**Regulation of Na\(^{+}\)-coupled Glucose Transport in LLC-PK\(_{1}\) Cells**

MESSAGE STABILIZATION INDUCED BY CYCLIC AMP ELEVATION IS ACCOMPANYED BY BINDING OF A M\(_{r}\) = 48,000 PROTEIN TO A URIDINE-RICH DOMAIN IN THE 3'-UNTRANSLATED REGION*

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In an exploration of the molecular basis of cyclic AMP-induced stabilization of Na\(^{+}\)/glucose cotransporter mRNA (SGLT1 isoform) accompanying cell differentiation in the pig kidney cell line LLC-PK\(_{1}\), we have identified a 48-kDa cytoplasmic protein factor, designated SG-URBP, which specifically binds a 120-nucleotide sequence within the 3'-untranslated region of the SGLT1 message. A 46-nucleotide uridine-rich element within this region appears necessary for specific binding, and the presence of the 3'-untranslated region is necessary for message stabilization by cyclic AMP. The binding activity of SG-URBP is up-regulated after cyclic AMP elevation and protein kinase A activation, whereas protein dephosphorylation either in vivo or in vitro is associated with loss of binding activity. The increase in SG-URBP binding activity correlates with an increase in the half-life of the SGLT1 message, suggesting a cause and effect relationship.

Glucose transport by mammalian cells is a highly regulated process. Two structurally, functionally, and genetically distinct classes of glucose transporters have been identified, the GLUT family of facilitative glucose transporters (Gould and Bell, 1993) and the SGLT family which mediates Na\(^{+}\)-coupled secondary active transport of glucose. SGLT1 transporters are restricted in tissue expression to the apical membranes of small intestine and renal proximal tubule and have been mapped to human chromosome 22 (Wright, 1993). SGLT1 also transports galactose and has been identified as the locus of the inherited defect glucose-galactose malabsorption (Turk et al., 1991).

Much of what is currently known about regulation of the expression of the SGLT type of glucose transporter has been derived from studies using the cell line LLC-PK\(_{1}\), derived from porcine renal proximal tubule (Amsler and Cook, 1982; Peng and Lever, 1993). This cell line permits the reconstitution of a fully functional polarized epithelium in vitro. The development of a confluent monolayer is accompanied by the appearance of a number of differentiated functions characteristic of this epithelium including the formation of tight junctions and microvilli, vasopressin responsiveness, transepithelial salt and water transport, several brush-border marker enzyme activities, and apical membrane Na\(^{+}\)-coupled glucose transport activity (reviewed in Lever (1989)). Elevation of intracellular levels of cyclic AMP or treatment with the differentiation inducer hexamethylene bisacetamide (HMBA),\(^1\) greatly increased both the levels of expression and rate of appearance of several of these differentiated functions including Na\(^{+}\)-coupled glucose transport (Amsler and Cook, 1982; Yoneyama and Lever, 1984; Peng and Lever, 1993; Yet et al., 1994). These agents appear to act by independent and synergistic mechanisms (Peng and Lever, 1993).

SGLT1 transcripts of 2.2 and 3.9 kb, differing in the length of the 3'-untranslated region (3'-UTR) are observed in LLC-PK\(_{1}\) cells as a result of alternative cleavage and polyadenylation (Ohta et al., 1990). We have recently demonstrated that the half-life of the larger transcript is increased by more than 8-fold after cyclic AMP elevation, indicating that post-transcriptional regulation exerts a major influence on SGLT1 expression (Peng and Lever, 1995). The observation that the 3'-UTR is necessary to observe this effect suggested that sequences within this region may regulate message stability in response to cyclic AMP.

In the present study, we extend these observations by identifying a cytoplasmic M\(_{r}\) = 48,000 mRNA-binding protein, designated SG-URBP, which binds a cognate sequence containing a uridine-rich element (URE) within the SGLT1 mRNA 3'-UTR. SG-URBP binding activity is greatly increased in extracts from LLC-PK\(_{1}\) cell cultures after treatment with cyclic AMP-elevating agents and is dependent on protein phosphorylation. SG-URBP appears to be a new member in the inventory of RNA-binding proteins which have in common the specific recognition of uridine-rich stability-determining elements in mRNA (Belasco and Brawerman, 1993; Chen and Shyu, 1995) and the only one identified thus far in this category which exhibits regulation by cyclic AMP-dependent protein phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**

\[^{32}P\]Uridine 5'-triphosphate, 3000 Ci/mmol, was from ICN. Nucleotide triphosphates were obtained from Boehringer Mannheim. Restriction enzymes were from either New England Biolabs, U.S. Biochemical Corp., or Promega. Both RNA polymerases T3 and T7 were from Stratagene. High quality RNase T1 was from Calbiochem. H-89 and H-7 were purchased from Seikagaku. Microcystin LR was from Calbiochem. Okadaic acid, potato acid phosphatase, and proteinase K were obtained from Sigma.

