The RNA secondary structure dependence of RNA-protein interactions and its implications for the post transcriptional regulation of gene expression

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Jörg Hackermüller

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This thesis presents a theoretical and computational analysis of RNA-protein interactions. I am convinced that biology can greatly benefit from theoretical concepts and computational techniques. Nevertheless, I think that best achievements will be made if theoretical, computational and experimental biology interact strongly and collaborate seamlessly. I was in the lucky position to work in such a collaboration. Nicole-Claudia Meisner's experiments delivered the data which were a prerequisite for computational predictions and allowed to verify these predictions eventually. Primarily, I am however grateful to her for numerous fruitful discussions and the excellent teamwork.

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Abstract

Controlled and specific recognition of RNA by ligands (especially proteins) is of great importance for many cellular processes, particularly in post transcriptional regulation of gene expression. RNA-ligand binding often depends crucially on the local RNA secondary structure at the binding site. We develop here a model that quantitatively predicts the effect of RNA secondary structure on effective RNA-ligand binding activities based on equilibrium thermodynamics and the explicit computations of partition functions for the RNA structures. A statistical test for the impact of a particular structural feature on the binding affinities follows directly from this approach. The formalism is extended to describing the effects of hybridizing small "modifier RNAs" to a target RNA molecule outside its ligand binding site.

We apply the developed methods to suggest a solution for an important unsolved question in AU-rich element (ARE) dependent regulation of mRNA stability. This pathway seems to be responsible for the accurate regulation of several thousand genes. While several negative regulators have been identified for this system, there is only a single, ubiquitously expressed protein known that upon binding stabilizes mRNAs in a highly stimulus and target specific manner, *HuR*. How the high level of specificity observed in mRNA stabilization by *HuR* is ensured is fundamentally unclear.

We derive an RNA sequence/structure motif for *HuR* binding from experimental *HuR*-RNA affinity data and show how modifier RNAs can be used to manipulate *in vitro* and endogenous *HuR*–mRNA association. Finally, we demonstrate the effectiveness and specificity of modifier RNAs for regulating *HuR* dependent mRNA stability in lysates of human peripheral blood mononuclear cells. We discuss our model and recent experimental findings demonstrating the effectivity of modifier RNAs in the context of the current research activities in the field of non-coding RNAs. We speculate that modifier RNAs might also exist in nature; if so, they present an additional regulatory layer for fine-tuning gene expression that could evolve rapidly, leaving no obvious traces in the genomic DNA sequences. Finally, we discuss the potential of modifier RNAs for applications in drug discovery and as tools in experimental biology.

Zusammenfassung

Die kontrollierte und spezifische Erkennung von RNAs durch Liganden, vor allem durch Proteine, ist für viele zelluläre Prozesse, insbesondere für die post–transkriptionale Regulation der Genexpression, von großer Bedeutung. Die Bindung eines Liganden an eine RNA ist in vielen Fällen von der Ausbildung einer bestimmten lokalen RNA Sekundärstruktur an der Bindungsstelle des Liganden abhängig. In dieser Arbeit wird die Entwicklung von Methoden beschrieben, die, basierend auf Gleichgewichtsthermodynamik und der expliziten Berechnung von Zustandssummen für RNA Strukturen, eine Vorhersage von effektiven RNA Ligand Affinitäten erlauben. Ein statistisches Testverfahren zur Bestimmung einer RNA Struktureigenschaft die für die Bindung eines bestimmten Liganden notwendig ist, baut direkt auf diesen Methoden auf. Wir erweitern den Formalismus um die quantitative Beschreibung des Effekts einer Hybridisierung zwischen RNA und "modifizierenden" RNAs außerhalb der Ligandenbindungsstelle auf die effektiven Affinitäten zwischen Ligand und RNA.

Wir verwenden die entwickelten Methoden, um eine Lösung für eine wichtige offene Frage in der Regulation der mRNA Stabilität durch AU-reiche Elemente vorzuschlagen. Diese Elemente dürften für eine präzise Regulation mehrerer tausend Gene verantwortlich sein. Während mehrere negativ regulierende Proteinliganden für diese Elemente beschrieben wurden, scheint es nur ein einziges ubiquitär exprimiertes Protein zu geben, das mRNAs durch Bindung an AU-reiche Elemente sehr spezifisch stabilisiert – *HuR*. Wie dieser hohe Grad an Spezifität in der Stabilisierung mehrerer tausend RNAs durch *HuR* ermöglicht wird ist ungeklärt.

