# HuR binds a cyclic nucleotide-dependent, stabilizing domain in the 3′ untranslated region of $Na^+$/glucose cotransporter (SGLT1) mRNA

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**Abstract Differentiation-dependent expression of the $Na^+$/glucose cotransporter (SGLT1) is accompanied by a large, cAMP-dependent increase in stability of its mRNA. Stabilization is mediated by protein binding to a critical uridine-rich element (URE) in its 3′ untranslated region. In the present study, we demonstrate that HuR, an RNA binding protein of the embryonic lethal abnormal vision family, binds the SGLT1 URE. HuR binding was increased after elevation of intracellular cAMP levels and was dependent on protein phosphorylation. This interaction was prevented by a substitution mutation previously shown to block cAMP-dependent reporter message stabilization. These results implicate HuR as a key mediator of cAMP-dependent SGLT1 mRNA stabilization. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* mRNA stability; Glucose transporter; cAMP; RNA binding protein; Kidney epithelial cell

## 1. Introduction

The $Na^+$/glucose cotransporter SGLT1 mediates active high-affinity transport of glucose in kidney and intestinal epithelial cells driven by $Na^+$ gradients across the membrane [1,2]. Owing to its key physiological role in maintaining glucose homeostasis, SGLT1 has been extensively characterized with respect to its transport mechanism and membrane topology [3,4]. It is part of a large transporter superfamily with over 35 homologous members transporting a variety of substrates in bacteria, yeast and mammalian cells [5]. The human SGLT1 gene contains 15 exons spanning 72 kb [6] and has been identified as the locus of inherited glucose–galactose malabsorption [7].

SGLT1 exhibits differentiation-regulated expression in the LLC-$PK_1$ pig kidney epithelial cell line [8,9]. SGLT1 mRNA is up-regulated post-transcriptionally in response to levels of cAMP [10]. Using wild-type and mutant reporter messages transfected into LLC-$PK_1$ cells, we have obtained direct evidence that a uridine-rich element (URE) in the 3′ untranslated region (UTR) of SGLT1 mediates cAMP-dependent stabilization of the message [11,12].

In the present study, we demonstrate that HuR, a ubiquitous RNA binding protein of the embryonic lethal abnormal vision family [13], forms a specific, cAMP-dependent complex with the URE sequence in SGLT1 mRNA. Our findings suggest that HuR plays a critical role in cAMP-dependent up-regulation of SGLT1 expression.

## 2. Materials and methods

### 2.1. Materials

3-Isobutyl-1-methylxanthine (IBMX) and potato acid phosphatase, type III, were obtained from Sigma (St. Louis, MO, USA). Plasmids T3 ARE-*fos* and pTet-Myc-over-HuR were obtained from Dr. Ann-Bin Shyu, University of Texas Medical School, Houston, TX, USA. An anti-HuR monoclonal antibody 19F12 was obtained from Dr. H. Furneaux, University of Connecticut Medical School, Farmington, CT, USA. An anti-c-myc tag $IgG_1$ monoclonal antibody purchased from Calbiochem (Ab1, cat # OP10L) recognizes residues 410–419 of the human c-myc sequence. A monoclonal antibody 4B10 to hnRNPA1 was obtained from Dr. Gideon Dreyfuss, University of Pennsylvania School of Medicine, and a monoclonal antibody to AUF1 (hnRNPD) was provided by Dr. Gary Brewer, UMDNJ-RW Johnson Medical School, Piscataway, NJ, USA. Monoclonal antibodies AC88 and H9010 to hsp90 were provided by Dr. David Toft, Mayo Clinic, Rochester, MN, USA, and monoclonal antibodies N27 and C92F3A5 to hsp70 were obtained from Dr. William Welch, University of California, San Francisco, CA, USA.