\(^1\) The abbreviations used are: HMBA, N,N'-hexamethylene bisacetamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; H-89, N-[1-(p-bromophenacyl)mamylaminoethyl]-5-isouquinolinesulfonamide; H-7, 1-(5-quinolinesulfonyl)-2-methylpiperezine; 3'-UTR, 3'-untranslated region; TPA, 12-o-tetradecanoylphorbol-13-acetate; kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); URE, U-rich element; ARE, AU-rich element; PAGE, polyacrylamide gel electrophoresis.

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Methods

Cell Culture—The porcine renal cell line LLC-PK1, clone G8 was maintained in a 50:50 mixture of Dulbecco's modified Eagle's medium: Ham's F-12 (ICN-Flow Laboratories) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 5 mM glutamine, 0.12% sodium bicarbonate, and 15 mM HEPES, pH 7.0, in 5% CO2, as described previously (Peng and Lever, 1993). Cells were plated at a density of 104 cells/cm2. The indicated inducers were added to cultures which had just reached a postconfluent state (3–4 days after plating). Iodoacetamide (BML), 1 mM, was added where indicated, for 4 days, its optimal time period for induction of SGLT1 mRNA levels and transport activity.

Construction of Plasmids Containing SGLT1 3'-UTR Domains—A renal SGLT1 cDNA clone (pPSGT-B1) kindly provided by Dr. David Rhoads (Massachusetts General Hospital, Boston, MA) contains a partial coding region (1818 bp) and the entire 3'-UTR (1832 bp) of pig kidney SGLT1 cDNA inserted in the pBluescript II SK(−) plasmid cloning vector (Stratagene) at the XbaI site. Plasmid p3UTR3, containing a 553-bp segment from nucleotides 1831–2384 was generated by deleting a 1276-bp Sau3AI fragment containing 151 nt of the pig SGLT1 3'-UTR (2562–2629) and 56 nt from pBluescript; p3UTR2, a 176-bp transcript synthesized from HindIII-linearized p3UTR2 containing 120 nt of the pig SGLT1 3'-UTR (2510–2629) and 56 nt from pBluescript; p3UTR2A, a 166-bp transcript synthesized from HindIII-linearized p3UTR2A containing 68 nt of the pig SGLT1 3'-UTR (2562–2629) and 98 nt from pBluescript; c-fos ARE, a 174-bp transcript synthesized from BglII-linearized p3ARE-WT containing 84 nt of human c-fos 3'-UTR and 80 nt of the rabbit β-globin coding region with 10 nt from pT7/73 α-19 (Shyu et al., 1989); AU8O, a 80-nt transcript synthesized from EcoRI-linearized pT7/73 cII containing 4 AUAUA repeats (Malter, 1989); T7/73 α-19A60, a 60-nt transcript synthesized from EcoRI-linearized pT7/73 α-19; GAPDH486, a 539-nt antisense transcript synthesized from XbaI-linearized pBGSAPDH486 containing 486 nt of rat glyceraldehyde-3-phosphate dehydrogenase coding region and 53 nt from pBluescript SK(−).

Sense-strand RNA transcripts were synthesized using T3 RNA polymerase (Stratagene) with the exception of probes AU8O, T7/73 α-19A60, and GAPDH486 which were transcribed using T7 RNA polymerase (Stratagene). After transcription, RNase-free DNase (RNQ1 DNase, Promega) was added and mixtures were incubated for an additional 30 min at 37°C to remove template DNA. Then, 20 μg of yeast RNA was added to each tube as carrier, followed by digestion with diethylpyrocarbonate-treated water to a final volume of 10 μl. Aliquots of 1 μl of each reaction mixture were removed and further diluted with 99 μl of diethylpyrocarbonate-treated water for trichloroacetic acid precipitation (Maniotis et al., 1989) to calculate the amount of cRNA produced. Labeled transcripts were extracted with phenol/chloroform and precipitated twice with 2 M ammonium acetate in 2.5 volumes of ice-cold ethanol at −80°C for at least 1 h. The final pellet was washed with 80% ice-cold ethanol, air-dried, dissolved in diethylpropylcarbonate-treated water, stored at −20°C, and used as soon as possible. The integrity of the transcripts was verified by agarose electrophoresis.

