A *cis*-dominant cyclic nucleotide-dependent regulatory domain in the 3′-untranslated region of Na^+/glucose cotransporter (SGLT1) mRNA

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Abstract A 122 nt uridine-rich sequence (URE) in the Na^+/glucose cotransporter (SGLT1) mRNA 3′-untranslated region is critical for cAMP-dependent message stabilization. Its function was investigated in LLC-PK_1 cells stably expressing β-globin reporter transcripts. Insertion of the SGLT1 URE downstream from an unrelated destabilizing sequence, the c-*fos* ARE, evoked cAMP-dependent message stabilization. Stabilization was blocked by a substitution mutation within the SLGT1 URE. These observations indicate that the SGLT1 URE is sufficient to transmit cAMP-dependent, *cis*-dominant mRNA stabilization in the presence of appropriate *trans*-acting factors and appears to function independently of the nature of the destabilizing domain.

Key words: mRNA stability; cAMP; Glucose transporter

1. Introduction

The 3′-untranslated region (3′-UTR) of a wide variety of genes contains highly conserved sequence elements of 100 nt or more which have been implicated in regulation of message stability and translation efficiency [1,2]. For example, adenylate/uridylate-rich elements (AREs) found in many highly labile mRNAs impart a potent destabilization when inserted into the 3′-UTR of stable reporter messages [3,4]. Stability regulation is mediated by the recognition of *cis*-acting signals on mRNAs by RNA binding proteins but the molecular basis for the specificity of this interaction and its regulation is poorly understood. This process is regulated by cell signaling pathways as a means to fine-tune levels of gene expression in response to changes in the cell environment or differentiation state [5].

Active glucose transport in kidney and intestine is mediated by SGLT1 Na^+/glucose cotransporters in the apical membrane. SGLT1 expression is differentiation-dependent, upregulated after cyclic AMP elevation [6,7] and impaired in a protein kinase A-deficient mutant [8]. Using deletion and mutation analysis, we have recently identified a uridine-rich regulatory sequence element (URE) in the 3′-UTR of the Na^+/glucose cotransporter SGLT1 mRNA which is critical for cAMP-dependent stabilization of the message [9].

In the present study, we demonstrate that the SGLT1 URE conveys cAMP-dependent message stabilization to a reporter message even in the presence of the c-*fos* ARE, a potent, unrelated destabilizing sequence. These observations indicate that the SGLT1 URE is a dominant, *cis*-acting functional unit and appears to act by a mechanism that is independent of the nature of the destabilizing domain(s) contained in the message.

2. Materials and methods

2.1. Materials

[α-^{32}P]uridine triphosphate (3000 Ci/mmol) was obtained from ICN (Costa Mesa, CA, USA). 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Sigma (St. Louis, MO, USA). The β-globin reporter plasmid pTet-BBB, which expresses β-globin under the control of a tetracycline-regulated promoter [10] and the plasmid T3 ARE *fos* [11] were obtained from Dr. Ann-Bin Shyu, University of Texas, Houston Medical School (Houston, TX, USA).

2.2. Cell culture

The porcine renal cell line LLC-PK_1 clone G8 and its stably transfected derivatives were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 2 mM glutamine, 0.37% sodium bicarbonate, 1% penicillin-streptomycin (50 units/ml) and 24 mM HEPES, pH 7.0 as described previously [12], with other additions as indicated.