Wir bestimmen ein RNA Sequenz/Struktur-Motif für die *HuR* Bindung und zeigen wie modifizierende RNAs verwendet werden können, um *in vitro* und zwischen endogener RNA und *HuR* die Komplexbildung zu manipulieren. In zellulären Lysaten können modifizierende RNAs effektiv und spezifisch für die Regulation der mRNA Stabilität verwendet werden.

Wir diskutieren den Zusammenhang unseres Modells und der experimentellen Ergebnisse mit aktuellen Forschungsaktivitäten im Bereich der nicht

proteinkodierenden RNAs und spekulieren, ob modifizierende RNAs auch natürlich vorkommen könnten. Wenn das der Fall ist, stellen modifizierende RNAs einen zusätzlichen Mechanismus für die Feinabstimmung der Genexpression dar, der rasch evolvieren könnte, ohne offensichtliche Signale in genomischen DNA Sequenzen zu hinterlassen. Letztendlich erörtern wir das Potential von modifizierenden RNAs in Anwendungen in der pharmazeutischen Forschung und als Werkzeug für die experimentelle Biologie.

Contents

Chapter 1

Introduction

Probably one of the most surprising findings of the genome sequencing projects [352, 200] was that the number of genes in the human and in the mouse genomes are roughly equal and that human and mouse genomes share about 99% of the protein coding genes, i.e. only several hundred human genes are unique [276, 237]. Genomic sequences of human and chimpanzee genomes differ only at about one percent of the nucleotides [188] and 99% of these differences are found in non-protein coding regions. Finally, cells of the same organism may appear and act so differently as neuronal cell and macrophage though sharing the identical genetic information. Obviously, it is not the genes themselves that makes us what we are but rather their regulation. Regulation not only determines the timepoint and extent a gene is expressed, it acts also on the information a gene contains and increases diversity of gene products by processes like alternative splicing or RNA editing.

Traditionally, the understanding of the regulation of gene expression has been reduced to mechanisms which switch on and off transcription [267]. Transcriptional regulation of gene expression is strongly related to the controlled interaction between proteins (transcription factors) and DNA, which was studied with intensity.

In the last decade it became however evident that many, if not the majority, of genes are regulated post-transcriptionally [151]. A plethora of different mechanisms modify the primary transcripts on their way to the protein (or non-coding RNA) that they eventually code for (see e.g. [109, 268] for recent reviews). Most of these control mechanisms involve specific RNA-protein interactions. Unlike genomic DNA, RNA is structurally diverse. Thus, unlike specific protein-DNA interactions, which rely predominantly on a direct readout of the sequence information, RNA-protein interactions depend crucially on the recognition of sequence and/or structural features of the RNA [146]. Examples of such RNA-protein interactions include the regulation of

viral life-cycles [27, 54, 195, 295], pre-mRNA processing [179], nuclear RNA export [79], and the control of RNA degradation [30] and stabilization [244].

An increasing number of functional features has been identified in eukaryotic mRNA, particularly in the untranslated regions [247]. Many of these motifs – often conserved between species – are required for specific and controlled mRNA-ligand interactions. Well-known motifs include internal ribosomal entry sites (IRES) in viral as well as cellular mRNAs, see e.g. [164], the *Rho*-independent termination signal (see [86] for a detailed computational analysis), or the iron responsive element (IRE) [154]. RNA secondary structure motifs are necessary in all these examples to enable the ligand to recognize (degenerate) sequence motifs. In some cases it is known that RNA secondary structure motifs without sequence constraints are recognized by regulatory proteins, see e.g. [246].

Despite the fact that the experimental findings summarized above (and many others not cited here) clearly indicate a pivotal role of RNA structure — and hence of the thermodynamics of RNA folding — in RNA ligand interactions, this topic has not yet been investigated systematically. In this contribution we derive a quantitative model for the effect of RNA secondary structure on RNA-ligand binding. We then use this theory to devise a statistical test for the involvement of specific RNA secondary structure features in RNA-ligand binding.