To synthesize unlabeled transcripts in quantity for competition experiments, transcription reactions were performed by the same procedure described above except [32P]UTP was replaced by 0.5 mM UTP, and the total volume of each reaction mixture was increased to 100 μl.

Preparation of Cell Lysates for RNA-Protein Binding Assay—LLC-PK1 monolayers grown in 6-cm culture dishes were washed twice with ice-cold phosphate-buffered saline and aspirated. Monolayers were lysed by direct addition to each dish of 1 ml of lysis buffer containing 10 mM HEPES, pH 7.4, 3 mM MgCl2, 40 mM KC1, 2 mM dithiothreitol, 5% glycerol, 0.5% Nonidet P-40, 8 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride for 15 min, as described by You et al. (1992). Nuclei and cell debris were removed by centrifugation at 10,000 × g for 5 min at 4°C, and cytoplasmic lysates were aliquoted and stored at −80°C until use. Protein was determined using a microwave version of the bicinchoninic (BCA) method (Atkins and Tuen, 1992) modified for use with 96-well microtiter plates.

UV-cross-linking Assay of RNA-Protein Interaction—The procedure described by You et al. (1992) was used with minor modifications. LLC-PK1, cytoplasmic lysates (10–40 μg of protein) and 32P-labeled RNA probe were incubated in a 10- or 20-μl reaction volume at room temperature for 15 min in a buffer containing 10 mM HEPES (pH 7.6), 3 mM MgCl2, 40 mM KC1, 2 mM dithiothreitol, 5% glycerol, 0.5% Nonidet P-40, 8 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride for 15 min, as described by You et al. (1992). Nuclei and cell debris were removed by centrifugation.

The procedure used. The RNA-protein mixtures containing equal amounts of cellular proteins were immediately transferred into wells of a 96-well microplate on ice and exposed to short-wave (254 nm) radiation in a UV Stratalinker 1800 (Stratagene) for 5 min on the automatic setting. The UV-cross-linked samples were then transferred into 1.5-ml microtubes, 1/10 volume of 4 X SDS sample buffer was added, and samples were boiled in boiling water for 3 min. Samples were resolved by SDS-PAGE (10% acrylamide). The dried gel was exposed to x-ray film at −80°C with double intensifying screens.
RESULTS

Cyclic AMP Elevation Is Accompanied by Formation of a 48-kDa RNA-Protein Complex—Our previous findings that the 3.9-kb SGLT1 transcript, which contains a 1.8-kb 3′-UTR, was stabilized by more than 8-fold after cyclic AMP elevation, whereas the half-life of the 2.2-kb transcript, which lacks the 3′-UTR, was unaffected (Peng and Lever, 1995), suggested that the 3′-UTR contains sequences which regulate the stability of this message in response to cyclic AMP. In order to identify putative trans-acting factors that may stabilize this transcript after cyclic AMP elevation by specifically interacting with cis-elements in the 3′-UTR, we produced by in vitro transcription four non-overlapping contiguous sense RNA probes (named 3UTR1, 3UTR2, 3UTR3, and 3UTR4) which encompassed the entire 3′-UTR of SGLT1 mRNA (Fig. 1). These probes were designed to segregate some of the sequence elements found in the SGLT1 3′-UTR which have been shown to regulate stability in other mRNAs (Belasco and Brawerman, 1993; Sachs, 1993). These include a 46-nt uridine-rich region (URE), which contains 68% uridine residues, present within 3UTR2, and three AUUUA motifs contained in probes 3UTR3 and 3UTR4, diagrammed in Fig. 1. RNA “band shift” assays using nondenaturing electrophoresis of the products of an RNA binding assay containing extracts from Nonidet P-40-solubilized LLC-PK₁ cells and the 32P-labeled sense RNA UTR probes revealed a unique band shift pattern for each probe with a signal strength proportional to the amount of cellular extract added and abolished by proteinase K, indicating that the observed bands were due to interactions of proteins with the labeled RNAs (data not shown). However, only in the case of probe 3UTR2 was a difference in band shift pattern and intensity noted when extracts from control and IBMX-induced cells were compared (not shown).