### 2.2. Cell culture

The porcine renal epithelial cell line LLC-$PK_1$ clone G8 exhibits a proximal tubule-specific phenotype [14]. Cells were cultured as described previously [15]. For experiments, cells were seeded at a density of $10^4$ cells/$cm^2$ and grown for 4 days to a post-confluent state, then the phosphodiesterase inhibitor IBMX was added with medium change to 1 mM final concentration to the indicated samples. Unless otherwise stated, cells were harvested at 96 h after addition of IBMX, with one medium change after 72 h of treatment.

LLC-TAK-B7, a clonal cell line stably expressing the tetracycline-regulated transactivator, was isolated from LLC-$PK_1$ clone G8 [11]. Cells were propagated in medium containing 30 ng/ml tetracycline to suppress expression from the plasmid.

### 2.3. Assay of RNA/protein complex formation

Radiolabeled RNA probe binding to protein was determined in nuclear and cytoplasmic extracts by the UV crosslinking and gel mobility shift assays we have described previously [11]. The indicated sense RNA probes were synthesized by in vitro transcription in the presence of [$\alpha$-$^{32}$P]uridine triphosphate (3000 Ci/mmol). Nucleotide numbers refer to the porcine SGLT1 sequence (accession no. M34044) [16]. Plasmids 122 nt/*Bam* and 122 nt m2/*Bam* [11], which contain a TT→GG substitution at nucleotides 2622–2623, were linearized with *Bbs*I and transcribed with T3 polymerase to yield wild-type and mutant 120 nucleotide (nt) RNA transcripts respectively, which contain SGLT1 nucleotides 2576–2695. The 47 nt transcript contains nucleotides 2596–2642 and was transcribed using T7 polymerase from a double-stranded synthetic oligonucleotide template containing the T7 promoter.

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*Abbreviations:* SGLT, $Na^+$-coupled glucose transporter; IBMX, 3-isobutyl-1-methylxanthine; UTR, untranslated region; URE, U-rich element; nt, nucleotide(s)

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Gel mobility supershift analysis was carried out as described previously [17]. Briefly, cytoplasmic or nuclear lysates were incubated for 15 min with the indicated $^{32}$P-labeled RNA probe, unprotected RNA was digested using ribonuclease T1 and then the indicated monoclonal antibody, or a non-specific control monoclonal antibody, was diluted 1:10 into the RNA/protein binding mixtures. Incubation was continued for an additional 30 min in the presence of one unit of RNasin and complexes were resolved on non-denaturing 6% polyacrylamide gels.

*2.4. Transfection of HuR cDNA*

Plasmid pTet-Myc-over-HuR contains full-length human HuR cDNA fused in frame with an N-terminal myc epitope tag, under regulation of the tetracycline-regulated promoter (tet-OFF) [18]. LLC-PK$_1$ clone B7, which stably expresses the tetracycline-regulated transactivator [11], was transiently transfected with pTet-Myc-over-HuR or the empty vector using the calcium phosphate procedure and cell culture conditions described previously [11]. Where indicated, cells were treated with 1 mM IBMX immediately after removal of DNA.

*2.5. Western blot analysis*

Cell extracts were prepared at 48 h after transient transfections described previously [17]. Samples of 15 μg protein were resolved by 10% acrylamide SDS–PAGE, transferred to nitrocellulose and then probed with a monoclonal antibody to the c-myc epitope tag at 1 μg/ml, followed by detection using a horseradish peroxidase-conjugated second antibody and the ECL Western blotting kit (Amersham Pharmacia) according to the manufacturer's instructions.

## 3. Results

*3.1. HuR binds a stability-regulating domain in SGLT1 mRNA*

A 120 nt URE at nucleotides 2576–2695 within the 3′ UTR of SGLT1 mRNA conveys a *cis*-dominant cAMP-dependent stabilization to chimeric globin reporter messages [11,12].

HuR has been strongly implicated in mRNA stabilization in the case of cytokine and early-response gene mRNAs [18,19] and has also been shown to bind regulatory elements in other categories of mRNAs [20–26]. Minimal sequence requirements for protein binding to the SGLT1 URE obtained by mutation and deletion analysis [11] are consistent with those defined for HuR binding to its target mRNAs [13].