2.3. Reporter plasmid construction

A gene coding for resistance to blasticidin (*Bsd*) was obtained by digesting the vector pCMV/Bsd (Invitrogen) with *Sal*I and *Xho*I. The *Bsd* fragment was then ligated into the *Xho*I site located upstream of the tetracycline promoter of pTet-BBB to form pTet-BBB/*Bsd*, which contained the *Bsd* gene in the opposite orientation to the tetracycline promoter. To test the influence of defined sequence elements on the stability of the β-globin message in vivo, test sequences were inserted into a unique *Bgl*II site of pTet-BBB/*Bsd* located in the 3′-UTR of β-globin. All SGLT1 nucleotide numbers refer to the pig SGLT1 sequence (accession no. M34044) [13]. Plasmid 122nt/*Bam* was prepared from p3UTR2Δ7 [14] by digestion with *Stu*I, religation in the presence of excess *Bam*HI linker followed by digestion with *Bam*H1 to produced a 145 bp fragment containing SGLT1 nucleotides 2576–2697. This was ligated into the *Bgl*II site of pTet-BBB/*Bsd* to create pTet-BBB/*Bsd*+122 nt. Plasmid p122nt/*Bam* was used as template for site-directed mutagenesis in order to construct p122 nt m2/*Bam* which contained a TT→GG substitution at nucleotides 2622–2623, as described previously [9]. This was digested with *Bam*HI and the 122 nt fragment was inserted into the *Bgl*II site in pTet-BBB/*Bsd* to create plasmid pTet-BBB/*Bsd*+122 nt m2. The 211 bp c-*fos* ARE fragment (nucleotides 3955–4165 of the human c-*fos* sequence) was excised from T3 ARE *fos* by digestion with *Eco*RI and *Hin*dIII and then blunt-end ligated into the above pTet-BBB/*Bsd* plasmids which had been pre-

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Abbreviations: SGLT, Na^+-coupled glucose transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; UTR, untranslated region; URE, U-rich element

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pared by digestion with *Pst*I, filled in using T4 DNA polymerase, treated with calf intestinal phosphatase and then purified by agarose electrophoresis, β-agarose digestion and ammonium sulfate precipitation. The 211 bp c-*fos* ARE fragment was also inserted by blunt-end ligation into the *Bgl*II site of pTet-BBB/*Bsd*. All constructs were verified by automated DNA sequencing.

2.4. Selection of double stable pTet-tTA/pTet-BBB LLC-PK$_1$ transfectant cell lines

A clonal LLC-PK$_1$ cell line LLC-TAK-B7 stably expressing the tetracycline-regulated *trans*-activator (pTet-tTA) was selected by G418 resistance [9] and then was stably transfected with the pTet-BBB/*Bsd* constructs described above using the calcium phosphate procedure we have described previously [9] followed by selection for 2 weeks in medium containing 500 ng/ml tetracycline, 500 μg/ml G418 and 8 μg/ml blasticidin.

2.5. Determination of chimeric mRNA decay rates

Stably transfected cell lines were grown to confluence in medium containing 30 ng/ml tetracycline, 8 μg/ml blasticidin and 500 μg/ml G418. Then 1 mM IBMX was added to the indicated cultures for 96 h, with refeeding at 72 h. Cells were washed twice then given fresh medium without tetracycline but containing 1 mM IBMX where indicated. After a 15 h pulse of expression from the plasmid, transcription was terminated by addition of 500 ng/ml tetracycline. At the indicated times, monolayers were washed twice with ice-cold phosphate-buffered saline and cytoplasmic RNA was extracted [15]. Samples of cytoplasmic RNA (10 μg) were analyzed by Northern blot using ^{32}P-labeled riboprobes for rabbit β-globin and porcine glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs to determine the kinetics of mRNA decay as we have described previously [9].

