Characteristics of AU-rich elements and involvement of the poly-(A) tail in stress-induced mRNA stabilization

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Hannover

Gayatri Gowrishankar

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1 ZUSAMMENFASSUNG

Posttranskriptionelle Mechanismen tragen zu schnellen durch Entzündungs-/Stress-Stimuli induzierten Änderungen der Genexpression bei. AU-reiche Elemente (AREs) sind Instabilitätsdeterminanten, deren Anwesenheit in den 3' untranslatierten Regionen die schnelle Degradation der mRNAs verschiedener "early response" Gene induziert, darunter Cytokine, Wachstumsfaktoren und Protoonkogene. Die Aktivierung des p38 MAP Kinase/MAPKAP Kinase 2 (MK2) Signalwegs in HeLa Zellen induziert, wie von unserer Gruppe gezeigt, selektiv die Stabilisierung ARE-enthaltender mRNAs. Dagegen führte die Einwirkung von UV-B Licht unabhängig von einem ARE und in p38/MK2 unabhängiger Weise zur Stabilisierung von mRNAs. Diese Beobachtungen warfen Fragen bezüglich der mRNA-Selektivität sowie der beteiligten Mechanismen beider Arten von mRNA-Stabilisierung auf.
Zur Untersuchung der für die schnelle Degradation und die Stimulus-induzierte Stabilisierung von mRNAs erforderlichen Eigenschaften von AREs wurde in dieser Arbeit das ARE in der mRNA des chemotaktischen Cytokins IL-8 definiert. Durch Messung der Degradationskinetik von β-Globin mRNAs, die Fragmente der 3' untranslatierten Region von IL-8 enthalten, wurde das ARE in einem 60 Nukleotide langen Fragment mit strukturell und funktionell zweiteiligem Aufbau lokalisiert: einer AUUUA-Motive enthaltenden Kerndomäne mit begrenzter eigener Destabilisierungsfunktion und einer Hilfsdomäne, welche die von der Kerndomäne ausgeübte Destabilisierung verstärkt. Die Aktivierung von p38/MK2 stabilisierte mRNAs, die das komplette ARE oder lediglich die Kerndomäne enthielten. Die Stabilisierung durch ein bekanntes ARE-bindendes Protein- HuR- erforderte andersartige Sequenzen, die im IL-8 ARE fehlen, aber in anderen AREs wie dem von *c fos* und von GMCSF vorliegen. Das weist auf die Existenz von Untergruppen von AREs hin, die sich in ihrer Reaktionsfähigkeit gegenüber verschiedenen Arten der mRNA-Stabilisierung unterscheiden. Die Analyse der Länge von poly-(A) Schwänzen ARE-enthaltender mRNAs zeigte an, dass die Aktivierung des p38/MK2 Signalwegs den Verlust des poly-(A) Schwanzes (Deadenylierung) hemmt, den initialen Schritt der mRNA-Degradation. Eine Rolle des poly-(A) Schwanzes und daran assoziierter Proteine bei der ARE-vermittelten mRNA-Degradation wurde durch Experimente mit mRNAs unterstützt, in denen der poly-(A) Schwanz durch eine Histon "stem loop" Sequenz ersetzt war. In Abwesenheit des poly-(A)

Schwanzes destabilisierte das ARE die mRNA nicht, und p38/MK2 Aktivierung hatte keinen Einfluss auf die mRNA-Stabilität, was auf einen Verlust der ARE-abhängigen Regulation hinwies. Die Deadenylierung von mRNAs wurde noch stärker in Zellen gehemmt, die UV-B Licht ausgesetzt wurden. Jedoch trat diese Hemmung unabhängig von einer p38/MK2 Aktivierung und auch in mRNAs ohne AREs auf. Eine Hemmung der mRNA-Degradation und Deadenylierung wurde bei weiteren zellulären Stressoren, Hitzeschock, osmotischen Schock und Einwirkung von H_2O_2, jedoch nicht bei γ-Strahlung beobachtet. Das spricht gegen die Beteiligung von DNA-Schädigung als primärem Ereignis bei dieser Reaktion auf UV Licht. Eine Bedeutung der UV-vermittelten mRNA-Stabilisierung für die Entzündungsreaktion wird nahegelegt durch starke Hemmwirkungen auf die Degradation und Deadenylierung der endogenen IL-8 mRNA und durch Microarray Analysen, welche in HeLa Zellen sowie in der Keratinozyten-Linie HaCaT die Stabilisierung einer Gruppe an Entzündungen beteiligter Transkripte nachweisen.

Diese Arbeit zeigt, dass mRNA-Stabilisierung durch verschiedene Auslöser in mechanistisch und bezüglich Transkriptselektivität unterschiedlicher Weise induziert wird. Weitere Experimente sind erforderlich, um die molekularen Prozesse und Gruppen von Ziel-mRNAs dieser Arten der mRNA-Stabilisierung zu definieren.

Schlagwörter: AREs, mRNA, Stabilisierung

1 ABSTRACT

Post-transcriptional mechanisms contribute to the rapid changes in gene expression induced by inflammatory/stress stimuli. AU-rich elements (AREs) are instability determinants whose presence in the 3' untranslated regions induces rapid degradation of the mRNAs of several early response genes, including cytokines, growth factors and proto-oncogenes. Activation of the p38 MAP kinase/MAPKAP kinase 2 (MK2) pathway in HeLa cells was shown by our group to selectively induce the stabilization of ARE-containing mRNAs, whereas exposure to UV light led to stabilization of mRNAs independently of an ARE and in a p38/MK2 independent manner. These observations raised questions concerning the mRNA selectivity as well as the mechanisms involved in both modes of mRNA stabilization. To investigate the features of AREs required for rapid degradation and for stimuli-induced stabilization of mRNAs, the ARE in the mRNA of the chemotactic cytokine IL-8 was defined in this study. By measuring the degradation kinetics of β-globin mRNA containing fragments of the IL-8 3'untranslated regions, the ARE was localized to a 60 nucleotide fragment with a structurally and functionally bipartite character: an AUUUA motif-containing core domain with limited destabilizing functions of its own and an auxiliary domain that enhances the destabilization exerted by the core domain. p38/MK2 activation stabilized mRNAs with the complete ARE or with the core domain alone. Stabilization by a well-known ARE-binding protein- HuR- required distinct sequences absent in the IL-8 ARE but present in other AREs like the *c fos* and GMCSF AREs. This suggests the existence of subsets of AREs differing in responsiveness to different modes of mRNA stabilization. Analyzing the poly-(A) tail lengths of ARE-containing mRNAs indicated that activation of the p38/MK2 pathway inhibits loss of the poly-(A) tail (deadenylation), the initial step in mRNA degradation. A role for the poly-(A) tail and its associated proteins in ARE-mediated mRNA degradation was supported by experiments with mRNAs in which the poly-(A) tail was replaced by a histone stem loop sequence. In the absence of a poly-(A) tail the ARE did not destabilize the mRNA, and p38/MK2 activation had no effect on mRNA stability, indicating that ARE-dependant regulation was lost. Deadenylation of mRNAs was more strongly impaired in cells exposed to UV-B light. However, this inhibition occurred independently of p38/MK2 activation and also in mRNAs lacking AREs. Among other cellular stresses heat shock, osmotic shock and exposure to H_2O_2

but not γ-radiation were also observed to inhibit mRNA degradation and deadenylation, which argues against the involvement of DNA damage as the primary event in this response of UV light. A role for the UV-mediated mRNA stabilization in the inflammatory response is suggested by strong inhibitory effects on the degradation and deadenylation of the endogenous IL-8 mRNA and by microarray analysis which revealed the stabilization of a group of transcripts involved in inflammation in both HeLa cells and in the keratinocyte cell line, HaCaT.

This study demonstrates that mRNA stabilization induced by different triggers differ mechanistically and with regard to transcript selectivity. More experiments are needed to define the molecular processes and sets of target mRNAs for these modes of mRNA stabilization.

Keywords: AREs, mRNA, stability

2 INTRODUCTION:

2.1 REGULATION OF GENE EXPRESSION: The transfer of genetic information from the level of the nucleotide sequence of a gene to the level of the amino acid sequence of a protein via the nucleotide sequence of RNA is termed gene expression. The entire process of gene expression in eukaryotes encompasses several steps including:

- Transcription
- Pre-mRNA processing
- Translation

Owing to the highly structured organization of eukaryotic systems, gene expression in eukaryotes needs to be tightly and precisely regulated in response to a variety of external cues. Due to the multitude of stages involved in gene expression, there are several potential regulatory sites including transcription, RNA processing, mRNA nuclear export and localization, mRNA decay, translation and posttranslational events. In addition to the long-established transcriptional mechanisms regulating gene expression, the involvement of posttranscriptional gene regulation events, in particular alterations in mRNA stability is increasingly recognized. In mammalian cells, mRNA half-lives can range from less than 20mins to 24hrs. These differences in mRNA decay rates have dramatic effects on the expression of specific genes, and allow the cell to effect rapid changes in transcript abundance. For example, the rate of mRNA turnover for individual mRNAs can vary in response to specific hormones *(Paek I 1987),* viral infection *(Nishioka Y 1977), (Rice AP 1983)*, as a consequence of differentiation *(Jack HM 1988)* or in response to inflammatory and stress stimuli *(Holtmann H 1999), (Blattner C 2000)*, *(Bollig F 2002)*. These inducible effects on mRNA stability have the potential to change cytoplasmic mRNA concentrations rapidly without alteration in transcriptional activity and to augment the magnitude of mRNA induction by coordinating changes in transcription and mRNA stability.

2.2 MECHANISM OF EUKARYOTIC mRNA DEGRADATION: All eukaryotic mRNAs bear terminal nuclear modifications at their 5' and 3' ends, in the form of the 7 methylated cap (^{7}mGpppN) structure at the 5' end and the poly (A) tail, which is a stretch of 100-250 adenine residues, at the 3'end. In eukaryotes mRNA degradation can occur via one of four related, yet distinct pathways. In most cases the degradation of the transcripts begins with the shortening of the poly (A) tail at the 3'end of the mRNA, a process titled deadenylation *(Shyu AB 1991) (Muhlrad D 1992)*. Three mRNA deadenylases have been identified. In yeast, the

predominant deadenylase complex contains two nucleases Ccr4p and Pop2p and several accessory proteins, Not1-Not5p, Caf4p, Caf16p, Caf40p and Caf130p *(Daugeron MC 2001), (Tucker M 2002), (Tucker M 2001)*. Additional deadenylases include the Pan2p/Pan3p complex, first identified in yeast and proposed to be involved in cytoplasmic deadenylation in addition to its role in the trimming of the poly (A) tails of nascent transcripts in the nucleus *(Boeck R 1996), (Brown CE 1996), (Tucker M 2001)*. Mammalian homologs of the yeast Pan2/Pan3 complex have also been recently identified and shown to have deadenylating activity in vitro *(Uchida N 2004)*. Another poly (A)-specific deadenylating nuclease- the RNase D homolog, PARN (poly (A) ribonuclease)- has been purified and biochemically characterized from both mammalian cells and *Xenopus laevis* oocytes *(Korner CG 1997), (Korner CG 1998).* An as yet unresolved issue is the relative importance of these individual deadenylases in different organisms, for different mRNAs and in different physiological situations.

In yeast, shortening of the poly (A) tail is followed by the removal of the 5' cap structure (decapping) by the decapping enzymes, Dcp1p and Dcp2p *(Decker CJ 1993), (Dunckley T 1999), (LaGrandeur TE 1998)*. This exposes the transcripts to digestion by the 5' to 3' exonuclease Xrn1p *(Muhlrad D 1994), (Hsu CL 1993)*. Similar mechanisms exist in mammalian cells as well as are evident from the conservation of the decapping enzymes, Dcp1p and Dcp2p, among eukaryotes *(Lykke-Andersen 2002), (van Dijk E 2002)*. Other observations also indicate that deadenylation precedes decapping in mammalian cells *(Couttet P 1997).*

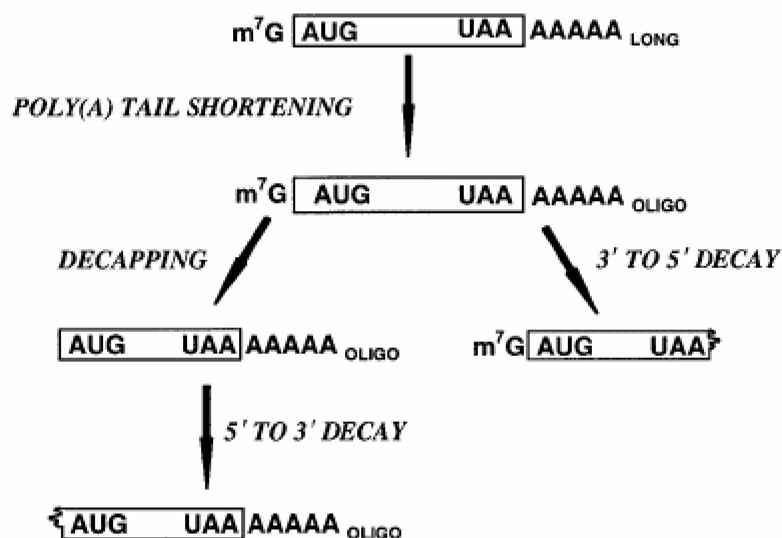


Figure 1:Deadenylation-dependent mRNA degradation pathways (Staton JM 2000)

The proteins involved in mRNA decapping and 5' to 3' exonucleolytic decay have been found to localize to specific cytoplasmic foci, referred to as P bodies. In yeast, GFP-tagged Dcp1p, Dcp2p, and Xrn1p have been localized to P bodies *(Sheth U 2003)*. In mammalian cells analogous foci have been identified where the mammalian decapping factors co-localize, *(van Dijk E 2002), (Cougot N 2004)*, attesting to the conservation of decay mechanisms amongst eukaryotes.

mRNAs can also be degraded in the 3' to 5' direction following deadenylation *(Muhlrad D 1995)*. 3' to 5' degradation of mRNAs is catalyzed by the exosome, a multisubunit complex of 3' to 5' exonucleases functioning in several RNA degradative and processing events *(Anderson AR 1998), (Mukherjee D 2002), (Rodgers ND 2002).*

Some eukaryotic mRNAs may also be degraded via endonucleolytic cleavage by a deadenylation independent process as has been shown for mammalian 9E3, transferrin receptor, *c-myc*, serum albumin, vitellogenin and *Xenopus* β-globin mRNAs *(Binder R 1994), (Cunningham KS 2000), (Stoeckle MY 1989), (Lee CH 1998).*

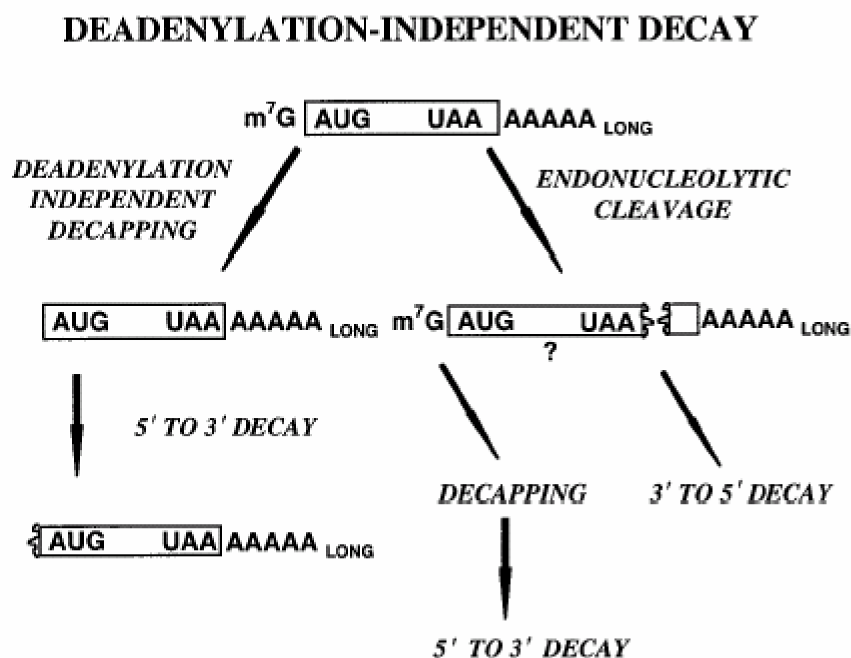


Figure 2: Deadenylation independent mRNA degradation pathways (Staton JM 2000)

Eukaryotic cells have also evolved quality control mechanisms, in the form of specialized pathways that degrade aberrant mRNAs containing either a premature translational stop codon (nonsense mediated decay) or those that do not contain a stop codon (non-stop decay). While the former involves decapping without prior removal of the poly (A) tail *(Muhlrad D 1994)*, the latter is effected via the cytoplasmic exosome and requires translation *(Frischmeyer PA 2002)*. Therefore it appears that one major function of mRNA decay, apart from its role in the regulation of gene expression, is quality control, in order to ensure that only mRNA having an appropriate structure, i.e., cap, start codon, stop codon, and poly (A) tail survive.

2.3 DETERMINANTS OF mRNA STABILITY: The cap and the poly (A) tail present at both ends of mRNAs play critical roles in mRNA translation and stability *(Gallie 1991), (Wickens M 1997)*. The role of the cap in mRNA stabilization has been demonstrated clearly first in *Saccharomyces cerevisiae*, and later in other eukaryotes, in which removal of the 5'-terminus represents part of a common degradation pathway for stable and unstable RNAs *(Tuite 1996), (Muhlrad D 1994), (Beelman CA 1996).*

The poly (A) tail inhibits mRNA decay through its interaction with the poly (A)-binding protein (PABP1) *(Bernstein P 1989)*. As well as binding poly (A), PABP1 interacts with a specific region of the translation initiation factor eIF4G, which in turn forms a ternary complex with the cap-binding protein eIF4E *(Gingras AC 1999)*. This interaction circularises the mRNA *in vitro*, *(Wells SE 1998)* and can simultaneously promote translation and stabilize mRNAs by protecting both ends of mRNAs from exo/endo nucleases *(Gao M 2000).*

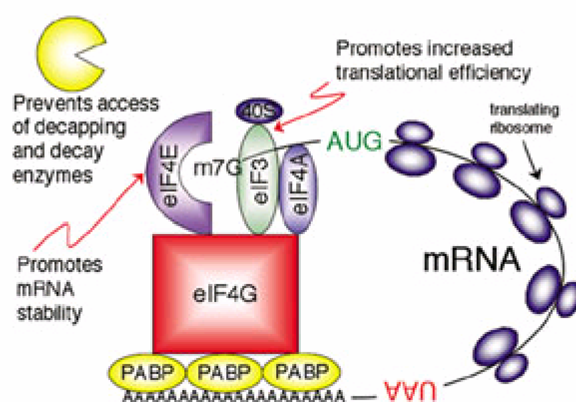


Figure 3: The closed-loop model for translation and mRNA stability. *(R.Lewis 2003)*

Apart from the cap and the poly (A) tail that function as determinants of generalized mRNA stability, there are specific sequence elements which determine the difference in decay rates between stable and unstable mRNAs. There is little doubt that unstable mRNAs contain instability determinants, but it is as yet unclear whether stable mRNAs contain discrete stabilizing determinants or are stable by default. With the exception of a few stabilizer sequences, as for example the cytosine rich element in the 3' UTR of the α globin gene *(Weiss IM 1995)*, and similar elements in a few other genes *(Russel JE 1996), (Holcik M 1997)*, most identified elements promote mRNA destabilization. These sequences include the well-known adenosine (A) + uridine (U)-rich elements (AREs) located in the 3'UTR of many short lived mammalian mRNAs.

2.3.1 AU-rich elements (AREs): The very short half-lives observed for transiently expressed genes, including early response genes such as lymphokines, cytokines and transcription factors is attributed to the presence of adenylate, uridylate-rich (AU-rich) instability elements (AREs) in their 3' untranslated regions (UTRs). In 1986, Shaw and Kamen observed that an

ARE in the 3'UTR of granulocyte-macrophage colony stimulating factor (GMCSF) mRNA could stimulate the degradation of the normally stable β-globin mRNA *(Shaw G 1986)*. Similarly the 3'UTR of the *c fos* mRNA, which contains a 69nt ARE, was also observed to reduce the stability of the β-globin mRNA *(Chen C 1995), (Chen CY 1995)*. Subsequently, AREs that function as RNA destabilizing elements were found in numerous mRNAs including that of certain hematopoietic cell growth factors, interleukins (eg. IL-6, IL-8, IL-3, IL-2) *(Winzen R 1999), (Stoecklin G 2000), (Lindstein T 1989)*, TNF α *(Lewis T 1998)* and some proto-oncogenes. Recent computational analysis of the 3'UTRs revealed that as many as 8% of human mRNAs contain AREs *(Bakheet T 2001)*. This finding suggests that AREs may account for the degradation of most unstable mRNAs.

AREs were initially grouped into three classes according to their sequence features and decay characteristics *(Chen C 1995), (Peng SS 1996)*. Class I AREs contain one to three scattered copies of the pentanucleotide AUUUA embedded within a U-rich region, found in *c fos* and *c myc* mRNAs. Class II AREs contain multiple overlapping copies of the AUUUA motif, only found in cytokine mRNAs. Class III AREs, such as the one in *c-jun* mRNA lack the hallmark AUUUA but contain a U-rich sequence. Bakheet and co-workers recently compiled a database of ARE containing mRNAs, and divided them into five groups *(Bakheet T 2001)*. In an attempt to reconcile both the experimental and computational classifications, Wilusz et al have formulated the table given below:

Class	Motif	Examples
I	WAUUUAW and a U-rich region	c-fos, c-myc
IIA	AUUUAUUUAUUUAUUUAUUUA	GM-CSF, TNF-α
IIB	AUUUAUUUAUUUAUUUA	Interferon-α
IIC	WAUUUAUUUAUUUAW	IL-2, cox-2
IID	WWAUUUAUUUAWW	FGF-2
III	U-rich, non-AUUUA	c-jun

Table 1: Classification of AREs. *(Wilusz CJ 2001)*

Where W can be either A/U. It is now becoming increasingly clear that it is the combination of several functionally and structurally distinct sequence motifs, that determines the ultimate

destabilizing ability of each particular ARE *(Peng SS 1996), (Chen CY 2002), (Winzen R 2004)*.

The physiological importance of AREs *in vivo* has been evaluated for very few mRNAs. It was reported as early as in 1989 that removal of the ARE stretch correlates with increased oncogenicity of the proto-oncogene *c fos* *(Raymond V 1989)*. It was also shown that deleting the AU-rich region of the TNF gene in mice led to the development of chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease *(Keffer J 1991)*, *(Kontoyiannis D 1999)*. Today it has been well established that several human cancer and inflammatory diseases, including certain B-cell lymphomas, neuroblastomas, and chronic inflammatory conditions are linked to ARE defects *(Conne B 2000)*. This is due to the fact that ARE-mediated changes in mRNA stability are important in processes that require transient responses such as cellular growth, immune response, cardiovascular toning, apoptosis and external stress-mediated pathways. Hence a stabilization of ARE mRNAs can cause a prolonged response that may subsequently lead to a diseased state.

Our understanding of the mechanism of ARE mediated mRNA turnover is far from complete. Some of the earliest observations from Chen and co-workers implied that AREs promote mRNA degradation by accelerating mRNA deadenylation *(Chen CY 1995)*. Recently it was shown that AREs could also act as potent stimulators of the decapping process *(Gao M 2001)* and recruit the major component of the 3' to 5' degradation pathway-the mammalian exosome-either via direct binding or through their interaction with destabilizing ARE-binding proteins *(Mukherjee D 2002), (Chen CY 2001), (Gherzi R 2004)*.

2.3.1.1 ARE-binding proteins: Numerous proteins have been described to bind AREs and designated as ARE binding proteins (ARE-BP) *(review: (Bevilacqua A 2003)*. Binding of these factors to transcripts bearing an ARE can have either a positive or negative effect on processes as diverse as stability, translation and sub-cellular localization of the mRNA.

2.3.1.1.1 HuR: One of the best-characterized ARE-BPs is HuR, a 36 kDa ubiquitously expressed member of the ELAV (embryonic lethal vision) family of RNA binding proteins *(Ma WJ 1996)*. There is evidence for the binding of HuR to ARE sequences *in vitro* *(Myer VE 1997), (Fan XC 1997)* and *in vivo* *(Atasoy U 2003), (Lopez de Silanes I 2004)*. Overexpression studies demonstrated that HuR could stabilize messages containing class I and class II (and to a lesser extent class III) AREs by a mechanism that involves protection of

the RNA body rather than slowing the rate of deadenylation *(Fan XC 1998), (Peng SS 1998), (Atasoy U 2003), (Levy NS 1998), (Rodriguez-Pascual F 2000)*. Other studies in which the HuR gene expression was downregulated by antisense RNA or siRNA approaches provided further evidence for the role of HuR in modulating mRNA turnover *(Wang W 2000), (Wang W 2000)*. Like other Hu-family proteins, HuR contains three RNA recognition motifs (RRMs) and a novel shuttling sequence, HNS, located in the hinge region between its second and third RRM, which is crucial for the nuclear-cytoplasmic shuttling property of HuR *(Fan XC 1998)*. HuR's ability to shuttle has led to the suggestion that HuR may initially become associated with target ARE-containing mRNAs in the nucleus and accompanies them into the cytoplasm where HuR is then able to exert its stabilizing actions *(Brennan CM 2001)*. While this hypothesis remains to be proven, there is a growing body of evidence that links the cytoplasmic presence of HuR with its mRNA stabilizing functions *(Wang W 2002), (Tran H 2003)*.

