Expression of the Na⁺-coupled glucose cotransporter SGLT1 is regulated post-transcriptionally at the level of mRNA stability. We have previously demonstrated that cAMP-dependent stabilization of the SGLT1 message was correlated with the protein phosphorylation-dependent binding of cytoplasmic proteins to a uridine-rich sequence (URE) in the 3′-untranslated region (UTR). In the present study, the regulatory role of the URE was demonstrated by inserting it into the 3′-UTR of a β-globin reporter minigene under the control of the tetracycline-regulator promoter. The resultant chimeric globin/SGLT1 mRNA expressed after transfection into LLC-PK1 cells exhibited a decreased half-life compared with the β-globin control, indicating that the URE serves a destabilizing function. Activation of protein kinase A stabilized the chimeric message but not the β-globin control, indicating the presence of a regulatory stabilizing sequence within the URE. A 38-kDa nucleocytoplasmic protein was identified that recognized a 12-nucleotide binding site within the URE. A mutation in this binding site that prevented protein binding assayed in vitro by UV cross-linking also prevented protein kinase A-dependent stabilization of the chimeric message assayed in vivo. These findings identify the interaction between a 38-kDa nucleocytoplasmic protein and a regulatory uridine-rich sequence in the 3′-UTR as critical for cAMP-mediated SGLT1 message stabilization.

Glucose is the major carbon and energy source for eukaryotic cells. Transport of glucose into mammalian cells is the rate-limiting step for its utilization and accordingly is a highly regulated process. Maintenance of a constant blood glucose level is essential for cellular homeostasis. In kidney, glucose is reabsorbed from the urinary filtrate by the action of several types of glucose transporters arranged in series along the proximal tubule. These function together in polarized epithelial cells to mediate transepithelial transport of glucose. SGLT1 (Na⁺/glucose) cotransporters in the apical membrane catalyze active glucose transport into the cell driven by the Na⁺ gradient. Glucose diffuses passively out of the cell into the circulation via basolateral GLUT facilitative transporters. High affinity glucose transporters SGLT1 and GLUT1 located in late proximal tubule scavenge the remainder of filtered glucose not reabsorbed in early proximal tubule. SGLT1 is also expressed in small intestine where it mediates absorption of dietary glucose and galactose.

Most of our current information concerning the regulation of SGLT1 expression has been obtained from studies using the polarized epithelial cell line LLC-PK1, derived from porcine kidney. SGLT1 is expressed in this cell line as shown by cDNA cloning and Northern blot analysis (6) and is highly regulated by the cell growth and differentiation state (5, 7, 8). Protein kinase A (PKA) and protein kinase C (PKC) exert opposing effects on SGLT1 mRNA stability and steady-state levels in confluent cultures. Activation of PKA results in SGLT1 message stabilization (9), whereas PKC activation by phorbol esters such as phorbol 12-O-tetradecanoate 13-acetate (TPA) results in rapid degradation of the SGLT1 message (8). PKA-stimulated message stabilization was associated with a protein phosphorylation-dependent binding of cytoplasmic protein(s) to a uridine-rich sequence (URE) in the 3′-UTR (10). Changes in SGLT1 mRNA half-life elicited by PKA activation correlate well with changes in steady-state levels of the transporter (9), indicating that message stability is a major determinant of expression level.

In the present study, we identify a cis-regulatory domain within the URE of the SGLT1 mRNA 3′-UTR that binds a 38-kDa nucleocytoplasmic protein and is required for PKA-mediated stabilization of this message. Our approach was to map the minimal protein-binding site using an in vitro assay of PKA-stimulated RNA-protein complex formation. This in vitro assay was also used to characterize key RNA sequence specificity determinants and identify a mutation that prevents protein binding. The in vivo effects of this mutation, placed in the context of the URE, on cAMP-mediated message stabilization were then tested by inserting it into a β-globin reporter minigene construct and transfecting it into LLC-PK1 cells. Results from this analysis, in combination with deletion studies, demonstrate that the SGLT1 URE contains distinct sequence domains that convey instability to the stable β-globin message as well as a regulatory domain that acts to stabilize the message in response to PKA activation.
Glucose Transporter mRNA Regulation

EXPERIMENTAL PROCEDURES

Materials—[α-32P]Uridine triphosphate (3000 Ci/mmol) was obtained from ICN (Costa Mesa, CA). Nucleotide triphosphates were from Roche Molecular Biochemicals. RNA polymerases T3 and T7 were purchased from Promega (Madison, WI). RNase T1 (aspergillus oryzae) was from Calbiochem (La Jolla, CA). 3-Isobutyl-1-methylxanthine (IBMX) and phenylmethysulfonyl fluoride (PMSF) were from Sigma Chemical Co. Plasmids pTet-tTA K, pUHC13-3, and pT3/T7 were purchased from Life Technologies, Inc., and pSVneo was from CLONTECH (Palo Alto, CA).

Cell Culture—The porcine renal cell line LLC-PK1, clone G8 was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 0.37% sodium bicarbonate, and 24 mM HEPES (pH 7.0 as described previously (11). For experiments, monolayers were seeded at a density of 10^4 cells/cm² and grown for 4 days (post-confluent stage) in this medium supplemented with 1% penicillin-streptomycin (50 units/ml), and then IBMX (final concentration, 1 mM) was added to the indicated samples with medium change. Unless otherwise stated, cells were harvested at 96 h after addition of IBMX with one medium change at 72 h after treatment.