This difference was more clearly apparent when RNA-protein incubations were UV-cross-linked after the RNase T1 digestion step to generate covalent bonds between the 32P-labeled sense RNA probe and associated proteins. The products were analyzed by SDS-PAGE in a label transfer experiment (Fig. 2). A 28-kDa RNA-protein complex was observed in samples derived from control cultures (lane 2) whereas samples derived from IBMX-induced cultures (lane 5) exhibited a 48-kDa RNA-protein complex accompanied by a loss of the 28-kDa complex. The presence of a 250× molar excess of specific competitor RNA (unlabeled 3UTR2 RNA) effectively blocked the appearance of these complexes (lanes 3 and 6), while addition of the same amount of nonspecific competitor RNA (a 529-bp unlabeled antisense glyceraldehyde-3-phosphate dehydrogenase RNA) had much less effect (lanes 4 and 7), indicating that these binding activities are specific. The formation of the 48-kDa complex was proportional to the amount of cellular extract added to the binding reaction and abolished if the binding reaction mixture was treated with proteinase K (Fig. 3). These results demonstrate the presence of a trans-acting protein factor in LLC-PK₁ cells which specifically interacts with the 3UTR2 mRNA fragment in response to IBMX treatment.

The formation of the 48-kDa complex was also induced by other cAMP-elevating agents such as 8-bromo-cAMP, dibutyryl cAMP, and forskolin (Fig. 4, lanes 4, 5, and 6). The IBMX-induced complex formation was reduced substantially when LLC-PK₁ cells were exposed to H-89 (lane 3), a specific protein kinase A inhibitor (Chijiwat al., 1990). Addition of the protein kinase C inhibitor H-7 (Hidaka et al., 1984) to cells produced a marginal inhibitory effect on the IBMX-induced 48-kDa complex (lane 8). These results strongly suggest that protein kinase A is involved in the regulation of the formation of the 48-kDa RNA-protein complex.

The differentiation inducer HMBA also promoted an increase in the half-life of the 3.9-kb SGLT1 message (Peng and Lever, 1995). HMBA, 5 μM, also induced an increase in the 48-kDa RNA-protein cross-linked complex (Fig. 4, lane 7) which was diminished by H-89 but not by H-7 (not shown), suggesting a possible role of cyclic AMP-protein kinase A in mediating this response.

A Phosphoprotein Is Required for Formation of the 48-kDa Complex—The possibility that the binding affinity of these protein factors for their cognate sites on the mRNA might be regulated by a reversible phosphorylation/dephosphorylation mechanism was tested. Cytoplasmic extracts from either IBMX-treated or untreated control cells were treated with protein acid phosphatase for 5 min to remove O-linked phosphates prior to binding assay. As shown in Fig. 5, phosphatase treatment resulted in a decrease in formation of both the 48-kDa and 28-kDa RNA-protein complexes. The presence of microcystin LR, a specific, potent inhibitor of phosphatase types I and 2A (Mackintosh et al., 1990), partially protected the RNA-protein complexes from acid phosphatase attack (Fig. 5, lanes 6–8) indicating that inhibition of complex formation was not
due to another contaminating enzymatic activity. The contribution of protein phosphorylation was also tested in vivo by addition of the specific phosphatase inhibitor, okadaic acid (Bialojan and Takai, 1988) to cells. Results shown in Fig. 6 indicate that okadaic acid, 2 μM, in the absence of IBMX produced the same increase in the formation of the 48-kDa RNA-protein complex as that produced by IBMX, but produced only a small additional increase in IBMX-treated cells. These observations suggest that the protein factor involved in the formation of the 48-kDa complex is regulated by protein phosphorylation and dephosphorylation.