2.3.1.1.2 AUF1: Another well-characterized ARE-BP is AU binding factor 1(AUF1) or heterogenous nuclear ribonucleoprotein (hnRNP D), the first ARE-BP to be identified. It was first isolated on the basis of its ability to induce *c myc* mRNA decay *in vitro* *(Zhang W 1993)*. Subsequently AUF1 has been implicated in the regulation of many ARE containing mRNAs including GMCSF *(Buzby JS 1999)*, Bcl-2 *(Lapucci A 2002)* and IL-10 *(Brewer G 2003)* amongst others. AUF1 exists as a family of four protein isoforms with apparent molecular masses of 37, 40, 42 and 45kDa that are derived from an alternatively spliced pre-mRNA *(Wagner BJ 1998)* and that differ in their sequences at their N- and/or C-termini *(Wilson GM 1999)*. All isoforms contain two N-terminal RRMs and a C-terminal domain containing Arg-Gly-Gly (RGG) motifs *(Dempsey LA 1998)*. All isoforms are also primarily localized in the nucleus but the p^{37} and p^{45} isoforms exhibited shuttling properties as revealed by heterokaryon studies *(Arao Y 2000)*. This ability to shuttle appears to be essential for AUF1 to exert its effects on ARE containing mRNA *(Chen CY 2004)*. Transfection studies have shown that AUF1 can function as an mRNA destabilizing/stabilizing factor in a cell type specific manner *(Loflin P 1999), (Xu N 2001)*. Laroia et al on the other hand have proposed a model wherein targeted degradation of AUF1 by the ubiquitin-proteasome pathway is associated with the acclerated turnover of GMCSF ARE containing mRNA *(Laroia G 1999)*. Other studies have implicated AUF1 in the turnover of *c fos* mRNA directed by its major coding region determinant (m-CRD) *(Grosset C 2000)* and in the accumulation of α-globin mRNA in erythrocytes *(Kiledjian M 1997)*. Recently Chen et al have demonstrated using cell-free decay systems that some ARE-BPs including AUF1 could recruit the mammalian exosome to

ARE containing RNA thereby mediating the degradation of ARE containing RNA *(Chen CY 2001)*. These contradictory results raise the hypothesis that the function of ARE-BPs may be conditioned by the complexes in which they are integrated, and by the physiological conditions that shape the formation of these complexes.

2.3.1.1.3 Tristetraprolin: Other ARE-BPs include Tristetraprolin (TTP), also known as Nup45, TIS11 or G0S24. TTP is the prototype of a group of CCCH tandem zinc finger (TZF) proteins, characterized by a CCCH zinc finger *(Thompson MJ 1996)*. Although TTP was originally thought to be a transcription factor, recent evidence indicates that TTP and its related proteins like Butyrate response factor 1 (BRF1) promote the turnover of ARE containing mRNA *(Carballo E 1998), (Carballo E 2000), (Stoecklin G 2002)*. Mice deficient in TTP develop a complex phenotype consisting of cachexia, dermatitis, conjunctivitis, destructive arthritis and autoimmunity *(Taylor GA 1996)*, all of which resembled earlier mouse models of TNF-α excess, thus implying a role for TTP in the regulation of TNF alpha synthesis, secretion, turnover, or action. Later it was demonstrated that TNF-α and GM-CSF mRNA are markedly stabilized in the cells from the TTP deficient animals *(Carballo E 1998; Carballo E 2000)*. The mechanism by which TTP destabilizes class II ARE containing mRNAs is not clearly understood but it was shown that the integrity of each of the two zinc finger motifs is required for binding and activity *(Lai WS 2002)*. It was also shown using cell-free systems that TTP and its related proteins could promote deadenylation and hence decay of ARE containing polyadenylated substrates by the mammalian deadenylase PARN *(Lai WS 2003)*. More recently it was shown that sequestering of TTP by 14-3-3 proteins in response to specific stimuli inhibited TTP dependent degradation of ARE containing transcripts *(Stoecklin G 2004)*.

2.3.1.1.4 KSRP: Additional ARE-BPs like the KSRP (K homology Splicing Regulatory Protein) have been purified *(Chen CY 2001)*. KSRP was originally identified as a component of a protein complex that assembles on an intronic *c-src* neuronal –specific splicing enhancer *(Min H 1997)*. It contains four RNA binding K homology (KH) motifs. KSRP was found by Chen et al to physically associate with the mammalian exosome *(Chen CY 2001)*, and that the isolated exosome then preferentially degrades ARE containing RNA. The group of Chen then went on to show that KSRP is required for rapid decay of ARE-containing mRNAs both *in vitro* and *in vivo*, and that the minimal active region is composed of the third and fourth KH motifs, which together mediate high-affinity binding to the ARE, whereas the third KH motif

alone is responsible for interaction with poly (A) ribonuclease, PARN and the exosome *(Gherzi R 2004)*.

Inspite of intensive study, the question as to how these proteins alter mRNA stability remains to a large extent unanswered. One possibility is that ARE-protein complexes alter interactions between PABP and poly (A), or between eIF4E and the 5' cap, thereby providing access to exonucleases or deadenylases.

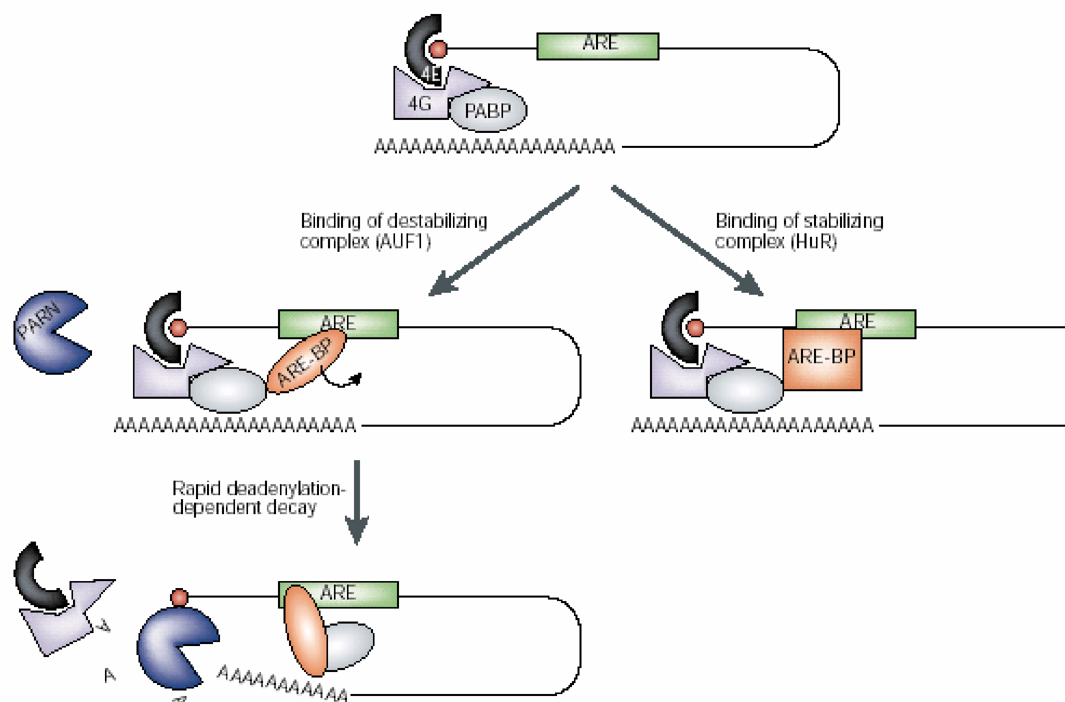


Figure 4: **Model to show how the AREs mediate mRNA destabilization and stabilization**. (Wilusz CJ 2001)

2.3.1.2 Regulated ARE-mediated mRNA stability: An emerging theme in post-transcriptional control of gene expression is that *trans*-acting factors like the ARE-BPs may play a critical role in modulating the rate of degradation of distinct mRNAs involved in cellular responses to environmental and/or metabolic changes. Consistent with this possibility are previous observations that some AREs are inactive in selected cell lines and that AREs can be differentially regulated in response to particular extracellular stimuli *(Nair AP 1999)*, *(Schiavone N 2000)*. Since small differences in half-lives can alter the abundance of a given mRNA, stabilization of otherwise labile ARE-containing mRNA contributes to their strong and rapid induction. This level of post-transcriptional control affords an additional degree of flexibility in the expression of a large number of transcripts that play essential roles in growth

control, differentiation, immune activation, and in rapid response to extracellular stimuli *(Koeffler HP 1988), (Ernst TJ 1989)*. Early reports described the altered turnover of ARE-containing mRNAs in response to extracellular as well as internally generated signals, such as phorbol esters, antibodies recognising CD3/CD28 surface receptors and TNF α *(Bickel M 1990), (Gorospe M 1993), (Lindstein T 1989)*. Protein kinase C (PKC) was specifically implicated in the enhanced stability of many labile mRNAs, such as those encoding p21 and IL-1 *(Gorospe M 1993), (Park JW 2001)*. The mitogen-activated protein kinases (MAPK) have also been implicated in regulating mRNA turnover. These include the Jun-N-terminal kinase (JNK) signalling pathway, which was found to participate in the stabilization of ARE-containing IL-3 and IL-2 mRNAs *(Chen CY 1998), (Ming XF 1998)*, phosphatidylinositol-3-kinase *(Ming XF 2001)*, and p38 mitogen-activated protein kinase pathways *(Dean JL 1999), (Miyazawa K 1998), (Ridley SH 1998), (Winzen R 1999)*.

2.3.1.2.1 p38 MAPK and mRNA stability: p38 belongs to the large family of mitogen-activated protein kinases. Stress signals, such as lipopolysaccharides (LPS) in lymphoid cells, heat shock and ultraviolet light or pro-inflammatory cytokines like IL-1 or TNF α can initiate signalling cascades resulting in the activation by dual tyrosine/threonine phosphorylation, of p38 *(Rouse J 1994) (Freshney NW 1994), (Han J 1994)*. Activation of this pathway affects a variety of cellular processes including cell division, apoptosis, invasiveness of cultured cells and the inflammatory response *(Xia Z 1995), (Takenaka K 1998), (Lee JC 1994)*. p38 MAP kinase activates many protein kinases including the MAP-kinase activated protein kinases 2, MK2 *(Stokoe D 1992)*.

Our own experiments and the work of others with cultured cells and with mice, indicate that activation of the p38 MAPK induces the stabilization of several ARE-containing endogenous mRNAs and reporter mRNAs containing different AREs including those of IL-3, IL-6, IL-8, TNF α, cyclooxygenase 2, and *c fos*, via the activation of its substrate MK2 *(Kotlyarov A 1999), (Lasa M 2000), (Neininger A 2002), (Winzen R 1999)*. In the case of TNF α alone, the ARE-mediated regulation via the p38 MAP kinase pathway appears to be at the level of translation *(Willeaume V 1995) (Kotlyarov A 1999), (Neininger A 2002)*. The mechanism by which the p38 MAPK/MK2 pathway controls the translation and stability of ARE-containing mRNA is as yet unknown but a working hypothesis is that this involves the phosphorylation of one or more proteins that bind to the ARE.

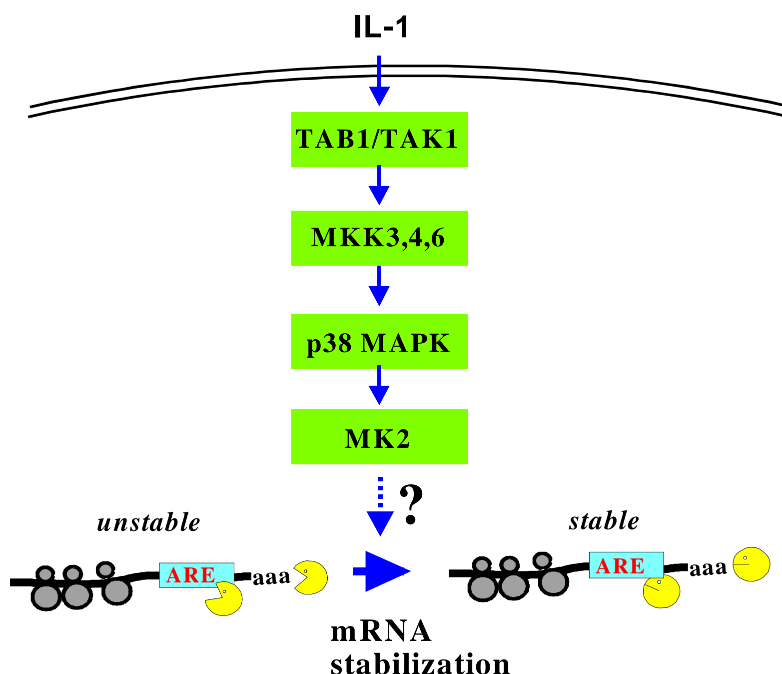


Figure 5: Scheme showing the role of the p38 MAPK/MK2 pathway in the stabilization of ARE-containing mRNAs.

p38 MAPK and its downstream substrate, MK2 have been reported to phosphorylate the ARE-BP tristetraprolin (TTP), yet the effect of this kinases on TTP activity is controversial *(Carballo E 2001)*, *(Mahtani KR 2001)*, *(Zhu W 2001)*. It was observed that phosphorylation promotes the nuclear export of TTP, an effect that is partly dependant on 14-3-3 proteins *(Johnson BA 2002)*. 14-3-3 proteins, by interacting with phospho-serine residues, are associated with a large number of partner proteins and thus influence a wide range of cellular processes *(Tzivion G 2001)*. Recently it was reported by Stoecklin et al that MK-2 induced phosphorylation of TTP at serines 52 and 178 promoted the assembly of TTP: 14-3-3 complexes and that this binding inhibits the degradation of ARE-containing transcripts *(Stoecklin G 2004)*.

Other data from Ming et al favor a model in which TTP promotes the destabilization of ARE-containing mRNA (in this case IL-3 mRNA) under resting conditions, and that HuR and other yet-to-be-identified ARE-BPs under the influence of specific signalling pathways (in this case PI3-K and p38 MAPK) overcome the TTP-mediated decay *(Ming XF 2001)*. While HuR itself is not a phosphoprotein, a critical remaining question is the mechanism by which HuR

exerts its stabilizing influence on labile mRNAs. Since HuR is predominantly localized in the nucleus, it has been proposed that the mRNA-stabilizing influence of HuR requires its translocation to the cytoplasm *(Atasoy U 1998), (Fan XC 1998), (Wang W 2000)*. An examination of signalling events that control HuR subcellular localization have uncovered the critical participation of the AMP-activated kinase (AMPK), also known as cellular sensor of metabolic stress *(Wang W 2002)*, the ERK pathway *(Yang X 2004)* and the p38 pathway itself *(Atasoy U 2003), (Tran H 2003).*

Other proteins that have shown to be phosphorylated by MK2 and selectively interact with AREs include hnRNP A0 *(Rousseau S 2002)* and PABP1 *(Bollig F 2003)*. Inspite of the expanding list of ARE-BPs that are being characterised, it remains to be seen how exactly the p38 MAPK/MK2 pathway regulates the stability of ARE-containing mRNAs.

2.4 UV LIGHT AND mRNA STABILITY: In addition to physiological stimuli, many adverse agents such as carcinogens, metal toxins, oxidants and radiation can induce signal transduction cascades and changes in gene expression. Ultraviolet (UV) radiation of various wavelengths has received much attention. The human population is intermittently yet chronically exposed to ultraviolet radiation (UVR) from the sun. UVR is primarily responsible for more than 1,000,000 cutaneous malignancies each year in the USA alone *(Miller DL 1994)*, making it the most efficient environmental carcinogen known. Acute and chronic effects of UVR on skin include inflammation, hyperpigmentation, hyperplasia and skin cancer *(Norris PG 1993), (Young AR 1993), (Gilchrest BA 1999), (Gilchrest BA 1996)*. The biological effects of UVR have been mainly attributed to the UVB (280-320nm) fraction, while the potentially dangerous UVC (100-290nm) radiations are absorbed by the ozone layer *(Madronich S 1998)*. UV-A (320-400nm) effects are primarily oxidative in nature.

Studies have shown that irradiating mammalian cells with UV-B light leads to transcriptional activation of immediate early genes such as *c fos* and *c jun* *(Devary Y 1991), (Buscher M 1988)*. This UV response is triggered through signal transduction, which originates from one of several primary UV target molecules: DNA lesions in the form of pyrimidine dimers and 6-4 photoproducts introduced through irradiation *(Blattner C 1998), (Schorpp M 1984), (Patrick 1977)*, through damaged ribosomal RNA *(Iordanov MS 1998)*, through the release of reactive oxygen species (ROS) *(Peus D 1999), (Peus D 2001), (Peus D 1999), (Klotz LO 2001)*, or through the inactivation of oxidation-sensitive protein tyrosine phosphotases *(Herrlich P 2000), (Knebel A 1996)*. UV irradiation activates several signal transduction

pathways including ERK, JNK and the p38 MAPK pathways *(Tyrrell 1996), (Bender K 1997)*.

While much is known about the transcriptional response to UV light, other levels of gene expression have, as yet, received less attention. Stabilization of protein is responsible for the UV-DNA lesion-induced increases in p53 and E2F-1 levels *(Blattner C 1999), (Blattner C 1999), (Maltzman W 1984)*. Other results indicate an increase in p53 translation following UV-C irradiation via binding of HuR *(Mazan-Mamczarz K 2003)*. In addition it was shown that UV irradiation induces phosphorylation of eukaryotic translation initiation factor 2 on the alpha-subunit (eIF2α) and inhibits protein synthesis in a dosage- and time-dependent manner *(Wu S 2002)*. Recently it has also been demonstrated that as a consequence of the abortive translation initiation that accompanies exposure to environmental stresses including UV irradiation, those mRNAs whose translation is aborted are routed to specific cytoplasmic foci referred to as Stress Granules (SGs) *(Kedersha N 2002), (Kedersha N 2000), (Kedersha N 1999)*. It has been proposed that these SGs could be sites of mRNA triage, where the fates of specific mRNA transcripts are determined by the activity of different RNA-binding proteins like HuR and TTP *(Stoecklin G 2004).*

Not much has been reported about the direct effects of UV light on mRNA stability. Blattner et al showed UV-C induced stabilization of *c fos* and other short-lived mRNA like *c jun*, *c myc*, IκBα and Kin17 *(Blattner C 2000)*. Similarly Wang et al reported the UV-C mediated stabilization of p21 mRNA by HuR *(Wang W 2000).* Our group showed that UV-B could induce stabilization of several short-lived reporter mRNAs-both ARE and non-ARE transcripts in a p38 MAPK independent manner *(Bollig F 2002)*.

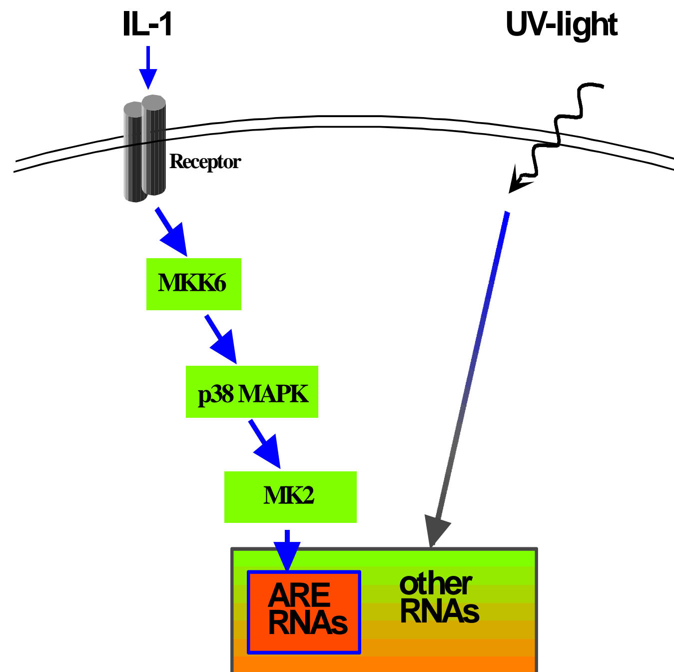


Figure 6: Scheme showing differences between UV mediated mRNA stabilization and the p38 MAPK/MK2 pathway mediated stabilization of ARE-containing RNAs

UV light is a potent inducer of inflammation like LPS and IL-1 and induces the expression of numerous cytokines and oncogenes *(Tyrrell 1996), (Herrlich P 1997)*. We believe that a part of this induction could be a result of mRNA stabilization. This idea has been reinforced by a recent report that reveals that mRNA stabilization and destabilization significantly influenced the expression of approximately 53% of stress-regulated genes out of a total of 1,152 genes examined using a cDNA array *(Fan J 2002)*.

2. 5 AIMS OF THE STUDY: The aim of this study was to obtain a deeper understanding of different modes of regulated mRNA turnover and their relationship to each other. Although much work has been done defining and characterizing the AU-rich elements, their mechanism of action is still unclear. The aim of the first part of the study was to identify sequence features of AREs that determine their destabilizing activity and their responsiveness to p38 MAPK/MK2 pathway induced stabilization using the IL-8 AU-rich element as the experimental model. The stabilization of ARE-containing RNA by the p38 MAPK/MK2 pathway could be effected via an inhibition of any one/more of the processes involved in mRNA degradation including deadenylation, decapping or exonucleolytic decay of the mRNA body. The aim of the second part of the study was to examine the effects of p38

MAPK activation on deadenylation, the first stage in the mRNA degradation process, and to thereby improve our understanding of the mechanism of action of the p38 MAPK/MK2 dependant stabilization of ARE-containing RNA. UV light stabilizes short-lived ARE- and non-ARE containing reporter transcripts in a p38 MAPK/MK2 pathway independent manner. The aim of the last part of the study was to similarly characterize the mechanism behind this stabilization by examining effects of UV light on deadenylation and to identify the signalling pathway/s that are involved in this effect of UV light.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Labware:

Plasticware, disposable material- including 2ml, 5ml and 10ml pipettes, tips and eppendorfs, and petri dishes for cell culture, cell culture flasks etc., were bought from the firms- *Eppendorf, Sarstedt, Greiner* and *Nunc.*

3.1.2 Equipment:

- **Electrophoresis-, Western blot- and Photographic systems:**
  Biometra -BioDoc Analyze UV transilluminator
  Biorad-Gel Doc 1000 System
  AGFA- Duoscan (scanner)
  Amersham Pharmacia Biotech- Electrophoresis Power supply- EPS301
  Amersham Pharmacia Biotech- Electrophoresis Power supply- EPS300
  GIBCO BRL Life technologies- Electrophoresis Power supply- ST305
  Hoefer Scientific Instruments- Mighty Small II SE250- chamber for PAGE
  SIGMA- Model Z37, 507-1-electophoresis chamber for big SDS PAGE gels.
  Peqlab biotechnologie- Semi-dry electroblotter
- **Microscope and Spectrophotometer:**
  Zeiss- Axiovert 40 CFL (fluoroscent microscope)
  Leica- DMIL (light microscope)
  Dynatech- MR6500 ELISA reader
  Shimadzu- Spectrophotometer UV-160A
  Pharmacia- Pharmacia LKB Ultrospec III-spectrophotometer
- **Assorted equipement:**
  Eppendorf- thermomixer 5436
  Roth- test tube thermostat TCR 100
  GFL- (-80ºC)
  Liebherr- Glassline (4ºC and -20ºC)
  LTF labortechnik-Stratolinker
  Omnilab- Heidolph MR 2002, MR 3001K-magnetic stirrers
  Omnilab- REAX 2000-vortex
  Bender and Hobein- Genie 2-vortex
  Samsung- M187DN-microwave

Lauda- E100-water bath with shaking

B.Braun Biotech international- horizontal shaker

Landgraf- thermocycler

Perkin Elmer- ABI PRISM 310 Genetic Analyzer (DNA sequencer)

Protec Optimax- Film Developer

Ziegra- Icemachine

Schott- CG840B- pH meter

- **Hybridization ovens:**

  MWG- Hybaid

  G.Kisker- Hybridization oven, Model 6/12

- **Weighing scales:**

  Omnilab- OL110-A

- **Bacterial culture:**

  New Brunswick scientific- Innova 4230 (Refrigerated incubator shaker)

  Shel lab- Incubator shaker

- **Cell culture:**

  Forma Scientific-CO_2 water-jacketed incubator

  Grant OLS 200- water bath in cell culture

  Heraeus instrumnts- 222 Hera safe (laminar hood)

- **Centrifuges:**

  Heraeus instruments- Labofuge 400R (centrifuge)

  Sigma- laboratory centrifuge 3K30

  Sigma- tabletop centrifuge 1-15

  Eppendorf- tabletop centrifuge 5415C

  Sorvall- RC5C

3.1.3 Chemicals and Solutions: All chemicals-powdered and solutions-, analytical grade or cell culture tested, were obtained from *Fluka*, *Merck*, *Serva*, *SIGMA-Aldrich*, *BIOMOL*, *ROTH* and *J.T.Baker*.