Given the importance of RNA secondary structure motifs for numerous regulatory RNA-ligand interactions – particularly in post transcriptional regulation of gene expression, alterations of RNA secondary structure open an interesting perspective for biotechnology. The effects of mRNA secondary structure modification on bacterial translation, for instance, are studied in [273]. Inhibition of ribozymes by means of oligonucleotide directed RNA misfolding has been demonstrated e.g. for group I introns and RNase P [100]. Oligomeric nucleic acid analogs were recently used to specifically inhibit IRES-dependent translation in hepatitis C virus [266], presumably by interfering with the IRES structure.

We therefore expand our theoretical framework to modeling the modification of the RNA secondary structure by means of hybridization of small oligonucleotides – modifier RNAs – outside the ligand binding motif. A recent study shows that such a mechanism is feasible *in vitro*: Isaacs *et al.* [168] demonstrate that translation of mRNAs that are not translatable because their ribosome binding site is inaccessible due to stable secondary structures can be activated by means of small artificial "transactivating RNAs". A related model system is described in ref. [379].

Over the recent years, a rapidly increasing number of different classes of non-coding RNAs has been identified that actively take part in mRNA pro-

cessing and expression regulation [159]: RNAseP, MRP RNA, spliceosomal RNA, signal recognition particle RNAs, and microRNAs all perform their function as part of RNA-protein complexes. In addition, there is a number of RNA-protein complexes, such as vaults [350] and Ro particles [341] that have been known for decades, but whose function has remained enigmatic so far. We propose that modifier RNAs might also occur naturally and might constitute another class of regulatory non-protein coding RNAs. We briefly discuss the impact of such an additional regulatory mechanism on our understanding of gene regulation and its evolution.

Post transcriptional regulation is particularly important for the controlled expression of genes that are regulated on a very short time-scale, e.g. genes involved in the early response to inflammatory stimuli, or genes that are critical for proper cell function like proto-oncogenes. A pathway that controls the expression of genes, particularly interesting for drug discovery applications, is the AU-rich element (ARE) dependent control of mRNA stability. These mRNA elements increase or decrease mRNA stability by interaction with stabilizing or destabilizing RNA binding proteins. While several destabilizing proteins have been identified, only one ubiquitously expressed positive regulator has been reported, *HuR*. An unsolved question is, how *HuR* can promote stimulus and target specific stabilization of potentially several thousand ARE target mRNAs.

We use the developed computational methods to define a sequence-structure RNA binding motif for *HuR* from experimental affinity data. We further design modifier RNAs for cytokine target mRNAs of *HuR* to modulate *HuR*-RNA binding. These modifier RNAs are validated experimentally by monitoring their effect on *HuR*-RNA association and on transcript stability. Finally, we propose a solution for the open question of specificity in *HuR* dependent mRNA stability control and discuss potential applications of modifier RNAs in experimental biology and drug discovery.

Chapter 2

RNA-protein interactions and post transcriptional regulation

2.1 Post transcriptional regulation of gene expression

In the following sections, we will briefly describe the various levels of post transcriptional regulation during gene expression. It is important to note, that though the term "levels" suggests some sequential order among those processes, they cannot be separated reliably on time scale. Rather, these processes are heavily connected and occur partially concomitantly [382]. The following enumeration of post transcriptional processes is not meant to be exhaustive. Rather, we aim to coarsely "position" the processes described in more detail in this work in the complex network of post transcriptional regulation.

2.1.1 Alternative splicing

Eukaryotic organisms - in contrast to prokaryotes - have their genes interspersed with sequence fragments which are not present in the mature transcripts of genes. These missing fragments are called *introns*, the sequence fragments which correspond to the mRNA are named *exons*. Pre-mRNA *splicing* is the process of intron removal. The intron exon structure of genes was originally discovered in *Adenovirus hexon* gene [306] and Walter Gilbert predicted that different combinations of exons could be spliced together to produce mRNA isoforms of a gene [129]. The questions of age, origin and purpose of introns have been the cause of vivid discussions in the community and are still far from being solved [130, 221, 90]. The presence of highly

conserved regions in introns, which are not necessarily involved in the regulation of splicing, indicates that introns are more than neutrally evolving trash sequences [26, 76].