A Uridine-rich Region Is Necessary for Formation of the 48-kDa RNA-Protein Complex—Since the 435-nt 3′UTR2 fragment contains a 46-bp uridine-rich region (68% U), at the 3′ portion (Fig. 1), the possibility that this U-rich element is a binding site for the protein factor forming the 48-kDa complex was examined. Results shown in Fig. 7 revealed that poly(U) (lane 8), but not poly(A), poly(G), or poly(C) (lanes 7, 9, and 10), competitively inhibited the formation of the 48-kDa RNA-protein complex, indicating that complex formation is uridine-dependent. Other U-rich RNAs were effective competitors. A synthetic 80-nt RNA (AU80) derived from a 63-nt plasmid T3/T7 α-19 sequence containing four reiterated AUUUA motifs (Malter, 1989) and a wild-type c-fos ARE RNA fragment (ARE-wt) which contains AUUUA elements with surrounding U-rich sequences (Shyu et al., 1989) were used as unlabeled competitors. As shown in Fig. 7, formation of the 48-kDa complex was inhibited in the presence of a 250-fold excess of either 3UTR2 RNA (lane 3), AU80 RNA (lane 4), or ARE-wt RNA (lane 6), but not by a 64-nt T3/T7 α-19 RNA (lane 5) which is same as AU80 except it lacks the four AUUUA motifs. Since 3UTR2 RNA does not contain an AUUUA motif, the specific competition of its protein binding by these AUUUA-containing RNAs would be attributed to their U-rich property.

**Fig. 3.** Protein concentration dependence and proteinase K sensitivity of 48-kDa complex formation. A, 32P-labeled 3UTR2 RNA was incubated with increasing amounts of cytoplasmic extracts from either control (lanes 2–5) or IBMX-treated (lanes 6–9) LLC-PK1 cells and analyzed by the UV-cross-linking assay. B, cytoplasmic lysates were preincubated with the indicated amounts of proteinase K at 37°C for 30 min prior to the UV-cross-linking assay.

**Fig. 4.** The 48-kDa RNA binding activity is abrogated by the protein kinase A inhibitor H-89. 32P-labeled 3UTR2 was incubated with cytoplasmic lysates (40 μg) from untreated control cells or cells treated for 4 days as indicated before UV-cross-linking assay. Lane 1, control; lane 2, 1 μM IBMX; lane 3, 1 μM IBMX plus 50 μM H-89; lane 4, 250 μM 8-bromo cAMP (8Br-cAMP); lane 5, 100 μM dibutyryl cAMP (dbcAMP); lane 6, 100 μM forskolin; lane 7, 5 μM HMBA; lane 8, 1 μM IBMX plus 40 μM H-7.

**Fig. 5.** Phosphatase treatment prevents formation of the 48-kDa RNA-protein complex. Potato acid phosphatase (PP) was preincubated with cytoplasmic lysates from IBMX-treated LLC-PK1 cells in the presence of increasing amounts of the phosphatase inhibitor microcystin LR (MICC-LR) for 15 min before addition of 32P-labeled 3UTR2 (1 ng) to initiate the RNA-protein binding reaction. After UV-crosslinking, the products were analyzed by 10% SDS-PAGE. The presence of MICC-LR partially protected against inhibition by potato acid phosphatase of 48-kDa complex formation (lanes 6–8) compared with extracts treated with potato acid phosphatase alone (lane 4). MICC-LR by itself had no significant effect on the RNA binding activity (lane 5) compared to that in uninduced control (lane 2) or IBMX-induced cells (lane 3). Lane 1, free 32P-labeled 3UTR2 probe without lysate.
motifs (Fig. 1). The c- probes 3′UTR3 and 3′UTR4 (not shown), which contain AUUUA 63 and 54 kDa were also observed to be cross-linked to SGLT1 respectively, were observed (Fig. 8, lane 1). 

As shown in Fig. 8, in the case of AU80, 3 RNA-protein complexes, with apparent molecular masses of 63, 54, and 40 kDa, were observed (lanes 1, 2, and 3). This indicates that the binding sites for both the 28-kDa and the 48-kDa protein factors must be in a region between the NspI site and the Stul site. A 5′ deletion up to the EcoO109I site, represented by probe 3UTR2Δ5, retained the same binding activity for both the 28-kDa and the 48-kDa complexes (Fig. 9, lanes 7 and 11) as the full-length 3UTR2 probe (lane 2). Probe 3UTRΔ7, a 120-nt Avall/Stul fragment, which contains the entire 46-nt URE, exhibits full ability to form the 28-kDa and 48-kDa complexes (Fig. 9, lanes 9 and 13); therefore, the binding site for both complexes must reside within this 120-nt region. 3UTRΔ7 was cut at a unique HindII site within the U-rich sequence to produce probes 3UTRΔ6 and 3UTRΔ8 (Fig. 10). Probe 3UTRΔ6, which contains the region upstream of the HindII site, conferred a very weak ability to form either the 28-kDa or 48-kDa complex (Fig. 9, lanes 8 and 12), while probe 3UTRΔ8, which contains the region downstream of the HindII site, could not form the 28-kDa complex and exhibited substantially reduced formation of the 48-kDa complex (lanes 10 and 14). Taken together, these data strongly indicate that the entire U-rich element (URE) is necessary for effective formation of both the 28-kDa and the 48-kDa complexes with SGLT1 mRNA, and binding is abolished if it is cut at the HindII site. The observation that increased 48-kDa binding activity was consistently accompanied by a reduced 28-kDa binding activity, yet both apparently map to the same 120-nt sequence (shown in Fig. 10C), suggests that both binding activities may recognize either the same site or of the protein, this finding suggests that neither AU80 nor c-fos ARE RNAs contain the cognate sequence recognized by protein(s) forming the 48-kDa complex with SGLT1 3UTR2.