3.1.4 Enzymes and Enzyme Buffers: All the enzymes used in the course of the study were obtained from *Invitrogen*, *MBI Fermentas*, *Roche*, and *Amersham Pharmacia*.

3.1.5 Buffers and Solutions:

Loading Buffer (DNA-, RNA- Gel electrophoresis)

50	% (v/v)	Glycerol
0.4	% (w/v)	Bromphenolblue
1	mM	EDTA pH 8,0

10X Detection solution (Northerns)

0.1	M	Tris-HCl
0.1	M	Sodium Chloride (NaCl)

pH set to 9.5

2X HEBS

16.4	g/l	NaCl
11.9	g/l	HEPES
0.21	g/l	Disodium hydrogen phosphate (Na_2HPO_4)

pH set to 7.12

Hybridization solution (for DIG based Northerns)

10.0	ml	Formaldehyde
5	ml	20 x SSPE
4	ml	10 x Blocking solution
0.2	ml	10% (w/v) N-Laurylsarcosine
0.02	ml	20% (w/v) SDS
.760	ml	H_2O

2X Maleic acid solution

0.1	M	Maleic acid
200	mM	NaCl

pH set to 7.5

10X MOPS

200	mM	3-(N-Morpholino)propanesulphonic acid
50	mM	Sodium acetate
10	mM	EDTA

pH set to 7.0

20X SSC

3	M	NaCl
300	mM	Tri-sodium citrate

pH set to 7.0

20X SSPE

3	M	NaCl
200	mM	Sodium dihydrogen phosphate (NaH_2PO_4)
20	mM	EDTA

pH set to 7.4

50X TAE

2	M	Tris
0,1	M	EDTA

pH set to 8.0 using acetic acid

TSS solution (bacterial transformation)

10	% (w/v)	Polyethylenglykol 6000
5	% (v/v)	dimethylsulfoxide (DMSO)
50	mM	Magnesium sulphate ($MgSO_4$)

in LB-Medium

Extraction Buffer (for mammalian genomic DNA preparation)

10	mM	Tris-HCl (pH 8.0)
0.1	mM	EDTA (pH 8.0)
0.5%	v/v	SDS

3.1.6 Ready-to-use buffers and solutions:

Dulbecco's modified eagle medium (DMEM)- *PAA*

Fetal Calf serum- *PAN*

L-Glutamine-*PAA*

Penicillin/Streptomycin-*PAA*

PBS-*PAA*

Trypsin-EDTA-*PAA*

Phenol/Chloroform/Isoamylalcohol- *ROTH*

1M Tris-HCl (pH from 7.2 to 8.0)- *Sigma*

3M Sodium acetate (pH 5.2)- *Sigma*

30% Acrylamide-bis acrylamide solution-*ROTH*

3.1.7 Inhibitors :

Actinomycin D -*Sigma*

Doxycycline -*Sigma*

Tetracycline -*Sigma*

SB203580 -*Calbiochem*

3.1.8 Other materials and substances:

Hybond N Membrane- *Amersham*

Immobilon PVDF membrane- *Millipore*

Neubauer cytometer- *Superior Marienfeld*

X ray film cassettes- *Applied Gene Technology Systems*

X ray films- *Kodak*

CSPD- *Roche diagnostics*

DIG-11-UTP- *Roche diagnostics*

Filter paper 3MM- *Whatmann*

3.1.9 Primers:

All primers were ordered from the company *MWG Biotech.*

Primers for site-directed mutagenesis of the ptetBBB and ptetBBB GMCSF vectors:

bG-Amut-Ava/1675sense:

5'-GAGCATCTGACTTCTGGCTAATGCATGAAATTTATTTTCATTGC-3'

bG-Amut-Ava/1718antisense:

5'-GCAATGAAAATAAATTTCATGCATTAGCCAGAAGTCAGATGCTC-3'

Primers for amplification of the 3'UTR of the human histone H1.3 gene:

hHis1-3Ava/1669sense:

5'-CCAATGCATGGCGGGACGTTCCCCT-3'

hHis1-3AvaNot/2638antisense:

5'-CCAATGCATGCGGCCGCGAGCCCCTGGGAAAATAAG-3'

Sequencing primer:

ptetBBB/s1563:

5'-GCAGGCTGCCTATCAGAAGG-3'

3.1.10 Bacterial strains:

- *E.coli* K12 LK111λ
- *E.coli* JM109
- *E.coli* XL1 blue

3.1.11 Cell lines:

- HeLa — ATCC CCL2 epithelial cervical carcinoma
- HeLa- tTa — HeLa cells stably transfected with the tetracycline sensitive Transactivator *(Gossen M 1992)*
- HaCaT — A spontaneously transformed human epithelial cell line from adult skin *(Boukamp P 1988)*. An aliquot of frozen cells were obtained from Dr. Detlef Neumann, Institute of Molecular Pharmacology, Medical School, Hannover.

3.1.12 Plasmids:

- ptetBBB — *(Xu N 1998)*
- ptetBBB GMCSF — *(Xu N 1998)*
- ptetBBB *c fos* — *(Winzen R 1999)*
- ptetBBB IL8 (972-1310) — *(Winzen R 1999)*
- ptet myc-HuR — a kind gift of Dr. A. B. Shyu, Houston, USA
- pCDNA3 $MKK6_{2E}$ — *(Winzen R 1999)*
- pCDNA3 $MK2_{K76R}$ — a kind gift of Dr. M. Gaestel, Hannover, Germany
- pCDNA3 hcatalase — a kind gift of Dr. S. Lortz, Hannover, Germany

- pUHD10 CAT-TIMP1 *(Bollig F 2002)*
- pEGFP-C1 Clontech

3.1.13 Kits:

- QuickChange^{TM} kit for site directed mutagenesis from *Stratagene.*
- Nucleospin RNA II (250 preps) for RNA isolation from *Macherey Nagel*
- Nucleospin A_x for plasmid maxi-preps from *Macherey Nagel*
- GFX^{TM} for plasmid mini-preps from *Amersham Biosciences*

3.2 METHODS

3.2.1 Cell culture:

3.2.1.1 Passaging and Handling: All cell culture techniques were performed under sterile conditions in the hood. Only disposable plastic ware-pipettes, 50ml falcon tubes, petri dishes, and culture flasks were used. The medium that was predominantly used for the culturing was Dulbecco's modified eagle medium (DMEM). Fetal calf serum (FCS), to a final concentration of 5% or 10%, L-Glutamine, penicillin and streptomycin were added to usually 500ml of the ready-to-use DMEM medium just before use. This medium was then stored at 4°C for some weeks. The medium was always prewarmed to 37°C before use. Similarly the 1XPBS prepared from the 10X stock, and the 1X trypsin-EDTA solution were also stored at 4°C, and prewarmed to 37°C before use. The cells were normally cultured in medium supplemented with 5% FCS. For passaging, the cells were allowed to reach confluence, and then the medium was discarded. The cells were washed carefully with warm PBS twice. Then an appropriate amount of trypsin-EDTA (according to the size of the culture flask or petri dish) was added to the cells. The cells were then incubated at 37°C for 2-3mins until they detached from the surface of the culture vessel. Fresh medium was immediately added to the cells, and the cells were split into an appropriate number of culture flasks according to the need and the period before the next passage.

3.2.1.2 Counting the cells: The cell count was determined using the Hemocytometer (Neubauer Chamber). An aliquot of the cell suspension obtained after trypsinizing the cells was diluted 1:1 with Trypan Blue (0.8% v/v in PBS). Trypan Blue is a cell permeable dye; while living cells are able to extrude the dye, dead cells are unable to do so and hence stain blue. To obtain an accurate count, the cells have to be uniformly distributed over the entire

chamber. The chamber is divided into 9 squares. Each square has a surface area of 1sq.mm and the depth of the chamber is 0.1mm. To get the final count in cells/ml, first divide the total count by 0.1mm (chamber depth) then divide the result by the total surface area counted.

3.2.1.3 Freezing and thawing cells: Cells that are to be frozen are cultured until they reach confluence. One $75cm^2$ flask of cells was prepared for one freezing ampoule. Each freezing ampoule was labeled with the name of the cell line and the date of freezing. The freezing medium contains:

70% growth medium
10% DMSO
20% FCS

The cells were trypsinized, and spun down by centrifugation. They were then resupended in the appropriate amount of ice cold freezing medium (1ml per ampoule) and transferred to the ampoule, which was then placed in a freezing box. The box was left at -80ºC for at least 24hrs. Thereafter the ampoules can be transferred to storage in liquid nitrogen.

For thawing cells, an ampoule was taken from the liquid nitrogen and allowed to thaw in warm water until only a small piece of ice is left. The cell suspension was transferred to a 15ml centrifuge tube. 1ml of cold FCS was added drop-wise to the cells, with gentle mixing. 10ml of growth medium containing 15% FCS was then added to the cells in portions with gentle mixing. The cells were spun down, and the cell pellet was resuspended in 1ml of the growth medium containing 15% FCS, after which the cell suspension was transferred to $75cm^2$ flask containing 25ml of the same growth medium and allowed to grow at the appropriate growth temperature until they reach confluence.

3.2.1.4 Transient transfections: Transient transfections of the HeLa tTA cells were done using the Calcium chloride-HEBS method. In brief, appropriate numbers of cells were seeded in petri dishes (4×10^6 for a dish 9cm in diameter) the day before the transfection was to be done, such that the cells reach a state of sub-confluence on the day of the transfection. The next day the medium in the dishes was replaced with fresh medium containing 10%FCS atleast an hour before the actual transfection was to be done. 250mM calcium chloride solution was prepared fresh from the 2.5M stock solutions. Then equal volumes of the 250mM calcium chloride solution and 2X HEBS solution (pH 7.12, set at RT) were tested against each other in a small glass tube for the formation of a thin white precipitate at the interface of the two solutions, taking care to add the prewarmed HEBS drop by drop along the

sides of the tube. Now the required amounts of the plasmids (30µg for a 9cm dish) to be transfected were added to the appropriate amounts of the calcium chloride solutions aliquoted in the glass tubes. Then equal volumes of the 2X HEBS solution were added individually to each tube, making sure a precipitate was formed in each case. Each mixture was allowed to stand at RT for 2mins, mixed well and then added to the cells to be transfected in the petri dishes. 4hrs after the transfection, a glycerol shock was given to the cells using a 10% glycerol solution prepared in warm DMEM. An appropriate amount of the 10% glycerol solution (2ml for a 9cm dish) was added to the cells, after removing the medium that was previously covering the cells. The glycerol-medium mixture was allowed to stand on the cells for 2mins. Then the glycerol was removed and the cells were washed twice with warm PBS and fresh medium added. 2hrs after the glycerol shock the cells were trypsinized and reseeded in parallel cultures as per the requirements for the assay to be carried out. Any assay that was to be done with the transfected cells was always done on the third day.

3.2.1.5 Preparation of lysates for RNA extraction: For the preparation of lysates, the transfected cells (about 24hrs after the transfection) were washed with cold PBS, and the appropriate amount of the RNA lysis buffer (from *Macherey Nagel*) was added to the cells, the lysates frozen in liquid nitrogen and stored at -80°C.

3.2.2 Bacterial transformations and cloning:

3.2.2.1 Preparation of competent cells: 3ml cultures of E.coli (LK111λ/JM109/XL1blue) were grown in LB overnight at 37ºC. The next day the 3ml cultures were inoculated into 100ml of LB each and allowed to grow at 37ºC in a shaker. In the meantime the transformation stop solution (TSS) was prepared fresh (ref section 3.1.5 for composition). When the OD_{600nm} reached 0.3-0.4, the cultures were spun down at 2000 RCF(g), 4ºC for 10mins. To each pellet 1/10 the volume of 1XTSS (10ml for a 100ml culture) was added, the pellets resuspended in the same and incubated on ice for 2mins. Then 200µl aliquots were made and stored at -80ºC.

3.2.2.2 Bacterial transformation: The competent cells were first thawed on ice. The 200µl of competent cells were taken in special blue-capped tubes. 5ngs of the plasmid DNA (in the case of a control plasmid like bluescript or 5µl of the ligation mix) was mixed with the competent cells. The mixture was left on ice for 30mins. Then the mixture was heat shocked at 42ºC for exactly 45secs. Then the samples were placed for 2mins on ice. Now 800µl of LB without ampicillin was added to each sample and the cells were allowed to recover for 1hr at

37ºC in a shaker. Then the cells were spun down in an eppendorf, 800µl of the supernatant was discarded and the pellets were resuspended in the remaining 200µl of LB. The 200µl suspensions were spread plated on LB plates containing the appropriate antibiotic (100µg/ml of ampicillin for LK111λ). The next day the plates were checked for the presence of transformed colonies. The transformation efficiency is calculated as the number of colonies per µg of plasmid DNA transformed.

3.2.2.3 Preparation of glycerol stocks: 3ml liquid cultures of the bacterial clones that were to be stored were grown O/N at 37ºC. The next day, 200µl of 100% glycerol stock solution was pipetted out into the cryovials, which had already been labeled. 800µl of the O/N cultures were added to the glycerol and mixed well using a vortex. They were then immediately frozen in liquid nitrogen and stored thereafter at -80ºC.

3.2.2.4 Plasmid preparations: Mini plasmid preparations were done using the *GFX* (ref section 3.1.13) kit according to the manufacturer's instructions. Maxi-preparations were also done using the Nucleobond kit from *Macherey Nagel*, according to the manufacturer's instructions.

3.2.2.5 Quick check of clones: This is a simple and easy procedure to test the size of plasmids for the presence or absence of an insertion or deletion. 50µl of phenol-chloroform mix and 10µl of the DNA loading dye were aliquoted into microfuge tubes. 100µl of overnight *E.Coli* liquid cultures grown in LB with or without Ampicillin for selection were added to the tubes. The mixtures were vortexed well and spun for 3mins in a microcentrifuge at RT. 20-30µl of the supernatant were loaded into a conventional 0.6-0.8% agarose gel.

3.2.3 MOLECULAR BIOLOGY

3.2.3.1 Isolation of RNA from mammalian cells: RNAs were isolated from mammalian cells in culture using the Nucleospin kit from *Macherey Nagel* (ref section 3.1.13). The principle behind the isolation was very simple. Cells were lysed using the lysis buffer provided with the kit. The appropriate amount of the lysate was added to Shredder columns to homogenize the lysate. Freshly prepared 70% ethanol was then added to the homogenized lysates to bind the nucleic acids. This mixture was then added to Columns, which had silica gel matrices that bound the RNA from the lysates. After suitable washes to remove contaminating DNA and proteins, pure RNA was eluted in 30-50µl of RNase free water.

3.2.3.2 Mammalian Genomic DNA extraction: HeLa tTA cells were grown in a flask until they were confluent. The cells were washed twice with ice cold PBS. Then using a policeman, the cells were scraped into 500µl of PBS. The collected cells were transferred into a 50ml centrifuge tube. The flask was washed again with 1ml of PBS and the remnant cells added to the collected cells. The cells were pelleted down by centrifuging at 1000 RCF(g) for 10mins at 4ºC. The cell pellet was resuspended in 5ml of cold PBS and the centrifugation repeated. Now the cells were resuspended in 2ml of TE (pH 8.0), and aliquoted into two-50ml Erlenmeyer's flasks. Now 10ml of extraction buffer was added to each flask. Proteinase K was added to a final concentration of 0.1mg/ml to each flask and mixed well using a pipette tip. The flasks were now incubated at 50ºC for 3hrs. The lysed cell suspensions were now transferred into two-50ml centrifuge tubes. 12ml of phenol-chloroform mixture was added to the suspensions and centrifuged at 5000 RCF(g) for 15mins at RT. The organic phase was discarded and 11ml of chloroform was added and the centrifugation repeated. The organic phase was again discarded and the chloroform extraction repeated. The aqueous phase was transferred to a fresh tube. 5ml of 7.5M ammonium acetate and 20ml of absolute alcohol was added to each tube, the tubes swirled gently until a clear stringy precipitate forms in the tubes. Now using a 1ml pipette the precipitates were collected and transferred to a fresh tube, the remnant alcohol removed, and the DNA allowed to dry for about 1/2hr. The dried DNA pellets were resuspended in about 500µl of DNase free water and incubated O/N in a shaker at 37ºC. to allow the DNA to dissolve. The concentration of the DNA was measured spectrophotometrically and the quality of the DNA checked on an agarose gel.

3.2.3.3 Spectrophotometric estimation of the concentration of nucleic acids: The concentration of nucleic acids was measured spectrophotometrically by measuring their absorption at 260nm. The concentrations were calculated using the following standard values:

$1\ OD_{260nm}$ = 40µg RNA/ml

$1\ OD_{260nm}$ = 50µg DNA/ml

3.2.3.4 Agarose gel electrophoresis for the separation of nucleic acids: For analysis of DNA samples, usually 1% agarose gels were made. 1g of agarose was dissolved by heating in a microwave in 100ml of 1XTAE. After allowing the melted agarose solution to cool to about 65ºC, about 2µl of a concentrated stock solution of ethidium bromide was added, mixed gently and poured immediately into the previously set up gel platform. The gel was allowed to solidify at RT. The DNA samples were mixed with the DNA loading buffer and loaded onto the wells. Then the gel was run in the electrophoresis chamber using 1X TAE as the running

buffer at the required current conditions (50-100V for 45-90mins). Usually a standard DNA marker was also loaded to control for the quality of the gels as well as to help estimate the size of the DNA fragments being examined. The gel was then photographed in a Gel Doc.

3.2.3.5 RNA gels: The required amount of agarose, depending on the % required, was dissolved in deionized water by boiling. Then the required amounts of 10X MOPS solution and formaldehyde solution were added and the gel poured immediately into the gel platform.

Total gel volume	300ml
Deionized water	214.8ml
Agarose	3g (1%)
37% formaldehyde	55.2ml
10X MOPS	30ml

Prepare 5-20µg of total RNA in deionized water. A master mix of the loading buffer was prepared

10X MOPS	1.5µl*X
37% Formaldehyde	3.75µl*X
1mg/ml Ethidium bromide	1µl*X
RNA loading dye	8.75µl*X

Where X is the no. of samples. Equal amounts of the RNA sample and the loading buffer were mixed with each other. The mixtures were vortexed briefly, then heated to 65ºC for 15mins, spun down, mixed well once again and loaded onto the gel/s. The gels were run at 120V until the dye front migrated halfway down the gel.

3.2.3.6 Northern transfer: The RNA gels were visualized under a transilluminator and photographed, so that a record of the quality of the RNA and the quantity (equal loading) can be made. Then the gels were rinsed in distilled water two times, 30 mins each time to wash off the excess formaldehyde fumes. The gels were then rinsed in 10X SSC for about 20 mins and in the meantime the transfer apparatus set up, using a glass dish, 10X SSC and a whatmann wick. The gels were blotted onto 0.45µm nylon membranes. The membranes were cut to the sizes of the gels to be transferred, and soaked briefly in 10X SSC. The membranes were then placed over the gels that had previously been placed on the transfer apparatus,

taking care to roll out air pockets if any. This was covered by two pieces of 3MM whatmann paper once again cut to the size of the gels which had also been briefly soaked first in distilled water and then in 10X SSC, followed by a stack of paper towels/diapers, and a weight was placed over the whole set-up. The transfer was allowed to take place for about 20hrs. Then the membranes were baked for about 10mins at 80ºC, and crosslinked using either a UV transilluminator (1min 15secs) or simply using a crosslinker.

3.2.3.7 Hybridization of Northern blots: The blots were presoaked in 2X SSC. The blots were now incubated with the pre-hybridization solution at 68ºC for atleast 2hrs the volume used depending on the size of the blots. After the pre-hybridzation, the blots were incubated with the hybridization solution at 68ºC O/N.

Pre-hybridization/Hybridization solutions (40ml)

Formamide	20ml
20X SSPE	10ml
10X Blocking solution	8ml
N-Laurylsarcosine	0.4ml
20% SDS	0.04ml
deionized water	1.52ml
*DIG labeled as RNA	100-200ng/ml

* only for the hybridization solution.

The first time that the probe was dissolved in the hybridization solution it was heated to 95ºC for 5mins. Then the hybridization solution was stored at -80ºC. Thereafter the hybridization solution was thawed at 75ºC for 15mins just before use.

After the O/N hybridization the blots were first rinsed under high stringency conditions (2X SSC, 0.1% SDS) at RT two times, 5mins each time. Then the blots were washed under low stringency conditions (0.1X SSC, 0.1% SDS) two times at 68ºC, 15mins each time. The blots were now incubated with the blocking buffer for 30mins. Now the blots were incubated with the blocking buffer containing the anti DIG-AP (alkaline phosphatase) conjugated antibody at a dilution of 1: 10,000 for another 30mins. Now the blots were washed two times, 15mins each time with the DIG washing solution (ref section 3.1.5). The blots were washed briefly in the DIG detection solution (ref section 3.1.5). The blots were now incubated with the

substrate solution, CSPD for 5mins, then placed in the film cassettes, and are now ready for exposures. Exposures were made by placing X-ray films over the blots in the cassettes, and after a suitable exposure period, the films were developed using a developer.

3.2.3.8 Analysis of poly (A) tail length: RNA samples were mixed with an equal amount of loading buffer (80% v/v formamide and 1mg/ml bromophenol blue in 1X TBE), incubated at 65ºC for 15mins and loaded onto denaturing polyacrylamide gels (4% w/v acrylamide, 7M urea). Running in 1XTBE was performed at about 10V/cms for variable times depending on the size of the RNA monitored. Semidry-electroblotting at 200mA for 1hr with 1XTBE as buffer was used to transfer the RNAs to a nylon membrane for northern blot analysis. Deadenylated transcripts were prepared *in vitro* by mixing total RNA (10µg) with oligo dTs (0.5µg) in a buffer containing 200mM KCl and 1mM EDTA (pH 8.0). The sample was incubated at 90ºC for 2mins followed by annealing at 25ºC for 10mins. Then two volumes of a solution containing 200mM KCl, 1mM EDTA (pH 8.0), 20mM tris-HCl (pH 8.0), 28mM $MgCl_2$, 20 units of RNase inhibitor, and 1 unit of RNase H were added. The sample was incubated at 37ºC for 30mins and precipitated with ethanol.

3.2.3.9 Creating density profiles from northern blots: Density profiles can be made for any northern blot, using the Aida Image Analyzer v 3.51. The profile gives the distribution of the signal intensity over a defined distance. The software requires scanned images of the blots as input. Different parameters can be chosen for making the profiles eg. the profiles made in this study (fig 18, section 4.3.1) were made by examining the transmisson of light in the scanned image. The height and width of the lanes to be analyzed can be set, and the same dimensions are then used for all the lanes.

3.2.3.10 Preparation of DIG labeled as RNA probe: The DIG labeled UTPs (DIG-11-UTP) were incorporated into an antisense RNA probe spanning a region of the gene of interest, which was generated by *in vitro* transcription using a template generated by restriction digestion of a plasmid or a cDNA template containing the gene of interest under the control of a T7/T3/SP6 promoter.

Linearised plasmid template/	1µg
cDNA template from RT-PCR	200ngs
5X transcription buffer	4µl
10mM DIG- (NTP)- mix	2µl

T7 RNA polymerase	2µl (40 units)
Deionized water	to 20µl

The transcription was allowed to take place for 2hrs at 37ºC. 20 units of DNaseI was added and the mix incubated for 30mins at 37ºC. Then 50µl of the Lithium chloride precipitation solution was added followed by 80µl of deionized water and 150µl of cold isopropanol and the mixture incubated at -20ºC for atleast 30mins. Then the RNA was spun down at 15,000rpm, for 15mins at 4ºC. The RNA pellet was then washed with 70% ethanol once again at 15,000rpm for 15mins at 4ºC. The pellet was then air dried and resuspended in an adequate amount of RNase free water. The concentration of the transcribed RNA was measured and the quality of the RNA was then visualized in an agarose gel.

