

Short communication

REGULATION OF THROMBOSPONDIN-1 EXPRESSION THROUGH AU-RICH ELEMENTS IN THE 3'UTR OF THE mRNA

ASA J. ROBERT MCGRAY, TIMOTHY GINGERICH, JAMES J. PETRIK
 and JONATHAN LAMARRE*

Department of Biomedical Sciences, University of Guelph, Guelph, Ontario,
 N1G 2W1, Canada

Abstract: Thrombospondin-1 (TSP-1) is a matricellular protein that participates in numerous normal and pathological tissue processes and is rapidly modulated by different stimuli. The presence of 8 highly-conserved AU rich elements (AREs) within the 3'-untranslated region (3'UTR) of the TSP-1 mRNA suggests that post-transcriptional regulation is likely to represent one mechanism by which TSP-1 gene expression is regulated. We investigated the roles of these AREs, and proteins which bind to them, in the control of TSP-1 mRNA stability. The endogenous TSP-1 mRNA half-life is approximately 2.0 hours in HEK293 cells. Luciferase reporter mRNAs containing the TSP-1 3'UTR show a similar rate of decay, suggesting that the 3'UTR influences the decay rate. Site-directed mutagenesis of individual and adjacent AREs prolonged reporter mRNA half-life to between 2.2 and 4.4 hours. Mutation of all AREs increased mRNA half life to 8.8 hours, suggesting that all AREs have some effect, but that specific AREs may have key roles in stability regulation. A labeled RNA oligonucleotide derived from the most influential ARE was utilized to purify TSP-1 ARE-binding proteins. The AU-binding protein AUF1 was shown to associate with this motif. These studies reveal that AREs in the 3'UTR control TSP-1 mRNA stability and that the RNA binding protein AUF1 participates in this control. These studies suggest that ARE-dependent control of TSP-1 mRNA stability may represent an important component in the control of TSP-1 gene expression.

Key words: Thrombospondin-1, mRNA stability, Angiogenesis, AU-rich element, Post-transcriptional, Gene expression

* Author for correspondence. Fax: 1-519-767-1450, e-mail: jlamarre@uoguelph.ca

Abbreviations used: ARE – adenine-uridine rich element; AUF1 – AU-binding factor-1; DRB – 5,6 dichloro-1-β-D-ribofuranosylbenzimidazole riboside; TSP-1 – thrombospondin-1; TTP – Tris-tetraproline; UTR – untranslated region

INTRODUCTION

Thrombospondin-1 (TSP-1) is a 450 kDa homotrimeric glycoprotein [1-3], expressed in a wide variety of cell types. TSP-1 is now recognized to participate in a range of cellular processes including proliferation, differentiation, cell adhesion, cell-cell and cell-matrix interactions, embryonic development, angiogenesis, nerve regeneration, inflammation, as well as in the growth and metastasis of tumours (reviewed in [4, 5]). This exceptional functional diversity may be a result of the wide range of cell surface receptors and matrix proteins to which it binds [6].

TSP-1 expression is highly regulated *in vitro* and *in vivo* by a number of specific factors [4, 7] and during different tissue processes such as development [8], folliculogenesis [9], inflammation, and tissue repair [10]. Changes in TSP-1 mRNA levels are observed *in vitro* following stimulation with mitogens, growth factors, and hormones [4, 6, 9, 11]. The best-characterized regulatory mechanisms for these changes in expression involve inducible changes in TSP-1 transcription [6]. However, changes in mRNA stability have recently been suggested to play a role in the modulation of TSP-1 gene expression [12-15].

mRNAs that are post-transcriptionally regulated often encode proteins with potent biological activities that peak shortly after an extracellular stimulus and then undergo rapid decay [16-19]. Changes in mRNA half life are often attributable to multiple copies of adenylate- and uridylylate-rich elements (AREs) within the 3' untranslated region (UTR) of the transcripts. Initially identified by Caput *et al.* [20], ARE sequences are found within the 3'UTR of genes implicated in the inflammatory response. Subsequent studies [21] with the 3'UTR of the GM-CSF mRNA revealed that transcript destabilization was specifically mediated by ARE sequences. Many reports have subsequently revealed that the destabilizing effects of these elements are due to changes in the binding of specific AU-binding proteins (AUBPs) [16, 20, 22-24]. Most AREs contain either the sequence AUUUA or a high prevalence of consecutive uridylylate and adenylate residues [18, 25].