Cognate Sequence Recognized by mRNA-binding Proteins—A series of deletions from either the 3′ or the 5′ end were constructed within the 435-nt region encompassed by the 3UTR2 probe in order to identify specific sequences involved in protein binding. 32P-labeled sense RNA transcripts were produced from these constructs and used for UV-cross-linking assay. The results shown in Figs. 9 and 10 indicate that transcripts 3UTR2Δ2, Δ3, and Δ4, which lack the U-rich element (URE) (Fig. 10), did not form either the 48-kDa or the 28-kDa complex (Fig. 9, lanes 4, 5, and 6). This indicates that the binding sites for both the 28-kDa and the 48-kDa protein factors must be in a region between the NspI site and the Stul site. A 5′ deletion up to the EcoO109I site, represented by probe 3UTR2Δ5, retained the same binding activity for both the 28-kDa and the 48-kDa complexes (Fig. 9, lanes 7 and 11) as the full-length 3UTR2 probe (lane 2). Probe 3UTRΔ7, a 120-nt Avall/Stul fragment, which contains the entire 46-nt URE, exhibits full ability to form the 28-kDa and 48-kDa complexes (Fig. 9, lanes 9 and 13); therefore, the binding site for both complexes must reside within this 120-nt region. 3UTRΔ7 was cut at a unique HindII site within the U-rich sequence to produce probes 3UTRΔ6 and 3UTRΔ8 (Fig. 10). Probe 3UTRΔ6, which contains the region upstream of the HindII site, conferred a very weak ability to form either the 28-kDa or 48-kDa complex (Fig. 9, lanes 8 and 12), while probe 3UTRΔ8, which contains the region downstream of the HindII site, could not form the 28-kDa complex and exhibited substantially reduced formation of the 48-kDa complex (lanes 10 and 14). Taken together, these data strongly indicate that the entire U-rich element (URE) is necessary for effective formation of both the 28-kDa and the 48-kDa complexes with SGLT1 mRNA, and binding is abolished if it is cut at the HindII site. The observation that increased 48-kDa binding activity was consistently accompanied by a reduced 28-kDa binding activity, yet both apparently map to the same 120-nt sequence (shown in Fig. 10C), suggests that both binding activities may recognize either the same site or of the protein, this finding suggests that neither AU80 nor c-fos ARE RNAs contain the cognate sequence recognized by protein(s) forming the 48-kDa complex with SGLT1 3UTR2.
The mechanisms responsible for maintenance of the differentiated phenotype in renal epithelial cells are poorly understood. Expression in cell culture of the high affinity Na+/glucose cotransporter (SGLT1), a marker for late proximal tubule (S3), is regulated by cell density, differentiation inducers, as well as a number of agonists of intracellular signaling pathways. We have recently demonstrated that post-transcriptional stabilization of the SGLT1 message plays a prominent role in differentiation-associated expression of this transporter induced by either cyclic AMP or the differentiation inducer HMBA (Peng and Lever, 1995). Message stabilization required the presence of the 3′-UTR, suggesting that cis-acting sequences in this region were involved. This mechanism would permit the cell to increase rapidly its commitment to synthesize a specialized end product of differentiation in response to various extracellular signals.