3.2.3.11 Molecular Biological techniques used for cloning:

3.2.3.11.1 Restriction Digestion: For any digestion reaction, the amounts of enzyme used or the duration of the incubation period would depend on the amount of plasmid DNA to be digested. An example of a typical digestion set-up is as is given below:

plasmid DNA	40µg
Enzyme buffer	30µl
Restriction enzyme	50 units
DD water	to 300µl

The reaction mixture was then incubated at a temperature usually 37ºC (with some exceptions for some enzymes) for 2-3 hrs. The digestion was checked by running the digested product with uncut controls of the same plasmid on a DNA agarose gel.

3.2.3.11.2 CIAP treatment and precipitation of the digested DNA: The digested DNA was then dephosphorylated (for cloning purposes) by treatment with Calf intestinal alkaline phosphatase (CIAP). Hence 5 units of CIAP was added to the previously digested DNA, and the mixture incubated for about 30mins at 37ºC. Now 300µl of phenol-chloroform was added to the reaction mix, and the mixture spun at 10,000rpm for 15mins at room temperature (RT). The upper aqueous phase was collected. To the organic phase 100µl of 10mM tris pH 7.6 was added and the mixture spun once again at 10,000rpm for 15mins at RT. The upper phase was collected and pooled with the previously collected aqueous phase. To the collected aqueous

phase 300µl of chloroform was added and the mixture spun down at 10,000rpm for 15mins at RT. Now the upper phase was once again collected. 30µl of 3M sodium acetate (pH 5.2) was added along with 1ml of absolute alcohol and the mixture incubated at -20ºC for at least 30mins. Now the mixture was spun down at 15,000rpm for 15mins at 4ºC. The supernatant was immediately discarded. To the pellet 400µl of isopropanol was added, and spun down again at 15,000 rpm for 15mins at 4ºC. The supernatant was discarded and the pellet washed one last time with about 400µl of 70% ethanol and finally air dried at 37ºC. The pellet was now resuspended in a specified amount of biochemically sterile water, depending on the amount of plasmid DNA initially taken-in this case about 60µl.

3.2.3.11.3 Gel extraction of the linearised vector: To the previously purified linearised vector DNA, an appropriate amount of the DNA loading buffer was added (30µl to 60µl of DNA). A 1% agarose gel was prepared and about 15µl of the Dye-DNA mixture was loaded in each of 6 wells. The gel was allowed to run until a good separation of the linearised vector from the other fragments resulting from the digestion was achieved. The gel was now visualized in a UV transilluminator and neat incisions were made using a scalpel, just behind the linearised vector, on the sides and just above. The gel was transferred back to the electrophoresis apparatus, which had been previously emptied off the buffer. Now a thin strip of preactivated DEAE (diethylaminoethyl) membrane was placed behind the cut piece of the gel in close contact with it. Another strip was placed above the cut piece. Now fresh buffer was poured into the electrophoresis chamber, and after ensuring that the gel is held tightly in place, with the help of a special device, the gel was allowed to run at 80V for exactly 13mins. Then the current was turned off, the buffer discarded and the strip of membrane behind the piece of gel was removed and placed in a clean eppendorf. About 400µl of the wash buffer containing 50mM tris pH 8.0 was added, and after pipetting up and down for a few minutes, the wash buffer was discarded and about 600µl of the elution buffer containing 50mM tris pH 8.0, 5mM EDTA and 1M sodium chloride was added to the membrane in the eppendorf. Now the eppendorf was incubated at 74ºC for 1 ½ hrs. Both the buffers had to be brought to RT from their storage at 4ºC before use. Now after the incubation 600µl cold isopropanol was added to a fresh eppendorf. The elution buffer containing the eluted vector DNA was added to this, and the mixture incubated -20ºC for ½hr. The tube was then spun at 15,000rpm for 15mins at 4ºC. The supernatant was discarded and the pellet was washed with 70% ethanol, air-dried and resuspended in an appropriate amount of DD H_2O.

3.2.3.11.4 Ligation: After the vectors were purified the next step was to determine the concentration of the vectors and compare it to the concentration of the inserts and then set up a ligation reaction. The ligation reactions were set up taking either a 1:1 or a 1:3 or a 1:5 ratio of molar concentrations of the vector: insert as follows:

Vector	50ngs
Insert	depends on size of insert
Ligation buffer	1µl
T4 DNA ligase	1.5µl (7.5 Weiss units)*
Deionized water	to 10µl

*0.01 Weiss units will ligate 1µg of DNA in 20mins

The reactions were incubated at 15ºC O/N. 5µl of the ligated mix was used for bacterial transformation.

3.2.3.11.5 RT-PCR: For the reverse transcription reaction, the following general protocol is followed;

Total RNA	1µg
5X RT buffer	4µl
0.1M DTT	2µl
10mM dNTPs	2µl
oligo(dT)s	1µl
Deionized DD H_2O	8.5µl
RNase inhibitor	1µl (40 units)
M-MLV reverse Transcriptase	0.5µl (100units)

The reaction mix was then incubated in the thermocycler at:

23ºC	10mins
37ºC	60mins
95ºC	5mins

For the PCR reaction the following general protocol was followed:

cDNA(RT product)	10µl
PCR buffer	9µl
50mM $MgCl_2$	1.8µl
Sense primer	10pmoles
antisense primer	10pmoles
deionized water	78µl
Taq polymerase	1µl (1.6units)

The reaction mix was overlayed with mineral oil before being placed in the Thermal cycler. For each PCR reaction the annealing temperature was chosen depending on the melting temperature of the primers. The normal program followed was as given below:

	95ºC	5mins
	85ºC	5mins (The Taq polymerase was added at this point)
40X	95ºC	1min
	55ºC	1sec (annealing temperature is varied)
	55ºC	1min
	72ºC	1sec
	72ºC	2min
	60ºC	7min
	4ºC	999min

3.2.3.11.6 PCR: Polymerase chain reactions are designed to amplify a DNA fragment of interest from the input template DNA, which could be either genomic DNA or plasmid DNA or cDNA from a reverse transcription. The general protocol for a simple PCR reaction would be as given below:

DNA template	10^6- 10^9 molecules
10X Taq polymerase buffer	10µl
50mM $MgCl_2$	3µl
100mM DTT	2µl

10mM dNTPs	2μl
sense primer	10pmoles
antisense primer	10pmoles
DD water	to 100μl
Taq polymerase	1μl (1.6units)

The reaction was overlayed with mineral oil and placed in the thermal cycler and the PCR conditions were in general as outlined above for the RT-PCR (section 3.2.3.10.5)

3.2.3.11.7 Site directed mutagenesis: The first step was to design appropriate primers with the desired mutations. Certain key points had to be remembered while designing the primers:

- the melting temperature (T_m) should be as high as 78ºC, to allow for the mismatching.
- It should be a 44mer
- two primers were designed- one for the sense strand and one for the antisense strands

Now the reaction mix was set up as follows:

Template DNA (plasmid)	5-50ngs
10X Pfu polymersase buffer	5μl
10mM dNTPs	1μl
sense primer	10pmoles
antisense primer	10pmoles
deionized water	to 50μl
Pfu polymerase	1μl (2.5units)

All the tubes were overlayed with 30μl of mineral oil. Then the tubes were placed in a thermal cycler and were subjected to the following PCR conditions:

	95ºC	30 secs
12X	95ºC	30 secs
	55ºC*	1 min
	68ºC	2 mins/ Kb of plasmid

The last three steps were carried out for 12 cycles.

* Annealing temperature varied depending on the primers

After the PCR, 25µl of PCR products from each reaction was taken in a separate eppendorf and 0.5µl (5units) of DpnI was added to each tube. DpnI has the ability to restrict only methylated DNA. Since the native template strand of the plasmids are methylated, this would leave only the mutated PCR generated plasmid DNA uncut. 3µl of the DpnI digested PCR products were used to transform LK111λ.

3.2.3.11.8 Sequencing: A typical sequencing reaction would include first setting up sequencing PCR.

Plasmid to be sequenced	0.3µg
Sequencing primer (sense)	8pmol
Deionized water	to 20µl
Sequencing premix	6µl

The reaction was overlayed with a small amount of mineral oil. The PCR conditions were as given below:

Denaturing	96ºC	30secs
Annealing	*	15secs
Extension	60ºC	4mins

* depends on the melting temperature of the primer. The PCR was done for 25 cycles. After the PCR, the probes were transferred to fresh eppendorfs without any mineral oil. All the steps that follow were done at room temperature and with HPLC purified water. To each probe 80µl of water, and 10µl of 3M sodium acetate (pH 5.2) and 250µl of 100% ethanol was added. The samples were spun down at 18,000 RCF(g) for 15mins at RT. The supernatants were removed. Now 250µl of freshly made 70% ethanol was added, and the samples were spun for 15mins at 18,000 RCF(g) at RT. The supernatants were removed, and the pellets were allowed to dry completely. Now 25µl of template suppression buffer was added, the tubes vortexed, spun briefly, incubated for 2mins at 95ºC, vortexed spun down again. The probes were kept on ice, transferred to sequencing tubes and placed in the sequencer.

3.2.3.12 DNA-*Microarray*-Analysis: The DNA-*Microarray*-analysis that has been done in this study was done in collaboration with PD Dr. Michael Kracht, Dr. Oliver Dittrich-Breiholz and Ms. Heike Schneider. The *Array* contains oligonucleotides complementary

to about 140 genes that have been chosen for their roles in inflammation and associated processes, after an intense survey of literature. To detect differentially expressed genes in two different mRNA populations, fluorescently labeled cRNA was generated from 5µg of total RNA. The total RNA for this purpose was prepared using the kit described under section 3.2.3.1 with an additional DNase I treatment. The labeled cRNA was then hybridized O/N at 42ºC to the *Array*. The following day the array was subjected to a series of SSC-stringency washes, dried and scanned using an Affymatrix 428 scanner. The resulting data was analyzed using a program provided by the company, *MWG Biotech and Biodiscovery*, with whose help the oligonucleotides in the array had been generated.

4 RESULTS

4.1 FEATURES OF AREs REQUIRED FOR mRNA DESTABILIZATION AND STABILIZATION BY THE p38 MAPK PATHWAY

AU rich sequences in the 3'UTR of many cytokine and proto-oncogene mRNAs have been identified as the *cis* elements responsible for their rapid degradation. The classical feature of AREs is the presence of the pentameric AUUUA motif. Based on the presence or absence of the pentamer, their number and organization, Chen and co-workers classified AREs into three groups (ref section 2.3.1). Although much work has been done, the salient features of a functional ARE have not been obvious especially those features of the AREs that are required for mRNA stabilization by the p38 MAP kinase/MK2 pathway. This question was addressed by investigating the AU rich sequences of the IL-8 mRNA.

IL-8 is a prototypic human chemokine; chemotactic for leukocytes and involved in physiological conditions like angiogenesis and inflammation.

972-atgttgtgaggacatgtggaagcactttaagtttttcatcataacataaattattttc
1031-aagtgtaacttattaacct**atttа**tt**atttа**tgt**atttatttа**agcatcaaatatttgtg
1091-caagaatttggaaaaatagaagatgaatcattgattgaatagttataaag
1141-atgttatagtaa**atttа**ttttattttagatattaaatgatgttttattagataaatttca
1201-atcagggtttttagattaaacaaacaaacaattgggtacccagttaaatt
1251-ttcatttcagatatacaacaaataatttttagtataagtacattattgtttatctgaaa-
1310

Figure 7: IL-8 mRNA organization. The 3'UTR of the IL-8 transcript contains several scattered AUUUA motifs, including a group of four (with two of them clustered), starting at position 1050, and one motif in an A- and U- rich environment starting at position 1153. A portion of the 3'UTR sequence is shown with the AUUUA (ATTTA, since it is a sequence from the genomic DNA) pentamers underlined. Genbank accession no. for IL-8 is Y00787.

A region in the 3'UTR of IL-8 RNA between nucleotides 972 and 1310 (fig 7), when inserted into the 3'UTR of a β-globin RNA, rendered the normally stable β-globin RNA unstable and

sensitive to stabilization by the p38 MAPK/MK2 pathway *(Winzen R 1999)*. The aim of the first part of the study was to define the IL-8 ARE and to identify its features required for destabilization and stabilization by the p38/MK2 pathway.

4.1.1 The tet-off system: The well-established tet-off system has been used to determine mRNA half-lives**.** In this system the gene of interest is cloned downstream of a tetracycline regulated promoter. These constructs are then transiently transfected into a cell line that constitutively expresses the tetracycline transactivator (tTA). The addition of tetracycline or its analog doxycycline stops transcription. Lysates are made at appropriate time intervals thereafter, RNAs prepared and examined in northern blots for half-life determination.

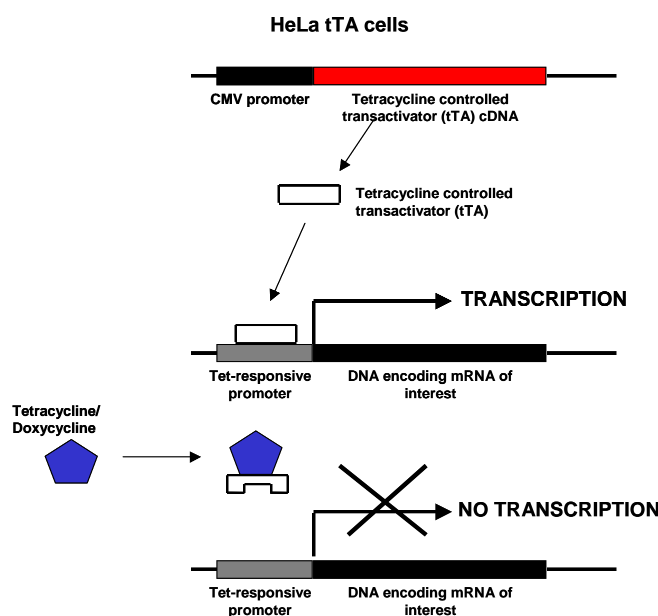


Figure 8: Schematic representation of the tet-off system: HeLa tTA cells obtained from Dr.Hermann Bujard, constitutively express the tetracycline controlled transactivator (tTA) *(Gossen M 1992)*. The transactivator (tTA) protein is a fusion between the tetracycline repressor (*tet*R) (from *E.coli*) and the activator domain of the *Herpes Simplex* transcription factor (VP16). The DNA of the reporter gene (as in our case β-globin) or another gene of interest (like the IL-8 DNA) is cloned under the control of a Tet-responsive promoter, which contains sequences for the binding of the tet repressor. The constructs are transfected in the HeLa tTA cells. In the absence of tetracycline or its analog doxycycline, tTA binds to and strongly activates the transcription of the reporter gene/gene of interest. In the presence of tetracycline/doxycycline the binding of the tTA to the promoter is abolished and transcription aborted.

4.1.2 Evidence for involvement of an ARE in controlling stability of the IL-8 mRNA:
Earlier results of the lab narrowed down the region in the 3'UTR of the IL-8 gene essential for the ARE mediated regulation to the region between nts 972-1132, using the tet-regulated β-globin mRNAs *(Winzen R 1999)*. The importance of this region was now confirmed using a full length IL-8 mRNA and deletion mutants therein. The full length IL-8 cDNA was expressed under a tetracycline-regulated promoter and the stability of the IL-8 mRNA monitored using the tet-off system. The half-life of the full length IL-8 mRNA was very short under the unstimulated control conditions (Figure 9, upper panel) and was markedly increased upon selective activation of the p38 MAP kinase pathway by cotransfection of pCDNA3 $MKK6_{2E}$, which expresses a constitutively active mutant of MKK6, the upstream activator of p38 MAPK. Deletions were then made from the 3'end of the IL-8 cDNA and the stability of the mRNAs examined. Deleting about 340nt from the 3'end had no affect on the regulation of the stability of the IL-8 mRNA (fig 9, middle panel). However deleting another 250nt, including a region encompassing four AUUUA motifs (nt1048-1076), resulted in the expression of an IL-8 mRNA with an extended half-life (fig 9, lower panel), which was unaffected by the activation of the p38 MAP kinase pathway. Hence the AUUUA- containing region appears to play an important role in destabilizing the IL-8 mRNA. As this IL-8 transcript is still only moderately stable, there may be other instability elements present further upstream. Most importantly the mRNA lacking the AU rich region had lost its responsiveness to p38 MAP kinase-induced stabilization.

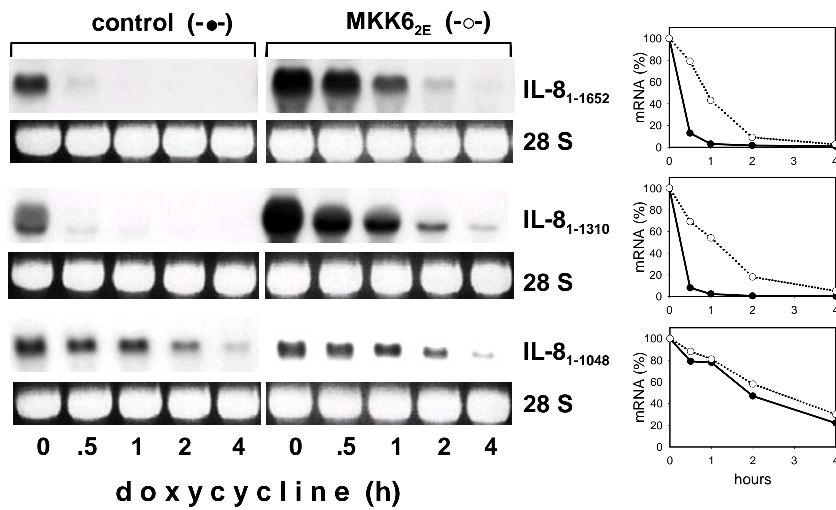
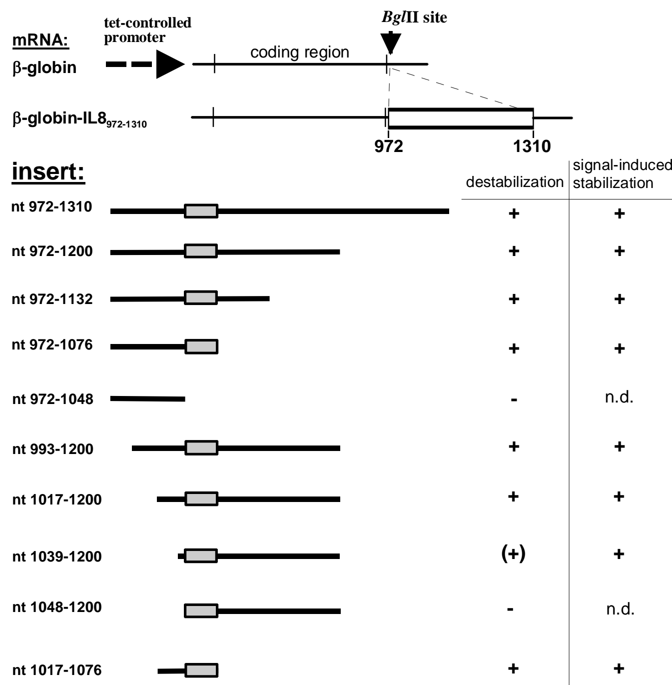


Figure 9: Evidence for involvement of an ARE in the regulation of IL-8 mRNA stability. HeLa-tTA cells were transfected with a vector expressing constitutively active MKK6 (MKK6$_{2E}$) or empty vector (control) and with tet-off plasmids encoding the complete IL-8 mRNA (IL-8$_{1-1652}$) and 3'-shortened forms with (IL-8$_{1-1310}$) or without (IL-8$_{1-1048}$) a region with four AUUUA motifs (see fig 7). Transcription was stopped by the addition of doxycycline (3µg/ml), lysates made at the indicated times, total RNA isolated and analyzed by northern blotting on 1% formaldehyde-agarose gels with an IL-8 antisense RNA probe. Ethidium bromide staining of the 28S rRNA is shown to allow comparison of the RNA amounts loaded. Results were quantified by a video analyzer system (amount of mRNA at the time of doxycycline addition [0 min] = 100%)

4.1.3 Identification of a 60nt regulatory ARE in the IL-8 3'UTR: Further localization of the regulatory ARE element in the IL-8 3'UTR was done by inserting fragments (generated by RT- PCR) shortened from both ends of the previously identified 160nt fragment (nt 972-1132) into the 3'UTR of the stable β-globin RNA, and the stability of the expressed mRNAs assayed under basal conditions and conditions where pCDNA3 $MKK6_{2E}$ had been co-transfected to activate the p38 MAPK pathway. The results from work done previously and those obtained during this study have been summarised (fig 10A) and show that destabilization was lost when shortening from the 3'end deleted the region containing the four AUUUA motifs (insertion of nt 972-1048). Shortening from the 5'end resulted in loss of regulation even before this region was reached. RNAs expressed from the β-globin reporter with an insert of nt 1017-1076 exhibited destabilization and $MKK6_{2E}$ induced stabilization comparable to the reporter with the insert nt 972-1310 (fig 10B, upper panel). Moreover expressing reporter RNAs in which this 60nt fragment between nt 1017-1076 had been deleted, increased the basal stability of these mRNA and led to loss of responsiveness to $MKK6_{2E}$, characteristics resembling those of a β-globin mRNA with no insertion (fig 10B, compare middle and lower panels). The minimal regulatory region within the 3'UTR of the IL-8 mRNA was thus identified as the region between nt 1017-1076.

A



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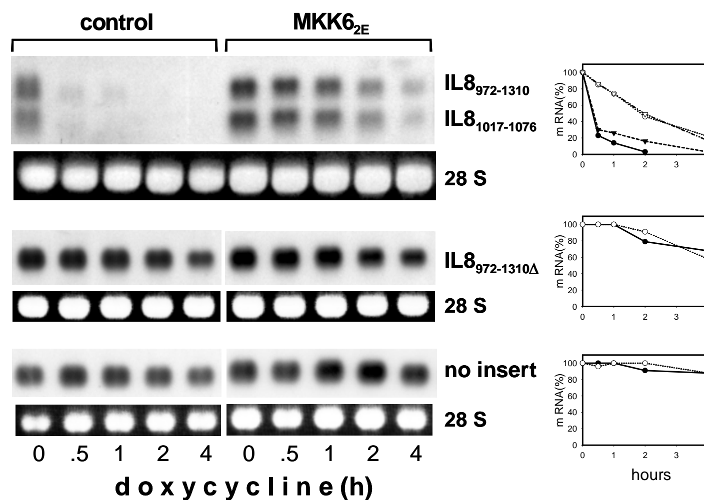


Figure 10: Localization of a regulatory ARE in the IL-8 mRNA. (A) Table summarizing results for basal decay and $MKK6_{2E}$-induced stabilization of β-globin-IL-8 hybrid mRNAs (n.d., not determined owing to a long basal half-life). RNAs were expressed from the plasmids ptetBBB containing the indicated IL-8 sequences inserted into the Bgl II site. The AUUUA-containing region is boxed. (B) Comparison of basal decay and $MKK6_{2E}$-induced stabilization was performed for β-globin mRNA without insertions or with IL-8 nt 972-1310, 1017-1076, or 972-1310 with nt 1017-1076 deleted (IL-$8_{972\text{-}1310\Delta}$). Decay kinetics were analyzed by using a β-globin antisense RNA probe and quantified as described in the legend to Fig. 9 (triangles, IL-$8_{972\text{-}1310}$; closed symbols, control; open symbols, $MKK6_{2E}$).

4.1.4 The IL-8 ARE consists of two functionally different domains, and the AUUUA-containing domain is sufficient for p38 MAP kinase-induced stabilization: Structurally in its 3' part, the nt 1017-1076 fragment has four AUUUA motifs, two of which overlap (fig 11A). The 5' part (nt 1017-1047) lacks AUUUA motifs. RNAs expressed from a construct containing only the 5'part (fig 10A, nt 972-1048) were stable, indicating that this region cannot destabilize on its own. This region was hence termed the auxiliary domain. Likewise expressing β-globin RNAs containing the 3'region with the AUUUA motifs, which we now call the core domain, resulted in very stable RNAs (fig 11B, upper panel). But because of the site of insertion in the β-globin construct, this fragment was located directly after the stop codon. There have been reports hinting at a possible effect of close proximity to the stop codon on the half-life of the expressed RNAs *(Xu N 1997).* To discount this possibility, RNAs were expressed from a construct in which a suitable spacing between the stop codon and the core domain was maintained, by inserting a random sequence identical in length to the auxiliary domain. The half-life of this RNA was examined. This mRNA (IL-8 R-CD)-in contrast to the one without spacing- clearly exhibited destabilization, although to a much lower extent as compared to that of RNA with the complete ARE (Fig 11B, compare IL-8 R-CD to IL-8 AD-CD). Thus the spacing between the stop codon and the destabilizing AU- rich sequence is apparently critical for the functioning of the ARE. Note that lack of destabilization of the RNA containing only the auxiliary domain (IL-8 nt 972-1048) was not spacing dependant, as in the case there was a distance of about 50nt between the auxiliary domain and the stop codon. Hence taken together, these results indicate that the 5' auxiliary domain does not destabilize on its own but enhances the moderate destabilization exerted by the core domain. Importantly activating the p38 MAP kinase cascade by co-transfecting pCDNA3 $MKK6_{2E}$, resulted in further stabilization of the RNA containing the random sequence plus the core domain (IL-8 R-CD Fig 11B). Thus, the AUUUA motif-containing core domain is sufficient to confer sensitivity to p38 MAP kinase-induced stabilization. Further, replacing every 10nt in the 5', central, or 3' part of the auxiliary domain with a 10nt random sequence indicates that the sequences most crucial for enhancing destabilization are located in the central part of the auxiliary domain (fig 11C).