Preparation of Cytosolic and Nuclear Cell Extracts—Monolayers of cells were washed three times with ice-cold phosphate-buffered saline and lysed on ice with buffer A containing 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.4% Nonidet P-40, 1 mM sodium pyrophosphate, 1 mM NaF, 0.5 mM sodium orthovanadate, and 0.5 mM phenylmethysulfonyl fluoride as modified from Schreiber et al. (12). The lysates were incubated on ice for 5 min and then centrifuged at 12,000 × g for 45 min at 4 °C. The supernatants were removed and recentrifuged at 12,000 × g for 15 min to remove any precipitate material. The pellets were washed once with 1 ml of ice-cold buffer A and then were extracted for 30 min at 4 °C in buffer B containing 20 mM HEPES, pH 7.9, 0.42 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethysulfonyl fluoride, 1 mM sodium pyrophosphate, 1 mM NaF, and 0.5 mM sodium orthovanadate. The supernatants were centrifuged at 12,000 × g for 20 min at 4 °C and stored in aliquots at −85 °C. The protein concentration of cytosolic and nuclear extracts was determined by the Micro BCA protein assay (Pierce) as described by Atkins and Tuen (13).

Synthesis of Sense RNA Fragments for Binding Studies—Plasmid DNAs were linearized with appropriate restriction enzymes and transcribed in the presence of [α-32P]uridine triphosphate (3000 Ci/mmol). The renal SGLT1 cDNA plasmid pPSGT-B1 (6) was kindly provided by Ohta et al. (12). (13). The lysates were incubated on ice for 5 min and then centrifuged at 12,000 × g for 45 min at 4 °C. The supernatants were removed and recentrifuged at 12,000 × g for 15 min to remove any precipitate material. The pellets were washed once with 1 ml of ice-cold buffer A and then were extracted for 30 min at 4 °C in buffer B containing 20 mM HEPES, pH 7.9, 0.42 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethysulfonyl fluoride, 1 mM sodium pyrophosphate, 1 mM NaF, and 0.5 mM sodium orthovanadate.

After transcription, RNase-free DNase (RQ1 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 as carrier, followed by dilution with diethylpropylcarbonate-treated water to a final volume of 100 μl. Labeled transcripts were extracted with phenol/chloroform and precipitated twice with 2 mM ammonium acetate (pH 7.5) and 0.5 vol 100% ethanol at −20 °C. 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 electrophoresis on 6% acrylamide urine gels.

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 1 mM UTP, and the total volume of each reaction mixture was increased to 100 μl. Also, the RNA transcripts were precipitated without addition of carrier yeast tRNA, and the concentration was determined by UV absorption at 260 nm.

UV Cross-linking Assay of RNA-Protein Interaction—Radiolabeled RNA probe binding to protein was determined by the UV cross-linking assay described previously (14) with minor modifications. Briefly, either cytosolic or nuclear cell extracts (40 or 20 μg, respectively) were incubated with 0.2 ng of [32P]-labeled RNA probe (1000 cpm) at room temperature for 15 min in 10 mM HEPES, pH 7.6, 5 mM NaCl, 40 mM KCl, 4 mM DTT, 0.5% Nonidet P-40, 10 ng/ml heparin, and 2% sodium dodecyl sulfate. The reaction mixture with RNAse T1 (20 units/ng RNA) for 30 min at room temperature. The RNA-protein complexes were cross-linked by exposing reaction mixtures on ice to 120,000 μJ short-wave radiation (254 nm) in a UV cross-linker 1800 (Stratagene) for 7 min. Samples were boiled for 3 min in SDS sample containing 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% sodium dodecyl sulfate, 10% glycerol, 0.1% bromphenol blue, and 0.5% β-mercaptoethanol and then resolved on 12% SDS-polyacrylamide gels. The gels were dried and exposed to x-ray film at −80 °C using an intensifying screen.

Northwestern Blot Analysis—Cytosolic proteins, 40–60 μg, were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell) using an ABN model SD 1000 electrotransfer unit. The membrane was washed twice for 20 min in phosphate-buffered saline, pH 7.4, and then protein renaturation was carried out by incubating the filter with a solution containing 12 mM HEPES, pH 7.6, 50 mM NaCl, 5% glycerol, and 1 mM EDTA at 4 °C for 3–6 h. The filter was then incubated with a blocking solution containing 12 mM HEPES, pH 7.6, 50 mM NaCl, 1 mM Denhardt's solution, and 100 μg/ml yeast tRNA for 2–3 h at room temperature. The indicated sense 32P-labeled RNA probe (2 ng) was added to 2–5 ml of blocking solution. After a constitution of 0.5–1.0 × 10^6 cpm/ml and incubated with the filter on a rocker at room temperature for 60 min. The blot was gently washed three times for 5 min each with a solution containing 50 mM NaCl and 12 mM HEPES. The blot was then air-dried and exposed to x-ray film at −80 °C with an intensifying screen.