The 3'UTR of hTSP-1 mRNA is 2,097 bases in length and contains 8 sites that conform to the broad definition of a potential ARE. Based on reports that TSP-1 gene expression is post-transcriptionally regulated, and on the presence of these AREs, we investigated whether these elements participate in the regulation of TSP-1 gene expression. Our studies strongly support a role for mRNA stability in the control of TSP-1 gene expression and reveal for the first time that multiple AREs are implicated in this post-transcriptional control mechanism, thus revealing a novel regulatory paradigm for this protein.

MATERIALS AND METHODS

Plasmid construction

The human TSP-1 3'UTR (Genebank: NM_003246.2) was generated by PCR using cDNA prepared from HepG2 cells. Thrombospondin mRNA contains

a prototypical poly-A signal AAUAAA, that is completed by an uridylate-rich sequence beginning 12 bases downstream. This second portion of the terminator element is encoded in genomic DNA but is not present in mRNA. To allow the 3'UTR to be cloned from cDNA, the second half of the terminator element was engineered into the reverse primer based on genomic sequence data (AADC01119899.1). The forward and reverse primers respectively were: *AACAGCTAGCTGGA****ACTATGGGCTTGAGAAAAC*** and: *AACAATCGATAGCATGGATACAGTAAGTATAAATATAAATTTCCATATGATTTATTGTTGTTCCTTGTACATAAGAAACAG*. Restriction sites are underlined, sequences complementary to the gene are shown in bold and the engineered terminator element is shown in italics. In order to prepare a reporter plasmid under the post-transcriptional control of the TSP-1 3'UTR, pTRE2hyg-Luc (Clontech), which expresses luciferase under the control of the *Tet* promoter, was digested with *EcoRV* and *PflMI*, and re-ligated upon itself in order to remove the β -globin 3'UTR and poly-A signal. This plasmid, and the TSP-1 3'UTR insert were then digested with *NheI* and *Clal* and ligated to produce pTRE2hyg-Luc-hTSP-1-3'UTR. For ease of description, this construct is referred to as Luc-hTSP1-3UTR.

Site-Directed mutagenesis of the hTSP-1 3'UTR

Mutation of each ARE present within the Luc-hTSP-3UTR was performed using the Quikchange II-E Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. For each mutation, the core pentamer AUUUA (ATTTA in DNA sequence) was mutated to GUUUG (GTTTG). The forward primers used are listed. The reverse primers are the reverse complements of the corresponding forward primer, (note that for AREs in close proximity, a single primer was used to mutate 2 adjacent sites). ARE's 1-8 located at bases (1,019-1,023), (1,145-1,149), (1,171-1,175), (1,214-1,218), (1,802-1,806), (1,811-1,815), (2,086-2,090), and (2,092-2,096) of the human TSP-1 3'UTR (NM_003246.2) were mutated using the following forward primers respectively:

mut1: ATGCTTTACGATGTAAAAT**GTTTG**TTTTTTACTTATTCTGGAAG

mut2,3: GATTGTAATACAGATT**GTTTG**TTA ACTCTGTTCTGCCTGGAAG**TTTG**GGCTTCATACGAAAG

mut4: GTTTGAGAGCAAGTAGTTGAC**GTTTG**TCAGCAAATCTCTTGCAAGAAC

mut5,6: CTCAGATTGTGTAGATATGCT**GTTTG**AATAG**TTTG**TCAGGAAATACTGCCTGTAG

mut7,8:GAACAACAATAAATCATATGGAAG**TTTG**T**TTTG**TACTTACTGTATCCATGCTTA.

