Mechanisms of CDE-dependent mRNA decay

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Bernd Rattenbacher
aus Deutschland

Basel, 2007

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel auf Antrag von

Professor Witold Filipowicz und Professor Christoph Moroni

Basel, den 23. Januar 2007

Prof. Dr. Hans-Peter Hauri
Dekan der Philosophisch-
Naturwissenchaftlichen Fakultät

Summary

The cytokine TNFα is a potent effector of inflammation, and is causally involved in toxic shock syndrome and rheumatoid arthritis. It is vital to maintain continuous control over TNFα levels under continuous control, which can be achieved in one way by regulating the decay rate of the TNFα mRNA. One cis-element, the AU-rich element (ARE) in the TNFα 3'UTR confers to rapid degradation of this message. When the element is knocked out in mice, TNFα is overproduced resulting in the corresponding pathology.

We recently described a second cis-element, the constitutive decay element (CDE), that is unique to TNFα and, compared to the ARE, is unresponsive to TNFα RNA stabilizing signals such as lipopolysaccharide (LPS) or phorbol esters. Furthermore a CDE-bearing reporter was degraded in Slow C cells that are defective in ARE dependent decay, implying that CDE mediated degradation is performed by a different mechanism. Through deletion mutation we were able to define a 40 nucleotide (nt) stretch (fragment O), located 42 nt downstream of the ARE, that is the minimal functional CDE. Any mutation to this minimal sequence was not only inactive in promoting decay, but also exhibited differences in protein binding compared to fragment O. The CDE binding proteins were assayed by UV-crosslinking assays, purified by biochemical fractionation and identified by mass spectrometry. The RNA-binding protein nucleolin was the prime candidate. We produced full length recombinant nucleolin with the baculovirus system, and could demonstrate that recombinant nucleolin binds in vitro to fragment O but not its mutated sequences by electrophoretic mobility shift assay. The nucleolin-CDE association was also observed with band shift and super shift assays in THP-1 human macrophage cytoplasmic extracts. Furthermore we could show by RNA immunoprecipitation assays in THP-1 cells, that nucleolin is specifically associated with endogenous TNFα mRNA in vivo, but not with the transcripts of IL-6 and IL-10, or GAPDH. To assess whether down regulation of nucleolin by RNA interference would affect

CDE mediated decay, we transfected nucleolin or control siRNAs into a human HT1080 GFP-CDE reporter cell line. By FACS we observed an increase in GFP protein levels with the nucleolin specific siRNAs but not with the control siRNAs, indicative of elevated CDE-reporter mRNA levels. Actinomycin D chase experiments confirmed stabilization of the CDE reporter with a 40% increase in reporter mRNA half-life from 96±2 min to 135±11 min, that was statistically significant. We conclude that nucleolin is associated with the CDE in vitro and in vivo and is be functionally involved in the CDE-mediated degradation pathway.

Table of contents

Abreviations

ARE	AU-rich element
AUBP	ARE binding protein
CDE	Constitutive decay element
CDEBP	CDE binding protein
Cpm	Counts per minute
FACS	Fluorescence activated cell sorter
GAPDH	Glycerine-aldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSH	Glutathion
GST	Glutathion-S-transferase
IL-2, 6 or10	Interleukin 2, 6 or 10
IP	Immunoprecipitation
LPS	Lipopolysaccharide
NCL	Nucleolin
NRE	Nucleolin recognition element
PAGE	Polyacrylamide gel electrophoresis
RNAi	RNA interference
RRM	RNA recognition motif
SiRNA	small interfering RNA
TeV	Tobacco etch virus
TNFα	Tumor necrosis factor alpha
UTR	Untranslated region

Less frequently used abbreviations are defined upon their first use in the text.

1. Introduction

1.1 Life cycle of an mRNA

Messenger RNA is transcription from the genomic DNA template by RNA polymerase II (Fig. 1). Whether a specific gene is transcribed or not depends upon the transcription factors and transcription enhancer factors present within a certain cell type or situation. By regulating these factors, the mRNAs that are needed in response to an external signal can be produced when required. To protect mRNA from rapid degradation, it is chemically modified on the 5' end by a 5'-methyl-guanosine cap structure that is attached co-transcriptionally and at the 3' end by the poly (A)-tail consisting of 150 to 250 adenosine residues which are added post-transcriptionally by the poly(A)-polymerase after cleavage of the nascent mRNA by an endonuclease at a specific signal sequence. During maturation of mRNA, alternative events can lead to heterogeneity for a particular mRNA species. One such event is alternative splicing. By choosing the exons to be included or omitted, different messages can arise from the same transcript and which may even have opposing effects. The current record holder in respect to alternative splice forms is the receptor molecule Dscam in Drosophila with 38016 putative splice forms. Dscam is involved in axon branching in Drosophila and it was shown that reduction of the maximal splice forms to 22176 by deletion resulted in axon branching defects (Chen, et al. 2006). Another example is the mRNA coding for the RNA-binding protein AUF1 that is expressed in four isoforms (Wagner et al. 1998). Two of the resulting isoforms, p37 and p42, stabilize their target transcripts whereas the p40 and p45 isoforms destabilize them (Raineri et al. 2004). Another step that can be regulated is the export of the mRNA. Some messages can be retained in the nucleus, and only be released into the cytoplasm upon receipt of a stimulus, as with the Lys2 mRNA in yeast (Das et al. 2006). Once exported, the mRNA can direct protein translation by ribosomes, a process which can

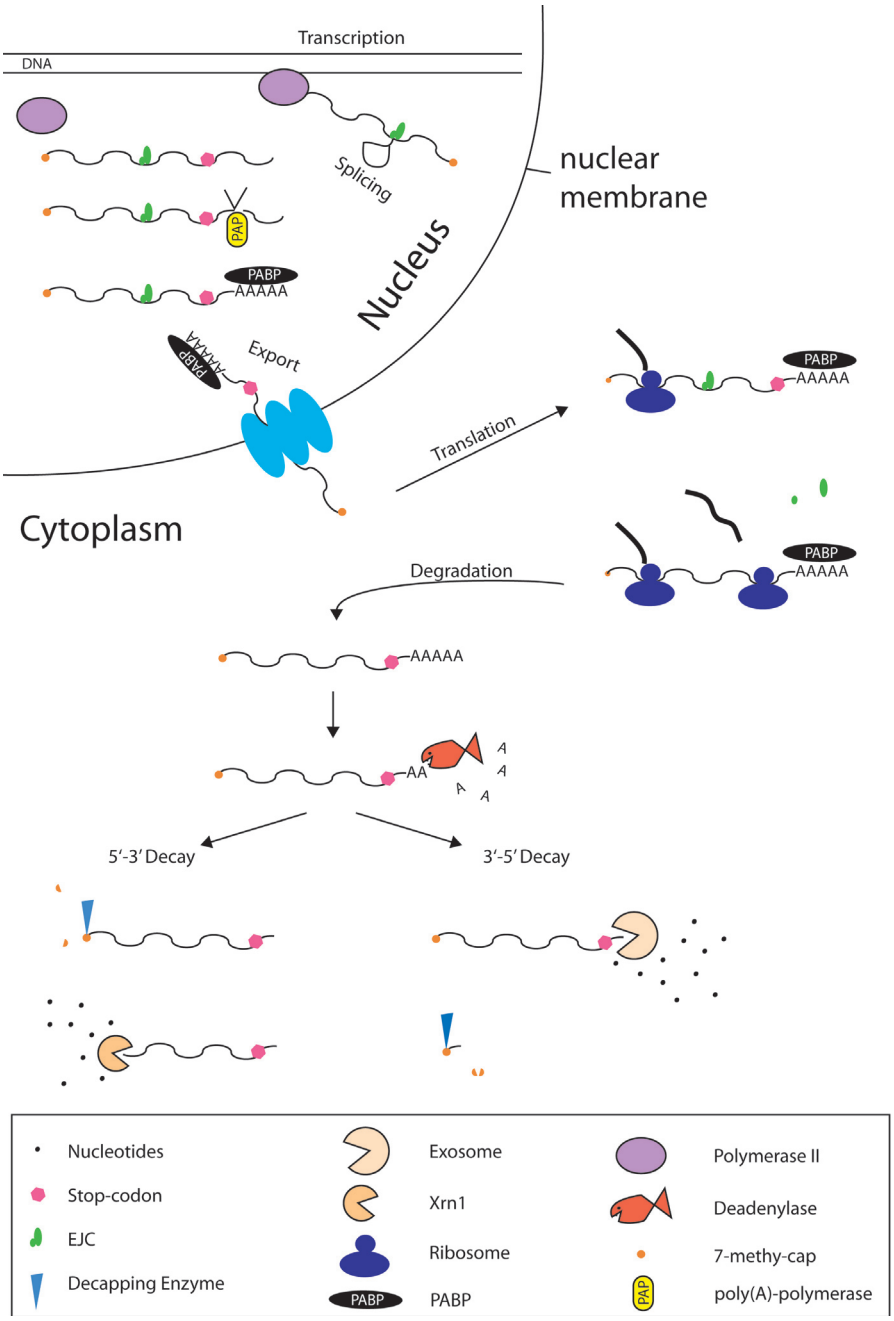


FIG. 1. Life cycle of a messenger RNA. mRNA is transcribed from genomic DNA by RNA polymerase II. The mRNA is then capped at the 5'end and spliced co-transcriptionally. The final step in mRNA maturation is polyadenylation by the poly(A) polymerase (PAP), which adds adenosine residues in a template independent fashion after an endonucleolytic cut at the polyadenylation site. The fully processed RNA is translation competent and is exported into the cytoplasm for translation on ribosomes. Finally, when the lifetime of an RNA is over, the mRNA is deadenylated and undergoes degradation by the 5'-3' or 3'-5' degradation pathway. In the 5'-3' degradation pathway, the 7-methylguansine-cap structure is cleaved by a decapping enzyme, and the unprotected mRNA is subsequently degraded completely by the exonuclease Xrn1. In the 5'-3' degradation pathway, first the poly(A) tail of the mRNA is removed by deadenylases and the RNA body is then degraded by the exosome. Finally the remaining 7-methyguanosine-cap structure is cleaved by a scavenging complex.

also be regulated by RNA binding proteins or special short micro RNAs (miRNA) (Piecyk et al. 2000; Reinhart et al. 2000; Pillai et al. 2005).

At the end of the mRNA's live cycle it needs to be degraded. Among the earliest known properties of mRNA after its discovery in 1961 in *E.coli* cells, was its instability with half-lives in *E.coli* ranging from 30 seconds to 20 minutes (Gros et al. 1961). In eukaryotic cells however, the mRNA half-lives are longer, from about 20 minutes up to more than 24 hours, and the degradation is executed by different nucleases. In eukaryotes, degradation of mRNA is commenced by two different pathways, the 5'-3' and the 3'-5' exonucleolytic decay pathways. In the 5'-3' decay pathway, the message is first decapped, and afterwards degraded by the exonuclease Xrn1 (Parker and Song 2004). In the 3'-5' pathway, the mRNA has first to be deadenylated by deadenylases before the RNA body can be degraded by the exosome. The last step in this pathway is the removal of the 7-methyl guanosine cap structure by a decapping enzyme (Parker and Song 2004). The decay kinetics of a specific RNA can be further influenced by proteins inducing stabilization or degradation of specific mRNAs (Ross 1996). In this work we will mainly concentrate on mRNA turnover, and not on the other aspects presented before.

1.2 Posttranscriptional regulation of gene expression

The genetic information of an organism has to be translated into effector molecules, which are mainly proteins. The multi-step process of expressing the genetic information from DNA to protein has to be regulated so that only the genes that are needed are expressed, and at an appropriate level of expression. For example; excess levels of a cleavage product of the amyloid precursor protein is a factor in Alzheimer's disease as reviewed by (Ghribi 2006), and various proteins with oncogenic potential are overexpressed in cancer. It is therefore of great importance to tightly regulate gene expression on multiple levels. The first stage of regulation is at the level of transcription. The transcription rate of certain genes can be

altered by extrinsic or intrinsic signals. The kinetics of transcription alone would result in a linear increase of mRNA in the presence of continuous signalling until transcription is stopped or all cellular resources are exhausted. To counteract this process, and therefore limit the amount of mRNA produced and at the same time replenish the stock of building blocks, the messages are degraded and so create an equilibrium between transcription and degradation. If the message is no longer required, the mRNA can be rapidly cleared after transcription is turned off. A good example to illustrate this process is the cytokine TNFα. Here the levels of TNFα mRNA increase over 4 hours when macrophages are challenged with LPS and decline thereafter again (Stoecklin et al. 2003). Controlling the availability of mRNA is a general way to produce certain proteins that are only required at a certain time-point and for a short period of time

1.3 Degradation of mRNA in eukaryotes

As previously mentioned, the half-lives of mRNAs transcribed from different genes can vary greatly and can be as short as 20 minutes to as long as 24 hours depending on the particular mRNA. It was debated for a long time whether mRNA is intrinsically labile and needs factors for stabilization or vice versa (Ross 1996). Eventually it became clear that RNA is intrinsically quite stable and needs cis- and trans-acting factors to be actively destabilized. On the other hand, super-stabilizing elements and factors have also been discovered, which will be discussed later. However, all mRNAs, independent of their lifetimes, appear to be subjected to common general mechanisms of decay that will be subsequently introduced.

1.3.1 Deadenylation

The prominent pathways of mRNA degradation in yeast and mammals start with the removal of the poly(A)-tail which is carried out by poly(A)-specific exonucleases. Initially, the poly(A)-tail is shortened by the Pan2/Pan3 complex (Sachs and Deardorff 1992; Boeck et al. 1996; Brown and Sachs 1998). In mammals it was reported that the Pan2/Pan3 complex

removes the poly(A) tail leaving 55 to 80 adenosine residues. This shortened poly(A)-tail is removed by the Ccr4-NOT complex (Yamashita et al. 2005), consisting of Ccr4p, which appears to be the main catalytic protein, that is tightly associated with Caf1p. These two core components of the CCR4-Not complex are members of the RNAse D exonuclease family, and are in complex with the proteins Not1-5p, Caf4, Caf40 and Caf130p as reviewed in (Denis and Chen 2003; Parker and Song 2004). Another poly(A)-specific exonuclease is PARN, which was isolated from calf thymus in 1998 and belongs to the RNAse D family of exonucleases, as CCR4 and the Caf-proteins (Korner et al. 1998). This protein however has no homologue in Saccharomyces cerevisiae or Drosophila melanogaster (Tucker et al. 2001). It was found to be mainly located in the nucleus, and knock down of PARN levels by small interfering RNAs did not have an effect on general deadenylation in the cytoplasm (Yamashita et al. 2005). Furthermore it was shown in vitro that PARN interacts with the 5'-^{7}methylGpppC-cap and the poly(A)-tail at the same time and suggests a link between translation and degradation (Dehlin et al. 2000; Gao et al. 2000; Martinez et al. 2001; Seal et al. 2005). After deadenylation of an mRNA, the RNA-body is open to total degradation.

1.3.2 Degradation of the RNA body

The RNA body can be degraded after deadenylation by two different enzymatic activities in the 5'-3' and 3'-5' exonucleolytic pathways.

The major degradation pathway in yeast is executed by 5'-3' exonucleases (Tucker et al. 2001; He et al. 2003; Fritz et al. 2004); however before these enzymes can act, the 7-methyl-guanosine cap structure has to be removed. The cap structure is cleaved by a complex consisting of the decapping enzymes Dcp1 and Dcp2, which are accompanied by the RNA helicase dhh1 (Muhlrad and Parker 1994; Fillman and Lykke-Andersen 2005). The exposed RNA body is subsequently digested by the Xrn1 and Rat1 proteins (Fritz et al. 2004). These two proteins are also present in higher eukaryotes, where the homologue of Rat1p is mainly localized in the nucleus.

A second pathway leads to 3' to 5' degradation of RNA via the exosome (Mitchell et al.

1996; Mitchell et al. 1997; Allmang et al. 1999b). The exosome is not only involved in the degradation of mRNA in the cytoplasm after deadenylation, but also plays a very important role in the maturation of ribosomal RNA in the nucleolus (Allmang et al. 1999a; Allmang et al. 2000), of small nucleolar RNA, of small nuclear RNA (Allmang et al. 1999a), and in the quality control of mRNAs that are aberrantly spliced (Hilleren and Parker 2003). In contrast to the hydrolytic exonuclease involved in the 5' to 3' RNA decay, these enzymes need ATP for phosphorolytic cleavage (Anderson and Parker 1998). The exosome is organized in a multimeric complex forming a ring-like structure (Fig. 2) (Lorentzen et al. 2005), similar to *E.coli* RNAse PH (Symmons et al. 2000). The core components of the human exosome are hRrp41p, hRrp42p, hRrp46p, OIP2, hMtr3p and PM/Scl75. This core associates with the proteins hRpr4p, hRpr40 and hCsl4p (Raijmakers et al. 2002a; Raijmakers et al. 2002b; Raijmakers et al. 2003; Raijmakers et al. 2004) that carry S1 RNA binding

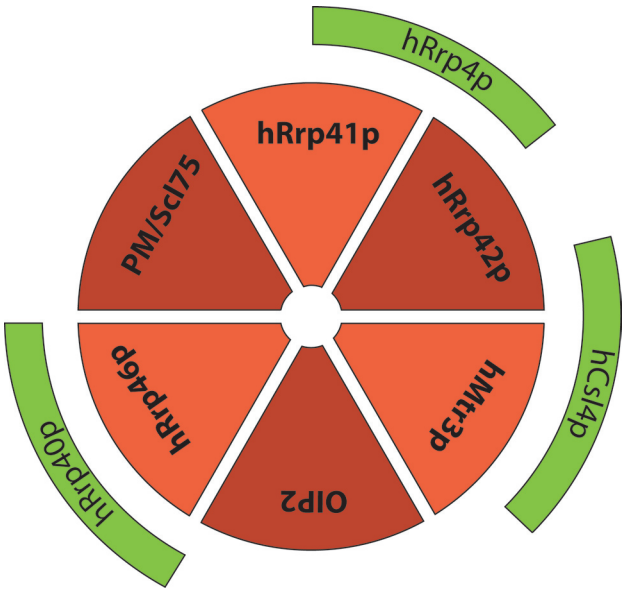


FIG. 2. The exosome. The core exosome consists of the phosphorolytic nucleases hRrp41, hRrp42p, hMtr3p, OIP2, hRrp46p and PM/Scl75, which form a ring-like structure. This core is surrounded by the hydrolytic nucleases hRrp4p, Rrp40p and the RNA-binding protein hCsl4p.

domains. Knockouts of the homologous components in yeast (Rrp4p, Rrp41p, Rrp44p and Ski4p/Csl4p) are viable, and reconstitution of the exosome with the corresponding human homologue of the deleted protein was possible, suggesting a strong conservation of this structure (Mitchell et al. 1997; Allmang et al. 1999b), as supported by the homologues of the exosome components in *Arabidopsis thaliana* (Chekanova et al. 2000; Chekanova et al. 2002), *Trypanosoma brucei* (Estevez et al. 2001; Estevez et al. 2003) and *Drosophila*

melanogaster (Andrulis et al. 2002). In addition, the exosome is associated with the Ski-complex in the cytoplasm (Ski2p, Ski3p, Ski8p) and Ski7p (Brown et al. 2000; Araki et al. 2001), in the nucleus (PM/Scl100, Rrp47p and hMtr4p) and in the nucleolus (Rrp14 and PM/Scl100) (de la Cruz et al. 1998; Allmang et al. 1999b; Jiang and Altman 2002; Mitchell et al. 2003). The human cytoplasmic Ski complex was suggested to play a bridging function between an mRNA and the exosome, thereby targeting the mRNA for degradation (Araki et al. 2001). The 7-methyl-guanosine cap structure remaining after 3'-5' degradation of the RNA-body is cleaved by the scavenger-decapping enzyme DcpS (Gu et al. 2004; Liu et al. 2004).

1.3.3 Deadenylation independent decay

Besides the common deadenylation dependent decay, other mechanisms are known to induce degradation of a messenger RNA independently of deadenylation such as RNA interference (RNAi), and quality control mechanisms such as nonsense mediated decay, and the special case of endonucleolytic cleavage of mRNA. These degradation pathways are specialized pathways and are briefly mentioned for the sake of completeness.

1.3.3.1 Small interfering RNA (siRNA) induced degradation

The siRNA-induced degradation is thought to be a defence mechanism against viral RNA (Galiana-Arnoux et al. 2006; van Rij et al. 2006). Double stranded RNA is recognized by dicer, which contains RNAse III domains besides two RNA binding domains, and processed into 21 to 25nt long siRNAs (Zhang et al. 2002) which are incorporated into the RNAi-induced silencing complex (RISC) review in (Hutvagner 2005). RISC can now recognize its target sequence through base pairing, as has been proven by mutating the target sequence (Pillai et al. 2005). The annealing of the siRNA with the target RNA activates an endonuclease within the RISC called AGO2, which cuts the target RNA (Meister et al. 2004) that is then degraded by the exosome and Xrn1 (Orban and Izaurralde 2005).

1.3.3.2 RNA-quality control mechanisms

1.3.3.2.1 Nonsense mediated decay (NMD)

Nonsense mediated decay (NMD) occurs when a premature stop codon (PTC) is introduced within the coding region of an mRNA by transcriptional error or other means. Translation of this transcript would produce a truncated and potentially harmful protein, making NMD an important step in mRNA quality control. During the first round of translation, the exon junction complexes (EJC) that are deposited in the step of splicing at exon-exon junctions are removed by the translating ribosome. If the ribosome now encounters a stop codon upstream of one of these EJCs, the "surveillance complex" consisting of the upf proteins are recruited. In mammalian cells it is believed that the message is degraded after it is decapped, independent of deadenylation, by the 5'-3' exonucleolytic pathway reviewed in(Weischenfeldt et al. 2005). In Drosophila, NMD is independent of the EJCs. When the ribosome encounters a PTC, it stalls, and recruits a yet unidentified endonuclease via the Upf proteins, which cleaves the RNA. The two parts of the RNA are then degraded via the exosome and Xrn1 (Gatfield et al. 2003; Gatfield and Izaurralde 2004).

1.3.3.2.2 Nonstop decay

The opposite situation is encountered in transcripts lacking a termination codon. In this case the affected cell could also produce an aberrant protein that may be harmful to the cell. This process, another mRNA quality control mechanism like NMD, was only recently described, as non-stop decay (Frischmeyer et al. 2002; Vasudevan et al. 2002). Here the ribosome continues to translate the poly(A)-tail, which leads to ribosome stalling and recruitment of the Ski complex (Ski2p, Ski3p and Ski8p) and Ski7p. Through binding of this complex, the exosome is recruited directly to the message and induces its degradation (van Hoof et al. 2002).

1.3.3.3 Endonucleolytic cleavage

Some messenger RNAs can undergo endonucleolytic cleavage and the resulting fragments are subsequently degraded via both the 3'-5' and the 5'-3' pathway in mammalian cells. This has been reported in the case of c-myc and IGF-II and the transferrin receptor (Mullner and Kuhn 1988; Binder et al. 1994; Beelman and Parker 1995; Lemm and Ross 2002).

1.4 Determinants of mRNA stability

Decay pathways alone however, do not provide an explanation as to, why different mRNAs can have half-lives ranging from 20 minutes to 24 hours. Short-lived messages mostly code for proto-oncogenes, proteins involved in apoptosis, cell cycle and cytokines that need to respond rapidly to changes in the environment, whereas long-lived messages tend to code for housekeeping genes, which are constitutively expressed. The half-life of a message is determined by cis-elements that either protects the message from degradation or renders the message very unstable. These elements can be found in the 5' UTR, the coding region and predominantly in the 3' UTR of a messenger RNA. We will now describe the different cis-elements along the messenger RNA

1.4.1 Decay regulating elements in the 5'UTR

Elements responsible for the stability of the chemokine KC (Tebo et al. 2000) and the IL-2 mRNA (Chen et al. 1998) have been identified in the 5' UTRs of these messages. After activation of a T-cell, the growth factor IL-2 needs to be stabilized. One element responsible for this is the Jun-kinase response element (JRE) located in the 5' UTR of the IL-2 message. It is bound by a complex of two proteins, nucleolin and YB-1, that are able to stabilize the message upon T-cells activation.

1.4.2 Decay regulatory elements in the coding region

The major coding region determinant (mCRD) is a decay element located in the coding region of c-fos, c-myc and β-tubulin. In β-tubulin, mRNA decay is coupled with translation. The first 13 nucleotides, which are translated, decide whether the transcript is degraded or translated. If sufficient levels of β-tubulin protein are present, the ribosome stalls after the fourth amino acid and the RNA is degraded, preventing an overproduction of tubulin (Gay et al. 1987; Yen et al. 1988; Gay et al. 1989). In c-fos, the mCRD consists of a long (320 nt) purine-rich sequence that is bound by a complex consisting of AUF1 p37, unr, the poly(A) binding protein (PABP), and the PABP interacting protein (Grosset et al. 2000). Unr was identified as the protein directly interacting with the purine-rich region and PABP, and is essential for CRD function. When translation is blocked, c-fos mRNA remains stable, but during ongoing translation, the deadenylase CCR-4 is recruited to unr and initiates the deadenylation of the message, inducing rapid degradation (Chang et al. 2004). In the case of the c-myc mRNA, the CRD is located in the exons 2 and 3 (Yeilding and Lee 1997). This sequence is bound by the CRD-binding protein, which protects the message from endonucleolytic cleavage (Prokipcak et al. 1994; Lemm and Ross 2002). There is also physiological evidence for the importance of this process during myoblast differentiation, where c-myc must be down regulated. The current decay-model is that the ribosome pauses in the absence of the CRD-binding protein, leaving the cleavage site unprotected, which can therefore be cleaved by an endonuclease (Bergstrom et al. 2006).

1.4.3 Decay elements in the 3'UTR

1.4.3.1 The iron response element

The iron response element (IRE) was the first element discovered regulating mRNA decay. It was defined as a loop structure (23 to 27 bases long) located in the 3' UTR of the transferrin receptor (TfR) and in the 5' UTR of ferritin where it controls translation. Both proteins are involved in regulating the iron level in the cell. At low iron levels, two iron dependent regulatory proteins, IRP1 and 2, bind to the IRE of the TfR-mRNA, stabilizing the message,

which leads to production of TfR and subsequently to iron uptake. At the same time, IRP1 and 2 bind to the ferritin 5'UTR, where they prevent translation and with that any further iron sequestration by ferritin. If sufficient iron levels are present again, iron binds to the IRPs and inactivates them, leading to reduced iron uptake and increased iron storage reviewed in (Rouault and Klausner 1997).

1.4.3.2 Stem loop structures

Another decay promoting element was found in the 3'UTR of the insulin growth factor-II (IGF-II) mRNA, which consisted of a stem loop. By an as yet unknown mechanism, the RNA is cleaved by an endonuclease and is subsequently degraded (Scheper et al. 1995). A stem-loop binding protein has been identified, but its role in the degradation of this mRNA could not be proven (Scheper et al. 1996; van Dijk et al. 2001).

A special case is histone H3 mRNA, an atypical RNA polymerase II transcript, which is not capped or polyadenylated. It also contains a short stem loop at its 3' end (Pandey and Marzluff 1987), which was implicated in cell cycle driven decay of the histone H3 message mediated by a stem loop binding protein (Whitfield et al. 2000). In an in vitro system, it could be shown that the histone H3 protein promotes its own decay but not the decay of control RNAs (Peltz and Ross 1987). It was reported that the histone H3 mRNA degradation is promoted by the exonuclease 3'hExo which specifically interacts with the histone H3 stem-loop (Yang et al. 2006).

1.4.3.3 The AU-rich element

The best-studied cis-element responsible for degradation is the AU-rich element (ARE), which is located in the 3'UTR of about 5% to 8% of all messenger RNAs (Bakheet et al. 2001; Bakheet et al. 2006). When this element is inserted in the 3' UTR of an α-globin reporter, the normally very stable α-globin message (half-life of more than 12h) becomes very unstable and is degraded in about half an hour (Shaw and Kamen 1986) illustrating nicely the power of this element. As the name AU-rich element already implies, the

sequences responsible for this rapid decay are rich in adenosine and uracil residues and are divided into three different subclasses. Classes I and II contain AUUUA pentamers, which can overlap to form AUUUAUUUA nonamers and are frequently found to be in an U-rich context (Chen and Shyu 1995). Class III AREs do not contain regular pentamers, but are generally U-rich (Peng et al. 1996). The different classes are distinguished by sequence criteria and do not necessarily reflect functional differences, as some ARE binding proteins, like HuR and KSRP, can bind to all three classes (Wein et al. 2003; Gherzi et al. 2004; Lopez de Silanes et al. 2004b). The functional differences and protein specificities of AU-rich elements in different mRNAs seem to be influenced by sequences surrounding the ARE (Brewer et al. 2004; Winzen et al. 2004), and by the conformation of the AU-rich sequence itself (Fialcowitz et al. 2005).

ARE-function is mediated through binding of specific proteins. Under normal conditions, decapping, deadenylation and exosomal degradation of the mRNA body are promoted by the ARE (Shyu et al. 1991; Xu et al. 1997; Gao et al. 2000; Chen et al. 2001; Mukherjee et al. 2002; Stoecklin et al. 2006). Different stimuli can abolish the rapid degradation of a message caused by an ARE. Over expression of the ras oncogene stabilizes ARE containing messages (Schmidlin et al. 2004), and deregulation of ARE mediated turnover can also contribute to oncogenic transformation (Nair et al. 1989; Marderosian et al. 2006). Stabilization of messages through the ARE also occurs under physiological conditions, such as T-cell activation (Lindstein et al. 1989) or, when mast cells respond to allergens (Wodnar-Filipowicz and Moroni 1990), which leads then to the production of cytokines relevant for the immune response, many of which are encoded by ARE-containing transcripts. Furthermore, the knockout of the TNFα-ARE leads to the overproduction of TNFα be increasing the mRNA half-life to 6 hours and causes rheumatoid arthritis (Kontoyiannis et al. 1999). It has been reported that the ARE not only controls the stability of a message, but also the translation of the encoded protein (Kontoyiannis et al. 1999; Piecyk et al. 2000). These various ARE-induced effects are mediated by ARE binding proteins (AUBPs) and will be discussed later in the introduction.

1.4.3.4 The constitutive decay element (CDE)

The constitutive decay element, the focus of study of this thesis, was recently discovered in our laboratory. It is unique to the TNFα 3' UTR, and its sequence, which may form a stem loop, is highly conserved in mammals. Insertion of the CDE in a heterogeneous reporter causes rapid degradation of the reporter construct. When point mutations are inserted into the putative stem-loop region, the reporter decay is abrogated. However complementary mutations are not sufficient to restore decay activity, indicating that the actual sequence of this region is also important. Furthermore it was shown that, unlike the ARE, the CDE is insensitive to stabilizing signals like LPS, TPA, ionomycin, and transfection of activated ras and MEKK6, hence the name constitutive decay element. This element appears to be essential in order to tightly control the production of TNFα (Stoecklin et al. 2003).

1.4.3.5 Stabilizing elements

Apart from destabilizing elements, stabilizing elements have been identified in α- and β-globin. The α- and β-globin proteins build the haemoglobin molecules in red blood cells, and have to be produced for the entire lifetime of these denucleated cells, and therefore their mRNA has to be extremely stable. The α-globin mRNA contains a pyrimidine-rich element in its 3' UTR, which is bound by the α-complex consisting of members of the αCP/hnRNP-E family and AUF1 (Wang et al. 1995c; Kiledjian et al. 1997). This complex interacts with the poly(A) binding protein to increase its binding efficiency to the α-globin poly(A)-tail, which is now protected from deadenylation (Wang et al. 1999; Wang and Kiledjian 2000c). The situation is similar in the case of β-globin. Here the element comprises of a half-stem, which needs to be opened by nucleolin in order to facilitate αCP binding and through this, protection from deadenylation (Jiang et al. 2006).

1.5 Protein factors involved in mRNA stability

As stated above, the ARE can target mRNA for rapid degradation. This cis-element is recognized by specific trans-acting factors that can induce rapid deadenylation, decapping and decay of the main RNA body. Another group of AUBPs can stabilize the ARE when a particular message needs to be expressed, usually after a stimulus, or during the cell cycle. A third group of AUBPs acts by controlling translation.

1.5.1 Destabilizing AUBPs

1.5.1.1 The Tis11/Zfp36 family

Tristetraprolin (TTP) is the founder member of a family of decay promoting proteins, although they possess no intrinsic nuclease activity. Four family members are known so far in mammals, TTP (Tis11, Zfp36p), butyrate response factor 1 (BRF1/Zfp36L1), butyrate response factor 2 (BRF2/Zfp36L2), and zinc finger protein 36 like 3 (Zfp36L3). The common feature of these four proteins is the possession of two unusual C-X_8-C-X_5-C-X_3-H zinc finger motifs that are preceded by the highly conserved YKTEL sequence (Varnum et al. 1991; Amann et al. 2003; Michel et al. 2003; Hudson et al. 2004; Blackshear et al. 2005). Homologous proteins are also found in yeast, frog and fish (Thompson et al. 1996; De et al. 1999).

1.5.1.1.1 Tristetraprolin (TTP/Tis11/Zfp36p/NUP475/GOS24)

TTP was simultaneously cloned by three different groups using independent approaches that uncovered different aspects of TTP expression. A partial cDNA sequence was found in a screen identifying TPA responsive genes (Varnum et al. 1989), it was also identified as a serum responsive gene in the nucleus (nuclear protein 475 (NUP475)) (DuBois et al. 1990), and finally as an early response protein induced by insulin. A characteristic proline-proline-proline-proline-glycine sequence, which is repeated three times in the protein, led to its being called tristetraprolin (TTP) (Lai et al. 1990). Consequently it was found to be inducible by other means for example with EGF, LPS and other growth stimulatory signals

(Carballo et al. 1998; Fairhurst et al. 2003). No function to the protein could be assigned to the protein until the generation of TTP knockout mice. Homozygous mice are viable, and apparently normal, but after a few months they begin to loose weight, and develop myeloid hyperplasia, polyartricular erosive arthritis, dermatitis, conjunctivitis, alopecia and autoimmunity (Taylor et al. 1996). This phenotype was similar to one induced by chronic application of the cytokine TNFα (Keffer et al. 1991), and could be completely suppressed by treatment with neutralizing TNFα antibodies, concluding that TTP knockout induces TNFα over expression (Taylor et al. 1996). Later it became clear that the knock out of TTP causes an increased stability of the TNFα and GMCSF mRNA. It is also involved in the regulation of interleukin 2 (Ogilvie et al. 2005), and the inducible nitric oxide synthetase (iNOS) (Linker et al. 2005). Other TTP binding mRNAs were identified by comparing RNA-immunoprecipitation followed by expression profiling of $TTP^{-/-}$ cells to the wild type. 250 transcripts were stabilized under knock out conditions, of which 23 carried AU-rich elements (Lai et al. 2006).

Mechanistically, TTP binds the AU-rich element via its two zinc finger domains (Lai et al. 1999) and thereby mediates degradation of the message (Carballo et al. 1998), and mutations in the zinc fingers abrogate ARE binding and decay (Lai et al. 2000). As TTP has no nuclease activity, it must be able to recruit the degradation machinery as was demonstrated by tethering experiments with TTP was fused to the MS2 protein (Lykke-Andersen and Wagner 2005). It could be shown that the N terminal portion of TTP interacts with and activates the decapping machinery (Fenger-Gron et al. 2005), deadenylases, and the exosome, thereby causing rapid degradation (Chen et al. 2001; Lykke-Andersen and Wagner 2005; Hau et al. 2006).

TTP activity is negatively regulated by external signals leading to phosphorylation of the serines 52 and 178 by MK2 (Stoecklin et al. 2004; Hitti et al. 2006). Inhibition of the p38 pathway leads to a relocalization from the cytoplasm to the nucleus. Furthermore, this phosphorylation causes the entry of TTP into stress granules by interaction with 14-3-3, and its decay promoting activity is reported to be inhibited (Stoecklin et al. 2004). This seems to be a controversial point, because another group did not find an influence of the p38

signalling-pathway on TTP function (Rigby et al. 2005). In addition it was also found that phosphorylation of TTP on these two sites causes stabilization of the protein (Brook et al. 2006).

1.5.1.1.2 Butyrate response factor 1 (BRF1/TIS11b/Zfp36L1/Berg36/ERF1/cMG1)

Butyrate response factor 1 (BRF1) was discovered in rat intestinal epithelial cells in early response after stimulation with the epidermal growth factor (EGF) (Gomperts et al. 1990). The human homologue was cloned later (Bustin and McKay 1999), and its expression can be induced by insulin, insulin-like growth factor 1 (IGF-1) (Corps and Brown 1995) and adrenocorticotropin (ACTH) (Chinn et al. 2002), and repressed by butyrate (Maclean et al. 1998). The function of BRF1 has recently been discovered by a genetic screen (Stoecklin et al. 2002). Here a GFP reporter was fused to the IL-3 3'UTR, which contains an ARE and renders the reporter unstable, and introduced into human HT1080 fibroblast cells. By treating these cells with a frame shift mutagen and selection for high GFP expression a cell line was isolated, slow C, in which the reporter transcript was stabilized. By reintroduction of a cDNA library from the parental cell line, BRF1 was cloned as a gene capable of restoring rapid mRNA decay. It was confirmed that in slowC cells, both alleles of BRF1 contained frame-shift mutations. BRF1 binds like TTP to AU-rich sequences via its zinc fingers and mutation of these leads to abrogation of degradation due to loss of RNA binding (Stoecklin et al. 2002). The mechanism by which BRF1 mediates decay is also similar to that of TTP via recruitment of deadenylases, the decapping machinery and the exosome to the targeted RNA (Lykke-Andersen and Wagner 2005). One of the BRF1 target genes was published to be VEGF (Ciais et al. 2004), but in our group, we have seen no effect of BRF1 on the stability of this putative target that could be confirmed using the $BRF1^{-/-}$ cell line. Nevertheless VEGF expression is increased in a $BRF1^{-/-}$ context (Raineri, unpublished data), but probably by an unknown mechanism unrelated to mRNA degradation. As with TTP, BRF1 is also responsive to extra cellular stimuli. Activation of the PI3 kinase-pathway by insulin leads to the phosphorylation of BRF1 on S92 and S203 by PKB/Akt, which results

in BRF1-14-3-3 binding, and inactivation of the BRF1 decay promoting activity. Similar to TTP, BRF1 protein is stabilized by the phosphorylation of these two sites (Schmidlin et al. 2004; Benjamin et al. 2006).

1.5.1.1.3 Butyrate response factor 2 (BRF2/Tis11d/Zfp36L2/ERF2)

Mouse BRF2 was discovered as the third member of the zinc finger protein 36 family (Varnum et al. 1991), and the human homologue a few years later (Nie et al. 1995). As with the other family members, it contains the highly conserved zinc finger region and is most identical to BRF1, with the exception of a unique extension at the 5' terminus. Therefore it was no surprise that BRF2 is also able to induce ARE-mediated degradation, as shown with the TNFα-ARE (Lai et al. 2000). Recently, the structure of the BRF2 zinc finger domain has been resolved by NMR in complex with an UUAUUUAUU nonamer, showing that each zinc finger interacts with an UUAU sequence (Hudson et al. 2004). It is suspected, because of the high level of identity between the zinc fingers, that the binding of TTP and BRF1 is similar although this remains to be proven. To elucidate BRF2 function, a knock out mouse has been produced that lacks the N-terminal 29 amino acids. The offspring were viable, in contrast to the BRF1 knock out mouse, and the only phenotype seen was female infertility. Transplantation of wild type ovaries was able to restore fertility (Ramos et al. 2004).