We utilized a gel mobility supershift assay to test whether endogenous HuR forms a complex with the 120 nt regulatory element of SGLT1 mRNA. LLC-PK$_1$ cell extracts were incubated with a $^{32}$P-labeled 120 nt SGLT1 URE riboprobe. After complex formation and digestion of unprotected RNA, samples were then incubated with either a monoclonal antibody to HuR or a control non-specific monoclonal antibody and resolved on non-denaturing gels. Nuclear extracts incubated with the control, non-specific monoclonal antibody formed several RNA/protein complexes with this probe whereas one major complex was observed using cytoplasmic extracts (Fig. 1A). A similar pattern was observed in the absence of antibody using the same RNA probe [11]. As reported previously [11], complex formation was increased in extracts from cells treated with the phosphodiesterase inhibitor IBMX.

Both nuclear and cytoplasmic extracts incubated with the anti-HuR monoclonal antibody exhibited a supershift of a major labeled complex (Fig. 1A). The amount of the supershifted complex (indicated by bracket) was increased in extracts from IBMX-treated cells and was also more abundant in nuclear extracts compared with cytoplasmic extracts. These observations indicate that endogenous nuclear and cytoplasmic HuR forms a cAMP-dependent complex with the SGLT1 URE.

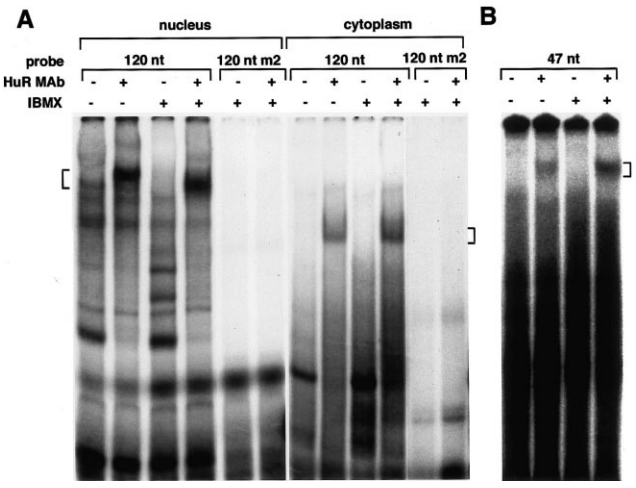


Fig. 1. HuR forms a cAMP-dependent complex with the SGLT1 URE sequence. Gel mobility supershift analysis was carried out by incubating extracts from control or IBMX-treated cells with the indicated sense RNA transcripts. After digestion of unprotected RNA, RNA/protein complexes were incubated with either a monoclonal antibody (MAb) to HuR (+) or a control, non-specific monoclonal antibody (−) before electrophoresis on non-denaturing polyacrylamide gels. A: Nuclear (15 μg protein) or cytoplasmic (40 μg protein) extracts were incubated with either the wild-type 120 nt RNA transcript or the mutant 120 nt transcript (120 nt m2). B: Cytoplasmic extracts (40 μg protein) were incubated with the 47 nt transcript.

Mutational analysis of the SGLT1 URE has provided a tool to examine the linkage between RNA/protein binding assayed in vitro and stability regulation assayed in vivo. cAMP-dependent stabilization of a globin reporter message bearing the SGLT1 URE in its 3′ UTR was blocked by a TT→GG substitution at nucleotides 2622–2623 within an essential pentameric uridine motif [11,12]. This mutation also prevented cAMP-dependent binding of a 38 kDa protein [11]. Incubation of either nuclear or cytoplasmic extracts from IBMX-treated cells with a $^{32}$P-labeled mutant transcript, 120 nt m2, which contained this mutant substitution, resulted in greatly reduced complex formation compared with the wild-type transcript and no supershift with the anti-HuR antibody (Fig. 1A). These findings indicate that HuR binding is prevented by a mutation which blocks cAMP-dependent stability regulation.

