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SURVEY AND SUMMARY

AU-rich elements and associated factors: are there unifying principles?

Carine Barreau, Luc Paillard and H. Beverley Osborne*

UMR 6061 CNRS Génétique et Développement, IFR 140 Génétique Fonctionnelle Agronomie et Santé, Université de Rennes 1, Faculté de Médecine, CS 34317, 35043 Rennes cedex, France

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ABSTRACT

The control of mRNA stability is an important process that allows cells to not only limit, but also rapidly adjust, the expression of regulatory factors whose over expression may be detrimental to the host organism. Sequence elements rich in A and U nucleotides or AU-rich elements (AREs) have been known for many years to target mRNAs for rapid degradation. In this survey, after briefly summarizing the data on the sequence characteristics of AREs, we present an analysis of the known ARE-binding proteins (ARE-BP) with respect to their mRNA targets and the consequences of their binding to the mRNA. In this analysis, both the changes in mRNA stability and the lesser studied effects on translation are considered. This analysis highlights the multitude of mRNAs bound by one ARE-BP and conversely the large number of ARE-BP that associate with any particular ARE-containing mRNA. This situation is discussed with respect to functional redundancies or antagonisms. The potential relationship between mRNA stability and translation is also discussed. Finally, we present several hypotheses that could unify the published data and suggest avenues for future research.

by their ability to target host mRNAs towards rapid degradation. In general, these mRNAs encode proteins that regulate either cell growth or the response of an organism to external factors such as micro-organisms, inflammatory stimuli and environmental factors. Such genes require a very precise control of their spatial and temporal expression patterns which is achieved, in addition to a transcriptional control, by a regulation of the translation and the stability of the mRNA (5–8). In resting or unstimulated cells the ARE-dependent degradation mechanism ensures a very low level of expression of these potent proteins. The importance of this repression in the resting state is testified by the observation that pathological states (cancers, chronic inflammations and auto-immune pathologies) are associated with a deregulation of the stability of ARE-containing mRNAs [(9–20), reviewed in (21,22)].

The sequence motifs that form an ARE were first identified within the 3'-untranslated regions (3'-UTR) of mRNAs encoding several cytokines or lymphokines (23) and, since, the list of mRNAs that contain such motifs has considerably lengthened. It has been estimated that 5–8% of human genes code for ARE-containing mRNAs; the corresponding proteins perform a variety of functions implicated in numerous transient biological processes (24,25). This gene-based analysis underscores the importance of AREs in the regulation of gene expression as only a subclass of AREs were taken into account.

INTRODUCTION

The regulation of mRNA stability and translation are essential in the control of gene expression [reviewed in (1,2–4)] and regulation of these two processes allows a cell to rapidly respond to changes in intracellular and extracellular stimuli. A number of sequence elements control the half-life of a mRNA either by stimulating or inhibiting degradation. In mammalian cells the sequence elements rich in adenosine and uridine, called AU-rich elements (AREs), were identified

DISCOVERY OF AREs AND SEQUENCE MOTIFS

AREs as mRNA destabilizing elements

AREs are sequence elements of 50–150 nt that are rich in adenosine and uridine bases. They are located in the 3'-UTRs of many but not all mRNAs that have a short half-life and have been identified by their capacity to provoke degradation of the host mRNA by a mechanism dependent on deadenylation [shortening of the poly(A) tail] [reviewed in (26,27)]. However, depending on the cellular context and the

*To whom correspondence should be addressed. Tel: +33 223 23 4523; Fax: +33 223 23 4478; Email: Beverley.osborne@univ-rennes1.fr

precise stimulus, the presence of an ARE can also lead to the stabilization of a mRNA (see below). For historical reasons it is generally considered that the term ARE is reserved for sequence elements that confer instability and/or contain AUUUA motifs. Consequently, there are regulatory RNA elements, rich in A and U nucleotides, that are not called AREs. The first functional demonstration of ARE-dependent mRNA degradation was obtained by studying, in transfected cells, the stability of a globin mRNA into which the ARE from the granulocyte-macrophage colony stimulating factor (GM-CSF) mRNA had been inserted (28). Since, inserting a putative ARE into the 3'-UTR of an otherwise stable reporter RNA has become the classical experimental approach to study cellular or artificial AREs. Many of these studies used Actinomycin D or 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB) to block transcription and thereby allow degradation rates to be measured. However, these drugs can cause a number of artefacts and even stabilize the mRNA (29). Wilson and Treisman (30) and Shyu *et al.* (31) have circumvented this problem by using the c-fos promoter to drive transient transcription of a reporter gene in transfected cells after serum stimulation. The use of the c-fos promoter to drive transient expression is however not amenable to all experimental situations. This led Xu *et al.* (32) to develop a protocol using the tetracycline-regulatory promoter system to produce a pulsed synthesis of the mRNA under study.