1.5.1.1.4 Zinc finger protein 36-like 3

This protein was only recently discovered on the X-chromosome in mouse by a database search for the tandem zinc finger motif. A homologue could be detected in rat, but not in humans. It is expressed in trophoblastic placenta and extra embryonic tissue, and GFP-Zfp36L3 fusion proteins could only be detected in cytoplasm. Like the other members, it is able to bind AU-rich elements and was shown to promote degradation of ARE-bearing messages when overexpressed. It was also able to promote degradation in vitro (Blackshear et al. 2005).

1.5.1.2 K homology-type splicing regulatory protein (KSRP/FBP2)

K homology-type splicing regulatory protein (KSRP) was originally identified in neurons involved in c-src splicing (Min et al. 1997). It contains four KH-type RNA binding motifs (for K-homology), which are essential for KH-protein driven differentiation in flies, worms and mammals (Adinolfi et al. 1999). KSRP was found to be ubiquitously expressed, and could be shown to interact with the TNFα, c-fos, pitx2 and IL-8 -AREs (Chen et al. 2001; Briata et al. 2003; Gherzi et al. 2004; Suswam et al. 2005). It was also implicated to play a role in the myoblast to myocyte transition by interacting with the AREs of myogenic transcripts (Briata et al. 2005), and in the regulation of human iNOS, together with TTP (Linker et al. 2005). KSRP copurified with the exosome and it is speculated, that KSRP might bridge the ARE-containing RNA and the exosome, and thus induce degradation (Chen et al. 2001). Further studies revealed that KSRP associates with the deadenylase PARN, the decapping enzyme Dcp2, and the exosomal protein Rrp4 via its KH2 domain (Gherzi et al. 2004; Chou et al. 2006). Recently it was found that over expression of KSRP in the BRF1$^{-/-}$ slow C cell line is able to restore ARE-mRNA degradation. KSRP activity is also regulated by phosphorylation via the p38 pathway, which leads to the inhibition of mRNA decay activity (Briata et al. 2005).

1.5.1.3 Polymyositis-scleroderma antigen (PM-Scl 75)

An ARE-binding protein that was found to be a core component of the exosome is the polymyositis-scleroderma antigen (PM-Scl75) (Mukherjee et al. 2002). It is believed that this interaction facilitates ARE-dependent decay. It could be shown in vitro that a 266 amino acid long region from the RNAse PH domain of PM-Scl 75 is responsible for the AU-specific binding. Furthermore, other components of the exosome such as OIP and Rrp41p are implicated in AU-rich RNA binding (Andersonet al. 2006). However the issue of specific AU-rich element binding of PM-Scl 75 is not generally accepted yet.

1.5.1.4 CUG binding protein 2 (NAPOR2/ETR-3/BrunoL3)

CUG binding protein 2 (CUGBP2) belongs to the CELF (CUGBP-Etr-3-like factor) family, which is thought to be involved in alternative splicing and RNA editing (Ladd et al. 2001), and was first identified as a protein binding CUG-repeats in myotonic dystrophy (Lu et al. 1999). It contains three RNA recognition motives (RRMs/RBPs), of which two are structured in a tandem repeat (Choi et al. 1998). This protein was shown to interact with the ARE of cyclooxygenase 2 (COX-2), where it stabilizes the message, but paradoxically inhibits translation (Mukhopadhyay et al. 2003).

1.5.1.5 RHAU

The RNA helicase RHAU was found as a protein binding the AU-rich element of the urokinase plasminogen activator (uPA). The alternative splice form of this protein is localized to the cytoplasm and affects the degradation of the uPA-ARE, for which the ATPase activity of the helicase is needed. RHAU was also found to interact directly with PARN, and the exosome, which then promotes decay (Tran et al. 2004).

1.5.2 Stabilizing AU-binding proteins

1.5.2.1 HuR (HuA)

HuR was originally identified in Drosophila melanogaster by a genetic screen in the embryonic lethal abnormal vision (elav) locus and is essential for neuronal development (Campos et al. 1985). It is a member of the ELAV family of RNA binding proteins, which contain three RRMs. Several mRNA targets could be identified for this protein, including IL-3, c-fos and TNFα amongst others by UV-crosslinking assays, bandshift, and RNA-immunoprecipitations followed by analysis on a genome array (Ma et al. 1996; Dean et al. 2001; Lal et al. 2004; Lopez de Silanes et al. 2004a). Unlike the other AUPBs discussed so far, HuR stabilizes ARE-containing RNAs when overexpressed (Fan and Steitz 1998b; Peng et al. 1998; Chen et al. 2001; Ming et al. 2001). This is supported by HuR knock down experiments by anti-sense RNA or RNAi techniques, which led to a destabilization of the messenger

RNAs tested (Wang et al. 2000a; Wang et al. 2000b; Raineri et al. 2004). Computational analysis indicates that HuR does not necessarily bind to classic AREs, but generally to U-rich regions (Lopez de Silanes et al. 2004b), and protects the message from degradation. HuR possesses three RRMs, where RRM 1 and 2 are in tandem. Deletion of the third RRM, which was suggested to bind the poly(A)-tail (Ma et al. 1997), abrogates stabilization of an ARE-containing message despite over expression (Fan and Steitz 1998b). The stabilization of certain messages, like the tumor suppressor p21, RhoB (Wang et al. 2000b; Westmark et al. 2005), or CD83 (Prechtel et al. 2006) was correlated to HuR presence in the cytoplasm, which is normally localized to the nucleus. The nuclear localization is caused by a nuclear localization signal (NLS) situated between RRMs two and three (Fan and Steitz 1998a), and it can be exported into the cytoplasm via the export protein CRM-1. This export is induced by various stimuli like heat shock (Gallouzi et al. 2003), UV-irradiation (Wang et al. 2000a), and other signals, which subsequently lead to the stabilization of ARE containing messages. It is believed that HuR is co-exported with the message thereby increasing its stability (Fan and Steitz 1998a; Fan and Steitz 1998b). A protein that co-purifies with HuR is the poly(A)-binding protein (PABP) and it is suggested that HuR stabilizes the message by increasing the affinity of PABP for the poly(A)-tail, and so protects the message from degradation (Nagaoka et al. 2006). Several other proteins have been found to associate with HuR, namely SETα/β (von Lindern et al. 1992), pp32 (Malek et al. 1990), APRIL (Mencinger et al. 1998), where SETα/β and pp32 were previously identified to inhibit the phosphates PP2A (Li et al. 1996; Saito et al. 1999), which may play a role in the regulation of ARE-mRNA decay. Furthermore pp32 and APRIL were found to interact with CRM-1. It is speculated that this interaction may modulate the export of HuR (Gallouzi and Steitz 2001).

HuR over expression was also implicated to play a role in tumorigenesis in colon cancer (Dixon et al. 2001), and tumors of the central nervous system (Nabors et al. 2001). In addition, HuR is implicated in muscle differentiation by stabilizing myogenin and MyoD, two important transcription factors involved in myogenesis when it is overexpressed (Figueroa et al. 2003), and it inhibits muscle differentiation when downregulated by RNAi

(van der Giessen et al. 2003). HuR also seems to play a role in the muscle wasting disease cachexia, where it regulates induced nitric oxide synthethase in an AMPK dependent manner (Di Marco et al. 2005). By a RNA-IP approach, the specific targets of HuR and AUF1 have been identified in HeLa cells (Lal et al. 2004). Besides its function in RNA stabilization, HuR was implicated in translational de-repression induced by micro RNAs on CAT-1 mRNA. In nonstarved HuH7 cells, the CAT-1 mRNA localizes to P-bodies (PB) though the action of microRNA miR-122. When anti-sense miR-122 is transfected, or the cells are deprived of amino acids, CAT-1 mRNA relocalizes from the PBs and is efficiently translated. This relocalization was committed by HuR binding the CAT-region D (Bhattacharyya et al. 2006).

1.5.2.2 AU-binding factor-1 (AUF1/hnRNP D)

AUF1 was originally identified as a promoter of ARE-mediated decay (Brewer 1991). It exists in four isoforms that are generated by alternative splicing of exon 2 and/or exon 7. The resulting isoforms are p37 (lacking exon 2 and 7), p40 containing exon 2, p42 containing exon 7, and p45, which possesses both alternative exons. The exon 7 plays a role in localization, so that the isoforms p42 and p45 are mainly located in the nucleus, whereas p37 and p40 are mainly in the cytoplasm. The isoforms lacking exon 7 are readily ubiquitinated and degraded by the proteasome, in contrast to p42 and p45 (Laroia and Schneider 2002). The absence of exon 2 has been associated with high ARE-binding affinity (DeMaria et al. 1997), and over expression of p37 and p42, lacking exon 2, antagonizes stabilization and leads to hemin-induced differentiation of K562 cells into erythroblasts (Loflin et al. 1999). When overexpressed, AUF1 also leads to increased degradation of ARE bearing messages in smooth muscle cells (Pende et al. 1996), monocytes (Sirenko et al. 1997) and peripheral blood mononuclear cells (Buzby et al. 1996; Buzby et al. 1999), however over expression of all four isoforms in 3T3 cells stabilizes ARE-messages (Xu et al. 2001), so the precise role of AUF1 in ARE-mediated decay is still not clear. Several models have been proposed where some isoforms cause stabilization, while the others cause degradation, depending on

the particular cell line and the type of ARE under investigation (Xu et al. 2001; Raineri et al. 2004). In contrast to HuR, AUF1 seems to induce stability by maintaining the conformation of its target RNA in a condensed conformation. This process is dependent upon the phosphorylation state of AUF1 S83 and S87 (Wilson et al. 2003a; Wilson et al. 2003b). AUF1 knockout mice have only recently been described. When these mice are challenged with endotoxins they display symptoms of severe toxic shock syndrome like vascular hemorrhage, intravascular coagulation and high mortality. This phenotype could be rescued by application of neutralizing antibodies against TNFα and interleukin 1 beta (IL-1β). Thus AUF1 plays a major role in the inflammatory response (Lu et al. 2006). Further targets for AUF1 in HeLa cells have also been recently identified by RNA immunoprecipitation (Lal et al. 2004).

Besides its function in ARE-mediated degradation, it is a part of the α-complex protecting the α-globin message from degradation (Kiledjian et al. 1997), and it also appears to play a role in the c-fos CRD driven degradation (Grosset et al. 2000). Besides RNA stability, other functions have emerged for AUF1, and it was reported to be involved in telomere maintenance (Eversole and Maizels 2000), transcriptional activation in EBV (Fuentes-Panana et al. 2000) and it may also regulate translation of the IL-6 message (Paschoud et al. 2006).

1.5.3 Translation regulatory AUBPs

1.5.3.1 T-cell intracellular antigen-1 (TIA-1)

The T-cell intracellular antigen-1 (TIA-1) functions as a regulator of translation and splicing (Del Gatto-Konczak et al. 2000). It possesses three RRMs and a G-rich region at the C-terminus. The RRM2 is capable of binding to U-rich sequences, whereas RRM3 seems to have no sequence specificity. RRM1 does not appear to bind to RNA at all (Dember et al. 1996). The C-terminal domain is related to the prion protein and can induce self-assembly into stress granules (Gilks et al. 2004). TIA-1 can specifically interact with the COX2- and TNFα-ARE via its RRMs (Piecyk et al. 2000; Dixon et al. 2003; Cok et al. 2004), and current reports state that it also plays together with HuR a role in regulating cytochrome c

expression (Kawai et al. 2006). Recently a number of target RNAs have been identified by RNA-immunoprecipitation. In silico analysis identified a consensus sequence and messages bearing this sequence in its 3'UTR undergo translational control (Lopez de Silanes et al. 2005). TIA-1 knock out mice have 50% embryonic lethality. In macrophages derived from TIA-1 knock out mice, elevated levels of TNFα protein was produced, but the amounts and half-life of the message were unchanged compared to macrophages derived from wild type mice. This indicates that TIA-1 controls TNFα production by inhibition of translation, as more TNFα mRNA was associated with polysomes in TIA-$1^{-/-}$ macrophages (Piecyk et al. 2000). This effect seems to be specific for TNFα, as GMCSF or interferon γ translation was unaffected. TIA-1 is also a nucleo-cytoplasmic shuttling protein, and is transported to the cytoplasm under stress conditions, where it organizes in stress granules (Kedersha et al. 1999). Whether this effect is physiological or pathological is not clear, because the stresses used, like arsenite or FCCP, will lead inevitably to the death of the cells. For review see (Kedersha and Anderson 2002).

1.5.3.2 TIA-1 related protein (TIAR)

TIAR is very similar to TIA-1 with respect to protein organization and translational regulation. It also consists of three RRMs and the prion-protein domain (Dember et al. 1996). It is also involved in translation control as well as in splicing (Le Guiner et al. 2001) and organization into stress granules as TIA-1 (Kedersha et al. 1999). mRNAs shown to be translationally controlled by TIAR are the iNOS (Fechir et al. 2005) and TNFα mRNA (Gueydan et al. 1999). Recently a large number of mRNA-targets for TIAR have been identified by RNA-immunoprecipitation, which include many proteins involved in translation (Mazan-Mamczarz et al. 2006). The mechanism by which TIA-1 and TIAR inhibit translation are yet unknown. In contrast to TIA-1, knockout mice of TIAR are 100% embryonic lethal (Beck et al. 1998).

1.6 Regulation of TNFα

1.6.1 Why did we choose TNFα mRNA as our model system?

Tumor necrosis factor alpha (TNFα) is a major mediator of inflammation, which is induced early after injury and infection. This factor is mainly produced by macrophages and T cells in a secreted or membrane bound form in response to various stimuli. Mice deficient in TNFα lack splenic primary B cell follicles and are unable to form organized follicular dentritic cell networks and germinal centers, implicating its relevance in the maturation of the humoral immune response (Pasparakis et al. 1996). Under physiological conditions, TNFα is released locally and transiently at the site of infection, whereas constitutive expression is pathological and may lead to rheumatoid arthritis, and other autoimmune disease (Keffer et al. 1991). A systemic release of TNFα, induced by stimulation of macrophages with lipopolysaccharide from the cell wall of gram-negative bacteria, mediates septic shock (Tracey and Cerami 1994; Karima et al. 1999), but not so in TNFα deficient mice.

1.6.2 Induction of TNFα expression

TNFα is regulated at virtually every step of its maturation process in order to ensure that only limited amounts of this toxic cytokine are produced. TNFα production is induced in response to LPS binding to the Toll-like receptor-4 receptor (TLR-4) (Poltorak et al. 1998; Hoshino et al. 1999) (Fig. 3). First, LPS complexes with the soluble LPS-binding protein (LBP) that circulates in the bloodstream (Schumann et al. 1990). The interaction of the LPS-LBP complex with TLR-4 is mediated by CD14 (Viriyakosol and Kirkland 1996; Muroi et al. 2002) and finally causes activation of NF-κB and other transcription factors (Collart et al. 1990; Trede et al. 1995; Yang et al. 1998; Myokai et al. 1999; Steer et al. 2000). Important signalling molecules are MyD88, which directly interacts with TLR-4 (Wesche et al. 1997), and IRAK4 (Li et al. 2002), a signalling molecule directly downstream of MyD88. MyD88$^{-/-}$ and IRAK 4$^{-/-}$ mice are resistant to LPS induced toxicity although NF-κB can still be activated via a MyD88 independent pathway (Kawai et al. 1999; Takeuchi et al. 2000; Horng et al. 2001; Suzuki et al. 2002; Fitzgerald et al. 2003). IRAK4 then activates IRAK1, and through

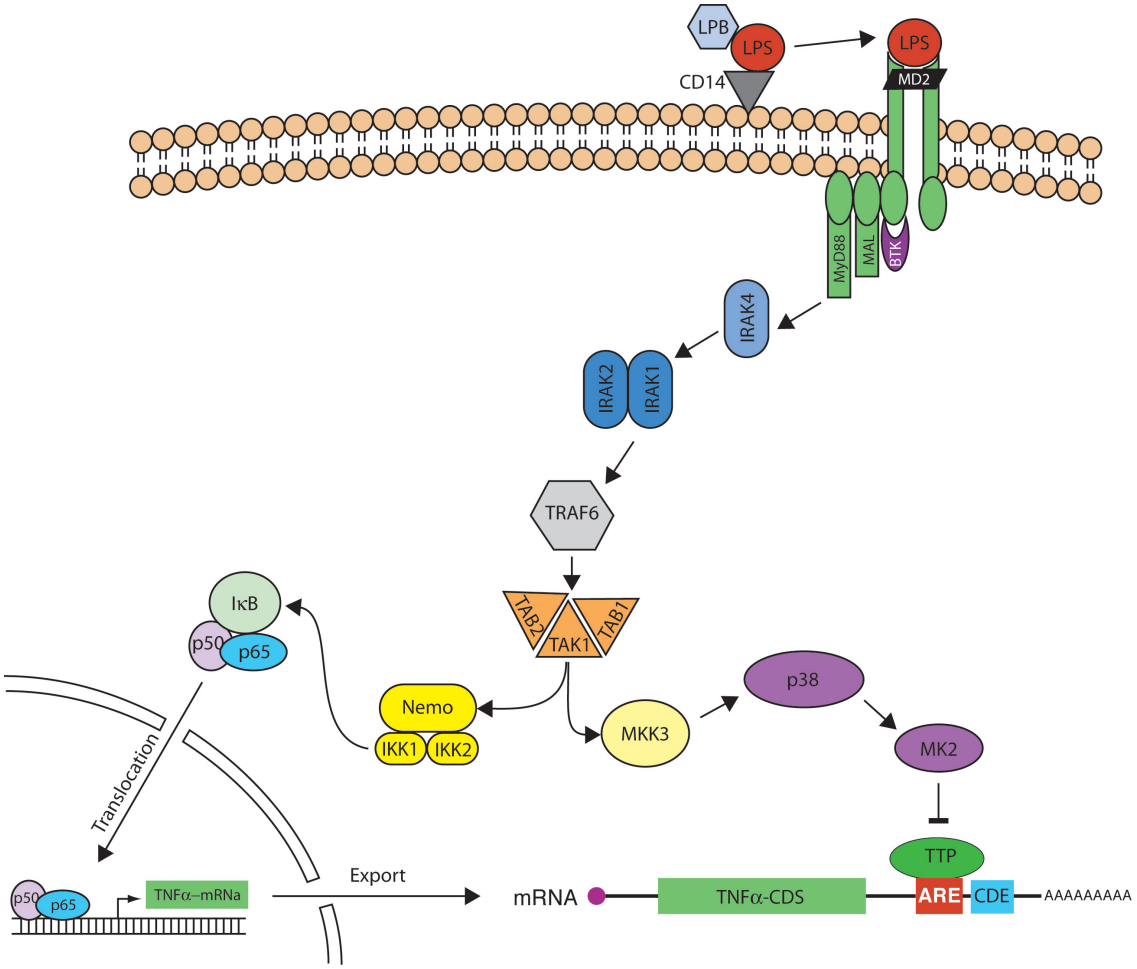


FIG. 3. Simplified model of the TOLL 4 receptor signalling. LPS is bound by LPB outside of the cell, and brought into contact with the TOLL4 receptor via CD14. The extrinsic signal is then transduced via MyD88, IRAK4, IRAK1 and TRAF6 to the TAK/TAB complex. This complex leads on one hand via IKK to the release of transcription NFκB into the nucleus and transcription of the TNFα mRNA, and on the other hand to the activation of the p38 pathway, which inhibits via MK2 the degradation of the TNFα mRNA by sequestering TTP.

this TRAF6 (Cao et al. 1996). Activated TRAF6 leads to an activation of MEK kinase and the IKK regulatory subunit NEMO, which result in NF-κB and c-jun promotion of TNFα mRNA transcription (Collart et al. 1990; Trede et al. 1995; Yang et al. 1998; Myokai et al. 1999; Steer et al. 2000). Furthermore MKK3 activates the p38-MK2 pathway which leads, as described before, to inactivation of TTP and TNFα mRNA stabilisation (Stoecklin et al. 2004; Hitti et al. 2006). This is reviewed in (Palsson-McDermott and O'Neill 2004).

1.6.3 Post-transcriptional regulation of TNFα

The TNFα mRNA contains in its 3' untranslated region (UTR) an AU-rich element (ARE) that interacts with various ARE-binding proteins that regulate its nuclear-cytoplasmic transport, decay activity and translation efficiency (Shyu and Wilkinson 2000; Wilusz et al. 2001). Tpl2-deficient mice retain TNFα in the nucleus upon LPS induction (Dumitru et al. 2000), indicating a deficiency in TNFα mRNA transport. To guarantee only a transient production of TNFα, its mRNA decay rate is tightly controlled. In lymphocytes stimulated with CD3 and CD28, the normally very labile TNFα mRNA becomes markedly stabilized (Lindstein et al. 1989), whereas in macrophages the issue is controversial. In reporter assays, where the TNFα coding sequence was replaced by the chloramphenicol acetyl transferase gene, no changes in reporter stability could be detected. The reporter RNA remained quite stable upon LPS treatment, but translation of CAT seemed to be increased (Han et al. 1990; Raabe et al. 1998). Subsequently the TNFα mRNA was explored by the same group. Here they found again, that after LPS induction, the CAT-reporter was quite stable, but endogenous TNFα-mRNA was rapidly degraded (Han et al. 1991; Brook et al. 2000), which could be supported by findings in our group. We could demonstrate that TNFα mRNA levels are induced by LPS over 6 hours and declines again thereafter, but the message remained unstable, whereas an isolated TNFα-ARE-sequence fused to a β-globin reporter was stabilized (Stoecklin et al. 2003). In contrast it was reported, that the TNFα mRNA is stabilized upon LPS treatment, and that the inhibition of the p38 MAP kinase pathway leads to degradation of TNFα mRNA, which was also confirmed in MK2 knockout mice (Han et al. 1991; Brook et al. 2000; Stoecklin et al. 2004; Hitti et al. 2006). There was even one group, which found that the TNFα-mRNA is stable independently of LPS stimulation (Han et al. 1990; Raabe et al. 1998).

TNFα ARE mediated decay in macrophages is mediated by TTP, which binds directly to the AU-rich sequence, and recruits factors mediating deadenylation, decapping and mRNA body decay (Chen and Shyu 1995; Stoecklin et al. 2001; Lykke-Andersen and Wagner 2005). Macrophages isolated from TTP-deficient mice produced high amounts of TNFα due to an

increase of its mRNA half-life (Carballo et al. 1998). Furthermore TTP was demonstrated to be phosphorylated and thereby inactivated by the p38 pathway (Stoecklin et al. 2004; Hitti et al. 2006), which is in turn activated via the TLR-4 signalling pathway leading to an increased stability of the TNFα message, as shown in MK2-deficient mice. Another factor reported to influence TNFα expression is AUF1, which seems to induce degradation of this message demonstrated in AUF1 knockout mice (Lu et al. 2006). When the TNFα-ARE is vacated by TTP and AUF1, HuR was found to associate with this ARE and cause stabilization of the TNFα message (Dean et al. 2001). Besides the ARE, the constitutive decay element (CDE) is located in the TNFα 3'UTR (Stoecklin et al. 2003). The CDE was discovered in slowC cells, which are unable to promote ARE dependent decay, but could rapidly degrade a reporter containing the entire TNFα 3'UTR independent of the AU-rich element. The CDE is located directly downstream of the TNFα-ARE, promotes constitutive degradation of the TNFα message, and is unresponsive to stabilizing agents such as LPS or phorbol esters (Stoecklin et al. 2003).

In addition to mRNA decay, TNF-α is regulated on the level of translation by TIA-1 which has high affinity to the TNFα ARE. In macrophages derived from TIA-1 deficient mice, higher levels of TNFα were produced compared to wild type macrophages from similar amounts of TNFα mRNA (Piecyk et al. 2000). This process is modulated by the mitogen activated protein kinase-2 (MAPKAP) (Kotlyarov et al. 1999).

1.7 Aim of the project

The project is aimed at understanding the mechanisms by which the TNFα message is controlled. Four different points were addressed during these studies:

1.7.1 Definition of the minimal active CDE

The smallest active constitutive decay element (CDE) was found to be 40nt long. The CDE as previously characterized by Stoecklin et al., was 80nt long with a half-life of roughly

two hours. In contrast, the complete 3'UTR sequence downstream of the ARE (element II) only had a half-life of 45 minutes (Stoecklin, Lu et al. 2003). By deletion mutagenesis, we were able to reduce the size of the CDE to 40nt, and mapped it to a region 42 nucleotides downstream of the last AUUUA pentamer. With this deletion we also restored the rapid decay phenotype of t1/2 = 45 minutes observed with the element II. Mutations introduced into this sequence could no longer promote decay.

1.7.2 Nucleolin binds to the CDE

The next issue was to identify a protein binding to the CDE. We were able to visualize an UV-crosslink activity at roughly 60 kD, which was competed by the CDE, but not by an ARE RNA. We purified this activity with a three-step purification procedure over heparin, affinity and anion exchange matrix. The crosslinking protein was identified by mass spectrometry as the RNA-binding protein nucleolin.

1.7.3 The nucleolin-CDE interaction is specific

Band shift assays with cytoplasmic macrophage extracts revealed that only the CDE can supershift with nucleolin, but not any of its mutants. This could be confirmed using recombinant full-length nucleolin produced with the baculovirus-expression system. To prove that nucleolin interacts with the TNFα mRNA in vivo, RNA-immunoprecipitation experiments were performed. Nucleolin was only able to pull down TNFα mRNA, and no other message tested.

1.7.4 Downregulation of nucleolin by RNAi slightly increased GFP-CDE-reporter stability

To provide functional evidence for the nucleolin-CDE interaction, nucleolin was downregulated by RNAi in a cell line expressing a GFP reporter linked to the CDE. By FACS analysis, increased GFP fluorescence was detectable when nucleolin was knocked down, which correlates with a slight but significant increase in the reporter half-life from 96 to 136 minutes.

2. Results

2.1 Part I: Identification of the smallest active constitutive decay element (CDE)

2.1.1 Definition of the constitutive decay element (CDE) by deletion mapping

This study focuses on the mRNA of tumor necrosis factor alpha (TNFα), which causes toxic shock syndrome and the autoimmune disease rheumatoid arthritis when its expression is dysregulated. As previously mentioned, the expression of TNFα is regulated at many steps, but in this study, we concentrated on RNA stability. As stated in the introduction, TNFα mRNA carries two destabilizing elements in its 3'UTR leading to its rapid degradation. One of them is the well-characterized AU-rich element (ARE) that is bound by the destabilizing proteins TTP and AUF1 (Taylor et al. 1996; Lu et al. 2006), and the stabilizing protein HuR (Dean et al. 2001) as well as the translation repressor TIA-1 (Piecyk et al. 2000). The other element is the only recently discovered CDE, which was defined as an 80nt long sequence directly adjacent and downstream to the ARE. This study utilizes as a starting point the fragment K, which contains 19 extra nucleotides including an AUUUA pentamer at the 5' end of fragment KΔAU. These extra nucleotides do not have an influence on decay kinetics as was shown by Stoecklin et al. (Stoecklin et al. 2003).

To map this element more precisely, we deleted the fragment K beginning from the 5' end to obtain the fragments M and O, which were then inserted downstream of a β-globin reporter (Fig. 4A). We assayed the decay activity of the various fragments by transient transfection into NIH3T3 B2A2 cells with the β-globin reporter alone (control) or carrying the various fragments followed by transcriptional arrest with actinomycin D (act. D) for 0, 30 and 60 minutes. As demonstrated in a representative experiment (Fig. 4B), the β-globin control or

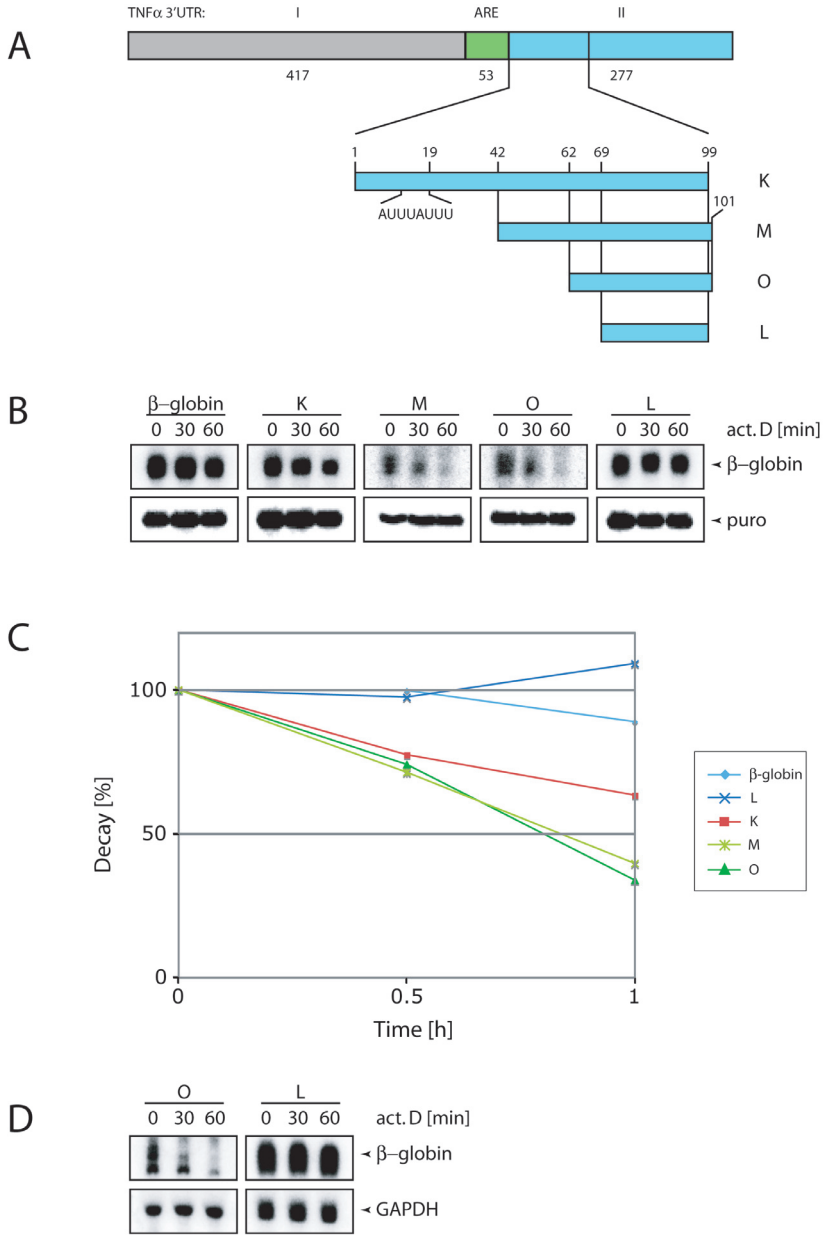


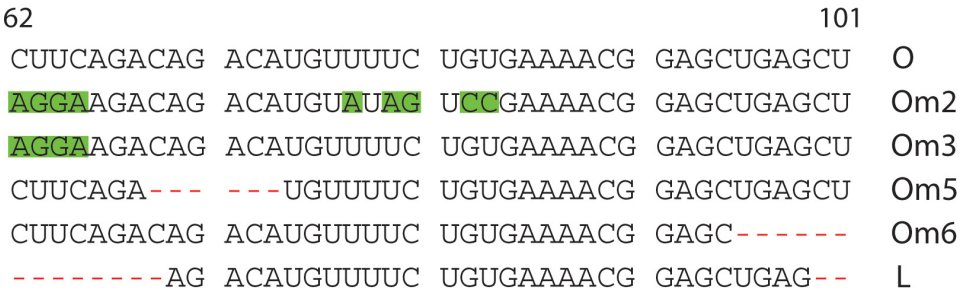
FIG. 4. Deletion mapping of the CDE within fragment K of the TNFα 3'UTR. (A) Fragments K, M, O and L were inserted into the 3'UTR of the β-globin reporter gene in plasmid puroMXβglobin. (B) After transient transfection into NIH 3T3 B2A2 cells, the stability of the reporter mRNAs was determined by actinomycin D (act. D) chase experiments. For each time point, cytoplasmic RNA was extracted and processed for northern blot analysis. Reporter RNA was detected using a radiolabelled SP6 generated probe specific for β-globin, and transfection efficiency and loading were controlled by rehybridizing with a probe against puromycin N-acetyltransferase (puro) expressed by the reporter plasmid. (C) Reporter levels were quantified with Quantity One software and normalized against the loading control. Percentage of decay was plotted against time of a representative experiment. (D) The β-globin O and L reporters were stably transfected into NIH 3T3 B2A2 cells by selection with 1μg/ml puromycin. The stability of the reporter in these cells was determined by act. D chase experiments and northern blot as described above.

fragment L bearing reporter were stable, whereas the fragment K promotes decay with an approximate half-life of two hours. Deletion of 41nt (fragment M), or 61nt (fragment O) from the 5' end led to a strong acceleration of the rate of decay, resulting in a half-life of approximately 45 minutes for both fragments. This corresponds to the half-life determined for the entire 277nt sequence downstream of the ARE, TNFα-E2 (Stoecklin et al. 2003) and suggests that this is the entire functional element. The 40nt long fragment O appears to be the minimal functional element that can still induce rapid reporter degradation, and a further 10nt deletion (fragment L) is sufficient to fully abolish activity. Fig. 4C shows the quantification of one representative experiment. Next, we stably transfected the β-globin-O and –L constructs into NIH3T3 B2A2 cells to see whether the stably transfected reporters behave the same as when transiently expressed (Fig. 4D). Here as well, the fragment O induced a very rapid decay with a half-life of less than 45 minutes. In this case fast deadenylation can be observed, which suggests, that the CDE-reporter is degraded via the 3'-5' exonucleolytic pathway.

2.1.2 Mutations in fragment O lead to loss of CDE activity

After defining fragment O as the minimal CDE, we next asked whether mutations in fragment O can affect its decay capability. In the CDE fragment K, which has a half-life of approximately two hours, decay activity was completely abolished by the introduction of a few point mutations (Stoecklin et al. 2003). Therefore we generated and analysed mutants of the CDE-fragment O by degradation assays. We cloned the sequences indicated in Fig. 5A into the puroMXβglobin vector. The green-boxed bases indicate the nucleotides which were mutated, whereas the red dashed lines indicate deletions in the fragment O. In the mutant Om2, we replaced five of the eight uridine residues by a different nucleotide, to assess the importance of the U-rich stretch for CDE-mediated decay. This mutant reporter was completely stable in in vivo decay assays (Fig. 5B). Within Om3, we only mutated the first four nucleotides, to see whether the mutations introduced into the putative stem in Om2 are responsible for the severe stabilization effect, but here too the reporter Om3 RNA was

A



B

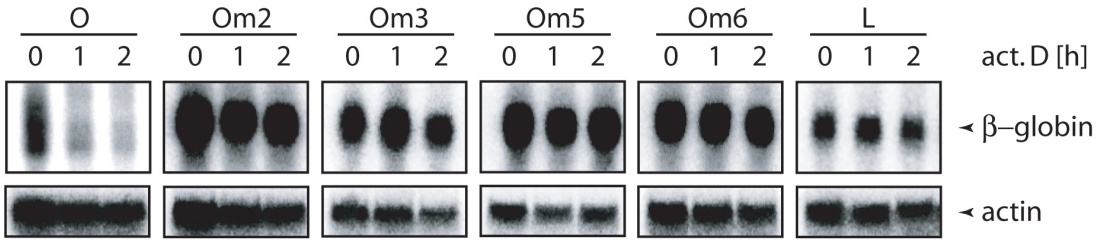


FIG. 5. Mutational analysis of fragment O, the minimal CDE. (A) Fragment O and its mutants Om2, Om3, Om5, Om6 and L were inserted into the 3'UTR of the β-globin reporter gene in plasmid puroMXβglobin. Nucleotide changes are indicated in green and deletions by red dashed lines. (B) After transient transfection into NIH 3T3 B2A2 cells, the stability of the reporter mRNAs was determined by act. D chase experiments. Northern blot analysis was performed as described in Fig. 1. The blot was reprobed against actin as loading control.

completely stable. Furthermore, this mutant demonstrates the importance of the 3' end of fragment O for promoting degradation, because L, from which the first six nucleotides are deleted, is also stable as reported by (Stoecklin et al. 2003). We were curious, if the non-U-rich sequences in this element also have an effect on stability. We deleted a 6nt part of fragment O to obtain mutant Om5, or the 6nt at the 3' end to generate Om6. Both mutants were very stable in vivo. These observations suggest that the sequence in fragment O is necessary to promote rapid degradation, and that this minimal CDE is sensitive to even slight changes in its sequence.