The HuR binding site was localized to a 47 nt region (nucleotides 2596–2642) localized within the 120 nt URE sequence. Results shown in Fig. 1B demonstrate that cytoplasmic complexes with the 47 nt RNA transcript were supershifted by the anti-HuR monoclonal antibody but not by the control monoclonal antibody. Furthermore, the supershifted complex was increased in cytoplasmic extracts from cells treated with IBMX. Similar results were obtained using nuclear extracts (data not shown). The 47 nt transcript was previously shown by UV crosslinking assay to form a single cAMP-dependent 50 kDa crosslinked RNA/protein complex in both nuclear and cytoplasmic extracts [11]. This complex consisted of a 38 kDa protein, identified by Northwestern analysis and by affinity isolation on a biotinylated RNA, plus a protected RNA fragment [11]. Taken together, these findings identify the 38 kDa protein as HuR (predicted molecular mass 36 kDa).

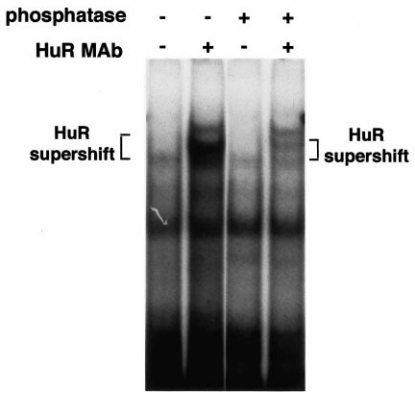


Fig. 2. HuR binding is decreased after phosphatase treatment. Nuclear (15 μg protein) extracts from IBMX-treated cells were incubated for 30 min at 37°C with 52 units/mg protein of potato acid phosphatase in 20 mM MOPS, pH 5.8 and 5 mg/ml bovine serum albumin. Then, aliquots were diluted into binding assay mixtures at pH 7.6 and incubated for 15 min with the $^{32}$P-labeled 120 nt SGLT1 probe, digested with five units of ribonuclease T1 for 30 min followed by 30 min incubation with the indicated monoclonal antibody (MAb). (+) A monoclonal antibody to HuR; (−) control, non-specific monoclonal antibody. Samples were immediately analyzed on non-denaturing 6% polyacrylamide gels.

We also tested antibodies to other proteins which bind stability-regulating sequences. hnRNPA1 forms a complex with HuR at a stability-regulating site in β-adrenergic receptor mRNA [25]. hnRNPD (AUF1), binds the c-*fos* AU-rich stability element (ARE) [27]. Monoclonal antibodies to hnRNPA1 and AUF1 each failed to supershift complexes formed with the 120 nt SGLT1 URE riboprobe in extracts from LLC-PK$_1$ cells. However these antibodies did supershift complexes formed with the 207 nt c-*fos* ARE transcript using the same extracts (data not shown). Heat shock proteins have been shown to bind RNA motifs involved in stability regulation [28]. Antibodies to heat shock proteins hsp70 and hsp90 failed to supershift proteins bound to the 120 nt SGLT1 URE riboprobe but did supershift proteins bound to the c-*fos* ARE riboprobe tested using the same extracts (data not shown).

### 3.2. HuR binding is protein phosphorylation-dependent

Results shown in Fig. 2 indicate that phosphatase treatment of nuclear extracts from IBMX-treated cells greatly diminished the supershifted HuR complex and also reduced formation of all complexes. A phosphorylation-dependence of the HuR supershifted complex was also observed using the 47 nt transcript (data not shown). These findings indicate that association of HuR and other proteins with the stability-regulating domain of SGLT1 mRNA is dependent on phosphorylation of protein(s) within the cell extracts.

