

# Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference

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## ABSTRACT

HT1080 cells stably expressing green fluorescent protein (GFP) linked to a 3' terminal AU-rich element (ARE) proved to be a convenient system to study the dynamics of mRNA stability, as changes in mRNA levels are reflected in increased or decreased fluorescence intensity. This study examined whether mRNA stability can be regulated by small interfering RNAs (siRNAs) targeted to AU-binding proteins (AUBPs), which in turn should reveal their intrinsic role as stabilizers or destabilizers of ARE-mRNAs. Indeed, siRNAs targeting HuR or BRF1 decreased or increased fluorescence, respectively. This effect was abolished if cells were treated with both siRNAs, thus indicating antagonistic control of ARE-mRNA stability. Unexpectedly, downregulation of all four AUF1 isoforms by targeting common exons did not affect fluorescence whereas selective downregulation of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> strongly increased fluorescence by stabilizing the GFP-ARE reporter mRNA. This observation was fully confirmed by the finding that only selective reduction of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> induced the production of GM-CSF, an endogenous target of AUF1. These data suggest that the relative levels of individual isoforms, rather than the absolute amount of AUF1, determine the net mRNA stability of ARE-containing transcripts, consistent with the differing ARE-binding capacities of the isoforms.

## INTRODUCTION

Regulation of mRNA turnover is a key mechanism determining the intracellular abundance of transcripts, thus contributing to the controlled production of the encoded gene products (1–3). So far, the best characterized cis-elements mediating control of mRNA stability are the AU-rich elements (ARE) contained in the 3' untranslated region (3'UTR) of cytokines, growth factors and proto-oncogenes (4,5). Presence of an ARE induces rapid shortening of the poly-A tail followed by exosomal degradation of the mRNA body (6–8). Transcripts

containing an ARE usually exhibit rapid decay in resting cells, but are readily stabilized upon exposure to exogenous signals. Importantly, defective ARE function causing increased transcript stability has been reported to be a pathogenic factor in human malignancies (9–11), experimental tumors (12) and inflammatory disorders (13,14).

Several proteins binding to the ARE, AU-rich element binding proteins (AUBPs) have been identified and shown to exert a defined role in ARE-mRNA turnover. HuR, a member of the embryonic lethal abnormal vision (ELAV) RNA binding protein family has been shown to stabilize ARE-mRNAs (15,16). Two closely related proteins also containing RNA recognition motifs (RRM), TIA-1 (T-cell internal antigen-1) and TIAR (TIA-1-related protein), bind the ARE and act as translational silencers (17). Tristetraprolin (TTP, *zfp-36*) the prototype of a family of zinc-finger proteins of the unusual Cys-Cys-Cys-His (CCCH) class promotes degradation of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (18,19), granulocyte-macrophage colony stimulating factor (GM-CSF) (20) and interleukin-3 (IL-3) mRNA (21). Butyrate response factor 1 (BRF1, *zfp-3611*), a second member of the same class of zinc finger proteins, has been identified using functional genetic screening (22) and was shown to mediate rapid decay of various cytokine mRNAs, including IL-3, IL-6, GM-CSF and TNF $\alpha$  (23). Finally, AUF1 was first identified as a promoter of rapid ARE-mRNA decay in extracts of K562 cells (24). However, upon purification and cloning, the recombinant protein lacked decay promoting activity (25). The analysis of AUF1 has been further complicated by the existence of four isoforms that arise by differential splicing (26). This family of proteins distinguishes a 37 kDa (p37<sup>AUF1</sup>) core protein, a 40 kDa protein (p40<sup>AUF1</sup>) containing an N-terminal 19 amino acid insertion (exon 2), a 42 kDa protein (p42<sup>AUF1</sup>) exhibiting a C-terminal 49 amino acids insertion (exon 7), and a 45 kDa protein (p45<sup>AUF1</sup>) with insertions of both exon 2 and exon 7. Presence or absence of these alternatively spliced exons confers distinct biological properties to individual AUF1 isoforms. Presence of exon 7 not only affects nucleocytoplasmic distribution (27,28), but also blocks ubiquitination of p42<sup>AUF1</sup> and p45<sup>AUF1</sup> (29). In contrast, the lack of exon 7 targets p37<sup>AUF1</sup> and p40<sup>AUF1</sup> to the ubiquitin-proteasome pathway, which results in rapid decay of ARE-mRNAs (30,31). Importantly, absence of exon 2 in p37<sup>AUF1</sup> and p42<sup>AUF1</sup> is associated with high affinity binding of these

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isoforms (26). In addition to its role in ARE-mRNA turnover, AUF1 has been proposed to be involved in multiple cellular processes such as telomere maintenance (32) and transcriptional activation (33). The fact that AUF1 was identified to be a component of the  $\alpha$ -globin mRNA stability complex (34) and to participate in mRNA decay directed by the *c-fos* major coding determinant (35) points to a more general function of this protein in mRNA turnover. With respect to ARE-mRNA turnover, the role of AUF1 has been extensively studied, but results were conflicting. Increased expression of AUF1 has been associated with rapid ARE-mRNA decay in peripheral blood mononuclear cells (PBMC) (36,37), smooth muscle cells (38) and monocytes (39). These data, together with the finding that forced expression of p37<sup>AUF1</sup> and p42<sup>AUF1</sup> antagonized stabilization of ARE-mRNAs accompanying hemin-induced erythroid differentiation of K562 cells (40) argue for an intrinsic destabilizing activity of AUF1. However, overexpression of all four AUF1 isoforms in NIH3T3 cells stabilized ARE-containing transcripts (41). It has not been resolved whether these disparities were due to the different cell types examined or changes in experimental conditions. Analysis of AUF1 knockout mice or studies using small interfering RNAs (siRNAs) targeted to AUF1 should help to clarify these issues.

The present study examined whether single or combined treatment of cells with siRNAs targeting HuR, BRF1 and AUF1 would affect ARE-mediated mRNA turnover. Our data indicate that BRF1 and HuR control ARE-mRNA stability in an antagonistic fashion. Furthermore, we present evidence that effects of AUF1 on ARE-containing transcripts depend on the expression level of individual AUF1 isoforms.