2.2 Part II: Purification and identification of nucleolin as the CDEBP

2.2.1 Detection of CDE-specific binding proteins

In contrast to the ARE, a CDE-bearing reporter is insensitive to RNA stabilizing signals emanating from extra-cellular stimuli such as phorbol esters, ionomycin, lipopolysaccharide (LPS), or over-expression of activated ras and activated MEK6 kinase (Stoecklin et al. 2003). This distinct behaviour suggests a different mechanism by which the CDE-bearing message is degraded. To elucidate the mechanism underlying CDE mediated decay, we tried to identify proteins specifically binding to the CDE-fragment K by UV-crosslinking assays. We incubated LPS-stimulated Raw 264.7 macrophage cytoplasmic extracts with an in vitro transcribed fragment of the CDE-K probe incorporating the crosslinking agent 4-thio-UTP and [α^{32}P]-ATP. We competed the radioactive probe with increasing amounts of unlabeled K- or unlabeled TNFα ARE- transcripts to score for the specificity of the K-probe protein interactions. The CDE-binding proteins were then crosslinked by ultraviolet radiation, and the complexes were analyzed by 7.5 to 15% SDS-PAGE and phosphorimaging. We could identify three activities binding the CDE at 200kD (complex 3), 90kD (complex 2) and 60kD (complex 1) (Fig. 6A). Only the 60kD band was sensitive to specific competition by the unlabeled probe K and not the ARE, indicating that complex 1 binds specifically to the CDE-K probe, whereas complex 2 and 3 are unspecific. As an alternative approach to discover specific CDE binding proteins, we performed electrophoretic mobility shift assays (EMSAs) und used here the shorter probe CDE-O and probes of the non-functional mutants of O identified above, which were incubated with LPS-stimulated Raw 264.7 macrophage cytoplasmic extract. The protein-RNA complexes were resolved by native gel electrophoresis (Fig. 6B.). The 40nt CDE-O probe is shifted into a low mobility complex A, whereas the 30nt long decay-inactive mutant probe L did not show a shifting complex. Om2 probe shows a weaker signal at the corresponding position for complex A, and with the mutant Om3, we could observed a complex with a higher mobility than complex A. The Om6 mutant probe

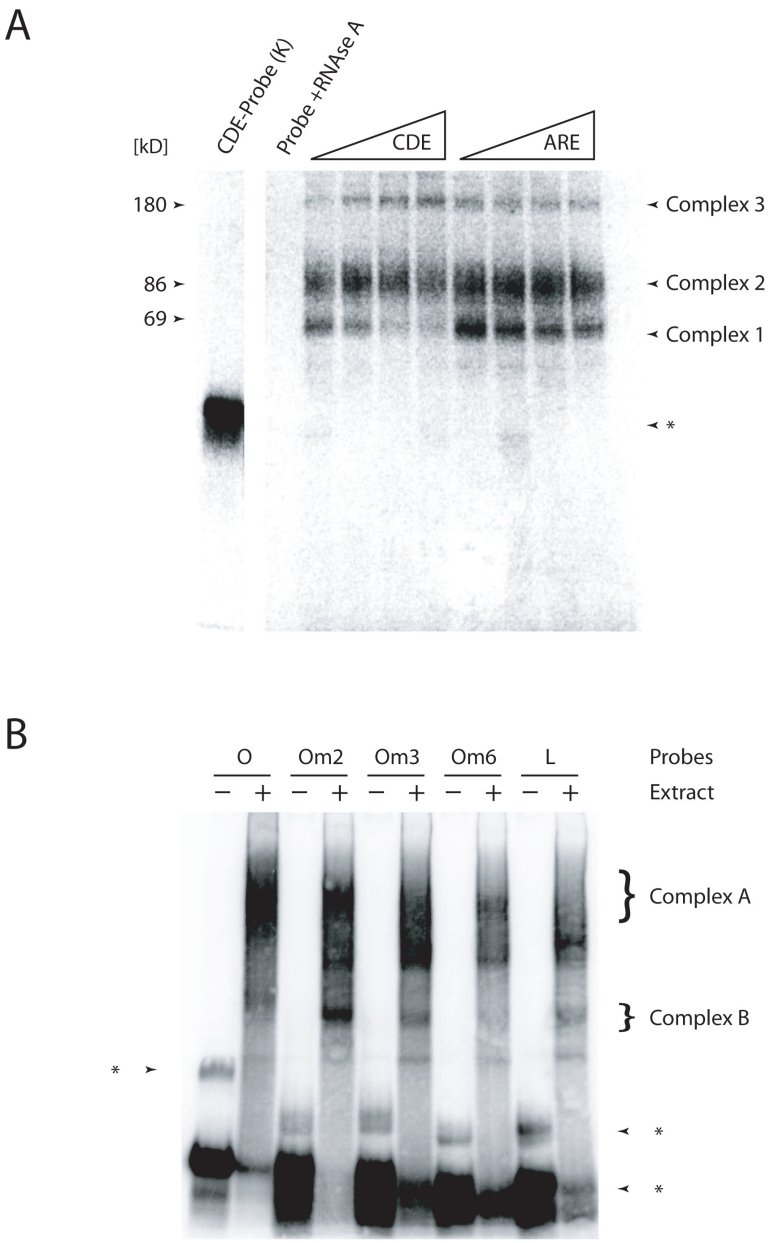


FIG. 6. Visualization of the CDE-binding proteins (CDE-BPs) by UV-crosslinking and electrophoretic mobility shift assays (EMSA). (A) The CDE-fragment K probe was transcribed in the presence of [α^{32}P]-ATP and 4-thio-UTP. 20kcpm of the probe were mixed with 0, 10, 100 or 1000 fold excess of specific competitor (unlabelled fragment K) or control competitor (unlabelled TNFα ARE). A mastermix containing 20µg of Raw 264.7 cytoplasmic extract was added and crosslinked to the probe on ice under a glass plate with 3,6 J/cm^2 of 312nm UV-light. Excess RNA was digested with RNAse A. Protein-RNA complexes were separated by a 7.5 to 15% gradient SDS-PAGE and visualized by phosphorimaging. The asterisk indicates the undigested probe. (B) For EMSA, radiolabelled fragment O, Om2, Om3, Om6 and L were incubated with buffer or with 10µg of Raw 264.7 cytoplasmic extract for 30 min on ice. Protein-RNA complexes were separated on a 4% native polyacrylamide gel and visualized by phosphorimaging. The asterisks indicate the free probe, whereby the upper form probably corresponds to a dimerized form of the probe.

associated, like L, only weakly with proteins and therefore no bandshift could be observed. These results suggest that the protein-complexes binding to the CDE-O differ qualitatively from the proteins binding to the mutated sequences, which correlates at least partly with the loss of decay activities of the mutants as shown in figure 5. These results encouraged us that it may be possible to isolate a regulatory protein with specificity for CDE-O, where the mutant sequences can serve as important negative controls.

2.2.2 Selection of suitable chromatography matrices to isolate the CDEBP

A prerequisite for chromatographic isolation of the specific 60kD complex 1, depicted in Fig. 6A, is to identify a suitable matrix which the protein of interest can bind and be eluted from. Specifically we were looking for chromatographic matrices to which the complex is able to bind under low salt, but not under high salt conditions (Fig. 7). Cytoplasmic extract from LPS stimulated Raw 264.7 macrophages was incubated with the strong anion exchange matrix Q sepharose, with the NAD analogue red sepharose, the NADPH analogue blue sepharose, heparin sepharose mimicking sugar backbones, or the non-polar matrix phenyl sepharose with either a concentration of 100mM KCl (even numbered lanes) or 500mM

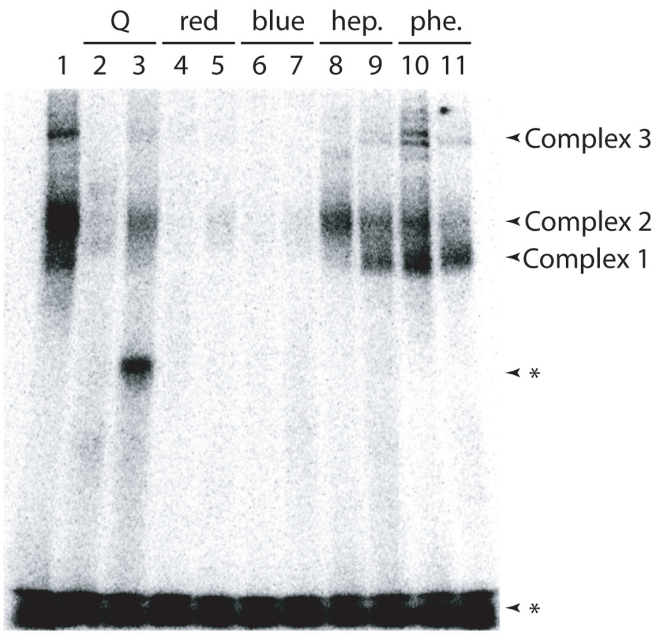


FIG. 7. Testing of different chromatography matrices to purify the CDEBP. Raw macrophage extract was incubated with Q sepharose, red sepharose, blue sepharose, heparin sepharose (hep.) or phenyl sepharose (phe.) for 30 min on ice in the presence of 100mM KCl (lanes 2,4,6,8,10) or 500mM KCl (lanes 3,5,7,9,11). The supernatants and an untreated control (lane 1) were subjected to UV-crosslink assay as described in Fig. 6, and separated by 7.5 to 15% gradient SDS-PAGE and visualized by phosphorimaging. The asterisks indicates the free probe, while the upper band in lane 3 corresponds to undigested probe.

KCl (odd numbered lanes). The supernatants of these matrices were then analysed by the UV-crosslinking system, using the CDE-fragment K as probe, to determine recovery of the previously identified complex 1. A crosslinked protein band should be observed at high salt conditions, and no crosslinked band under low salt conditions, from the supernatants of the binding reactions. The phenyl sepharose matrix works in a contrary manner, because hydrophobic interactions are strengthened under high salt conditions. As a positive control (lane 1), we used untreated extract, were we could again detect the three complexes as in Figure 6 A. Complex 1 was able to adsorb to the Q sepharose matrix and to the heparin sepharose matrix under low salt conditions (lanes 2 and 8), but not under high salt conditions (lane 3 and 9). In contrast, red sepharose and blue sepharose could adsorb the protein even under high salt conditions, indicating that the protein cannot be eluted from the matrix, while phenyl sepharose was incapable of binding this protein at all. The properties of Q sepharose and heparin sepharose make these two matrices suitable for the purification of complex 1. The protein binds to the matrix under low salt conditions, and can be eluted out with high salt. For our future work we selected heparin sepharose, followed by an affinity purification and anion exchange chromatography.

2.2.3 Overview of the CDE binding protein purification procedure

The approach leading to successful purification of the CDE binding protein is depicted in Fig. 8. An overview of the 4-step procedure is described briefly here with detailed results following. Raw 264.7 macrophage cells were stimulated with LPS, lysed and precleared by centrifugation to obtain the cleared cytoplasmic extract. In step 1 of the purification, the extract was applied to a heparin sepharose column, which resembles the sugar backbone of RNA, and enables us therefore to select for nucleotide binding proteins. After washing, protein was eluted with 1M KCl. In step 2 the proteins eluted from the heparin sepharose column were bound to streptavidin sepharose beads precoated with 5’-biotinylated CDE-fragment K, or with streptavidin sepharose alone as a control. With this CDE-K matrix, we introduced an affinity purification step, where specific binding proteins should be selected,

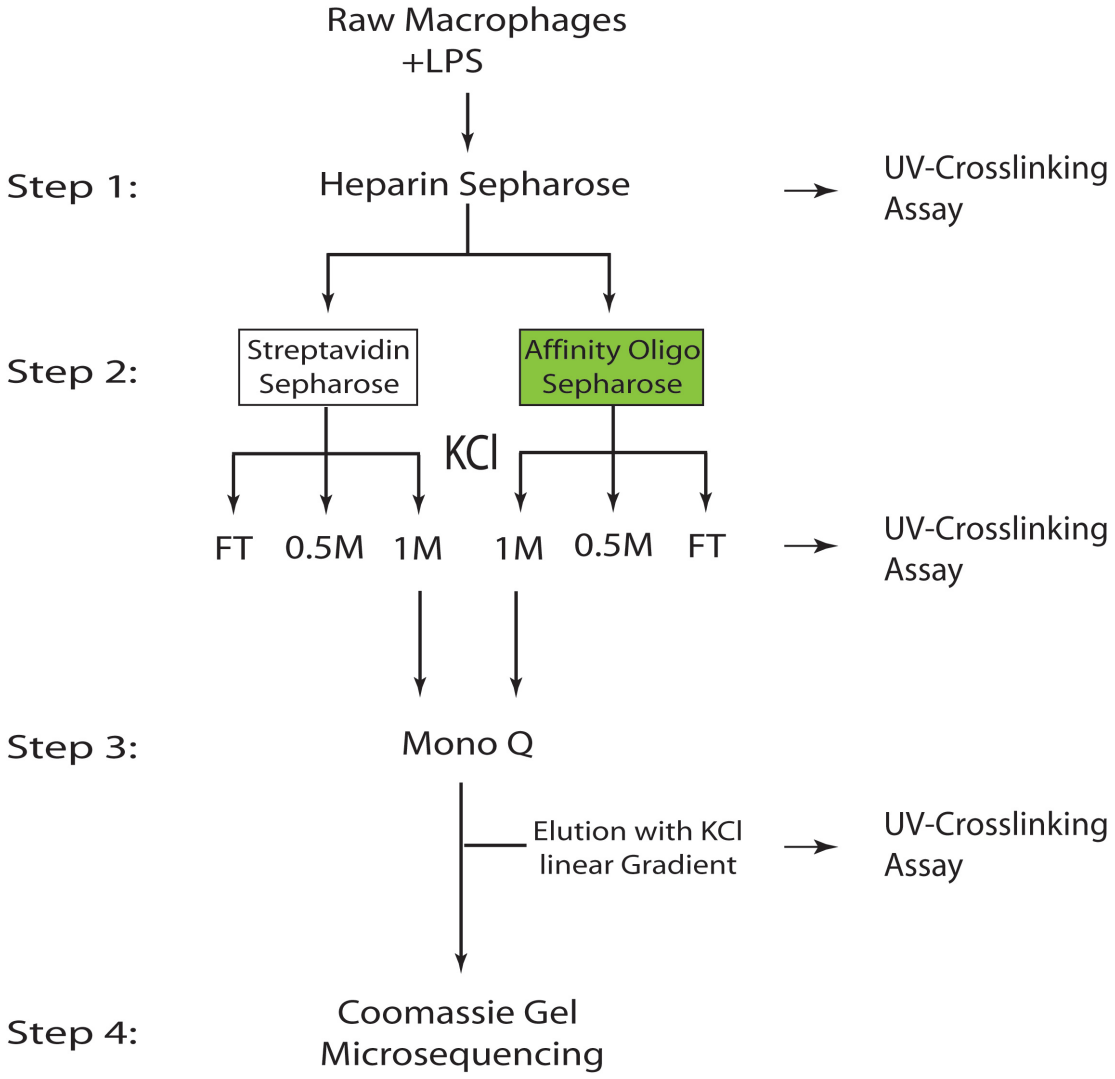


FIG. 8. Overview of the CDE-binding protein purification procedure. Raw 264.7 macrophages were stimulated with LPS, lysed and precleared by centrifugation. In step 1, the extract was loaded on a HR 10/10 heparin sepharose column, washed, and eluted with 1M KCl buffer. In step 2, the eluate was bound either to streptavidin sepharose (control), or affinity sepharose (streptavidin sepharose preincubated with 5'-biotinylated fragment K). The CDE-binding activity was eluted stepwise with 500mM or 1M KCl. The latter fractions (control or affinity) were loaded on a Mono-Q column in step 3 and the protein was eluted with a linear gradient ranging from 250mM to 1M KCl. All steps were controlled for the presence of the CDE-binding protein by UV-crosslinking assays. In step 4, the CDE-binding proteins were visualized on a coomassie gel and the bands were cut out and identified by mass spectrometry.

whereas most other nucleotide binding proteins will be washed away. After elution of the specific binding proteins, we applied step 3 where the eluted protein was purified over a Mono-Q anion exchange column. From this matrix, the proteins were eluted over a linear

KCl gradient ranging from 250mM to 1M KCl. In step 4, the fractions containing the CDE binding protein were analysed by SDS-PAGE and individual proteins were visualized by coomassie blue staining. The protein bands were cut out of the gel and analysed by mass spectrometry. The experimental procedures and the single steps of the purification are explained in detail below.

2.2.3.1 First step: Heparin sepharose chromatography

For large-scale purification, approximately five litres of growing Raw 264.7 macrophage culture were stimulated with 5µg/ml LPS for two hours and harvested by centrifugation. The cells were lysed in 30ml of hypotonic lysis buffer and disrupted with a dounce homogenizer with 30 strokes of a tight pestle. After preclearing the extract by centrifugation at 15000g for 10 minutes, we obtained 550mg of cytoplasmic protein and adjusted the KCl concentration to 100mM. 60mg of the extract were applied per run to a HR 30/10 heparin sepharose column using a FPLC machine at a flow rate of 4ml per minute and fractions were taken every minute (Fig. 9A). The flow through fractions 1 to 9 were pooled and the column was washed with 10 bed volumes of 100mM KCl buffer that was collected separately as the wash fraction, and was eluted first with 250mM KCl buffer (fractions 10 to 13) and in a second step with 1M KCl (fractions 16 to 18). The red curve depicts the conductivity, and the blue curve the OD_{280} corresponding to the protein amount. Fractions 10 to 13 were pooled as the 250mM KCl fraction, and fractions 16 to 18 as the 1M KCl fraction and the proteins were analysed by SDS-PAGE followed by silver staining (Fig. 9B), and UV-crosslinking using a radioactive CDE probe (Fig. 9C). Most of the protein was present in the flow through fraction (Fig. 9B, lane 2), and in the 250mM KCl step fraction (lane 4), whereas the wash fraction (lane 3) and the 1M KCl fraction (lane 5) contained no detectable protein. The gel was dried and exposed to a phosphorimager screen (Fig. 9C). Complex 1 was barely visible in the input fraction (lane 1), and not at all in the flow through and wash fractions (lanes 2 to 4). However, complex 1 was highly enriched in the 1M KCl fraction (lane 5), although no protein was detectable by silver staining. This can be explained by the higher sensitivity

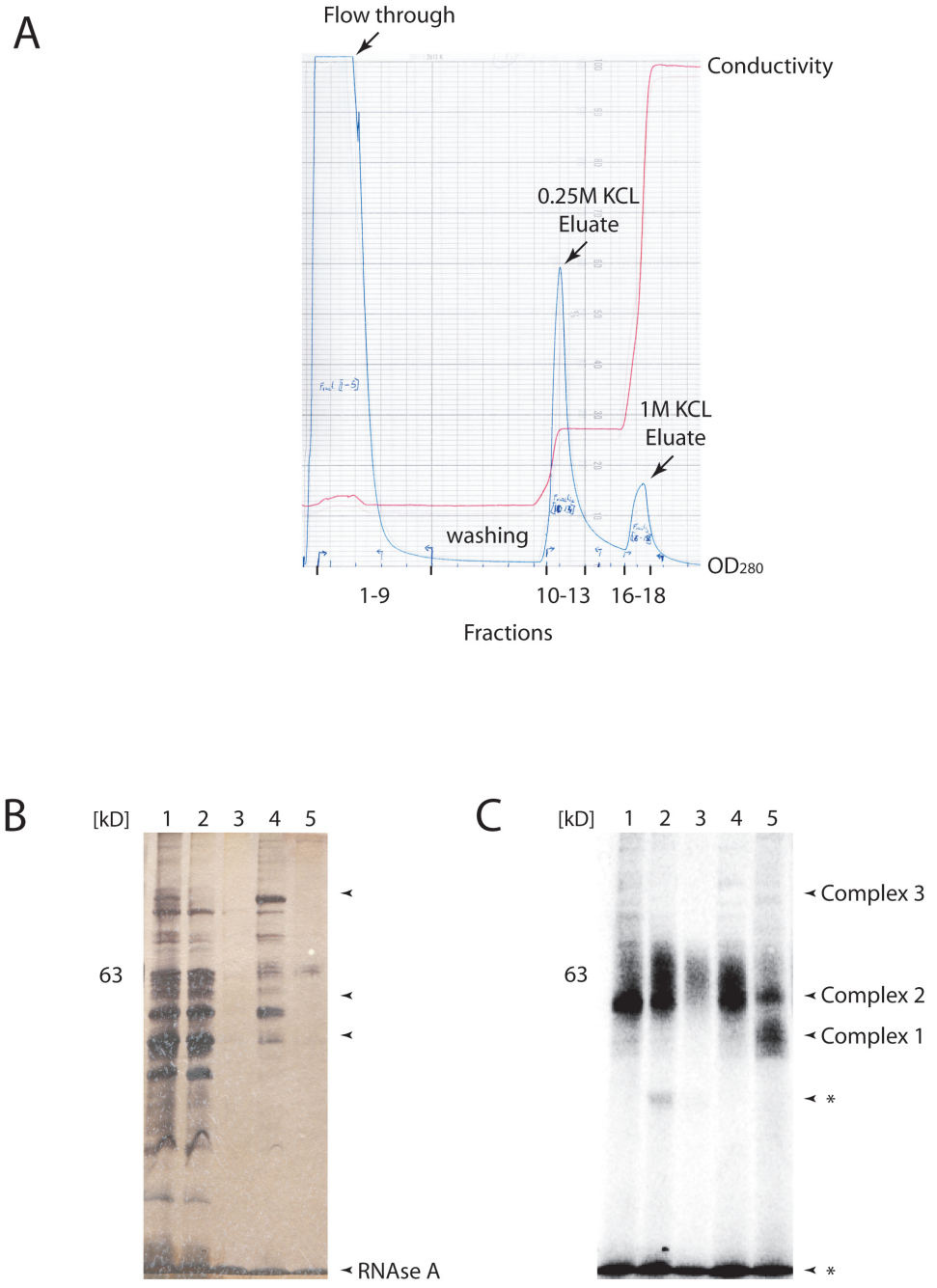


FIG. 9. Step 1: Purification of the CDE-BP over a heparin sepharose column. (A) Raw 264.7 cytoplasmic extracts were applied to a HR10/10 heparin sepharose column using a FPLC-machine. The red curve shows the conductivity and the blue curve the OD_{280}. The flowthrough (Fractions 1 to 9), a wash fraction, the 250mM KCl fraction (Fractions 10 to 13) and the 1M KCl fraction (Fractions 16 to18) were collected. (B and C) Raw 264.7 extract (lane 1), flow through (lane 2), a wash fraction (lane 3), a 250mM KCl fraction (lane 4) and a 1M KCl fraction (lane 5) were subjected to UV-crosslinking with the radiolabelled CDE-probe K as described in Fig. 6, and separated by 7.5% to 15% gradient SDS-PAGE. (B) Protein bands were visualized by silver staining, and (C) protein-RNA complexes by phosphorimaging. The asterisks indicates the free probe, and the undigested probe in lane 2 (upper band).

of the UV crosslinking assay compared to silver staining, and is also an indication that the protein we are trying to purify is still functional, a necessary requirement for the next step of purification.

2.2.3.2 Second step: CDE affinity purification

The 1M KCl fraction from Fig. 9 was dialysed against buffer A pH 8.0 containing 100mM KCl. One half of the eluate was bound to streptavidin sepharose beads precoated with 5' biotinylated CDE-K, and the other half was incubated with streptavidin sepharose alone as a control, and rotated overnight at 4°C. Both samples were washed identically and in parallel with a buffer containing 500mM KCl and eluted with 1M KCl. Each of these fractions was tested by UV-crosslinking with a radioactively labelled CDE probe followed by SDS-PAGE. In the silver stained gel (Fig. 10A), most protein remained in the supernatant (lanes 2 and 5), whereas no protein was eluted with 500mM and 1M KCl from the streptavidin control (lanes 3 and 4). From the affinity matrix some protein could be eluted with 500mM KCl (lane 6), but no detectable amounts were visible in the 1M KCl fraction (lane7). After phosphorimager exposure (Fig 10. B), only a slight band can be observed at the position of complex 1 in the input and control supernatant (lanes 1 and 2), but the activity was strongly enriched in the 1M KCl elution fraction (lane 7) of the affinity matrix. The activity was depleted completely in the affinity matrix flow through (lane 5), and was barely visible in the 500mM KCl wash fraction (lane 6). Complex 1 could not be pulled down with the control beads (lanes 3 and 4), indicating that the CDE binding protein does not interact with the matrix alone. The 1M KCl fraction from the affinity chromatography was used for further testing to confirm that a CDE-specific protein was obtained. Before proceeding in the purification procedure it was necessary to confirm whether this crosslink-activity is specifically binding to the CDE-O, but not fragment L, as observed in Fig. 6B. In an electrophoretic mobility shift assay (EMSA), we incubated ^{32}P labelled CDE-O (promoting decay in vivo) and fragment L (inactive) with the 1M KCl affinity elution fraction and separated the complexes on a 4% non-denaturing polyacrylamide gel (Fig. 10C). With the CDE-O, a complex could be detected that is absent

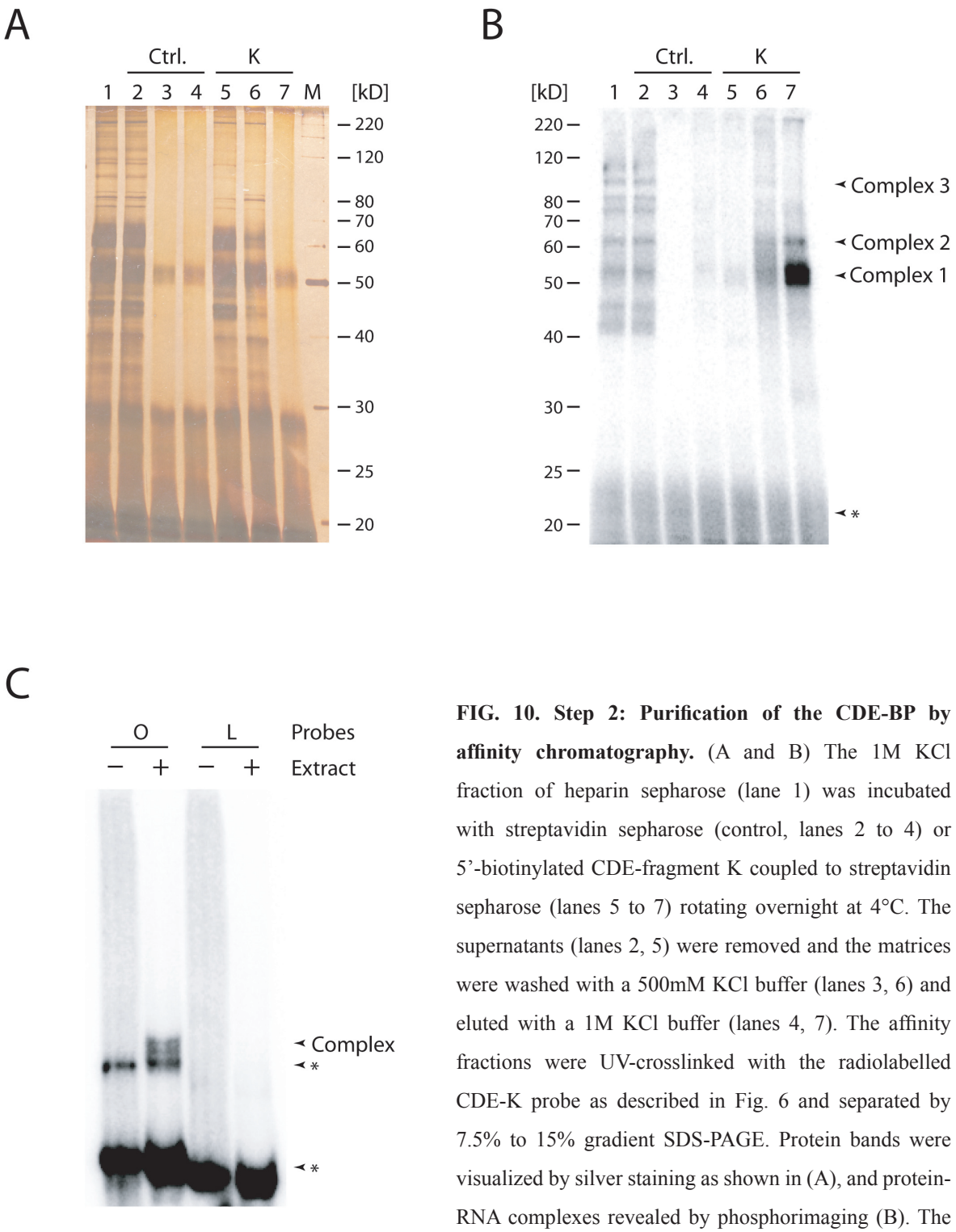


FIG. 10. Step 2: Purification of the CDE-BP by affinity chromatography. (A and B) The 1M KCl fraction of heparin sepharose (lane 1) was incubated with streptavidin sepharose (control, lanes 2 to 4) or 5'-biotinylated CDE-fragment K coupled to streptavidin sepharose (lanes 5 to 7) rotating overnight at 4°C. The supernatants (lanes 2, 5) were removed and the matrices were washed with a 500mM KCl buffer (lanes 3, 6) and eluted with a 1M KCl buffer (lanes 4, 7). The affinity fractions were UV-crosslinked with the radiolabelled CDE-K probe as described in Fig. 6 and separated by 7.5% to 15% gradient SDS-PAGE. Protein bands were visualized by silver staining as shown in (A), and protein-RNA complexes revealed by phosphorimaging (B). The asterisks indicates the free probe. (C) For EMSA, the affinity fraction or buffer were diluted and mixed with radiolabelled CDE-probe O or L and incubated for 30min on ice. Protein-RNA complexes were separated on a 4% native polyacrylamide gel. The complexes were visualized by phosphorimaging. The asterisks indicate the free probe, where the upper band indicates a putative dimerized probe.

when incubated with the water control, or when the affinity purified protein is incubated with fragment L. This result encouraged us to continue with the third step of purification and confirms the correlation of the in vivo decay activities of the CDE-O and mutant L to the activity visualized by the UV-crosslinking experiments.

2.2.3.3 Third step: Anion exchange chromatography

The elution fraction from the affinity purification (Fig. 10A and B, lane 7) or streptavidin sepharose (Fig. 10A and B, lane 4), was diluted 1:10 with buffer A to reduce the KCl concentration to 100mM, and applied to a prepacked Mono-Q column, using an FPLC machine, which was subsequently washed with a 250mM KCl containing buffer. The bound proteins were eluted with a linear KCl gradient ranging from 250mM to 1M KCl, and 0.5ml fractions were collected. In the Mono-Q fractions derived from the streptavidin control eluate no crosslink activities were visible (data not shown). The results obtained with Mono-Q chromatography, after affinity-K purification, are shown in Fig. 11A. Fractions 2 to 5 contained the flow through and 6 to 15 the 250mM KCl wash fractions. In fractions 16 to 23, collected from the gradient elution, several protein "peaks" were visible (indicated by black arrows). The Mono-Q fractions were assayed by UV-crosslinking using the radioactively labelled CDE-K probe, to identify the fractions containing the CDE binding protein, and to estimate the proteins size (Fig. 11B). We could detect four crosslinking activities in fractions 17 to 21, with molecular weights of 45, 50, 70 and 100kD. The bands at 45 and 50kD would correspond to complex 1, although the size of this complex is smaller than observed in Fig. 6A. Possible reasons could be proteolytic degradation of the protein, a discrepancy between the different sets of protein markers used, or the influence of the elevated amounts of KCl in these fractions. Furthermore, we co-eluted the band at 70kD, probably corresponding to complex 2, , which binds non-specifically to the CDE as it could not be competed by unlabelled CDE-K and TNFα-ARE_{53} in Fig. 6A. Nevertheless, we decided to identify the protein by mass spectrometry.

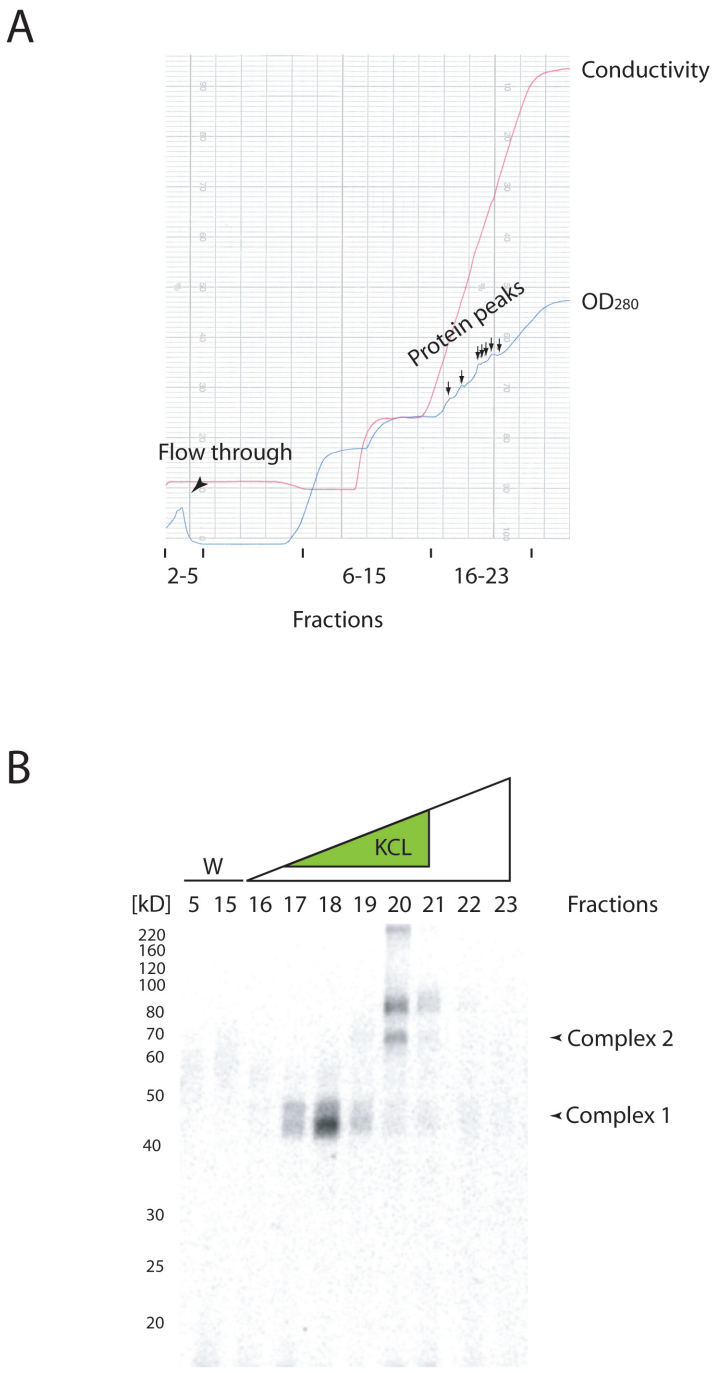


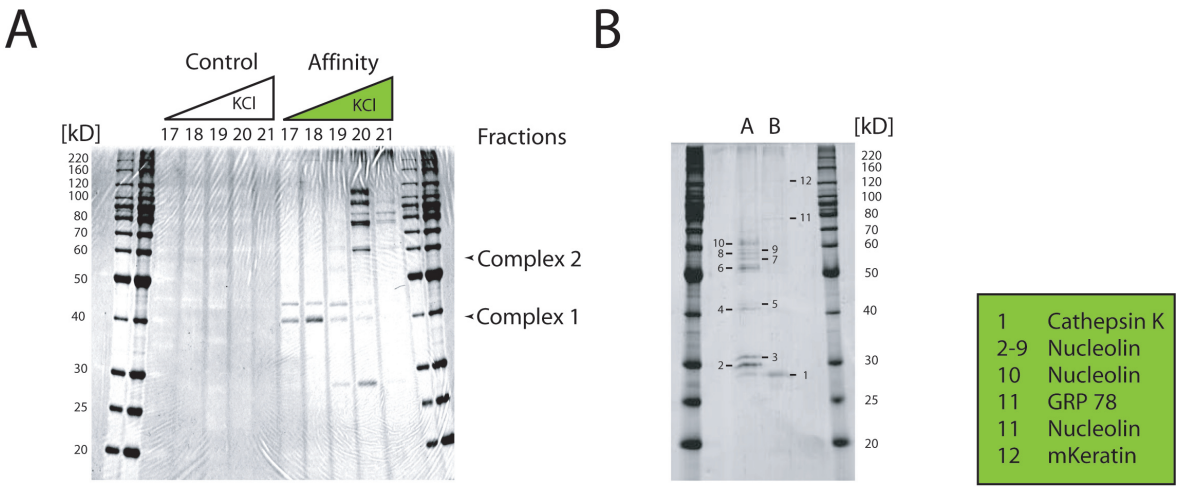
FIG. 11. Step 3: Purification of the CDE-BP by anion exchange chromatography. (A) The affinity purified fractions (Fig. 10) were applied to a HR 5/5 Mono-Q column using a FPLC-machine. The flow through (fractions 2 to 5) was collected, and the column was washed subsequently with 100mM and 250mM KCl (Fractions 6-14). Proteins were eluted from the column with a linear KCl gradient ranging from 250mM to 1M KCl and fractions 15 to 23 were collected. (B) Fractions 5 and 15 to 23 were subjected to UV-crosslinking using a radiolabelled CDE-K probe as described in Fig. 6, separated by 7.5% to 15% gradient SDS-PAGE. Protein-RNA complexes were visualized by phosphorimaging.

2.2.3.4 Fourth step: Mass spectrometry

The proteins from the Mono-Q fractions 17 to 21, derived from the 1M KCl eluate of the streptavidin control or the CDE-affinity purification, were precipitated, alkylated and resolved by a 7.5 to 15% gradient SDS-PAGE and visualized by coomassie blue staining (Fig. 12A). While the fractions from the Mono-Q column loaded with the streptavidin control fraction were devoid of protein, ten bands were visible in the affinity-derived Mono-Q fractions. The two bands visible in fractions 17 to 19 between the 40 and 50kD marker could correspond to the complex 1, where the protein amounts correlate with the crosslinking intensity of Fig. 11B. Another band in fraction 20 ran at the level of the 60kD marker, which would correspond to the non-specific complex 2 that was also visible in the UV-crosslinking assay (Fig 11B).

In a second experiment following the same purification scheme (Fig. 12B), we pooled the fractions 17 to 19 in lane A, and 20 to 21 in lane B, and after precipitation and alkylation, the purified proteins were visualized by coomassie staining. All visible bands were cut out and sequenced by MALDI-TOF and tandem mass spectrometry (MSMS) by Dr. Evers and Mr. Roeder at F. Hoffmann La-Roche, Ltd. Band 1 was identified as the protease cathepsin K, band 11 as the protein chaperone GRP 78, and band 12 as mouse keratin. The other seven bands corresponded to the RNA-binding protein nucleolin and its degradation products.

Nucleolin consists of an alternating basic acidic domain at the N-terminus, a central domain with four RNA recognition motives (RRMs), and a C-terminus with an RGG domain (Bourbon et al. 1988). The structure of the entire protein and the fragments identified by mass spectrometry are schematically depicted in Fig. 12C. The peptides identified in the bands 2 to 5 are all located in the region of the RRM1 and 2, whereas bands 6 to 9 and 11 contained peptides from all four RRMs. Peptides from the N-terminus or the RGG domain could not be identified. Band 10 could be identified by MSMS only. In this purification experiment we did not obtain full-length nucleolin, which would run at a molecular weight of 110,000.



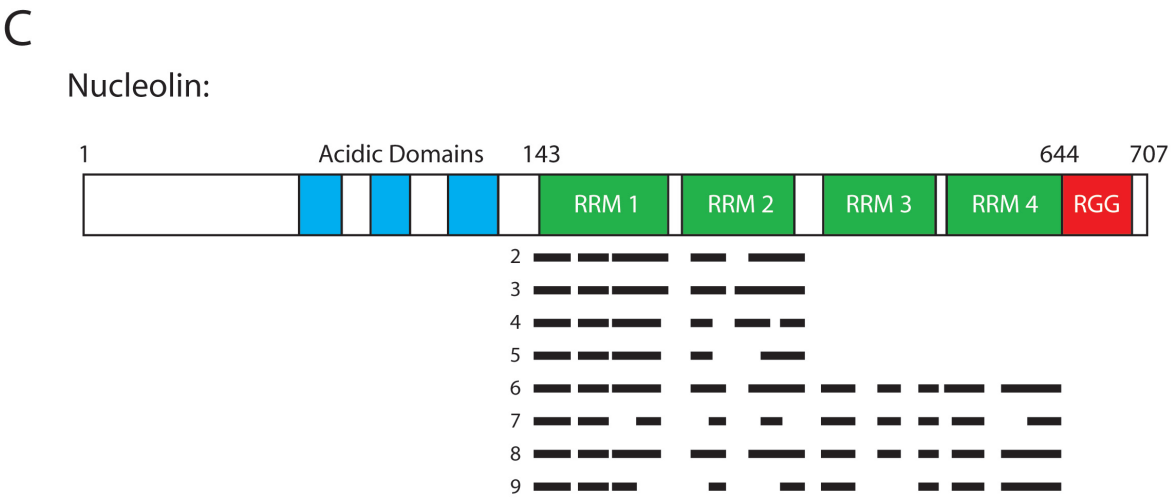


FIG. 12. Step 4: Nucleolin was identified as a CDE-BP by mass spectrometry. (A) Protein from the Mono-Q fractions were precipitated, alkylated and loaded on a 7.5% to 15% SDS-polyacrylamide gradient gel. Protein was visualized by coomassie staining. (B) From a repeat purification, fractions 17 to19 were collected in pool A, and fractions 20 to 21 in pool B. The pools A and B were precipitated, alkylated and analysed by 7.5% to 15% gradient SDS-PAGE. Protein was visualized by coomassie staining and the bands 1 to 12 were cut out and identified by MALDI-TOF and MSMS. The table shows all 12 bands identified. (C) Nucleolin is schematically depicted. It contains three acidic domains in the N-terminal domain, 4 RRMs in the central part, and a C-terminal RGG domain. The peptides identified from bands 2 to 5 were distributed over the RRMs 1 and 2 and bands 6 to 9 over the RRMs 1 to 4. The peptides are indicated by black bars.