These mutations yielded the following plasmid constructs: Luc-hTSP1-3'UTR(mut1), Luc-hTSP1-3'UTR(mut2,3), Luc-hTSP1-3'UTR(mut4), Luc-hTSP1-3'UTR(mut5,6), Luc-hTSP1-3'UTR(mut7,8). The Luc-hTSP1-3'UTR(mut all) plasmid was generated by sequentially utilizing each primer set. Plasmids were digested using *DpnI* (Stratagene) and correct sequences of all mutant inserts were confirmed by dye-terminator sequencing at the University of Guelph Laboratory Services Centre.

Cell culture

Human transformed embryonal kidney (HEK293 cells) expressing the tetracycline-responsive transactivator protein (Clontech) were cultured in DMEM (Sigma) supplemented with 10% Fetal Bovine Serum (Sigma). Cells were maintained in the presence of 1 µg/ml doxycycline (ICN Biomedicals) throughout cell culture except when deprived for experiments as described.

Luciferase/TSP-1 3'UTR mRNA reporter experiments

Cells were grown on 10 cm plates to 40% confluency then transfected with 1 µg of the Luc-hTSP-3UTR reporter construct or a mutated derivative reporter construct using 10 µl of Lipofectamine 2000 (Invitrogen). 24 hours after transfection, media was replaced with serum-free media for 18h. Plates were then treated with 10 µg/ml doxycycline in order to stop new transcription from the reporter plasmids. RNA was isolated at various time points after doxycycline treatment.

Endogenous hTSP-1 mRNA decay analysis

Cells were cultured on 10 cm plates to 70% confluency then treated with 32 µg/ml of the transcriptional inhibitor 5,6 dichloro-1-β-D-ribofuranosylbenzimidazole riboside (DRB) (Sigma). RNA was isolated at various time points and subjected to quantitative RT-PCR as described below.

RNA isolation, reverse transcription and quantitative (real-time PCR)

Total RNA was isolated using Trizol (Invitrogen) as per the manufacturer's instructions and DNase treated using DNA-free (Ambion). 3 µg of RNA was reverse transcribed with poly-T using the Superscript II First Strand Synthesis System (Invitrogen). Real-Time PCR was performed in a Roche Light Cycler using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Primers (Invitrogen) for Real-Time PCR were: Tsp1 5'CGCCATCAGGGTA-AAGAACT3' and 5'GGGGTTGGTG-TCCCAGTAG3' Luciferase: 5'GCGCGTTATTTATCGGAGTTG3' and 5'CCGGGAGGTAGATGAGATGTG3' glucose-6-phosphate dehydrogenase (hG6PD): 5'TGGAACCG-GGACAACATC3' and 5'CAACAC-CTTGACCTTCTCATCAC3'. Samples were normalized to hG6PD. Standard curves for each gene were generated using 5 serial dilutions of cDNA with each dilution performed in quadruplicate. Real-Time PCR data was calibrator normalized and efficiency corrected using the Relative Quantification Software (Roche).

RNA pulldown experiments

HEK293 cells were grown to 80-90% confluence in two 10 cm dishes. The cells were washed 2x with cold MilliQ water and each plate was scraped into 700 µl of lysis buffer (10 mM HEPES, 20 mM NaCl, 60 mM KCl, 0.5 µM ZnSO₄, 4 mM MgCl₂, 0.3 mM CaCl₂) with the following additives: 1 EDTA-Free Protease Inhibitor Tablet (Roche) per 5 ml, 1.0 mM sodium orthovanadate, 1mM NaF, 1% of each of Phosphatase Inhibitor Cocktail I and Phosphatase Inhibitor