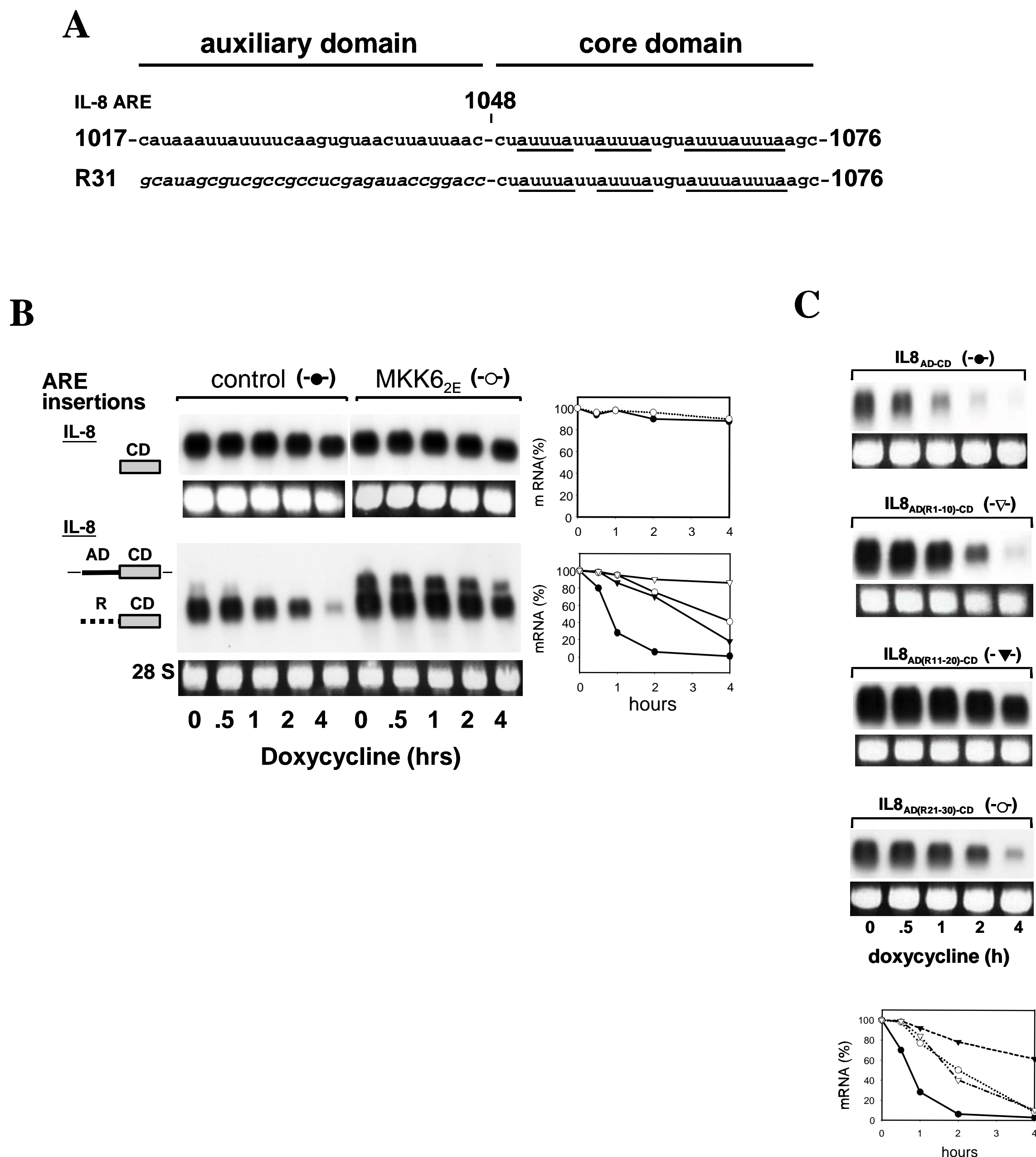


Figure 11: **Two-domain structure of the IL-8 ARE.** (A) Sequence of the IL-8 ARE as shown with AUUUA motifs underlined. The random sequence used to replace the auxiliary domain (R31) is shown in italics. (B) Basal decay and MKK6$_{2E}$-induced stabilization were analysed for β-globin mRNAs with domains of IL-8 AREs as indicated (CD, core domain; AD, auxiliary domain; R, random sequence; triangles IL-8 R-CD; closed symbol, control; open symbols MKK6$_{2E}$). (C) Destabilizing activity of the IL-8 ARE (IL-8$_{AD-CD}$) and derivatives in which the auxiliary domain was mutated by exchanging 10nt of its 5' part (IL-8$_{AD(R1-10)-CD}$), its central part (IL-8$_{AD(R11-20)-CD}$), or its 3' part (IL-8$_{AD(R21-30)-CD}$) with a random sequence (GCCGCCUCGA). mRNA stability was assayed and quantified as described in the legend to fig 9.

4.1.5 mRNA stabilization by HuR is selective and can be imposed by a distinct auxiliary domain: Experiments from the group show that an ARE binding protein, HuR interacts with both the IL-8 and GMCSF AREs in gel shift assays *(Winzen R 2004)*. It has been shown by several groups that HuR binds to and stabilizes ARE-containing RNAs (ref section 2.3.1.1.1). There have also been some reports claiming a role for HuR in p38 MAPK/MK2 mediated stabilization of ARE-containing RNA *(Atasoy U 2003), (Tran H 2003)*. Hence the role of HuR in the p38/MK2 pathway induced mRNA stabilization was investigated. Overexpression of myc-tagged HuR, in HeLa tTA cells cotransfected either with ptetBBB IL-$8_{1017\text{-}1076}$ (ref section 4.1.3), ptetBBB GMCSF or ptetBBB *c fos*, the latter two expressing β-globin RNA containing either the GMCSF ARE *(Winzen R 1999)* or the *c fos* ARE, revealed that degradation of the IL-8 ARE-containing RNA was not affected by overexpression of myc-tagged HuR (Fig 12A, upper panel). Whereas clear stabilization was observed for the RNA containing the ARE of GMCSF, and-in line with the observations of others *(Chen CY 2002)*- even stronger stabilization occurred for an RNA containing the *c fos* ARE (fig 12A, middle and lower panels). Thus the inability of HuR overexpression to stabilize an IL-8 ARE containing reporter suggested that HuR does not account for the p38 MAPK/MK2 effect on mRNA stability.

Since our own results and those of Shyu and co-workers *(Chen CY 1994)* indicate that all the above mentioned AREs have bipartite structures with auxiliary and core domains, we asked if the differences in HuR induced stabilization as opposed to p38/MK2 induced stabilization were dependant on particular domains. Constructs were made in which the auxiliary domain of the IL-8 ARE was replaced with those of the GMCSF ARE or the *c fos* ARE. The RNA containing a chimeric ARE with the auxiliary domain of the GMCSF ARE fused to the core domain of the IL-8 ARE was stabilized significantly by HuR overexpression (Fig 12B, upper panel). Even stronger stabilization was observed for the RNA containing the IL-8 core domain fused to the auxiliary domain of the *c fos* ARE (Fig 12B, lower panel). This domain lacks AUUUA motifs and was recently identified by Shyu and co-workers as the major domain responsible for HuR induced stabilization in the *c fos* ARE *(Chen CY 2002)*. Thus, unresponsiveness of the IL-8 ARE to HuR-induced stabilization results from the lack of a HuR-responsive auxiliary domain. These results show different sequence requirements for stabilization by HuR as opposed to that due to p38MAPK/MK2 activation.

This hypothesis was further substantiated by some co-transfection studies. RNAs containing the *c fos* auxiliary domain fused to the IL-8 ARE core domain were stabilized by the

expression of $MKK6_{2E}$ (Fig 12C). While this effect-expectedly- was inhibited by the co-expression of a dominant negative form of MK2 ($MK2_{K76R}$), stabilization by the overexpression of HuR was not. This rules out the possibility that MK2 is involved in the effect of HuR. This and the involvement of different ARE domains in each of the two effects indicate that stabilization of ARE-containing mRNAs by HuR overexpression and that by p38 MAP kinase activation occur via different mechanisms.

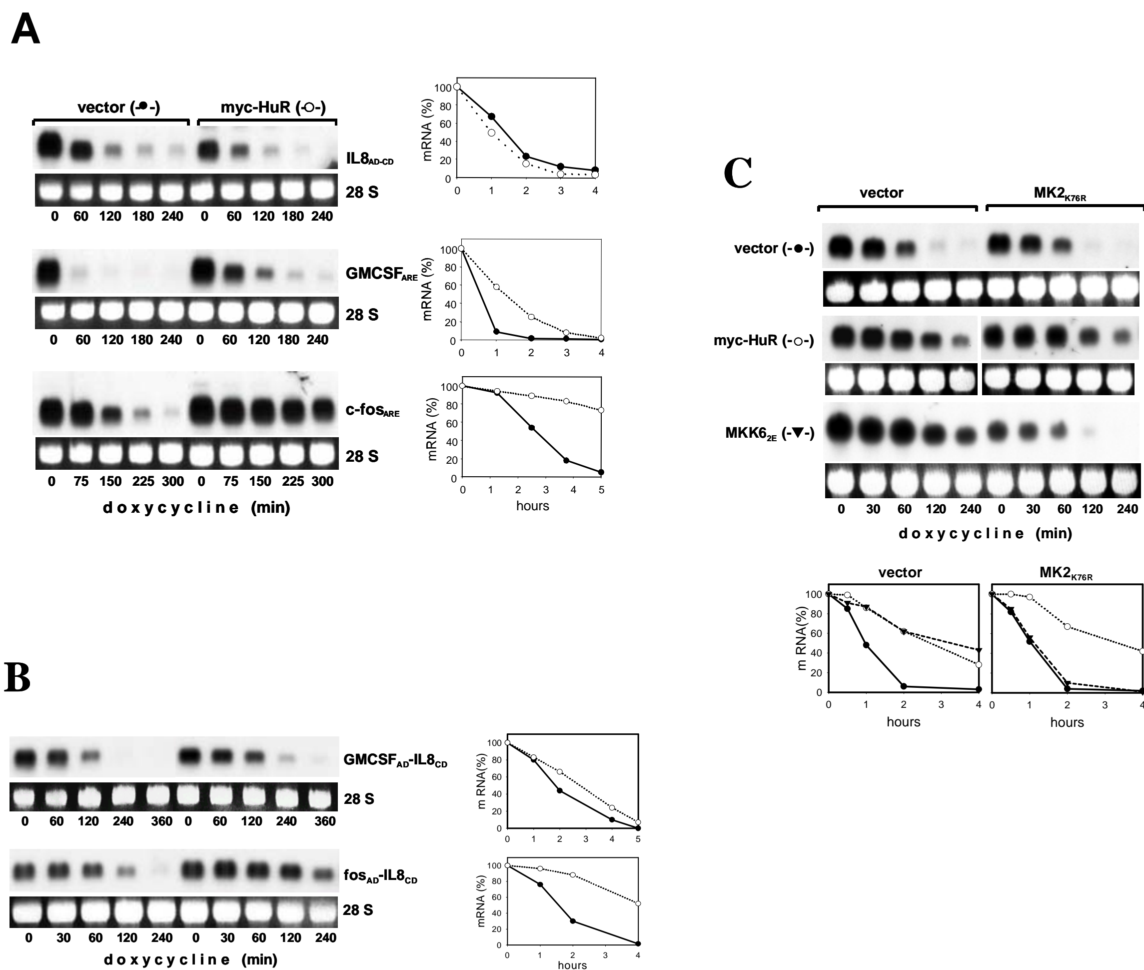


Figure 12: **mRNA stabilization by HuR is selective and can be imposed by auxiliary domains.** β-globin mRNAs containing the ARE of IL-8, GM-CSF or *c fos* (A) or chimeric AREs consisting of the core domains of the IL-8 ARE and the auxiliary domain of the GM-CSF or *c fos* AREs (GM-CSF$_{AD}$-IL-8$_{CD}$ or *fos*$_{AD}$-IL-8$_{CD}$, respectively) (B) were co-expressed with empty vector or myc-HuR as indicated. (C) Cells were transfected with plasmids encoding the β-globin – *fos*$_{AD}$-IL-8$_{CD}$ mRNA and myc-HuR or MKK6$_{2E}$ together with empty vector or an expression vector for MK2$_{K76R}$. mRNA stability was determined and quantified as described in the legend to fig 9.

4.2 ROLE OF THE POLY (A) TAIL IN mRNA DEGRADATION AND ITS REGULATION BY THE p38 MAPK PATHWAY

Deadenylation is considered the first step in the degradation of ARE-containing mRNAs (ref section 2.3.1). The p38/MK2 pathway can therefore stabilize ARE-containing RNA either by affecting deadenylation or by affecting later stages in the mRNA degradation machinery. In this part of the work, this question has been addressed.

4.2.1. Oligo(dT)/RNase H- Northern Assay: In order to determine differences in poly (A) tail lengths, the Tet-off system has been used in combination with a method involving RNase H digestion of total RNA to generate deadenylated reference RNA. RNase H has the ability to recognize RNA: DNA hybrids and cleave the RNA part of the hybrid. Total RNA prepared from eukaryotic cells is treated with RNase H in the presence or absence of oligo(dT)s. The oligo(dT)s hybridize to the poly (A) tail of the poly (A) tailed mRNA population, the RNase H recognizes these RNA: DNA hybrids and cleaves the RNA portion i.e the poly (A) tail leaving behind deadenylated RNAs (fig 13). These deadenylated RNA species prepared *in vitro* are compared to full-length mRNAs with poly (A) tails in a high-resolution gel. Total RNA when analyzed under these conditions and compared to the single band representing the deadenylated RNA marker, appears as a smear representing mRNAs captured at different stages of deadenylation. Hence the gradual disappearance of the longer polyadenylated species at the upper end of the smear can be followed over time.

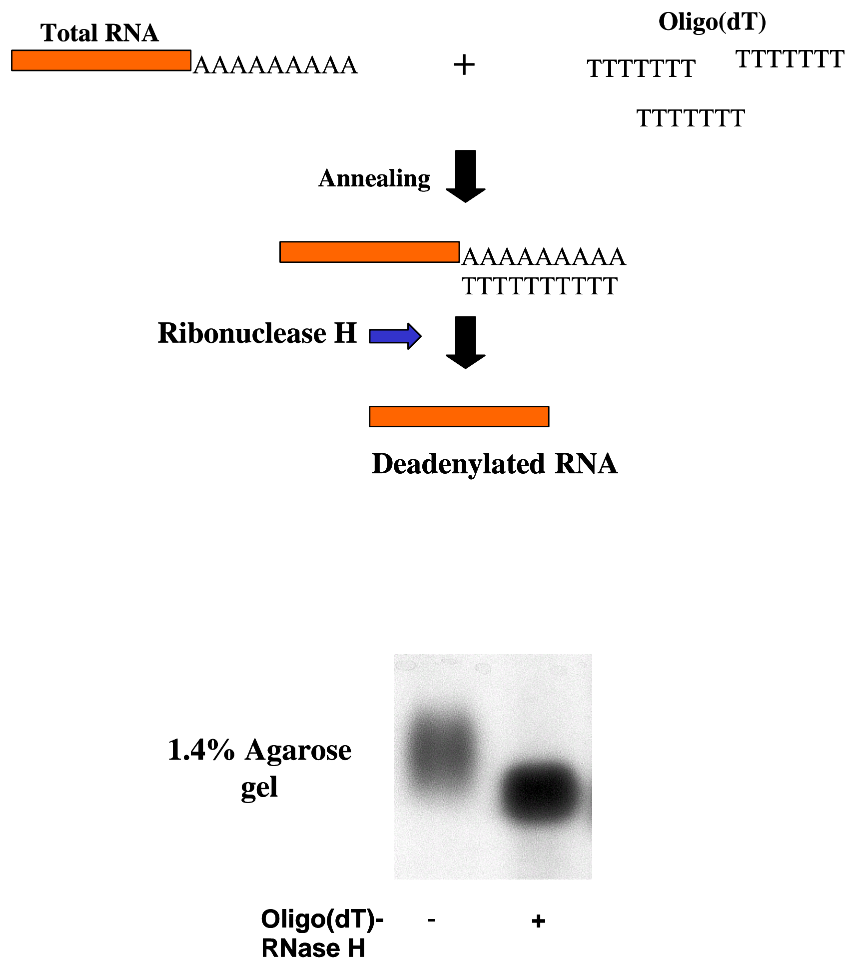


Figure 13: Schematic representation and an experimental example of the oligo (dT)-RNase H digestion/northern assay to compare poly (A) tail lengths. Total RNA from cells transfected with ptetBBB GMCSF was mixed with oligo(dT)'s and the annealing was carried out at 25ºC. Ribonuclease H (RNase H) was then added and the digestion done at 37ºC for 30mins, following which the digested RNA was precipitated and examined along with an aliquot of undigested RNA on 1.4% formaldehyde-agaorose gel by northern blotting with a β-globin antisense RNA probe.

4.2.2 Use of denaturing polyacrylamide gels allows a finer resolution of poly (A) tail lengths: Although the differences between polyadenylated and deadenylated RNAs were visible on the 1.4% agarose gels, the resolution was not sufficient to make statements concerning rates of deadenylation. Denaturing polyacrylamide gels have been used routinely in molecular biology to visualize short RNA fragments generated *in vitro*. There have been other reports where these systems have been used to observe differences in the polyadenylation state *(Decker CJ 1993)*, *(Salles FJ 1999)*.

A protocol was thus generated based on these reports and optimized to suit our requirements. 4% acrylamide/7M urea gels were used. The RNAs from the gels were blotted onto nylon membranes by semi-dry electroblotting. A more detailed version of the protocol can be found under the 'Methods' chapter (ref section 3.2.3.8). RNAs were prepared from HeLa tTA cells transfected with ptetBBB *c fos* and examined on denaturing acrylamide gels. The separation that was achieved using the acrylamide system was far better than that obtained using the agarose gels (fig 14).

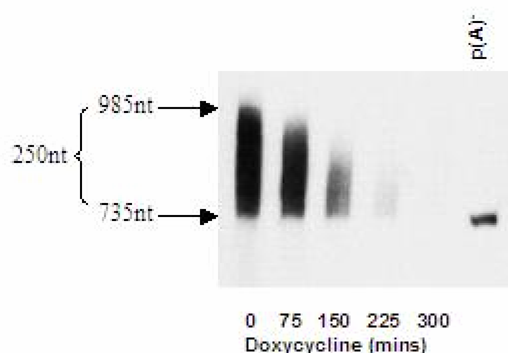


Figure 14: An experimental example of the resolution achieved using denaturing acrylamide gels. HeLa tTA cells were transfected with ptetBBB *c fos*. Transcription was stopped by the addition of doxycycline, lysates were prepared at the indicated time intervals, RNAs extracted and analyzed by northern blotting on 4%acrylamide/7M urea gels with a β-globin antisense RNA probe. Deadenylated standard prepared by RNase H treatment is shown ($p(A)^-$).

4.2.3 Activation of the p38 MAPK pathway inhibits the deadenylation of the IL-8 ARE-containing β-globin RNA: The high resolution denaturing poly acrylamide gels were then used to examine differences in the poly (A) tail length of the β-globin reporter RNA having the ARE-containing region of the IL-8 3'UTR (nt 972-1310) that has been defined in section 4.1.

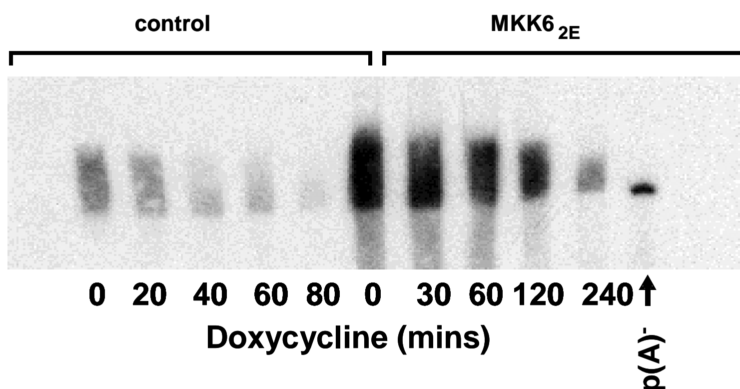


Figure 15: p38 MAPK activation inhibits the deadenylation and degradation of an IL-8 ARE-containing β-globin RNA. HeLa tTA cells were transfected with ptetBBB IL-$8_{972\text{-}1310}$ and cotransfected with either an empty vector (control) or with pCDNA3 $MKK6_{2E}$ ($MKK6_{2E}$). The next day transcription was stopped by the addition of doxycycline. Short time intervals of 20 mins were chosen for the unstimulated controls in contrast to the longer kinetics for the stimulated cells. RNAs were prepared, run alongside deadenylated BBB IL-$8_{972\text{-}1310}$ RNAs on the denaturing polyacrylamide gels ($p(A)^-$) and analyzed by northern blotting as described in the legend to fig 14.

The degradation and deadenylation of the IL-8 ARE-containing β-globin RNAs was much slower in the cells where the p38 MAP kinase pathway had been activated, taking into account the longer kinetics for lysate preparation in the latter (fig 15, compare the 80min time point in the left panel with the 240min in the right). Thus the activation of the p38 MAPK pathway appears to inhibit the rapid deadenylation of ARE-containing mRNA.

4.2.4 Loss of ARE-dependant regulation in the absence of a poly (A) tail: Another approach to investigating the importance of the poly (A) tail and associated proteins in ARE mediated mRNA degradation would be to generate ARE-containing RNAs without a poly (A) tail and then analyze ARE-dependant regulation of mRNA stability. Histone mRNAs are the only known eukaryotic mRNA that do not end in a poly (A) tail. Instead they end in a conserved stem loop that acts as a functional replacement of the poly (A) tail, participating in the export, stability and translatability of these mRNA. Hence the introduction of the histone stem loop (HSL) into the 3'UTR of the β-globin RNA in the plasmids ptetBBB and ptetBBB GMCSF would generate a functional RNA without a poly (A) tail, enabling us to look at mRNA degradation in the presence and absence of the poly (A) tail.

So the polyadenylation signals AAUAAA in the constructs ptetBBB and ptetBBB GMCSF were destroyed by site directed mutagenesis, simultaneously generating an Ava III restriction site (fig 16). A region of the histone H1.3 mRNA, that included the 15bp histone stem loop

(HSL), the histone downstream element- a purine-rich sequence 9-14nt 3' of the cleavage site and flanking sequences *(Cotten M 1988), (Mowry KL 1989), (Neu-Yilik G 2001)* was introduced into the 3'UTR of the β-globin RNA in the plasmids ptetBBB and ptetBBB GMCSF.