ARE sequence features

The first AREs identified highlighted the presence of AUUUA pentamers, often overlapping, and frequently found within U-rich regions of the 3'-UTR [reviewed in (26)]. Many studies have since shown that the AUUUA motif and a certain uridine enrichment are two important characteristics of an ARE, but also, that they cannot fully explain the destabilizing activity of an ARE (33–37). The minimal sequence motif necessary to increase the turnover of chimeric mRNAs is the nonamer UUAUUUA(U/A)(U/A) (36,37), but only a modest effect on the stability of a reporter mRNA is produced by the insertion of a single nonamer in the 3'-UTR (36,37). Systematic mutagenesis of the UUAUUUAUU motif in GM-CSF ARE confirmed that two or four copies of the nonamer motif were more efficient in promoting deadenylation and instability than a single copy of this motif (38). However, mutagenesis of the single UUAUUUAUU present in the c-fos ARE does not significantly decrease its ability to destabilize a mRNA (33) suggesting that the UUAUUUA(U/A)(U/A) motif may be essential for only a subset of AREs. This notion is reinforced by the observation that a consensus recognition sequence for the ARE-binding proteins (ARE-BPs) HuR and TIA-1, derived from large scale screens of the endogenous targets, although U-rich did not contain a UUAUUUAUU motif (39,40).

Classification of AREs

Based on the number and the distribution of AUUUA pentamers, AREs have been grouped into three classes (26). A number of these are listed in Table 1 and are grouped according to the two classification methods that have been proposed (24,26). Class I AREs contain several dispersed copies of the AUUUA motif within U-rich regions. Class II AREs possess at least 2 overlapping UUAUUUA(U/A)(U/A) nonamers.

Bakheet *et al.* (24,25) have constructed a database of mRNAs containing class II AREs and these regulatory elements were divided into five groups, three of which are presented in Table 1. The classification in this ARE database (<http://rc.kfshrc.edu.sa/ared/>) is based on the repetition pattern of the AUUUA pentamer.

Class III AREs are much less well defined, they are U-rich regions but contain no AUUUA motif. The best documented example of a type III ARE is that situated within the 3'-UTR of c-jun mRNA (35,41,42).

It is necessary to note that no real consensus sequence has yet been precisely defined for any class of ARE and, furthermore, these ARE classifications are based neither on the associated proteins nor on biological functions. However, it is interesting to note that most of the mRNAs containing class II AREs encode cytokines whereas mRNAs encoding transcription factors and cell cycle regulatory proteins mostly contain class I and occasionally class III AREs (Table 1). Also inhibition of transcription with Actinomycin D or DRB stabilized mRNAs containing class I or II AREs (29) but had only a modest effect on the decay kinetics of a mRNA with a class III ARE (41). Therefore, although the initial classification was not based on biological function it could be that they group together mRNAs controlled by similar pathways or encoding proteins with associated functions.

ROLE OF ARE-BPs IN RNA DEGRADATION

A great deal of attention has been given to cellular proteins that bind to AREs as regulators of mRNA stability. Identification of ARE-BPs and analysis of their contributions to the ARE-dependent degradation of targeted mRNAs started ~15 years ago and is still continuing [reviewed in (27,43,44)]. As would be expected for proteins involved in controlling the stability of proto-oncogenes or cytokines, changes in the expression level of certain ARE-BPs have been implicated in the development of cancers [reviewed in (22)].

Although the AREs present in the many individual mRNAs listed in Table 1 are different in sequence, it is obvious from the summarized data that most are able to bind more than one ARE-BP. In some instances [e.g. c-myc, c-fos, GM-CSF, tumour necrosis factor α (TNF- α) and cyclooxygenase 2 (Cox-2)] the same ARE-containing mRNA can bind to many of the known ARE-BPs. Furthermore, many of the ARE-BPs have been observed to bind to multiple mRNAs and this binding crosses the ARE classification limits. Therefore, at present no clear segregation of certain ARE-BPs or structurally related ARE-BPs with specific types of AREs appears possible. It should also be noted that in many cases listed in Table 1 where an association between an ARE-BP and multiple mRNAs has been reported, these demonstrations depended principally on *in vitro* analyses such as UV-crosslinking or electrophoretic mobility shift assays (EMSA) (see table in Supplementary Data). Only a few studies have analysed associations between endogenous mRNAs and proteins (indicated by an asterisk in Table 1). Despite this reserve, the multiplicity of possible associations between ARE-BPs and AREs brings forward the question of binding specificity and functional redundancy/additivity or antagonism that is discussed latter in this survey.

Table 1. ARE-binding proteins that associate with AREs in mammals

mRNAs Class	Motif	Examples	ARE-BPs AUFI hnRNP D	HuR HuA	Hel-N1 HuC HuB	TTP	BRF1 TIA-1	TIAR KSRP AUH GAPDH Hsp70	Hsp110	hnRNP A1	hnRNP A2	hnRNP A3	hnRNP C	hnRNP L	CUG-BP1 CELF1 ETR-3	CUG-BP2 CELF2 ETR-3	Nucleolin TINO PAIP2
I	1 to 5 dispersed AUUUA motifs, in an U-rich context	c-myc	(46) (47) (154) (171)	(155) (156)	(157)* (78)	(78)		(158) (163)	(159)								
		c-fos	(42,67) (47) (53) (154) (162) (155)	(63) (67) (70) (71) (103) (155)	(157)	(160) (161)		(90) (93)									
		Beta1-AR PTH Interferon-gamma MyoD p21	(162) (56)					(163) (159)									
		Cyclin A Cyclin B1 Cyclin D1 PAL-2 NOS II/iNOS	(48)* (167)	(68)* (48)* (69) (70) (48)* (70) (166) (64) (102) (103) (101) (138)		(164)		(102)									
IIA	(AUUU) ₃ A	GM-CSF	(42) (47) (54)*		(157)	(84) (85) (101)	(138)	(158) (163)	(159)	(168)			(168)				
		TNF-alpha	(167) (94) (167) (169)	(65) (74) (85) (139)*		(18) (84) (85) (86) (89)	(89) (120) (139)* (170)										
IIIB	(AUUU) ₄ A	Interferon-alpha															
IIC	(A/U)(AUUU) ₃ A(A/U)	Cox-2	(171) (172)	(71) (139)* (172) (173)		(87)	(121) (139)* (172)			(150)* (172)	(172)				(150)*		
		IL-2 IL-3 bcl-2	(167) (174) (176)	(100)* (155)		(82)	(92)	(159) (159)	(168)						(175)	(176)	
III	No AUUUA, U-rich region	Interferon-beta VEGF c-jun GLUT1 p53		(62)		(78) (78)											(177)*
		Id			(72)										(178)	(153)	
		hsp70 Myogenin NF-M		(99)* (137)*	(179)												
		GAP-43		(181)* (68)*	(73)*	(180)											