## MATERIALS AND METHODS

### Cell culture and transfection

The previously described HT1080-derived reporter cell line HTwt16 (21) was cultured in Iscove's Modified Dulbecco Medium containing 10% fetal calf serum (FCS), 50  $\mu$ M 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C/7% CO<sub>2</sub>. In addition, NIH 3T3 cells stably expressing the GFP-ARE (3T3GFP-ARE) were generated and cultured as described above.

For transfections,  $1.5 \times 10^5$  cells were plated per well of a 6-well plate and cultured overnight. Thereafter, siRNAs were transfected at a final concentration of 240 nM in the presence of serum free medium (OptiMEM, Invitrogen) using Oligofectamine (Invitrogen) as the carrier. Analyses were performed 48–72 h after transfection.

### Oligoribonucleotides

The following 19mer oligoribonucleotide duplexes containing a 2-nt deoxythymidine overhang at each 3' end were purchased from Xeragon. BRF1, 5' CAA GAU GCU CAA CUA UAG U; HuR (oligo A), 5' UAA AGU AGC AGG ACA CAG C; HuR (oligo B), 5' CAC GCU GAA CGG CUU GAG G (42); AUF1 (exon 1), 5' GAU UGA CGC CAG UAA GAA C; AUF1 (exon 2/oligo A), 5' ACG AGG AGG AUG AAG GCC A (the first 16 nt are located in exon 1, the last 3 nt in exon 2); AUF1 (exon 2/oligo B), 5' GCA GCG ACG GCA CAG CGG G; AUF1 (exon 3), 5' GAU CCU AUC ACA GGG

CGA U; AUF1 (exon 7), 5' CUG GAA CCA GGG AUA UAG U;  $\beta$ -globin, 5' CAA GAA AGU GCU CGG UGC C.

Sequences were derived from the human sequences of HuR mRNA (accession number BC003376), BRF1 mRNA (accession number NM\_007564), AUF1 mRNA (accession number NM\_031370) and  $\beta$ -globin mRNA (accession number V00497.1). Blast search was performed on all the sequences to avoid cross-reaction with other genes.

### Flow cytometry

FACS analysis of  $1-2 \times 10^4$  cells was performed using a FACScan (Becton Dickinson) and the Cellquest software. GFP fluorescence was excited at 488 nm and emission was measured at 510 nm. To analyze the difference in fluorescence between medium and siRNA treated cells, Kolmogorow-Smirnow statistics calculating the greatest difference (D) in fluorescence between these two treatments were performed. The mean  $\pm$  standard deviation (SD) of D for 4–9 independent experiments was calculated and Student's *t*-test comparing D of  $\beta$ -globin and specific siRNA-treated cells was performed.

### Northern blot analysis

To analyze mRNA levels in cells treated with specific siRNAs, total RNA was harvested 72 h after transfection using Trizol<sup>®</sup> (Invitrogen). Total RNA (10–15  $\mu$ g) was resolved on 1% agarose-formaldehyde gels, which were subsequently blotted onto Hybond-N+ membranes (Amersham) using 20 $\times$  SSC. To detect HuR, filters were hybridized in the presence of 50% formamide (43) using a [ $\alpha$ -<sup>32</sup>P]GTP-labeled SP6 transcribed probe spanning nt 121–488 of the murine HuR cDNA (accession number NM\_010485). To detect BRF1, control  $\beta$ -actin and G3PDH, PCR fragments of the respective cDNAs (BRF1, nt 524–1019, accession number X99404; G3PDH, nt 589–1246, accession number M33197; murine  $\beta$ -actin, nt 554–815, accession number X03765) were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP using Klenow enzyme (New England Biolabs) and the gene specific 3' primers. Filters were hybridized overnight at 65°C in 0.5 M sodium phosphate buffer pH 7.2 containing 1% BSA (Fraction V, Sigma), 7% SDS and 5 mM EDTA, and subsequently washed at 55°C each in 2 $\times$  SSC/0.2% SDS and 0.2 $\times$  SSC/0.2% SDS.

To analyze mRNA stability of the GFP-ARE reporter, cells were treated 72 h after transfection with 5  $\mu$ g/ml of actinomycin D. Cytoplasmic RNA was harvested at the indicated time-points (44) and northern blot analysis performed using an SP6 transcribed [ $\alpha$ -<sup>32</sup>P]GTP-labeled probe spanning a XbaI-EcoRI fragment containing the murine IL-3 3'UTR as described.

Signal intensities were quantified using a Personal Molecular Imager<sup>®</sup> FX (Bio-Rad) and the Quantity One<sup>™</sup> software (Bio-Rad). To normalize for loading differences, signal intensities were normalized to the  $\beta$ -actin or G3PDH control.

### Western blot analysis

Western blot analysis was performed as recently described (21). Briefly, 30–50  $\mu$ g of total cell lysates in RIPA buffer (BRF1 and AUF1) or 15  $\mu$ g of cytoplasmic cell lysate (HuR), prepared with the NE-PER<sup>®</sup> nuclear cytoplasmic extraction reagents (Pierce), were resolved on 4–20% gradient polyacrylamide gels (Anamed Electrophoresis GmbH) and blotted

onto Immobilon-P (Millipore) membranes. To detect BRF1, a polyclonal antibody was generated in rabbits immunized with a C-terminal BRF1 peptide (SDSPTLDNSRRLPIFSRLSI-SDD; Neosystem) covalently linked to KLH. Antibodies were subsequently affinity purified using the BRF1 peptides employed in immunization. Monoclonal mouse-anti human HuR (Santa Cruz), polyclonal rabbit-anti human AUF1 (Upstate Biotechnology) and monoclonal mouse-anti bovine  $\alpha$ -tubulin antibodies (Molecular Probes) served to detect the respective genes. An alkaline phosphatase-coupled goat-anti-rabbit IgG (Southern Biotechnology Associates Inc.) or a horseradish-peroxidase-coupled goat-anti-mouse IgG (DAKO) were used as secondary antibodies. Development was performed using CDP-Star (Roche) or ECL Advance (Amersham), respectively. For quantification, autoradiographs were scanned and analyzed by densitometry using the Quantity One<sup>®</sup> software (Bio-Rad).