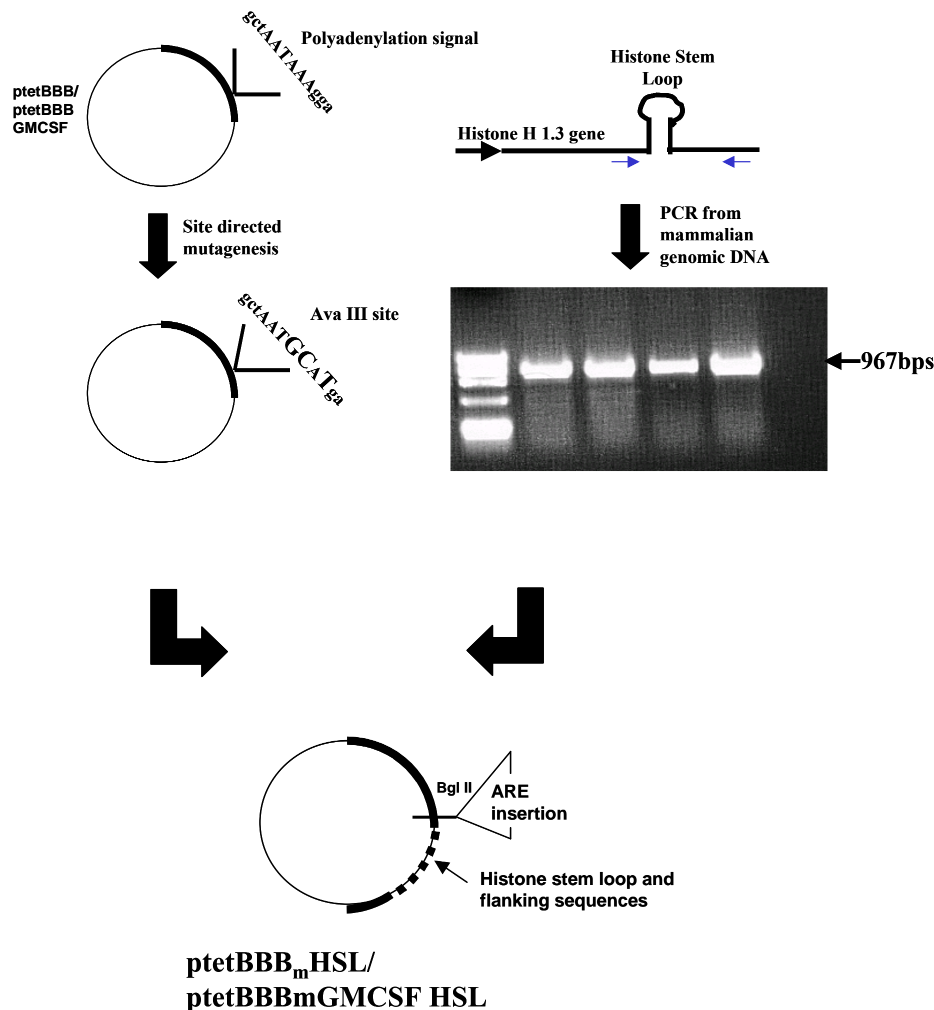


Figure 16: Cloning of the histone stem loop into ptetBBB and ptetBBB GMCSF. Two bases of the polyadenylation signals-the two adenines following the thymine (AATAAA) and the guanine following the signal in the plasmids, ptetBBB and ptetBBB GMCSF, were mutated to create an Ava III restriction site (ATGCAT) by site directed mutagenesis using the QuikChange™ kit from Stratagene. A 967bp fragment from the histone H1.3 gene was PCR amplified from genomic DNA isolated from HeLa cells and cloned into the newly generated Ava III sites in the plasmids ptetBBB and ptetBBB GMCSF to create the plasmids ptetBBB$_m$HSL and ptetBBB$_m$GMCSF HSL. The Bgl II site in the plasmid ptetBBB into which ARE sequences were inserted is about 70bp upstream from the Ava III site into which the Histone stem loop sequences were inserted.

The efficacy of mutating the polyadenylation signal was then tested by checking the RNAs expressed from these plasmids for the presence or absence of a poly (A) tail. It was clear from the results that the RNA expressed from these constructs ran as a single band, in contrast to the smear representing a heterogenous population of mRNAs with varying poly (A) tail

lengths for polyadenylated RNA. Also the band remained unchanged after oligo(dT)-RNase H digestion (fig 17A). The half-lives of these mRNA without a poly (A) tail were then analyzed for destabilization by an ARE and alterations in the presence or absence of p38 MAPK activation. In the presence of the poly (A) tail, the GMCSF ARE-containing β-globin RNA is rapidly degraded when compared to the β-globin RNA without an insert and stabilized by activation of the p38 MAPK pathway by $MKK6_{2E}$ expression (fig 17B). In the absence of the poly (A) tail for the RNAs with the histone stem loops, the half-life of the GMCSF ARE-containing RNA becomes comparable to that of the β-globin RNA without an insert and unresponsive to p38 MAPK activation (fig 17B). Hence the ARE-dependant regulation of mRNA stability appears to be lost in the mRNAs without a poly (A) tail. This result suggested a role for the poly (A) tail and its associated proteins in ARE-dependant rapid mRNA degradation (more in discussion).

A

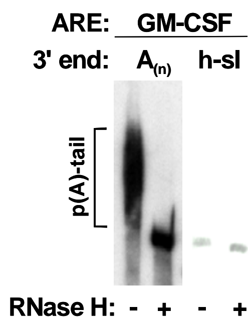
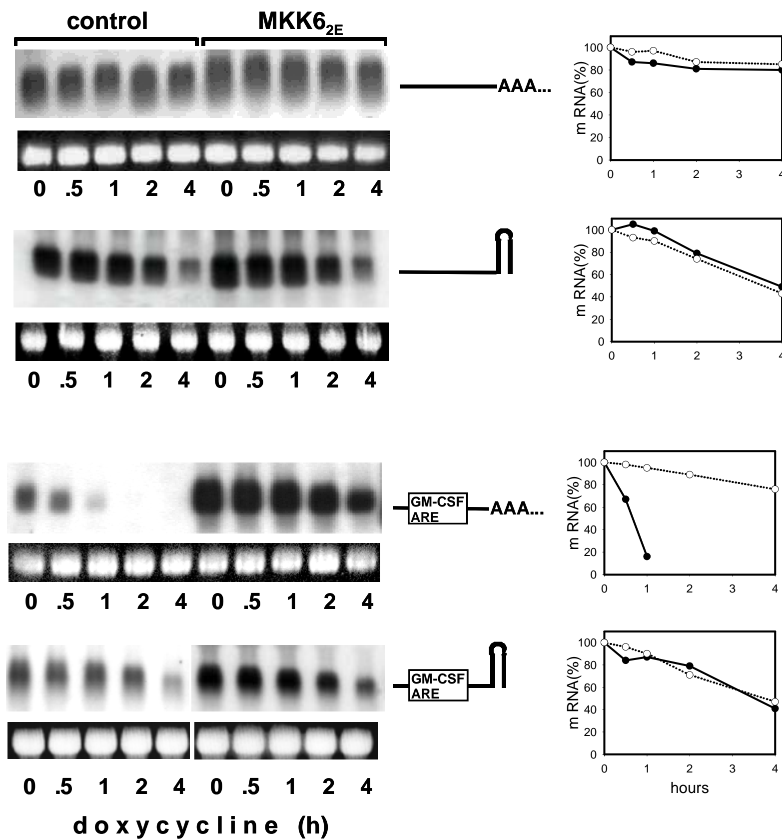


Figure 17: Loss of ARE-dependant regulation in the absence of a poly (A) tail. (A) Absence of a poly (A) tail in mRNAs containing a histone mRNA-derived stem loop was confirmed by polyacrylamide gel electrophoresis of total RNA from cells expressing β-globin RNAs with the GMCSF ARE with the poly (A) tail (A^n) or with the histone stem loop (h-sl) before and after digestion with RNase H plus oligo(dT). (B) Decay kinetics was determined for polyadenylated and nonadenylated, histone stem loop containing β-globin RNAs without or with the GMCSF ARE. Basal decay and $MKK6_{2E}$-induced stabilization were assayed as described in the legend to fig 9.

B



4.3 UV LIGHT AND mRNA STABILITY

Work done in our group indicated that like activation of the p38 MAP kinase/MK2 pathway, exposure to UV-B light led to an inhibition of mRNA degradation *(Bollig F 2002)*. Unlike the p38 MAPK/MK2 pathway whose effect is limited to AU-rich transcripts and independently of it, UV light stabilizes short-lived mRNAs either containing or lacking AU-rich elements. The signaling involved in the effect of UV-light, and the effect of UV light on mRNA deadenylation, have been addressed in this part of the work.

4.3.1 Effect of UV-B on mRNA degradation and deadenylation: Since UV light inhibits mRNA degradation; we asked if this inhibition of degradation was accompanied by an inhibition of deadenylation. Two mRNA species were expressed in HeLa tTA cells using the tet-off system: the β-globin reporter with the *c fos* ARE and the TIMP1 mRNA which lacks ARE sequences in its 3'UTR and contains a short insertion of the CAT gene in its 5'UTR to distinguish it from endogenous transcripts. RNAs were prepared from cells exposed to UV-B and also from cells in which the ptetBBB *c fos* constructs had been cotransfected with ptet myc-HuR, which expresses the well-known RNA binding protein-HuR. HuR has been described in the literature as an ARE binding protein (refer section 2.3.1.1.1), shown to be involved in stabilization of *c fos* ARE-containing β-globin RNA in overexpression studies, but without causing any inhibition of the process of deadenylation *(Peng SS 1998)*. Moreover HuR was also shown to be involved in the UV-C mediated stabilization of p21 mRNA *(Wang W 2000)*.

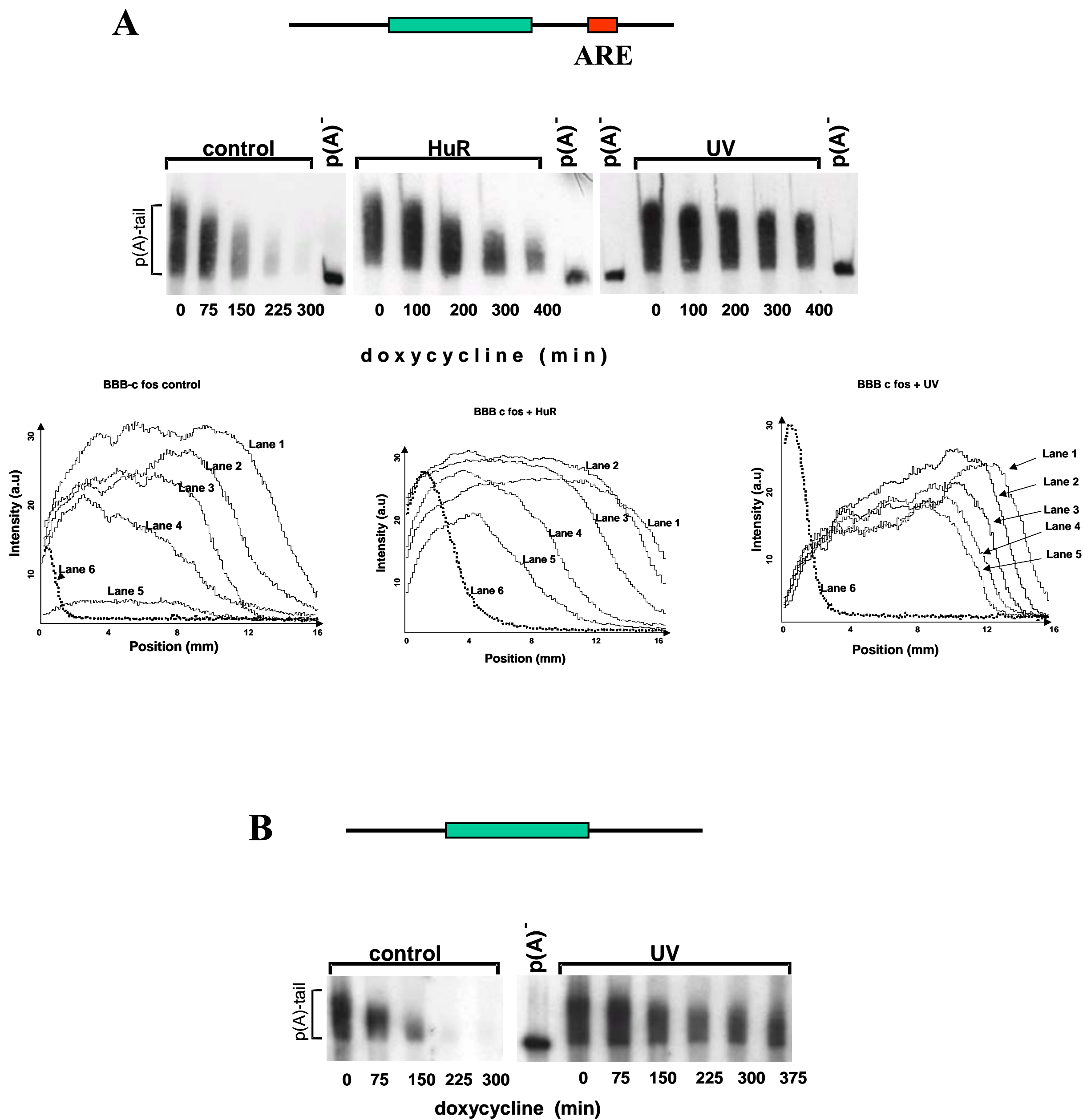


Figure 18: Effect of UV-B on mRNA degradation and deadenylation. HeLa tTA cells were transfected with (A) petBBB *c fos* and empty vector (control) or ptet-myc HuR (HuR), and (B) pUHD10 CAT TIMP1. Following the transfection, the cells were split into parallel cutures in 25cm^2 flasks. The next day, one set of these cultures was exposed to 1200 J/m^2 of UV-B (UV) and after 2hrs at 37ºC, transcription was stopped by the addition of doxycycline, total RNA isolated and examined on 4% denaturing polyacrylamide gels as described in the legend to fig 14. Density profiles made using the Aida Image Analyzer v 3.51 are shown below (A) (ref section 3.2.3.9). The intensity peak at position 0 represents the peak formed by the deadenylated RNA

Exposure to UV light caused a strong inhibition of both the degradation and the accompanying deadenylation of the *c fos* ARE-containing β-globin reporter and the non-ARE containing CAT TIMP1 reporter RNA (fig 18A and B). This contrasted to the stabilization imposed by the overexpression of HuR, where there was an inhibition of degradation with only a marginal effect on the process of deadenylation (fig 18A middle panel). While this effect of HuR on deadenylation coincided with the observations of Peng et al. *(Peng SS 1998)* this result argues against the involvement of HuR in the UV effect.

4.3.2 Effect of other cell stresses on mRNA degradation and deadenylation: The effect of UV light on mRNA degradation and deadenylation could be specific to UV light or may be a phenomenon common to other cellular stresses. Hence the effects of other cellular stresses like heat shock on both ARE and non-ARE containing reporters were examined. Three different heat shock temperatures, 40ºC, 42ºC, and 44ºC, were tested using the *c fos* ARE-containing β-globin reporter. There was clearly a temperature dependant increase in the inhibition of the degradation and deadenylation of the *c fos* ARE containing β-globin RNA, with the maximum inhibition at 44ºC (fig 19A). Hence all other heat-shock experiments were performed at 44ºC. As was seen in the case of UV light, heat shock at 44ºC also led to a strong inhibition in the degradation and deadenylation of the non-ARE containing CAT TIMP1 transcripts (fig 19B). Thus like UV light, heat shock at 44ºC led to a generalized RNA stabilization of both ARE and non-ARE containing reporter transcripts, whether by a similar mechanism or via activation of the same signaling pathways remains to be seen.

The effect of another direct cell stress-osmotic stress on mRNA metabolism was then examined. There was an inhibition in the degradation and deadenylation of both the ARE and non-ARE containing reporters but to a lesser extent as compared to both UV light and heat shock, indicating that perhaps different cellular stresses affected mRNA degradation to varying degrees (fig 19C).

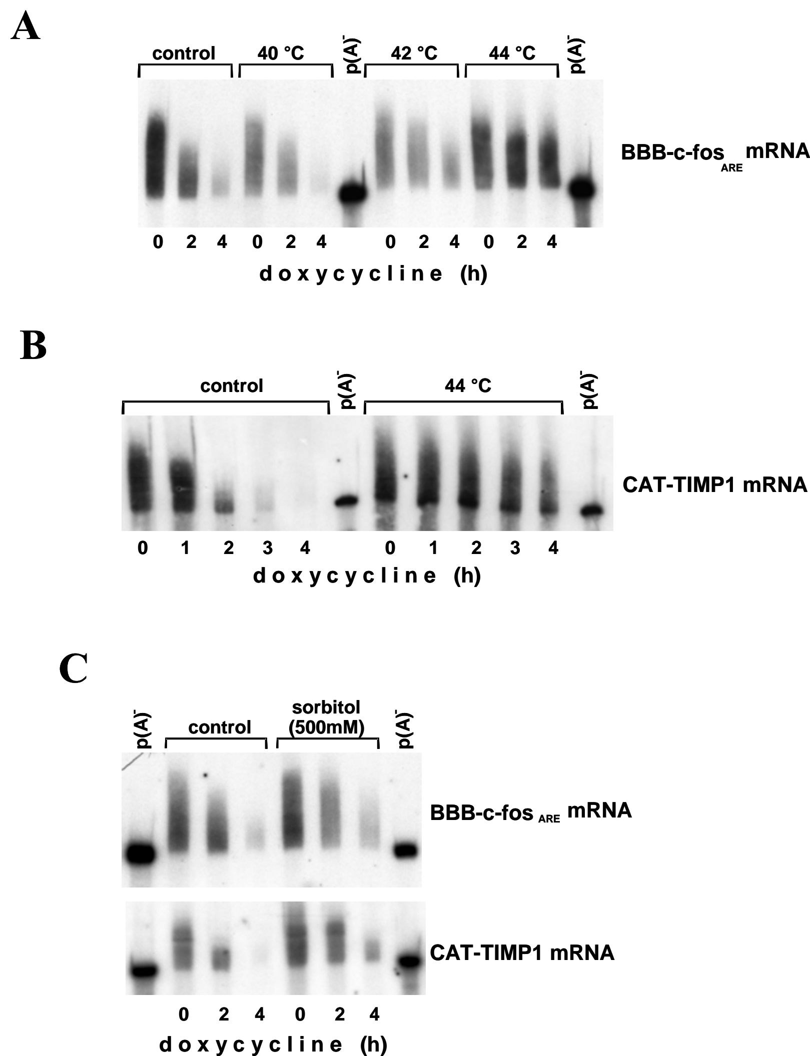


Figure 19: Effect of heat shock and osmotic shock on mRNA degradation and deadenylation. HeLa tTA cells were transfected with ptetBBB *c fos* or pUHD10 CAT TIMP or both as indicated. The transfected cells were kept at 37ºC (control) or exposed to the indicated heat shock temperatures for 1hr (A) and (B) or 500mM sorbitol applied to the transfected cells for 30mins at 37ºC and washed away with warm PBS (C). Transcription was stopped by the addition of doxycycline. Degradation and deadenylation kinetics was then determined as described in the legend to fig 14.

The effects of two other cellular stresses - H_2O_2 and γ radiation on mRNA stability were then checked. While the application of H_2O_2 would lead to the release of Reactive Oxygen Species (ROS) within the cells, exposure to γ radiation would lead to DNA damage. Hence by examining the effects of these two cellular stresses on mRNA stability, the involvement of pathways that are activated either by ROS or by DNA damage, in the inhibition of mRNA degradation and deadenylation by UV light can be examined (ref discussion). The results show that there was no effect of all three doses of γ radiation tested, on mRNA degradation

and deadenylation (fig 20 right panel). But exposure to H_2O_2, had an effect that was similar to UV light in terms of the inhibitions of both mRNA degradation and deadenylation (fig 20 left panel). These results indicate that ROS may be involved in the effect of UV light and argue against the involvement of DNA damage in the inhibition of mRNA degradation and deadenylation by UV light (more in discussion).

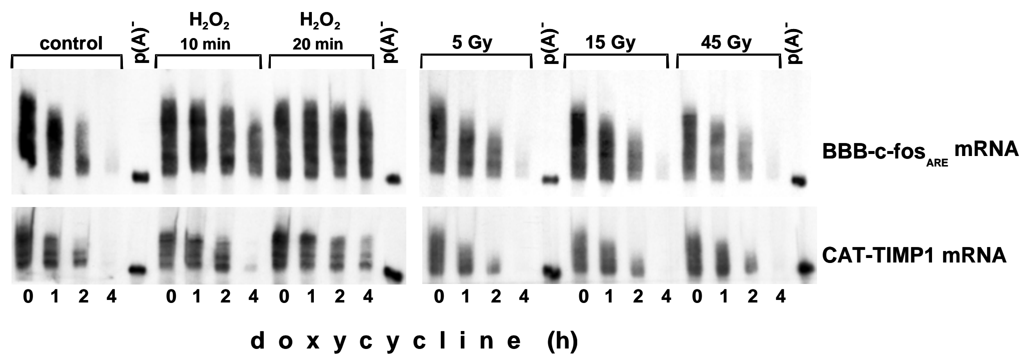


Figure 20: Effect of H_2O_2 and γ-radiation on mRNA degradation and deadenylation. HeLa tTA cells were co-transfected with ptetBBB *c fos* and pUHD10 CAT TIMP and the transfected cells left untreated (control) or exposed to the indicated doses of γ radiation or to 1mM H_2O_2 for either 10mins or 20mins. The stressed cells were allowed to recover for 1/2hr at 37ºC before stopping transcription with doxycycline. Degradation and deadenylation kinetics was determined as described in the legend to fig 14.

4.3.3 Lack of involvement of ROS in UV light mediated mRNA stabilization: The use of antioxidants like N-acetyl cysteine (NAC) or butylated hydroxy anisole (BHA) or enzymatic antioxidants like catalase to inhibit ROS generation and effects induced by exposure to H_2O_2 *(Peus D 1999)* has previously been documented. Hence such antioxidant pre-treatment would enable us to clarify the involvement of ROS in UV light mediated mRNA stabilization. HeLa tTA cells were therefore subjected to a pretreatment with both- NAC and BHA and then exposed to UV-B or H_2O_2. The results indicate that a combined pre-treatment with NAC and BHA could partially inhibit the H_2O_2 mediated increase in mRNA stability but had no effect on the UV light mediated increase in mRNA stability (fig 21).

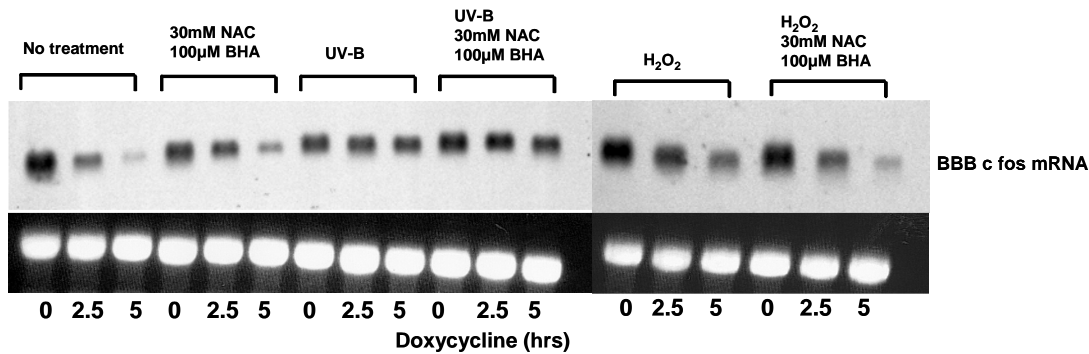


Figure 21: Use of antioxidants to investigate the involvement of ROS in UV mediated mRNA stabilization. HeLa tTA cells were transfected with ptetBBB *c fos*, and left untreated (no treatment) or exposed to UV-B or treated with 0.5mM H_2O_2 without or with prior treatment to 30mM NAC and 100µM BHA for 1/2hr, or as control, treated only with both antioxidants. Transcription was stopped by the addition of doxycycline. Decay kinetics was determined on agarose gels, as described in the legend to fig 9 with a β-globin antisense RNA probe.

Expression of the human catalase in HeLa tTA cells in combination with the *c fos* ARE-containing β-globin reporter RNA, followed by exposure to UV-B and H_2O_2 showed that like the antioxidants-NAC and BHA, the effect of H_2O_2 on mRNA degradation and deadenylation could be partially inhibited. But the UV-mediated inhibition of both these processes could not be blocked by catalase expression (fig 22). This confirms that ROS may not be directly involved in the effect of UV light but is rather suggestive of a scenario wherein these two cellular stresses exert their effects on mRNA turnover independently, perhaps through common downstream events. More experiments are needed to confirm this hypothesis.

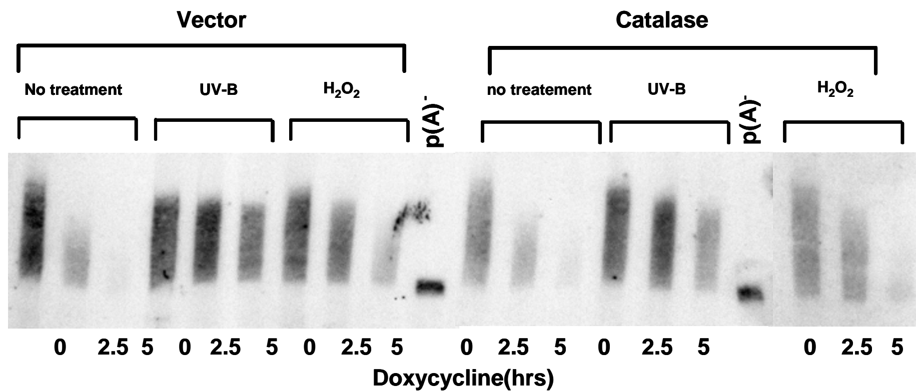


Figure 22: Effect of catalase expression on UV- mediated inhibition of mRNA degradation and deadenylation. HeLa tTA cells were transfected with ptetBBB cfos and empty vector or with pCDNA3 h catalase, and the transfected cells left untreated or exposed to UV-B or to 0.5mM H_2O_2 where indicated. Transcription was stopped by the addition of doxycycline and degradation and deadenylation kinetics determined as described in the legend to fig 14.