A matrix presentation has been used to represent the identified associations between ARE-BPs and ARE-containing mRNAs. The mRNAs containing identified functional AREs are listed vertically and are grouped according to the classifications proposed by (24) and (26). The ARE-BPs are displayed horizontally. Where appropriate the different names used to denote the same protein or mRNA are given. The lists of ARE-containing mRNAs and of ARE-BPs are not exhaustive and only direct interactions have been considered. Where the experimental methods used identified endogenous interactions, these are indicated by an asterisk. Data on the experimental methods are presented in the Supplementary data. Numbers correspond to listed references.

Table 2. Effect of ARE-BPs on the stability and translation of ARE-containing mRNAs

ARE-BPs	mRNA stability		Protein expression Translational efficiency		Abundance	Down regulated
	Increase	Decrease	Increase	Decrease	Up regulated	
AUF1	c-myc (42) c-fos (42,67) PTH (56) GM-CSF (42) TNF-alpha (42)	c-myc (46) c-fos (53) p21 (48) Cyclin D1 (48) GM-CSF (53,54) IL-3 (55)				GM-CSF (55) IL-3 (55)
HuR	c-fos (59,63,67) MyoD (68) p21 (48,68,69) Cyclin A (70) Cyclin B1 (70) Cyclin D1 (48) NOS II/iNOS (64) GM-CSF (59) TNF-alpha (65,74,139) Cox-2 (71,139) IL-3 (55,66) VEGF (62) Myogenin (68)		p53 (99,137)	TNF-alpha (139) Cox-2 (139)	p21 (69) Cyclin A (70) Cyclin B1 (70) NOS II/iNOS (64) GM-CSF (55) Cox-2 (71,173) IL-3 (55) VEGF (173) p53 (99,137)	TNF-alpha (139)
Hel-N1	TNF-alpha (74) GLUT1 (72)		NF-M (73) GLUT1 (72)		NF-M (73) GLUT1 (72)	
HuD	GAP-43 (75–77)				GAP-43 (75,76)	
TTP		c-fos (90) GM-CSF (18, 81,83–85, 91) TNF-alpha (18,81,83–86,89,90) Cox-2 (87) IL-2 (82,90) IL-3 (18, 66, 83,84, 88) TNF-alpha (89,93) IL-3 (55,92,93)				GM-CSF (81) TNF-alpha (80) IL-2 (82) IL-3 (88)
BRF1						GM-CSF (55) IL-3 (55)
TIA-1				TNF-alpha (120) Cox-2 (121)		TNF-alpha (120) Cox-2 (121)
KSRP		c-fos (90,93) NOS II/iNOS (102) TNF-alpha (90,93) IL-2 (90,93) c-jun (93)				NOS II/iNOS (102)
CUG-BP2	Cox-2 (150)			Cox-2 (150)		Cox-2 (150)
Nucleolin	bcl-2 (175)					
TINO		bcl-2 (176)				
PAIP2	VEGF (177)				VEGF (177)	

The listed ARE-BPs correspond to those in Table 1 for which effects on mRNA stability and protein expression levels (translational efficiency or steady-state abundance) have been reported. The mRNAs whose stability is increased (stabilized) or decreased (degraded) are indicated for each ARE-BP. The stability of TNF- α and Cox-2 mRNA or NF-M mRNA were not modified by TIA-1 or Hel-N1, respectively. The several mRNAs for which increased or decreased expression of the encoded protein has been reported are similarly indicated. Increased or decreased translational efficiency was measured either by polysome analysis or methionine incorporation. Changes in protein expression classified as abundance were determined on steady-state levels either by western analysis or measurement of associated enzymatic activities. Numbers correspond to listed references.

In a number of cases, the consequences of ARE-BP binding to a particular mRNA have been studied either with respect to the changes in mRNA stability or translation, (Table 2). For a small proportion of the ARE-BPs the effects on mRNA stability and translation have been investigated simultaneously. The results from these studies are summarized below for AUF1, the Hu family, in particular HuR, and Tristetraprolin (TTP) that have been the most extensively studied.

AUF1

This protein was the first ARE-BP to be isolated and for which a role in controlling mRNA stability was demonstrated (45–47). AUF1, also named hnRNP D (heterogeneous nuclear ribonucleoprotein D), is essentially nuclear (47,48), but it can

shuttle between the nuclear and cytoplasmic compartments (49–52). It binds to both class I and II AREs but no example of a class III ARE associated with AUF1 has been reported (Table 1).