Cocktail II (Sigma) and 1.5 mM DTT. The cells were sonicated in a Fisher model 100 sonic dismembrator (Fisher Scientific) using setting 8 for 5 pulses of 2 seconds each. Lysis was confirmed by microscopy. The lysate was cleared by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge for 10 min at 4°C. 200 mg Dynabeads M-280 (Invitrogen) were washed and re-suspended in binding buffer as described by the manufacturer. 500 pmoles of a 5'-biotinylated RNA oligonucleotide containing the sequence 5' UUUACGAUGUAAAAUAUUUAUUUUUUACUUAUU 3' (Sigma Proligo) derived from bases 1,004 to 1,036 of the 3'UTR of human TSP-1 or a 5'-biotinylated RNA oligonucleotide containing the control sequence 5'-CGACUCCCGCAUCGACCACGGCUACGAAUGAGACCUC-3' combined with 400 units RNase OUT were incubated with Dynabeads for 10 min at room temperature. Bead/oligonucleotide complexes were then magnetically separated and resuspended in blocking buffer (10% BSA in lysis buffer plus 400 units RNase out) for 5 minutes at room temperature. Complexes were washed once in lysis buffer plus 400 units RNase OUT (Invitrogen), 0.5% DNase (Ambion) and resuspended in 100 µl of the same buffer to which 100 µg of yeast tRNA (Invitrogen) was added. Complexes were then mixed with 60 µg of cell lysate and incubated with rocking for 1 hour at 21°C. Complexes were then washed by magnetic separation twice using the lysis buffer with additives and 4x in buffer alone. Beads were resuspended in 150 µl of lysis buffer. 40 µl of each pulldown product was used for western blotting to evaluate the presence of AUF1 (Abcam, 1: 1500 dilution). Total cell lysates alone (60 µg, not subjected to pulldown) was analyzed as a control.

Statistical analysis

Data is presented as the mean +/- standard error of the mean (SEM) of 3 independent replicates. Statistical significance was determined using a two-way analysis of variance (ANOVA) and, in cases where a p value of < 0.05 was calculated, a Bonferroni post-test was utilized to verify the statistical significance.

RESULTS AND DISCUSSION

The 3' UTR of TSP-1 mRNAs contain multiple, conserved AREs

Thrombospondin-1 is a potent, multifunctional, extracellular protein that is markedly and rapidly regulated at the level of gene expression [4, 6]. Several previous studies have speculated that AREs within the TSP-1 3'UTR might be involved in the control of TSP-1 and TSP-2 gene expression [13, 14, 26]. Since AU-rich elements in the 3'UTR often participate in RNA stability regulation, we evaluated the TSP-1 3'UTR for the presence of these sequence elements. Comparative analysis of the TSP-1 3'UTR from human, mouse and rat revealed considerable sequence conservation, with over 70% sequence identity between the three species, and greater than 83% between any two species (Fig. 1). Of particular note was the presence of 6 conserved putative AREs within the 3'UTR

of each species, and the presence of an additional 2 elements in the human TSP-1 3'UTR. Two different consensus polyadenylation signals were present, located at bases 2,070 and 2,087 in the human, 2,076 and 2,093 in the mouse and 1,950 and 1,967 in the rat 3'UTR sequences. The overall sequence identity of the TSP-1 3'UTR between the different species coupled with the presence and conservation of AU-rich elements strongly suggested that the TSP-1 mRNA may be subject to post-transcriptional regulation.

<u>ARE #1</u>			
Human	1,012	GTAA <u>AA</u> TATTTATTTT 1,030	
Mouse	1,007	GTAATATATTTATTTGTT 1,025	
Rat	900	GTAATATATTTATTTTCC 918	
<u>ARE #2</u>			
Human	1,138	ACAGATTATTTATTA <u>ACTC</u> 1,156	
Mouse	1,131	ACAGATTATTTATTA <u>ACCC</u> 1,149	
Rat	1,023	ACAGATTATTTATTA <u>ACCC</u> 1,041	
<u>ARE #3</u>			
Human	1,164	CCTGGAAATTTAGGCTTCA 1,182	
<u>ARE #4</u>			
Human	1,207	AGTTGACATTATCAGCAA 1,225	
<u>ARE #5 & 6</u>			
Human	1,798	GCTATTTAAATAATTTATCAG 1,818	
Mouse	1,791	GCTATTTAAATAATTTATCAG 1,811	
Rat	1,675	GCTATTTAAATAATTTATCAG 1,695	
<u>ARE #7 & 8</u>			
Human	2,082	GAAATTTATATTTATA 2,097	
Mouse	2,089	GAAGTTTATATTTATA 2,104	
Rat	1,963	GAAGTTTATATTTAT 1,977	
<u>ARE (Mouse & Rat Only)</u>			
Mouse	260	AGCGCCTATTTACTTCCCA 278	
Rat	258	AGCGACTATTTACTTCCCA 276	

Fig. 1. Conserved AREs in the 3'untranslated regions from human, rat and mouse TSP-1. The cDNA sequence of the TSP-1 3'UTR from human, rat and mouse was compared *in silico*. ARE sequences from all three species are indicated as are AREs present only in human and only in rat and mouse. Overall sequence identity was greater than 70% for all three sequences and greater than 83% for any two sequences.