3. Results

3.1. Analysis of sequence domains which influence mRNA stability

In order to monitor the rate of mRNA decay, we have used the tetracycline-regulated promoter system to drive a transcriptional burst of reporter mRNA expression in stably transfected LLC-PK$_1$ cell populations. The stable β-globin message has been widely used as a reporter message to analyze the effects of destabilizing sequence domains within the 3′-UTRs of various unstable mRNAs [3]. Plasmid pTet-BBB encodes a β-globin minigene under control of the tetracycline-regulated promoter (tet-OFF) [10,16]. Test sequences are inserted into the unique *Bgl*II site located at the junction of the translated and untranslated regions of the β-globin gene. In the presence of the tetracycline-regulated transcriptional activator (pTet-pTA), transcription of the chimeric β-globin mRNA is switched on by removal of tetracycline from the medium and rapidly switched off following tetracycline addition, permitting the analysis of mRNA decay characteristics and half-life. This strategy offers important advantages over the use of transcription inhibitors since tetracycline does not affect mammalian cell physiology nor does it influence the transcription or stability of endogenous cellular mRNAs. The use of stable transfectants avoids non-physiological side effects and variations in expression associated with transient transfection methods. Furthermore, expression can be monitored in confluent cell lines whereas transient transfection requires subconfluent, actively dividing cells.

LLC-PK$_1$ cell populations stably expressing both the tetracycline-regulated *trans*-activator pTet-pTA and the indicated pTet-BBB/*Bsd* β-globin reporter plasmids, were used for analysis of mRNA decay. Uncloned populations of resistant cells were used in order to minimize effects due to clonal differences in integration of the plasmid. Analysis of the kinetics of expression after tetracycline removal indicated that after a 3 h lag, expression levels increased linearly for 16 h, reaching 50–60% of maximum after 15 h (data not shown). In the presence study, an expression period of 15 h was used in order to achieve reporter mRNA levels sufficient for analysis of decay.

3.2. A destabilizing domain and a regulatory cAMP-dependent stabilizing domain map within a 122 nt sequence (nucleotides 2576–2697)

Previously, we have identified a 435 nt uridine-rich domain (URE) within the 3′-untranslated region of the Na^+/glucose cotransporter SGLT1 mRNA that conveys cAMP-dependent message stabilization to a reporter β-globin message assayed in transient transfectants [9]. In the absence of cAMP elevation, this sequence has a destabilizing effect. A UU→GG substitution mutation at nucleotides 2262–2263 within this domain resulted in loss of cAMP-dependent stabilization and also reduced protein binding to this sequence. Protein binding sites were localized to a 122 nt uridine-rich sequence within the 435 nt segment [9] but effects of the 122 nt sequence on stability have not previously been tested. Results shown in Figs. 1 and 2 indicate that β-globin transcripts bearing either the wild-type 122 nt SGLT1 sequence or the mutant 122 nt m2 sequence containing the UU→GG substitution mutation at nucleotides 2262–2263 decayed with similar rates in control, untreated cultures ($t_{1/2}$ = 5.5 h). These decay rates significantly exceeded those of reporter transcripts lacking these sequences ($t_{1/2} > 12$ h), indicating the presence of destabilizing sequences. After treatment with the phosphodiesterase inhibitor IBMX in order to elevate cyclic AMP levels, transcripts bearing the wild-type 122 nt sequence became stable ($t_{1/2} > 13$ h), with over 90% of the mRNA remaining at 10 h after inhibition of transcription. Mutant transcripts (122 nt m2) were only minimally stabilized after IBMX treatment, manifest as an increased lag period of 3 h before initiation of decay of the mRNA body. The decay phase exhibited a half-life of 5.7 h, unchanged from that of control, untreated cultures.

3.3. cis-Dominance of the cAMP-dependent 122 nt SGLT1 regulatory domain in the presence of the c-fos ARE sequence

We next asked whether the cAMP-dependent stabilizing function of the 122 nt regulatory domain was specific for the endogenous destabilizing activity present within this sequence or whether it could exert its effects on a different category of destabilization element. For this purpose, we chose the 211 nt c-*fos* ARE as the test sequence. The c-*fos* ARE is a potent destabilizing element found in the 3′-UTRs of a variety of highly labile mRNAs [3,15]. Several key sequence features responsible for its destabilizing function have been defined [17,18] and it behaves as a functional destabilizing element where inserted into a variety of reporter constructs [10,19].