### 3.3. Binding of expressed recombinant HuR

We next tested whether transiently expressed recombinant HuR recognized the SGLT1 stability-regulating sequence (Fig. 3). Since protein binding to the SGLT1 URE is cAMP- and phosphorylation-dependent, we tested recombinant HuR expressed in IBMX-treated LLC-PK$_1$ cells rather than the purified GST–HuR fusion protein. An epitope tag was used to distinguish recombinant HuR from endogenous HuR. Myc-tagged HuR expressed from plasmid pTet-Myc-over-HuR has previously been shown to bind the c-*fos* ARE [18]. LLC-PK$_1$ clone B7 cultures stably expressing the tetracycline-regulated transactivator [11] were transiently transfected with pTet-Myc-over-HuR in the presence of tetracycline. After 15 h, DNA and tetracycline were removed to permit expression from the plasmid and cultures were maintained an additional 48 h in the presence or absence of 1 mM IBMX.

Nuclear extracts from transfected cells were assayed by gel mobility shift using the $^{32}$P-labeled 120 nt SGLT1 URE probe and a monoclonal antibody to the myc epitope tag. Fig. 3A demonstrates that a diffuse supershifted complex, denoted by the bracket, was observed in nuclear extracts from IBMX-treated cells transfected with pTet-Myc-over-HuR after incubation of the binding mixture with the anti-myc tag antibody. This band was not observed in the absence of IBMX treatment, or in samples incubated with the control monoclonal antibody, nor was it observed in nuclear extracts from cultures transfected with the empty vector. The diffusiveness of the supershifted band may be due to proteolysis of the myc epitope tag in cell extracts despite the presence of protease inhibitors.

Nuclear extracts from transiently transfected cells were also assayed by UV crosslinking. Formation of the single 50 kDa RNA/protein crosslinked complex with the 47 nt transcript was greatly increased in nuclear extracts from IBMX-treated cultures transiently expressing recombinant myc-HuR com-

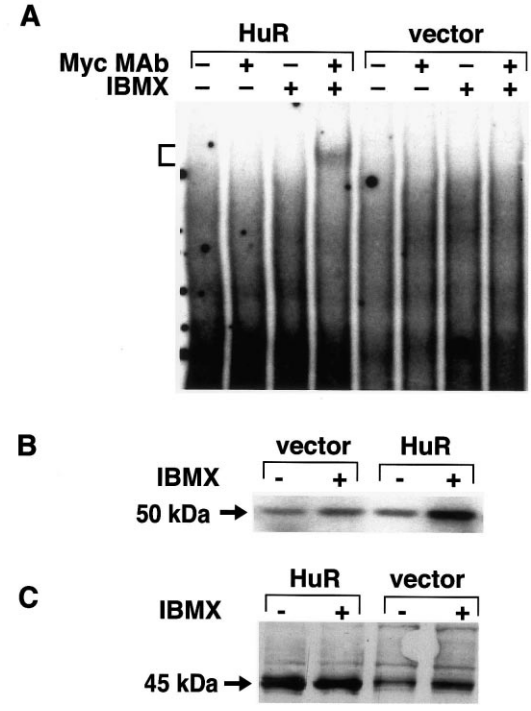


Fig. 3. Ectopic expression of myc-tagged recombinant HuR. LLC-PK$_1$ cells stably expressing the tetracycline-regulated transactivator [11] were transiently transfected with plasmid pTet-Myc-over-HuR (HuR) or the empty vector (vector). Nuclear extracts were prepared from control or IBMX-treated cells at 48 h after transfection. A: Gel mobility supershift analysis was carried out by incubating extracts (15 μg protein) with the $^{32}$P-labeled 120 nt SGLT1 URE RNA probe, followed by digestion of unprotected RNA and incubation with either a monoclonal antibody (MAb) to the myc tag epitope (+) or a control, non-specific monoclonal antibody (−). The supershifted band is indicated by the bracket. B: Extracts (20 μg) were also assayed by UV crosslinking using the 47 nt SGLT1 URE probe. C: Western blot analysis of nuclear extracts (15 μg protein) from transfected cells using the anti-myc tag antibody.

pared with the endogenous binding activity assessed using vector controls and compared with myc-HuR-transfected cultures not treated with IBMX (Fig. 3B). These results indicate that expressed recombinant HuR formed a crosslinked RNA/protein complex co-migrating with that formed by the endogenous RNA binding activity, identified by gel mobility shift experiments (shown in Fig. 1) as HuR. Both endogenous and recombinant RNA binding activities are activated after cAMP elevation.