The causality of the correlation between AUF1-binding and decreased mRNA stability has been demonstrated by experiments using either over expression (53,54) or depletion (by siRNAs) (48,55) of AUF1 in cells or modulation of the AUF1 content in cytoplasmic extracts (46) (Table 2). In one study (55) the increased stability of IL-3 mRNA caused by a siRNA knock-down of AUF1 led to an increased expression of the encoded protein. This is coherent with AUF1 targeting these mRNAs for degradation and consequently decreasing the expression of the encoded proteins. However, AUF1-binding has also been associated with increased mRNA

stability (Table 2). In the case of Parathyroid hormone mRNA (56), which contains a class I ARE (Table 1), the effect is seen in a particular situation, cytoplasmic extracts of rat parathyroid. In the study by Xu *et al.* (42), the stabilizing effect of AUF1 over expression (all isoforms) in NIH-3T3 cells was most evident for class II AREs (TNF- α and GM-CSF), less pronounced for the class I AREs (c-myc and c-fos) and absent for class III ARE (c-jun and a synthetic non-AUUUA ARE). As indicated by Xu *et al.* (42) the effect of AUF1 on mRNA stability may be cell type specific: destabilizing in K562 proerythroblast cells (46,53) and stabilizing in NIH-3T3 fibroblast cells (42). However, Sarkar *et al.* (54) reported that over expression of the p37 isoform of AUF1 did not stabilize reporter mRNAs containing the GM-CSF ARE in a variety of cell type including NIH-3T3 cells. At present there is no clear explanation for this discrepancy.

Hu family

The mammalian genome encodes four closely related proteins (HuR/HuA, Hel-N1, HuC and HuD) that are part of a super family of elav-related proteins (57). HuR, which is expressed ubiquitously, has been the most extensively studied. The expression of the other Hu proteins is restricted to neurons except for Hel-N1 that is also expressed in gonads [reviewed in (58)]. HuR, like AUF1, has a predominantly nuclear localization and can shuttle between nucleus and cytoplasm (59,60). This shuttling may have a functional role as HuR has been shown to serve as an adaptor for the nuclear export of a class I ARE mRNA, c-fos (61).

HuR has been observed to bind to a large number of RNAs of all three ARE classes (Table 1). For a number of these mRNAs, the effect on their stability has been studied by modulating intracellular HuR either by ectopically expressing or over expressing HuR (59,62–68) or by siRNA or anti-sense RNA knock-down (48,55,62,64,69–71) (Table 2). In all these cases the data show that HuR-binding and increased mRNA stability are causally related.

Over expression of both Hel-N1 (72–74) and HuD (75–77) have also been correlated with an increased stability of the target mRNA and expression of the encoded protein (Table 2). The binding of HuC to c-myc and VEGF mRNAs was not correlated with a functional change to the mRNA (78).

TTP

In contrast to AUF1 and HuD the binding specificity of TTP appears to be restricted to mRNAs containing class II AREs (Table 1). Another difference is that TTP is predominantly cytoplasmic (18,79). The demonstration that TTP-binding can cause degradation of the target mRNA has been greatly helped by the use of TTP knockout mice. In TTP deficient mice increased protein production and/or mRNA was observed for TNF- α in foetal liver derived macrophages (80), for TNF- α and GM-CSF in bone marrow precursor cells and macrophages (18,80,81) and for IL-2 in splenocytes and T-cells (82). Further *in vitro* studies from several laboratories showed that the degradation of mRNAs encoding GM-CSF (18,83–85), TNF- α (18,83,86), Cox-2 (87) and IL-3 (18,66,83,88) was dependent on the amount of TTP expressed. Experiments using cell free extracts confirmed (83–85,89,90) and extended these observations to c-fos (90) mRNAs. TTP

mutants that do not bind to the target mRNA are ineffective in stimulating degradation (84,85) whereas tethering the domain of TTP required to activate degradation to a mRNA enhances the degradation of an otherwise stable reporter mRNA (91). By a systematic mutation of TTP-binding sites in GM-CSF ARE, Lai *et al.* (38) showed that maximum deadenylation and degradation rates requires the binding of two TTP molecules to the mRNA.

FUNCTIONAL RELATIONSHIPS BETWEEN ARE-BPs

Redundant or additive effects

As indicated in Table 2, most of the identified ARE-BPs, with the exception of the Elav-like proteins (HuR, Hel-N1 and HuD), CUG-BP2, Nucleolin and PAIP2, have been implicated in the degradation of mRNAs containing AREs. Some of these factors are co-expressed in the same cells and show overlapping binding affinities [reviewed in (27,44)]. However, the depletion of specific ARE-BPs can cause the stabilization of sub-populations of ARE-containing mRNAs in certain cell types (18,81,92,93). Nevertheless, it is not clear whether these proteins are functionally redundant or if each protein targets specific sub-populations of ARE-containing mRNAs for degradation. One possible example of a partial functional redundancy between two ARE-BPs is that of KSRP and BRF1. *In vitro* and *in vivo* studies have shown that a loss of either KSRP or BRF1 proteins from HT1080 or HeLa cells leads to a partial stabilization of a reporter mRNA containing an ARE. However, the simultaneous decrease of both proteins increases, additively, the stability of the reporter mRNA (93) indicating that the effect may be more additive than redundant. However, the relative importance of the two proteins, with respect to controlling mRNA stability, was cell type specific suggestive of a complex interplay with other factors.