### Detection of GM-CSF by ELISA

Supernatants of triplicate cultures treated with medium or individual siRNA were harvested 72 h after transfection. GM-CSF was determined by ELISA (Pharmingen) according to the manufacturers instructions.

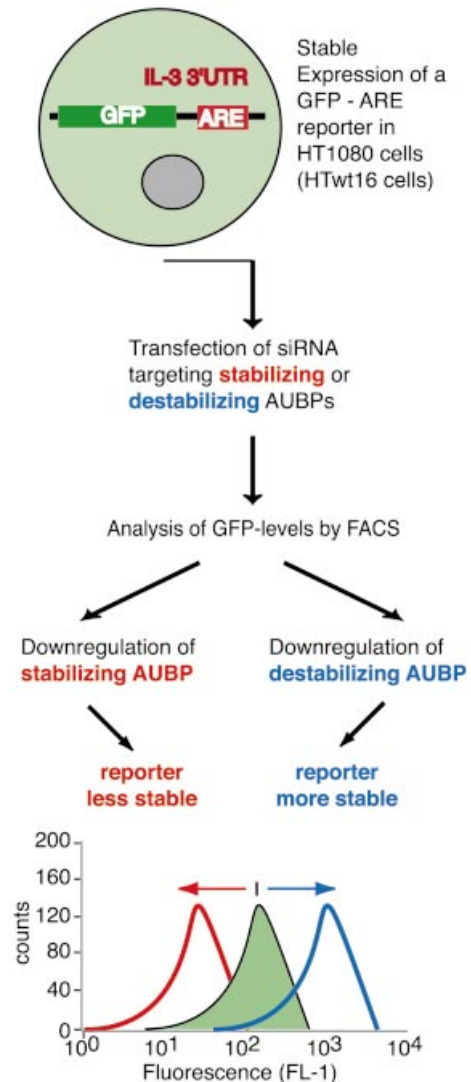
## RESULTS

### SiRNA targeting HuR and BRF1

We recently showed that siRNA targeting BRF1 increased mRNA stability of a GFP-ARE reporter and, concomitantly, fluorescence of transfected cells by FACS. Indeed, these siRNAs allowed distinction between cells with two wild-type BRF1 alleles from cells exhibiting a heterozygous inactivation of BRF1, solely based on the extent of the fluorescence shift (22). These data encouraged us to extend this approach to further AUBPs, arguing that if changes in GFP-ARE transcript stability are faithfully reflected in altered fluorescence intensity, this approach will enable the assessment of ARE-mRNA turnover rates in live cells without requiring the use of transcriptional inhibitors. As exemplified in Figure 1, downregulation of AUBPs stabilizing or destabilizing ARE-mRNAs will decrease or increase fluorescence intensity, respectively.

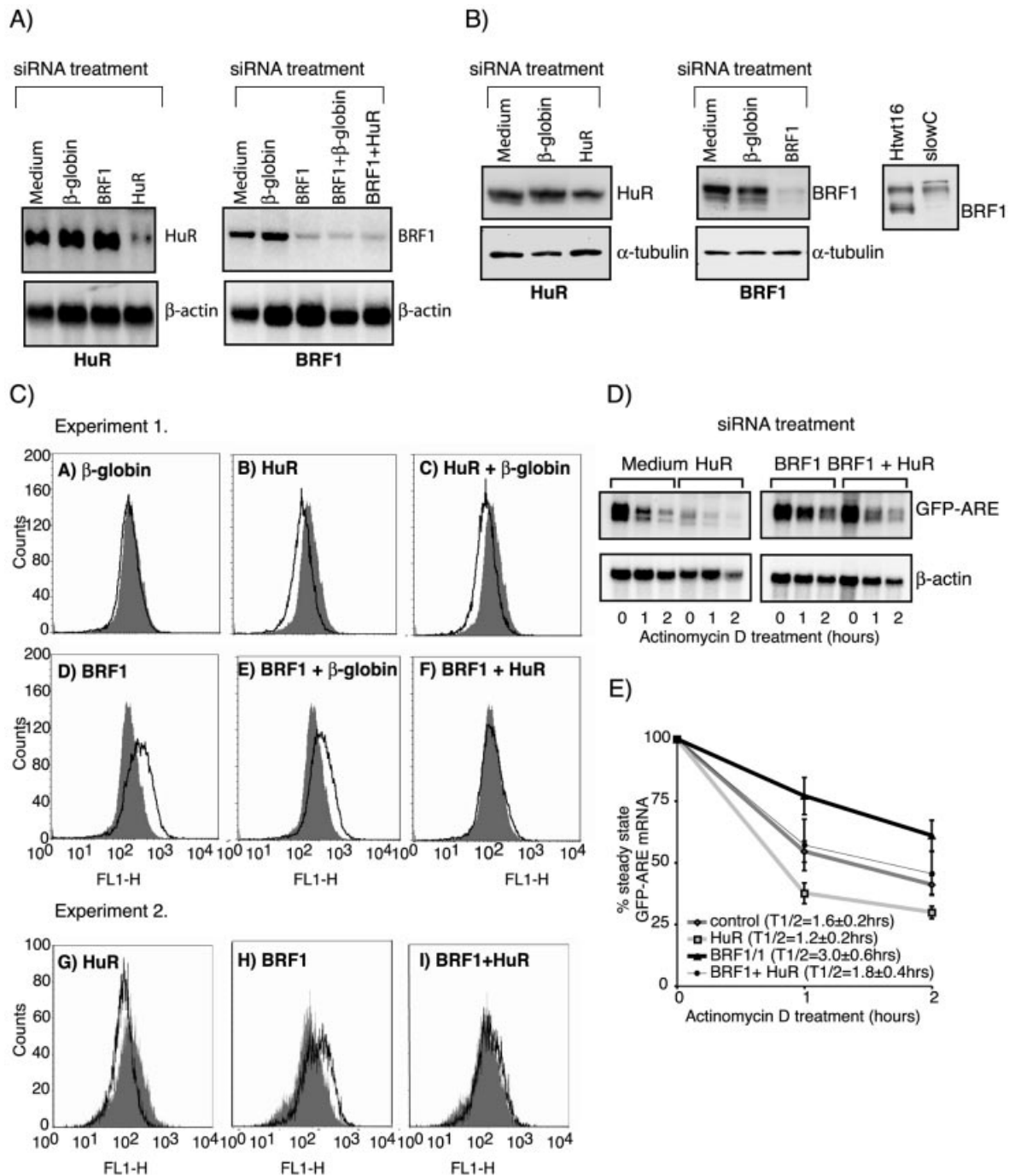
First, we investigated HuR, an AUBP known to stabilize ARE-mRNAs (15,16). A siRNA targeting HuR was designed that reduced HuR mRNA levels to 40% of medium or  $\beta$ -globin siRNA treated controls. By comparison, treatment of cells with BRF1 siRNA alone or in combination with  $\beta$ -globin or HuR siRNAs reduced BRF1 mRNA levels to 25% of controls (Fig. 2A). An additional HuR siRNA that had recently been described by others (42) reduced HuR protein levels to 60% of controls by western blot (Fig. 2B). Efficient inhibition of BRF1 by siRNA was also established in western blots using a polyclonal anti-BRF1 antibody generated in rabbits immunized with a C-terminal peptide of BRF1. Specific recognition of BRF1 by this antibody was confirmed by the absence of the signal in mutant slowC cells, which lack BRF1 expression compared to parental Hwtw16 cells, which express wild-type BRF1 (22) (Fig. 2B).