Our present results show that the 3′-UTR of porcine renal Na+/glucose cotransporter SGLT1 mRNA contains a U-rich region (URE) that exhibits a specific, cyclic AMP-dependent and protein phosphorylation-dependent interaction with a cytoplasmic protein of apparent Mr = 48,000 which we have named SG-URBP. The simplest hypothesis to explain our results is that SG-URBP is a phosphoprotein in its activated state, and its binding affinity to SGLT1 mRNA is directly activated by protein kinase A-mediated phosphorylation. Alternatively, protein kinase A may be indirectly involved via a kinase cascade nor can we rule out the possibility that phosphorylation of another protein may be involved in activation of SG-URBP. The active phosphorylated form of SG-URBP would be hydrolyzed to an inactive (or low affinity) dephosphorylated form by an unidentified protein phosphatase. Our UV-cross-linking assay is only capable of assaying SG-URBP in its activated, RNA binding state, and, in the absence of specific antibodies, the putative inactive form cannot be measured. Direct demonstration of the mechanism will require purification of SG-URBP.

We have identified a 120-base region within the 3′-UTR of the renal SGLT1 message (Fig. 10C) which specifically interacts with SG-URBP to form the 48-kDa RNA-protein complex. This region contains a 46-nt U-rich element (68% U), and binding activity is effectively competed only by poly(U), unlabeled 3′UTR2 RNA, and other U-rich RNA sequences, but not by poly(A), poly(G), and poly(C), or irrelevant RNA sequences which contain no U-rich element. Furthermore, cleavage of this segment within the U-rich region abolishes the ability to form the complex. These data suggest that the U-rich element may be involved directly in RNA-protein interaction. Sequence data for this region of the 3′-UTR of human, rabbit, or rat SGLT1 are not available for comparison with the pig sequence in order to determine whether this URE motif is conserved across species.

Uridine-rich elements involved in destabilization of several nuclear transcription factor, cytokine, or lymphokine mRNAs have been divided into two broad categories (Chen and Shyu, 1994); those containing 1-3 copies of AUUUA motifs within the U-rich region, in some cases forming overlapping copies of the...
Glucose Cotransporter mRNA Regulation

| Table I | Correlation between the increased half-life of the SGLT1 3.9-kb message and the increased formation of the 48-kDa complex |
|---------|-----------------------------------------------------------------------------------------------------------------|
| Treatment | Half-life of the 3.9-kb SGLT1 mRNA* | Formation of 48-kDa RNA-protein complex^b |
| Control | 1 | 1 |
| IBMX, 1 mM | 8.6 | 5-10 |
| HMBA, 5 mM | 2.6 | 3 |

* Half-life was determined 4 days after addition of HMBA or IBMX and data are from Peng and Lever (1995).

^b UV-cross-linking assay was carried out in parallel using lysates from late-confluent cells treated with HMBA or IBMX for 4 days, and levels of the 48-kDa complex were estimated by scanning densitometry. Values are expressed as fold increase relative to untreated controls.

UUAUUUA(U/A)(U/A) nonamer, and those which lack the AUUUUA motif. The SGLT1 URE falls within the latter category since it lacks an AUUUUA motif within the U-rich element. Although three AUUUUA motifs are present further downstream (Fig. 1), they do not participate in SG-URBP binding.

The functional significance of SG-URBP binding to the U-rich mRNA sequence was not directly demonstrated in the present study. SG-URBP binding activity was activated by cyclic AMP-elevating agents and by phosphatase inhibitors. The increase in half-life (8.6-fold) of SGLT1 3.9-kb mRNA after cyclic AMP elevation (Peng and Lever, 1995) correlates well with the 5-10-fold increased formation of the SG-URBP complex under parallel conditions (Table I). Our previous results demonstrated that inhibitors of protein synthesis did not block stabilization of the message after cyclic AMP elevation (Peng and Lever, 1995). We propose that the post-transcriptional stabilization of SGLT1 mRNA in response to agents that elevate intracellular cAMP levels is mediated, at least in part, by the interaction between SGLT1 mRNA and its specific binding protein, SG-URBP. The ability of SG-URBP to bind its target site on the mRNA would be activated by protein phosphorylation, directly or indirectly mediated by protein kinase A. LCL-145 cells express two distinct but similar protein kinase A catalytic subunits encoded by different genes and both R1, and R1 regulatory subunits (Adavani et al., 1987). A protein kinase A-deficient mutant of this cell line was deficient in Na^+/glucose cotransporter expression in addition to alterations in other differentiated properties (Amsler et al., 1991). The U-rich sequence (URE) identified in this study encompasses the specific binding site for SG-URBP. This sequence may serve as a component of the degradation machinery and participate in the selective degradation of SGLT1 mRNA. It is downstream from the first polyadenylation site suggesting it may function in the nucleus as well as the cytoplasm. The URE may be a nuclear hypersensitive site or a binding site for a destabilizing factor which directs nucleases to the region. A potential hairpin loop region occurs upstream from the URE but does not appear to participate in SG-URBP binding. Presumably, the binding of phosphorylated SG-URBP would mask the U-rich sequence and protect the message from degradation.