Chromatographic Fractionation of Binding Activity—Nuclear extracts (105.6 μg of protein) were prepared from five roller bottles of IBMX-treated confluent cells and then fractionated by ammonium sulfate precipitation. Proteins precipitated between 30 and 60% ammonium sulfate were collected by centrifugation at 12,000 × g for 15 min, dialyzed overnight against 0.4 M Tris-HCl, pH 8.0, 1 mM DTT, 1 mM phenylmethysulfonyl fluoride, 1 mM sodium pyrophosphate, 1 mM NaF, and 0.5 mM sodium orthovanadate with one buffer change, and then applied to a 123-ml diethylaminoethyl cellulose (DE52) column equilibrated with 0.4 M Tris-HCl, pH 8.0. The column was eluted in a gradient of 0.2–0.6 M KCl at a flow rate of 0.48 ml/min. Fractions (1 ml each) were collected beginning after 20 min of elution, and 10 μl of each fraction was assayed for RNA binding using the UV cross-linking assay.

Affinity Purification Using a Biotinylated Specific RNA Probe—Streptavidin-conjugated magnetic beads were washed three times with binding buffer TENS500 (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 500 mM NaCl). Biotinylated RNA probes were prepared in vitro transcription with T7 polymerase as described above except transcription mixtures contained 5 mM each of ATP, GTP, and UTP, 2.5 mM CTP, and 2.5 mM biotin-14 CTP. The indicated biotinylated RNA probe (200 pmol) was then complexed with 1 mg of magnetic beads in 1 ml of binding buffer by incubating at 37 °C for 4 h on a rocker. Then the streptavidin-conjugated beads were washed three times with wash buffer TEN1000 (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM NaCl).

Confluent LLC-PK1 cells on two 15-cm dishes were treated with 1 mM IBMX for 72 h and then switched to Dulbecco's modified Eagle’s medium deficient in methionine, cysteine, and cystine supplemented with 10% dialyzed fetal bovine serum, 2 mM glutamine, and 1 mM IBMX.
After 1 h culture in 35S-deficient medium, 35S-labeled methionine/cysteine was added to each dish. Tetracycline was then added to one dish of each isolated clonal cell line LLC-TAK-B7 exhibited a 50-fold increase in luciferase activity at 48 h after removal of tetracycline and was chosen as the host cell line for tetracycline-regulated ectopic gene expression. The kinetics of reappearance of luciferase activity following the removal of tetracycline was also analyzed. After a lag of 16 h, luciferase activity increased linearly for 16 h, reaching 50–60% of maximum at 15 h after the removal of tetracycline (data not shown).

**RESULTS**

**Nucleocytoplasmic Distribution of RNA Binding Activities**—A 223-nt uridine-rich region (nucleotides 2576–2697) in
the 3′-UTR of porcine SGLT1 mRNA (Fig. 1) has been implicated in the stabilization of this message after PKA activation (10). For this analysis, 32P-labeled sense RNA transcripts within a uridine-rich region of the SGLT1 3′-UTR were utilized. After binding, digestion of unprotected RNA with ribonuclease T1, and covalent cross-linking of the RNA-protein complex catalyzed by UV irradiation, labeled RNA-protein complexes were visualized after resolution by SDS-PAGE. The 122-nt RNA fragment recognized a cytoplasmic RNA binding activity that was stimulated after treatment of cells with cAMP elevating agents such as forskolin or IBMX (10). Stimulation was blocked by the PKA inhibitor H89 and was dependent on protein phosphorylation.

We investigated the possibility that nuclear RNA-binding proteins may also bind the SGLT1 URE sequence. A 47-nt RNA probe (nucleotides 2597–2643), consisting of a uridine-rich domain within the 122-nt region, formed a single 50-kDa complex using either nuclear or cytoplasmic extracts (Fig. 2A). Direct comparison of nuclear and cytoplasmic binding to the 47-nt probe assessed by band intensity under identical conditions indicated that the specific activity of SG-URBP was much greater in the nucleus than in the cytoplasm and was stimulated after treatment of cells with the cAMP phosphodiesterase inhibitor IBMX (Fig. 2A). Probe 3UTR2, a 435-nt fragment (nucleotides 2263–2697) overlapping the 122-nt region, gave a similar pattern of multiple IBMX-stimulated bands to that observed using the 122-nt probe in both cytoplasmic (Fig. 2B) and nuclear (not shown) extracts. No complex formation was observed in either nuclear or cytoplasmic extracts if the 122-nt region was deleted from probe 3UTR2 (not shown). Therefore, the regions immediately flanking the 122-nt site did not contribute to protein binding.

Mapping the Minimal Cognate Sequence—To further localize the binding site within the 47-nt RNA sequence, we generated a series of sense RNA fragments from corresponding synthetic double-stranded oligonucleotides containing a T7 promoter (Fig. 1). Fragments labeled with α-32P-labeled UTP by in vitro transcription with T7 polymerase were incubated with nuclear and cytoplasmic extracts from control and IBMX-treated cells and analyzed by the UV cross-linking assay (Fig. 2). As we have reported previously (10), complex formation with either the 3UTR2 transcript or the 122-nt transcript (previously named 3UTR2Δ) was greatly increased in cytoplasmic extracts from IBMX-treated cells compared with controls (Fig. 2B). The U-rich region represented by the 47-nt transcript contains two pentameric uridine motifs separated by 7 nucleotides (Fig. 1). Deletion of residues from the 5′ end of the 47-nt transcript to form a 28-nt transcript, which contains both uridine pentamers, retained 50-kDa complex formation in both cytoplasmic and nuclear extracts (Fig. 2, B and C). Further deletion from
the 5′ end with removal of both pentameric uridine motifs to form a 23-nt transcript abolished complex formation in both nuclear and cytoplasmic extracts. A homologous 32-nt transcript (HSGLT; Fig. 1) consisting of nucleotides 1098–1129 from exon 15 of the human genomic SGLT1 sequence (GenBank accession number L29339) also exhibited IBMX-stimulated formation of the 50-kDa complex both in nuclear and cytoplasmic extracts from LLC-PK1 cells (Fig. 2, B and C). HSGLT contained both pentameric uridine motifs present in the wild-type porcine sequence but differed in flanking residues.