2.2.4 Nucleolin can be precipitated with RNA coated beads

Nucleolin was discovered as a highly expressed protein in the nucleus (Orrick et al. 1973; Prestayko et al. 1974), and is divided into three functionally distinct parts. The N-terminus contains three acidic domains, which are interspersed by basic stretches and is reported to be a protein interaction domain. The central part contains four RRMs, which are only 10% identical to each other. The RRMs make specific contact with different target sequences like the Jun-kinase responsive element (JRE) (Chen et al. 2000), the bcl-2 ARE (Sengupta et al. 2004) or the well characterized nucleolin recognition element (NRE) which is found in the rRNA precursor and is involved in ribosome biogenesis (Serin et al. 1996). The NRE consensus sequence was defined by the SELEX method as a short stem loop (Ghisolfi-Nieto et al. 1996), and this sNRE (for SELEX-NRE) is bound by nucleolin with high affinity. The C-terminal domain contains RGG-repeats that are relevant for protein-protein interaction with ribosomal proteins and hnRNP A1 (Cartegni et al. 1996; Bouvet et al. 1998) and can also bind non-specifically to RNA (Ghisolfi et al. 1992), which makes it necessary to rigorously confirm the binding specificity of nucleolin to a target sequence. Initially, we wanted to know whether nucleolin is the only protein binding to the CDE-fragment O. We incubated Raw 264.7 macrophage extract with beads precoated with either a 5' biotinylated sNRE oligonucleotide or the biotinylated fragment CDE-K and uncoated beads were used as a control. After the binding reaction, the supernatant was removed and the beads thoroughly washed. Proteins associated with the oligomers were eluted with 1M KCl, and the protein fractions were analysed by EMSA using radiolabelled CDE-O as the probe (Fig. 13A). Depletion with the CDE and NRE matrices resulted in greatly diminished or absent protein-RNA complexes A and B in the depleted supernatants (NRE and CDE) compared to the input and the control (Ctr) supernatant. The band shift activity could be recovered in the elution fractions from the CDE and NRE coated beads, see Fig. 13A and B, but not in the elution fraction of the control (Ctr) lane. By western blot with a nucleolin-specific antibody (Fig. 13B), we could show that nucleolin was indeed removed from the cytoplasmic lysate. It was only detected in the input, the control supernatant and in the pellets of the NRE and

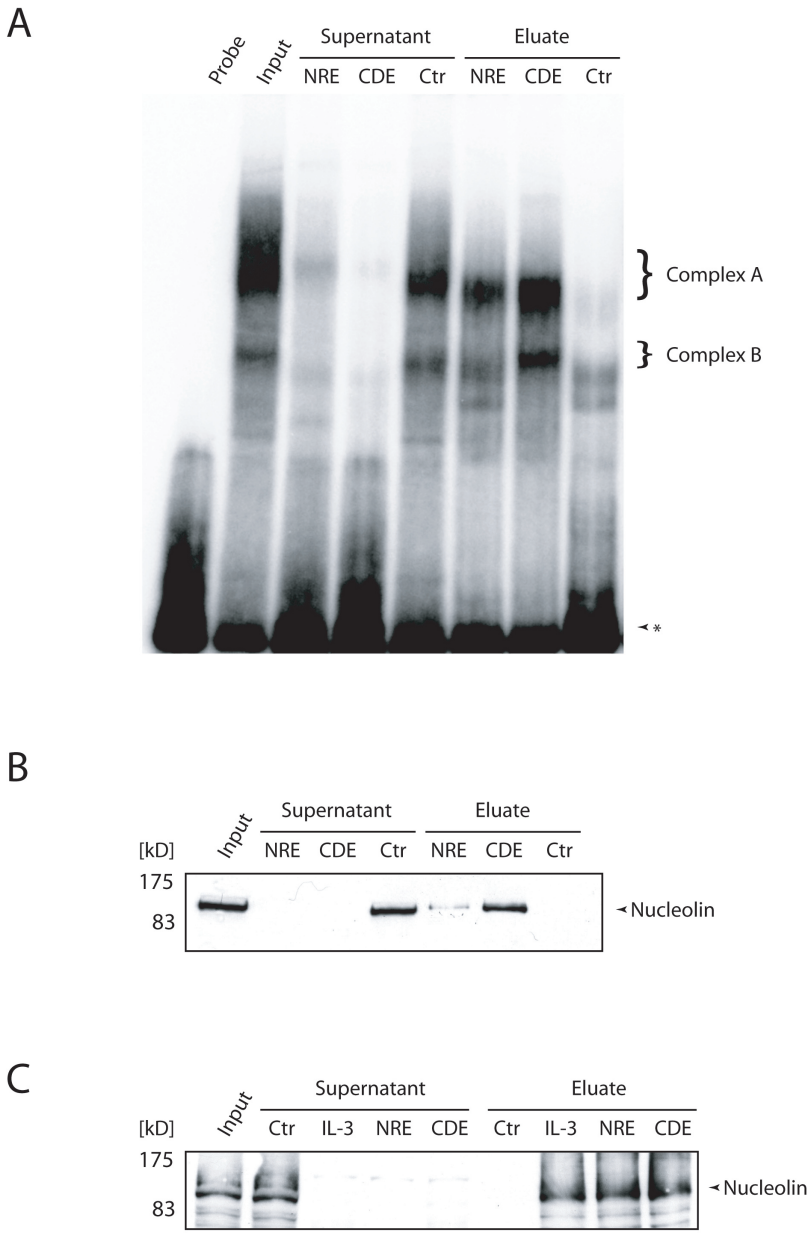


FIG. 13. Nucleolin can be precipitated with RNA coated beads from Raw 264.7 lysates. (A) Raw 264.7 macrophage extracts were incubated with 5'-biotinylated sNRE coupled to streptavidin sepharose, 5'-biotinylated CDE-O coupled to streptavidin sepharose or streptavidin sepharose alone overnight at 4°C. The supernatants were transferred to a new tube and the beads were washed several times. The bound protein was eluted with a 1M KCl buffer (Eluate). Raw cytoplasmic extract (Input), a buffer control (Probe) and the fractions were incubated with a radiolabelled CDE-O-probe for 30 min on ice. Protein-RNA complexes were separated on a 4% native polyacrylamide gel and visualized by phosphorimaging. The asterisks indicate the free probe. (B) The nucleolin levels in the different fractions were determined by western blot using a nucleolin-specific antibody. (C) The pull-down was repeated as in (A), except that a control, corresponding to 40nt of the IL-3 3'UTR, was included (IL3). The input, the different supernatants and eluates were analysed by western blot using a nucleolin-specific antibody.

CDE affinity matrices. Nucleolin eluted from the sNRE seems to be under-represented. One explanation could be that the elution from the sNRE beads was not quantitative due to its high affinity for nucleolin. It was also observed, that full-length nucleolin was eluted in this pull down assay, whereas in the purification strategy used to identify nucleolin, only fragments could be obtained. It is possible that extensive handling during the purification process, results in some degradation of nucleolin, whereas the comparatively simple depletion experiments only take a short time, resulting in an intact protein. In this experiment no other protein than nucleolin appears to be associated with the CDE and we concluded that nucleolin is a likely candidate to be associated with the CDE. Subsequently a similar approach using a 40nt long 5'biotinylated sequence from the IL-3 3'UTR (IL-3) as a non-specific precipitation control in western blot was performed (Fig. 13C). As shown before, the NRE and the CDE coated beads cleared nucleolin from the supernatant compared to the streptavidin control (Ctr), but, unfortunately, so did the IL-3 3'UTR-sequence (IL-3). Nucleolin could again be recovered from the beads (IL-3, NRE and CDE) but not from the control (Ctr). This raised again the question as to whether the nucleolin-CDE interaction is really specific, and not merely mediated by the non-specific binding activity of the RGG domain.

2.3 Part III: Functional assays

2.3.1 Nucleolin binds to CDE-O, but not the mutants

As the pull down experiment demonstrated, it is critical to prove that the nucleolin-CDE interaction is specific. To resolve this problem, we decided to test the CDE-O and the derived O-mutant sequences in bandshift experiments, as we had earlier shown in this work that the affinity-purified protein could discriminate between the CDE-O and fragment L RNA (Fig. 10C). Here we tried to repeat this result with cytoplasmic extract and the wild type and mutant probes of O, and to prove whether any activities observed are identical with nucleolin, we would try to super shift the complex with a anti-nucleolin antibody. If the CDE-O probe binds to nucleolin and not to its mutants, this would indicate specificity of

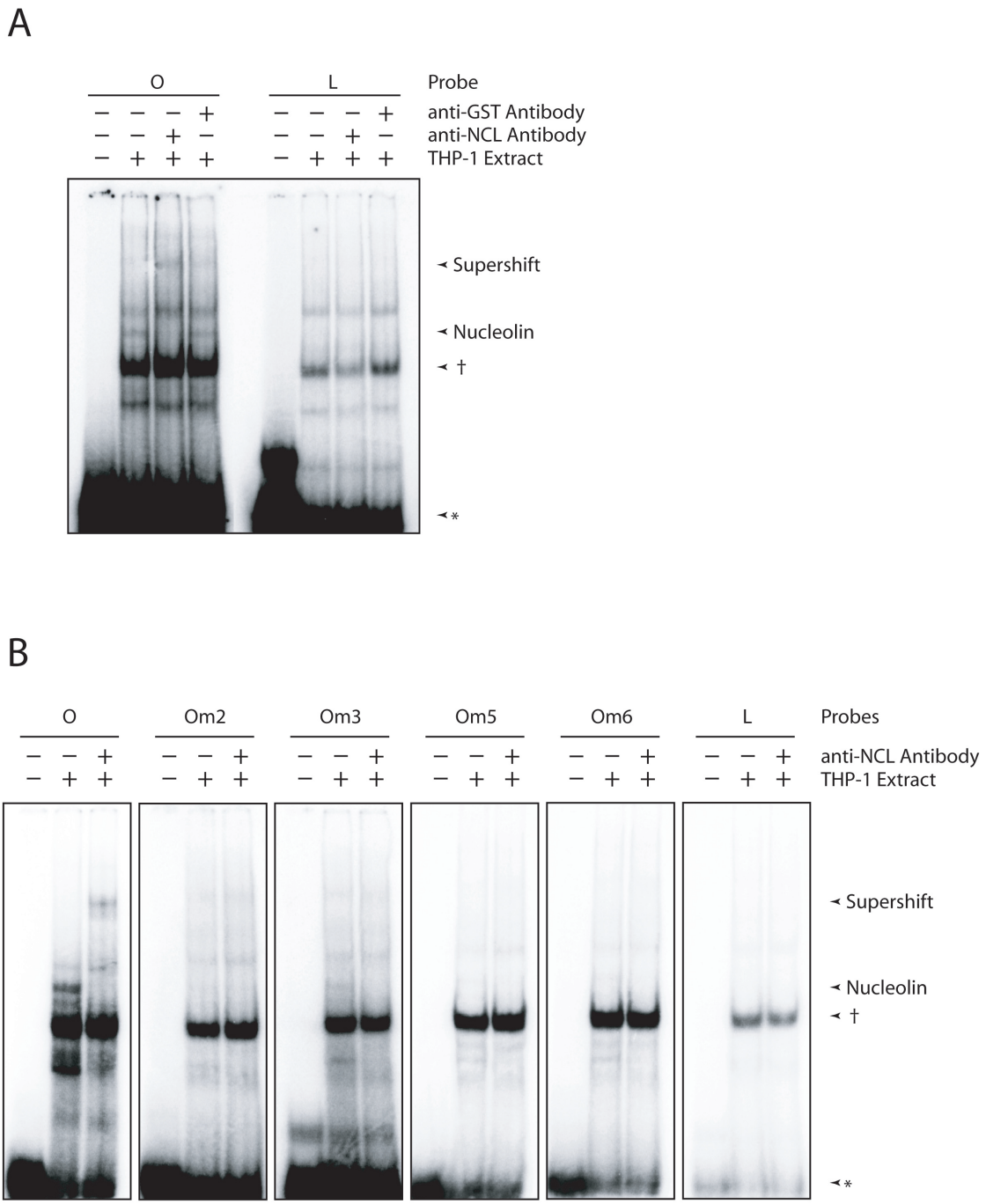


FIG. 14. Nucleolin binds specifically to fragment O. (A) 10μg THP-1 human macrophage extract was preincubated with anti-nucleolin (NCL) or anti-glutathione-S-transferase (GST) antibody for 30 min on ice, and then mixed with 10kcpm of radiolabelled CDE-probe O or L, and incubated for a further 30min on ice. Protein-RNA complexes were separated on a 4% native polyacrylamide gel. The complexes were visualized by phosphorimaging. Asterisks indicate the free probe, and the cross indicates a non-specific band. (B) EMSA was repeated as in (A) using CDE-probe O and the mutants Om2, Om3, Om5, Om6 and L, and a nucleolin-specific antibody for supershift. Asterisks indicate the free probe, and the cross indicates a non-specific band.

nucleolin for the CDE-O. Therefore 10µg of THP-1 extract was incubated with 10kcpm of radiolabelled probes O and L (Fig. 14A). Several non-specific protein-RNA complexes were observed that bound to both L and O. However, there was one band that was only observed with the CDE-O probe. To confirm that this is nucleolin, we used a mouse monoclonal anti-nucleolin antibody (MS3 Santacruz) and a control anti-GST antibody to perform a super shift. The band unique to O could be super shifted with the anti-nucleolin antibody, but not with the anti-GST control antibody, proving that the band unique to O is indeed nucleolin. To correlate the in vitro binding of nucleolin with the in vivo activity of the different mutants, we repeated the band shift experiments using 10kcpm of radiolabelled probes of all the mutant-O sequences and 10µg THP-1 cytoplasmic extract (Fig. 14B). We observed that nucleolin binds strongly to the fragment O, but only weakly or not at all to any of the mutant sequences. Binding of nucleolin to fragment O was confirmed by super shift assays with a specific anti-nucleolin antibody. Therefore we conclude that nucleolin binding in vitro correlates with rapid CDE mediated mRNA decay in vivo.

2.3.2 Preparation of recombinant nucleolin

To investigate the nucleolin-CDE binding further using well-defined reagents, we decided to produce recombinant nucleolin using the baculo-virus expression system.

An overview of the recombinant GST-nucleolin production from vector to protein is depicted in Fig. 15A. Nucleolin was amplified by PCR from Raw macrophage cDNA with the primers Nucleolin-baculo and Nucleolin-AntiS, and was inserted together with a tobacco etch virus (TeV) protease recognition site into the donor plasmid pFastbac GST (Brondani et al. 2005). This vector was transformed into DH10Bac *E.coli* cells, which contained a bacmid encoding for the baculovirus genome that was modified by insertion of the lacZ gen and the attTn7 recombination site. The GST-TeV-Nucleolin DNA recombined with the attTn7 site via its Tn7 recombination site disrupting the lacZ gene. Positive GST-TeV-nucleolin bacmid clones were isolated by selection with kanamycin, gentamycin and tetracycline followed by blue-white selection. The bacmid was then extracted from the bacteria and transfected into High5

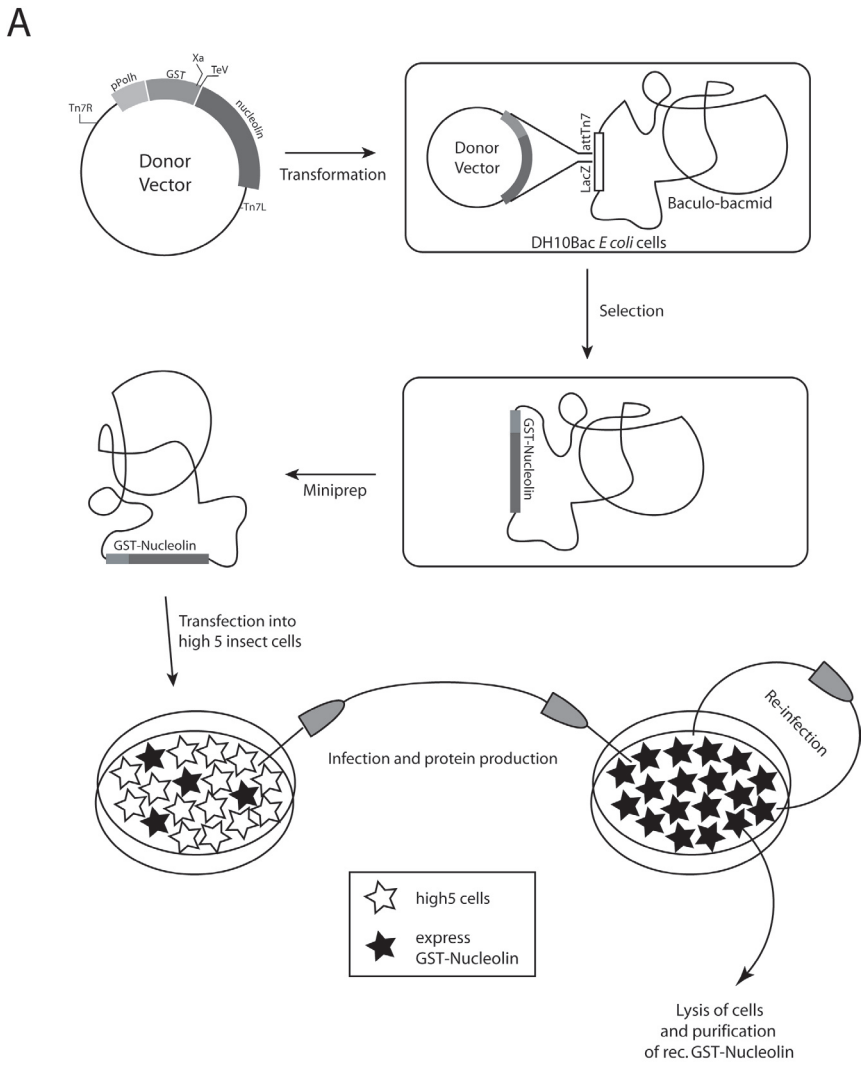


FIG. 15. Expression of recombinant nucleolin with the baculovirus expression system. (A) The GST-fastbac donor vector (Brondani et al. 2005) containing the full-length nucleolin sequence was transformed into DH10Bac E.coli cells containing the baculovirus genome. The GST-nucleolin fusion sequence was integrated into the baculovirus genome via its Tn7 recombination sites, and after selection the recombinant baculo-bacmid was extracted from the E.coli cells and transfected into High5 insect cells for baculovirus production. The virus-containing supernatant was harvested and High5 cells were infected repeatedly to obtain high expression of the recombinant protein. (B) Uninfected cells (lane 1,2), or cells infected with two different baculovirus clones (lane 3 to 6) were lysed and centrifuged to obtain soluble fraction (lanes 1,3,5) and an insoluble pellet (lane 2,4,6). The expression of recombinant GST-nucleolin was shown by western blot.

insect cells, which produced high amounts of the recombinant virus. The cells were lysed after the second round of infection, after sufficient protein is produced. To confirm GST-TeV nucleolin production (Fig 15. B.), High5 cells were infected with two independent virus productions, namely baculo-clone1 (lane 3 and 4) and baculo-clone2 (lane 5 and 6) or a GST control virus (lane 1 and 2). The cells were lysed and the insoluble fraction was spun down in a centrifuge. An aliquot of the soluble (odd numbered lanes) and the insoluble fraction (even numbered lanes) were analysed by western blot with an antibody specific for nucleolin. Both clones, but not the control contained the recombinant protein. Unfortunately, most of the desired fusion protein was insoluble (lanes 4 and 6), and could not be brought into solution. We purified the recombinant nucleolin from the soluble fraction with GST sepharose.

2.3.3 Recombinant nucleolin binds strongly to fragment O, but not to Om2 or L

Apart from its known target sequences, nucleolin is also able to bind RNA non-specifically by virtue of its RGG domain (Ghisolfi et al. 1992). Therefore we wanted to determine if recombinant nucleolin can discriminate between the fragment O and mutated CDE sequences. We expressed the construct shown in Fig. 15A. as well as a GST control construct in High5 insect cells using the approach described above. The GST or GST-nucleolin expressing High5 cells were lysed, and GST or GST-nucleolin was bound to GSH-sepharose. After three rounds of washing and one round of equilibration with the TeV buffer, nucleolin could be eluted by TeV protease cleavage, resulting in untagged, soluble nucleolin. Fractions from every working step were analysed by western blot using an anti-nucleolin specific antibody (Fig. 16A. data for GST control not shown). GST nucleolin was present in the input and in the supernatant (lanes 1 and 2), but not in the wash and equilibration fractions (lanes 3 to 6). A nucleolin band could be detected after elution by TeV cleavage for 1h at 4°C, which had a higher mobility than the GST-nucleolin fusion protein and corresponded to untagged nucleolin (lane 7). To assess the purity of the recombinant protein, the eluted nucleolin (lane N) and the GST control (lane C) were viewed on a coomassie gel (Fig. 16B). The control lane is bare, but in the eluted fraction a band corresponding to the size of nucleolin

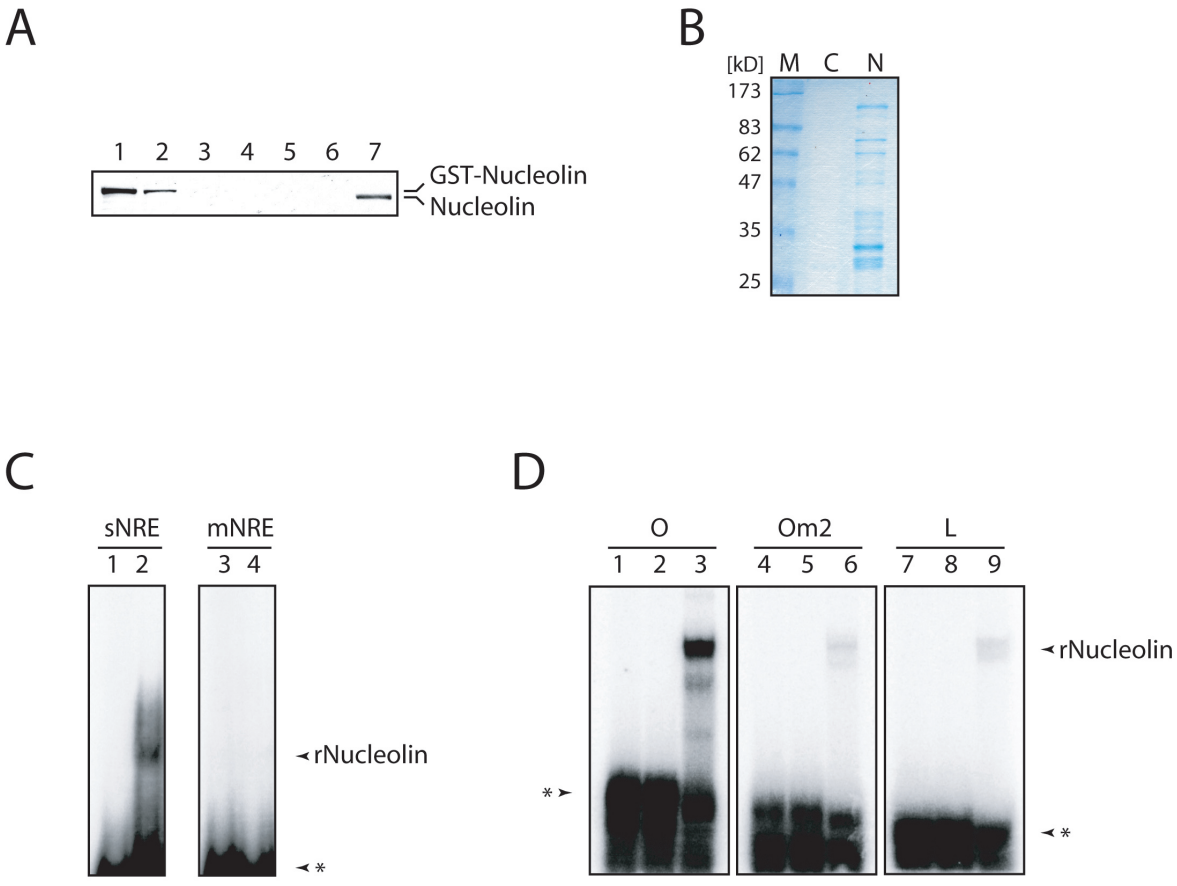


FIG. 16. Purification and testing of recombinant nucleolin. (A) High5 extracts containing recombinant GST-Nucleolin (lane 1) were bound to glutathione sepharose matrix. The supernatant (lane 2) was removed and the matrix was washed three times (lanes 3 to 5). After equilibration with TeV-protease buffer (lane 6), nucleolin was released by TeV-protease (lane 7). The fractions were separated by SDS-PAGE, and nucleolin was detected by western blot using a specific anti-nucleolin antibody. (B) GST-control (lane C) and nucleolin eluates (lane N) were analysed by SDS-PAGE and coomassie blue staining. (C) Recombinant nucleolin was tested by EMSA as described in Fig. 4, using radioactively labelled SELEX-selected nucleolin recognition element (sNRE) and a mutant NRE (mNRE) as probes. (D) The same experiment was performed using radiolabelled probes for O, Om2 and L. Asterisks indicate the free probe.

was observed together with other bands, which may be either copurified proteins from insect cells or degradation products of nucleolin. We next wanted to know if the purified full-length nucleolin is biologically active. This was tested in an EMSA using 100ng of the recombinant nucleolin. For a positive control we used 5kcpm of radiolabelled selex nucleolin recognition element (sNRE, a well established nucleolin target), and a mutant NRE sequence (mNRE) originally referred to as NS26A18C as a specificity control (Fig.

16C) (Johansson et al. 2004). The sNRE was defined in a selex experiment as a nucleolin target sequence binding with high affinity to nucleolin (Ghisolfi-Nieto et al. 1996). Similar sequences were previously defined on the pre-ribosomal RNA, a physiological target of nucleolin (Serin et al. 1996). The affinity of sNRE and NRE-mutants were later tested by biacore assays to assess the nucleotides relevant for nucleolin-NRE binding. There the K_D value of sNRE was determined at 2 to 5nM and for the mNRE at more than 50µM, making them ideal controls for this experiment (Johansson et al. 2004). As expected, a shift could be observed with the sNRE probe (lane 2) indicating proper folding and biological activity of the recombinant nucleolin, but not with the mNRE (lane 4). The probes alone are shown in lane 1 and lane 3 respectively. Finally, we wanted to demonstrate that recombinant nucleolin is able to distinguish between radiolabelled fragment O, L and Om2 probes (Fig. 16D). In a band shift assay, we observed a shift of the probe O with 100ng of recombinant nucleolin (lane 3), but not with a GST-control fraction (lane 2). Probes Om2 and L showed greatly reduced nucleolin binding (lane 6 and 9). The control fractions were not able to induce a band shift with any probe (lanes 2, 5, and 8). Lanes 1, 4 and 7 show the integrity of the radioactively labelled probes. These results support our data obtained with THP-1 extracts and the correlation between nucleolin binding and in vivo CDE activity.

2.3.4 Nucleolin interacts with TNFα mRNA in vivo

The results so far demonstrate that nucleolin can interact with the functional CDE in vitro, and shows no or greatly reduced binding to inactive CDE-mutants. To test whether nucleolin and the CDE indeed interact in vivo, we performed RNA-immunoprecipitation assays with endogenous nucleolin and TNFα mRNA. It would strengthen our binding data, if endogenous TNFα mRNA could be pull-down together with nucleolin. For this we prepared cytoplasmic extract from THP-1 human macrophages, which was precleared by centrifugation. 2mg of this extract were added to protein G sepharose beads precoated with the specific antibodies against the myc epitope (control), the ELAV protein HuR and nucleolin (Lopez de Silanes et al. 2004a; Takagi et al. 2005) in the presence of RNAse inhibitors. HuR was previously

shown to bind the TNFα-ARE, and was therefore used as a positive control. After the extract was incubated with the coated beads for 2h at 25°C, the supernatant was removed, and the beads were washed ten times with buffer NT2. Finally the RNA and protein was eluted from the beads with buffer NT2 containing 0.1 % SDS at 65°C. Western blot was performed to test whether HuR and nucleolin can be pulled down under these conditions (Fig. 17A). In the input and myc supernatant fraction, nucleolin, HuR and GAPDH were all detected by their corresponding antibodies. HuR was greatly reduced in the supernatant of the HuR coated beads, but could be recovered from the HuR pellet, indicating it is a very effective precipitating antibody. Nucleolin was not so effectively pulled down by its specific antibody. Substantial amounts of nucleolin are still visible in the supernatant, and only 20 to 50% of nucleolin could be pulled down with the nucleolin specific antibody. GAPDH, our negative control protein, was not precipitated by any of the antibodies used, and the myc control antibody was not able to pull down nucleolin, HuR or GAPDH, indicating that the washing of the beads was sufficient. Although the pull-down of nucleolin was incomplete, it was nevertheless sufficient to proceed with RNA-IP and isolation of RNA. For the RNA-IP experiment, we prepared extracts from unstimulated and LPS stimulated THP-1 macrophages (4h). LPS induces the levels of TNFα mRNA and that of our control cytokines IL-6 and IL-10, which are barely visible under unstimulated conditions. Another effect of LPS on macrophages is that HuR, the positive control, translocates from the nucleus to the cytoplasm, so that more HuR bound TNFα message can be detected. The RNA-IP was performed on these lysates. After the RNA-IP was performed as described above, the eluted RNA was purified using the RNeasy mini kit (Qiagen). The RNA from the input, and from the precipitated RNP complexes were reverse transcribed to cDNA. From this cDNA we amplified the various messages by 35 rounds of PCR (Fig. 17B). GAPDH specific primers were used as a contamination control. This house keeping gene is highly expressed, and would indicate immediately if the beads were not sufficiently washed. Therefore, it should only be detectable in the input (I), but not in the pellet (P), as was the case in this experiment. A further control was a parallel pull-down using an anti-myc antibody. This antibody is not

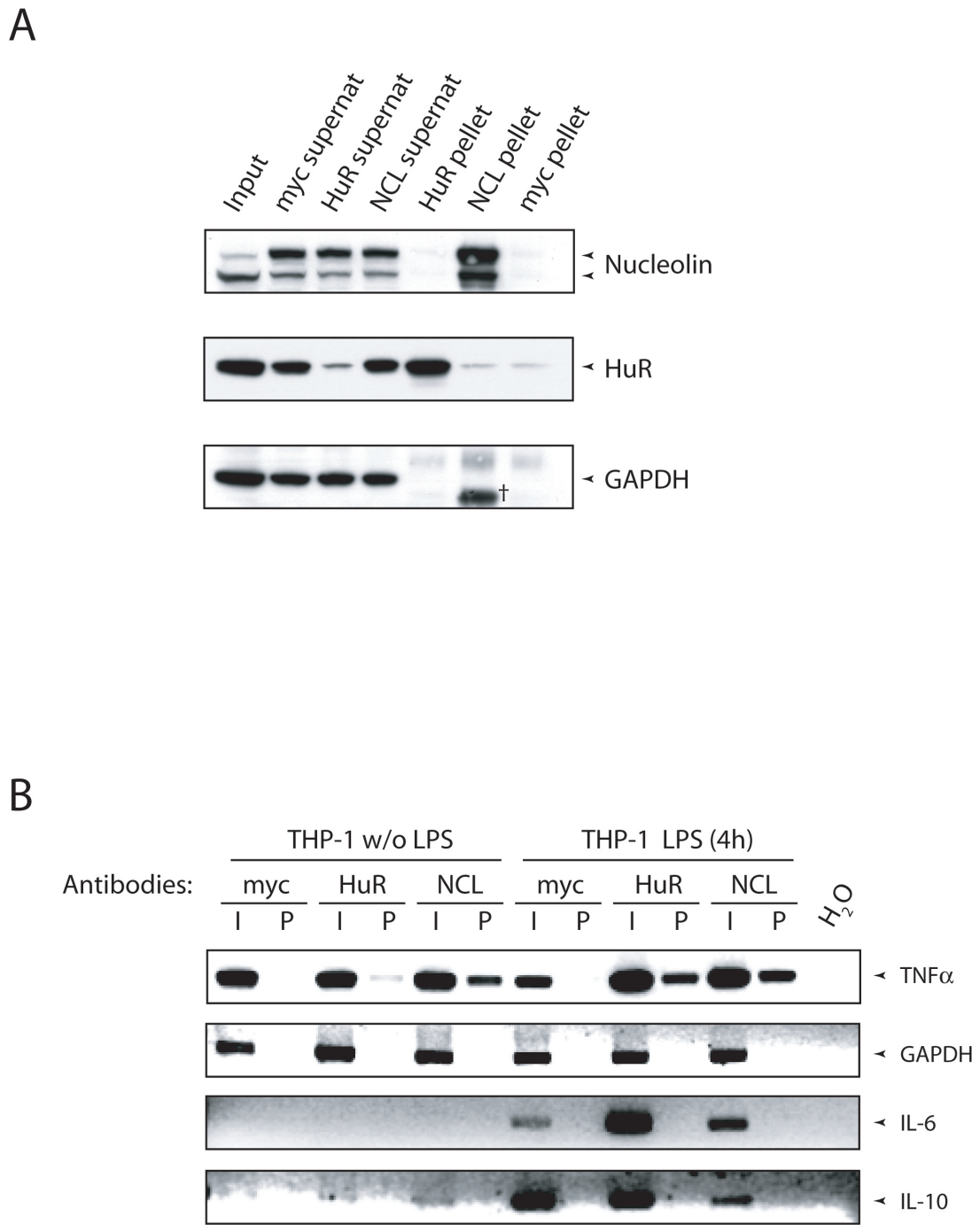


FIG. 17. The TNFα mRNA interacts with nucleolin in vivo. (A) THP-1 cell extracts were incubated with anti-myc, anti-nucleolin or anti-HuR coated protein G-sepharose beads for two hours at room temperature. Supernatants were removed and beads were washed ten times. Protein was eluted from the beads with 0.1% SDS at 65°C. The input, supernatants and protein of the pellets were analysed by western blot. The cross marks an unspecific band. (B) THP-1 cells were treated as above, and RNA was eluted from beads with 0.1% SDS and 10μg proteinase K at 50°C. RNA from inputs (I) and pellets (P) was purified, and RT-PCR was performed using specific primers for TNFα, GADPH, IL-6 and IL-10.

expected to pull down any message due to the absence of a binding target protein in the THP-1 cells, and this proved to be the case from the lack of any message from the pellet (P). IL-6 and IL-10 are cytokines that contain an ARE in their 3'UTR. Both messages are expressed in LPS stimulated cells, but were not pulled down by the nucleolin or HuR specific antibodies. These two messages acted as a specificity control for HuR, to exclude artificial interactions that are established after the cells were disrupted. HuR and nucleolin both displayed a significant association to the TNFα message, which was increased after LPS stimulation. This indicates that nucleolin indeed is associated with the TNFα mRNA in vivo and supports our hypothesis that nucleolin may be a regulator of TNFα-mRNA turnover. This encouraged us to investigate further the involvement of nucleolin in CDE-mediated decay.

2.3.5 Knockdown of nucleolin with siRNA causes an increase of GFP protein in a GFP-CDE reporter cell line

Having confirmed the interaction between nucleolin and the CDE, a RNA-destabilizing element, we wished to observe the effect of nucleolin depletion on a CDE-bearing reporter gene. Nucleolin levels were down-regulated by siRNA in HT1080 cells stably transfected with a GFP-CDE reporter (Fig. 18A), and the behaviour of this reporter was monitored by analysing the changes in GFP-levels by fluorescence activated cell sorting (FACS) (Benjamin et al. 2004). Double transfection of two different nucleolin specific siRNAs, NCL-2 (lane 4) and NCL-3 (lane 5), resulted in a substantial reduction in nucleolin protein levels, whereas a reduction of nucleolin levels could not be observed with a HuR (lane 3), a BRF1 (lane 2) specific siRNA or an untransfected control (lane 1). The protein levels of GAPDH were not influenced by any of the siRNAs. Interestingly, the levels of HuR was not only reduced by the HuR specific siRNA (lane 3), but also by the nucleolin specific siRNAs (lanes 4 and 5) for unknown reasons, albeit not as strongly. A decrease in translation induced by nucleolin down regulation could be responsible for this effect, although this was not observed with the GAPDH protein. One could even speculate that nucleolin my influence HuR expression. By FACS analysis we observed that transfection of both siRNAs directed against nucleolin (green) were able to cause an increase in GFP expression levels compared

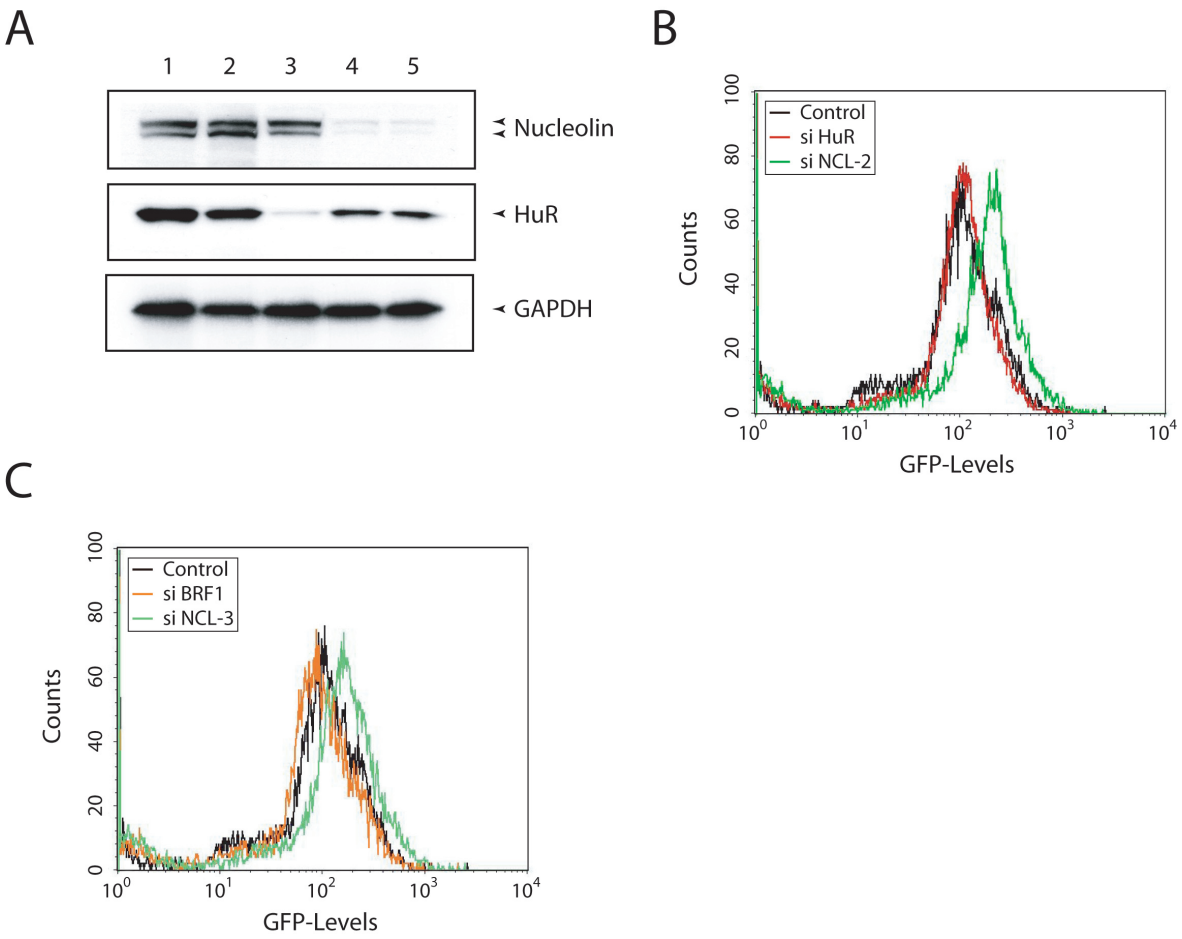


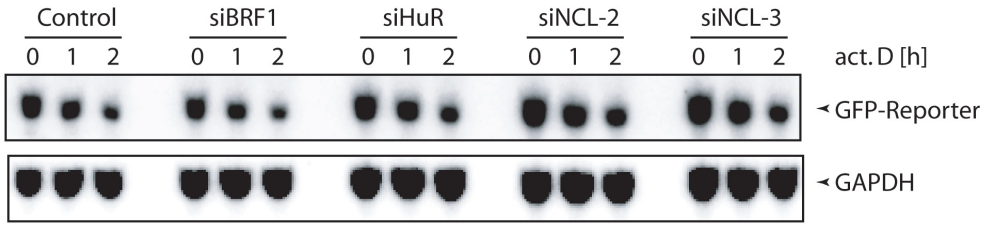
FIG. 18. Knockdown of nucleolin causes an increase in GFP protein levels in GFP-CDE reporter cells. (A) HT1080 GFP-CDE reporter cells were transfected twice with siRNA against BRF1 (lane 2), HuR (lane 3) and two different siRNAs against Nucleolin (lane 4 and 5) or not at all (lane 1). Total cell lysates were harvested 72h after transfection, and separated by SDS-PAGE. Knockdown of the respective genes was confirmed by western blot. (B and C) GFP levels of the control and siRNA transfected cells were analysed by FACS.

to the untransfected control (black), the si-BRF1 (orange) or si-HuR (red) treated cells (Fig 18.B and C). Furthermore, both nucleolin specific RNAs had a similar effect in increasing GFP expression levels thus reducing the possibility of an off-target effect caused by siRNA transfection. Although this result is consistent with our hypothesis, it remains to be prove, if nucleolin influences GFP-reporter mRNA stability or if the observed increase of GFP-expression is caused by other means.