Endogenous TSP-1 mRNA is normally subject to rapid decay

The presence of AREs within the 3'UTR of target transcripts is frequently associated with rapid turnover of the associated mRNA. We therefore proceeded to evaluate the half-life of TSP-1 mRNA in HEK293 cells. Following the inhibition of transcription with DRB, a rapid decline in the cellular level of TSP-1 mRNA was observed, as determined by quantitative, real-time PCR (Fig. 2). Analysis of the decay kinetics of the TSP-1 mRNA in HEK 293 cells revealed a transcript half-life of approximately 2.0 h, with overall mRNA levels reduced to below 30 percent of those observed at time 0 over a period of 6 h (Fig. 2A).

This indicated that the TSP-1 mRNA is likely to be subject to regulation at the post-transcriptional level, and that the normal steady-state level of TSP-1 mRNA is affected by the relatively short half-life of the mRNA.

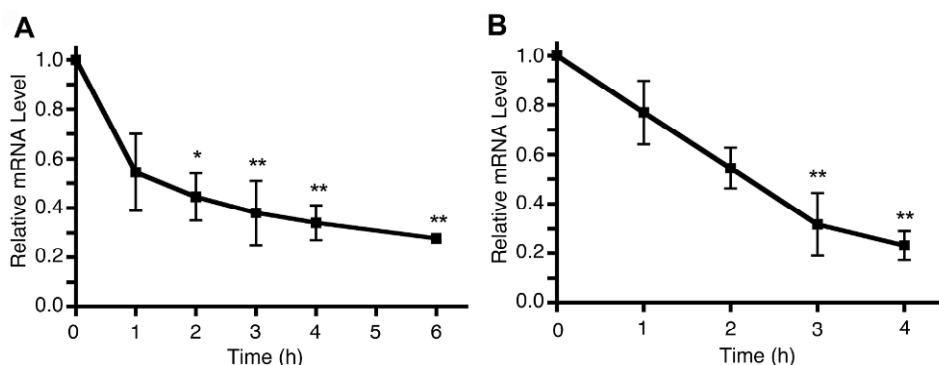


Fig. 2. Comparison of endogenous TSP-1 mRNA decay and decay of a luciferase/TSP-1 3'UTR reporter construct. A – Analysis of endogenous TSP-1 mRNA decay. Subconfluent HEK 293 cells were cultured for 24 hours then treated with 32 $\mu\text{g/ml}$ DRB (time 0 h). mRNA was isolated at the times indicated and TSP-1 mRNA levels were evaluated by quantitative PCR. Values represent the mean \pm SEM of triplicate samples from 3 separate experiments. B – Decay of luciferase reporter mRNA containing the TSP-1 3'UTR. HEK293 cells were transfected with a plasmid containing a tetracycline-responsive luciferase cDNA bearing the 3'UTR of human TSP-1. After selective transcriptional inhibition with doxycycline, RNA was collected at the specified time points and analysed for luciferase gene expression by quantitative PCR. Values for endogenous TSP-1 mRNA decay are presented for comparison. Results represent the mean \pm SEM of triplicate cultures from 3 separate experiments. Significantly different from the time 0 control at (*) $p < 0.05$ and (**) $p < 0.01$.