Gilbert's speculation was finally very relevant. Splicing is indeed not only a constitutive process in gene expression. During splicing, alternative spice sites may be used, leading to shortened or elongated exons or entire exons may be skipped. While some variations in splicing are due to aberrant splice sites (particularly frequently occurring in tumor cell lines) the majority of splice variants is due to a highly regulated process, termed *alternative splicing*. The process of alternative splicing was originally identified in myoglobin [103, 7, 11] and it was considered a rather exotic phenomenon. Traditionally, alternative splicing has been thought to play a role for about 5% of the human genes [319]. Today, alternative splicing is understood to be involved in the expression of 40% to 60% of the genes in the human genome [252, 41, 177, 249], however, in a survey based on 700 expressed sequences, a fraction of 99% has been detected [253]. Consequently, alternative splicing is a very important biological process, particularly in development and cell differentiation. The regulation of alternative splicing is increasingly but not yet fully understood [323, 52].

2.1.2 RNA editing

The removal of introns is not the only observed difference between genomic and mRNA sequence. All modifications of RNA that change its coding capacity and are different from splicing, capping and 3' processing (poly adenylation, degradation) are summarized as *RNA editing*. Originally, RNA editing referred to the process of inserting or deleting uridines in mRNAs in mitochondria of kinetoplastid *protozoa* [28]. In its current understanding, RNA editing means the insertion, deletion or modification of nucleotides in mRNAs [184]. The processes investigated at most detail are C to U editing in mammals [64] and A to I editing in higher eukaryotes [210] - where I is read as a G by the translation machinery. RNA editing is strongly connected with other post transcriptional mechanisms, e.g. edits ADAR2, an adenosine deaminase, splice sites in its own pre-mRNA to regulate alternative splicing [297]. Maybe the most important process from a biomedical perspective involving RNA editing is antibody isotype switching in B cells. This class switch recombination is ultimately dependent on a cytidine deaminase, which is induced *in vitro* by *IL4*, *LPS* and *CD40L* [353]. A bioinformatic analysis of RNA editing sites is presented in [46].

2.1.3 Capping and poly-adenylation

Eukaryotic mRNAs are marked at their 5' ends by the addition of a methylated guanosine *cap*. Precisely, all RNA polymerase II transcripts (those include all protein coding mRNAs and a significant part of functional RNAs) are equipped with a cap which is necessary for sufficient cytoplasmic stability and initiation of translation (unless the mRNA does not form an internal ribosomal entry site - IRES), stimulates splicing, 3' end formation and nuclear export[214]. The capping process occurs co-transcriptionally and is dependent on RNA polymerase II. However, also the transcriptional elongation is dependent on capping activity – this seems to act as a checkpoint that holds up elongation until the nascent transcript has been capped [382].

Nearly all mature eukaryotic mRNAs exhibit a tail of poly adenosine at their 3' end. Analogously to the cap this *poly(A) tail* improves nuclear export and translation efficiency and is a major determinant of mRNA stability (see below). An enzymatic activity for the formation of poly(A) from adenosine triphosphate (ATP) has been found in thymus nuclei [105] long before the first demonstration of poly-adenylated mRNA sequences[106, 85]. The poly(A) tail is formed in conjunction with transcription termination by a large enzymatic machinery. Briefly, the nascent mRNA is cut between the conserved signal `AAUAAA` and G/U-rich sequence elements and the poly(A) stretch is transferred to the mRNA. Though it is possible to separate cleavage and poly(A) addition *in vitro*, it is believed, that both processes occur tightly coupled *in vivo* [71]. Like capping, poly-adenylation activity is required for successful transcription. This seems to ensure that the transcription machinery is only released from the template after synthesis of a full length mRNA [382].

Many mRNAs contain several poly-adenylation sites which allow *alternative poly(A) site usage*. This process, analogously to alternative splicing, leads to the formation of mRNA isoforms with different lengths of 3' untranslated regions (UTRs). 3' UTRs carry many functional elements important for post transcriptional regulation, which can be in- or excluded by alternative poly(A) site usage. Alternative splicing does only interfere with alternative poly-adenylation if alternative terminal exons exist. Within 3'UTRs alternative splicing occurs rarely, as only 1% to 10% of eukaryotic genes contain introns in 3' UTRs [279]. How the poly(A) site is selected remains elusive, it has been proposed recently that the speed of transcriptional elongation determines the poly(A) site [78].