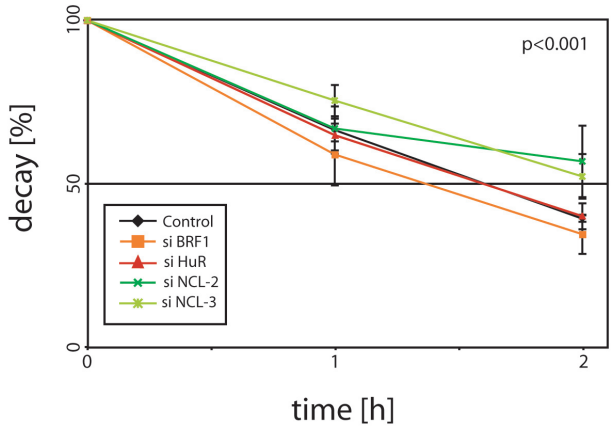
2.3.6 Effect of nucleolin downregulation on the GFP-CDE reporter mRNA stability

The shift in GFP protein levels observed in Fig. 18B and C may be due to reasons other than a change in the decay rate of this reporter, such as increased transcription or translation rates. To clarify this question, we performed actinomycin D mRNA decay chase experiments. We transfected 15cm dishes of HT1080 GFP-CDE cells with the previously described siRNAs. After 48 hours, the cells were split equally into three 10cm dishes, and after 72 hours the cells were treated with actinomycin D to arrest transcription. Cells were harvested over a time course for total RNA extraction. The RNA was analysed by northern blotting with a specific probe against the TNFα 3'UTR. This experiment was repeated several times, and a typical experiment is shown in Fig. 19A. We observed that the reporter from cells treated with both the nucleolin specific siRNAs decayed slower than in the untreated cells or cells transfected with the control siRNA oligomer. The amount of endogenous GAPDH mRNA was not changed under any of the conditions. The amount of GFP reporter mRNA left after one or two hours with actinomycin D was normalized against the GAPDH control. The mean of all experiments indicated in table 1 was calculated and plotted against time (Fig. 19B). The half-lives and standard deviations were calculated (table 1), and the statistical significance was calculated by the student (t) test. Although the changes in decay rate are not dramatic, down-regulation of nucleolin (green) increases the CDE-reporter half-life by more than 30 percent from 96 to 134 minutes, compared to the down-regulation of BRF1 (orange), HuR (red) or untransfected cells (black). This 30% increase in half-life resulted in a 3 to 4 fold increase in protein levels, which would be sufficient in the case of TNFα to induce autoimmune disease or rheumatoid arthritis over time.

A



B



	$t_{\frac{1}{2}}$ (min)	STDev	N. exp.
Control	96	+/- 2	4
siBRF1	84	+/- 6	5
siHuR	96	+/- 5	5
siNCL-2	134	+/- 11	5
siNCL-3	136	+/- 6	4

FIG. 19. Knockdown of nucleolin influences GFP-CDE reporter RNA half-life. (A) The siRNA transfected HT1080 GFP-CDE reporter cells (Fig. 18) were treated with act. D for 0, 1 or 2 hours. Total RNA was extracted, and analysed by northern blot. Reporter RNA was detected using a radiolabelled probe specific for the TNFα 3'UTR. To control loading, the membranes were rehybridized with a probe against GAPDH. (B) Reporter levels were quantified with Quantity One and normalized against the loading control. Percentage of decay was plotted against time. The student(t) test was applied to determine the statistical significance.

3. Discussion

3.1 The CDE is a rapid decay element

In this work we have investigated the function of the constitutive decay element (CDE) that is located downstream of the ARE in the 3' UTR of the TNFα message. The CDE was originally discovered in slowC cells that are deficient in ARE dependent decay due to a frame shift mutation in both alleles of the AU-binding protein BRF1. A reporter containing the entire 3' UTR of TNFα was degraded rapidly, with a half-life of about 45 minutes, whereas a reporter containing just the defined ARE sequence was stable for over more than 6 hours in these cells. By deletion mutagenesis, the CDE could be mapped to an 80-nucleotide long sequence (KΔAU) located 20 nucleotides downstream of the TNFα-ARE_{53}. It contains a highly conserved region with a putative stem loop region, and was degraded with a half-life of 1.7 hours, approximately 2.5 times slower than the complete 3'UTR in slowC cells (Stoecklin et al. 2003). In the present work, we were able to further reduce the size of the active CDE to a minimum of 40 nucleotides (fragment O) by deletion mapping (Fig. 4). Surprisingly, a reporter mRNA containing this minimal CDE was degraded even more rapidly than the KΔAU-fragment, and followed similar kinetics as the entire region downstream of the ARE with a half-life of 45 minutes. It is therefore conceivable, that the longer KΔAU fragment contains a sequence that negatively influences the decay activity, either via an altered mRNA secondary structure, or by means of active stabilization via stabilizing proteins. Another possibility could be the deletion of two bases at the 3' terminus, which were included in CDE-O but not in the KΔAU fragment (Fig. 4A). When we stably introduced the reporter constructs bearing the decay-promoting CDE-fragment O and the inactive fragment L into NIH 3T3 cells, we were able to confirm the rapid decay kinetics from transient transfection experiments, and the increased reporter expression in stable cell

lines allowed us to observe rapid deadenylation of fragment O. Deadenylated products could already be detected at time point zero, hinting at the power of the CDE in mediating rapid decay (Fig 4D).

By M-fold RNA structure prediction, a putative stem-loop was discovered in the CDE. Mutations in the loop region and the stem rendered the slow-decaying CDE fragment K non-functional, which however could not be reversed by restoring the base pairing by complementary mutations (Stoecklin et al. 2003). It is also striking that the fragment O is very U-rich, which is a common feature of destabilizing motifs (Chen and Shyu 1995; Peng et al. 1996), and we presumed that the CDE-O might act as a class-III ARE. We also postulated, that the 5' region that is present in O but absent in L was responsible for the inactivity of fragment L in promoting decay. Therefore we introduced mutations in three of the seven Us of the stem and loop implicated by Stoecklin et al. and changed the first four nucleotides from CUUC to AGGA in the CDE-O (Om2) (Fig 5A). In mutant Om3 only the first four residues were changed in order to assess the relevance of the first six nucleotides that were deleted in L compared to O. Two additional mutants were create, which retained the U-rich region of the CDE, Om6 where the 3' terminal six nucleotides were deleted, and Om5 which contains an internal deletion of six nucleotides. All mutants were subsequently tested for decay promoting activity. It was clear that the introduction of any mutation into fragment O leads to a loss of function of this cis-element (Fig. 5B) so that not only the entire length of 40 bases is required for CDE activity, but also the specific sequence. This also excludes the possibility that the CDE is a class III-ARE, as in this case, the mutations in Om5 and Om6 would not have had any effect on CDE-mediated decay.

3.2 Nucleolin was identified as the CDE binding protein

3.2.1 Identification of a specific CDE binding protein

To deepen our understanding of the mechanism underlying CDE-mediated decay, we decided to identify the trans-acting factors interacting with this cis-element. By UV-crosslinking experiments with the CDE-K, we were able to visualize a protein at approximately 60kD, named complex 1, that could be competed with a CDE but not with an ARE probe, indicating the specificity of the protein towards the CDE (Fig. 6). It was also of interest to test whether the CDE differs from its mutants with respect to protein binding and whether this correlates with the CDE-activity. This was tested by electrophoretic mobility shift assay. A low mobility complex A formed with the CDE and was still visible in the mutant Om2, but became progressively weaker with the other mutants. Mutant L showed only a low association with protein. One could speculate at this point that the affinity of the CDE-binding protein seemed to be higher for the CDE than for the mutants, which was proven in later experiments.

3.2.2 Purification and identification of nucleolin

Encouraged by the binding results above, we tried to purify complex 1 CDE-binding activity. We chose different chromatography media, and assayed for the unbound protein under low or high salt conditions, which we were able to visualize by UV-crosslink experiments (Fig. 7). With blue and red sepharose matrices, the protein was bound even under high salt conditions, making them unsuitable for the purification procedure as elution from this matrix was not possible. Phenyl sepharose was also unsuitable as it was not able to bind complex 1 at all. This left Q and heparin sepharose, which were both able to bind the activity under low salt but not under high salt conditions. We started to purify the binding activities over two affinity steps, initially with heparin sepharose (Fig. 9), which is able to adsorb proteins specifically binding to sugar residues, and an affinity matrix generated by linking the biotinylated CDE to streptavidin beads (Fig. 10), which should efficiently interact with the

CDE specific protein. After the affinity purification, the fraction was checked for its ability to bind to the CDE-fragment O, but not CDE-fragment L before proceeding further. By EMSA we observed that radioactive CDE-O, but not the CDE-L probe, was shifted confirming enrichment of the CDE-binding activity. However the observed complex was much smaller than complex A in Fig. 6. Due to the high grade of purification obtained after the affinity step by the harsh washing and elution steps, the protein complex A could have been disrupted and proteins not directly or strongly associated with the CDE may have been washed away. An alternative explanation could be proteolytic degradation of complex A during the purification procedure, giving rise to a higher mobility complex. One has to note that both the heparin and the affinity matrices are also weak cation-exchange matrices due to their negative charge, which allow for non-specific binding of positively charged proteins. To reduce the amount of the non-specifically binding proteins, we employed an anion exchange matrix in a final purification step to repel the positively charged proteins (Fig. 11). It has to be noted that the size of the complexes 1 and 2 appear to vary relative to the protein markers. One reason could be that different sets of markers were used in different experiments, which may cause the discrepancy between the complex 1 at 60kD as in Fig. 6 compared to 50kD in the other figures. Another possible reason could be the proteolytic degradation mentioned earlier. Furthermore, we also copurified with this procedure the protein-RNA complex 2, which was thought to bind non-specifically to the CDE as shown with the UV-crosslink competition assay in Fig. 6.

Candidate CDE-binding proteins were identified by mass spectrometry (Fig 12). Besides the often encountered protein contaminant keratin, peptides were identified from the acidic protease cathepsin K, involved in osteoporosis reviewed in (Yamashita and Dodds 2000), the protein-chaperone GRP78, which helps in correctly folding of the tertiary structure of certain proteins (Ng et al. 1992), and the RNA binding protein nucleolin reviewed in (Ginisty et al. 1999). Although only fragments of nucleolin and no full-length protein were identified, it appears the most likely candidate to be involved in CDE-binding.

Using a similar approach, nucleolin and YB-1 were purified as interacting proteins of the jun-

kinase responsive element (JRE). Here the two JRE-crosslinking activities at 50 (p50) and 100kD (p100) were first purified over a anion exchange column, which separated p50 from p100. p100 was further purified via heparin sepharose and an affinity-RNA step, whereas p50 was gel-filtrated over Superose 6B and an affinity-RNA step (Chen et al. 2000).

The various nucleolin bands that we identified could be divided into two groups. The first group, bands 2 to 5, contain peptides from the RRMs 1 and 2, and the second group from band 6 to 9, contain peptides from the RRMs 1 to 4 of nucleolin. Although the peptides identified from both groups respectively are similar, the sizes of the different bands vary greatly, from 30kD (band 2) to 40kD (band 5), or from 50kD (band 6) to 65kD (band 9). This size difference could be due to an extension on the N-terminal side, where no peptides could be identified by MALDI-ToF. Furthermore nucleolin was identified at the positions of complex 1 and complex 2 (Fig. 11), which was thought to bind non-specifically to the CDE and raises the question of how specific the CDE-nucleolin interaction really is.

3.3 The chaperone GRP78

GRP78/BiP is a chaperone associated with the endoplasmatic reticulum and is induced by inflammatory stress. It is involved in folding of secreted and membrane proteins (Ng et al. 1992). No evidence has been found so far that it plays any role on proteins involved in RNA degradation, but interestingly it has been reported to display anti-inflammatory properties. It can be found as an autoantigen in patients suffering from rheumatoid arthritis, which is induced by TNFα as discussed before, and seems to counteract the inflammatory response by stimulation of macrophages to release anti inflammatory cytokines (reviewed in Panayi and Corrigall 2006).

3.4 The multifunctional protein nucleolin

3.4.1 Structure of nucleolin

Nucleolin/C23, which was initially discovered in 1973 as a spot on a two-dimensional gel (Orrick et al. 1973; Prestayko et al. 1974) is a highly expressed protein and can constitute up to 10% of nucleolar protein (Bugler et al. 1982). Homologous proteins have been found in eukaryotes from yeast to man (Table 1). The human nucleolin gene is organized in to 14 exons and 13 introns located on chromosome 2 (Srivastava et al. 1990). It consists of a highly acidic N-terminal domain separated by basic stretches (Fig. 12C) and is highly phosphorylated by different kinases (reviewed in Ginisty et al. 1999). The central part of nucleolin in higher animals contain 4 RNA recognition motifs (RRMs), through which

Organism	Name	Mol. Mass	RRMs	References
Human	Nucleolin	76.3 (100)	4	(Srivastava et al. 1989)
Mouse	Nucleolin	76.7 (105)	4	(Bourbon et al. 1988)
Rat	Nucleolin	77.1 (110)	4	(Bourbon and Amalric 1990)
Hamster	Nucleolin	77.0 (100)	4	(Bourbon and Amalric 1990)
Chicken	Nucleolin	75.6	4	(Maridor et al. 1990)
Xenopus laevis	Nucleolin	75.52 (95) 72.20 (90)	4	(Caizergues-Ferrer et al. 1989; Rankin et al. 1993)
Alfalfa	NucMs1	67.1 (95)	2	(Bogre et al. 1996)
Pea	Nucleolin	64.8 (90)	2	(Tong et al. 1997)
Onion	Nop64A Nop61A	(64) (61)	2	(de Carcer et al. 1997)
Arabidopsis thaliana	FMV3bp	57.9	2	(Didier and Klee 1992)
Tetrahymena thermophilia	Nopp52	51.7	2	(McGrath et al. 1997)
S. pombe	gar2	53	2	(Gulli et al. 1995)
S. cerevisiae	Nsr1p	44.5 (67)	2	(Lee et al. 1992)

Table 1. Nucleolin and nucleolin like proteins in several organisms. Apparent molecular masses given in brakets

nucleolin is able to make specific contact to its RNA-target. The C-terminal domain contains many Arg-Gly-Gly (RGG) repeats, which are interspersed by other amino acids. The length of these RGG domains are not conserved between the species, and can bind to RNA in a non-specific way due to the high positive charge (Ghisolfi et al. 1992). This region is methylated on the arginine residues (Lischwe et al. 1982), which is thought to counteract non-specific nucleolin binding.

3.4.2 Localization of nucleolin

Nucleolin is ubiquitously expressed in most cells throughout the organism (Maridor et al. 1990) and is mostly located in the nucleolus. For nuclear localization, the N-terminal domain contains a nuclear localization sequence. The nucleolar localization however is dependent upon nucleolin-RNA interaction (Schwab and Dreyer 1997), where the RGG domain in combination with at least one RRM is required as was shown by mutations in the RNA recognition motives (Creancier et al. 1993). By electron microscopy, it could be detected mostly in the dense fibrillar region of the nucleolus (Escande et al. 1985; Biggiogera et al. 1990), and is relocalized during mitosis into cytoplasmic nucleolus-derived foci and pre-nucleolar bodies (Gas et al. 1985; Dundr et al. 1997) that also contain other proteins involved in rRNA processing (Hernandez-Verdun and Gautier 1994). By heterokaryon assays, it was shown that nucleolin is also able to shuttle between the nucleus and the cytoplasm (Borer et al. 1989; Schmidt-Zachmann and Nigg 1993) and that the N-terminus of nucleolin is required for this, although it does not seem to be actively exported (Schmidt-Zachmann and Nigg 1993). Specific circumstances have been described, such as poliovirus infection (Waggoner and Sarnow 1998) or in rat IEC-6 cells grown without laminin (Yu et al. 1998), where nucleolin can relocalize to the cytoplasm in high amounts, while low levels of nucleolin always seem to be present in the cytoplasm.

3.4.3 Functions of nucleolin

Nucleolin appears to be involved in many processes ranging from splicing to being a receptor on the plasma membrane (review in Ginisty et al. 1999). Here we only describe the functions most acknowledged in the field, namely ribosomal biogenesis and posttranslational regulation.

3.4.3.1 Ribosome biogenesis

Nucleolin is suggested to be involved in rRNA transcription, although no concrete evidence has yet been offered. All the studies suggesting this were based on in vitro experiments and even then, only correlations could be drawn between a functional role and the absence or presence of nucleolin (review in Ginisty, Sicard et al. 1999). In yeast, it could be demonstrated that the nucleolin homologues Nsr1p and gar2 (Table 1) are required for the correct processing of rRNA and disruption of the genes led to a deficit in production of 18S rRNA and 40S ribosomal subunits (Lee et al. 1992; Gulli et al. 1995). In mouse, nucleolin was implicated in the first step of pre-rRNA splicing. It was shown to bind co-transcriptionally (Lazdins et al. 1997) to two sequence motifs in the rRNA, the NRE (Serin et al. 1996) and the ECM (Ginisty et al. 2001), and subsequently recruit the U3 snoRNP and other factors that promote rRNA processing in vitro (Ginisty et al. 1998). A very well studied aspect here is the interaction of nucleolin with the nucleolin recognition element (NRE). The NRE was discovered by a SELEX experiment as a hairpin with the conserved loop sequence (U/G)CCCC(A/G) (Ghisolfi-Nieto et al. 1996). This sNRE motif is bound specifically by the RRMs 1 and 2 of nucleolin (KD = 1-5 nM), as was shown by bandshift assays, NMR and biacore experiments (Allain et al. 2000a; Allain et al. 2000b; Finger et al. 2003; Finger et al. 2004; Johansson et al. 2004), whereas the NRE-motifs from the 5'ETS region of mouse pre-rRNA (b1NRE and b2NRE) only bound with a K_{Ds} of 1μM (Johansson et al. 2004). From these NMR studies it became clear that the RRMs 1 and 2 do not interact with each other and bind on opposite sides of the stem-loop formed by the sNRE and stabilizes it (Allain et al. 2000a). In addition, the linker between RRM 1 and 2 seems to play a role in this interaction

(Finger et al. 2004). According to the data obtained by NMR, nucleolin is proposed to act as an RNA-chaperone (Allain et al. 2000a). The binding of nucleolin to the ECM is not well studied yet, but it is clear that this interaction requires all four RRMs of nucleolin (K_D = 50nM) (Ginisty et al. 2001).

3.4.3.2 Post-transcriptional regulation

Besides the roles in rRNA maturation and ribosome biogenesis, nucleolin is implicated in several other processes. A few reports have shown nucleolin playing a role in regulation of mRNA stability and translation. For example nucleolin is suggested to be involved in the stabilization of the amyloid precursor protein (APP) mRNA. It was shown to bind, together with hnRNPC, to a 29 nucleotide long sequence that was responsible for degradation of the APP transcript (Zaidi and Malter 1995). Nucleolin is also associated with the 3'UTR of the human renin mRNA together with YB-1 and other proteins. Here the forskolin induced stabilization of the renin mRNA is correlated with increased levels of nucleolin, YB-1 and other partners associating to the renin 3'UTR (Skalweit et al. 2003). Nucleolin also seems to play a role in maintaining the stability of the β-globin mRNA. Nucleolin is reported to bind to the 3'UTR of endogenous β-globin by RNA-IP assays, and a model proposes that it facilitates the binding of the known stabilizing complex α-CP and so contributes to super stabilization of the β-globin mRNA (Jiang et al. 2006). A fourth case where nucleolin is involved in mRNA stabilization is for the anti-apoptotic gene bcl2. Nucleolin is reported to bind the ARE of bcl2 (Sengupta et al. 2004), not to the AUUUA pentamers itself, but to a 50nt long intervening sequence (Eleanor Spicer, personal communication). It was shown by in vitro decay assays that extracts from okadaic acid stimulated cells can rapidly degrade a bcl2-ARE probe and the addition of a recombinant truncated nucleolin fragment (residues 284-707, corresponding to RRMs 1 to 4) inhibited this degradation. It was proposed in a model that the stimulation with okadaic acid and taxol led to fragmentation of nucleolin and thus enables the degradation machinery to destroy the unprotected bcl2 mRNA (Sengupta et al. 2004). It could also be shown that increased cytoplasmic localization of nucleolin correlates with bcl-2 stabilization

in chronic lymphoid leukemia (CLL) cells (Otake et al. 2006). Recently an RNA-aptamer has been developed for treatment in cancer patients having upregulated bcl2 levels. It could also be shown that increased cytoplasmic localization of nucleolin correlates with bcl-2 stabilization in CLL cells. This aptamer inhibits the nucleolin-bcl2 mRNA interaction and leads to rapid degradation of this RNA in vivo. Fortunately, this aptamer does not interfere with ribosome biosynthesis because it is restricted to the cytoplasm allowing the in vivo biological effect to be ascertained (Daniel Fernandez, personal communications). Nucleolin was also implicated together with YB-1 in the stabilization of the IL-2 message. Both proteins were shown to associate with the JRE-sequence in the IL-2 5'UTR, but not to mutant motifs by UV crosslinking and immunoprecipitation experiments. It was demonstrated in a cell free system, that nucleolin and YB-1 are required for the stabilization of the IL-2 mRNA. When one or the other protein was depleted from the extracts, a JNK signal was not able to stabilize the IL-2 mRNA. Furthermore after anti-sense depletion of YB-1 or nucleolin, IL2 mRNA could no longer be stabilized by MEKK, and that over expression of nucleolin in N9 cells, which are unresponsive to MEKK induced stabilization of an IL-2 reporter, could restore the stabilizing action of MEKK (Chen et al. 2000).

In addition, nucleolin does not only seem to play a role in mRNA stabilization, but also in translational regulation of certain messages. It is claimed to enhance translation of the matrix metallo-protease 9 (Fahling et al. 2005), and in the translation suppression of p53 (Takagi, Absalon et al. 2005). As nucleolin appears to perform many functions, it is therefore very important to rigorously prove a putative interaction of nucleolin, especially as reports have suggested that the RGG domain of nucleolin can interact with RNA non-specifically (Ghisolfi et al. 1992).

3.5 The nucleolin-CDE binding is specific

As stated above, the nucleolin-RNA association is not always sequence specific. Therefore we needed to demonstrate that nucleolin is binding specifically to the CDE-O by pull-down assays using biotinylated RNAs of the sNRE, the CDE-O and a 40nt stretch of RNA from the IL-3 3'-UTR as negative control. Unfortunately, we found that nucleolin associates with all three RNAs and is pulled down with equal efficiency (Fig. 13). To clarify the situation, we decided to perform further bandshifts comparing the CDE-O with L and the fragment O mutants. The experiments were performed in THP-1 human macrophages, as the commercially available antibodies capable of supershifting a nucleolin CDE complex only recognize human nucleolin. We could show that in THP-1 macrophage extracts a specific band is observed with the CDE-probe that is supershifted with an anti-nucleolin, but not with the GST-control antibody. This specific band is absent when the CDE-L probe or the other O-mutants are employed with the notable exception of mutant Om3, which still retains some nucleolin binding (Fig 14). The binding intensity of Om3 to nucleolin was however greatly reduced compared to CDE-O, whereas the non-specific activity was able to bind the probes in comparable amounts. It should be mentioned that fragment L associates poorly with nucleolin even when excessive amounts of radioactively labelled probe is used. This encouraged us to continue and concentrate on recombinant nucleolin, in order to further investigate the specificity of the nucleolin-CDE binding. Recombinant GST-nucleolin was produced using the baculovirus expression system (Fig. 15), and the purified GST-nucleolin fusion protein was capable of binding the NRE sequence only after cleavage of the GST-tag. To our knowledge this is the first production of functional recombinant full-length nucleolin, which is a rather refractory protein to express (Fig. 16). The recombinant nucleolin was able to bind the SELEX selected NRE, but not a mutant NRE containing two nucleotide substitutions (Johansson et al. 2004). It could also discriminate between the CDE-O probe and the mutant probes Om2 and L, which show greatly reduced binding to the recombinant nucleolin. With the full-length recombinant nucleolin, it was clear that CDE-O associates for better with nucleolin than any of its mutants. This result nicely correlates with the in

vivo decay activities of the CDE-O and mutant reporters, and argues that nucleolin might be involved in promoting constitutive decay of the TNFα-message.

To prove interaction of endogenous nucleolin with endogenous TNFα mRNA in vivo, we performed RNA-IP (Fig. 17). Using specific antibodies against nucleolin and HuR, we were able to pull down the respective proteins in sufficient amounts from LPS-stimulated and unstimulated THP-1 extracts as demonstrated (Lopez de Silanes et al. 2004a). HuR was previously shown to associate with the TNFα-ARE (Dean et al. 2001) and served as a positive control, whereas an anti-myc-epitope antibody that is not expected to interact with the TNFα mRNA was employed as a negative control. With the anti-HuR antibody, we were indeed able to co-immunoprecipitate detectable amounts TNFα mRNA, which was increased in LPS stimulated extract as HuR exits the nucleus and TNFα mRNA levels are increased upon LPS stimulation (Stoecklin et al. 2003). With the anti-nucleolin antibody, we were also able to precipitate the TNFα mRNA, but in much higher amounts, taking into consideration that the anti-nucleolin antibody was less efficient than the anti-HuR antibody. Furthermore we observed no contaminating mRNAs that were pulled down, unlike the case in many other publications. We conclude that nucleolin is directly interacting with the TNFα-mRNA in vivo, most probably with the CDE as suggested by EMSA in vitro.

3.6 CDE mediated decay is influenced by nucleolin

As stated above, the binding of nucleolin to the CDE-O and its mutant sequences correlates exactly with the observed in vivo activity. The CDE-O reporter is rapidly degraded and binds nucleolin, whereas the mutant sequence containing reporters Om2, Om3, Om5 and L are stable and show greatly reduced nucleolin binding. To obtain evidence showing that nucleolin influences CDE-dependent decay, we down-regulated nucleolin by RNAi to10% of its original level by two rounds of transfection over 72h in a GFP-CDE reporter cell line. We could not observe differences in growth of the si-nucleolin transfected cells versus the control, although we were concerned that knockdown of nucleolin could interfere with

ribosomal biosynthesis and growth as was seen in the knockout of the Nrs1 and gar2 proteins in yeast (Lee et al. 1992; Gulli et al. 1995). Presumably 72 hours is too short to see an effect on growth from reduced amounts of ribosomes, in addition the knockdown was only up to 90%, perhaps leaving enough nucleolin to produce sufficient ribosomes. The GFP-reporter levels were analysed by FACS, where increased levels of GFP could be observed when nucleolin was down regulated compared to the controls. This increase of protein levels may be the result of different processes like transcriptional upregulation, translational enhancement or mRNA stability. Proceeding from our previous results, this we suggest that nucleolin might be involved in the destabilization of the GFP-CDE reporter, therefore the decay kinetics of the reporter after nucleolin siRNA treatment were analysed in several experiments. We observed that the decay activity of the CDE-reporter was indeed reduced after si-nucleolin treatment compared to the controls. When calculated, the reporter half-lives from si-treated cells significantly increased from 96 minutes (control) to 134 minutes (si-nucleolin treated cells). We can explain this modest effect by the fact that nucleolin is highly expressed, and that even after down regulation there might be sufficient nucleolin present to promote decay thereby preventing us from observing a stronger effect. Furthermore the cell line used contains the TNFα-CDE embedded in a β-globin UTR context, which was recently reported to be stabilized by nucleolin (Jian et al. 2006) and would counteract our effect. Another explanation would be that nucleolin functions as an RNA-chaperone on the CDE making it accessible for the degradation machinery. In this case we would also expect the effect to be small as RNA folding is a dynamic process, where nucleolin would only speed up the conformational changes required for degradation.

To summarize the work presented in this thesis we propose the following working hypothesis (Fig. 20): Nucleolin is constitutively associated with the TNFα-CDE independently of LPS stimulation. When nucleolin is associated with the CDE, the CDE-bearing mRNA is degraded rapidly, most probably by enhanced deadenylation as was seen in the stably transfected NIH3T3 cells (Fig. 4D), although activation of other proteins of the decay machinery cannot be excluded. Based on RNAi experiments, and taking into consideration the other functions

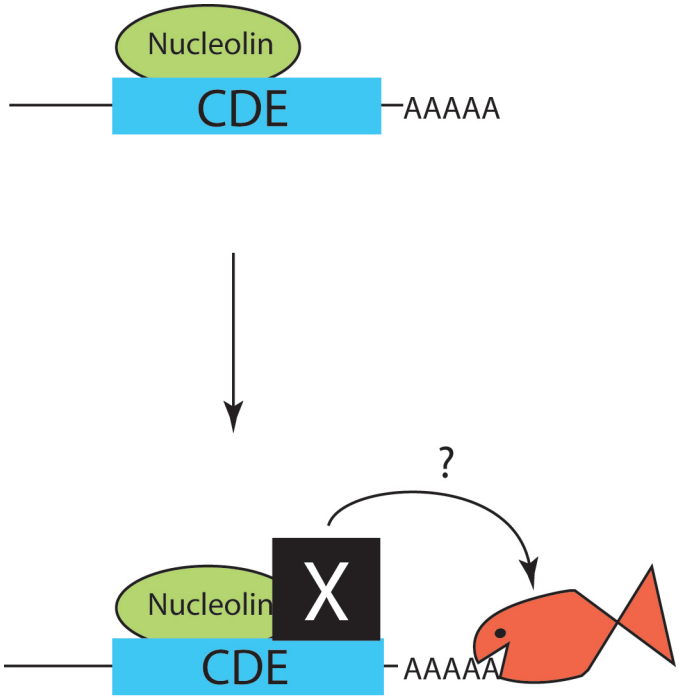


FIG. 20. Working hypothesis of CDE-mediated decay. Nucleolin is constantly bound to the CDE and eventually recruits an unknown factor X, which is able to stimulate deadenylases (red fish).

of nucleolin in the cytoplasm such as stabilization of the IL-2 and bcl-2 mRNAs (Chen et al. 2000; Sengupta et al. 2004; Otake et al. 2006) or the translational inhibition of the p53 mRNA (Takagi et al. 2005), we suggest that nucleolin needs to associate with an effector protein(s) (X) that efficiently induces CDE-mediated degradation. In order that nucleolin-CDE binding only executes one of the many roles that nucleolin is capable of, the association between nucleolin and the factor X needs to be RNA dependent, and the associated protein would then be responsible for degradation.

3.7 Posttranscriptional regulation of TNFα mRNA decay

The issue of regulation of TNFα mRNA expression in macrophages is very complex and involves many cis- and trans-acting factors (Fig. 21). We, among others, have found that endogenous TNFα mRNA in Raw 264.7 macrophages is unstable, and only minimal amounts

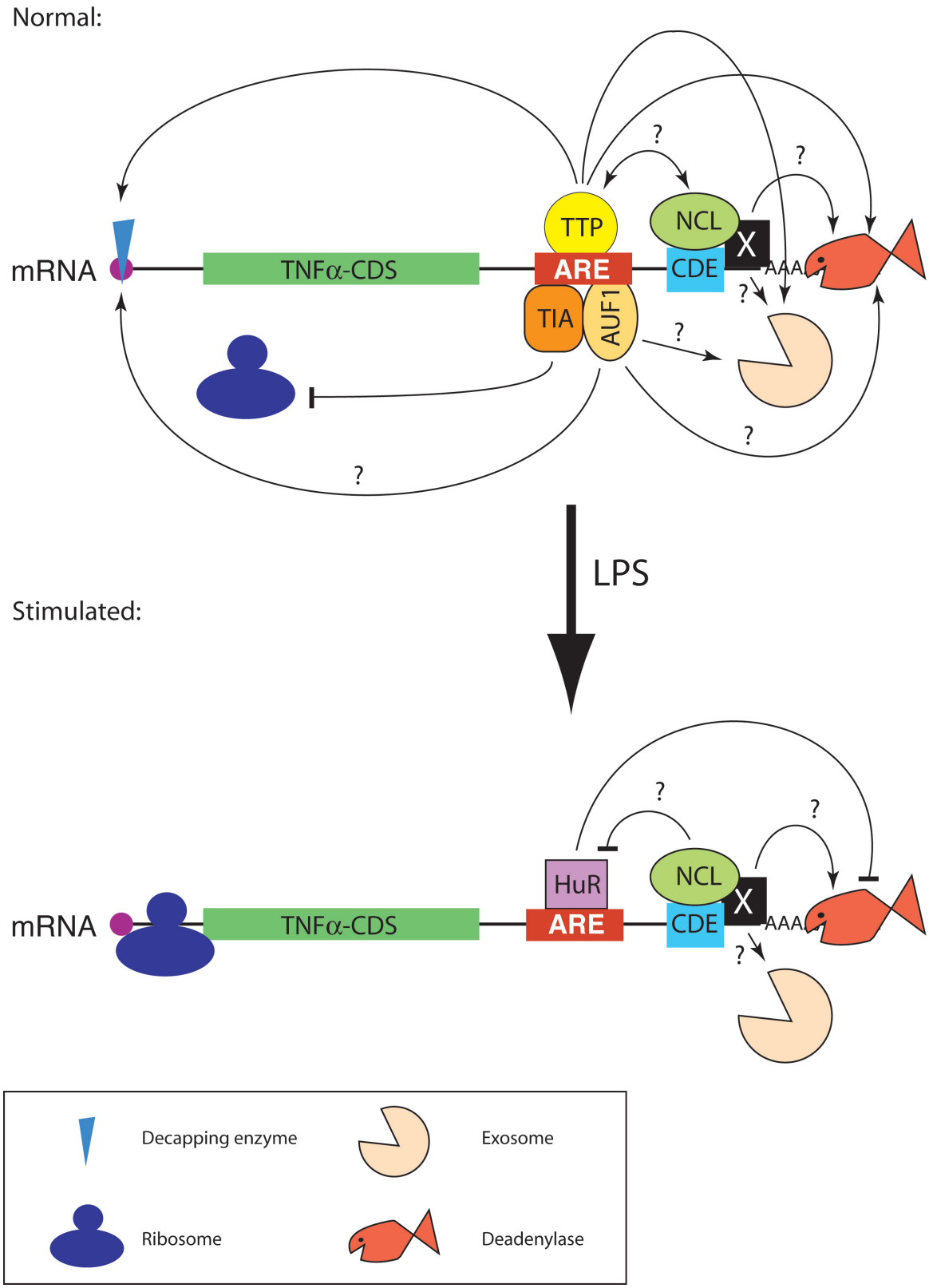


FIG. 21. Current model of the posttranscriptional regulation of the TNFα-mRNA. For detailed description see text.

of TNFα protein is expressed (Han et al. 1990; Han et al. 1991; Stoecklin et al. 2003). It has been suggested that TNFα mRNA is degraded in unstimulated cells by the ARE-binding proteins TTP (Taylor et al. 1996) and AUF1 (Lu et al. 2006). TTP was reported to associate with components of the degradation machinery responsible for deadenylation, decapping and RNA body degradation (Lykke-Andersen and Wagner 2005), but the mode of action of AUF1 remains unclear. Furthermore it was demonstrated in knock out mice that TIA-1 inhibits translation of TNFα in unstimulated cells (Piecyk et al. 2000).

Another element involved in TNFα mRNA decay is the CDE, which causes rapid decay of a reporter bearing this element (Stoecklin et al. 2003). We found that nucleolin is associated with the CDE and might also play a role in TNFα mRNA degradation by a yet unknown mechanism. We hypothesise that nucleolin associates with an unknown factor X or even with factors responsible for ARE-mediated decay like TTP or AUF1 and thus induces TNFα mRNA decay.

In macrophages stimulated by LPS, the issue of TNFα mRNA stability is controversial. It is unclear whether AUF1 remains associated with the TNFα mRNA after LPS stimulation, but there is general agreement that TTP and TIA-1 dissociate from the TNFα ARE (Anderson et al. 2004), and are replaced by the stabilizing protein HuR (Dean et al. 2001). Some reports suggest that the TNFα mRNA is stabilized hereafter (Han et al. 1991; Brook et al. 2000; Stoecklin et al. 2004; Hitti et al. 2006) while others state that it remains unstable (Han et al. 1991; Brook et al. 2000). In our hands the TNFα mRNA remains unstable even after LPS stimulation through the ARE-independent action of the CDE (Stoecklin et al. 2003). We suggest that nucleolin is constantly associated with the CDE and hypothesize that it helps a factor X in recruiting the decay machinery to the TNFα mRNA thereby overriding the stabilizing action of HuR.