Reporter mRNAs carrying the TSP-1 3'UTR demonstrate similar decay kinetics to endogenous TSP-1 mRNA

Different mRNA transcripts demonstrate a wide range in rates of decay, with half-lives ranging from a few minutes (eg. many cytokines, transcription factors) to greater than 24 hours (β -globin) [16-18]. Based on the relatively rapid decay of TSP-1 mRNA and the presence of multiple AU-rich elements in the transcript, we hypothesized that the 3'UTR was likely to be an important participant in TSP-1 mRNA decay. We therefore generated a luciferase reporter plasmid under the transcriptional control of a tetracycline response element (TRE) and carrying the hTSP-1 3'UTR downstream of the ORF. HEK 293 cells stably expressing the tetracycline transactivator (tTA) (which leads to selective inhibition of gene expression in the presence of doxycycline) were transfected with this plasmid. After treatment with doxycycline, cultures were evaluated for reporter mRNA levels and the half-life of the reporter transcript was determined. The half-life of the mRNA generated from this plasmid was similar to that observed for endogenous TSP-1 mRNA in this cell type (Fig. 2B) which

strongly suggested that the reporter mRNA construct containing the TSP-1 3'UTR recapitulates important control elements present in the endogenous TSP-1 mRNA. It is important to note that the mechanism of transcriptional inhibition is different for each transcript (DRB vs. doxycycline) which results in "global" versus transcript-specific inhibition. Nevertheless, the overall similarities in decay rate strongly supported the possibility that similar regulatory mechanisms controlled decay in each case. In essence, the same regulatory elements were likely to be present in both the 3'UTR of the TSP-1 mRNA and the 3'UTR of the reporter, indicating that the reporter system could be utilized as an appropriate model system to further define the *cis*-acting elements involved. Since we had already established that conserved AREs were present throughout the 3'UTR, we focused our investigation on these elements of the TSP-1 transcript.

Mutation of AREs within the Luc-hTSP-3UTR construct alters the stability of transcribed reporter mRNAs

AU-rich elements represent important regulatory domains in the 3'UTR of many genes that are subject to rapid changes in expression, particularly rapid decay [16, 17, 19]. No specific association between the number or type of AREs and overall transcript stability has been established, nor is it clear whether there is a "threshold" effect, beyond which greater numbers of AREs fail to influence the rate of transcript decay. However, it is postulated that the presence of multiple AREs is associated with faster transcript decay [18, 27]. Several important recent studies have indicated that post-transcriptional regulation of TSP-1 is important in the overall regulation of gene expression [12, 15], and the 3'UTR has been specifically implicated [15]. However, no role for AREs in the control of TSP-1 mRNA stability has been reported in the literature.

Based on these earlier studies, we examined which, if any, of the AREs that we had identified in the TSP-1 3'UTR were likely to participate in the rapid decay process identified for the TSP-1 mRNA using the reporter construct that we had developed. To this end, we utilized site-directed mutagenesis to generate a plasmid in which some or all putative AREs were mutated (Luc-hTSP1-3'UTR(MutAll)). Transient transfection of HEK293/tTA cells with either the Luc-hTSP1-3'UTR reporter construct or the Luc-hTSP1-3'UTR(MutAll) construct, followed by treatment with doxycycline revealed a marked prolongation of the half-life (8.8 h) of the reporter mRNA generated from the mutant plasmid (Fig. 3). We then prepared a series of mutant plasmids in which individual and/or adjacent AREs were mutated. Transient transfection of these reporter plasmids into HEK293/tTA, followed by doxycycline treatment (to inhibit transcription and allow analysis of the decay rate), revealed different effects of each ARE on the decay of reporter constructs (Fig. 3).

Mutation of any ARE within the TSP-1 3'UTR had some effect on the overall stability of the corresponding transcript (i.e. stability of the transcript was increased to some extent). The effect of individual mutations was highly

variable, depending on the ARE involved. Two mutations in particular, Luc-hTSP1-3'UTR(mut1) and Luc-hTSP1-3'UTR (mut5,6), had marked overall effects on transcript stability (Fig. 3) and resulted in significantly slower transcript decay rates (half-lives of 4.4h and 3.6 h respectively), suggesting that they were likely to be the most important determinants of TSP-1 mRNA stability. Less affected were transcripts bearing mutations in other AREs ($T_{1/2}$ of 2.2 h, 2.8 h and 2.4 h for Mut2,3, Mut4 and Mut7,8 respectively).