Functional cooperation is also a possibility that needs to be taken into account. For example, the transition between a condensed and an open conformation of the RNA associated with AUF1 is dependent on the phosphorylation state of AUF1 (94). Potentially, this structural transition could affect the binding properties of other ARE-BPs.

Antagonistic effects

A study of the tissue specific expression of several ARE-BPs in the mouse showed that high levels of expression of AUF1 and HuR occurred in the same tissues (95). Some ARE-containing mRNAs, such as those encoding c-fos, GM-CSF, the cyclin dependent kinase inhibitor p21, IL-3 and cyclin D1 are destabilized in the presence of AUF1 and stabilized by HuR (Table 2). Consequently, it is possible that the relative cytoplasmic concentrations of functionally antagonistic ARE-BPs define the stability of a particular mRNA. Supporting this hypothesis is a RNA interference (RNAi) study in which the decreased half-life of IL-3 mRNA caused by HuR depletion is neutralized by co-depletion of another ARE-BP, BRF1, that is known to mediate the rapid degradation of several cytokines (55). Similarly, the increased expression of endogenous GM-CSF associated with the RNAi

mediated knock-down of BRF1 or certain isoforms of AUF1 is partially repressed by the co-transfection of siRNAs directed against HuR (55). Lal *et al.* (48) have directly investigated if functional interactions can exist between AUF1 and HuR. Using a micro-array based screen they showed that these two proteins can co-exist on the same mRNA when it is in the nuclear compartment. However, this association is no longer observed when the mRNA has been exported into the cytoplasm. HuR is found associated with mRNAs in polysomes and AUF1 is only found in polysome free fractions. This confirms earlier studies showing that HuR and AUF1 partition differently between polysomes and polysome free fractions (96,97). When the association of these two proteins with cyclin D1 and p21 mRNAs were studied both *in vitro* and *in vivo* (48), the binding sites were found to be distinct and non-overlapping which renders possible the simultaneous binding of these two proteins to the same mRNA. However, the depletion of either AUF1 or HuR caused a reciprocal increase in the association of the other protein with the mRNA, by binding to the site of the depleted factor.

Therefore, the degradation rate of a mRNA could be determined, at least in part or for certain mRNAs, by an equilibrium or a balance between stabilizing and destabilizing factors in the cytoplasm. This balance would be modulated by physiological stimuli that could, for instance, affect the nuclear/cytoplasmic distribution of the factors. In many cell types the majority of HuR is localized in the nucleus but it is observed to translocate to the cytoplasm in a cell cycle dependent manner (70,98) or after stimulation of the cells (69,71,99,100) and during skeletal myogenesis (68). AUF1 is also a predominantly nuclear protein and can shuttle between the nucleus and the cytoplasm (49,51,52). The sub-cellular localization of AUF1 is influenced by heat shock (50).

AUF1 and HuR are not the only factors whose relative amounts may be important. In the case of T-lymphocytes, after activation both HuR and TTP appear in the cytoplasm (101). According to a number of *in vitro* studies, these two proteins, that have opposing effects on ARE-dependent mRNA degradation (Table 2), present binding specificities that are distinct but overlapping, notably for GM-CSF, TNF- α , IL-2 and COX-2 mRNAs (Table 1). Furthermore, by co-transfection of NIH-3T3 cells with expression plasmids for TTP and HuR, Ming *et al.* (66) showed that TTP antagonises the HuR induced stabilisation of a reporter mRNA containing the IL-3 ARE.

Finally, a complex antagonistic interplay between KSRP, TTP and HuR has recently been reported by Linker *et al.* (102). They showed that KSRP targets the human inducible nitric oxide synthase (iNOS), also called nitric oxide synthase II (NOS II), mRNA for rapid degradation by binding to an ARE that is also recognized by HuR (64) and a competition between these two proteins for the ARE in the iNOS mRNA was demonstrated. Cytokine stimulation of DLD-1 cells, that increases the stability of iNOS mRNA, reduced KSRP-binding while HuR-binding increased. TTP is also an actor in this scenario. Contrary to the general picture, the over expression of TTP in DLD-1 cells stabilizes the iNOS mRNA (103). However, in this case TTP does not bind to iNOS mRNA but participates in the RNA-protein complex via an interaction with KSRP that appears to inhibit KSRP directed degradation of iNOS mRNA. Comparing the data in Tables 1 and 2 brings

to light several other ARE-BPs reported to regulate the stability of a mRNA but to which binding has not been demonstrated (AUF1/IL-3, Hel-N1/TNF- α , TTP/IL-3, BRF1/GM-CSF and KSRP/IL-2). This suggests that a re-evaluation of whether the observed ARE-BP induced changes are direct effects or due to the interaction with another ARE-BP is required.

Hence, there are significant data to support the notion that variations in the relative amounts and potentially the binding affinities of AUF1, KSRP, BRF1, TTP and HuR could determine both the identity of the targeted mRNAs and their cytoplasmic fate. By including the association with common or distinct co-factors, this regulation could be further fine tuned. We propose that the same scenario also applies to other combinations of ARE-BPs. The corollary to this is that understanding how a cell differentially regulates the stability of diverse ARE-containing mRNAs has become a question of how the relative cytoplasmic expression levels of the different ARE-BPs are controlled.