We first demonstrated that the c-*fos* ARE promoted destabilization of the reporter β-globin transcript in stably transfected LLC-PK$_1$ cells (Figs. 1, 2). As expected, ARE-bearing transcripts were rapidly degraded, with a half-life of 3.2 h, in the absence of IBMX. After 10 h, only 25% of the initial transcripts remained. Surprisingly, IBMX treatment led to partial stabilization of ARE-mediated decay, with 60% of transcripts remaining after 10 h. Stabilization of c-*fos* ARE-containing messages by cAMP elevation has not been previ-

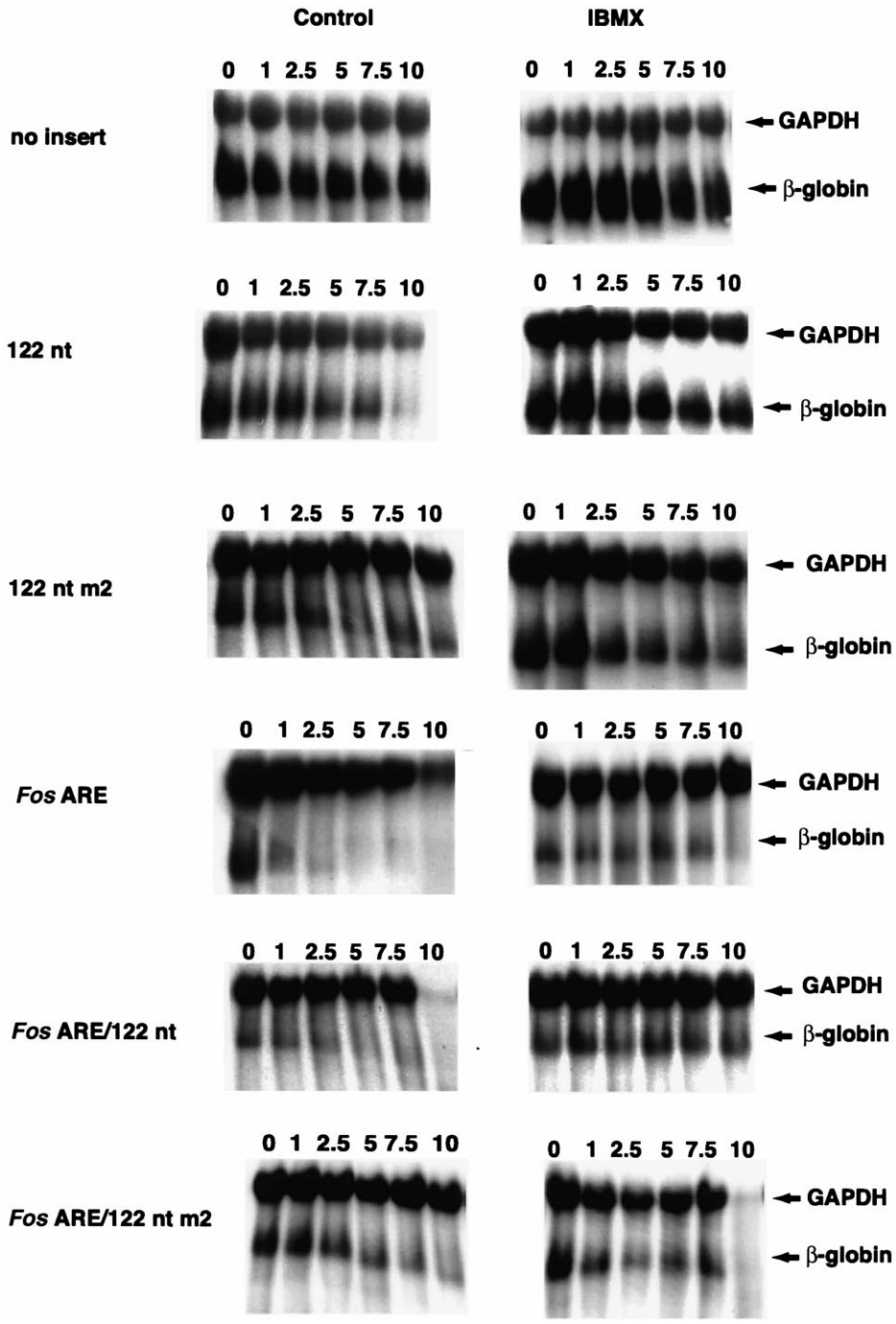

Fig. 1. Cyclic AMP-dependent stabilization of β-globin mRNA by the 122 nt SGLT1 regulatory domain in the presence of the c-*fos* ARE is decreased by mutation in a critical uridine pentamer within the SGLT1 URE. Stably transfected LLC-PK_1 cell lines exhibiting tetracycline-regulated expression of chimeric β-globin mRNAs containing the indicated insert in the 3′-UTR were analyzed for β-globin mRNA decay after growth in the presence or absence of 1 mM IBMX. Blots were simultaneously hybridized with riboprobes for GAPDH and β-globin mRNA.

ously described, although cAMP elevation has been reported to regulate decay of messages bearing similar AU-rich motifs [20,21].