Next, we determined by FACS whether siRNA treatment affected GFP expression. The results of two independent



**Figure 1.** Reporter system employed to reveal effects of decreased AUBP expression on ARE-mRNA stability. HT1080 cells stably expressing a GFP-ARE reporter (HTwt16) are transfected with siRNA targeting AUBP. Decreasing the level of an AUBP that stabilizes ARE-mRNAs should result in decreased fluorescence by FACS. In contrast, downregulation of an AUBP that destabilizes ARE-mRNAs should result in increased fluorescence.

experiments are shown in Figure 2C. For quantification, the greatest difference (D) in fluorescence between the curves obtained from cells treated with medium or siRNA was calculated using Kolmogorov-Smirnov statistics (Table 1). In experiment 1, no significant differences were observed between cells transfected with medium or a non-specific  $\beta$ -globin control siRNA (Fig. 2C, panel A). However, transfection of HuR siRNA decreased fluorescence (panel B) and BRF1siRNA increased fluorescence as previously reported (panel D) (22). To evaluate whether HuR siRNA might antagonize the stabilizing effect of BRF1 siRNA, cells were treated with combinations of siRNAs. Combined treatment with HuR or BRF1 and a non-specific  $\beta$ -globin siRNA led to the same shift in fluorescence as treatment with HuR or BRF1 siRNA alone (panel C and E). In contrast, combined treatment with BRF1 and HuR siRNA abolished the effects of the single



**Figure 2.** HuR and BRF1 siRNA have opposing effects on fluorescence and GFP-ARE mRNA stability in Hwtw16 cells. (A) Northern blot analysis of HuR and BRF1 expression in Hwtw16 cells treated for 72 h with control  $\beta$ -globin, HuR (oligo A), BRF1 siRNA or their combinations. (B) Western blots of 15  $\mu$ g of cytoplasmic and 30  $\mu$ g of total cell lysate to analyze cells treated with HuR (oligo B) or BRF1 siRNA. As control for the specificity of the anti-BRF1 antibody, absence of the BRF1 signal in slowC cells lacking BRF1 expression is shown in comparison to Hwtw16 cells, which express wild-type BRF1. (C) FACS analysis of cells treated for 72 h. In experiment 1 (A–F) the shaded areas represent medium and the overlays siRNA-treated cells; HuR (oligo A) was employed in panels B and F. In experiment 2 (G–I) the shaded curves represent cells treated with  $\beta$ -globin control and the overlays represent cells treated with specific BRF1 and/or HuR (oligo B) siRNA. (D) To evaluate the effects of siRNA on GFP-ARE mRNA stability, cells were exposed to actinomycin D for the indicated time-points 72 h after transfection of medium,  $\beta$ -globin control, BRF1 and/or HuR (oligo A) siRNA. Values were normalized to the  $\beta$ -actin control. (E) Quantification of the mean  $\pm$  SEM of at least three independent experiments is shown and the half-life ( $T_{1/2}$ ) of the GFP-ARE reporter for each individual treatment is indicated.

siRNAs (panel F). We verified these findings with a second HuR siRNA (Fig. 2C, experiment 2). Again, HuR siRNA decreased fluorescence of transfected cells compared to

$\beta$ -globin siRNA-treated control cells (panel G) and annihilated the increase in fluorescence observed in cells treated solely with BRF1 siRNA (panels H and I).

**Table 1.** Treatment of Hwt16 cells with HuR siRNA decreases fluorescence, an effect that is reversed upon combined treatment with BRF1 siRNA

siRNA treatment	<i>n</i> <sup>a</sup>	Fluorescence <sup>b</sup>	$D_{\text{Kolmogorov-Smirnov}}$ <sup>c</sup> mean $\pm$ SD	<i>P</i> <sup>d</sup>
$\beta$ -globin	13	no effect	0.07 $\pm$ 0.04	
HuR	9	decreased	0.23 $\pm$ 0.08	<0.05
HuR + $\beta$ -globin	4	decreased	0.24 $\pm$ 0.23	<0.05
BRF1	9	increased	0.31 $\pm$ 0.12	<0.05
BRF1 + $\beta$ -globin	5	increased	0.29 $\pm$ 0.11	<0.05
HuR + BRF1	5	no effect	0.07 $\pm$ 0.03	= 0.9

<sup>a</sup>Number of experiments.<sup>b</sup>Effect on fluorescence of medium compared to siRNA-treated cells. For statistical analysis only HuR (oligo A) was taken into consideration.<sup>c</sup>Greatest distance (D) between curves obtained from medium- and siRNA-treated cells.<sup>d</sup>Student's *t*-test comparing D of cells treated with control  $\beta$ -globin and specific siRNA.