MECHANISMS AND PATHWAYS OF ARE-DEPENDENT DEGRADATION

Rapid deadenylation is a prelude to ARE-dependent degradation

In yeast, deadenylation [shortening of the 3' poly(A) tail] is followed by decapping (removal of the 5' cap structure) and degradation by 5'-3' exonucleases [reviewed in (2,43,104)]. In mammals, deadenylation is also the first and rate-limiting step in the degradation of many ARE-containing mRNAs both *in vivo* (30,105–108) and *in vitro* (109) and decapping can follow this initial event (110). Several *in vitro* studies have shown that the kinetics with which a mRNA is deadenylated varies between the ARE classes. Reporter mRNAs containing class I and III AREs are deadenylated in a synchronous manner, whereas for mRNAs containing class II AREs the poly(A) tails are shortened asynchronously (29,33,35). It is not clear at present what the biological significance of these differences may be as the overall stability of the mRNA does not appear to be affected.

Following deadenylation two separate cellular entities have been implicated in mRNA degradation, the exosome and GW or processing bodies (P-bodies). *In vitro* the degradation of the decapped mRNA occurs principally by the exosome associated 3'-5' exonucleases (111). However, the presence of the 5'-3' exonuclease Xrn1 in P-bodies implies that this route is also active in mammalian cells (112).

Exosome

The study of mRNA degradation using *in vitro* systems has shown that 3'-5' degradation of mRNAs, including those containing AREs, requires a large multi-protein complex called the exosome [reviewed in (113)]. Mukherjee *et al.* (111) suggested that certain subunits of the human exosome specifically bind to AREs causing an ARE-dependent degradation of mRNAs. Other studies have shown that several ARE-BPs, for instance TTP and KSRP, are physically associated *in vitro* with the exosome and are required so that the exosome can preferentially degrade ARE-containing mRNAs (90,93). Also TTP can stimulate the deadenylase [poly(A)

ribonuclease] (PARN) in cell extracts and this confers a specificity towards ARE-containing mRNAs (85). In cytoplasmic extracts, the ARE-BP KSRP simultaneously associates with PARN and the exosome (93). These results imply that both TTP and KSRP can recruit to a mRNA the cellular factors necessary for both deadenylation and degradation via the exosome, thereby ensuring a rapid and preferential elimination of the transcript.

P-bodies

P-bodies, first identified in yeast (114), contain mRNA decapping (Dcp1 and Dcp2) and degradation (Xrn1) factors. In mammalian cells, these same factors are found in GW bodies (112,115–117). It has been recently shown that, in mammalian cells, stress granules and P-bodies are dynamically linked and that mRNAs can be transferred between these two entities (112,118). Stress granules, that accumulate in response to particular physiological signals sequester untranslated mRNAs [reviewed in (119)] but they do not contain decapping factors (112). Therefore, stress granules act as an anti-chamber to P-bodies and hold mRNA in a translationally repressed state awaiting the decision to either recycle the mRNA towards polysomes or direct it to P-bodies for degradation (112,118). Several ARE-BPs are associated with either stress granules or P-bodies. TIA-1 and TIAR are an integral part of stress granules and HuR and TTP can be recruited to both [reviewed in (119)]. Interestingly, the translational repression of TNF- α and Cox-2 mRNAs has been correlated with TIA-1 expression (Table 2) (120,121). Furthermore, in HeLa cell extracts, the presence of an ARE in the mRNA stimulates decapping (122) and the proteins TTP and BRF1 are associated with decapping enzymes (91).

Coupling of ARE-dependent degradation and translation

The question of whether ARE-dependent degradation of mRNAs and translation are coupled is still controversial. Evidence both for and against this coupling have been reported for a number of cellular models. In some cases inhibiting translation of reporter RNAs containing the GM-CSF or c-fos ARE either pharmacologically [(28,123), reviewed in (124)] or by mutating the initiation codons (125) or inserting stable secondary structures (123,126,127), stabilized the mRNA. Also the deadenylation kinetics conferred by c-fos ARE was modified by blocking translation with a stable stem-loop structure (29). In contrast, in other studies inhibiting the translation of mRNAs containing c-fos, GM-CSF or c-jun AREs by introducing a stem-loop structure or an Iron Responsive Element in the 5'-UTR did not stabilize the mRNA (29,41,128). In addition to mRNAs, snRNA can also be targeted for rapid deadenylation by an ARE-dependent process (129), which implicitly supports the model that ARE-dependent deadenylation and decay does not require translation of the host mRNA.

It is now known that stress granules and P-bodies are affected by drugs that inhibit translation; cycloheximide and emetine that 'freeze' polysomes cause disassembly of stress granules and P-bodies whereas puromycin that dismantles polysomes promotes the assembly of stress granules (112,130). This indicates a need to re-evaluate at least some of the earlier data using drugs to inhibit translation.