Next, the potential interplay between the c-*fos* ARE and the 122 nt SGLT1 regulatory domain was tested using stable transfectants expressing reporter β-globin transcripts bearing both the c-*fos* ARE and the wild-type and mutant 122 nt SGLT1 regulatory domains (Figs. 1, 2). The 211 nt c-*fos* ARE sequence [22] was placed at 14 nt upstream of the SGLT1 122 nt sequence. In the absence of IBMX, both c-*fos* ARE/SGLT1 122 nt and c-*fos* ARE/SGLT1 122 nt m2 β-globin chimeric transcripts decayed with the same half-life of 6.2 h, indicating that the UU→GG substitution mutation in the 122 nt m2 sequence did not influence destabilization. This decay rate was approximately 2-fold slower than that of transcripts bearing the c-*fos* ARE alone under these conditions but similar to that of transcripts bearing the 122 nt sequence. In IBMX-treated cultures, reporter transcripts bearing the c-*fos* ARE/122 nt wild-type sequence exhibited a pronounced stabilization, with over 80% of the transcripts

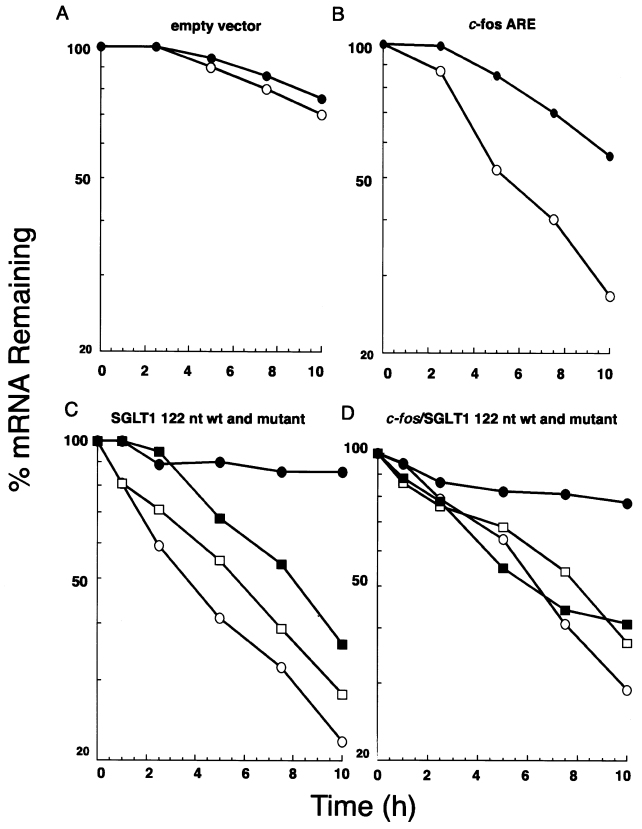

Fig. 2. Kinetic analysis of decay of chimeric β-globin mRNAs. Northern blots depicted in Fig. 1 were quantitated using a phosphorimager. Values for β-globin mRNA in each of the indicated constructs were normalized to those for GAPDH mRNA. Circles, wild-type; squares, 122 nt m2; open symbols, control, no addition; filled symbols, IBMX-treated.