To evaluate whether changes in fluorescence were accompanied by altered mRNA stability of the GFP-ARE reporter, actinomycin D chase experiments were performed. The results of a representative experiment are shown in Figure 2D and quantification of at least three independent experiments for each treatment is exhibited in Figure 2E. Cells treated with HuR siRNA exhibited reduced GFP-ARE mRNA levels and a slightly accelerated decay that was reflected in a decrease of the GFP-ARE half-life ( $t_{1/2} = 1.2 \pm 0.2$  h) compared to controls ( $t_{1/2} = 1.6 \pm 0.2$  h). The stabilization exerted by BRF1 siRNA ( $t_{1/2} = 3.0 \pm 0.6$  h) was reversed by combined treatment with HuR siRNA ( $t_{1/2} = 1.8 \pm 0.4$  h). In all actinomycin D chase experiments performed, we observed the decay of the GFP-ARE mRNA reporter to be fastest during the first hour of treatment. Actinomycin D has been shown to itself stabilize ARE-mRNAs (45), an effect that may be responsible for the observed impediment of decay at later time points. Together, changes in fluorescence intensity in cells treated with BRF1, HuR and combinations of these two siRNAs were qualitatively reflected in altered decay rates of the GFP-ARE reporter mRNA.

### SiRNA targeting AUF1

The role of AUF1 in ARE-mediated mRNA decay is complicated by the existence of four isoforms and both destabilizing and stabilizing effects have been reported (40,41). To differentially decrease selected AUF1 isoforms we targeted siRNA to exons 1 and 3, which are common to all four isoforms, to exon 2, which is contained in p40<sup>AUF1</sup> and p45<sup>AUF1</sup>, and to exon 7, which is present in p42<sup>AUF1</sup> and p45<sup>AUF1</sup> (Fig. 3A). First, to evaluate whether the selected siRNA would specifically reduce the targeted isoforms we analyzed their distribution by western blot and quantified the respective bands by densitometry (Fig. 3B). In all experiments performed, we observed a non-specific band (marked by an asterisk) at ~55 kDa whose signal intensity correlated to the  $\alpha$ -tubulin loading control as determined by densitometry (data not shown). The p37 and p45 isoforms were clearly distinguished in control cells (lanes 1 and 2). However, p40<sup>AUF1</sup>, which is highly abundant in these cells, resolved as a very broad band obscuring simultaneous detection of the weakly expressed p42<sup>AUF1</sup>. As expected, both siRNAs targeting exons

1 and 3 decreased expression of all four isoforms (lanes 3 and 6). Two independent oligoribonucleotide duplexes targeting exon 2 of AUF1 reduced only expression of p40<sup>AUF1</sup>, the most abundant isoform in this cell line, and p45<sup>AUF1</sup> (lanes 4 and 5). In contrast, expression of p37<sup>AUF1</sup> was comparable to medium or  $\beta$ -globin siRNA-treated controls. Of note, the low abundant p42 isoform became visible only after selective removal of p40<sup>AUF1</sup>, in cells treated with exon 2-specific siRNA. Finally, siRNA targeting exon 7 specifically reduced p45<sup>AUF1</sup>. The concomitant decrease of p42<sup>AUF1</sup> could not be analyzed (lane 7), due to the reasons mentioned above. In conclusion, the AUF1 siRNA employed allowed selective and efficient inhibition of the targeted isoforms, with p45<sup>AUF1</sup> being reduced to 10–27% of controls by the different siRNA.

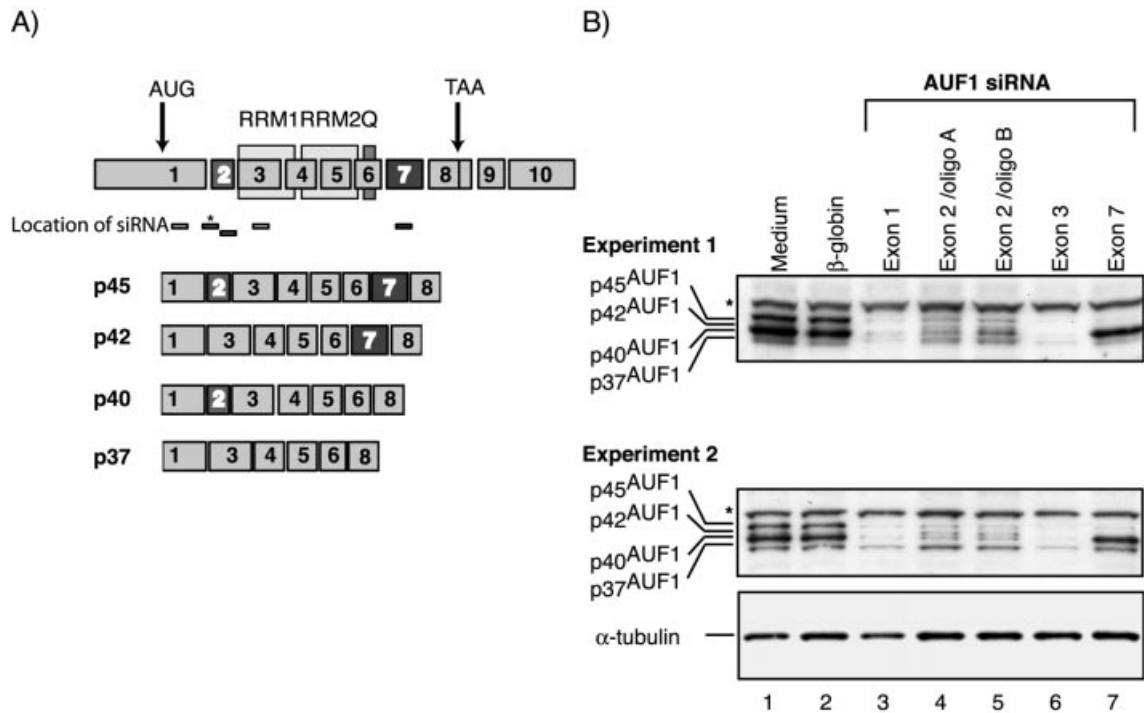
Next, we investigated how the different AUF1 siRNA affected fluorescence of transfected cells by FACS. Figure 4A shows a representative experiment and quantification is exhibited in Table 2. Decreasing the expression of all four isoforms by targeting exon 1 or 3 did not significantly alter fluorescence compared to  $\beta$ -globin siRNA-treated controls (panels B and E). Downregulation of both p42<sup>AUF1</sup> and p45<sup>AUF1</sup> led in some experiments to a slight, not consistently observed and statistically not significant, decrease of fluorescence compared to controls (panel F and Table 2). Together, these data might indicate that AUF1 does not affect expression of the GFP-ARE reporter in this cell line. However, we observed a significant increase of fluorescence in cells exhibiting reduced p40<sup>AUF1</sup> and p45<sup>AUF1</sup> expression after treatment with two independent exon 2-specific oligoribonucleotide duplexes (panels C and D). To substantiate these findings we performed a titration experiment, treating cells with 240–1.92 nM of the different AUF1 siRNA employed. Whereas siRNA targeting exons 1, 3 or 7 did not increase fluorescence of transfected cells at any of the concentrations tested, both siRNA targeting exon 2 increased fluorescence, with a minimal effect still being observed at 9.6 nM (data not shown).