4.3.4 The effect of UV light does not require activation of the p38 MAP kinase pathway:

UV light activates several signaling cascades including the p38 MAP kinase pathway. Our results with the transcript selectivity of the two modes of stabilization indicated that the p38 MAP kinase pathway might not be involved in the UV mediated stabilization of mRNA. Although stabilization of mRNA by UV light had been shown previously to be independent of p38 MAPK activation *(Bollig F 2002)*, inhibition of mRNA deadenylation could still be a consequence of this activation. Hence SB 203580, the p38MAPK inhibitor was applied to the cells prior to UV exposure or heat shock and the effects on mRNA deadenylation examined. Blocking the p38 MAP kinase activation using the SB inhibitor could not reverse the inhibition of mRNA degradation and deadenylation induced by both UV light and by heat shock (fig 23). These results corroborate earlier results and rule out the requirement of prior activation of the p38 MAP kinase for the UV light mediated inhibition of mRNA degradation and deadenylation.

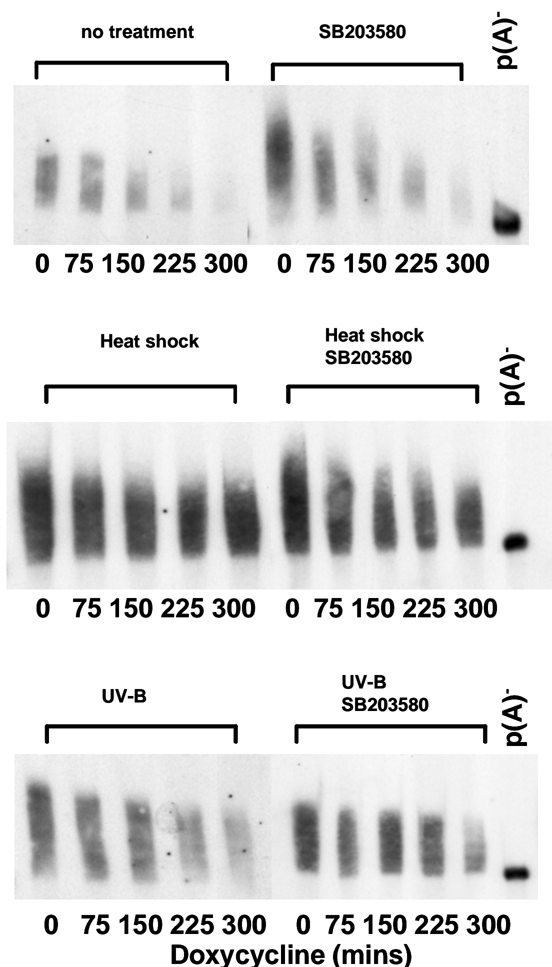


Figure 23: The effect of UV light on mRNA deadenylation and degradation does not require activation of the p38 MAPK. HeLa tTA cells were transfected with ptetBBB *c fos*. The transfected cells were either left untreated, or incubated with 2μM SB 203580 for 30 mins, exposed to UV-B or heat-shocked at 44ºC without or with a 30mins pre-incubation in the presence of the SB inhibitor. Transcription was stopped with doxycycline. Degradation and deadenylation kinetics was determined as described in the legend to fig 14.

4.3.5 Deadenylation and degradation are inhibited by low doses of UV light that do not inhibit general protein synthesis: mRNA stabilization is normally expected to increase mRNA levels and thereby increase protein synthesis. On the other hand exposure to UV light inhibits general protein synthesis *(Wu S 2002)*, *(Deng J 2002)*. Results from the group showed that significant inhibition of protein synthesis occurred only after exposure to 2400 J/m^2 of UV light *(Beniam 2003)*. HeLa tTA cells were exposed to different doses of UV light and the effects on mRNA degradation and deadenylation examined to see whether the effects of UV light on translation and mRNA stabilization required different doses of UV light.

UV-induced stabilization and inhibition of deadenylation correlated and were maximal already at 600 J/m^2 (fig 24), a dose that is 4 fold lower than the dose of UV light at which effects on protein synthesis were observed. Thus mRNAs stabilized at low doses of UV light can indeed be translated into protein.

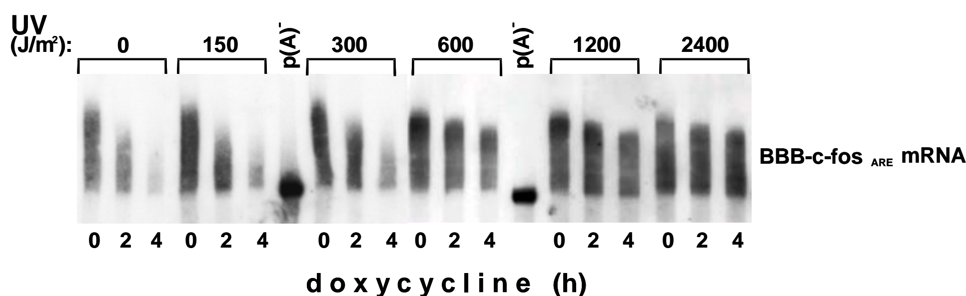


Figure 24: mRNA deadenylation and degradation are inhibited by low doses of UV light. HeLa tTA cells were transfected with ptet BBB *c fos* and the transfected cells exposed to different doses of UV light as indicated. Transcription was stopped by the addition of doxycycline. Degradation and deadenylation kinetics was determined as described in the legend to fig 14.

4.3.6 UV light inhibits the degradation and deadenylation of the endogenous IL-8 mRNA: The effects of UV light on the stability of the endogenous IL-8 mRNA had previously been examined and documented *(Beniam 2003)* by our group. Hence we analyzed the effects of UV light on the deadenylation of the IL-8 mRNA. In the HeLa cells IL-8 production can be induced by exposure to the pro-inflammatory cytokine, IL-1. The poly (A) tail length distribution during induction was analyzed using the acrylamide gels. In accordance with previous results the induction was transient, with a sharp peak at 1hr after the addition of IL-1, and gradual disappearance of the RNA thereafter. At the 1hr time point when the IL-8 mRNA was freshly transcribed; most of the mRNA was highly polyadenylated (fig 25). By 2hrs post-IL-1 addition, an even distribution of poly (A) tail lengths was seen. At 4hrs, when most of the IL-8 mRNA was degraded, the shorter oligo-adenylated and largely deadenylated species were more prominent, attesting to the rapid degradation and deadenylation of this well known ARE-containing RNA.

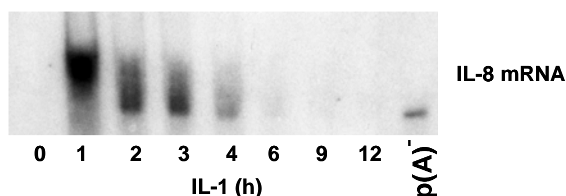


Figure 25: Induction of IL-8 mRNA in HeLa cells by IL-1. 1ng/ml of IL-1 was added to HeLa tTA cells in culture. Lysates were made at the indicated times, RNAs prepared and analyzed by northern blotting on acrylamide gels using an IL-8 antisense RNA probe. An aliquot of the total RNA from the 1hr time point was digested with RNase H to prepare the deadenylated refernce $p(A)^-$.

Deadenylation of the IL-8 mRNA following exposure to UV light was then analyzed in an actinomycin D chase experiment. In the absence of exposure to UV light, the IL-8 mRNA was rapidly deadenylated (fig 26 left panel). Exposure to UV light after the addition of IL-1caused a prolonged inhibition in both the degradation and deadenylation of the IL-8 mRNA (fig 26 middle panel). Even when the cells were exposed to UV light prior to IL-1 stimulation, the effect of UV light on the inhibition of mRNA degradation and deadenylation could be reproduced (fig 26 right panel). This last experiment indicated that the effect of UV light on mRNA degradation and deadenylation was not via any direct modification of the RNAs themselves, as under these experimental conditions the IL-8 RNAs that were stabilized had been generated one hr after exposure to UV light.

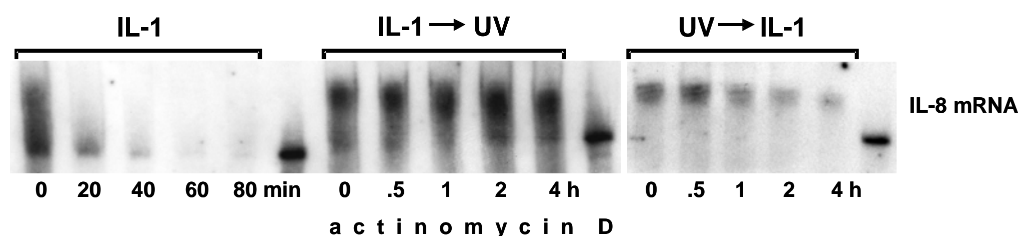


Figure 26: Effect of UV light on the deadenylation and degradation of the endogenous IL-8 mRNA. HeLa cells were stimulated with 1ng/ml of IL-1 without (IL-1) or with exposure to UV-B, 1hr after (IL-1→UV) or 1hr before (UV→IL-1) the addition of IL-1. 3hrs after the addition of IL-1 transcription was stopped by the addition of 5µg/ml of actinomycin D, a general transcription inhibitor. Degradation and deadenylation kinetics was determined as described in the legend to fig 14 with an antisense IL-8 RNA probe.

Exposure to UV light causes the transcriptional induction of several stress response genes. To test whether the effect of UV light on mRNA degradation requires the transcriptional induction of gene expression, transcription was stopped by the addition of actinomycin D immediately after the exposure to UV light. As the inhibition of mRNA degradation and deadenylation proceeded unabated under these conditions (fig 27), these results indicate that the inhibition of mRNA degradation and deadenylation by UV light is independent of ongoing transcription.

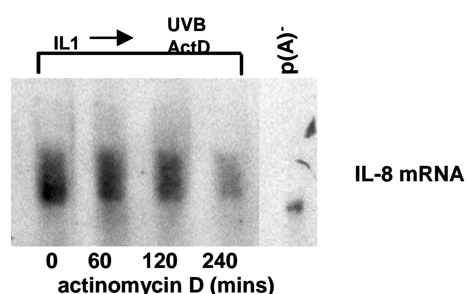


Figure 27: Effect of UV light is independent of ongoing transcription. HeLa tTA cells were stimulated with IL-1 for 3hrs, exposed to UV-B and 5µg/ml actinomycin D added immediately to stop transcription. Degradation and deadenylation kinetics was determined as described in the legend to fig 14 with an antisense IL-8 RNA probe.

4.3.7 UV light stabilizes other short-lived mRNA transcripts involved in the inflammatory response: The effects of UV light on the IL-8 mRNA suggested a role for mRNA stabilization by UV light in the inflammatory response. This hypothesis was examined by analyzing the effects of UV light on the stability of mRNAs on a larger scale using the microarray facility at the Institute of Molecular Pharmacology, Hannover Medical School in collaboration with PD. Dr. Michael Kracht and Dr. Oliver Dittrich-Breiholz. This microarray contains oligonucleotides complementary to about 140 mRNA that have been chosen for their roles in inflammation and associated processes. The effects of UV light on the mRNA of these genes from HeLa tTA cells was determined by comparing the differences before and after the addition of actinomycin D with and without exposure to UV-B. The reproducibility of the results was confirmed by doing a second experiment. Selected genes whose expression is regulated by UV light at the level of mRNA stability are shown in the table. Of all the cells types in our body, the epidermal keratinocytes are maximally exposed to UV light. Several studies involving effects of UV light are carried out using primary keratinocytes or keratinocyte cell lines like the HaCaT cells *(Chen W 2001), (Ashida M 2003).* Therefore

mRNA from HaCaT cells were also analysed on the same microarrays. The profiles obtained overlap with those obtained using the HeLa cells.

Since all the genes on the array have crucial roles to play in inflammation and related process, the regulation of the mRNA stability of a group of these genes by UV light indicates that this regulation could indeed be important for the inflammatory response to UV stress.

Accesion no.	Genname InflHum	Ist experiment: HeLa tTA 2hIL1→ 3h act D	Ist experiment: HeLa tTA 2hIL1→UV 3h act D	Ist experiment: Fold Stabilization	2nd experiment: HeLa tTA 2hIL1→ 3h act D	2nd experiment: HeLa tTA 2hIL1→UV 3h act D	2nd experiment: Fold Stabilization
NM_020529	nfkbia	3	18	5.9	3	20	6.6
NM_000600	il6	14	75	5.5	17	66	3.8
NM_003467	cxcr4	7	28	3.8	8	37	4.7
NM_000584	cxcl8	18	71	4.0	24	64	2.7
NM_001165	birc3	20	64	3.2	18	53	2.9
NM_002228	jun	20	41	2.0	6	35	5.5
NM_002923	rgs2	16	33	2.0	11	42	3.9
NM_021009	ubc	27	61	2.3	32	70	2.2
NM_000963	ptgs2	25	72	2.8	44	66	1.5
NM_002729	hhex	28	45	1.6	16	42	2.6
NM_006290	tnfaip3	23	34	1.5	14	36	2.5
NM_002229	junb	31	38	1.3	18	34	1.9
NM_015675	gadd45b	45	61	1.3	34	46	1.4

TABLE 2: Examination of UV-dependant stabilization of mRNA on a large scale using an inflammatory microarray. HeLa tTA cells were stimulated with 1ng/ml of IL-1 for 2hrs, and transcription stopped by the addition of actinomycin D with and without a subsequent exposure to UV-B. Lysates were made 3hrs later and RNAs were prepared for microarray analysis as outlined in methods section 3.2.3.1. The results shown were calculated from the raw data and expressed as the percentage of RNA left in the cytoplasm after the addition of actinomycin D with and without UV exposure. The fold stabilization by UV exposure is also shown. The table shows results from two independent experiments, for a group of transcripts, which were selected using certain criteria: the transcripts selected had to have short half-lives (% of RNA left after act D addition should be less than 50), and should show a significant increase in amount after UV exposure.

Accession no.	Genname InflHum	Ist experiment HaCaT 2hIL1→ 3h act D	HaCaT 2hIL1→UV 3h act D	Fold Stabilization	2nd experiment HaCaT 2hIL1→ 3h act D	HaCaT 2hIL1→UV 3h act D	Fold Stabilization
NM_000584	cxcl8	13	59	4.4	12	52	4.5
NM_000576	il1b	16	71	4.5	15	63	4.2
NM_002228	jun	9	27	3.0	6	36	6.4
NM_000758	csf2	32	83	2.6	13	73	5.5
NM_020529	nfkbia	6	15	2.4	5	20	4.3
NM_021009	ubc	18	55	3.0	25	74	2.9
NM_000575	il1a	19	41	2.1	13	41	3.1
NM_006290	tnfaip3	21	32	1.5	16	29	1.9
NM_002198	irf1	32	39	1.2	16	29	1.9

TABLE 3: The effect of UV light on the stability of mRNA from HaCaT cells. HaCaT cells (see section 3.1.11) were stimulated with 1ng/ml of IL-1 for 2hrs, and transcription stopped by the addition of actinomycin D with and without a subsequent exposure to UV-B. Lysates were made 3hrs later and RNAs were prepared for microarray analysis as outlined in methods section 3.2.3.1. The results have been expressed as outlined in the legend to table 2 for two independent experiments.

5 DISCUSSION:

In this study different aspects of two different kinds of regulated mRNA turnover have been examined: the p38 MAPK/MK2 dependant stabilization of ARE-containing mRNA and the UV-B dependant stabilization of ARE and non-ARE containing mRNA. Both kinds of mRNA stabilization are activated by inflammatory/stress signals and contribute to the expression of genes involved in inflammation like the cytokine genes.

5.1 Structural and functional analysis of the IL-8 ARE: In the first part of the study, the AU-rich element of the IL-8 mRNA was characterized. AU-rich sequences present in the 3'UTR of the IL-8 mRNA have been shown to be involved in the post-transcriptional control of its expression *(Winzen R 1999)*. While previous studies demonstrated this using the β-globin reporter expressed with the tet-off system, the importance of AU-rich sequences for regulating IL-8 mRNA stability has been now confirmed, using full-length and truncated IL-8 RNA constructs (fig 9). The destabilizing potential of AU-rich sequences has for the most part been attributed to the presence of one or more AUUUA motifs. However it has been observed that an AUUUA motif-containing region alone was of low destabilizing potential *(Chen CY 1994)*, *(Xu N 1997)*, *(Winzen R 1999)*, *(Yu Y 2001)*. Defining features of the IL-8 ARE required for regulated mRNA stability, we report the importance of a 30nt flanking sequence, which enhances the moderate destabilizing potential exerted by the AUUUA-motif containing region of the IL-8 ARE.

The minimal regulatory element in the IL-8 3'UTR is thus a 60nt region with a bipartite structure, having an AUUUA containing sequence-the core domain and a 30nt upstream AU-rich sequence termed the auxiliary domain (fig 10 and 11). A similar structure has been reported for the ARE of *c fos* although in this case the U-rich auxiliary domain is 3' to the AUUUA motif containing core domain *(Chen CY 1994)*. On the other hand studies from this lab and others showed that the GMCSF ARE is organised like the IL-8 ARE containing clusters of overlapping AUUUA motifs in the 3'part and AU-rich sequences 5' to the AUUUA motifs that contribute to destabilization *(Winzen R 2004)*, *(Xu N 1997)*. Although the existence of auxiliary domains has not been explored for many AREs, evidence has been presented that additional sequences outside the AUUUA motif-containing region, which may be functional equivalents of such auxiliary domains, are necessary to observe the rapid degradation exerted by the AREs. These may be present either upstream as in the TNF-α

mRNA *(Stoecklin G 2001)* or downstream as in the COX-2 mRNA *(Lasa M 2000)*. Hence the organisation of these domains in AREs appears to vary.

Although much work has been done characterizing features of AREs that are required for degradation, few other studies have characterized the sequence features of AREs, which determine the control of its function by signaling pathways. While the auxiliary domain of the IL-8 ARE is necessary to enhance destabilization exerted by the IL-8 ARE core domain, it is dispensable for the stabilizing effect of the p38/MK2 pathway (fig 11). This potential of auxiliary domains to enhance destabilization exerted by the AUUUA-motif containing core domain becomes particularly important in the event of mutations destroying the individual AUUUA motifs *(Winzen R 2004)*, *(Chen CY 1994)*. The minimal functionally relevant ARE motif has been variously defined as UUAUUUA(A/U)(A/U) or the nonamer UUAUUUAUU *(Lagnado CA 1994), (Zubiaga AM 1995)*, or more recently in a computational derivation as WWWUAUUUAUWWW where W can be either A/U *(Bakheet T 2001)*. In the IL-8 ARE, the second and third AUUUA motifs form the centres of sequences fulfilling these criteria. Interestingly, mutants in which both of these AUUUA motifs were destroyed still mediate destabilization and stabilization by the p38 MAPK pathway *(Winzen R 2004).* Even when the individual AUUUA motifs of the IL-8 ARE had been systematically destroyed singly or in various combinations *(Winzen R 2004)* as long as there was measurable destabilization through an ARE, activation of the p38/MK2 pathway induced stabilization. These observations thus favor a model in which the p38/MK2 pathway inhibits the destabilization exerted by the AREs, perhaps by inactivating or releasing a bound destabilizing ARE-BP (ref fig 29). This has been shown for the TNF α mRNA, where activation of the p38/MK2 pathway phosphorylates the destabilizing ARE-BP, TTP (ref section 2.3.1.1.3) *(Stoecklin G 2004)*. The phosphorylation allows the sequestering of TTP by 14-3-3 proteins. Thereby the p38/MK2 pathway contributes to the induction in TNF α production in response to LPS stimulation.

Since the first observations that implied a role for the p38 MAPK pathway in the regulation of the half-lives of a number of AU-rich mRNA including cyclooxygenase 2 (COX2), TNF-α, IL-3, IL-6, IL-8, MIP1- α, GM-CSF, VEGF, and uPA *(Brook M 2000), (Holtmann H 1999), (Ming XF 2001), (Montero L 1999), (Pages G 2000), (Stoecklin G 2001),* it has been seen that not all AREs mediate stabilization through this pathway. A number of mRNAs fulfilling the criteria of AREs as defined by the ARED database (ref section 2.3.1) are not stabilized in a p38 MAP-kinase dependant manner in response to LPS as an activator of p38/MK2

signaling *(Frevel MA 2003), (Tebo J 2003)*. There may be several reasons for this. Certain AREs may lack features that make them unresponsive to stabilization by p38 MAPK activation. It is also possible that this may include not just aspects of the primary sequence but also changes involving secondary structure. The presence of additional destabilizing elements apart from the AREs may be another reason. This has been shown for the TNF-α mRNA (ref fig 28). Its 3'UTR has a special element called the constitutive decay element (CDE) downstream of its ARE, which is unresponsive to stabilization by the p38/MK2 pathway *(Stoecklin G 2003)*. The CDE can thus destabilize the TNF- α mRNA in an ARE-independent and p38/MK2 independent manner. In this study two observation suggest the presence of additional destabilizing elements like the CDE: 1) IL-8 mRNA lacking the AU-rich sequences exhibit an extended half-life compared to 20mins for an RNA with ARE sequences (fig 9), yet appears to exhibit moderate instability (half-life of 2hrs) and 2) the activation of the p38 MAPK could only extend the half-life of the full length ARE-containing IL-8 mRNA from 20mins to about 1hr (fig 9 upper panel). Unlike the CDE in the TNF-α mRNA these additional destabilizing elements in the IL-8 mRNA, cannot by themselves destabilize leaving the IL-8 mRNA still responsive to stabilization by the p38 MAPK pathway (ref fig 28). The presence of such elements and their interplay with the AREs would therefore be an important factor in determining the responsiveness of AREs to different signaling mechanisms.

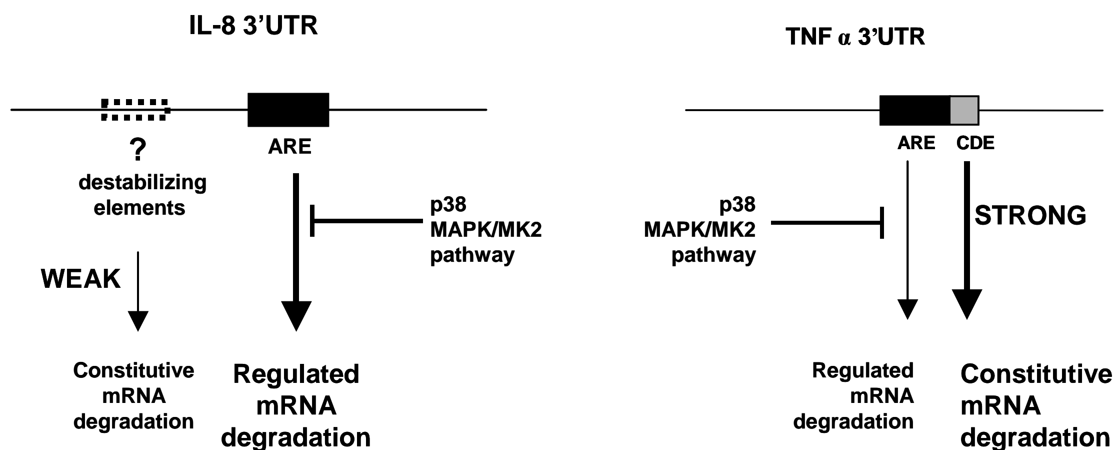


Figure 28: Scheme showing the impact of constitutive destabilizing elements on regulated mRNA turnover. Destabilization exerted by the IL-8 and TNF-α AREs can be blocked by activation of the p38/MK2 pathway. There may exist additional destabilizing elements in the IL-8 3'UTR like the CDE in the TNF-α mRNA. These elements are probably not responsive to stabilization by the p38/MK2 pathway. In the case of the TNF-α mRNA, the CDE has a strong effect and the net effect on mRNA stability is thus constitutive mRNA degradation. For the IL-8 mRNA, evidence suggests that these additional elements exert only a weak effect. Hence the net effect would be a predominance of regulated mRNA turnover through the AREs, limited to a small extent by the presence of these elements.