Additional deletions within the URE region established that of the two pentameric uridine motifs shown in the diagram in Fig. 1, only the 5′ proximal one was necessary for 50-kDa complex formation. The 12-nt transcript (nucleotides 2620–2631), in which the 3′ proximal pentameric uridine motif was deleted but the 5′ proximal uridine pentamer was retained, represented the minimal sequence sufficient for IBMX-stimulated 50-kDa complex formation. The 41-, 38-, and 29-kDa transcripts, which lacked the 5′ proximal uridine pentamer but contained the 3′ proximal one, were not able to form the 50-kDa complex in either nuclear extracts (Fig. 2C) or cytoplasmic extracts (not shown). The 122-, 47-, 28-, and 19-nt transcripts, which contained both uridine pentamers, exhibited 50-kDa complex formation (Fig. 2). The 23- and 24-nt transcripts, which lacked both uridine pentamers, were ineffective in complex formation.

Protein binding required single-stranded sense RNA. The 50-kDa complex formation was observed using a sense 12-nt transcript (Fig. 2C) but not with the antisense 12-nt transcript or with double-stranded RNA annealed from sense and antisense 12-nt transcripts (not shown).

Specificity of RNA-Protein Complex Formation—We had previously shown that binding of cytoplasmic proteins to the 435-nt 3UTR2 probe was reduced by addition of an excess of unlabeled specific competitor RNAs but not by nonspecific RNAs (10). Furthermore, binding to 3UTR2 was competed by poly(U) RNA but not by other ribohomopolymers (10). Fig. 3 demonstrates that binding of nuclear proteins to either the human HSGLT or porcine 47-, 28-, or 12-nt 32P-labeled probes is competitively inhibited by a 250-fold molar excess of unlabeled 47-, 28-, 19-, and 12-nt RNA transcripts, all of which contain sequence elements sufficient for 50-kDa RNA-protein complex formation. By contrast, a 250-fold molar excess of either of the unlabeled 41-, 23-, and 29-nt transcripts, which lack these recognition elements, did not compete for binding to these probes. Similar results were obtained using cytoplasmic extracts (not shown). These competitive interactions among RNA probes that contain the 5′ uridine pentamer indicate that these probes recognize the same cellular factor. The inability of RNA probes lacking this uridine pentamer to competitively inhibit binding confirms the requirement for this motif in specific complex formation.

A 250-fold molar excess of poly(U) RNA competitively inhibited 50-kDa complex formation with the 122-nt transcript in both nuclear and cytoplasmic extracts as well as binding of nuclear proteins to the 47- and 28-nt transcripts (Fig. 4). Poly(A), poly(C), and poly(G) were ineffective as competitors. These results indicate that uridine residues play an important role in complex formation in both the nucleus and cytoplasm.

Stimulation of Nuclear and Cytoplasmic Binding Activities—We investigated the kinetics of activation of the URE binding activity in both nucleus and cytoplasm after treatment of confluent monolayers with IBMX for comparison with untreated control monolayers. Using either the 47-nt probe (Fig. 5A) or the 12-nt probe (Fig. 5B), 50-kDa complex formation in nuclear extracts was stimulated by IBMX within 1 h of addition and stimulation was maintained for 96 h. Similar results were obtained using nuclear extracts from forskolin-treated cells (Fig. 5C). In cytosolic extracts from control, untreated cells, a transient stimulation of complex formation was observed at time points up to 24 h after medium change, but complex formation was greatly diminished at later time points up to 96 h (Fig. 5, A and B). By contrast, in cytosolic extracts from IBMX-treated cells, complex formation, assayed with either the 47-nt (Fig. 5A) or the 12-nt (Fig. 5B) probes, was reproducibly maximal at 96 h relative to controls. Similar findings were obtained using the 122-nt probe (not shown). These findings indicate that PKA activation resulted in a rapid (<1 h) activation of binding activity in both the nucleus and cytoplasm, which is maintained up to 96 h.

Treatment of LLC-PK1 cells with 0.1 μM TPA results in rapid degradation of the SGLT1 message as a result of PKC activation (8). To investigate the possible role of the URE region in the SGLT1 3′-UTR in mediating PKC-stimulated SGLT1 message decay, we assayed RNA-protein complex formation in extracts from control and 0.1 μM TPA-treated cells using the 47-nt probe. At several exposure times, no significant difference in 50-kDa band intensity was observed between control and TPA-treated cells using nuclear extracts (Fig. 5D) or cytoplasmic extracts (not shown), nor were additional complexes detected. These observations indicate that TPA-mediated message destabilization is not associated with changes in protein binding to the URE.
**Protein Kinase Activators.** Clear fractions after treatment with protein kinase activators. A and B, cytoplasmic and nuclear extracts from confluent cultures treated with 1 mM IBMX for the indicated times and untreated controls were assayed by UV-cross-linking with an [a-32P]UTP-labeled sense 47-nt probe (A) or 12-nt probe (B). C, confluent cultures were treated with 100 µM forskolin for the indicated times for comparison with untreated controls and cultures treated with 1 mM IBMX (I), before isolation of nuclear extracts and assay by UV cross-linking using the 47-nt probe. D, nuclear extracts from cultures treated with 0.1 µM TPA for the indicated times and untreated controls were assayed by UV cross-linking using the 47-nt probe.