3.8 Outlook

The work presented in this thesis provides insight into the mechanism of CDE-mediated TNFα mRNA degradation via nucleolin binding but at the same time raises further questions which will be addressed in the future. The model in Fig. 21 displays the complexity of posttranscriptional regulation of the TNFα mRNA, and the function of the CDE has to be evaluated further. Among the outstanding remaining questions are: Which degradation pathway is activated by the CDE? Can the CDE-mediated decay be influenced by external signals? What other factors are involved? Is there an interplay between the ARE and the CDE?

By further characterizing the CDE and its interaction with nucleolin, hopefully we will learn more about CDE-mediated decay and the very complex posttranscriptional regulation of the TNFα mRNA.

4. Materials and Methods

4.1 Solutions

4.1.1 Cell culture media

M2 Medium	Iscove's Modified Dulbecco's Medium (IMDM), 10% fetal calf serum (FCS), 50µM 2-mercapto-ethanol, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin.
Phosphate Buffered Saline (PBS)	10mM Phosphate buffer pH 7.4, 2.7mM KCl, 137mM NaCl
Propidium iodide solution	5µg/ml propidium iodide in PBS
Luria-Bertani Medium (LB)	20g Lennox L Broth base (Invitrogen) in 1000ml H_2O dist. Autoclave 20min at 120°C
LB-Amp	100µg/ml ampicillin in LB
LB-Kan	25µg/ml kanamycin in LB
LB-Agar	20g LB Broth base, 15g Select Agar in 1000ml H_2O dist. Autoclave 20min at 120°C, cool down and add the appropriate antibiotic and pour the plates
Baculo select agar plates	LB agar containing 50µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, 40 µg/ml IPTG

4.1.2 Solutions for SDS-gel electrophoresis and western blotting

10x SDS running buffer	250mM Tris HCl pH 7.5, 2.5M Glycine, 1% Sodium Dodecylsulfate (SDS)
5x Sample buffer	250mM Tris HCl pH 6.8, 500mM Dithiothreithol (DTT), 0.1% Bromophenol Blue, 50% Glycerol
Blotting buffer	1x SDS running buffer, 20% Methanol
TBS (Tris buffered saline)	50mM Tris HCl pH 7.5, 150mM NaCl
TBST	add 0.1% Tween-20 to TBS

TBST Blocking buffer	add 2% blocking Agent (Amersham-Pharmacia) to TBST
Western blot stripping solution	62.5mM Tris/HCl pH 6.7, 2% SDS, 100mM 2-mercaptoethanol
SDS-Gel fixing solution	45ml Methanol, 45ml H_2O, 5ml acetic acid
Coomassie stain solution	0.25mg Coomassie Brilliant Blue R-250 (Fluka), 45ml Methanol, 45ml H_2O dist., 10ml Acetic acid
Sensitizing solution	0.02% $Na_2S_2O_3$ in H_2O
$AgNO_3$ solution	0.1% $AgNO_3$ in H_2O
Developer	12.5g Na_2CO_3, and 150µl formaldehyde in 500ml H_2O

4.1.3 Solutions for DNA Gel electrophoresis

50x TAE buffer	242g Tris base, 57.1ml Acetic acid, 18.6g EDTA
5x DNA Loading buffer	0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 15% Ficoll Type 400 (Pharmacia)

4.1.4 Solution for RNA preparation and northern blotting

Solution I (Lysis buffer)	10mM Tris HCl pH7.5, 150mM NaCl, 1.5mM $MgCl_2$, 0.65% NP-40
Solution II (Extraction buffer)	10mM Tris HCl pH7.5, 10mM EDTA pH8, 350mM NaCl, 1% SDS, 7M Urea: store in the dark at room temperature
10x MOPS buffer	41.8g 4-Morpholinepropane-sulfonic acid (MOPS), 6.8g Na-Acetate·3 H_2O, 20ml 0.5M EDTA in 1000ml H_2O dist.
2x RNA loading buffer	7.2ml Formamide, 1.6ml 10xMOPS buffer, 2.6ml Formaldehyde (37%), 1.8ml dMilliQ H_2O, 1ml 80% Glycerol (in H_2O), 100µl 1% Bromphenol blue, 14µl Etidium Bromide (Sigma): store at –20°C
Gel solution	1.5% Agarose, 1x MOPS, 1% Formaldehyde
20x SSC	3M NaCl, 0.4 M Trisodiumcitrate, pH 7.0
50x Denhardt's solution	1% Ficoll, 1% Polyvinylpyrolidine, 1% BSA
Hybridization buffer (DNA probe)	500mM Sodium phosphate buffer pH 7.2, 7% SDS, 1% BSA, 2mM EDTA
Hybridization buffer (RNA probe)	50% formamide, 5x SSC, 5x Denhardt's, 5mM EDTA, 10mM PIPES pH 6.4, 0.4g/ml yeast RNA, 1x SDS

Northern stripping buffer	0.5% SDS in H_2O

4.1.5 Solutions for UV cross-linking assays

10x DNK buffer	500mM Tris/HCl pH 7.8, 25mM $MgCl_2$, 1mM EDTA, 50mM DTT, 10mM spermidine
Gel elution buffer	0.5M Ammonium acetate, 10mM Magnesium acetate, 1mM EDTA, 0.1% SDS
5x Reaction buffer	20mM Hepes pH 7.4, 6mM $MgCl_2$, 2mM DTT, 200mM KCl, 12% Glycerol, 6.5mM ATP, 25mM Creatine Phosphate
Dilution buffer	10mM Hepes pH 7.4, 3mM $MgCl_2$, 1mM DTT, 100mM KCl, 6% Glycerol

4.1.6 Solutions for RNA-EMSA

5x EMSA buffer	50mM HEPES/NaOH pH7.6, 15mM $MgCl_2$, 200mM KCl, 25% Glycerol, 10mM DTT
EMSA lysis buffer	10mM HEPES/NaOH pH7.6, 3mM $MgCl_2$, 40mM KCl, 5% Glycerol, 2mM DTT, 0.5% NP-40 and 1x complete protease inhibitor (Roche)
10x Bandshift buffer (BSB)	450mM Tris-borate pH8.0
EMSA running buffer	45mM Tris-borate pH8.0 and 0.05% TritonX-100

4.1.7 Buffers for Chromatography

Hypotonic Lysis buffer	10mM KCl, 2mM MgCl2, 1mM EDTA, 1mM EGTA, 1x complete protease inhibitors (Roche), 1mM PMSF, 1mM NaF, 0.4mM Na_3VO_4
Buffer A	50mM Tris pH 8.0, 2mM $MgCl_2$, 1mM DTT, 6% glycerol
Buffer B	50mM Tris pH 8.0, 1M KCl, 2mM $MgCl_2$, 1mM DTT, 6% glycerol
GST-Wash buffer	10mM PBS pH 7.4, 2mM $MgCl_2$ and 0.2% Triton X-100
GST-Wash buffer 500	PBS pH 7.4, 2mM $MgCl_2$ and 0.2% Triton X-100, 500mM NaCl
TeV-buffer	50mM Tris/HCl pH 8.0, 0.5mM EDTA, 1mM DTT

4.1.8 Solutions to prepare cellular protein extracts

Hypotonic Lysis buffer	10mM KCl, 2mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1x complete protease Inhibitors (Roche), 1mM PMSF, 1mM NaF, 0.4mM Na_3VO_4
RIPA lysis buffer	10mM Sodium phosphate buffer pH 7.2, 150mM NaCl, 1% Triton X-100, 1% Sodium desoxycholate, 0.1% SDS, 1x complete protease inhibitors (Roche)
Extraction buffer	25mM Tris pH 8.0, 2mM $MgCl_2$, 0.1% Triton X100

4.1.9 Solutions for RNA-IP

RNA-IP lysis buffer	100mM KCl, 5mM $MgCl_2$, 10mM Hepes/KOH, pH 7.0, 0.5% Nonidet P-40 Add at the time of use: 1mM DTT, 100units/ml RNaseIn (Promega), 0.2% Vanadyl ribonucleoside complexes (VRC) (Sigma), 0.2mM PMSF, 1x Complete Inhibitor cocktail (Roche)
NT2-buffer	50mM Tris/HCl, pH 7.4, 150mM NaCl, 1mM $MgCl_2$, 0.05% Nonidet P-40

4.1.10 Cell lines and bacterial strains

Mammalian cell lines

HT1080	Human fibrosarcoma cell line
HT1080 GFP-E2	HT1080 containing the MXh GFP-TNFα element II construct
Raw 264.7	Mouse macrophages
THP-1	Human macrophages
B2A2	Mouse fibroblast cell line derivative of NIH 3T3 cells expressing a tetracycline repressor (Xu, Loflin et al. 1998)
B2A2 -O	B2A2 cells stably transfected with pMXb-O plasmid
B2A2 -L	B2A2 cells stably transfected with pMXb-L plasmid
HEK 293	Human embryonal kidney cell

Insect cell lines

High 5	Invitrogen

Bacterial strains

DH5a	Invitrogen
DH10 bac	Invitrogen

4.1.11 Primers

Primers for cloning

TV242	5’-GATCGACCCAGTGTGGGAAGCTGTCTTCAGACAGACATG TTTTCTGTGAAAACGGAGCTGAGCTG-3’
TV243	5’-GATCCAGCTCAGCTCCGTTTTCACAGAAAACATGTCTGTC TGAAGACAGCTTCCCACACTGGGTC-3’
TV246	5’-GATCCTTCAGACAGACATGTTTTCTGTGAAAACGGAGCTG AGCTG-3’
TV247	5’-GATCCAGCTCAGCTCCGTTTTCACAGAAAACATGTCTGTC TGAAG-3’
Om2	5’-GATCAGGAAGACAGACATGTATAGTCCGAAAACGGAGCT GAGCTG-3’
Om2AS	5’ GATCAGCTCAGCTCCGTTTTCGGACTATACATGTCTGTCT TCCTG-3’
Om3	5’-GATCAGGAAGACAGACATGTTTTCTGTGAAAACGGAGCTG AGCTG-3’
Om3AS	5’-GATCAGCTCAGCTCCGTTTTCACAGAAAACATGTCTGTCTT CCTG-3’
Om5	5’-GATCCTTCAGATGTTTTCTGTGAAAACGGAGCTGAGCTG-3’
Om5AS	5’-GATCAGCTCAGCTCCGTTTTCACAGAAAACATCTGAAGG-3’
Om6	5’-GATCCTTCAGACAGACATGTTTTCTGTGAAAACGGAGG-3’
Om6AS	5’-GATCCTCCGTTTTCACAGAAAACATGTCTGTCTGAAGG-3’
M2138	5’-GATCCTCTATTTATATTTGCACTTATTATTTATTATTTATTT ATTATTTATTTATTTGA-3’
M2139	5’-GATCTCAAATAAATAAATAATAAATAAATAATAAATAATA AGTGCAAATATAAATAGAG-3’
M2354	5’-GATCCAGACTGTTTTCTGTGAAAACGGAGCTGA-3’
M2355	5’-GATCTCAGCTCCGTTTTCACAGAAAACATGTCTG-3’
TV250	5’-AATTCAGATTACGATATCCCAACGACCGAAAACCTGTATTT TCAGGGCAG-3’
TV251	5’-GATCCTGCCCTGAAAATACAGGTTTTCGGTCGTTGGGATAT CGTAATCTG-3’

Nucleolin-baculo	5’-GAAGATCTCTATGGTGAAGCTCGCAAAG-3’
Nucleolin-AntiS	5’-GCTCTAGACTATTCAAACTTCGTCTTCTTTCC-3’

Primers for sequencing

pSP73-100	5’-GAGCAGATTGTACTGAGAG-3’
M2016	5’-GTGCTGGTTATTGTGCTG-3’
GST	5’-GGTGATCATGTAACCCATCCTG-3’
NCL-1	5’-GTACCAACGCTTTTGCTTGTGC-3’
NCL-2	5’-GCACAAGCAAAAGCGTTGGTAC-3’
NCL-3	5’-GCAGAAGATGATGATGAGGAAG-3’
NCL-4	5’-GGAAGAAAAGCAGGGGGGCAGAA-3’
NCL-5	5’-CCTTAAAAGAATCATTTGAGGGC-3’

Primers for probe preparation

SP6-sNREs	5’-ATTTAGGTGACACTATAGAAGAGGAAATCCCGAAGTACTC-3’
SP6-sNREa	5’-GAGTACTTCGGGATTTCCTCTTCTATAGTGTCACCTAAAT-3’
SP6-NS26A18Cs	5’-ATTTAGGTGACACTATAGAAGAGGAAAGCCCAAAGTCCTC-3’
SP6-NS26A18Ca	5’-GAGGACTTTGGGCTTTCCTCTTCTATAGTGTCACCTAAAT-3’
M2353	5’-ACAGTCCATGCCATCACTGC-3’
M1170	5’-ACTGTGTTGGCATAGAGGTC-3’
M1171	5’-ACATCAAAGAGAAGCTGTGC-3’

Primers for PCR

BR5	5’-CACCACTTCGAAACCTGG-3’
BR6	5’-CAATGAGTGACAGTTGGTCAC-3’
BR16	5’-GATGCAATAACCACCCC-3’
BR17	5’-GAGGTAAGCCTACACTTTCCAAG-3’
BR18	5’-GAAGCTTCCATTCCAAGC-3’
BR19	5’-GCAACTTCCATCTCCTGG-3’

M2352	5’-TGATGGTACATGACAAGGTGC-3’
M2353	5’-ACAGTCCATGCCATCACTGC-3’

Biotinylated RNAs for pulldown and affinity purification

CDE-K	Bio-5’-UUAUGAAUGUAUUUAUUUGGAAGGCCGGGGUGUCC UGGAGGACCCAGUGUGGGAAGCUGUCUUCAGACAGACAUC UUUUCUGUGA-3’
CDE-O	Bio-5’-CUUCAGACAGACAUGUUUUCUGUGAAAACGGAGCU GAGCU-3’
sNRE	Bio-5’-AGGCCGAAAUCCCGAAGUAGGCC-3’
IL-3-UTR	Bio-5’-GAUGUGAGUACUGGGGCCAUGUUCAUUUGU-3’

siRNAs

BRF1/1s	5’-CAAGAUGCUCAACUAUAGAUU-3’
BRF1/1a	5’-UCUAUAGUUGAGCAUCUUGUU-3’
HuR1s	5’-CUACCUCCCUCAGAACAUGUU-3’
HuR1a	5’-CAUGUUCUGAGGAGGUAGUU-3’
NCL-2s	5’-GAAAUAAAGUUACCUUGGAUU-3’
NCL-2a	5’-UCCAAGGUAACUUUAUUUCUU-3’
NCL-3s	5’-GAAAGAAGACGAAGUUUGAUU-3’
NCL-3a	5’-UCAAACUUCGUCUUCUUUCUU-3’

4.2 Methods

4.2.1 Plasmid construction

puroMXβglobin, puroMXβ-TNF-α-K and puroMXβ-TNF-α-L

Vectors were described previously (Stoecklin et al. 2003).

pSP73-K

This vector was constructed by Min Lu by inserting a PCR fragment of CDE-K into the pSP73 vector (Min Lu, unpublished data).

FIG. 22.Vector map of the puroMXβglobin vector.

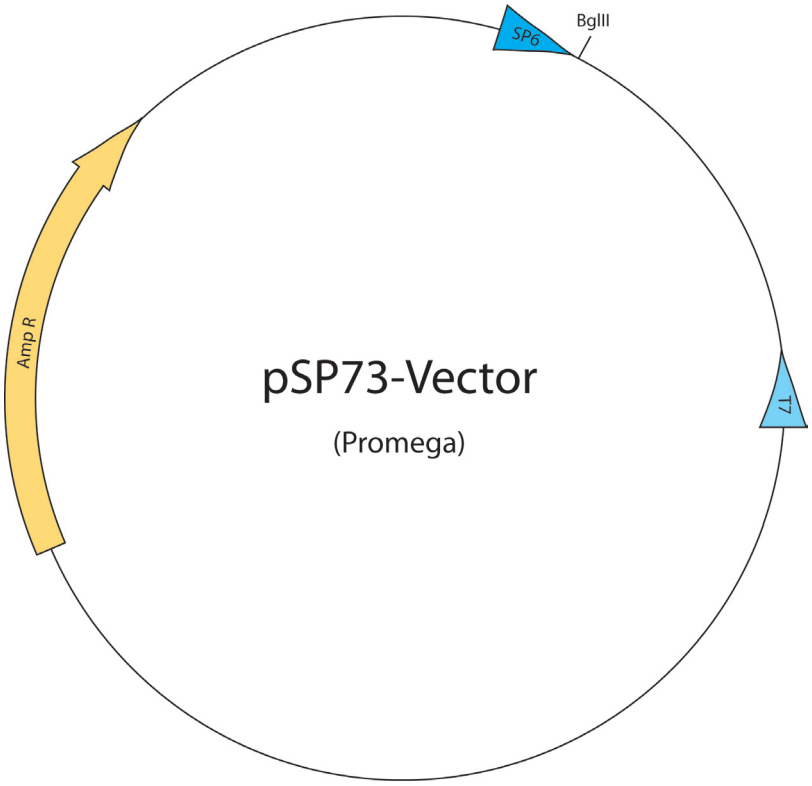


FIG. 23. The pSP73 vector (Promega). All fragment for probe production were cloned into the *BglII*-site in front of the SP6 promotor.

Vectors for CDE-mapping experiments

To generate the β-globin reporter constructs and the pSP73 constructs for probe preparation, 3µg of the vector puroMXβglobin (Fig. 22) or pSP73 (Fig. 23; Promega) was linearized *BglII* (NEB), and subsequently gel purified. To phosphorylate and anneal the oligomers for the inserts, 100pmol of complementary DNA-oligomers were mixed and incubated with 10U polynucleotide kinase (NEB) in the corresponding buffer containing 0.2mM ATP for 1h at 37°C and subsequently heated to 95°C for 5min and cooled down slowly. Equimolar amounts of cut plasmid and insert were used for ligation with the Quick ligation kit (NEB) following the manufacturers protocol. The following DNA-oligomers were used:

puroMXβ-TNF-α-M	pSP73	TV242	TV243
puroMXβ-TNF-α-O	pSP73-O	TV246	TV247
puroMXβ-TNF-α-Om2	pSP73-Om2	Om2	Om2AS
puroMXβ-TNF-α-Om3	pSP73-Om3	Om3	Om3AS
puroMXβ-TNF-α-Om5	pSP73-Om5	Om5	Om5AS
puroMXβ-TNF-α-Om6	pSP73-Om6	Om6	Om6AS
puroMXβ-TNF-α-L	pSP73-L	M2354	M2355
	pSP73-ARE	M2138	M2139

pFastbacGST-TeV-Nucleolin

The murine nucleolin gene was amplified from cDNA with the primers nucleolin-baculo and nucleolin-AntiS. The PCR-product was cut with 20U of the restriction enzymes *Bgl*II and *Xba*I (NEB) and gel purified; 3µg of the pFastbacGST vector (Brondani et al. 2005) was cut with 20U *EcoR*I and *Xba*I (NEB) and the oligonucleotide sequences TV250 and TV251, which possess compatible ends for the *EcoR*I and *Bgl*II cutting sites, were phosphorylated and annealed as described above. Subsequently equimolar amounts of the three fragments were ligated using the Quick ligation kit (NEB).

All plasmids created were positively selected by colony PCR and confirmed by sequencing.

4.2.2 Transformation of *E.coli*

Ligation reactions or 40 to 100ng of pure plasmid were incubated with 50µl of competent DH5α *E.coli* (Invitrogen) cells for 20 min on ice and subsequently heat shocked for 2 min at 42°C. The bacteria were allowed to recover for 30 minutes in 450µl LB medium at 37°C and afterwards collected by centrifugation. The supernatant was removed leaving only 50µl, which were then streaked on LB-Amp plates and incubated overnight at 37°C.

4.2.3 Colony PCR of transformants

Colonies were picked with a sterile pipette tip and diluted in 25µl H_2O. The tip was afterwards transferred in a correspondingly labeled culture tube containing LB-Amp selective medium. A PCR Master Mix (1x PCR Buffer (Qiagen), 0.2mM dNTPs each (Roche), 400nM primer pSP73, 400nM specific reverse primer (see table primers), 0.2U Taq Polymerase (Qiagen) in a total volume of 25µl) was prepared and added to each diluted colony.

PCR-program: The bacteria were lysed for 5min at 94°C. 25 cycles with 30sec denaturation at 94°C, 30sec annealing at 50°C and 30sec extension at 72°C were performed. As a final cycle, 5min extension at 72°C was added then ramped to 4°C. 10µl of each extension product was loaded on a 3% NuSieve agarose (Sigma) gel to identify transformants with the correct insertion. The inoculated colonies of positive transformants were incubated overnight at 37°C.

4.2.4 Isolation of plasmid DNA

Gel purification

The gel slice of interest was cut out of the gel, and the DNA was extracted using the QIAquick Gel extraction kit (Qiagen) following the manufacturers protocol. The DNA was eluted in 30µl of H_2O.

Miniprep

4ml of LB-AMP medium was inoculated with an *E.coli* colony and grown for 12h to 14h at 37°C. The bacteria were transferred in a 1.5ml Eppendorf tube and collected by

centrifugation in a tabletop centrifuge at 11000g for 30sec. The plasmid was isolated using the QIAprep spin Miniprep kit (Qiagen) following the manufacturers protocol.

Maxiprep

One colony with the correct sequence was inoculated into 250ml of LB-Amp selective medium and incubated overnight at 37°C. The cells were pelleted by centrifugation in a Sorvall RC3C centrifuge (rotor 19) at 3500rpm for 20min. Plasmid extraction was performed following the QIAGEN Plasmid Maxi Kit protocol and DNA was dissolved in 400µl of TE buffer (Qiagen). The concentrations of the plasmids were determined by measuring the OD_{260} in a quartz cuvette before storing at -20°C.

4.2.5 Sequencing

The plasmids were sequenced using the ABI Prism 310 Genetic Analyzer (Applied Biosystems) or the CEQ 8000 (Beckman Coulter). The sequencing reaction was performed according to the manufacturers protocol. The pFastbac GST-TeV-nucleolin vector was sequenced with the primers GST, NCL-1, NCL-2, NCL-3, NCL-4 and NCL-5, the puroMXβ-TNF-α with M2016, and the pSP73 constructs with the pSP73-100 primers.

4.2.6 Cell culture and transfection

Cell culture

All mammalian cell lines were grown in M2 medium with regular medium change. Adherent cells were split 1:5 every second day. Suspension cells were diluted 1:3 every second day. High5 insect cells were grown at room temperature in JX-400 medium in a spinner culture. For transfection, the cells were plated at 90% density into a F-25 bottle. The cells adhered to the dish within 20min after plating and were ready for transfection.

Transfection of mammalian cells

Plasmids were transfected into cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturers instruction.

To obtain stable integration of puroMXβ-TNFα constructs into NIH3T3 B2A2 cells, the positive clones were selected by addition of 1µg/ml puromycin. Expression was tested by northern blot.

Transfection of High5 insect cells

High5 cells were transfected with 10µl of the bacmid DNA and 10µl cell Fectin (Invitrogen). Both reagents were individually diluted in 500µl FX-400 medium and then mixed. The transfection reaction was added to a F-25 bottle of High5 cells in 1ml medium. The cells were incubated for 4 hours at 28°C followed by medium change. Two days after transfection the baculovirus containing supernatant was collected and stored at 4°C.

Transfection of siRNA

To downregulate nucleolin, HuR and BRF1 by siRNA, a 90% confluent 15 cm dish of HT1080 GFP-E2 cells was washed with Optimem (Invitrogen), and 8ml of Optimem was added afterwards. 200µl of Lipofectamine 2000 (Invitrogen) was diluted in 4ml Optimem and in a separate vial, 200µl of a 20µM solution of the corresponding siRNA was added to 4ml Optimem. The Lipofectamin 2000 and siRNA dilutions were mixed and after incubation for 20min, the solution was added dropwise to the cells. After exactly 4h incubation time the transfection mix was removed and fresh M2 medium was added. 24h after the first transfection, the cells were transfected again in the same manner. 48h after the first transfection the cells were split into three 10cm dishes for decay assay and one 6 well for FACS analysis.

4.2.7 Propidium iodide staining and flow cytometry (FACS)

The cells were trypsinized, washed with PBS and collected by centrifugation. The cells were resuspended in a 5µg/ml propidium iodide solution. The samples were analyzed using the FACScan and Cellquest software (Beckton Dickinson).

4.2.8 Western blotting

10µg of complete cell lysate or 100ng of the recombinant protein were resolved on a Tris-Glycine-Gradient-Gel 4-20% (Anamed). Immobilon-P membranes (Millipore) were prepared for blotting by rinsing with methanol and rehydrating them for 5min in H_2O. Blotting was performed in 1x blotting buffer for 1h at 80V in a mini-blot apparatus (BioRad). After blotting the membrane was incubated in TBST blocking buffer for 1h on a shaker to reduce unspecific antibody binding. Membranes were incubated with the appropriate antibody overnight at 4°C or 1h at room temperature on a shaker. Membranes were rinsed and washed 3x with TBST for 30min. As a secondary antibody, horseradish-peroxidase-coupled goat anti-mouse antibody (DAKO) or goat-anti-rabbit antibody (Southern Biotechnology Associates) in blocking agent were added and membranes were incubated for 45min at 37°C. Membranes were washed as described above. To detect the specific signal, ECL reagent (Amersham Pharmacia) was added to the blot, which was then exposed to autoradiographic film (Hyperfilm MP, Amersham Pharmacia).

For directly horseradish-peroxidase-coupled rabbit-anti-GAPDH antibody (Abcam), the incubation time was 45min at room temperature, followed by washing and exposure to film.

For stripping and reprobing of western blots, the membrane was incubated for 30min at 50°C in stripping solution. The blot was washed subsequently 4 times for 10min with TBST and blocked with TBST-blocking buffer.

4.2.9 RNA preparation

Isolation of cytoplasmic RNA

To prepare cytoplasmic RNA, cells were washed with PBS, trypsinized, collected in 10ml M2 medium and centrifuged at 1000g for 5min. The cell pellet was resuspended in 400µl ice-cold solution I and transferred to an Eppendorf tube. Cells were vortexed and centrifuged at 10000g for 1min at 4°C. The clear lysate was carefully transferred to a previously prepared Eppendorf tube containing 400µl solution II and 400µl of phenol/chlorophorm/

isoamylalcohol (25:24:1) at 4°C. Tubes were vortexed vigorously (the solution gets cloudy and white), incubated for 10min at 65°C and centrifuged at 10000g for 20min at room temperature. The upper (aqueous) phase was carefully transferred to a new Eppendorf tube. After incubating samples for ≥2h at –20°C, 800µl isopropanol was added to precipitate the RNA. Tubes were centrifuged at 10000g for 20min at 4°C. The white RNA pellet was carefully washed once with 500µl 80% ethanol. Residual ethanol was removed by evaporation in a speed vacuum centrifuge for 5-10min. The RNA pellet was dissolved in 50µl RNAse free H_2O for 10min at 65°C. To determine the RNA concentration, OD_{260} of 1:400 diluted samples was measured and calculated accordingly: 1 OD_{260} corresponds to 40µg/ml RNA.

Isolation of total RNA

Total RNA was isolated using the RNeasy kit (Qiagen) following the manufacturers instructions.

4.2.10 RNA analysis by northern blotting

Sample preparation

To analyze RNA levels of specific transcripts, 25 to 40µg of RNA were diluted in 20µl RNAse free H_2O and 2x RNA loading buffer was added to each sample at an equal volume. The samples were heated for 20min at 65°C before loading them onto the gel. The RNA was separated by electrophoresis in a 1.8% agarose MOPS gel. The RNA was transferred overnight onto a Hybond N+ membrane (Amersham Pharmacia) by capillary force using 20x SSC. The membrane was rinsed with 0.1x SSC, 0.1% SDS, and crosslinked at 1200 µJ/cm^2.

Probe preparation

RNA-probes

Probes for fragment K: pSP73 K vector was cut with *BglII*, and the probe was transcribed from 100µg/ml template DNA in 40µCi α[^{32}P] UTP, 500µM CTP, 500µM ATP, 500µM GTP-

and 20U of T7 RNA-polymerase (Promega).

Probes for β-globin were generated by SP6 transcription from the pSP73-β-globin vector linearized with EcoRI (Stoecklin, Lu et al. 2003).

DNA-probes

The template for the β-actin probe was generated by PCR-amplification of actin from cDNA with the primers M1170 and M1171, and for GAPDH with the primers M2352 and M2353. The puromycin N-acetyltransferase (puro) probe was generated by amplification of the puromycin gene from pBABE puro as described in (Stoecklin et al. 2001). The probes were generated from 25ng template DNA in 40μCi [^{32}P]dCTP, 500μM dATP, 500μM dGTP, 500μM dTTP- and 20U of Klenow fragment in the corresponding buffer (Promega), using 1mM of the primers M1171 for the β-actin probe, M2353 for the GAPDH probe and random hexamers for the puro probe.

Hybridization

The membrane was blocked in hybridization buffer for at least 30min at 65°C for DNA probes in DNA hybridization buffer, and for RNA probes in RNA hybridization buffer at 55°C. After blocking the membrane, the appropriate probe was added and hybridized overnight at the indicated temperature. Blots hybridized with RNA probes were washed 30min with 2xSSC, 0.2%SDS, 30min with 0.2xSSC, 0.2%SDS and 30min with 0.1xSSC and 0.1%SDS at 65°C. Solutions were prewarmed to 65°C.

Blots hybridized with DNA probes were washed two times 20min with 2xSSC, 0.2%SDS and once 10 min with 0.1xSSC and 0.1%SDS at 42°C. The blots were exposed to phosphorimager plates (Biorad), and read out in the Personal Molecular Imager FX (Biorad).

4.2.11 UV-Crosslinking assay

Preparation of protein extracts

Raw 264.7 mouse macrophages were stimulated for 2h with 5μg/ml LPS and harvested

by centrifugation at 1500 rpm for 3min and washed once with ice-cold hypotonic lysis buffer. Cells were resuspended in 3 volumes of ice-cold hypotonic lysis buffer containing 1x Complete protease inhibitor mix, 1mM NaF, 1mM $NaVO_4$, 1mM PMSF and incubated for 10min on ice. Cells were snap frozen in liquid nitrogen, thawed at room temperature and disrupted in a dounce homogenizer by 30 strokes. The nuclei were centrifuged down at 600g for 5min. The cytoplasmic supernatant was transferred to a new tube and cleared by centrifugation at 14000g for 20min. The cytoplasmic lysate was supplemented with 10% glycerol and 100mM KCl and frozen at –80°C until required.

Preparation of 4-thio-UTP

To phosphorylate 4-thio-UDP, 10mM 4-Thio-UDP (Sigma), 20mM GTP were incubated with 5 units nucleoside-5'-diphosphate kinase (Sigma) in 1x DNK-buffer for 2h at room temperature in the dark. The reaction was stopped by adding 5mM EDTA and 0.1% SDS, and the enzyme was digested with 1mg/ml proteinase K at 37°C for 15min. The reaction was adjusted to 500µl with 0.1%SDS solution and 1/10 volume of Na-acetate was added. After phenol extraction with 1 volume of phenol, the nucleotides were precipitated with 1 volume of ethanol/acetone (1:1) for 30min at –20°C. The nucleotides were collected by centrifugation at 18000g for 30min at 4°C, dried in a speedvac and resuspended in an appropriate volume of nuclease free water. Approximately 50% of the 4-thio-UDP solution was converted to 4-thio-UTP (4-thio-UTP/GTP mix). The solution must be permanently kept in the dark all the time.

In vitro transcription of UV-Crosslinking probes

For this reaction 100µg/ml template DNA, 40µCi $\alpha[^{32}P]$ATP, 500µM CTP, 50µM ATP, 500µM 4-thio-UTP/GTP-mix, 20U RNasin (Roche) and 20U of SP6 RNA-polymerase were mixed in 1x transcription buffer (Roche) and incubated for at least 2h at 37°C. Template DNA was removed by adding 10µg/ml DNAse RQ1 (Promega) for 15min at 37°C. 1/10 volume of Na-acetate and 20µg tRNA were added to the reaction and extracted with 1

volume of phenol/chloroform/isoamyl alcohol (25:24:1). The probe was precipitated with 3 volumes of ethanol at –20°C for at least 20min and centrifuged at 18000g for 20min. The pellet was washed once with 80% ethanol and dried down in the speedvac.

Probe purification

The sample was resuspended in 10µl RNA loading buffer at 65°C for 5min and afterwards purified over a 5% acrylamide, 7M urea, 1x TBE gel. To visualize the probe, the gel was exposed for 1min to an autoradiographic film (Hyperfilm MP). The transcript was cut out of the gel using the film as a guide template. The gel slice was cut in pieces and the labeled RNA was eluted in 400µl elution buffer for either 2h at 42°C or overnight at 37°C. The supernatant was transferred to a new tube, and the gel slices were washed with 200µl elution buffer at 42°C for 30min. The supernatant was added to the new tube and 1/10 volume of 3M Na-acetate and 2.5 volumes ethanol were added to the sample, and the probe was precipitated at –20°C for 30min. The labeled RNA was collected by centrifugation at 18000g for 30min, dried, and resuspended in RNAse free water. The specific activity of the probe was measured in a scintillation counter.

Protein binding and crosslink

20µg protein extract or the corresponding amount of the chromatography fractions, 10µg heparin sulfate, 1x reaction buffer were mixed in dilution buffer and incubated for 30min at 30°C. A volume corresponding to 50000cpm of probe was added to the crosslinking reaction and incubated for 10min at 30°C. The tubes were put on ice, covered by a glass plate and crosslinking was achieved by a dose of 2700mJ/cm^2 with 312nm UV-light in a Strata linker (Stratagen). The non-crosslinked RNA was digested with 10µg RNase A per reaction at 37°C for 20min. The protein-RNA complexes were boiled for 5min with 1x protein loading dye and separated on a 10% SDS-polyacrylamide gel. The radioactively labeled protein-RNA complexes were visualized by phosphor imaging. Adapted from: (McBratney and Sarnow 1996)

4.2.12 Staining of the protein gels

Silver staining

The protein gel was fixed for 30min in a 45% methanol/ 5% acetic acid solution and washed afterwards for 1h with several changes of water. The gel was sensitized for 2min with a 0.02% Sodium thio sulfate solution, rinsed twice with water and stained with a 0.1% Ag nitrate solution for 20min. The gel was again rinsed twice with water and once with developer (12.5g $NaCO_3$, and 150µl formaldehyde in 500ml water). The gel was developed until the bands were clearly visible, stopped by adding fixing solution and dried in a gel dryer.

Coomassie blue staining

The protein gel was fixed for 30min in a 45% methanol/ 5% acetic acid solution and stained with Coomassie blue staining solution (Invitrogen) for 1h at room temperature. The gel was destained with water until bands were clearly visible against the background.

4.2.13 Chromatography

Selection of the appropriate chromatography matrices

2x 100µl of the various chromatographic media (50% slurry): Q-Sepharose, Red-Sepharose, Blue-Sepharose, Heparin-Sepharose and Phenyl-Sepharose, were washed three times with water. One part was equilibrated with 50mM Tris/HCl pH8.0 and 100mM KCl, the other part with 50mM Tris/HCl pH8.0 and 500mM KCl. To each matrix, 150µg lysate was added containing either 100mM or 500mM KCl. The protein was adsorbed for 1h at 4°C. The beads were spun down at 2000rpm for 2min and the supernatants were transferred to new tubes. For the UV-Crosslinking experiments 10µl of the supernatants were used.

Heparin-Sepharose chromatography (FPLC)

A HR10/30 column was packed with Heparin-Sepharose and equilibrated with buffer A/ 10% buffer B (= 100mM KCl) and 50mg of protein were loaded onto the column. The flow rate was 4ml per minute. The column was washed for 15 minutes with this buffer, and then

buffer B concentration was raised to 25% (= 250mM KCl) for 7.5min, followed by a further increase to 100% (= 1M KCl) for 7.5min. The column was re-equilibrated with 10% buffer B. 4ml fractions were collected. The 1M KCl fractions were pooled and dialyzed against buffer A supplemented with 100mM KCl.

Affinity chromatography

Streptavidin sepharose was pre-coated with a biotinylated RNA-oligomer Bio-CDE-K in PBS for 20min at room temperature, with uncoated streptavidin beads serving as control. The beads were washed three times with PBS and afterwards equilibrated with buffer A supplemented with 100mM KCl. Fractions from the heparin sepharose column were added to the beads and incubated overnight at 4°C. The beads were washed with 5 bead volumes of buffer A containing 500mM KCl and the proteins were eluted with three bead volumes of buffer B. The same procedure was used for affinity-pulldown from cytoplasmic extracts using bio-CDE-O, bio-sNRE and bio-IL3-UTR.

Mono-Q chromatography (FPLC)

The affinity eluate was diluted 1:10 in buffer A and loaded onto a HR5/5 column loaded with Q-sepharose beads at a flow rate of 1ml/min with buffer A, 10% buffer B. The column was washed with 5 column volumes of this buffer and afterwards buffer B concentration was raised to 25% and washed again with 5 column volumes. The bound proteins were eluted with a gradient from 25%-100% buffer B over 8min. The column was cleaned with 8 volumes 100% buffer B and re-equilibrated with 10% buffer B. 500µl fractions were collected.

4.2.14 Mass spectrometry

The samples were precipitated with 15% tri-chloro-acetic-acid at 4°C for 30 minutes, precipitated by centrifugation for 15min at 18000g, and resuspended in 20µl sample buffer containing 10mM DET. The pH was adjusted to 7.5 with 1M tris/HCl pH 8.0 and reduced by boiling for 10min at 95°C. After the sample cooled down to room temperature, it was

reduced with iodoacetamide for 60min at room temperature in the dark. The samples were analysed by a 7.5 to 15% gradient SDS-PAGE, followed by coomassie staining.

Mass spectrometry was performed by Stefan Evers and Daniel Röder at F. Hoffmann-La Roche, Ltd as in: (Grunenfelder et al. 2001; Lee et al. 2002). In short: Protein bands were cut out of the gel, destained and digested with trypsin. The masses of the peptides after proteolytic digestion were determined with a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Reflex3, Bruker, Billerica, MA). Protein homology searches were done by Blast-2. If the identification of a band could not by achieved by this method, it was determined by nano-spray-tandem mass spectrometry on a quadrupole time-of-flight mass spectrometer (Qstar Pulsar 1;ABI/MDS Sciex, Toronto, Canada).

4.2.15 Preparation of recombinant nucleolin

One liter of High 5 cells were infected with 150ml viral supernatant and incubated for 42h at 28°C. The cells were collected by centrifugation at 600g for 10min. The supernatant was retained at 4°C for further infections at 4°C. The cells were washed once in PBS, spun down at 600g for 10min and the PBS was removed completely. The cells were lysed in four pellet volumes of extraction buffer for 30min at 4°C and sonicated three times for 10sec. The insoluble fraction was removed by centrifugation at 20000 rpm in a Sorvall SS34 rotor for 30min. The supernatant was transferred to a new tube and 2ml of GSH-sepharose beads (50% slurry) were added and GST-Nucleolin was allowed to bind overnight at 4°C tumbling end over end. The beads were collected at 1000g for 2min and the supernatant was removed. The beads were washed with 10 bead volumes of GST-wash buffer, 10 bead volumes of GST-wash buffer 500 and again with 10 bead volumes of GST-wash buffer. The column was equilibrated with 4 volumes of TeV-buffer, and nucleolin was eluted in 1ml TeV-buffer containing 500U TeV-protease (Invitrogen) for 1h at 4°C by tumbling end over end. The beads were again washed with 1ml TeV-buffer and the eluates were pooled. The eluates were supplemented with 10% glycerol and frozen as 50µl aliquots in liquid nitrogen. The recombinant nucleolin was stored at –80°C.