2.1.4 Nuclear export

Nuclear export of mRNAs occurs through large structures embedded in the nuclear membranes, called nuclear pore complexes (NPC). Presumably, NPCs are the gates to and from the nucleus for all cellular macromolecules. mRNAs are not transported nakedly through the pore complex, but as messenger ribo-nucleoprotein (mRNP). Transport of macromolecules through NPCs requires the binding of export factors which are specific for the type of cargo they transport. mRNAs are transported by members of the evolutionary conserved family of NXF proteins [172] or NXT1 (p15) [157]. These proteins form hetero-dimers, but not all metazoan mRNAs require both factors for export. Nuclear export seems to be dependent on most other post transcriptional regulation mechanisms occurring in the nucleus. Splicing seems to stimulate the export of certain mRNAs [224], in yeast the assembly of export factors occurs co-transcriptionally [208]. 3' end processing seems to be a prerequisite for nuclear export [217], however, mRNAs retained in the nucleus appeared to be hyper-adenylated [176]. A connection with mRNA stability regulation is indicated by the finding that deleting a component of the nuclear exosome, can partially reduce the retention of mRNA in the nucleus [381, 217].

2.1.5 mRNA stability

A prerequisite of an effective regulation of gene expression is the instability of mRNA. Regulatory processes upstream of translation control would not be effective for any processes that have a shorter response time than the average lifetime of the regulated RNA species. mRNAs are rather unstable compared to other biomacromolecules like proteins or DNA. However, there is a huge diversity in transcript stabilities, ranging from a few minutes to many hours. The stability of a particular mRNA species reflects its function and the time characteristics of processes this species is involved in. Transcripts of highly expressed genes (e.g. β-globin [299]) or transcripts where translation is delayed, like maternal mRNAs in oocytes [327] are highly stable. Contrary, mRNAs which are expressed as a fast response to external stimuli are degraded rapidly [311].

mRNA stability control is consequently a central and integrative level of post transcriptional regulation [143, 250]. The life time of an RNA molecule is influenced by the effectiveness of upstream processes, like pre-mRNA splicing, capping and poly-adenylation. It depends on various elements in the mRNA which are recognized by trans-acting factors. The stability of an mRNA is a function of the overall structure of the RNP, the mRNA has formed

during and after transcription. Early work on the stability of transcripts has been performed in *Xenopus* oocytes, studying the influence of the poly-A tail on RNA half-life [265]. Messenger RNA degradation and stabilization is dependent on many different cis-elements in the RNA and trans-acting factors. We will review here the most relevant and prominent ones and those where the molecular mechanism is well understood.

Constitutive pathways of transcript degradation

Two types of exonucleolytic activities are found in eukaryotic cells, 5' to 3' decay and 3' to 5' decay. 5' to 3' degradation is prevented by the cap which is incorporated in a stabilizing complex with *eIF4E* , a translation initiation factor [282]. 5' to 3' degradation following an endonucleolytic cleavage of the mRNA can, however, not be prevented by the cap.

Most eukaryotic mRNAs are poly-adenylated. 3' terminal stretches of poly(A) are bound by the highly expressed poly(A)-binding protein *PABP* [135], which protects the transcript from 3' to 5' decay *in vitro*. Degradation of mammalian mRNAs starts often by de-adenylation. Also, poly(A) facilitates translation and mRNA stability and translation are highly interconnected. These findings led to the conclusion that poly(A) and *PABP* play a key role in the prevention of transcript degradation.

Poly-adenylated transcripts that are actively translated are subject to poly(A) shortening, which is dependent on at least one poly(A) ribonuclease (PARN) in mammals. Such a de-adenylation is usually the initial step of the de-adenylation-dependent pathway of mRNA degradation [191]. In yeast, de-adenylation is followed by removal of the cap, which then results in both 5' to 3' and 3' to 5' exonucleolytic degradation [25]. In mammals, a similar pathway exists involving e.g. the human yeast homologue *Dcp2*, however, this pathway seems to be restricted to regulated decay [72]. The prevalent form of degradation appears to be 3' to 5' degradation. After de-adenylation, the transcript is degraded by the exosome, the remaining residual cap structure is hydrolyzed by the *DCpS* scavenger de-capping enzyme[185].