5.2 Distinct yet overlapping subsets of AREs can be stabilized by distinct mechanisms of mRNA stabilization: In this study, a difference in sequence requirements for stabilization through the p38/MK2 pathway as opposed to stabilization due to increased cytoplasmic HuR is demonstrated. Gel shift studies in our group indicated that HuR binds to both GMCSF and IL-8 AREs *(Winzen R 2004)*. HuR has been described in literature as a stabilizing ARE-BP (ref section 2.3.1.1.1) and recently its cytoplasmic presence has been linked to the p38/MK2-induced stabilization of ARE transcripts *(Atasoy U 2003), (Tran H 2003)*. Hence HuR may be involved in the stabilization of IL-8 ARE-containing RNA by the p38/MK2 pathway. The results in fig 12A argue against this possibility. While the destabilizing effect of the AUUUA motif-containing region of the IL-8 ARE is sensitive to stabilization by the p38/MK2 pathway, it does not suffice to allow stabilization by HuR. Rather a separate domain from the *c fos* or GMCSF AREs can impose stabilization through HuR as shown for the chimeric ARE constructs (fig 12B). The lack of involvement of MK2 in HuR mediated stabilization of the *c fos* AD-IL-8 CD chimeric ARE transcripts also suggests that stabilization by overexpression of HuR operates via a mechanism distinct from that used by the p38/MK2 pathway (fig 12C). These results thus indicate the existence of subsets of AREs: some like the IL-8 ARE apparently sensitive to specific signaling pathways like the p38/MK2 and others like the *c fos* or GMCSF AREs that are responsive to both modes of mRNA stabilization. It is also possible that there exists a subset of AREs that are only responsive to stabilization by HuR.

As discussed previously the p38/MK2 pathway stabilizes RNA by inhibiting the function of destabilizing ARE-BP. The binding to and subsequent stabilization of certain ARE-containing RNAs by HuR represents another mechanism of mRNA stabilization, wherein the binding of stabilizing ARE-BPs leads to the stabilization of mRNA (ref fig 29). A recent report by Lal et al showed that the RNA binding proteins- HuR and AUF1 bind several common target transcripts on both distinct, non-overlapping sites and on common sites in a competitive fashion *(Lal A 2004)*. That report complements the displacement model outlined in the introduction, whereby decay-promoting and stability-promoting ARE-BPs are thought to compete for the same binding site *(Ming XF 2001)*. So the binding of HuR could alter interactions of bound destabilizing ARE-BPs and therefore stabilize certain ARE-containing RNA. In the case of the IL-8 ARE, where there is binding to HuR but no stabilization, an as yet unidentified destabilizing ARE-BP may compete with the bound HuR. The affinity of HuR for the IL-8 ARE may be too low to displace this destabilizing ARE-BP. Alternatively HuR may have to bind in a distinct way and/or in combination with certain other proteins to stabilize the IL-8 RNA. Irrespective of the reason, the absence of a stabilizing effect of HuR on IL-8 ARE-containing mRNA argues against increased levels of HuR in the cytoplasm as the sole mechanistic basis for p38/MK2-induced stabilization.

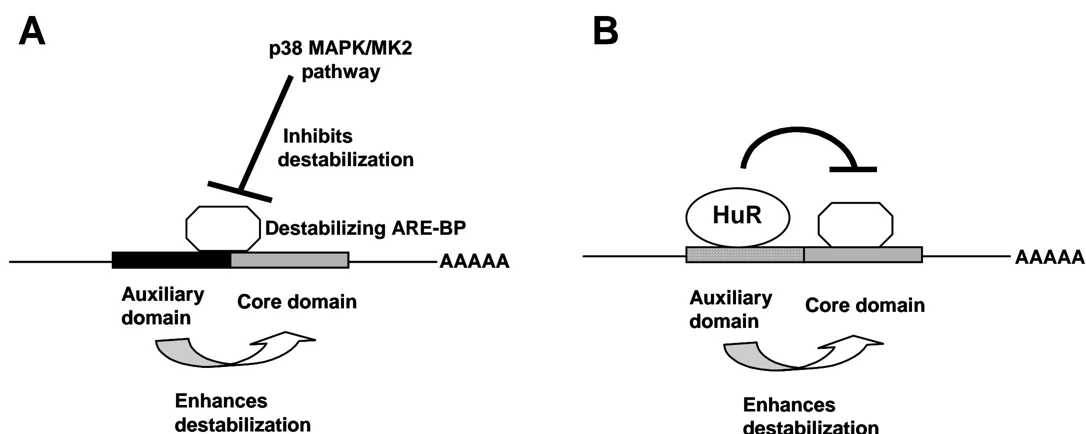


Figure 29: Models to explain the structure-function relationship in AREs. AREs appear to have bipartite structures with AUUUA-containing core domains and AU-rich or U-rich auxiliary domains. Auxiliary domains cannot destabilize on their own but enhance the moderate destabilizing potential of the core domains. (A) Stabilization by the p38/MK2 pathway inhibits ARE-dependent rapid mRNA degradation perhaps by affecting bound destabilizing ARE-BPs. (B) HuR, a stabilizing ARE-BP requires distinct sequences present in auxiliary domains of certain AREs and stabilizes possibly by displacing or affecting in some other way previously bound destabilizing ARE-BPs.

5.3 Role for the poly (A) tail in regulated ARE-dependent mRNA degradation: The result in figure 15 shows that stabilization of an ARE-containing reporter by the p38/MK2 pathway is accompanied by inhibition of deadenylation as has been reported recently for COX2 ARE-containing reporters *(Dean JL 2003).* A role for the poly (A) tail in ARE-mediated regulation of mRNA stability is supported by the results shown in fig 17, where replacing the poly (A) tail with a histone stem loop sequence abolished ARE-dependant regulation of mRNA turnover. Additional evidence in support of this hypothesis is provided by the observation that the poly (A) binding protein, PABP1, can be phosphorylated *in vitro* by MK2 the downstream substrate of p38 *(Bollig F 2003).* A physical interaction between an ARE in the 3'UTR of an mRNA with PABP1 could disturb the 'closed loop' (ref section 2.3, fig 3) structure that forms as a result of interactions between PABP1 and the translation initiation factor eIF4G. This would then expose the RNA to exonucleases. In support of this model, it has been shown by other groups that PABP1 interacts with AU-rich elements *(Wiklund L 2002)* with affinity constants in the nM range *(Sladic RT 2004).* Further support comes from another report, which showed using gel mobility shift assays that the ARE of GMCSF interferes with the association between PABP1 and the poly (A)-tail *(Grosset C 2004)* and interferes with translation and mRNA degradation. Hence the p38/MK2 pathway could phosphorylate PABP1, alter its association with the AREs thereby causing a stabilization of ARE-containing RNA. Further experiments are needed to substantiate this hypothesis.

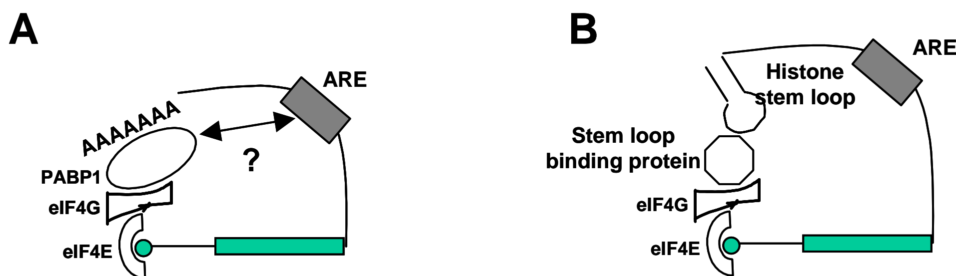


Figure 30: Speculative model to explain the role of the poly (A) tail in ARE-dependant regulation of mRNA stability. (A) The association of poly (A) binding protein 1 (PABP1) to the poly (A) tail or to eIF4G may be impaired in the presence of AREs. This would affect the formation of the closed loop structure contributing to the rapid degradation of ARE-containing mRNA. (B) In the presence of the histone stem loop PABP1 is replaced by the stem loop binding protein (SLBP). Interactions between the SLBP and eIF4G may be insensitive to ARE-dependant regulation of mRNA degradation.

5.4 UV light and mRNA stability: Unlike the stabilization mediated by the p38 pathway, which appears to be specific to a subset of ARE-containing transcripts, stabilization by UV light affects both ARE and non-ARE containing reporter transcripts *(Bollig F 2002)* and the endogenous ARE-containing IL-8 mRNA as has been shown in fig 18 and fig 26 and by others in the group *(Beniam 2003)*. Activation of the p38 MAPK inhibits the rate of deadenylation. The effects of UV-B on mRNA deadenylation were therefore examined and figures 18 and 26 show that exposure to UV-B leads to a strong inhibition in the deadenylation of both ARE- and non-ARE containing reporters, as well as an inhibition in the deadenylation of the endogenous IL-8 mRNA. To our knowledge this is the first report documenting this particular effect of UV light. Since deadenylation is the first step in mRNA turnover (ref section 2.2), logic dictates that an inhibition of deadenylation would be the cause for an inhibition of degradation. However these experiments are insufficient to prove that these two effects share a causal relationship. Therefore it is still possible that they are two independent effects of UV light that occur in parallel. The significance of an inhibition of deadenylation for the mRNA stabilization by exposure to UV light or by the activation of the p38/MK2 pathway can only be obtained by using additional approaches like examining the effects of UV exposure or p38 MAPK activation on known deadenylases.

5.5 Investigating the signalling involved in UV-B mediated mRNA stabilization: UV irradiation activates several signal transduction pathways including the p38 MAPK pathway (ref section 2.4). From our previous studies it was concluded that the activation of the p38 MAPK pathway alone or in combination with the JNK and NF-κB pathways was not enough

to observe UV-B mediated stabilization of the non-ARE reporters *(Bollig F 2002)*. This coincides with observations from Blattner et al who could not prevent UV-C mediated stabilization with the use of the SB203580 *(Blattner C 2000)*. We now observe that inhibiting the activation of p38 MAPK by using the pyridinyl imidazole SB203580 could also not inhibit the effect of UV light on mRNA deadenylation (fig 23). The inhibition of mRNA degradation and deadenylation by UV light could be prevented by high concentrations of another protein kinase inhibitor, staurosporine (results not shown), suggesting that the effect of UV light involves a phosphorylation event. But at lower concentrations where specificity towards certain kinases like PKC is expected *(Matsumoto K 1998)*, (check staurosporine product details under www.biocompare.com), the effect of UV light on mRNA degradation and deadenylation could not be inhibited. Moreover we and others have tested various other protein kinase inhibitors but could not prevent UV-induced mRNA stabilization (data not shown) and *(Blattner C 2000)*. These observations suggest that the effect of UV-B on mRNA degradation and deadenylation may involve an as yet unidentified signalling pathway or a combined activation of several signalling pathways.

As another approach to narrowing down the signalling pathway/s involved, we examined the source of the signalling that is needed to observe UV-induced mRNA stabilization. In addition to events triggered by DNA damage, it has become quite evident that there exist other, non-nuclear sources for the signalling (ref section 2.4), *(Bender K 1997)*. The results in fig 20 indicate that signalling initiated by γ-radiation induced DNA damage is not sufficient to inhibit mRNA degradation and deadenylation. But this does not exclude that signals initiated by the specific type of UV-induced DNA damage are involved in the effect of UV light on mRNA stabilization. Apart from UV light, other stresses like heat shock and ER stress have been reported to have global effects on mRNA turnover *(Fan J 2002)*, *(Kawai T 2004)*. The results in fig 19 show that heat shock at 44ºC and osmotic stress impair the degradation and deadenylation of ARE- and non-ARE containing reporters. As heat shock is not associated with DNA damage, this supports the notion that the inhibition of mRNA degradation and deadenylation by UV light may not be a general consequence of DNA damage. On the other hand exposure to H_2O_2 led to an inhibition of mRNA deadenylation and degradation in a manner similar to that of UV light (fig 20). It has been suggested that reactive oxygen species (ROS) may be key regulators of UV-induced signalling pathways (ref section 2.4). But the results in figures 21 and 22 indicate that inhibiting signalling initiated by free radicals with the antioxidants, N-acetyl cysteine (NAC) and butylated hydroxy anisole (BHA) or the expression of human catalase could not inhibit the effects of UV light on mRNA stability. It

was also examined if UV light exerts its effects on mRNA stability by a direct modification of the RNA molecules as it has been reported that UV light can cause site-specific damage of the 28s rRNA *(Iordanov MS 1998)*. But as was shown in figure 26, the effects of UV light on mRNA degradation and deadenylation were reproducible on endogenous IL-8 RNA that was generated after exposure to UV light. This study thus rules out direct modification of RNA molecules or the involvement of ROS in the UV-mediated inhibition of mRNA degradation and deadenylation, but has helped identify a group of cellular stresses apart from UV light-like heat shock, osmotic shock and H_2O_2 that have similar effects on mRNA degradation and deadenylation. If they do so by affecting the same downstream target remains an interesting question.

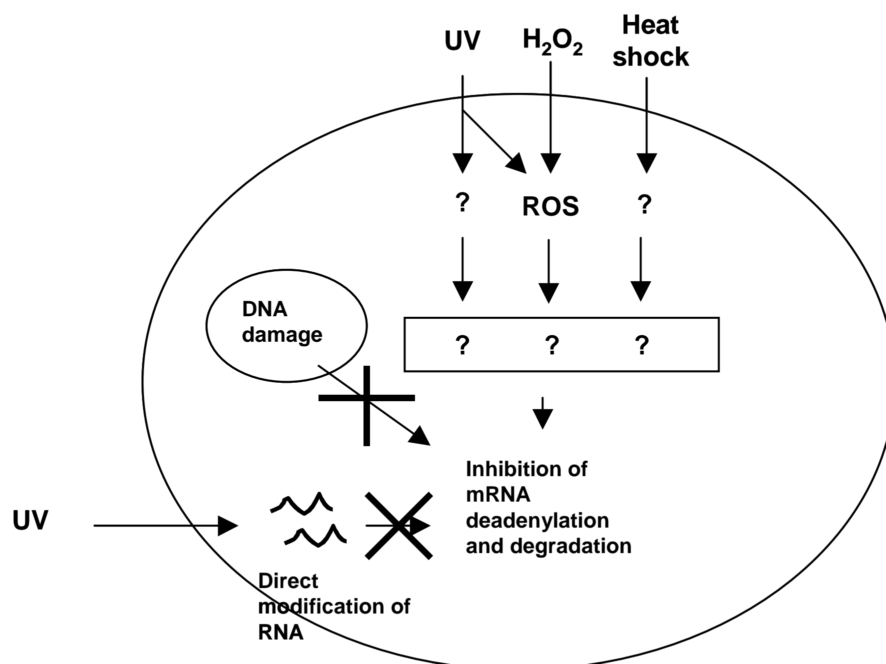


Figure 31: Scheme showing the possible sources of signalling for the effect of UV light on mRNA deadenylation. The inhibition of mRNA degradation by UV light appears not to be a consequence of general DNA damage or a direct modification of the RNA molecules. Nor is it dependant on ROS. Stresses like UV, heat and H_2O_2 , all of which lead to an inhibition of mRNA degradation may activate a common signalling pathway or may activate distinct signalling events that end in the same effect on mRNA stability.

5.6 Physiological relevance of UV-B mediated mRNA stabilization: The relationship between mRNA degradation and translation has always been a point of contention amongst scientists. Studies by Kedersha et al showed that several environmental stresses lead to the accumulation of untranslated RNA in cytoplasmic foci termed stress granules as a consequence of the translational shut down that accompanies exposure to environmental

stresses (ref section 2.4) *(Kedersha N 2002), (Kedersha N 2000)*. But there are contradictory reports as to the fate of the mRNA in these granules. While the group of Paul Anderson suggest that stress granules are closely related to mRNA turnover *(Stoecklin G 2004)*, others have shown that factors involved in mRNA degradation and deadenylation accumulate in foci distinct from the stress granules *(Cougot N 2004)*. Additional results (results not shown) showed that expressing the transdominant inhibitors of stress granule formation, TIA1ΔRRM and TIARΔRRM *(Kedersha NL 1999)*, did not affect UV-dependant mRNA stabilization. This suggests that the UV effect may not be related to the stress granules.

UV light like other environmental stresses leads to a generalized inhibition of protein synthesis which effect presumably requires phosporylation of the translation initiation factor, eIF2α. eIF2α was shown to be phosphorylated by both UV-C and UV-B *(Wu S 2002)*, *(Deng J 2002), (Jiang HY 2004)*. Results in fig 24 show that the effects of UV-B on mRNA degradation and deadenylation occur with the same dose response relation, which falls within the range of the minimal erythemic doses of UV-B light in man. The inhibition of translation on the other hand required higher doses of UV light *(Beniam 2003)*. This thus explains why prolonged stabilization of the endogenous IL-8 mRNA (ref fig 26) is accompanied by increased protein synthesis *(Beniam 2003)*.

Stabilization of RNA by UV light may be an important ATP-conserving mechanism used by cells to compensate for the general decrease in RNA synthesis that occurs in response to UV exposure *(Rockx DA 2000)*. This involves phosphorylation of the C-terminal domain of RNA polymerase II and its subsequent proteasome dependant degradation *(Rockx DA 2000), (Kuznetsova AV 2003), (Lee KB 2002)*. UV-light can stabilize several short-lived transcripts (ref section 2.4) *(Bollig F 2002)*, *(Blattner C 2000), (Wang W 2000).* A common feature of the mRNAs of genes involved in the inflammatory response is their short half-lives. Hence a stabilization of these mRNA by UV light could well be crucial for controlling the extent and duration of gene expression during the inflammatory response. The results with the IL-8 mRNA (fig 26) and the microarray results where there appears to be a stabilization of a group of transcripts including IL-6, Cxcr4 and jun (ref table 2) support this hypothesis. Further studies need to be done with primary keratinocytes to confirm the role of UV-dependant mRNA stabilization in the inflammatory response of the skin.

5.7 PERSPECTIVES:

In the course of this study different modes of mRNA stabilization have been examined: stabilization induced by activation of the p38/MK2 pathway, by increased expression of HuR and by exposure to UV-B, all of which have been suggested to be inter-related. What has emerged is the diversity in the requirements for each mode of stabilization. While the p38/MK2 pathway is specific to a certain group of AREs some of which have bipartite structures, stabilization by overexpression of HuR requires distinct domains that are present in the *c fos* and GMCSF AREs and absent in the IL-8 ARE, thus defining distinct subsets of AREs. The structural and functional importance of these domains for different AREs needs to be examined in future studies. UV-induced stabilization on the other hand does not require the presence of AREs. Both the activation of the p38/MK2 pathway and exposure to UV light lead to an inhibition of mRNA deadenylation, while the overexpression of HuR as seen in our studies and reported earlier has if at all only a marginal effect on deadenylation. More direct evidence in the form of effects on currently known deadenylases needs to be looked at to confirm these differences. The downstream effector of the p38/MK2 pathway responsible for mediating the inhibition of degradation has remained elusive to this date although some evidence has been presented by Stoecklin et al *(Stoecklin G 2004)*. Likewise the signalling events downstream of exposure to UV-B that lead to mRNA stabilization remain unknown. Hence although with the help of this study some answers have been obtained, more questions have been raised and await answering.

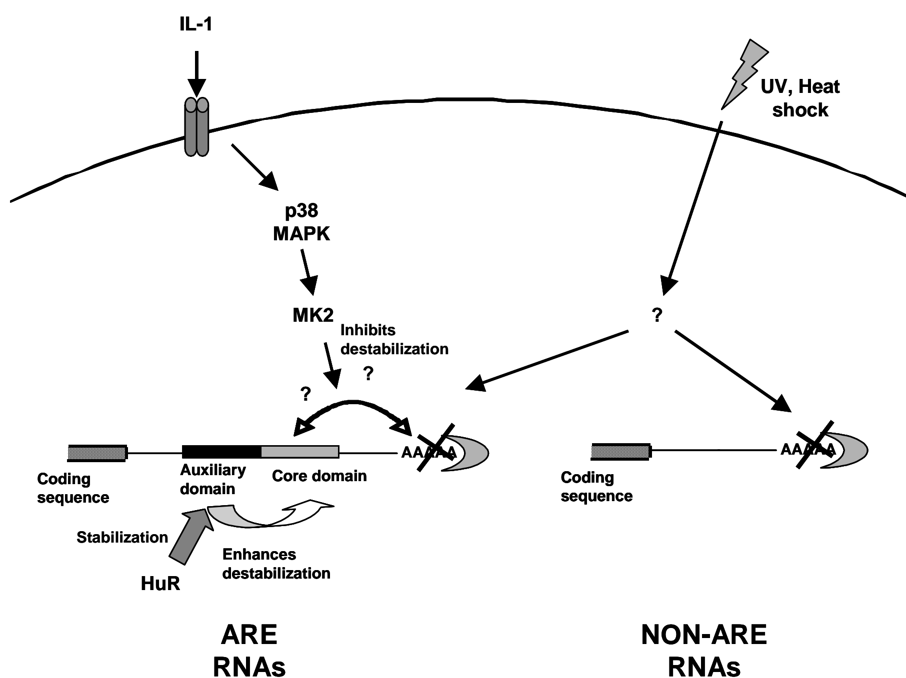


Figure 32: Schematic representation of the summary on the different modes of mRNA stabilization examined in this study. p38/MK2-dependant mRNA stabilization is specific to ARE-containing RNA, some of which have bipartite structures-with auxiliary and core domains and appears to inhibit the rate of mRNA deadenylation. HuR-dependant stabilization affects a distinct but overlapping subset of AREs and appears to act through the presence of auxiliary domains in these AREs. Here the stabilization appears to have no or minimal effects on deadenylation. Direct cellular stresses like UV light and heat shock through an as yet unidentified pathway lead to a stabilization of both ARE and non ARE-containing RNA. This stabilization is accompanied by a strong inhibition of deadenylation.

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7 ABBREVIATIONS

%	Percent
α	alpha
β	beta
A	Adenine
ARE	AU-rich element
ARE-BP	ARE-binding protein
bp	base pairs
˚C	degrees celsius
cDNA	complementary DNA
cm^2	squared centimeter
CSPD	chemiluminescent substrate
DEAE	Diethylaminoethyl
dH_2O	distilled water
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DIG	Digoxygenin
DNase	deoxyribonuclease
dNTPs	deoxyribonucleoside triphosphate mix
DTT	Dithiothreitol
EtBr	Ethidium Bromide
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetracetic acid
eIF	eukaryotic initiation factor
ERK	extracellular signal-regulated kinase
et al	and others
FCS	fetal calf serum
Fig	figure
g	gram
g/l	gram per liter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GMCSF	granulocyte/macrophage colony stimulating factor
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HEBS	Hepes buffered saline
h/hrs	hours
IL	interleukin
JNK	*c jun* N-terminal kinase
Kb	kilobase (pair)
kDA	kilodalton
LB	luria-bertoni
LPS	lipopolysaccharide
µ	micro (10^{-6})
m	milli (10^{-3})
M	molar
mA	milliampere
mM	millimolar
MAPK	mitogen activated protein kinase
MEKK	MAPK/ERK kinase kinase
mg	milligram

min/s	minute/s
MKK	MAP kinase kinase
ml	milliliter
mol	Mole (6.023 x 10^{23} molecules)
MMLV	mouse mammary leukemia virus
mRNA	messenger RNA
NaCl	Natrium chloride
nm	nanometre
nt	nucleotide
OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PABP1	poly (A) binding protein 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmoles	picomoles (10^{-12} moles)
RCF (g)	relative centrifugation force
ref	refer to
RNA	ribonucleic acid
RNase	ribonuclease
ROS	Reactive oxygen species
rpm	revolutions per minute
RRM	RNA recognition motifs
RT	reverse transcription/room temperature
secs	seconds
SAPK	stress activated protein kinase
SDS	Sodium dodecyl sulphate
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TEMED	N, N, N’, N’- tetramethlenediamine
TIMP	tissue inhibitor of matrix metallo-protease
TNF	tumour necrosis factor
Tris	Tris (hydroxymethyl)-aminoethane
U	units
UTR	untranslated region
UV	ultraviolet light
V	volt
v/v	volume by volume
w/v	weight by volume
X	times

8 LEBENSLAUF

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9 LIST OF PUBLICATIONS

Articles:

- Distinct Domains of AU rich elements exert different functions in mRNA destabilization and stabilization by p38 MAP kinase or HuR
  Reinhard Winzen, Gayatri Gowrishankar*, Frank Bollig, Natalie Redich, Klaus Resch, and Helmut Holtmann*
  * equal contribution
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- Inhibition of mRNA degradation and deadenylation by ultraviolet light and other types of cell stresses
  Gayatri Gowrishankar, Reinhard Winzen*, Oliver Dittrich-Breiholz, Frank Bollig, Beniam Ghebremedhin, Natalie Redich, Birgit Ritter, Klaus Resch, Michael Kracht, and Helmut Holtmann*
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Posters:

1. Distinct domains of the IL-8 ARE involved in rapid mRNA decay and inducible stabilization.
   Gayatri Gowrishankar, Reinhard Winzen, Natalie Redich, Frank Bollig, Klaus Resch, Helmut Holtmann
   Structure, function and dynamics of RNA-protein complexes, Göttingen, Germany: 17.09-20.09.03 pg 72.

2. Control of cytokine mRNA stability by the p38 MAP kinase/MAPKAP kinase 2 pathway.
   Gowrishankar G, Winzen R, Bollig F, Redich N, Resch K, Holtmann H.
   10th International TNF superfamily Conference, Lausanne, Switzerland: 29.09-02.10.04 P72 pg 96.

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