TRANSLATIONAL REGULATION BY AREs AND ARE-BPs

In addition to a reduction in protein synthesis consequential to mRNA degradation, AREs can also repress the translation of host mRNA (Table 2). The 3'-UTR of Interferon- β , that contains an ARE, strongly inhibits the translation not only of the endogenous mRNA but also of reporter mRNAs containing this region in both rabbit reticulocyte lysates and in *Xenopus* oocytes (131,132). Injection of mRNAs into *Xenopus* oocytes, a biological model in which mRNAs are particularly stable, was also used to show that the AREs of GM-CSF and c-fos mRNAs inhibit the translation of the host mRNA in the absence of mRNA degradation (133). Another example of an ARE-containing mRNA that is regulated by many processes (stability, deadenylation and translational initiation) is that of TNF- α (134–136). In resting macrophages TNF- α mRNA is in a translationally inactive form (136). In response to endotoxins, the translational repression of a reporter mRNA containing the TNF- α 3'-UTR is relieved and expression of the reporter protein increases importantly; this is not due to an increase in the cytoplasmic concentration of the mRNA (135). Similarly, the translational repression conferred on a reporter mRNA by the AU-rich 3'-UTR of p53 mRNA is relieved by UV-irradiation (137). Finally, Grosset *et al.* (138) showed, also by using a reporter mRNA, that the ARE of GM-CSF mRNA can repress translation independently of the effect of this element on mRNA stability.

Among the various studies of ARE-BPs only a few have addressed the question of their role in the translational regulation of ARE-containing mRNAs (Table 2). The protein TIA-1 has been described as a translational inhibitor of some labile mRNAs such as TNF- α and Cox-2 (120,121). Using mRNAs identified in a large scale screen for TIA-1 associated mRNA, Lopez de Silanes *et al.* (40) extended this role for TIA-1 as a translational repressor to a large number of mRNAs. However, in this study the TIA-1-binding sites within the target mRNAs did not contain AUUUA motifs.

In contrast, Hel-N1 has been shown to activate translation of GLUT1 and NF-M mRNAs in 3T3L1 and hNT2 cells, respectively (72,73). Recently, HuR was reported to act as both a positive and a negative translational regulator. In carcinoma cells UV-irradiation (137) or expression of the tumour suppressor Von Hippel-Lindau (VHL) gene (99) causes an increase in HuR expression and binding to the 3'-UTR that are positively correlated with an increased translation (polysome recruitment or pulse-chase assay) of p53 mRNA. In contrast, over expression of HuR in stressed macrophages inhibited the polysome recruitment of TNF- α and Cox-2 (139) (Table 2). Interestingly, HuR induced changes in the expression of p53 (99,137) occurred in the absence of any change in the abundance of the corresponding mRNA whereas for TNF- α and Cox-2 mRNAs opposing changes in mRNA stability and translation were observed (139). It should be noted that in all these studies particular experimental conditions were used: UV-induced stress (137); over expression of the von Hippel-Lindau tumour suppressor protein (99); induced inflammatory response in macrophages (139).

The inhibitory effects of ARE-BPs on mRNA translation could be mediated by two mechanisms. In oocytes and early embryos a direct correlation has been established between the

adenylation status of a mRNA and its presence in polysomes; the polyadenylated mRNAs being translated more efficiently [reviewed in (140)]. This relationship has also been observed in many lower and higher eukaryotes [reviewed in (141)]. Cap-dependent recruitment of ribosomes is mediated by the translation initiation complex eIF4F composed of several proteins including eIF4E and eIF4G. The cap structure (m^7GpppX), present at the 5' end of eukaryotic cellular mRNAs is recognised by eIF4E. eIF4G acts as a scaffold protein that binds both eIF4E, at the 5' extremity of the mRNA, and the poly(A) binding protein1 (PABP1) associated with the 3' poly(A) tail [reviewed in (142)]. The association between eIF4E/eIF4G and the 5' cap is stabilized when eIF4G also binds to PABP1 (143,144). Therefore, ARE-dependent inhibition of translation could ensue from the deadenylation of the target mRNA leading to a loss of PABP1 bound to the mRNA which, in turn, would destabilise the eIF4E-cap interaction. A variant of this model would be that the ARE-BP binds to eIF4E, competitively inhibiting the interaction of eIF4E with eIF4G. Such mechanisms have been discussed for *Xenopus* maskin and *Drosophila* Cup [reviewed in (145)], two proteins that are indirectly recruited to the 3'-UTR of specific mRNAs. Although no direct interaction between an ARE-BP and eIF4E has been reported to date, it is intriguing that AUF1 is part of a multimeric complex containing eIF4G (50).

An alternative mechanism brings into play stress granules. As described above stress granules contain untranslated mRNAs and are associated with several ARE-BPs. This suggests a route for translational arrest by an ARE-BP mediated targeting of specific mRNAs into stress granules. However, in the examples given above translation is repressed in the resting state and activated after a cellular stimulus that can be assimilated to an environmental stress. Therefore, it is necessary to posit that at least some ARE-containing mRNAs would be constitutively targeted to stress granules. Relief of TNF- α (135) or p53 (137) translational arrest by endotoxins or UV-irradiation, respectively would then correspond to an export of these mRNAs out of the stress granules. In this context it is interesting to note that stress-induced nascent transcripts are excluded from stress granules in both plants (146) and mammalian cells (119).

ARE THERE UNIFYING PRINCIPLES?