4.2.16 RNA-electrophoretic mobility shift assay

Probe preparation

The templates for transcription were obtained by PCR amplification of the pSP73 O, Om2, Om3, Om5, Om6 and L vectors with the primers pSP73-100 and TV247, Om2AS, Om3AS, Om5AS, Om6AS or M2355 respectively. The templates of sNRE and mNRE were obtained by annealing the oligonucleotide pairs SP6-sNREs and SP6-sNREa or SP6-NS26A18Cs and SP6-NS26A18Ca. The radioactively labeled transcripts were generated by in vitro transcription with the SP6 maxiscript kit (Ambion) in the presence of 40µCi α[^{32}P]UTP, following the manufacturers protocol. The probes were gel purified as described in 3.2.11.4

Sample preparation

Cytoplasmic extracts from Raw264.7 or THP-1 cells were obtained by lysis with EMSA lysis buffer for 10min on ice. The insoluble fraction was precipitated in an Eppendorf table top centrifuge at 600g for 10min, and the cytoplasmic extract was transferred to an new tube.

Binding reaction and electrophoresis

RNA-Protein complexes were formed for 30min at 4°C with either 10µg cytoplasmic extract or 100ng purified nucleolin in EMSA buffer supplemented with 1µg/reaction of heparin sulfate and polyAU (Sigma), and 10 kcpm of ^{32}P labeled transcript. In shift assays with recombinant protein, also 1µg of BSA was also added per reaction. For supershift assays 0.5µg of anti-GST-antibody (Pharmingen) or anti-nucleolin-antibody (MS3 Santa Cruz) were added 30min before the addition of the radioactive probe.

The complexes were electrophoresed on a 4% polyacrylamide gel containing 45mM Tris-borate pH8.0 in EMSA running buffer.

4.2.17 RNA-IP

Preparation of the RNA-IP

Approximately 10^8 cells were washed 3 times with ice cold PBS and pelleted in a centrifuge at 1200 rpm for 5min. The pellets were resuspended in 1 volume of RNA-IP lysis buffer by repeated pipetting and incubated 5min at 4°C. Aliquots of 250µl were frozen in liquid nitrogen and stored at –150°C. Before use, samples were thawed on ice and centrifuged for 10min at 14000g. The supernatant was transferred to a new tube. This step was repeated once. Protein G sepharose beads (Amersham Pharmacia) were washed 3 times with water and incubated for at least 1h with 4 volumes of buffer NT2 containing 5% BSA. 4µg of anti nucleolin antibody (MS3 Santa Cruz) or 4µg of anti-tubulin antibody (Santa Cruz) were added to 250µl of slurry resulting in 50µl pelleted bead volume and tumbled end over end for 18h at 4°C. The antibody-coated beads were washed 4 times with 1ml buffer NT2 and resuspended in 850µl NT2 supplemented with 30µl 0.5M EDTA, 10µl 100mM DTT, 5µl VRC (200mM, Sigma), and 5µl RNAseIn (200U). The beads were mixed shortly before adding 100µl of RNA-IP lysate. The sample was mixed again shortly and centrifuged at 2500 rpm for two minutes. 100µl of supernatant representing the total cellular mRNA, were transferred to a new tube and tumbled end over end alongside the IP-reaction at 25°C for 2h. The beads were washed 10 times with 1ml buffer NT2 and resuspended in 100µl NT2 buffer supplemented with 0.1% SDS and 30µg proteinase K (Tenenbaum et al. 2002). The reaction was incubated 30min at 55°C. The RNA was extracted with the RNeasy kit (Qiagen) following the manufacturers protocol. 350µl of buffer RLT containing 2-mercapto-ethanol were added and the reaction was mixed shortly. The beads were pellet at 14000g for 2min and the supernatant was transferred to a new tube. After addition of 250µl ethanol, the sample was loaded onto the RNeasy spin column. After washing, the RNA was eluted with 30µl RNAse-free water.

Reverse transcription

For reverse transcription 5µg of total RNA or a corresponding amount from the RNA-IP were mixed with 100pmol oligo dT_{15} primer and adjusted to a total volume of 13µl with H_2O. The samples were heated to 65°C for 5min and put on ice for 2min. 4µl of 5x 1st strand buffer, 1µl 100mM DTT, 1µl (40U) RNAseIn and 1µl Superscript III reverse transcriptase (Invitrogen) were added. The reaction was mixed and incubated at 50°C for 1h and the RT inactivated at 70°C for 15min. The RNA was removed by adding 1µl of DNAse-free RNAse (Roche) for 20min at 37°C. The reaction was stored at –20°C

PCR amplification of the immunoprecipitated cDNAs

To amplify the targets, 2µl cDNA was added to a mix containing 1x PCR Buffer (Qiagen), 2.5mM forward primer and 2.5mM reverse primer, 400µM of each dNTP and 0.2U of Taq polymerase (Qiagen). 35 cycles with 45sec denaturation at 94°C, 45sec annealing at 55°C and 45sec extension at 72°C were performed, followed by 10min of elongation at 72°C. TNF-α was detected with the primers BR5 and BR6, IL-6 with BR16 and BR17, IL-10 with BR18 and BR19, and GAPDH with M2352 and M2353. The PCR products were analyzed on a 1% Agarose-TAE gel.

5. Acknowledgements

I would like to thank Prof. Christoph Moroni for giving me the great opportunity to do my PhD thesis in his laboratory on this very interesting and challenging topic, and for his advice in times of need. I would also like to thank Dr. Stefan Evers and Daniel Röder for many rounds of protein sequencing to identify nucleolin. I want to say thank you as well to all the members of our research group for helpful discussions, especially to Min Lu who helped me whenever there was need and to Dr. Don Benjamin for his advice and help on the manuscript.

I also would like to thank Dr. Georg Stöcklin for helpful discussion and Dr. Witold Filipowicz for his advice and help on my project.

A great thanks goes to my parents who always supported me and finally to my wife Karin, I got to know in Prof. Moroni's laboratory, for her constant support and for enriching my life.

6. References

6.1 A-B

Adinolfi, S., C. Bagni, M. A. Castiglione Morelli, F. Fraternali, G. Musco and A. Pastore (1999). "Novel RNA-binding motif: the KH module." Biopolymers **51**(2): 153-64.

Allain, F. H., P. Bouvet, T. Dieckmann and J. Feigon (2000a). "Molecular basis of sequence-specific recognition of pre-ribosomal RNA by nucleolin." The EMBO journal **19**(24): 6870-81.

Allain, F. H., D. E. Gilbert, P. Bouvet and J. Feigon (2000b). "Solution structure of the two N-terminal RNA-binding domains of nucleolin and NMR study of the interaction with its RNA target." J Mol Biol **303**(2): 227-41.

Allmang, C., J. Kufel, G. Chanfreau, P. Mitchell, E. Petfalski and D. Tollervey (1999a). "Functions of the exosome in rRNA, snoRNA and snRNA synthesis." The EMBO journal **18**(19): 5399-410.

Allmang, C., P. Mitchell, E. Petfalski and D. Tollervey (2000). "Degradation of ribosomal RNA precursors by the exosome." Nucleic acids research **28**(8): 1684-91.

Allmang, C., E. Petfalski, A. Podtelejnikov, M. Mann, D. Tollervey and P. Mitchell (1999b). "The yeast exosome and human PM-Scl are related complexes of 3' --> 5' exonucleases." Genes & development **13**(16): 2148-58.

Amann, B. T., M. T. Worthington and J. M. Berg (2003). "A Cys3His zinc-binding domain from Nup475/tristetraprolin: a novel fold with a disklike structure." Biochemistry **42**(1): 217-21.

Anderson, J. R., D. Mukherjee, K. Muthukumaraswamy, K. C. Moraes, C. J. Wilusz and J. Wilusz (2006). "Sequence-specific RNA binding mediated by the RNase PH domain of components of the exosome." RNA (New York, N.Y **12**(10): 1810-6.

Anderson, J. S. and R. P. Parker (1998). "The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex." The EMBO journal **17**(5): 1497-506.

Anderson, P., K. Phillips, G. Stoecklin and N. Kedersha (2004). "Post-transcriptional regulation of proinflammatory proteins." Journal of leukocyte biology **76**(1): 42-7.

Andrulis, E. D., J. Werner, A. Nazarian, H. Erdjument-Bromage, P. Tempst and J. T. Lis (2002). "The RNA processing exosome is linked to elongating RNA polymerase II in Drosophila." Nature **420**(6917): 837-41.

Araki, Y., S. Takahashi, T. Kobayashi, H. Kajiho, S. Hoshino and T. Katada (2001). "Ski7p G protein interacts with the exosome and the Ski complex for 3'-to-5' mRNA decay in yeast." Embo J **20**(17): 4684-93.

Bakheet, T., M. Frevel, B. R. Williams, W. Greer and K. S. Khabar (2001). "ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins." Nucleic Acids Res **29**(1): 246-54.

Bakheet, T., B. R. Williams and K. S. Khabar (2006). "ARED 3.0: the large and diverse AU-rich transcriptome." Nucleic Acids Res **34**(Database issue): D111-4.

Beck, A. R., I. J. Miller, P. Anderson and M. Streuli (1998). "RNA-binding protein TIAR is essential for primordial germ cell development." Proc Natl Acad Sci U S A **95**(5): 2331-6.

Beelman, C. A. and R. Parker (1995). "Degradation of mRNA in eukaryotes." Cell **81**(2): 179-83.

Benjamin, D., M. Colombi and C. Moroni (2004). "A GFP-based assay for rapid screening of compounds affecting ARE-dependent mRNA turnover." Nucleic Acids Res **32**(11): e89.

Benjamin, D., M. Schmidlin, L. Min, B. Gross and C. Moroni (2006). "BRF1 Protein Turnover and mRNA Decay Activity Are Regulated by Protein Kinase B at the Same Phosphorylation Sites." Molecular and cellular biology **26**(24): 9497-507.

Bergstrom, K., J. C. Urquhart, A. Tafech, E. Doyle and C. H. Lee (2006). "Purification and characterization of a novel mammalian endoribonuclease." J Cell Biochem **98**(3): 519-37.

Bhattacharyya, S. N., R. Habermacher, U. Martine, E. I. Closs and W. Filipowicz (2006). "Relief of microRNA-mediated translational repression in human cells subjected to stress." Cell **125**(6): 1111-24.

Biggiogera, M., K. Burki, S. H. Kaufmann, J. H. Shaper, N. Gas, F. Amalric and S. Fakan (1990). "Nucleolar distribution of proteins B23 and nucleolin in mouse preimplantation embryos as visualized by immunoelectron microscopy." Development **110**(4): 1263-70.

Binder, R., J. A. Horowitz, J. P. Basilion, D. M. Koeller, R. D. Klausner and J. B. Harford (1994). "Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening." The EMBO journal **13**(8): 1969-80.

Blackshear, P. J., R. S. Phillips, S. Ghosh, S. B. Ramos, E. K. Richfield and W. S. Lai (2005). "Zfp36l3, a rodent X chromosome gene encoding a placenta-specific member of the Tristetraprolin family of CCCH tandem zinc finger proteins." Biology of reproduction **73**(2): 297-307.

Boeck, R., S. Tarun, Jr., M. Rieger, J. A. Deardorff, S. Muller-Auer and A. B. Sachs (1996). „The yeast Pan2 protein is required for poly(A)-binding protein-stimulated poly(A)-nuclease activity." The Journal of biological chemistry **271**(1): 432-8.

Bogre, L., C. Jonak, M. Mink, I. Meskiene, J. Traas, D. T. Ha, I. Swoboda, C. Plank, E. Wagner, E. Heberle-Bors and H. Hirt (1996). "Developmental and cell cycle regulation of alfalfa nucMs1, a plant homolog of the yeast Nsr1 and mammalian nucleolin." Plant Cell **8**(3): 417-28.

Borer, R. A., C. F. Lehner, H. M. Eppenberger and E. A. Nigg (1989). "Major nucleolar proteins shuttle between nucleus and cytoplasm." Cell **56**(3): 379-90.

Bourbon, H. M. and F. Amalric (1990). "Nucleolin gene organization in rodents: highly conserved sequences within three of the 13 introns." Gene **88**(2): 187-96.

Bourbon, H. M., B. Lapeyre and F. Amalric (1988). "Structure of the mouse nucleolin gene. The complete sequence reveals that each RNA binding domain is encoded by two independent exons." J Mol Biol **200**(4): 627-38.

Bouvet, P., J. J. Diaz, K. Kindbeiter, J. J. Madjar and F. Amalric (1998). "Nucleolin interacts with several ribosomal proteins through its RGG domain." J Biol Chem **273**(30): 19025-9.

Brewer, B. Y., J. Malicka, P. J. Blackshear and G. M. Wilson (2004). "RNA sequence elements required for high affinity binding by the zinc finger domain of tristetraprolin: conformational changes coupled to the bipartite nature of Au-rich MRNA-destabilizing motifs." J Biol Chem **279**(27): 27870-7.

Brewer, G. (1991). "An A + U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro." Mol Cell Biol **11**(5): 2460-6.

Briata, P., S. V. Forcales, M. Ponassi, G. Corte, C. Y. Chen, M. Karin, P. L. Puri and R. Gherzi (2005). "p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP controls the stability of select myogenic transcripts." Molecular cell **20**(6): 891-903.

Briata, P., C. Ilengo, G. Corte, C. Moroni, M. G. Rosenfeld, C. Y. Chen and R. Gherzi (2003). "The Wnt/beta-catenin-->Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs." Molecular cell **12**(5): 1201-11.

Brondani, V., Q. Schefer, F. Hamy and T. Klimkait (2005). "The peptidyl-prolyl isomerase Pin1 regulates phospho-Ser77 retinoic acid receptor alpha stability." Biochem Biophys Res Commun **328**(1): 6-13.

Brook, M., G. Sully, A. R. Clark and J. Saklatvala (2000). "Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade." FEBS letters **483**(1): 57-61.

Brook, M., C. R. Tchen, T. Santalucia, J. McIlrath, J. S. Arthur, J. Saklatvala and A. R. Clark (2006). "Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways." Mol Cell Biol **26**(6): 2408-18.

Brown, C. E. and A. B. Sachs (1998). "Poly(A) tail length control in Saccharomyces cerevisiae occurs by message-specific deadenylation." Molecular and cellular biology **18**(11): 6548-59.

Brown, J. T., X. Bai and A. W. Johnson (2000). "The yeast antiviral proteins Ski2p, Ski3p, and Ski8p exist as a complex in vivo." Rna **6**(3): 449-57.

Bugler, B., M. Caizergues-Ferrer, G. Bouche, H. Bourbon and F. Amalric (1982). "Detection and localization of a class of proteins immunologically related to a 100-kDa nucleolar protein." European journal of biochemistry / FEBS **128**(2-3): 475-80.

Bustin, S. A. and I. A. McKay (1999). "The product of the primary response gene BRF1 inhibits the interaction between 14-3-3 proteins and cRaf-1 in the yeast trihybrid system." DNA Cell Biol **18**(8): 653-61.

Buzby, J. S., G. Brewer and D. J. Nugent (1999). "Developmental regulation of RNA transcript destabilization by A + U-rich elements is AUF1-dependent." J Biol Chem **274**(48): 33973-8.

Buzby, J. S., S. M. Lee, P. Van Winkle, C. T. DeMaria, G. Brewer and M. S. Cairo (1996). "Increased granulocyte-macrophage colony-stimulating factor mRNA instability in cord versus adult mononuclear cells is translation-dependent and associated with increased levels of A + U-rich element binding factor." Blood **88**(8): 2889-97.

6.2 C-F

Caizergues-Ferrer, M., P. Mariottini, C. Curie, B. Lapeyre, N. Gas, F. Amalric and F. Amaldi (1989). "Nucleolin from Xenopus laevis: cDNA cloning and expression during development." Genes & development **3**(3): 324-33.

Campos, A. R., D. Grossman and K. White (1985). “Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis.” J Neurogenet **2**(3): 197-218.

Cao, Z., J. Xiong, M. Takeuchi, T. Kurama and D. V. Goeddel (1996). “TRAF6 is a signal transducer for interleukin-1.” Nature **383**(6599): 443-6.

Carballo, E., W. S. Lai and P. J. Blackshear (1998). “Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin.” Science **281**(5379): 1001-5.

Cartegni, L., M. Maconi, E. Morandi, F. Cobianchi, S. Riva and G. Biamonti (1996). “hnRNP A1 selectively interacts through its Gly-rich domain with different RNA-binding proteins.” J Mol Biol **259**(3): 337-48.

Chang, T. C., A. Yamashita, C. Y. Chen, Y. Yamashita, W. Zhu, S. Durdan, A. Kahvejian, N. Sonenberg and A. B. Shyu (2004). “UNR, a new partner of poly(A)-binding protein, plays a key role in translationally coupled mRNA turnover mediated by the c-fos major coding-region determinant.” Genes Dev **18**(16): 2010-23.

Chekanova, J. A., J. A. Dutko, I. S. Mian and D. A. Belostotsky (2002). “Arabidopsis thaliana exosome subunit AtRrp4p is a hydrolytic 3’-->5’ exonuclease containing S1 and KH RNA-binding domains.” Nucleic Acids Res **30**(3): 695-700.

Chekanova, J. A., R. J. Shaw, M. A. Wills and D. A. Belostotsky (2000). “Poly(A) tail-dependent exonuclease AtRrp41p from Arabidopsis thaliana rescues 5.8 S rRNA processing and mRNA decay defects of the yeast ski6 mutant and is found in an exosome-sized complex in plant and yeast cells.” J Biol Chem **275**(42): 33158-66.

Chen, B. E., M. Kondo, A. Garnier, F. L. Watson, R. Puettmann-Holgado, D. R. Lamar and D. Schmucker (2006). „The molecular diversity of Dscam is functionally required for neuronal wiring specificity in Drosophila.“ Cell **125**(3): 607-20.

Chen, C. Y., F. Del Gatto-Konczak, Z. Wu and M. Karin (1998). “Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway.” Science **280**(5371): 1945-9.

Chen, C. Y., R. Gherzi, J. S. Andersen, G. Gaietta, K. Jurchott, H. D. Royer, M. Mann and M. Karin (2000). “Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation.” Genes & development **14**(10): 1236-48.

Chen, C. Y., R. Gherzi, S. E. Ong, E. L. Chan, R. Raijmakers, G. J. Pruijn, G. Stoecklin, C. Moroni, M. Mann and M. Karin (2001). “AU binding proteins recruit the exosome to degrade ARE-containing mRNAs.” Cell **107**(4): 451-64.

Chen, C. Y. and A. B. Shyu (1995). “AU-rich elements: characterization and importance in mRNA degradation.” Trends in biochemical sciences **20**(11): 465-70.

Chinn, A. M., D. Ciais, S. Bailly, E. Chambaz, J. LaMarre and J. J. Feige (2002). “Identification of two novel ACTH-responsive genes encoding manganese-dependent superoxide dismutase (SOD2) and the zinc finger protein TIS11b [tetradecanoyl phorbol acetate (TPA)-inducible sequence 11b].” Mol Endocrinol **16**(6): 1417-27.

Choi, D. K., T. Ito, Y. Mitsui and Y. Sakaki (1998). “Fluorescent differential display analysis of gene expression in apoptotic neuroblastoma cells.” Gene **223**(1-2): 21-31.

Chou, C. F., A. Mulky, S. Maitra, W. J. Lin, R. Gherzi, J. Kappes and C. Y. Chen (2006). “Tethering KSRP, a decay-promoting AU-rich element-binding protein, to mRNAs elicits mRNA decay.” Molecular and cellular biology **26**(10): 3695-706.

Ciais, D., N. Cherradi, S. Bailly, E. Grenier, E. Berra, J. Pouyssegur, J. Lamarre and J. J. Feige (2004). “Destabilization of vascular endothelial growth factor mRNA by the zinc-finger protein TIS11b.” Oncogene **23**(53): 8673-80.

Cok, S. J., S. J. Acton, A. E. Sexton and A. R. Morrison (2004). "Identification of RNA-binding proteins in RAW 264.7 cells that recognize a lipopolysaccharide-responsive element in the 3-untranslated region of the murine cyclooxygenase-2 mRNA." J Biol Chem **279**(9): 8196-205.

Collart, M. A., P. Baeuerle and P. Vassalli (1990). "Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B." Molecular and cellular biology **10**(4): 1498-506.

Corps, A. N. and K. D. Brown (1995). "Insulin and insulin-like growth factor I stimulate expression of the primary response gene cMG1/TIS11b by a wortmannin-sensitive pathway in RIE-1 cells." FEBS Lett **368**(1): 160-4.

Creancier, L., H. Prats, C. Zanibellato, F. Amalric and B. Bugler (1993). "Determination of the functional domains involved in nucleolar targeting of nucleolin." Mol Biol Cell **4**(12): 1239-50.

Das, B., S. Das and F. Sherman (2006). "Mutant LYS2 mRNAs retained and degraded in the nucleus of Saccharomyces cerevisiae." Proceedings of the National Academy of Sciences of the United States of America **103**(29): 10871-6.

de Carcer, G., A. Cerdido and F. J. Medina (1997). "NopA64, a novel nucleolar phosphoprotein from proliferating onion cells, sharing immunological determinants with mammalian nucleolin." Planta **201**(4): 487-95.

De, J., W. S. Lai, J. M. Thorn, S. M. Goldsworthy, X. Liu, T. K. Blackwell and P. J. Blackshear (1999). "Identification of four CCCH zinc finger proteins in Xenopus, including a novel vertebrate protein with four zinc fingers and severely restricted expression." Gene **228**(1-2): 133-45.

de la Cruz, J., D. Kressler, D. Tollervey and P. Linder (1998). "Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3' end formation of 5.8S rRNA in Saccharomyces cerevisiae." Embo J **17**(4): 1128-40.

Dean, J. L., R. Wait, K. R. Mahtani, G. Sully, A. R. Clark and J. Saklatvala (2001). "The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR." Molecular and cellular biology **21**(3): 721-30.

Dehlin, E., M. Wormington, C. G. Korner and E. Wahle (2000). „Cap-dependent deadenylation of mRNA." The EMBO journal **19**(5): 1079-86.

Del Gatto-Konczak, F., C. F. Bourgeois, C. Le Guiner, L. Kister, M. C. Gesnel, J. Stevenin and R. Breathnach (2000). "The RNA-binding protein TIA-1 is a novel mammalian splicing regulator acting through intron sequences adjacent to a 5' splice site." Mol Cell Biol **20**(17): 6287-99.

DeMaria, C. T., Y. Sun, B. J. Wagner, L. Long and G. A. Brewer (1997). "Structural determination in AUF1 required for high affinity binding to A + U-rich elements." Nucleic Acids Symp Ser(36): 12-4.

Dember, L. M., N. D. Kim, K. Q. Liu and P. Anderson (1996). „Individual RNA recognition motifs of TIA-1 and TIAR have different RNA binding specificities." J Biol Chem **271**(5): 2783-8.

Denis, C. L. and J. Chen (2003). "The CCR4-NOT complex plays diverse roles in mRNA metabolism." Progress in nucleic acid research and molecular biology **73**: 221-50.

Di Marco, S., R. Mazroui, P. Dallaire, S. Chittur, S. A. Tenenbaum, D. Radzioch, A. Marette and I. E. Gallouzi (2005). "NF-kappa B-mediated MyoD decay during muscle wasting requires nitric oxide synthase mRNA stabilization, HuR protein, and nitric oxide release." Mol Cell Biol **25**(15): 6533-45.

Didier, D. K. and H. J. Klee (1992). “Identification of an Arabidopsis DNA-binding protein with homology to nucleolin.” Plant Mol Biol **18**(5): 977-9.

Dixon, D. A., G. C. Balch, N. Kedersha, P. Anderson, G. A. Zimmerman, R. D. Beauchamp and S. M. Prescott (2003). “Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1.” The Journal of experimental medicine **198**(3): 475-81.

Dixon, D. A., N. D. Tolley, P. H. King, L. B. Nabors, T. M. McIntyre, G. A. Zimmerman and S. M. Prescott (2001). “Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells.” J Clin Invest **108**(11): 1657-65.

DuBois, R. N., M. W. McLane, K. Ryder, L. F. Lau and D. Nathans (1990). “A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence.” J Biol Chem **265**(31): 19185-91.

Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias and P. N. Tsichlis (2000). „TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway.“ Cell **103**(7): 1071-83.

Dundr, M., U. T. Meier, N. Lewis, D. Rekosh, M. L. Hammarskjold and M. O. Olson (1997). “A class of nonribosomal nucleolar components is located in chromosome periphery and in nucleolus-derived foci during anaphase and telophase.” Chromosoma **105**(7-8): 407-17.

Escande, M. L., N. Gas and B. J. Stevens (1985). “Immunolocalization of the 100 K nucleolar protein in CHO cells.” Biol Cell **53**(2): 99-109.

Estevez, A. M., T. Kempf and C. Clayton (2001). “The exosome of Trypanosoma brucei.” Embo J **20**(14): 3831-9.

Estevez, A. M., B. Lehner, C. M. Sanderson, T. Ruppert and C. Clayton (2003). “The roles of intersubunit interactions in exosome stability.” J Biol Chem **278**(37): 34943-51.

Eversole, A. and N. Maizels (2000). “In vitro properties of the conserved mammalian protein hnRNP D suggest a role in telomere maintenance.” Mol Cell Biol **20**(15): 5425-32.

Fahling, M., A. Steege, A. Perlewitz, B. Nafz, R. Mrowka, P. B. Persson and B. J. Thiele (2005). “Role of nucleolin in posttranscriptional control of MMP-9 expression.” Biochim Biophys Acta **1731**(1): 32-40.

Fairhurst, A. M., J. E. Connolly, K. A. Hintz, N. J. Goulding, A. J. Rassias, M. P. Yeager, W. Rigby and P. K. Wallace (2003). “Regulation and localization of endogenous human tristetraprolin.” Arthritis research & therapy **5**(4): R214-25.

Fan, X. C. and J. A. Steitz (1998a). “HNS, a nuclear-cytoplasmic shuttling sequence in HuR.” Proc Natl Acad Sci U S A **95**(26): 15293-8.

Fan, X. C. and J. A. Steitz (1998b). “Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs.” Embo J **17**(12): 3448-60.

Fechir, M., K. Linker, A. Pautz, T. Hubrich and H. Kleinert (2005). „The RNA binding protein TIAR is involved in the regulation of human iNOS expression.“ Cellular and molecular biology (Noisy-le-Grand, France) **51**(3): 299-305.

Fenger-Gron, M., C. Fillman, B. Norrild and J. Lykke-Andersen (2005). “Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping.” Molecular cell **20**(6): 905-15.

Fialcowitz, E. J., B. Y. Brewer, B. P. Keenan and G. M. Wilson (2005). “A hairpin-like structure within an AU-rich mRNA-destabilizing element regulates trans-factor binding selectivity and mRNA decay kinetics.” J Biol Chem **280**(23): 22406-17.

Figueroa, A., A. Cuadrado, J. Fan, U. Atasoy, G. E. Muscat, P. Munoz-Canoves, M. Gorospe and A. Munoz (2003). "Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes." Mol Cell Biol **23**(14): 4991-5004.

Fillman, C. and J. Lykke-Andersen (2005). "RNA decapping inside and outside of processing bodies." Current opinion in cell biology **17**(3): 326-31.

Finger, L. D., C. Johansson, B. Rinaldi, P. Bouvet and J. Feigon (2004). "Contributions of the RNA-binding and linker domains and RNA structure to the specificity and affinity of the nucleolin RBD12/NRE interaction." Biochemistry **43**(22): 6937-47.

Finger, L. D., L. Trantirek, C. Johansson and J. Feigon (2003). "Solution structures of stem-loop RNAs that bind to the two N-terminal RNA-binding domains of nucleolin." Nucleic Acids Res **31**(22): 6461-72.

Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha and D. T. Golenbock (2003). "LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF." J Exp Med **198**(7): 1043-55.

Frischmeyer, P. A., A. van Hoof, K. O'Donnell, A. L. Guerrerio, R. Parker and H. C. Dietz (2002). "An mRNA surveillance mechanism that eliminates transcripts lacking termination codons." Science **295**(5563): 2258-61.

Fritz, D. T., N. Bergman, W. J. Kilpatrick, C. J. Wilusz and J. Wilusz (2004). "Messenger RNA decay in Mammalian cells: the exonuclease perspective." Cell biochemistry and biophysics **41**(2): 265-78.

Fuentes-Panana, E. M., R. Peng, G. Brewer, J. Tan and P. D. Ling (2000). "Regulation of the Epstein-Barr virus C promoter by AUF1 and the cyclic AMP/protein kinase A signaling pathway." J Virol **74**(17): 8166-75.

6.3 G-K

Galiana-Arnoux, D., C. Dostert, A. Schneemann, J. A. Hoffmann and J. L. Imler (2006). "Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila." Nat Immunol **7**(6): 590-7.

Gallouzi, I. E., C. M. Brennan and J. A. Steitz (2003). "Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock." Rna **9**(11): 1410.

Gallouzi, I. E. and J. A. Steitz (2001). "Delineation of mRNA export pathways by the use of cell-permeable peptides." Science **294**(5548): 1895-901.

Gao, M., D. T. Fritz, L. P. Ford and J. Wilusz (2000). "Interaction between a poly(A)-specific ribonuclease and the 5' cap influences mRNA deadenylation rates in vitro." Molecular cell **5**(3): 479-88.

Gas, N., M. L. Escande and B. J. Stevens (1985). "Immunolocalization of the 100 kDa nucleolar protein during the mitotic cycle in CHO cells." Biol Cell **53**(3): 209-18.

Gatfield, D. and E. Izaurralde (2004). "Nonsense-mediated messenger RNA decay is initiated by endonucleolytic cleavage in Drosophila." Nature **429**(6991): 575-8.

Gatfield, D., L. Unterholzner, F. D. Ciccarelli, P. Bork and E. Izaurralde (2003). "Nonsense-mediated mRNA decay in Drosophila: at the intersection of the yeast and mammalian pathways." Embo J **22**(15): 3960-70.

Gay, D. A., S. S. Sisodia and D. W. Cleveland (1989). "Autoregulatory control of beta-tubulin mRNA stability is linked to translation elongation." Proc Natl Acad Sci U S A **86**(15): 5763-7.

Gay, D. A., T. J. Yen, J. T. Lau and D. W. Cleveland (1987). "Sequences that confer beta-tubulin autoregulation through modulated mRNA stability reside within exon 1 of a beta-tubulin mRNA." Cell **50**(5): 671-9.

Gherzi, R., K. Y. Lee, P. Briata, D. Wegmuller, C. Moroni, M. Karin and C. Y. Chen (2004). "A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery." Molecular cell **14**(5): 571-83.

Ghisolfi, L., G. Joseph, F. Amalric and M. Erard (1992). "The glycine-rich domain of nucleolin has an unusual supersecondary structure responsible for its RNA-helix-destabilizing properties." J Biol Chem **267**(5): 2955-9.

Ghisolfi-Nieto, L., G. Joseph, F. Puvion-Dutilleul, F. Amalric and P. Bouvet (1996). "Nucleolin is a sequence-specific RNA-binding protein: characterization of targets on pre-ribosomal RNA." Journal of molecular biology **260**(1): 34-53.

Ghribi, O. (2006). "The role of the endoplasmic reticulum in the accumulation of beta-amyloid peptide in Alzheimer's disease." Current molecular medicine **6**(1): 119-33.

Gilks, N., N. Kedersha, M. Ayodele, L. Shen, G. Stoecklin, L. M. Dember and P. Anderson (2004). "Stress granule assembly is mediated by prion-like aggregation of TIA-1." Mol Biol Cell **15**(12): 5383-98.

Ginisty, H., F. Amalric and P. Bouvet (1998). "Nucleolin functions in the first step of ribosomal RNA processing." Embo J **17**(5): 1476-86.

Ginisty, H., F. Amalric and P. Bouvet (2001). "Two different combinations of RNA-binding domains determine the RNA binding specificity of nucleolin." J Biol Chem **276**(17): 14338-43.

Ginisty, H., H. Sicard, B. Roger and P. Bouvet (1999). "Structure and functions of nucleolin." J Cell Sci **112 (Pt 6)**: 761-72.

Gomperts, M., J. C. Pascall and K. D. Brown (1990). "The nucleotide sequence of a cDNA encoding an EGF-inducible gene indicates the existence of a new family of mitogen-induced genes." Oncogene **5**(7): 1081-3.

Gros, F., H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Risebrough and J. D. Watson (1961). "Unstable ribonucleic acid revealed by pulse labelling of Escherichia coli." Nature **190**: 581-5.

Grosset, C., C. Y. Chen, N. Xu, N. Sonenberg, H. Jacquemin-Sablon and A. B. Shyu (2000). "A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a c-fos RNA coding determinant via a protein complex." Cell **103**(1): 29-40.

Grunenfelder, B., G. Rummel, J. Vohradsky, D. Roder, H. Langen and U. Jenal (2001). „Proteomic analysis of the bacterial cell cycle." Proc Natl Acad Sci U S A **98**(8): 4681-6.

Gu, M., C. Fabrega, S. W. Liu, H. Liu, M. Kiledjian and C. D. Lima (2004). "Insights into the structure, mechanism, and regulation of scavenger mRNA decapping activity." Mol Cell **14**(1): 67-80.

Gueydan, C., L. Droogmans, P. Chalon, G. Huez, D. Caput and V. Kruys (1999). "Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor alpha mRNA." J Biol Chem **274**(4): 2322-6.

Gulli, M. P., J. P. Girard, D. Zabetakis, B. Lapeyre, T. Melese and M. Caizergues-Ferrer (1995). "gar2 is a nucleolar protein from Schizosaccharomyces pombe required for 18S rRNA and 40S ribosomal subunit accumulation." Nucleic Acids Res **23**(11): 1912-8.

Han, J., T. Brown and B. Beutler (1990). "Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level." The Journal of experimental medicine **171**(2): 465-75.

Han, J. H., B. Beutler and G. Huez (1991). "Complex regulation of tumor necrosis factor mRNA turnover in lipopolysaccharide-activated macrophages." Biochimica et biophysica acta **1090**(1): 22-8.

Hau, H. H., R. J. Walsh, R. L. Ogilvie, D. A. Williams, C. S. Reilly and P. R. Bohjanen (2006). "Tristetraprolin recruits functional mRNA decay complexes to ARE sequences."

He, F., X. Li, P. Spatrick, R. Casillo, S. Dong and A. Jacobson (2003). "Genome-wide analysis of mRNAs regulated by the nonsense-mediated and 5' to 3' mRNA decay pathways in yeast." Molecular cell **12**(6): 1439-52.

Hernandez-Verdun, D. and T. Gautier (1994). "The chromosome periphery during mitosis." Bioessays **16**(3): 179-85.

Hilleren, P. J. and R. Parker (2003). "Cytoplasmic degradation of splice-defective pre-mRNAs and intermediates." Molecular cell **12**(6): 1453-65.

Hitti, E., T. Iakovleva, M. Brook, S. Deppenmeier, A. D. Gruber, D. Radzioch, A. R. Clark, P. J. Blackshear, A. Kotlyarov and M. Gaestel (2006). "Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element." Molecular and cellular biology **26**(6): 2399-407.

Horng, T., G. M. Barton and R. Medzhitov (2001). "TIRAP: an adapter molecule in the Toll signaling pathway." Nat Immunol **2**(9): 835-41.

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda and S. Akira (1999). "Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product." J Immunol **162**(7): 3749-52.

Hudson, B. P., M. A. Martinez-Yamout, H. J. Dyson and P. E. Wright (2004). "Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d." Nat Struct Mol Biol **11**(3): 257-64.

Hutvagner, G. (2005). "Small RNA asymmetry in RNAi: function in RISC assembly and gene regulation." FEBS Lett **579**(26): 5850-7.

Jiang, T. and S. Altman (2002). "A protein subunit of human RNase P, Rpp14, and its interacting partner, OIP2, have 3'-->5' exoribonuclease activity." Proc Natl Acad Sci U S A **99**(8): 5295-300.

Jiang, Y., X. S. Xu and J. E. Russell (2006). "A nucleolin-binding 3' untranslated region element stabilizes beta-globin mRNA in vivo." Mol Cell Biol **26**(6): 2419-29.

Johansson, C., L. D. Finger, L. Trantirek, T. D. Mueller, S. Kim, I. A. Laird-Offringa and J. Feigon (2004). "Solution structure of the complex formed by the two N-terminal RNA-binding domains of nucleolin and a pre-rRNA target." J Mol Biol **337**(4): 799-816.

Karima, R., S. Matsumoto, H. Higashi and K. Matsushima (1999). "The molecular pathogenesis of endotoxic shock and organ failure." Molecular medicine today **5**(3): 123-32.

Kawai, T., O. Adachi, T. Ogawa, K. Takeda and S. Akira (1999). "Unresponsiveness of MyD88-deficient mice to endotoxin." Immunity **11**(1): 115-22.

Kawai, T., A. Lal, X. Yang, S. Galban, K. Mazan-Mamczarz and M. Gorospe (2006). "Translational control of cytochrome c by RNA-binding proteins TIA-1 and HuR." Molecular and cellular biology **26**(8): 3295-307.

Kedersha, N. and P. Anderson (2002). “Stress granules: sites of mRNA triage that regulate mRNA stability and translatability.” Biochemical Society transactions **30**(Pt 6): 963-9.

Kedersha, N. L., M. Gupta, W. Li, I. Miller and P. Anderson (1999). “RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules.” J Cell Biol **147**(7): 1431-42.

Keffer, J., L. Probert, H. Cazlaris, S. Georgopoulos, E. Kaslaris, D. Kioussis and G. Kollias (1991). “Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis.” Embo J **10**(13): 4025-31.

Kiledjian, M., C. T. DeMaria, G. Brewer and K. Novick (1997). “Identification of AUF1 (heterogeneous nuclear ribonucleoprotein D) as a component of the alpha-globin mRNA stability complex.” Molecular and cellular biology **17**(8): 4870-6.

Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli and G. Kollias (1999). “Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies.” Immunity **10**(3): 387-98.

Korner, C. G., M. Wormington, M. Muckenthaler, S. Schneider, E. Dehlin and E. Wahle (1998). „The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of Xenopus oocytes.“ The EMBO journal **17**(18): 5427-37.

Kotlyarov, A., A. Neininger, C. Schubert, R. Eckert, C. Birchmeier, H. D. Volk and M. Gaestel (1999). „MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis.“ Nature cell biology **1**(2): 94-7.

6.4 L-N

Ladd, A. N., N. Charlet and T. A. Cooper (2001). “The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing.” Mol Cell Biol **21**(4): 1285-96.

Lai, W. S., E. Carballo, J. R. Strum, E. A. Kennington, R. S. Phillips and P. J. Blackshear (1999). “Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA.” Mol Cell Biol **19**(6): 4311-23.

