifted with the REN-MUT probe (Fig. 5C, lanes 7 and 8). The 3′-end of the C-rich motif was absent in REN-MUT, likely explaining the reduced binding (Fig. 1F). Binding was competed with excess unlabeled REN-WT RNA (Fig. 5C, lane 11), but not with vector (lane 12). Upon UV cross-linking, cleaved CP1 bound definitively to the REN-WT probe (Fig. 5D, lane 1), but less so when the REN-SRE was absent (lane 2). Immunoblotting revealed cleaved CP1 protein at ~43 kDa (Fig. 5D, lane 4). These diverse data strongly support CP1 being a major component of the ~43-kDa RPC in Fig. 4B. Analysis of HADHB, HuR, and CP1 levels showed that each increased in response to forskolin (Fig. 5E).

Association of HADHB, HuR, and CP1 with REN mRNA in Vivo—Antibodies to HADHB, HuR, and CP1 co-immunoprecipitated REN mRNA from Calu-6 cells (Fig. 6, A and B, lanes 5 and 6). However, no REN-specific PCR product was seen with unrelated antibody (p21WAF1) (Fig. 6A, lane 7) or no antibody (beads alone) (Fig. 6A, B, and lane 4). Controls were: positive (assay of supernatant following immunoprecipitation (Fig. 6, A and B, lanes 1–3) and plasmid REN DNA (data not shown) and negative (without RT (A and B, lanes 8–11 and lanes 7–10, respectively) and water (A and B, lanes 12 and 11, respectively)). These data establish that HADHB, HuR, and CP1 interact closely with REN mRNA in Calu-6 cells.

Effect on Renin of RNAi Directed at HuR and HADHB mRNAs—Transfection of specific siRNAs in Calu-6 cells led to a substantial down-regulation of both HADHB and HuR, with no effect on actin (Fig. 7A, upper and lower panels, respectively, lane 2). Transfection with nonsense siRNA had no effect on
HADHB, HuR, or actin levels (Fig. 7A, lane 3). For HADHB siRNA, renin protein increased (Fig. 7A, upper panel, center row, lane 2), whereas for HuR siRNA, a decrease in renin was seen (lower panel, center row, lane 2). This provides functional data consistent with the notion that HADHB and HuR modulate renin mRNA stability, resulting in changes in renin protein levels.

To evaluate the effect of HADHB and HuR on renin mRNA stability, we performed ActD chase experiments on Calu-6 cells transfected with HADHB or HuR siRNA. In control cells (no siRNA transfected), the half-life of renin mRNA was ~4.5 h (Fig. 7B). However, in the presence of HADHB siRNA, the renin mRNA half-life was increased to >12 h (Fig. 7B), consistent with the increase in total renin protein (Fig. 7A) and the theory that HADHB destabilizes the renin mRNA. In contrast, with HuR siRNA, the renin mRNA half-life was reduced to ~2.5 h (Fig. 7B), consistent with the renin protein levels (Fig. 7A) and the notion that HuR stabilizes renin mRNA.

Effect of Thiolaese Inhibition on Renin Protein Accumulation—To extend our finding that 4-BCA reduced the binding of proteins to renin mRNA (Fig. 4B), we examined the effect of 4-BCA on renin protein levels. We found that addition of 4-BCA to Calu-6 cells resulted in significantly increased renin protein accumulation over the course of several hours (Fig. 8A).

Localization of HADHB, HuR, and CP1—Three-dimensional imaging microscopy showed colocalization of HADHB with mitochondrial proteins (Fig. 9A). Some HADHB was in the cytoplasm and in the nucleus within nucleoli. In contrast, HuR was widely expressed in the nucleus, but not in nucleoli, and there was little in the cytoplasm (Fig. 9B). CP1 was also in the nucleus and partly colocalized with SR proteins in speckles (Fig. 9C), which are subregions where RNA-processing factors are stored or assembled (46, 47). Nonspecific staining, assessed by incubating cells with rabbit or mouse IgG instead of primary antibody as well as by incubating with no primary antibody, was negligible (data not shown). Forskolin treatment did not change the localization patterns seen (data not shown).

Localization of HADHB within the Kidney—Immunohistochemistry of mouse kidney sections with anti-HADHB antibody demonstrated particularly strong staining in the afferent arteriole proximal to the glomerulus (Fig. 10), i.e. in the juxtaglomerular cells, the major physiological source of renin. As expected, HuR, which is expressed ubiquitously, was distributed evenly across all cell types in the kidney (data not shown).

DISCUSSION

Although renin mRNA is regulated primarily at the level of mRNA turnover, the molecular mechanisms involved have remained elusive. Here we demonstrated that the renin 3′-UTR can regulate a heterologous reporter and that a novel AU-rich cis-element of 34 nucleotides (REN-SRE) can convey its regulatory effect to the SV40 poly(A) sequence. We then identified and characterized a novel set of renin mRNA-binding proteins: HADHB, HuR, and CP1. The binding of these to renin mRNA was regulated by forskolin, which increased whole cell levels of each. We also showed that HuR, HADHB, and CP1 each associate with renin mRNA in vivo. Each of these proteins is differentially distributed in Calu-6 cells: HADHB mainly in mitochondria; HuR in the nucleus; and CP1 broadly throughout the cell, but mostly in the nucleus. Moreover, our RNAi experiments provide functional data that support the notion that HADHB destabilizes renin mRNA, whereas HuR acts to increase renin mRNA stability.