**Northwestern Blot Analysis**—In the above studies, protein binding was detected as a covalent complex with its cognate mRNA transcript. To directly identify cytoplasmic proteins that may have affinity for the SGLT1 URE, we carried out Northwestern blotting of proteins from control and IBMX-treated cells (Fig. 6). Proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, allowed to renature, and then incubated with 32P-labeled sense RNA probes. This method did not require ribonuclease digestion or UV cross-linking. Probe 3UTR2 and the 122-nt probe each recognized a 38-kDa protein and a 70-kDa protein that exhibited increased binding activity in IBMX-treated cell extracts (Fig. 6A). Another major band at 29 kDa was also observed. Probes of 47 nt and smaller were ineffective using this protocol because of technical difficulties in retaining label on the blot after washing. The 38- and 70-kDa bands detected in IBMX-treated cells were greatly reduced if cell extracts were treated with potato acid phosphatase before electrophoresis (Fig. 6B). Addition of the phosphatase inhibitor microcystin LR protected against these effects of phosphatase treatment (Fig. 6B).

These observations identify a major cytoplasmic 38-kDa URE-binding protein that exhibits increased binding activity in IBMX-treated cells as well as a requirement for protein phosphorylation. A 50-kDa RNA-protein complex is detected by these probes in cytoplasmic extracts after UV cross-linking; the increased size represents the contribution of cross-linked RNA. A 50-kDa cross-linked complex is observed with probes ranging in size from 435 to 12 nt, suggesting that the same minimal protected size of bound RNA remains after T1 ribonuclease digestion.

**Mutational Analysis of the Binding Site within the URE**—The importance of uridine residues and the 5’ proximal uridine pentamer in RNA-protein binding suggested by competition and deletion analysis was further explored using mutational analysis. We tested whether one or both uridine pentamers is required for binding and whether the flanking nucleotide residues also play a role in binding specificity. Introduction of two G substitutions in the 5’ proximal uridine pentamer (mutant 28-nt m5) had no discernable effect. A transcript containing these U → G substitutions in both pentamers (mutant 28-nt m3) also exhibited greatly reduced 50-kDa complex formation. Two U → G substitutions within the three uridine residues at the 5’ end of the 28-nt probe (28 nt m1) had only minimal effect on complex formation (Fig. 7A). C → A substitution of one or both of the residues flanking the 3’ proximal uridine pentamer (mutants 28-nt m4 and 28-nt m7) did not affect complex formation. Substitution of both flanking residues of the 3’ proximal uridine pentamer from CUUUUC → AUUUUG, the same residues flanking the 5’ proximal uridine pentamer, also did not affect complex formation (28-nt m6). Substitution of G and C residues flanking both uridine pentamers to A to create two AUUUUA motifs proximal uridine pentamer (mutant 28-nt m5) had no discernable effect. A transcript containing these U → G substitutions in both pentamers (mutant 28-nt m3) also exhibited greatly reduced 50-kDa complex formation. Two U → G substitutions within the three uridine residues at the 5’ end of the 28-nt probe (28 nt m1) had only minimal effect on complex formation (Fig. 7A). C → A substitution of one or both of the residues flanking the 3’ proximal uridine pentamer (mutants 28-nt m4 and 28-nt m7) did not affect complex formation. Substitution of both flanking residues of the 3’ proximal uridine pentamer from CUUUUC → AUUUUG, the same residues flanking the 5’ proximal uridine pentamer, also did not affect complex formation (28-nt m6). Substitution of G and C residues flanking both uridine pentamers to A to create two AUUUUA motifs proximal uridine pentamer (mutant 28-nt m5) had no discernable effect. A transcript containing these U → G substitutions in both pentamers (mutant 28-nt m3) also exhibited greatly reduced 50-kDa complex formation. Two U → G substitutions within the three uridine residues at the 5’ end of the 28-nt probe (28 nt m1) had only minimal effect on complex formation (Fig. 7A). C → A substitution of one or both of the residues flanking the 3’ proximal uridine pentamer (mutants 28-nt m4 and 28-nt m7) did not affect complex formation. Substitution of both flanking residues of the 3’ proximal uridine pentamer from CUUUUC → AUUUUG, the same residues flanking the 5’ proximal uridine pentamer, also did not affect complex formation (28-nt m6). Substitution of G and C residues flanking both uridine pentamers to A to create two AUUUUA motifs proximal uridine pentamer (mutant 28-nt m5) had no discernable effect. A transcript containing these U → G substitutions in both pentamers (mutant 28-nt m3) also exhibited greatly reduced 50-kDa complex formation. Two U → G substitutions within the three uridine residues at the 5’ end of the 28-nt probe (28 nt m1) had only minimal effect on complex formation (Fig. 7A). C → A substitution of one or both of the residues flanking the 3’ proximal uridine pentamer (mutants 28-nt m4 and 28-nt m7) did not affect complex formation. Substitution of both flanking residues of the 3’ proximal uridine pentamer from CUUUUC → AUUUUG, the same residues flanking the 5’ proximal uridine pentamer, also did not affect complex formation (28-nt m6). Substitution of G and C residues flanking both uridine pentamers to A to create two AUUUUA motifs
(mutant 28-nt m7) similarly had no effect on complex formation. Substitution to C of both residues flanking the 5’ proximal uridine pentamer (mutant 19-nt m3) had no appreciable effect (see Fig. 10B, bottom panel). Deletion of one uridine residue from the 5’ proximal pentamer caused reduced complex formation (not shown).