Regulated decay by de-adenylation and de-capping

Many determinants regulate the decay of transcripts. The decay is initiated either by endonucleolytic cleavage (see below) or by de-adenylation followed by de-capping. The exact interplay between poly(A) tail, cap and trans-acting factors is still enigmatic. However, it has been demonstrated recently, that *eIF4E* and *PABP* compete for binding to the cap. In presence of *eIF4E* the lower affinity binder *PABP* is displaced from the cap. However, *PABP*

may still interact with the cap indirectly by binding to *eIF4G*, which interacts with *eIF4E*. Both *eIF4E* and *PABP* inhibit de-capping in complex and individually [362]. It has been speculated that after translation and initial de-adenylation the *eIF4E–eIF4G–PABP* complex might be disrupted and *PABP* gains direct access to the cap. Removal of *eIF4E* would ensure no re-initiation of translation, while the transcript would still be protected from degradation at the site of translation [185]. De-capping would occur after transit to distinct foci of de-capping - which have been recently identified in yeast [322].

Degradation by endonucleolytic cleavage

For a number of eukaryotic mRNAs, degradation is known to be initiated by endonucleolytic cleavage independent of de-adenylation. Among those mRNAs are transcripts of insulin-like growth factor 2 [310], the transferrin receptor [32], *c-myc* [281], serum albumin [149], vitellogenin [80], α-globin [363] and *Xenopus* β-globin [38, 328]. There does not seem to be a great overlap among the various cleavage sites in these mRNAs, so that the existence of a number of specific endonucleases can be assumed [72]. After endonucleolytic cleavage, the 5' fragment is degraded by 3' to 5' decay, whether the 3' fragment is degraded by 5' to 3' activities or by the exosome is not resolved. Some of the known endonucleases are constitutively active and degradation initiation is controlled by the accessibility to the cleavage site [143]. Others, like the mammalian RNase L are directly regulated [216]. Endonucleolytic cleavage is also the prior mechanism of degradation induced by functional RNAs. These mechanisms are discussed in section 2.3.

RNA surveillance

A major function of RNA decay in eukaryotic cells is to provide a quality control system for the correct transcription, splicing and processing of mRNAs. The cell has developed several pathways for the rapid degradation of aberrant mRNAs summarized by the term *RNA surveillance*.

The probably best studied among these mechanisms is nonsense mediated decay (NMD): transcripts which contain premature translation termination codons are recognized and degraded. Studies performed in yeast on the degradation of PGK1 mRNAs with stop codons close to the 5' terminus revealed that degradation is initiated by de-adenylation independent de-capping [257]. In this "leaky surveillance" model, the probability that a premature stop codon causes degradation would decrease with the distance of the stop codon from the 5' terminus. More recent efforts to perform computational model-

ing of NMD, based on experimental data, could not corroborate the leaky surveillance model. Rather, all nonsense-containing transcripts are equally well recognized as aberrant but de-capping occurs with position dependent efficiency. Additionally, it was shown that NMD involves simultaneous de-capping and de-adenylation [50].

How are premature stop codons or frame shift mutations detected and where does detection and degradation occur? Premature stop codons can be detected either by finding a stop codon upstream of an intron or by an "initial round of translation". Stop codons upstream of introns can be detected also in the cytoplasm, because the intron position remains tagged after intron removal by the exon-exon-junction complex [203]. However, for highly expressed protein mRNAs that carry a dominant negative frame-shift or a premature stop codon, degradation in the cytoplasm might be too late. Also, there is some evidence for a co-transcriptional detection of aberrant mRNAs leading to accumulation of nuclear unspliced mRNA [256] or increased alternative splicing which skips the offending mutation [355]. Finally, studies suggest that there is a significant fraction of translation occurring in the nucleus [166, 230]. Based on these findings it has been recently proposed that aberrant transcripts might be detected in the nucleus by an initial translation followed leading to a nuclear variant of nonsense mediated decay [368].