Western blot analysis using the anti-myc tag antibody demonstrated increased levels of a 45 kDa protein in nuclear extracts from myc-HuR-transfected cells compared with vector controls (Fig. 3C). This size is in agreement with that previously determined for myc-tagged HuR expressed from this vector [18] and confirms expression of this recombinant protein. Background staining of a slower-migrating band observed in cultures transfected with the empty vector was also noted. Expressed levels of immunodetectable protein were not appreciably influenced by IBMX treatment. Therefore increased RNA/protein complex formation after IBMX treatment of myc-HuR-expressing cells reflects cAMP-dependent activation of the RNA binding activity of myc-HuR rather than increased levels of the protein.

## 4. Discussion

The transition from an actively dividing, undifferentiated cell population to a functionally and morphologically differentiated renal epithelial cell monolayer is accompanied by an 8-fold increase in half-life of SGLT1 mRNA mediated by a URE in its 3′ UTR [10–12,29]. We have utilized gel mobility supershift analysis to identify HuR as one of the nucleocytoplasmic proteins which interacts with the SGLT1 URE.

Our results provide the first demonstration that HuR binding to its cognate site in the 3′ UTR of an mRNA is stimulated after intracellular cAMP elevation and is dependent on phosphorylation of protein(s) in the cell extract. Importantly, a *cis*-acting substitution mutation within a critical uridine pentamer in the URE which prevented cAMP-dependent stabilization of a chimeric reporter message also prevented HuR binding. This observation indicates that cAMP-dependent binding of HuR is critical for cAMP-dependent SGLT1 message stabilization.

HuR was initially shown to bind AREs in the 3′ UTRs of proto-oncogene, growth factor, cytokine and lymphokine mRNAs [30]. Subsequently, the list of its target mRNAs has been expanded to include the β-adrenergic receptor mRNA [25], cyclin mRNAs [20], the cyclin kinase inhibitor p21 mRNA [21], plasminogen activator inhibitor mRNA [31] and nitric oxide synthase mRNA [23]. The presence of a binding site for HuR in the 3′ UTRs of diverse mRNAs suggests that their decay rates can be co-ordinately regulated in response to cell signaling pathways.

The direct involvement of HuR in message stabilization was demonstrated using antisense constructs [20,22,23], a cell-free mRNA decay system [32] and by ectopic overexpression [18,19,22,23,33]. Stabilizing effects of HuR overexpression could not be tested in LLC-$PK_1$ cells, since cAMP elevation resulted in maximal stabilization of reporter transcripts bearing the SGLT1 URE, with over 90% of transcripts remaining at 10 h after inhibition of transcription [12].

A key property of HuR is its ability to shuttle between the nucleus and cytoplasm [19,34]. HuR may bind its target mRNAs in the nucleus and accompany them to the cytoplasm, providing protection from degradation. The recent identification of a novel, specific endonuclease that cleaves within a HuR binding site and is specifically inhibited by HuR [35] suggests that HuR may bind near an endonuclease recognition site in order to render it inaccessible to endonuclease action.

The interaction between HuR and its target mRNAs was regulated by three proteins known to be protein phosphatase 2A inhibitors [36]. These findings, taken together with our demonstration that HuR binding to the SGLT1 URE is inhibited by dephosphorylation of cellular protein(s) and increased after cAMP elevation, point to a key role for a regulatory cascade and protein phosphorylation/dephosphorylation in RNA stabilization by HuR.

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