Nuclear extracts from IBMX-treated cells were fractionated by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (Fig. 7C). Fractions were assayed using the UV cross-linking assay. Binding activity for the wild-type 28 nt probe (Fig. 7C) as well as the 47-, 19-, and 12-nt RNA probes (not shown) exhibited the same elution profile with peak activity in fraction 63. These results reinforced conclusions from deletion studies that these probes recognized the same RNA-binding protein. Probe 28-nt m7, which contained two AUUUUA motifs, also exhibited peak binding in fraction 63 (Fig. 7C). Probe 28-nt m2 did not show binding activity in any fraction (not shown), consistent with its inability to bind proteins in the total cell extract.

Taken together, our results indicate that the 5’ proximal uridine pentamer is critical for protein recognition but that the nucleotides immediately flanking it do not appear to contribute to binding specificity. Furthermore, these mutation data reinforce conclusions from deletion analysis in Fig. 2 that the 3’ proximal uridine pentamer is not essential for 50-kDa complex formation with either cytoplasmic or nuclear extracts.

Using increased electrophoretic resolution and a 120-nt RNA transcript encompassing nucleotides 2576–2695, 50- and 57-kDa cross-linked complexes were observed in both nuclear and cytoplasmic extracts, and two additional major complexes of 68 and 120 kDa were observed using nuclear extracts (Fig. 8). Because the 120-nt transcript recognized multiple nuclear proteins, we introduced the same UU → GG substitution present in 28-nt m2 to create mutant transcript 120-nt m2. Both cytoplasmic and nuclear 50-kDa complex formation were abolished in 120-nt m2. This mutation did not affect the formation of the nuclear 57-kDa complex but did reduce formation of the larger nuclear complexes.

Binding activity of the wild-type and mutant 120-nt transcripts was also compared in the absence of cross-linking using electrophoretic mobility shift assay in nondenaturing gels (Fig. 9). This analysis permits the detection of multi-protein complexes and avoids possible artifacts because of sequence requirements for UV-catalyzed cross-linking. At least five major complexes were observed in nuclear extracts from IBMX-treated cells using the wild-type 120-nt probe but only one
major complex using the 120-nt m2 probe.

Effect of Secondary Structure—The possible role of secondary structure of the 120-nt RNA transcript in protein binding activity was tested by boiling the probe for 10 min and then either quickly cooling it on ice or slowly cooling it for 30 min at room temperature before assay of binding activity using nuclear extracts (Fig. 8B). Because it was necessary to assay binding activity at 16 °C instead of room temperature to prevent renaturation during assay, an alteration in the relative band intensities is observed in the control sample at the lower assay temperature. These results indicate that when renaturation of the RNA transcript was slowed by quick cooling, binding activity was greatly reduced, indicating a requirement for RNA secondary structure in protein recognition.

Isolation of RNA-binding Proteins Using a Specific Biotinylated RNA—The 19-nt probe was double-labeled with 32P and biotin by in vitro transcription in the presence of both [α-32P]UTP and biotin-14 CTP and tested using the UV cross-linking assay (Fig. 10C). Biotinylation did not affect the ability of this probe to form the 50-kDa complex. However, attempts to use the wild-type 19-nt probe for affinity purification on streptavidin-conjugated beads were hindered by degradation of the probe in the presence of nuclear extracts. However, probe 19-nt m3, which contains two C substitutions flanking the uridine pentamer, also exhibits 50-kDa complex formation unaffected by biotinylation (Fig. 10, B and C) but is much more resistant to degradation. Unlabeled biotinylated 19-nt m3 probe was able to competitively inhibit binding of 32P-labeled unbiotinylated and biotinylated wild-type 19-nt and 19-nt m3 probes (Fig. 10C), indicating that the same protein was recognized.

Nuclear extracts were prepared from IBMX-treated cells biosynthetically labeled with [35S]methionine. Fig. 10A demonstrates that an 35S-labeled band (indicated by arrow) with an apparent molecular mass of 38 kDa was bound to biotinylated probe 19-nt m3. The estimated 38-kDa size of this band agrees with that obtained by Northwestern blot analysis of cytoplasmic extracts. A 27-kDa band was also eluted. A 27-kDa band was also noted by Northwestern blot (Fig. 6) and appears to represent the protein component of a 28-kDa RNA-protein complex formed in extracts from control cultures not treated with IBMX (10). The 28-kDa complex is down-regulated after IBMX treatment and recognizes the same 12-nt minimal RNA-binding site shown in Fig. 1 as shown by deletion analysis. These bands were not observed in the absence of RNA or using a biotinylated probe 28-nt m2, which, as shown in Fig. 7, does not bind the 38-kDa protein. A number of higher molecular mass bands were eluted that represent nonspecific binding to the bead because they are observed in the absence of added RNA (lane N).