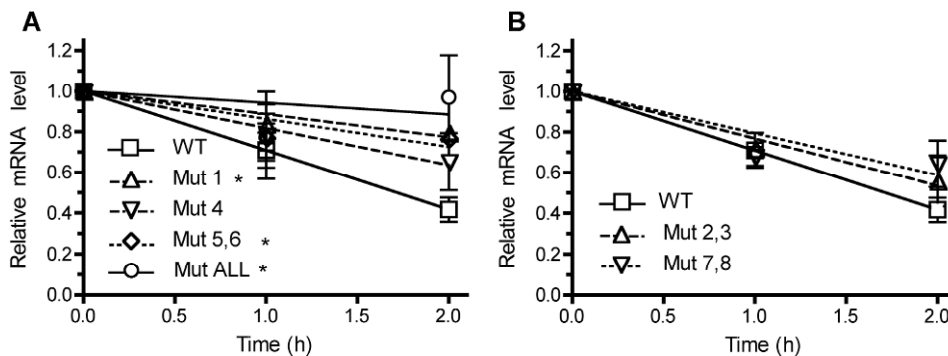


Fig. 3. Effect of mutation of different AREs and ARE combinations within the TSP-1 3'UTR on the rate of mRNA decay. HEK293/tTA cells were transfected with plasmids containing a tetracycline-responsive luciferase cDNA bearing either the wild type 3'UTR of human TSP-1 or one in which individual or multiple putative AREs have been mutated as indicated in the sidebar on the graph. Following selective transcriptional inhibition with doxycycline, RNA was collected at specific time points and analyzed for luciferase gene expression by quantitative PCR. Results represent the mean \pm SEM of triplicate cultures. A – Constructs that demonstrated a delayed half-life after mutation of ARE indicated. B – Constructs that showed an unaltered half-life after mutation. * Significantly different from wild-type ARE decay rate, $p < 0.05$.

These studies indicated that no single ARE is sufficient to regulate the relatively rapid observed rate of TSP-1 mRNA decay; no single mutation recapitulated the rate observed in the complete absence of functional AREs. This suggests that the overall effects of AREs within the TSP-1 3'UTR are additive, and the overall decay rate ultimately depends on the presence of most or all of the elements identified. However, there is clearly a hierarchy of importance; some AREs, such as that found between bases 1,019-1,023 of the TSP-1 3'UTR (Mut1) clearly have very potent individual roles in TSP-1 mRNA stability control, as mutation of this site alone leads to significant changes in reporter transcript decay rates. Our observation that multiple AREs within the TSP-1 gene are necessary for maximal decay suggests some redundancy within TSP-1 AREs in the control of transcript stability.

It is important to note here that miRNAs, including those induced by c-myc, also appear to participate in the control of TSP-1 mRNA stability in some cell types [12, 28]. This may provide a mechanism whereby the TSP-1 mRNA decay rate can be rapidly modulated by external stimuli. Although miRNAs were not the

focus of the current study, they should clearly be considered in the general context of TSP-1 mRNA stability in cells and tissues and is clearly an important subject for further investigation. It is also noteworthy that elements in both the 3' and 5'UTRs are likely to be important in the control of TSP-1 mRNA stability, particularly when regulated by external stimuli such as glucose levels [15]. Studies [15] have also suggested that the TSP-1 3'UTR alone actually inhibits the expression of reporter constructs that are not simultaneously under the control of the TSP-1 5'UTR. The mechanism behind this finding remains to be determined.

Oligonucleotides derived from the TSP-1 3'UTR bind to AUF1, a known ARE binding protein