Actinomycin D chase experiments (Fig. 4B) revealed that cells exhibiting an increased fluorescence due to selective reduction of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> indeed showed a slower decay rate of the GFP-ARE reporter compared to  $\beta$ -globin siRNA- or medium-treated controls. The half-life of the GFP-ARE reporter mRNA in cells treated with AUF1 siRNA targeting exon 2 varied between individual experiments, but a consistent increase was observed compared to controls. The reduced decay rate of the GFP-ARE mRNA in cells thus treated was reflected in an accordingly increased steady-state level of the reporter, which was, however, 15–20% higher than expected. This discrepancy between the predicted and the observed increase was within the variation of the experiment. In contrast, cells exhibiting reduced levels of all four AUF1 isoforms or selectively of p42<sup>AUF1</sup>/p45<sup>AUF1</sup> showed decay rates of the reporter that were within the range of the controls.

In addition, NIH 3T3 cells stably expressing the GFP-ARE reporter also exhibited only an increased fluorescence if treated with AUF1 (exon 2a), but not AUF1 (exon 3) siRNA (data not shown).

### Effects of HuR, BRF1 and AUF1 siRNA on endogenous GM-CSF expression

To validate these data we wished to investigate whether these siRNAs influenced expression of a physiological endogenous



**Relative Signal Intensities of Individual AUF1 Isoforms (Exp. 2)**

	% Signal Intensity of Control <sup>1)</sup>					
	Control	Exon 1	Exon 2a	Exon 2b	Exon 3	Exon 7
p45	100%	10%	27%	23%	10%	17%
p40/p42	100%	7%	27%	28%	5%	96%
p37	100%	37%	108%	87%	25%	117%

1) The integrated volumes of the AUF1 signals of experiment 2 were normalized to the  $\alpha$ -tubulin control and expressed as percentage of the average signal obtained in the medium and  $\beta$ -globin treated control samples.

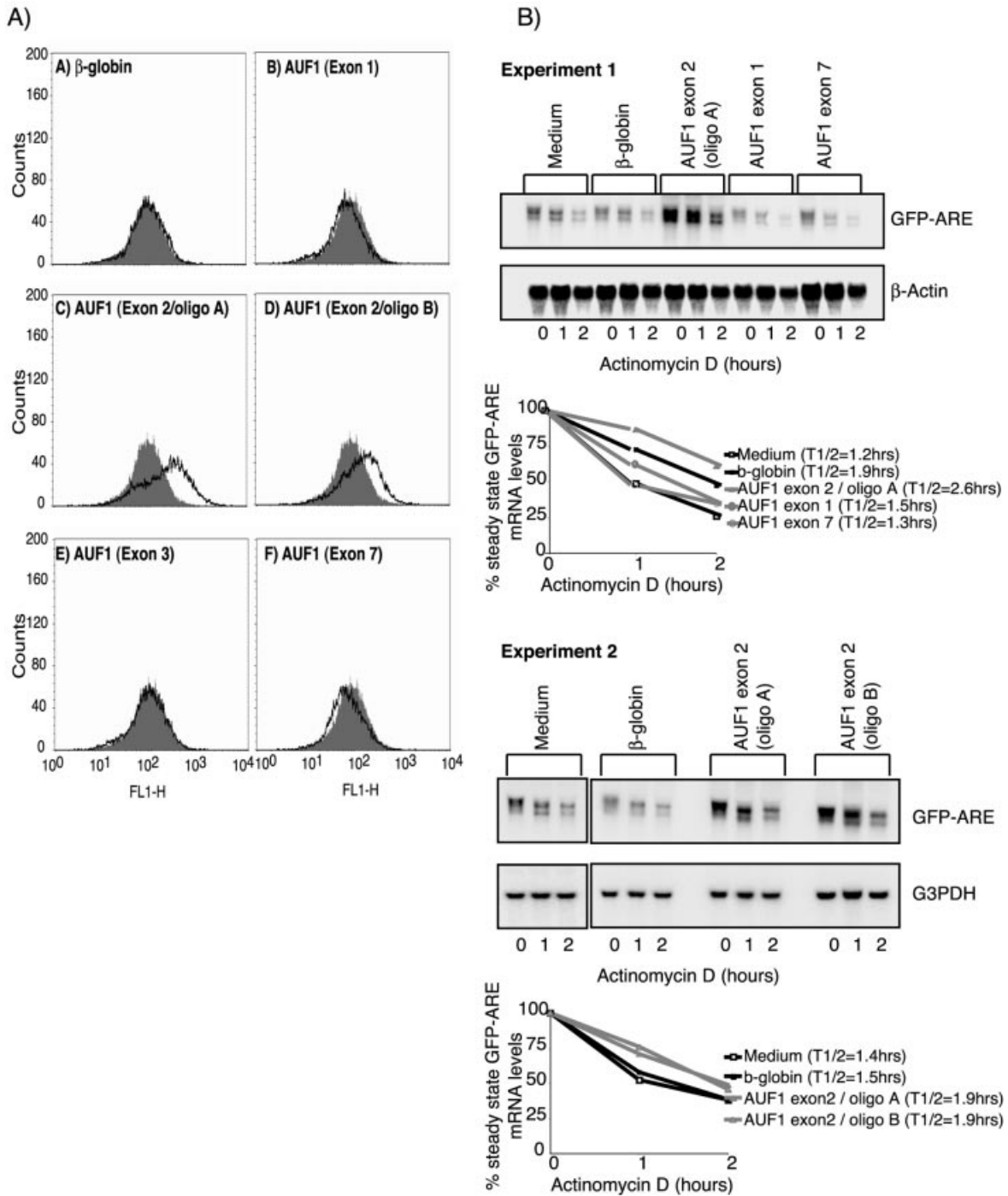
**Figure 3.** Exon-specific targeting of AUF1 allows selective reduction of specific AUF1 isoforms in Htw16 cells. (A) Schematic drawing of the mRNAs coding for the different AUF1 isoforms and the localization of the siRNA employed. Location of the RRM1 and the glutamine rich (Q) stretch are indicated. The siRNA AUF1 (exon 2/oligo A) marked by an asterisk targets 16 bp of exon 1 and 3 bp of exon 2. (B) Two independent western blots of cells treated for 72 h with AUF1 siRNA targeting exon 1 (lanes 3), exon 2 (lanes 4 and 5), exon 3 (lanes 6) and exon 7 (lanes 7). The asterisk marks a non-specific band at ~55 kDa that was observed in all experiments performed, and whose signal intensity correlated to the  $\alpha$ -tubulin loading control in individual samples. Quantitative analysis of experiment 2 is shown in the table.