The 3UTR2 Sequence (Bases 2263–2697) Contains Both Destabilizing Elements and PKA-dependent Stabilizing Ele-
ties and half-life. This system offers important advantages over the use of actinomycin D to block transcription because tetracycline does not affect cell physiology or influence transcription of endogenous cellular mRNAs. Actinomycin D has been reported to influence mRNA decay rates (19) and the nucleocytoplasmic distribution of RNA-binding proteins such as HuR (20). Furthermore, other inducible promoter systems such as c-fos require serum induction conditions that may influence the evaluation of cell signaling effects on message decay.

To optimize and standardize tetracycline regulation of transcription, the tetracycline-regulated transactivator plasmid pTet-pTA was stably transfected into LLC-PK1 clone G8 cells by co-transfection with a plasmid encoding a neomycin resistance gene. Stable transfecants were selected using G418 and cloned by single-cell plating. Clonal lines were screened for tetracycline-regulated expression of a transiently transfected plasmid pUHC13-3 in which luciferase transcription is under the regulation of the tetracycline-regulated promoter. A cell line LLC-TAK-B7 was chosen as host cell line for these studies based on its 50-fold increase in luciferase expression after removal of tetracycline.

The 3'UTR2 region of the SGLT1 3'-UTR was inserted into the BglII site of pTet-BBB to construct pTet-BBB + 3UTR2. Plasmids were transiently transfected into LLC-TAK-B7 cells. After 24 h, β-globin expression was induced by removal of tetracycline. After 15 h of expression in the presence and absence of 1 mM IBMX as indicated, transcription was terminated by addition of 500 ng/ml tetracycline, and cells were harvested at the indicated times for isolation of cytoplasmic RNA. Samples were analyzed by Northern blot with quantitation by a PhosphorImager. Blots were sequentially hybridized with riboprobes for β-globin mRNA synthesis was then initiated by removal of tetracycline for 15 h in the presence and absence of 1 mM IBMX. Then transcription was terminated by addition of 500 ng/ml tetracycline, and cells were harvested at the indicated times for isolation of cytoplasmic RNA. Samples were analyzed by Northern blot with quantitation by a PhosphorImager. Blots were sequentially hybridized with riboprobes for β-globin mRNA. For half-life determinations, β-globin mRNA values were normalized to GAPDH. Open circles, IBMX-treated; filled circles, control; uninduced, cells were maintained in the presence of tetracycline to prevent expression. Poly(A)+ RNA was prepared by treating an aliquot of the zero time RNA sample with oligo(dT) and ribonuclease H to remove the poly(A) tail. Estimated molecular mass values of the transcripts at zero time were: pTet-BBB, 1 kb; pTet-BBB + UTR2, 1.45 kb. Values for the poly(A)+ samples were: pTet-BBB, 0.75 kb; pTet-BBB + UTR2, 1.2 kb.

To demonstrate the obligatory role of the URE region of the SGLT1 3'-UTR in regulating message stability in response to PKA activation, we utilized tetracycline-regulated expression of β-globin mRNA as a reporter message (Fig. 11). The stable β-globin message has been extensively used to analyse regulation of mRNA turnover by AU-rich sequence elements in various proto-oncogenes, lymphokines and cytokine 3'-UTRs (18). Test sequences are inserted into the unique BglII site located at the junction of the β-globin translated and 3'-untranslated regions in plasmid pTet-BBB (15). pTet-BBB encodes a β-globin minigene containing two introns and three exons. Transcription from this vector under the control of the tetracycline-regulated promoter (tet-Off) in the presence of a tetracycline-regulated transcriptional activator is switched on in the absence of tetracycline, yielding a chimeric β-globin mRNA. Transcription is rapidly switched off after the addition of tetracycline, permitting the analysis of RNA decay proper.
for cyclic nucleotide-dependent protein binding assayed in vitro and for cyclic nucleotide-dependent stabilization of a reporter message assayed in vivo. A 38-kDa nucleocytoplasmic protein that specifically binds this site in a PKA-stimulated, protein phosphorylation-dependent manner was identified by Northwestern blot analysis as well as by affinity purification using a specific biotinylated RNA. These studies provide direct evidence that binding of this protein to its site in the URE is necessary for cyclic nucleotide-dependent message stabilization.

Furthermore, our results suggest that the cAMP regulatory element within the 3′-UTR recognized by this protein is distinct from sequences that promote destabilization. Binding activity was predominantly found in the nucleus although also present in the cytoplasm, suggesting a role in both cellular compartments.

A complex interaction between cis-acting sequences within the message coding or noncoding regions and trans-acting protein factors in the nucleus and cytoplasm has been implicated in regulating the decay of a number of different mRNAs (21). However, there is currently no information on how this interaction influences mRNA degradation or how the process is regulated. Evidence that ongoing translation is required for destabilization has been reported in certain cases (22, 23). Poly(A) shortening initiates mRNA degradation (21), and dephosphorylation is subject to regulation by cis-acting sequences in the 3′-UTR (24).