U-rich elements found within the 3′-UTRs of some short-lived mRNAs such as c-fos and c-myc play an important role in regulation of turnover of these mRNAs (Belasco and Brawerman, 1993; Chen and Shyu, 1995). Several U-rich element binding activities have been identified. These include a 59-kDa binding activity that interacts with a c-myc ARE in Balb/c3T3 cells (Alberta et al., 1994), a 37-kDa serum-inducible, translation-dependent protein that recognizes the c-fos ARE in NIH 3T3 cells (You et al., 1992), and a human elav-like neuronal protein 1 (Hel-N1) which specifically binds to a U-rich region in the 3′-UTR of c-myc mRNA (Levine et al., 1993) and forms a 28-kDa RNA-protein complex. We have observed a 28-kDa binding activity which also interacts with the URE of the SGLT1 3′-UTR. Whether this 28-kDa protein is a kidney cell counterpart of Hel-N1 or a different protein remains unknown. We observed that an increase in the 48-kDa SG-URBP binding activity after cyclic AMP elevation was consistently accompanied by a corresponding reduction in the formation of the 28-kDa complex. Both binding activities recognize the same 120-base region and are competed by U-rich RNAs. Therefore, these two RNA binding activities are reciprocally regulated by cyclic AMP and may share a common binding site.

Several AU-rich mRNA-binding protein activities can be modulated by reversible phosphorylation/dephosphorylation. The adenosine-uridine binding factor AUBF (Malter 1989) is a phosphoprotein which specifically binds to AUUUUA motifs of several lymphokine, cytokine, and oncogene mRNAs (Gillis and Malter, 1991) after activation of protein kinase C by TPA and is proposed to contribute to agonist-induced stabilization of these labile mRNA species (Malter and Hong, 1991; Rajagopal and Malter, 1994). Two cytoplasmic proteins of 65 and 45 kDa reported to stabilize prostaglandin endoperoxide synthase II (COX II) mRNA in response to IL-1b and TPA in renal mesangial cells specifically bind the 3′-UTR in a phosphorylation-dependent manner (Srivastava et al., 1994).

A group of AU-rich element (ARE) mRNA-binding proteins in the Mr range of 32,000–40,000 (Brewer, 1991; Vakalopoulos et al., 1991; Bohijanen et al., 1991, 1992; Bickel et al., 1992; Port et al., 1992; Huang et al., 1993) bind to target mRNAs containing both AUUUUA pentameric motifs and flanking U-rich domains. Within this category is a 35-kDa protein up-regulated by isoproterenol which has been implicated in agonist-mediated destabilization of β-adrenergic receptor mRNA (Huang et al., 1993). However, AUUUUA motifs are neither always necessary nor always sufficient to target an mRNA for degradation (Shyu et al., 1989; Kabrick and Housman, 1988; You et al., 1992; Albert et al., 1994). Comparative analysis has found an obvious correlation between mRNA half-life and uridine content in the immediate vicinity of the AUUUUA motif (Alberta et al., 1994). It appears that U-richness is the most prominent characteristic of these AU elements. Binding of AUBF, a 44-kDa protein, to an artificial 80-bp reiterating AUUUUA RNA transcribed from probe AU80, was stimulated in extracts from cyclic AMP analog-treated adipocytes (Stephens et al., 1992), but enhanced binding to the naturally occurring message was not demonstrated. Our results suggest that AU80 and c-fos ARE RNAs do not specifically interact with SG-URBP even though both can effectively compete with the binding of SG-URBP to SGLT1 3′-URE mRNA. Since SG-URBP differs from other known mRNA-binding proteins not only in apparent molecular size, but also in activation by agents that elevate intracellular cyclic AMP, it is probably a new member of the U-rich region mRNA-binding protein inventory.

At present, we do not know if SG-URBP can also interact with a subset of mRNAs other than SGLT1 to coordinately influence their stability in response to cyclic AMP. Such a possibility would extend the Second Messenger Hypothesis for cyclic AMP-mediated regulation to the post-transcriptional level and involve mRNA-binding phosphoproteins as components of this intracellular cascade mechanism.

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