ARE-dependent mRNA decay is mediated, at least in part, by interactions with specific binding proteins (AUBPs). AUF1 is a well-studied AUBP that binds to known AREs and typically facilitates rapid decay [18, 19, 29, 30]. The observed ARE-dependent decay pattern of TSP-1 mRNA prompted us to determine whether the most influential ARE in the 3'UTR of TSP-1 was capable of interacting with AUF1, which could then suggest involvement in the decay process (Note: HEK 293 cells lack TTP family members, the other AUBPs most often implicated in decay [17, 31]). To this end, we performed pulldown experiments using biotinylated RNA oligonucleotides, followed by western-blot analysis. These studies revealed that an oligoribonucleotide containing the most influential (in terms of stability) ARE sequence (ARE#1 in Fig. 1) within the TSP-1 3'UTR bound the mRNA destabilizing protein AUF1 in lysates of HEK293 cells (Fig. 4). Two AUF1 isoforms were found to bind to the target sequence and were tentatively identified as p37 and p40 AUF1, based exclusively on the predicted molecular weights of the bands observed. Preferential binding to the 40 kDa AUF1 isoform was noted, as indicated by differences between band intensities in the whole cell lysate (lane 3) and the pulldown (lane 1) samples. This suggests that individual isoforms may bind preferentially and mediate the pattern of decay observed. Significant levels of other AUF1 isoforms (42 kDa and 45 kDa) were not detected in the cellular lysates, suggesting that either the antibody does not recognize these, or that they are not expressed under these conditions. Further studies will be necessary to confirm whether these are indeed the isoforms predicted, and whether they mediate the observed effects.

These studies are the first to demonstrate a physical interaction between an ARE within the TSP-1 3'UTR and any AUBP. Our observation that AUF1 binds to a TSP-1 ARE suggests that the TSP-1 mRNA is probably regulated similarly to other ARE-bearing transcripts [29, 30]. Our finding that AUF1 associates with the TSP-1 mRNA is consistent with the observed inherent instability of endogenous and reporter TSP-1 mRNAs and with our finding that mutation of an AUF1 binding site enhances transcript stability. It is important to note, however, that the present studies have not attempted to evaluate all the binding

proteins that might potentially interact with the 3'UTR of the TSP-1 mRNA, nor do they indicate which of the proteins is the most important or relevant. We have, however, convincingly demonstrated that a known, abundant, and well-characterized binding protein associates with at least one functionally-relevant ARE in the TSP-1 3'UTR. Whether other proteins bind under these or other conditions and regulate mRNA stability, as seen for other transcripts [32], is a subject of active investigation.

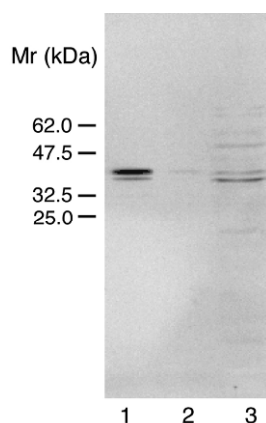


Fig. 4. AUF1 binding to a functional ARE in the TSP-1 3'UTR. Cellular lysates from HEK293 cells were subjected to biotinylated RNA oligonucleotide pulldown assays followed by western blot analysis for specific AUBPs as described in the methods. Whole cell lysates were examined as controls. Representative western blot for AUF1. Lane 1 – Pulldown assay utilizing the RNA oligonucleotide corresponding to the ARE located between nucleotides 1004 to 1036 (ARE#1) of the TSP-1 3'UTR (WT). Lane 2 – Pulldown assay using the control scrambled RNA sequence. Lane 3 – HEK293 total cellular lysate.

Overall, the results of the present study demonstrate that ARE-mediated changes in mRNA stability are potentially important facets in the control of TSP-1 gene expression. We have also demonstrated that multiple AREs in the 3'UTR of the TSP-1 mRNA appear to participate in the control of TSP-1 mRNA stability, that distinct AREs have independent effects on the stability of the TSP-1 transcript and that collectively, these AREs potently influence TSP-1 mRNA stability. Furthermore, we have shown that the RNA binding protein AUF-1 associates with at least one of several AREs in the TSP-1 3'UTR. These sites, and their associated binding proteins, represent potential candidates for participation in signaling events that modulate TSP-1 gene expression. Since TSP-1 has been widely implicated in angiogenesis [4] and other aspects of cancer biology, and abnormalities in the expression of RNA binding proteins such as AUF1 and TTP have recently been linked with the malignant phenotype [23, 31], associations between the mechanisms controlling TSP-1 gene expression in normal versus neoplastic cellular behaviour are likely to emerge as these pathways are investigated further.

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