HADHB, which has 3-ketoacyl-CoA thiolase activity (48, 49), has not previously been identified as an RNA-binding protein. However, several other metabolic enzymes are known to bind RNA, including glutamate dehydrogenase, NAD+−dependent isocitrate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, thymidylate synthase, dihydrofolate reductase, cathepsin E, enoyl-CoA hydratase, and thiolase (8). A number of these enzymes are NAD+/NADH coenzyme-dependent; and interestingly, the NAD+-binding fold of glyceraldehyde-3-phosphate dehydrogenase, which comprises a βαβ structure (Rossmann fold), is sufficient to confer RNA binding to AU-rich sequences (50). We found that thiolase activity may be important in renin production because the thiolase inhibitor 4-BCA increased renin progressively in cultured Calu-6 cells. Also of interest, a weak 4-BCA-sensitive band at ~39 kDa obtained upon UV cross-linking is the size of human 3-oxoacyl-CoA thiolase. In S. peregrina, 3-oxoacyl-CoA thiolase binds to sarcotoxin IIA mRNA in a region (15) that has high homology to the distal end of the renin 3′-UTR. Its effect on mRNA stability in S. peregrina has not been tested, but tested. Consequently, further identification and characterization of the proteins binding to ~29 and...
~39 kDa should provide greater insight into the REN mRNA 3’-UTR-multiprotein complex.

In the mitochondrial inner membrane, HADHB and very long chain acyl-CoA dehydrogenase are responsible for catalyzing the CoA esters produced by carnitine palmitoyltransferase II (51, 52). HADHB is part of a multienzyme complex composed of four α-subunits (HADHA) with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities and four β-subunits (HADHB) with 3-ketoacyl-CoA thiolase activity (48, 49). Hydratase and dehydrogenase activities are seen in various RBPs (50, 53), and our findings now implicate thiolase activity as well. Because the half-life of REN mRNA in Calu-6 cells is 4.2 h (54), a 3-fold increase in renin protein accumulation over 8 h in response to thiolase inhibition suggests that REN mRNA degradation mediated by thiolase activity may represent a significant proportion of REN mRNA turnover. Our RNAi data showing that reduction of HADHB levels leads to a marked increase in renin protein expression and a lengthening of the REN mRNA half-life are entirely consistent with the above hypothesis. Interestingly, inhibition of mitochondrial protein synthesis increases the stability of nuclear encoded mRNA transcripts, suggesting that mitochondrial proteins can affect mRNA processing (55). However, the exact mode by which this occurs is unknown.

Most RNA regulatory proteins such as HuR and CP1, although mostly nuclear, can also be present in the cytoplasm, at least transiently (56). In Calu-6 cells, however, we found HuR only in the nucleus, suggesting a role here in REN mRNA stabilization, whereas CP1 was present in both the nucleus and cytoplasm. HADHB is located on the inner mitochondrial membrane, yet our data support an ability to bind RNA in response to thiolase inhibition. Several other mitochondrial proteins can also bind RNA, including AUH (53), Rna14p in yeast (57), glutamate dehydrogenase (58), and 3-oxoacyl-CoA thiolase from S. peregrina (15). Taken together with our data, RNA binding appears to be a common feature of a variety of mitochondrial

![Image](image_url)
proteins, thus generating novel potential links between mitochondrial processes such as β-oxidation and mRNA metabolism. Metabolic enzymes, including glyceraldehyde-3-phosphate dehydrogenase, can bind to 3′-UTRs, consistent with a possible role for metabolic enzymes in RNA processing (16). Moreover, components of the citric acid cycle can control binding of IRP-1 to the transferrin receptor mRNA (59). Although mostly mitochondrial, we also saw HADHB in the cytoplasm and nucleoli. The 5-ketoacyl-CoA thiolase precursor protein is distributed equally between the cytosol and mitochondria (60), making its interaction with mRNA feasible. Studies to examine

the time course of HADHB, HuR, and CP1 binding to REN mRNA, the whole cell functional consequence, and the interactions between the three proteins will provide further insight into these novel interactions.

Interestingly, an alternative transcript of renin (renin A) is

transported into mitochondria in the adrenal gland, and a role in the regulation of aldosterone biosynthesis has been hypothesized (61). The transcripts of mitochondrially targeted proteins such as renin A have been proposed to reside in the cytosol in close proximity to mitochondria (62), suggesting that such transcripts could come in contact with mitochondrial proteins.
The consequences of HADHB binding to REN mRNA in the context of the whole animal is unknown. 3-Ketoacyl-CoA thiolase is, however, within a candidate locus for blood pressure control in genetically hypertensive rat strains (63), so a link between thiolase proteins and blood pressure regulation may warrant exploration.

What could the role of HuR be in the control of REN mRNA turnover? Our RNAi data suggest a previously unrecognized important role. HuR plays a critical role in the decay of a variety of short-lived mRNAs, stabilizing them, and also has an important role. HuR plays a critical role in the decay of a variety of transcripts, including α-globin, tyrosine hydroxylase, and erythropoietin (68)’s role in the control of REN mRNA turnover. Finally, these novel cis-trans interactions involving the 3′-UTR of REN mRNA provide an array of new potential targets for therapeutics based on modulation of post-transcriptional gene expression.

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FIG. 10. Immunolocalization of renin and HADHB in the kidney to juxtaglomerular cells. Immunostaining (dark-brown precipitate) was detected using peroxidase/diaminobenzidine. Nuclei were counterstained with hematoxylin. A, renin; B, HADHB. Scale bars = 500 μm.

The 3′-UTR/mRNA-binding proteins/Stability/Expression of Renin mRNA
3'–UTR/Binding Proteins/Stability/Expression of Renin mRNA

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