Although much attention has focused on the role of AU-rich RNA destabilizing elements that are present in the 3′-UTRs of many labile transiently expressed RNAs such as those encoding proto-oncogenes, lymphokines, and cytokines (18), a much wider diversity of mRNAs is subject to post-transcriptional regulation. Examples of stabilization or destabilization of specific mRNAs by PKA (25–27) or PKC activation (8, 28, 29) have been described, but the sequences and trans-acting factors that mediate stability regulation have been identified in only a few cases. The involvement of specific RNA-binding proteins in regulated mRNA turnover has been inferred from indirect correlation evidence. The proteins were shown, using RNA gel mobility shift or UV catalyzed RNA-protein cross-linking, to bind in vitro to mRNA sequences that conferred altered stability in vivo when inserted into reporter genes and transcribed into cells. Several proteins meeting these criteria have been purified, and their cDNAs have been cloned (30–32). In the case of HuR (33, 34) and HnRNP D (35), ectopic overexpression of their cDNAs was shown to alter decay of a reporter globin message containing their cognate binding site. However, interpretation of these studies is not unequivocal because protein overexpression could alter decay by nonphysiological means, e.g. by saturating the decay machinery, activating low affinity or nonspecific pathways, or altering the normal nucleocytoplasmic distribution. Interestingly, in vivo UV cross-linking failed to detect binding of hRNF D to poly(A)+ RNA, although HuR binding was detected (36). Agonist destabilization of hamster β-adrenergic receptor mRNA was disrupted by point mutations that prevented protein binding to an AU-rich region in its 3′-UTR (37); these proteins were subsequently identified as hnRNP A1 and HuR (38). However, hnRNP A1 has been shown to bind to an unusually diverse array of RNA sequences (39), whereas HuR has typically been implicated in message stabilization rather than destabilization (33).

Sequence analysis of the 3′-UTRs of a wide variety of orthologous genes revealed stretches of 100 nucleotides or more that were highly conserved for over 300 million years of evolution, suggesting an essential function (40, 41). Porcine and human SGLT1 3′-UTRs exhibit 75% nucleotide sequence identity, a value comparable with that of their coding regions. An RNA
transcript based on the homologous region in the human SGLT1 gene also recognized the same binding activity in LLC-PK1 cell extracts and contained the pentameric uridine motif, indicating that human SGLT1 may be regulated by a similar mechanism.

In addition to a demonstrated role in regulating mRNA stability, the 3’-UTR has been implicated in regulating mRNA translation efficiency (42). This may be mediated by interaction of the poly(A) tail and its poly(A)-binding protein with the initiation factor eIF4G in the cap-binding protein complex via a loop structure (43), as demonstrated by studies in yeast (44) and mammalian cells (45). The poly(A)-binding protein shuttles between the nucleus and cytoplasm and may play a role in transport of mRNPs particles to the cytoplasm (46).

The identity of the 38-kDa RNA-binding protein described here is unknown. We have detected nuclear and cytoplasmic proteins that form a 50-kDa complex with our 47-nt probe in a wide variety of cell lines that do not express SGLT1 including COS cells, MDCK cells and 3T3 cells, suggesting a wider role. The cis-acting sequences described in this report differ from those described in the 3’-UTR of other messages whose stability is regulated by cAMP. Agonist destabilization of the β-adrenergic receptor mRNA was associated in hamster with protein binding to a 20-nt AU-rich sequence containing an AUUUUA motif flanked by U-rich regions (37) and in human with binding to an AU-rich nonamer UAUAUAUUAU (47). Cyclic nucleotide destabilization of type-1 plasminogen activator-inhibitor mRNA was associated with a predominantly A-rich sequence (26), whereas a C-rich sequence was implicated in limiting hormone/human choric gonadotropin receptor mRNA regulation (48). The cAMP-regulated stabilizing region in the lactate dehydrogenase-A 3’-UTR was identified as AUAUUUUCUGUAUUAUAUGUGU (49). A search of sequence data bases for sequences matching the consensus 12-nt minimal cis-acting sequence identified in our study revealed that it occurs rarely, nor was it found in other cAMP-regulated messages. Our observation that protein binding to the 120-nt fragment is secondary structure-dependent suggests that a search for potential binding sites in other messages involving more than the simple presence of short primary sequence motifs. A requirement for specific hairpin loop structures for RNA-protein binding has been noted for many mRNAs, notably the iron-responsive elements (50), where a combinatorial approach has been used to select stem-loop structures with high affinity for iron regulatory factor. A combinatorial approach has also been used to select AU-rich element consensus sequences (51).

Both nuclear and cytoplasmic binding activities were rapidly activated within 1 h of addition of IBMX or forskolin and were abolished by phosphatase treatment. These observations suggest that binding of the 38-kDa protein to the URE is dependent on nuclear and cytoplasmic protein phosphorylation events triggered by PKA. Addition of the purified PKA catalytic subunit to unstimulated cell extracts in the presence of substrates did not activate 50-kDa complex formation, suggesting that the 38-kDa protein is not phosphorylated directly by PKA but rather is activated by a protein kinase cascade triggered by PKA. It is possible that binding of the 38-kDa protein to the SGLT1 URE is initiated in the nucleus and accompanies the message to the cytoplasm, providing continuous protection from the cellular decay machinery. Efforts are currently under way to determine the identity of the 38-kDa protein and other components of the multiprotein complex and to elucidate their individual role in nuclear and cytoplasmic events that regulate the stability and translatability of the SGLT1 message.

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