Lai, W. S., E. Carballo, J. M. Thorn, E. A. Kennington and P. J. Blackshear (2000). “Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to Au-rich elements and destabilization of mRNA.” The Journal of biological chemistry **275**(23): 17827-37.

Lai, W. S., J. S. Parker, S. F. Grissom, D. J. Stumpo and P. J. Blackshear (2006). “Novel mRNA Targets for Tristetraprolin (TTP) Identified by Global Analysis of Stabilized Transcripts in TTP-Deficient Fibroblasts.” Mol Cell Biol **26**(24): 9196-208.

Lai, W. S., D. J. Stumpo and P. J. Blackshear (1990). “Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein.” J Biol Chem **265**(27): 16556-63.

Lal, A., K. Mazan-Mamczarz, T. Kawai, X. Yang, J. L. Martindale and M. Gorospe (2004). “Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs.” Embo J **23**(15): 3092-102.

Lapeyre, B., M. Caizergues-Ferrer, G. Bouche and F. Amalric (1985). “Cloning of cDNA encoding a 100 kDa nucleolar protein (nucleoline) of Chinese hamster ovary cells.” Nucleic Acids Res **13**(16): 5805-16.

Laroia, G. and R. J. Schneider (2002). "Alternate exon insertion controls selective ubiquitination and degradation of different AUF1 protein isoforms." Nucleic Acids Res **30**(14): 3052-8.

Lazdins, I. B., M. Delannoy and B. Sollner-Webb (1997). "Analysis of nucleolar transcription and processing domains and pre-rRNA movements by in situ hybridization." Chromosoma **105**(7-8): 481-95.

Le Guiner, C., F. Lejeune, D. Galiana, L. Kister, R. Breathnach, J. Stevenin and F. Del Gatto-Konczak (2001). "TIA-1 and TIAR activate splicing of alternative exons with weak 5' splice sites followed by a U-rich stretch on their own pre-mRNAs." J Biol Chem **276**(44): 40638-46.

Lee, S. J., S. Evers, D. Roeder, A. F. Parlow, J. Risteli, L. Risteli, Y. C. Lee, T. Feizi, H. Langen and M. C. Nussenzweig (2002). „Mannose receptor-mediated regulation of serum glycoprotein homeostasis." Science **295**(5561): 1898-901.

Lee, W. C., D. Zabetakis and T. Melese (1992). "NSR1 is required for pre-rRNA processing and for the proper maintenance of steady-state levels of ribosomal subunits." Mol Cell Biol **12**(9): 3865-71.

Lemm, I. and J. Ross (2002). "Regulation of c-myc mRNA decay by translational pausing in a coding region instability determinant." Mol Cell Biol **22**(12): 3959-69.

Li, M., A. Makkinje and Z. Damuni (1996). "Molecular identification of I1PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A." Biochemistry **35**(22): 6998-7002.

Li, S., A. Strelow, E. J. Fontana and H. Wesche (2002). "IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase." Proc Natl Acad Sci U S A **99**(8): 5567-72.

Lindstein, T., C. H. June, J. A. Ledbetter, G. Stella and C. B. Thompson (1989). "Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway." Science **244**(4902): 339-43.

Linker, K., A. Pautz, M. Fechir, T. Hubrich, J. Greeve and H. Kleinert (2005). "Involvement of KSRP in the post-transcriptional regulation of human iNOS expression-complex interplay of KSRP with TTP and HuR." Nucleic acids research **33**(15): 4813-27.

Lischwe, M. A., K. D. Roberts, L. C. Yeoman and H. Busch (1982). "Nucleolar specific acidic phosphoprotein C23 is highly methylated." The Journal of biological chemistry **257**(24): 14600-2.

Liu, S. W., X. Jiao, H. Liu, M. Gu, C. D. Lima and M. Kiledjian (2004). "Functional analysis of mRNA scavenger decapping enzymes." Rna **10**(9): 1412-22.

Loflin, P., C. Y. Chen and A. B. Shyu (1999). "Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element." Genes Dev **13**(14): 1884-97.

Lopez de Silanes, I., J. Fan, C. J. Galban, R. G. Spencer, K. G. Becker and M. Gorospe (2004a). "Global analysis of HuR-regulated gene expression in colon cancer systems of reducing complexity." Gene Expr **12**(1): 49-59.

Lopez de Silanes, I., S. Galban, J. L. Martindale, X. Yang, K. Mazan-Mamczarz, F. E. Indig, G. Falco, M. Zhan and M. Gorospe (2005). "Identification and functional outcome of mRNAs associated with RNA-binding protein TIA-1." Mol Cell Biol **25**(21): 9520-31.

Lopez de Silanes, I., M. Zhan, A. Lal, X. Yang and M. Gorospe (2004b). "Identification of a target RNA motif for RNA-binding protein HuR." Proc Natl Acad Sci U S A **101**(9): 2987-92.

Lorentzen, E., P. Walter, S. Fribourg, E. Evguenieva-Hackenberg, G. Klug and E. Conti (2005). "The archaeal exosome core is a hexameric ring structure with three catalytic subunits." Nat Struct Mol Biol **12**(7): 575-81.

Lu, J. Y., N. Sadri and R. J. Schneider (2006). „Endotoxic shock in AUF1 knockout mice mediated by failure to degrade proinflammatory cytokine mRNAs." Genes Dev **20**(22): 3174-84.

Lu, X., N. A. Timchenko and L. T. Timchenko (1999). "Cardiac elav-type RNA-binding protein (ETR-3) binds to RNA CUG repeats expanded in myotonic dystrophy." Hum Mol Genet **8**(1): 53-60.

Lykke-Andersen, J. and E. Wagner (2005). „Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1." Genes Dev **19**(3): 351-61.

Ma, W. J., S. Cheng, C. Campbell, A. Wright and H. Furneaux (1996). "Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein." J Biol Chem **271**(14): 8144-51.

Ma, W. J., S. Chung and H. Furneaux (1997). "The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA." Nucleic Acids Res **25**(18): 3564-9.

Maclean, K. N., I. A. McKay and S. A. Bustin (1998). "Differential effects of sodium butyrate on the transcription of the human TIS11 family of early-response genes in colorectal cancer cells." Br J Biomed Sci **55**(3): 184-91.

Malek, S. N., A. I. Katumuluwa and G. R. Pasternack (1990). "Identification and preliminary characterization of two related proliferation-associated nuclear phosphoproteins." J Biol Chem **265**(22): 13400-9.

Marderosian, M., A. Sharma, A. P. Funk, R. Vartanian, J. Masri, O. D. Jo and J. F. Gera (2006). "Tristetraprolin regulates Cyclin D1 and c-Myc mRNA stability in response to rapamycin in an Akt-dependent manner via p38 MAPK signaling." Oncogene **25**(47): 6277-90.

Maridor, G., W. Krek and E. A. Nigg (1990). "Structure and developmental expression of chicken nucleolin and NO38: coordinate expression of two abundant non-ribosomal nucleolar proteins." Biochim Biophys Acta **1049**(2): 126-33.

Martinez, J., Y. G. Ren, P. Nilsson, M. Ehrenberg and A. Virtanen (2001). „The mRNA cap structure stimulates rate of poly(A) removal and amplifies processivity of degradation." The Journal of biological chemistry **276**(30): 27923-9.

Mazan-Mamczarz, K., A. Lal, J. L. Martindale, T. Kawai and M. Gorospe (2006). "Translational repression by RNA-binding protein TIAR." Mol Cell Biol **26**(7): 2716-27.

McBratney, S. and P. Sarnow (1996). "Evidence for involvement of trans-acting factors in selection of the AUG start codon during eukaryotic translational initiation." Molecular and cellular biology **16**(7): 3523-34.

McGrath, K. E., J. F. Smothers, C. A. Dadd, M. T. Madireddi, M. A. Gorovsky and C. D. Allis (1997). "An abundant nucleolar phosphoprotein is associated with ribosomal DNA in Tetrahymena macronuclei." Mol Biol Cell **8**(1): 97-108.

Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng and T. Tuschl (2004). „Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs." Mol Cell **15**(2): 185-97.

Mencinger, M., I. Panagopoulos, J. A. Contreras, F. Mitelman and P. Aman (1998). "Expression analysis and chromosomal mapping of a novel human gene, APRIL, encoding an acidic protein rich in leucines." Biochim Biophys Acta **1395**(2): 176-80.

Michel, S. L., A. L. Guerrerio and J. M. Berg (2003). „Selective RNA binding by a single CCCH zinc-binding domain from Nup475 (Tristetraprolin)." Biochemistry **42**(16): 4626-30.

Min, H., C. W. Turck, J. M. Nikolic and D. L. Black (1997). "A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer." Genes Dev **11**(8): 1023-36.

Ming, X. F., G. Stoecklin, M. Lu, R. Looser and C. Moroni (2001). "Parallel and independent regulation of interleukin-3 mRNA turnover by phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase." Molecular and cellular biology **21**(17): 5778-89.

Mitchell, P., E. Petfalski, R. Houalla, A. Podtelejnikov, M. Mann and D. Tollervey (2003). "Rrp47p is an exosome-associated protein required for the 3' processing of stable RNAs." Mol Cell Biol **23**(19): 6982-92.

Mitchell, P., E. Petfalski, A. Shevchenko, M. Mann and D. Tollervey (1997). "The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-->5' exoribonucleases." Cell **91**(4): 457-66.

Mitchell, P., E. Petfalski and D. Tollervey (1996). "The 3' end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism." Genes & development **10**(4): 502-13.

Muhlrad, D. and R. Parker (1994). "Premature translational termination triggers mRNA decapping." Nature **370**(6490): 578-81.

Mukherjee, D., M. Gao, J. P. O'Connor, R. Raijmakers, G. Pruijn, C. S. Lutz and J. Wilusz (2002). "The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements." The EMBO journal **21**(1-2): 165-74.

Mukhopadhyay, D., C. W. Houchen, S. Kennedy, B. K. Dieckgraefe and S. Anant (2003). "Coupled mRNA stabilization and translational silencing of cyclooxygenase-2 by a novel RNA binding protein, CUGBP2." Mol Cell **11**(1): 113-26.

Mullner, E. W. and L. C. Kuhn (1988). "A stem-loop in the 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm." Cell **53**(5): 815-25.

Muroi, M., T. Ohnishi and K. Tanamoto (2002). "Regions of the mouse CD14 molecule required for toll-like receptor 2- and 4-mediated activation of NF-kappa B." J Biol Chem **277**(44): 42372-9.

Myokai, F., S. Takashiba, R. Lebo and S. Amar (1999). "A novel lipopolysaccharide-induced transcription factor regulating tumor necrosis factor alpha gene expression: molecular cloning, sequencing, characterization, and chromosomal assignment." Proceedings of the National Academy of Sciences of the United States of America **96**(8): 4518-23.

Nabors, L. B., G. Y. Gillespie, L. Harkins and P. H. King (2001). "HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs." Cancer Res **61**(5): 2154-61.

Nagaoka, K., T. Suzuki, T. Kawano, K. Imakawa and S. Sakai (2006). "Stability of casein mRNA is ensured by structural interactions between the 3'-untranslated region and poly(A) tail via the HuR and poly(A)-binding protein complex." Biochim Biophys Acta **1759**(3-4): 132-40.

Nair, A. P., I. D. Diamantis, J. F. Conscience, V. Kindler, P. Hofer and C. Moroni (1989). "A v-H-ras-dependent hemopoietic tumor model involving progression from a clonal stage of transformation competence to autocrine interleukin 3 production." Molecular and cellular biology **9**(3): 1183-90.

Ng, D. T., S. S. Watowich and R. A. Lamb (1992). "Analysis in vivo of GRP78-BiP/substrate interactions and their role in induction of the GRP78-BiP gene." Mol Biol Cell **3**(2): 143-55.

Nie, X. F., K. N. Maclean, V. Kumar, I. A. McKay and S. A. Bustin (1995). "ERF-2, the human homologue of the murine Tis11d early response gene." Gene **152**(2): 285-6.

6.5 O-S

Ogilvie, R. L., M. Abelson, H. H. Hau, I. Vlasova, P. J. Blackshear and P. R. Bohjanen (2005). "Tristetraprolin down-regulates IL-2 gene expression through AU-rich element-mediated mRNA decay." Journal of immunology (Baltimore, Md. **174**(2): 953-61.

Orban, T. I. and E. Izaurralde (2005). "Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome." Rna **11**(4): 459-69.

Orrick, L. R., M. O. Olson and H. Busch (1973). "Comparison of nucleolar proteins of normal rat liver and Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis." Proc Natl Acad Sci U S A **70**(5): 1316-20.

Otake, Y., S. Soundararajan, T. K. Sengupta, E. A. Kio, J. C. Smith, M. Pineda-Roman, R. K. Stuart, E. K. Spicer and D. J. Fernandes (2006). "Overexpression of nucleolin in chronic lymphocytic leukemia cells induces stabilization of bcl-2 mRNA." Blood.

Palsson-McDermott, E. M. and L. A. O'Neill (2004). "Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4." Immunology **113**(2): 153-62.

Panayi, G. S. and V. M. Corrigall (2006). "BiP regulates autoimmune inflammation and tissue damage." Autoimmun Rev **5**(2): 140-2.

Pandey, N. B. and W. F. Marzluff (1987). "The stem-loop structure at the 3' end of histone mRNA is necessary and sufficient for regulation of histone mRNA stability." Molecular and cellular biology **7**(12): 4557-9.

Parker, R. and H. Song (2004). "The enzymes and control of eukaryotic mRNA turnover." Nature structural & molecular biology **11**(2): 121-7.

Paschoud, S., A. M. Dogar, C. Kuntz, B. Grisoni-Neupert, L. Richman and L. C. Kuhn (2006). "Destabilization of interleukin-6 mRNA requires a putative RNA stem-loop structure, an AU-rich element, and the RNA-binding protein AUF1." Molecular and cellular biology **26**(22): 8228-41.

Pasparakis, M., L. Alexopoulou, V. Episkopou and G. Kollias (1996). "Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response." J Exp Med **184**(4): 1397-411.

Peltz, S. W. and J. Ross (1987). "Autogenous regulation of histone mRNA decay by histone proteins in a cell-free system." Mol Cell Biol **7**(12): 4345-56.

Pende, A., K. D. Tremmel, C. T. DeMaria, B. C. Blaxall, W. A. Minobe, J. A. Sherman, J. D. Bisognano, M. R. Bristow, G. Brewer and J. Port (1996). "Regulation of the mRNA-binding protein AUF1 by activation of the beta-adrenergic receptor signal transduction pathway." J Biol Chem **271**(14): 8493-501.

Peng, S. S., C. Y. Chen and A. B. Shyu (1996). "Functional characterization of a non-AUUUA AU-rich element from the c-jun proto-oncogene mRNA: evidence for a novel class of AU-rich elements." Mol Cell Biol **16**(4): 1490-9.

Peng, S. S., C. Y. Chen, N. Xu and A. B. Shyu (1998). "RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein." Embo J **17**(12): 3461-70.

Piecyk, M., S. Wax, A. R. Beck, N. Kedersha, M. Gupta, B. Maritim, S. Chen, C. Gueydan, V. Kruys, M. Streuli and P. Anderson (2000). "TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha." The EMBO journal **19**(15): 4154-63.

Pillai, R. S., S. N. Bhattacharyya, C. G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand and W. Filipowicz (2005). "Inhibition of translational initiation by Let-7 MicroRNA in human cells." Science **309**(5740): 1573-6.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-8.

Prechtel, A. T., J. Chemnitz, S. Schirmer, C. Ehlers, I. Langbein-Detsch, J. Stulke, M. C. Dabauvalle, R. H. Kehlenbach and J. Hauber (2006). „Expression of CD83 is regulated by HuR via a novel cis-active coding region RNA element." J Biol Chem **281**(16): 10912-25.

Prestayko, A. W., G. R. Klomp, D. J. Schmoll and H. Busch (1974). "Comparison of proteins of ribosomal subunits and nucleolar preribosomal particles from Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis." Biochemistry **13**(9): 1945-51.

Prokipcak, R. D., D. J. Herrick and J. Ross (1994). "Purification and properties of a protein that binds to the C-terminal coding region of human c-myc mRNA." J Biol Chem **269**(12): 9261-9.

Raabe, T., M. Bukrinsky and R. A. Currie (1998). "Relative contribution of transcription and translation to the induction of tumor necrosis factor-alpha by lipopolysaccharide." The Journal of biological chemistry **273**(2): 974-80.

Raijmakers, R., W. V. Egberts, W. J. van Venrooij and G. J. Pruijn (2002a). "Protein-protein interactions between human exosome components support the assembly of RNase PH-type subunits into a six-membered PNPase-like ring." Journal of molecular biology **323**(4): 653-63.

Raijmakers, R., W. V. Egberts, W. J. van Venrooij and G. J. Pruijn (2003). "The association of the human PM/Scl-75 autoantigen with the exosome is dependent on a newly identified N terminus." The Journal of biological chemistry **278**(33): 30698-704.

Raijmakers, R., Y. E. Noordman, W. J. van Venrooij and G. J. Pruijn (2002b). "Protein-protein interactions of hCsl4p with other human exosome subunits." Journal of molecular biology **315**(4): 809-18.

Raijmakers, R., G. Schilders and G. J. Pruijn (2004). "The exosome, a molecular machine for controlled RNA degradation in both nucleus and cytoplasm." European journal of cell biology **83**(5): 175-83.

Raineri, I., D. Wegmueller, B. Gross, U. Certa and C. Moroni (2004). "Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference." Nucleic acids research **32**(4): 1279-88.

Ramos, S. B., D. J. Stumpo, E. A. Kennington, R. S. Phillips, C. B. Bock, F. Ribeiro-Neto and P. J. Blackshear (2004). "The CCCH tandem zinc-finger protein Zfp36l2 is crucial for female fertility and early embryonic development." Development (Cambridge, England) **131**(19): 4883-93.

Rankin, M. L., M. A. Heine, S. Xiao, M. D. LeBlanc, J. W. Nelson and P. J. DiMario (1993). "A complete nucleolin cDNA sequence from Xenopus laevis." Nucleic Acids Res **21**(1): 169.

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz and G. Ruvkun (2000). "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans." Nature **403**(6772): 901-6.

Rigby, W. F., K. Roy, J. Collins, S. Rigby, J. E. Connolly, D. B. Bloch and S. A. Brooks (2005). "Structure/function analysis of tristetraprolin (TTP): p38 stress-activated protein kinase and lipopolysaccharide stimulation do not alter TTP function." Journal of immunology (Baltimore, Md. **174**(12): 7883-93.

Ross, J. (1996). "Control of messenger RNA stability in higher eukaryotes." Trends Genet **12**(5): 171-5.

Rouault, T. and R. Klausner (1997). "Regulation of iron metabolism in eukaryotes." Curr Top Cell Regul **35**: 1-19.

Sachs, A. B. and J. A. Deardorff (1992). "Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast." Cell **70**(6): 961-73.

Saito, S., M. Miyaji-Yamaguchi, T. Shimoyama and K. Nagata (1999). "Functional domains of template-activating factor-I as a protein phosphatase 2A inhibitor." Biochem Biophys Res Commun **259**(2): 471-5.

Scheper, W., P. E. Holthuizen and J. S. Sussenbach (1996). "The cis-acting elements involved in endonucleolytic cleavage of the 3' UTR of human IGF-II mRNAs bind a 50 kDa protein." Nucleic Acids Res **24**(6): 1000-7.

Scheper, W., D. Meinsma, P. E. Holthuizen and J. S. Sussenbach (1995). "Long-range RNA interaction of two sequence elements required for endonucleolytic cleavage of human insulin-like growth factor II mRNAs." Mol Cell Biol **15**(1): 235-45.

Schmidlin, M., M. Lu, S. A. Leuenberger, G. Stoecklin, M. Mallaun, B. Gross, R. Gherzi, D. Hess, B. A. Hemmings and C. Moroni (2004). "The ARE-dependent mRNA-destabilizing activity of BRF1 is regulated by protein kinase B." The EMBO journal **23**(24): 4760-9.

Schmidt-Zachmann, M. S. and E. A. Nigg (1993). „Protein localization to the nucleolus: a search for targeting domains in nucleolin." J Cell Sci **105 (Pt 3)**: 799-806.

Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias and R. J. Ulevitch (1990). "Structure and function of lipopolysaccharide binding protein." Science **249**(4975): 1429-31.

Schwab, M. S. and C. Dreyer (1997). "Protein phosphorylation sites regulate the function of the bipartite NLS of nucleolin." Eur J Cell Biol **73**(4): 287-97.

Seal, R., R. Temperley, J. Wilusz, R. N. Lightowlers and Z. M. Chrzanowska-Lightowlers (2005). "Serum-deprivation stimulates cap-binding by PARN at the expense of eIF4E, consistent with the observed decrease in mRNA stability." Nucleic acids research **33**(1): 376-87.

Sengupta, T. K., S. Bandyopadhyay, D. J. Fernandes and E. K. Spicer (2004). "Identification of nucleolin as an AU-rich element binding protein involved in bcl-2 mRNA stabilization." The Journal of biological chemistry **279**(12): 10855-63.

Serin, G., G. Joseph, C. Faucher, L. Ghisolfi, G. Bouche, F. Amalric and P. Bouvet (1996). "Localization of nucleolin binding sites on human and mouse pre-ribosomal RNA." Biochimie **78**(6): 530-8.

Shaw, G. and R. Kamen (1986). "A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation." Cell **46**(5): 659-67.

Shyu, A. B., J. G. Belasco and M. E. Greenberg (1991). "Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay." Genes & development **5**(2): 221-31.

Shyu, A. B. and M. F. Wilkinson (2000). "The double lives of shuttling mRNA binding proteins." Cell **102**(2): 135-8.

Sirenko, O. I., A. K. Lofquist, C. T. DeMaria, J. S. Morris, G. Brewer and J. S. Haskill (1997). "Adhesion-dependent regulation of an A+U-rich element-binding activity associated with AUF1." Mol Cell Biol **17**(7): 3898-906.

Skalweit, A., A. Doller, A. Huth, T. Kahne, P. B. Persson and B. J. Thiele (2003). „Posttranscriptional control of renin synthesis: identification of proteins interacting with renin mRNA 3'-untranslated region." Circulation research **92**(4): 419-27.

Srivastava, M., P. J. Fleming, H. B. Pollard and A. L. Burns (1989). "Cloning and sequencing of the human nucleolin cDNA." FEBS Lett **250**(1): 99-105.

Srivastava, M., O. W. McBride, P. J. Fleming, H. B. Pollard and A. L. Burns (1990). "Genomic organization and chromosomal localization of the human nucleolin gene." J Biol Chem **265**(25): 14922-31.

Steer, J. H., K. M. Kroeger, L. J. Abraham and D. A. Joyce (2000). "Glucocorticoids suppress tumor necrosis factor-alpha expression by human monocytic THP-1 cells by suppressing transactivation through adjacent NF-kappa B and c-Jun-activating transcription factor-2 binding sites in the promoter." The Journal of biological chemistry **275**(24): 18432-40.

Stoecklin, G., M. Colombi, I. Raineri, S. Leuenberger, M. Mallaun, M. Schmidlin, B. Gross, M. Lu, T. Kitamura and C. Moroni (2002). "Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover." The EMBO journal **21**(17): 4709-18.

Stoecklin, G., M. Lu, B. Rattenbacher and C. Moroni (2003). „A constitutive decay element promotes tumor necrosis factor alpha mRNA degradation via an AU-rich element-independent pathway." Molecular and cellular biology **23**(10): 3506-15.

Stoecklin, G., T. Mayo and P. Anderson (2006). "ARE-mRNA degradation requires the 5'-3' decay pathway." EMBO Rep **7**(1): 72-7.

Stoecklin, G., P. Stoeckle, M. Lu, O. Muehlemann and C. Moroni (2001). "Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements." RNA (New York, N.Y **7**(11): 1578-88.

Stoecklin, G., T. Stubbs, N. Kedersha, S. Wax, W. F. Rigby, T. K. Blackwell and P. Anderson (2004). "MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay." The EMBO journal **23**(6): 1313-24.

Suswam, E. A., L. B. Nabors, Y. Huang, X. Yang and P. H. King (2005). "IL-1beta induces stabilization of IL-8 mRNA in malignant breast cancer cells via the 3' untranslated region: Involvement of divergent RNA-binding factors HuR, KSRP and TIAR." International journal of cancer **113**(6): 911-9.

Suzuki, N., S. Suzuki, G. S. Duncan, D. G. Millar, T. Wada, C. Mirtsos, H. Takada, A. Wakeham, A. Itie, S. Li, J. M. Penninger, H. Wesche, P. S. Ohashi, T. W. Mak and W. C. Yeh (2002). "Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4." Nature **416**(6882): 750-6.

Symmons, M. F., G. H. Jones and B. F. Luisi (2000). "A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation." Structure **8**(11): 1215-26.

6.6 T-Z

Takagi, M., M. J. Absalon, K. G. McLure and M. B. Kastan (2005). "Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin." Cell **123**(1): 49-63.

Takeuchi, O., K. Takeda, K. Hoshino, O. Adachi, T. Ogawa and S. Akira (2000). "Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades." Int Immunol **12**(1): 113-7.

Taylor, G. A., E. Carballo, D. M. Lee, W. S. Lai, M. J. Thompson, D. D. Patel, D. I. Schenkman, G. S. Gilkeson, H. E. Broxmeyer, B. F. Haynes and P. J. Blackshear (1996). "A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency." Immunity **4**(5): 445-54.

Tebo, J. M., S. Datta, R. Kishore, M. Kolosov, J. A. Major, Y. Ohmori and T. A. Hamilton (2000). "Interleukin-1-mediated stabilization of mouse KC mRNA depends on sequences in both 5'- and 3'-untranslated regions." J Biol Chem **275**(17): 12987-93.

Tenenbaum, S. A., P. J. Lager, C. C. Carson and J. D. Keene (2002). "Ribonomics: identifying mRNA subsets in mRNP complexes using antibodies to RNA-binding proteins and genomic arrays." Methods **26**(2): 191-8.

Thompson, M. J., W. S. Lai, G. A. Taylor and P. J. Blackshear (1996). "Cloning and characterization of two yeast genes encoding members of the CCCH class of zinc finger proteins: zinc finger-mediated impairment of cell growth." Gene **174**(2): 225-33.

Tong, C. G., S. Reichler, S. Blumenthal, J. Balk, H. L. Hsieh and S. J. Roux (1997). "Light regulation of the abundance of mRNA encoding a nucleolin-like protein localized in the nucleoli of pea nuclei." Plant Physiol **114**(2): 643-52.

Tracey, K. J. and A. Cerami (1994). "Tumor necrosis factor: a pleiotropic cytokine and therapeutic target." Annual review of medicine **45**: 491-503.

Tran, H., M. Schilling, C. Wirbelauer, D. Hess and Y. Nagamine (2004). "Facilitation of mRNA deadenylation and decay by the exosome-bound, DExH protein RHAU." Mol Cell **13**(1): 101-11.

Trede, N. S., A. V. Tsytsykova, T. Chatila, A. E. Goldfeld and R. S. Geha (1995). "Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B." Journal of immunology (Baltimore, Md. **155**(2): 902-8.

Tucker, M., M. A. Valencia-Sanchez, R. R. Staples, J. Chen, C. L. Denis and R. Parker (2001). "The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae." Cell **104**(3): 377-86.

van der Giessen, K., S. Di-Marco, E. Clair and I. E. Gallouzi (2003). "RNAi-mediated HuR depletion leads to the inhibition of muscle cell differentiation." J Biol Chem **278**(47): 47119-28.

van Dijk, E. L., J. S. Sussenbach and P. E. Holthuizen (2001). "Kinetics and regulation of site-specific endonucleolytic cleavage of human IGF-II mRNAs." Nucleic Acids Res **29**(17): 3477-86.

van Hoof, A., P. A. Frischmeyer, H. C. Dietz and R. Parker (2002). "Exosome-mediated recognition and degradation of mRNAs lacking a termination codon." Science **295**(5563): 2262-4.

van Rij, R. P., M. C. Saleh, B. Berry, C. Foo, A. Houk, C. Antoniewski and R. Andino (2006). "The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster." Genes Dev **20**(21): 2985-95.

Varnum, B. C., R. W. Lim, V. P. Sukhatme and H. R. Herschman (1989). "Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate." Oncogene **4**(1): 119-20.

Varnum, B. C., Q. F. Ma, T. H. Chi, B. Fletcher and H. R. Herschman (1991). "The TIS11 primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual Cys-His repeat." Mol Cell Biol **11**(3): 1754-8.

Vasudevan, S., S. W. Peltz and C. J. Wilusz (2002). "Non-stop decay--a new mRNA surveillance pathway." BioEssays **24**(9): 785-8.

Viriyakosol, S. and T. N. Kirkland (1996). "The N-terminal half of membrane CD14 is a functional cellular lipopolysaccharide receptor." Infect Immun **64**(2): 653-6.

von Lindern, M., S. van Baal, J. Wiegant, A. Raap, A. Hagemeijer and G. Grosveld (1992). „Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene." Mol Cell Biol **12**(8): 3346-55.

Waggoner, S. and P. Sarnow (1998). "Viral ribonucleoprotein complex formation and nucleolar-cytoplasmic relocalization of nucleolin in poliovirus-infected cells." J Virol **72**(8): 6699-709.

Wagner, B. J., C. T. DeMaria, Y. Sun, G. M. Wilson and G. Brewer (1998). "Structure and genomic organization of the human AUF1 gene: alternative pre-mRNA splicing generates four protein isoforms." Genomics **48**(2): 195-202.

Wang, W., M. C. Caldwell, S. Lin, H. Furneaux and M. Gorospe (2000a). "HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation." Embo J **19**(10): 2340-50.

Wang, W., H. Furneaux, H. Cheng, M. C. Caldwell, D. Hutter, Y. Liu, N. Holbrook and M. Gorospe (2000b). "HuR regulates p21 mRNA stabilization by UV light." Mol Cell Biol **20**(3): 760-9.

Wang, X., M. Kiledjian, I. M. Weiss and S. A. Liebhaber (1995). "Detection and characterization of a 3' untranslated region ribonucleoprotein complex associated with human alpha-globin mRNA stability." Molecular and cellular biology **15**(3): 1769-77.

Wang, Z., N. Day, P. Trifillis and M. Kiledjian (1999). "An mRNA stability complex functions with poly(A)-binding protein to stabilize mRNA in vitro." Molecular and cellular biology **19**(7): 4552-60.

Wang, Z. and M. Kiledjian (2000c). "The poly(A)-binding protein and an mRNA stability protein jointly regulate an endoribonuclease activity." Molecular and cellular biology **20**(17): 6334-41.

Wein, G., M. Rossler, R. Klug and T. Herget (2003). „The 3'-UTR of the mRNA coding for the major protein kinase C substrate MARCKS contains a novel CU-rich element interacting with the mRNA stabilizing factors HuD and HuR." Eur J Biochem **270**(2): 350-65.

Weischenfeldt, J., J. Lykke-Andersen and B. Porse (2005). „Messenger RNA surveillance: neutralizing natural nonsense.“ Curr Biol **15**(14): R559-62.

Wesche, H., W. J. Henzel, W. Shillinglaw, S. Li and Z. Cao (1997). “MyD88: an adapter that recruits IRAK to the IL-1 receptor complex.” Immunity **7**(6): 837-47.

Westmark, C. J., V. B. Bartleson and J. S. Malter (2005). “RhoB mRNA is stabilized by HuR after UV light.” Oncogene **24**(3): 502-11.

Whitfield, M. L., L. X. Zheng, A. Baldwin, T. Ohta, M. M. Hurt and W. F. Marzluff (2000). “Stem-loop binding protein, the protein that binds the 3’ end of histone mRNA, is cell cycle regulated by both translational and posttranslational mechanisms.” Mol Cell Biol **20**(12): 4188-98.

Wilson, G. M., J. Lu, K. Sutphen, Y. Suarez, S. Sinha, B. Brewer, E. C. Villanueva-Feliciano, R. M. Ysla, S. Charles and G. Brewer (2003). “Phosphorylation of p40AUF1 regulates binding to A + U-rich mRNA-destabilizing elements and protein-induced changes in ribonucleoprotein structure.” J Biol Chem **278**(35): 33039-48.

Wilson, G. M., J. Lu, K. Sutphen, Y. Sun, Y. Huynh and G. Brewer (2003). “Regulation of A + U-rich element-directed mRNA turnover involving reversible phosphorylation of AUF1.” J Biol Chem **278**(35): 33029-38.

Wilusz, C. J., M. Wormington and S. W. Peltz (2001). “The cap-to-tail guide to mRNA turnover.” Nature reviews **2**(4): 237-46.

Winzen, R., G. Gowrishankar, F. Bollig, N. Redich, K. Resch and H. Holtmann (2004). “Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR.” Mol Cell Biol **24**(11): 4835-47.

Wodnar-Filipowicz, A. and C. Moroni (1990). “Regulation of interleukin 3 mRNA expression in mast cells occurs at the posttranscriptional level and is mediated by calcium ions.” Proc Natl Acad Sci U S A **87**(2): 777-81.

Xu, N., C. Y. Chen and A. B. Shyu (1997). “Modulation of the fate of cytoplasmic mRNA by AU-rich elements: key sequence features controlling mRNA deadenylation and decay.” Mol Cell Biol **17**(8): 4611-21.

Xu, N., C. Y. Chen and A. B. Shyu (2001). “Versatile role for hnRNP D isoforms in the differential regulation of cytoplasmic mRNA turnover.” Mol Cell Biol **21**(20): 6960-71.

Xu, N., P. Loflin, C. Y. Chen and A. B. Shyu (1998). “A broader role for AU-rich element-mediated mRNA turnover revealed by a new transcriptional pulse strategy.” Nucleic acids research **26**(2): 558-65.

Yamashita, A., T. C. Chang, Y. Yamashita, W. Zhu, Z. Zhong, C. Y. Chen and A. B. Shyu (2005). “Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover.” Nature structural & molecular biology **12**(12): 1054-63.

Yamashita, D. S. and R. A. Dodds (2000). “Cathepsin K and the design of inhibitors of cathepsin K.” Curr Pharm Des **6**(1): 1-24.

Yang, X. C., M. Purdy, W. F. Marzluff and Z. Dominski (2006). “Characterization of 3’hExo, a 3’ exonuclease specifically interacting with the 3’ end of histone mRNA.” J Biol Chem **281**(41): 30447-54.

Yang, Y., J. F. Chang, J. R. Parnes and C. G. Fathman (1998). “T cell receptor (TCR) engagement leads to activation-induced splicing of tumor necrosis factor (TNF) nuclear pre-mRNA.” The Journal of experimental medicine **188**(2): 247-54.

Yeilding, N. M. and W. M. Lee (1997). “Coding elements in exons 2 and 3 target c-myc mRNA downregulation during myogenic differentiation.” Mol Cell Biol **17**(5): 2698-707.

Yen, T. J., D. A. Gay, J. S. Pachter and D. W. Cleveland (1988). “Autoregulated changes in stability of polyribosome-bound beta-tubulin mRNAs are specified by the first 13 translated nucleotides.” Mol Cell Biol **8**(3): 1224-35.

Yu, D., M. Z. Schwartz and R. Petryshyn (1998). „Effect of laminin on the nuclear localization of nucleolin in rat intestinal epithelial IEC-6 cells.“ Biochem Biophys Res Commun **247**(1): 186-92.

Zaidi, S. H. and J. S. Malter (1995). “Nucleolin and heterogeneous nuclear ribonucleoprotein C proteins specifically interact with the 3’-untranslated region of amyloid protein precursor mRNA.” The Journal of biological chemistry **270**(29): 17292-8.

Zhang, H., F. A. Kolb, V. Brondani, E. Billy and W. Filipowicz (2002). “Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP.” The EMBO journal **21**(21): 5875-85.

7. Appendix

7.1 Figures and Table

Introduction:

Results:

Discussion:

Material and Methods:

7.2 Curriculum vitae

Person:

Bernd Rattenbacher
born on the December 29th 1974
married, no children
Dipl. Biologist

Education

1981-85	Primary school in Mering
1985-94	Gymnasium bei St. Anna, Augsburg
1994-96	Rudolf-Diesel-Gymnasium, Augsburg Finished school with the Abitur

Military Service

07/01/1996-04/31/1997	During my military service I worked as a navigation mechanic at the electronic group at the JaBoG 32 in Lagerlechfeld. I had extra training in first aid and pyrtotechnics

University:

11/1/97 to 8/13/01	Education in Biology at the LMU-Munich Oral exams in the subjects: zoology, biochemistry, immunology and microbiology
11/13/2000-8/13/2001	Diploma thesis in biochemistry at the Genecenter and Institute for Biochemistry at the LMU Munich, in Prof. Rudolf Grosschedl's laboratory Title: Post transcriptional regulation of Lef-1 Finished my diploma with the mark of 1.3
04.03.2002-02.2007	PhD thesis in biochemestry at the Institute for Medical Microbiology at the University of Basel in Prof. Christoph Moroni's laboratory Title: Mechanisms of CDE-dependent mRNA decay

Research experience:

08/01/1994-08/20/1994	Practical course at the clinic in Großhadern, Munich in Dr. Wolfgang Gebhard's laboratory
09/01/1997-10/31/1997 03/02/1998-04/09/1998 03/01/1999-05/31/1999	Practical courses at the Max-Planck-Institute for Biochemistry, Munich at Dr. Axel Ullrich's laboratory
08/01/1998-09/30/1998	Practical courses at Virgene Pharmaceuticals AG, Munich
11/01/1998-03/01/1999	Practical courses at Virgene Pharmaceuticals AG, Munich
08/01/1999-08/31/1999	Practical courses at Virgene Pharmaceuticals AG, Munich
09/17/2001-02/28/2002	Junior scientist at Axxima Pharmaceuticals AG, Munich
05/08/2000-08/07/2000	Practical course at the Genecenter, Munich, in Prof. Rudolf Grosschedl's laboratory

Publications:

Papers:	Stoecklin G., Lu M., Rattenbacher B., and Moroni C.: A Constitutive Decay Element Promotes Tumor Necrosis Factor Alpha mRNA Degradation via an AU-Rich Element-Independent Pathway. Mol. Cell. Biol. 2003, 23:3506-3515
Posters:	Meeting of the RNA society, Vienna, 2003 Title: Identification and purification of a TNFa Constitutive Decay Element (CDE) binding protein.
	Meeting of the RNA society, Madison Wisconsin, 2004 Title: Nucleolin recognizes the TNFa Constitutive Decay Element (CDE).
	Meeting of the RNA society, Banff, 2005 Title: TNFa Constitutive Decay Element (CDE) induces rapid deadenylation.
	EMBO Workshop on RNA turnover, Arolla, 2005 Title: Nucleolin is binding the TNFa-Constitutive Decay Element.
	Meeting of the RNA society, Seattle, 2006 Title: A Possible Role for Nucleolin in CDE-mediated TNFa mRNA Decay.

Other qualifications	
Languages:	German, English and French
Others:	Knowlege of MS-Word, Excel, Powerpoint, Adobe Photoshop, Illustrator, In Design coach for judo referee for judo
Hobbies and private interests:	judo and chess

7.3 Declaration

I declare that I wrote this thesis „Mechanisms of CDE-dependent mRNA decay“ with the help indicated and only handed it in to the faculty of science of the University of Basel and to no other faculty and no other university.

Bernd Rattenbacher