remaining after 10 h. By contrast, reporter transcripts bearing the c-*fos* ARE/122 nt m2 sequence exhibited no increase in stability after IBMX treatment. Even the partial cAMP-dependent stabilization observed in transcripts containing the c-*fos* ARE alone was not observed for the chimeric c-*fos* ARE/SGLT1 122 nt m2 sequence. These observations indicate that the cAMP-dependent mRNA stabilizing activity associated with the 122 nt SGLT1 URE is functionally dominant in the presence of the c-*fos* ARE. The specific contribution of this element is demonstrated by the observation that the substitution mutation within the 122 nt sequence completely blocks cAMP-mediated stabilization.

4. Discussion

In the present study, we demonstrate using stable transfectants of LLC-PK$_1$ cells that the SGLT1 URE conveys cAMP-regulated cytoplasmic message stabilization to chimeric transcripts containing an unrelated destabilizing sequence, the well-characterized c-*fos* ARE. Our results also indicate that a potent destabilizing domain and a cAMP-dependent stabilizing domain reside in close proximity within a 122 nt sequence domain in the 3′-UTR of the SGLT1 message. Close proximity between destabilizing and cAMP-dependent stabilizing domains has also been described in the case of lactate dehydrogenase-A subunit mRNA, which contains both activities within a 22 nt sequence in the 3′-UTR [23]. A close proximity between destabilizing and stabilizing domains has also been described in the case of the c-*fos* ARE [22].

Analysis of 3′-UTR sequence elements implicated in cAMP-dependent mRNA stabilization in various messages has not revealed a consensus sequence motif which is generally characteristic of cAMP-dependent regulatory domains, although most described to date are enriched in uridine residues. In the case of porcine SGLT1, the minimal 12 nt binding site for a 38 kDa protein implicated in stability regulation is AUUUUUGGUUAA [9]. An RNA transcript based on the homologous human SGLT1 sequence containing the pentameric uridine motif also recognized the same binding activity in LLC-PK$_1$ cell extracts, indicating that human SGLT1 may be regulated by a similar mechanism [9]. The cAMP-dependent stabilizing region in the lactate dehydrogenase-A 3′-UTR was identified as AUAUUUUCUGUAUUAUAUGUGU [23]. Agonist destabilization of hamster β-adrenergic receptor mRNA was associated with a 20 nt AU-rich sequence [21] containing a critical AUUUUA motif which can tolerate the loss of one or gain of several uridine residues as determined by mutation and by comparison of the homologous region in different species [24]. By contrast, cAMP destabilization of type-1 plasminogen activator-inhibitor mRNA was associated with a predominantly A-rich sequence [25].

The mechanism of cAMP-dependent effects on message stability is not understood. Proteins which bind the SGLT1 URE sequence are most abundant in the nucleus but also found in the cytoplasm [9]. Stability-enhancing proteins may first bind the URE domain in the nucleus after protein kinase A activation and protein phosphorylation. They would then accompany the message into the cytoplasm, acting to block or displace destabilizing factors such as ribonucleases. Protein phosphorylation is required for protein binding to the SGLT1 URE, suggesting the involvement of a regulatory cascade [9,14].

Efforts are currently underway to identify the RNA binding proteins which interact with the SGLT1 URE to increase SGLT1 mRNA stability and the signaling pathways which regulate this interaction. These studies will provide insight into the series of events leading to increased active glucose resorption in kidney and intestine and increased blood glucose levels [26] following activation of protein kinase A.

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