target gene. A recent cDNA microarray analysis had revealed that HT1080 cells lacking wild-type BRF1 (22) expressed at least six-fold higher GM-CSF mRNA and protein levels compared to cells with two intact BRF1 alleles, thus identifying GM-CSF as a BRF1 target gene (unpublished data). Therefore, HT1080 cells were treated for 72 h with the different siRNAs and secreted GM-CSF was subsequently determined by ELISA. Treatment with BRF1 siRNA increased GM-CSF production, an effect that was reduced upon simultaneous addition of HuR but not of  $\beta$ -globin siRNA (Fig. 5A). Extending this study to AUF1, a known regulator of GM-CSF expression (46), we found an even more powerful effect than with BRF1 siRNA. Of note, only selective reduction of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> increased GM-CSF expression, whereas reduction of all four isoforms or p42<sup>AUF1</sup>/p45<sup>AUF1</sup> again had no effect (Fig. 5B). The increase in GM-CSF expression in cells treated with AUF1 (exon 2a) siRNA was

partially reversed if cells were simultaneously treated with HuR siRNA (Fig. 5C). Therefore, not only do the relative levels of stabilizing and destabilizing AUBPs regulate expression of GM-CSF but, surprisingly, also the relative levels of individual AUF1 isoforms.

## DISCUSSION

The present study was designed to evaluate siRNA targeting AUBPs in the investigation of ARE-mRNA turnover. This seemed to be a timely objective as, with the exception of TTP (47), no AUBP knockout mice models have been reported. Results gained from loss-of-function experiments are needed to validate data previously obtained by overexpression of AUBPs. This is of particular importance because these experiments did not provide unifying conclusions (see below).



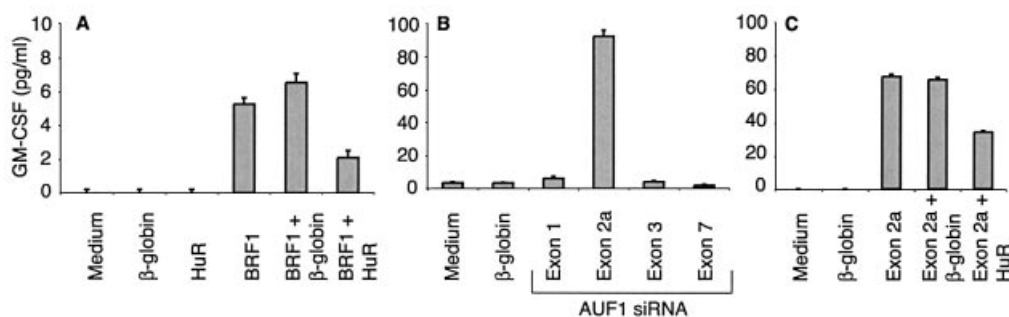
**Figure 4.** Selective downregulation of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> increases fluorescence and stabilizes the GFP-ARE reporter in Hwt16 cells. (A) FACS analysis of cells treated for 72 h with medium or siRNA as indicated. (B) To evaluate the effects of siRNA on GFP-ARE mRNA stability, cells were exposed to actinomycin D for the indicated time-points 72 h after transfection of siRNA.

Indeed, treatment of Hwt16 cells with siRNA targeting HuR, a protein known to stabilize ARE-mRNAs (15,16) decreased fluorescence intensity and promoted decay of the GFP-ARE-mRNA reporter, thus confirming HuR to be a stabilizer of ARE-mRNAs. BRF1 siRNA induced the expected increase in fluorescence intensity and stabilized the GFP-ARE reporter, effects that were nullified in cells treated with a combination of HuR and BRF1 siRNAs. Inactive

siRNAs have been claimed to compete with active siRNAs in a sequence-independent manner (48). However, non-specific effects were ruled out in our experiments, showing that cells treated with a combination of BRF1 and control  $\beta$ -globin siRNA exhibited the same shift in fluorescence as cells treated solely with BRF1 siRNA. Thus, we concluded that HuR and BRF1 control mRNA stability of the GFP-ARE reporter in an antagonistic fashion, presumably by competing for the ARE.