Despite the large amount of published work on AREs and ARE-BPs since the initial description of AREs in 1986 (23), we are still largely in the dark as to the precise molecular pathways to which these *cis*-acting sequence elements target mRNAs for degradation and/or translational arrest. This is not particular to ARE-dependent degradation and we would posit that part of the difficulty in describing these molecular machines is inherent in their nature. It is only recently that molecular data on the characteristics of localised factories implicated in the regulation of gene expression have been obtained. This is true for both cytoplasmic and nuclear processes. The co-localization of transcribed genes in the nucleus is now being documented (147) and the nuclear processing (capping, splicing and polyadenylation) of pre-mRNAs is also coordinated within particles [reviewed in (148,149)]. We have already described the cytoplasmic stress granules and P-bodies

that respectively sequester mRNAs in response to particular physiological signals or achieve particular processing events. Several ARE-BPs are associated with the exosome, stress granules and P-bodies (see above).

One very plausible role for ARE-BPs is to act as traffic markers that channel targeted mRNAs into specific pathways. This is easy to envisage for ARE-BPs implicated in enhancing the degradation or imposing a translational arrest on an mRNA. These observed effects could be two steps in the same pathway, first sequestering the mRNA away from the translation machinery and then shuttling it into a degradation pathway. The transfer of mRNAs into stress granules and then to P-bodies is precisely such a pathway. If control was exerted between these two steps then some mRNAs could be translationally arrested but not degraded. Cox-2 mRNA is an example of this (150).

Several models by which an ARE-BP could enhance protein expression can also be envisaged. Evidently, if the binding of the ARE-BP stabilizes the target mRNA then an increase in the synthesis of the encoded protein will probably ensue. Enhanced translation could also result from relieving repression. Consider a mRNA that is translationally repressed but not degraded. If following a particular cellular stimulus the mRNA leaves the inhibitory complex and enters into polysomes, translation will increase. For some mRNAs stress granules can achieve this task (112).

The functional analyses of ARE-BPs both *in vitro* and *in vivo* have led to the identification of a large number of target mRNAs (Tables 1 and 2). Many of the *in vitro* experiments are performed using the same cellular models. Hence the large number of ARE-BPs binding to the same mRNA suggests that many redundant factors have evolved which, although not impossible, is rather surprising. We favour an alternative hypothesis that, in fact, these proteins are not redundant but only appear to be so due the experimental design(s) that are feasible at present. As mentioned above, ARE-BPs are probably integral components of large functional complexes. In such complexes they will interact with some but probably not all of the other components. The functions of many regulatory proteins have been studied by either over expressing or depleting the factor(s) of interest, both of which can cause several 'unnatural' consequences. For instance, certain proteins when over expressed act as dominant negative mutants. However, such considerations should not be taken to exclude the possibility that in a physiological normal situation any one ARE-containing mRNA can associate with more than one ARE-BP. Indeed, several examples have already been discussed. Furthermore, the association of an ARE with several ARE-BP with either complementary or antagonistic consequences would allow targeting of these mRNA into different processing pathways. Furthermore, several ARE-BPs could combine together in a single complex and cooperatively define the functional outcome for a mRNA.

FUTURE DIRECTIONS

It is now obvious that further characterization of ARE-BPs and their functions will require a description of the cellular complexes in which they are normally found. Furthermore, the composition of these RNA-containing complexes is probably

not static but may be cell cycle dependent, change with localization of the associated mRNA or as a function of external stimuli. This adds a temporal aspect to ARE-dependent regulation. Accordingly, a first necessary step towards understanding ARE-dependent regulation is the isolation of ribonucleoprotein complexes containing ARE-BPs from unmodified cells. One interesting study in this vein is that of Lal *et al.* (48) who showed that HuR and AUF1 can simultaneously bind to many mRNAs in the nucleus but that they are associated with distinct mRNA populations in the cytoplasm. This brings to light a second facet of ARE-BPs that has not been systematically considered; the intracellular localization of these proteins. For the majority of ARE-BPs whose localization has been studied, this is predominantly nuclear. This is the case for HuR and AUF1 whose most well described functions are, respectively, to stabilize and destabilize mRNAs, presumably in the cytoplasm. In unperturbed cells how is this achieved? Do these ARE-BPs piggy-back on targeted mRNAs that are predestined to be stable or rapidly degraded and how is this destiny defined in the nucleus? Intriguingly, a 'nuclear history' is required for one of the AUF1 isoforms to exert its cytoplasmic function (151). Localization of ARE-BPs within the cytoplasm should also be high on the priority list for future research. HuR, TIA-1, TIAR and TTP are already identified as components of stress granules (112,152) and the interaction between stress granules and P-bodies is promoted by TTP and BRF1 (112). A complete study of stress granule and P-body dynamics, associated with precise measurements of ARE-dependent mRNA translation, deadenylation and decay and the localization of the related ARE-BPs is now possible and should yield results of high interest.

From this survey it is apparent that many questions remain to be answered and, as indicated above, light will probably only start to be shed by the conjunction of complementary approaches. For instance, highly specific biochemical analyses of the composition of ribonucleoprotein complexes containing ARE-BPs and analysis of the binding affinities of ARE-BPs for the various target AREs would certainly yield data on any redundant, antagonistic or cooperative binding between ARE and BPs. Finally, *in situ* localization methods coupled with the biochemical data on the composition of ARE-BP complexes and micro-array profiling of expressed and translated mRNAs would provide the data to develop a more overall functional map of the pathways into which AREs and the associated ARE-BPs direct specific mRNAs either constitutively or in response to particular cellular events or external stimuli.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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