**Table 2.** Exon specific targeting of AUF1 reveals downregulation of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> to increase fluorescence of transfected cells

siRNA treatment	Isoforms targeted	<i>n</i> <sup>a</sup>	Fluorescence <sup>b</sup>	D <sub>Kolmogorov-Smirnov</sub> <sup>c</sup> mean ± SD	<i>P</i> <sup>d</sup>
β-globin		7	no effect	0.09 ± 0.04	
AUF1 exon 1	p37, p40, p42, p45	4	no effect	0.09 ± 0.05	= 1
AUF1 exon 2 (oligo A)	p40, p45	7	increased	0.46 ± 0.09	<0.05
AUF1 exon 2 (oligo B)	p40, p45	4	increased	0.29 ± 0.09	<0.05
AUF1 exon 3	p37, p40, p42, p45	4	no effect	0.1 ± 0.08	= 0.9
AUF1 exon 7	p42, p45	4	no effect	0.12 ± 0.03	= 0.2

<sup>a</sup>Number of experiments.<sup>b</sup>Effect on fluorescence of medium compared to siRNA-treated cells (Fig. 4A).<sup>c</sup>Greatest distance (D) between curves obtained from medium and siRNA-treated cells.<sup>d</sup>Student's *t*-test comparing D of cells treated with control β-globin and specific siRNA.**Figure 5.** Downregulation of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> increases GM-CSF secretion of transfected Hwt16 cells. Secreted GM-CSF was measured by ELISA in the culture supernatant of cells treated for 72 h with BRF1 and HuR (oligo A) siRNA (A), AUF1 siRNA (B) and AUF1 (exon 2a) and HuR (oligo A) siRNA (C).

Experiments revealing that BRF1 and HuR also exerted opposing effects on expression of GM-CSF, an endogenous BRF1 target gene recently identified by cDNA microarray, further validated our findings.

In contrast to BRF1 and HuR, the role of AUF1 in ARE-mRNA turnover has not been fully clarified. Conclusions, of whether this protein acts as a stabilizer or destabilizer of ARE-mRNAs, are based on indirect evidence associating AUF1 abundance with ARE-mRNA stability (36,38,39,49) and on experiments using AUF1 overexpression, which provided ambiguous results. In NIH 3T3 cells, all four isoforms stabilized ARE-mRNAs (41). Other studies, however, showed that both p37<sup>AUF1</sup> and p40<sup>AUF1</sup> promoted decay in several cell lines investigated, including NIH 3T3 (50). Also, in K562 cells forced expression of AUF1 antagonized stabilization of ARE-mRNAs (40). The reasons for these discrepancies are not clear. Short of performing a gene knockout experiment, exon-specific targeting of AUF1 by siRNA might provide a more physiologic approach to functionally dissect this gene. At first sight, our finding that two different siRNAs efficiently inhibiting expression of all four AUF1 isoforms did not exert an effect on cellular fluorescence or mRNA stability at any of the concentrations tested would argue that, at least in HT1080 cells, AUF1 is not involved in steady-state control of ARE-mRNA turnover. However, this possibility was rejected because the decrease of p40<sup>AUF1</sup>/p45<sup>AUF1</sup> in cells treated with exon 2-specific siRNA strongly induced cellular fluorescence and stabilized the GFP-ARE reporter mRNA. This seems to be mainly due to reduction of the most abundant p40 isoform rather than to the decrease in p45<sup>AUF1</sup> because downregulation

of p42<sup>AUF1</sup> together with p45<sup>AUF1</sup> did not significantly affect fluorescence of transfected cells. We wish to stress that the AUF1 siRNA employed had the same effect on GM-CSF, a gene known to be controlled by AUF1 (46). Again, downregulation of all four isoforms had no effect, whereas selective reduction of p40<sup>AUF1</sup> and p45<sup>AUF1</sup> strongly stimulated GM-CSF production. Based on these results, one might draw the conclusion that p40<sup>AUF1</sup>, like BRF1, acts as a destabilizer of ARE-mRNAs. However, we would like to offer an alternative explanation.

The four AUF1 isoforms differ considerably in their ARE-binding affinities *in vitro*, with the rank order of p37>p42>p45>p40 (26) and the two isoforms with the highest binding affinity exert the most profound effect on ARE-mRNA stability (40,41). Thus, we propose that in HT1080 cells the highly abundant yet low-affinity p40<sup>AUF1</sup> competes with p37<sup>AUF1</sup> and p42<sup>AUF1</sup> for ARE-binding. After selective downregulation of p40<sup>AUF1</sup>, p37<sup>AUF1</sup> and p42<sup>AUF1</sup> gain access to the ARE and stabilize transcripts. This model does not exclude that post-transcriptional modifications of AUF1 might play an additional role, as the affinity of p40<sup>AUF1</sup> for the ARE has been shown to be affected by phosphorylation (51–53). Our proposition that alterations in ARE-mRNA stability might be induced by changes in the relative rather than the absolute levels of AUF1 isoforms would explain why the uniform reduction of all four isoforms had no effect. A key factor may well be the ratio of p37<sup>AUF1</sup> and possibly p42<sup>AUF1</sup> to p40<sup>AUF1</sup>. This notion is supported by two previous observations. First, the decreased stability of the β-adrenergic receptor mRNA in the failing compared to normal human heart has been



associated with an increased expression of both p40<sup>AUF1</sup> and p45<sup>AUF1</sup> (38). Second, differential expression of AUF1 isoforms accompanied the decreased half-life of GM-CSF mRNA in neonatal compared to adult PBMC (36,37). Both p37<sup>AUF1</sup> and p40<sup>AUF1</sup> were decreased in the latter, whereas expression of p42<sup>AUF1</sup> was comparable in neonatal and adult PBMC. If our model is correct, it would follow that mRNA splicing might ultimately influence ARE-mRNA stability by determining the relative levels of individual AUF1 isoforms.

The fact that simultaneous targeting of HuR and p40<sup>AUF1</sup>/p45<sup>AUF1</sup> partially cancelled the effect of solely targeting p40<sup>AUF1</sup>/p45<sup>AUF1</sup> clearly indicates that the relative levels of the individual AUF1 isoforms can only be one determinant of ARE-mRNA stability. We propose that the quantity of all stabilizing (HuR) and destabilizing AUBPs (TTP, BRF1, BRF2), together with the relative amount of the individual AUF1 isoforms, determines the net stability of ARE-containing transcripts in a balanced fashion. Thus, silencing a single destabilizing factor will tip the balance towards increased stabilization despite the fact that other ARE-destabilizing factors may still be present. In return, decreasing the expression of any stabilizing factor will favor destabilization despite the potential presence of additional stabilizing AUBPs.

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