An analogous, but mechanistically different surveillance mechanism has been identified in yeast that leads to the detection of "nonstop" transcripts, i.e. mRNAs lacking any stop codon [124]. The process seems to be initiated when the ribosome reaches the 3' terminus of the transcript. How degradation is initiated is not clear, probably the displacement of stabilizing factors from the 3'UTR and the disruption of the circular interaction between poly(A) tail and cap lead to decay. Nonstop associated degradation depends on the exosome and an exosome associated protein (*Ski7p*) that binds to an empty aminoacyl-RNA binding site of the ribosome [349].

Besides aberrantly spliced alternative transcripts which end up in NMD, improper alternative splicing is thought to result in two major forms of defects. Either, pre-mRNAs are unable to assemble into a spliceosome complex or they are defective in one of the two transesterification reactions. Surprisingly, the first are efficiently exported from the nucleus and are degraded in the cytoplasm by de-adenylation dependent 5' to 3' decay. The latter, lariat shaped RNAs, which are protected from degradation by the 2' to 5' branch structure, are removed by an endonuclease (*Dbr1p*). The linearized RNAs are exported from the nucleus and degraded in the cytoplasm by a 5' to 3' exonuclease (*Xrn1p*). In the absence of lariat debranching, the RNAs are degraded 3' to 5' by the cytoplasmic exosome [158].

Cis-elements and *trans*-acting factors controlling mRNA stability

Several sequence and/or structure elements that determine transcript stability have been identified in mRNAs. These elements are not localized to a particular region of mRNAs, rather they can be found throughout the body of transcripts.

Elements in the 5'UTR Stability elements in the 5'UTR are less common than those in the 3'UTR. IL-2 mRNA stability is not only determined by ARE elements in the 3'UTR but also by a *JNK*-response element (JRE) in the 5'UTR. Two RNA binding proteins, nucleolin and *YB-1* bind the JRE specifically, leading to stabilization upon T-cell activation [60]. *KC* chemokine mRNA, which also exhibits an ARE element in the 3'UTR, contains a 68nt region in the 5'UTR that is required for stabilization. Transacting factors interacting with this region to promote stabilization are unknown [340]. Otherwise unstable *c-myc* mRNA is stabilized in certain lymphoma and plasmacytoma cells by translocation of immunoglobulin introns into the *c-myc* 5'UTR [178]. Mitochondrial stability and translation of cytochrome b mRNA depends on the interaction with *Cbp1*, which binds to the triplet `CCG` in the 5'UTR [169]. Glucose dependent destabilization of the succinate dehydrogenase complex in yeast is conferred by the 5'UTRs of SDH1, SDH2 and SUC2 mRNAs [87, 222].

Several studies from eukaryotes and bacteria show, that 5'UTR secondary structure is an important determinant of stability. Nitrate reductase mRNA in *Chlorella vulgaris* [49], *aprE* leader RNA in *Bacillus subtilis* [147], *PapA* in *Escherichia Coli* [43] and a DNA gyrase mRNA in *Mycobacterium smegmatis* [347] are protected from degradation by formation of a hairpin structure in the 5'UTR.

Elements in the coding region A few examples are known where mRNA stability is determined by elements in the coding region. The destabilization of *c-myc* during differentiation has been attributed to a coding sequence element in exon 3 [372]. A protein has been identified that binds this region and protects *c-my* mRNA from endonucleolytic cleavage [281]. More recent studies indicate an involvement of elements in exons 2 and 3 in *c-myc* downregulation during myoblast differentiation [378].

c-fos mRNA contains two coding region determinants of mRNA stability, CRD-1 and CRD-2 [63, 312]. CRD-1 is bound by a complex of proteins, including well known RNA destabilizing proteins *Unr*, *PABP*, *PAIP-1*, *AUF-1* (p37 isoform), *NSAP1* [141]. A recent study suggests that interaction between *Unr*, *PABP* and CRD-1 recruits the poly(A) nuclease *CCR4*.

Upon translation initiation, *CCR4* can access the poly(A) tail and initiate degradation [59]. Additional destabilizing elements in coding regions have been reported for fushi tarazu mRNA in *Drosophila* [170], IL1-F7b and IL-18 mRNAs in human blood monocytes [45]. Stabilizing elements have been identified in the coding regions of *PGK1* and *TEF1/2* mRNAs [298].

Elements in the 3'UTR Most *cis*-acting mRNA stability elements have been identified in the 3'UTR. Also, some of the 3'UTR elements occur in many functionally different mRNAs and form classes of *cis*-acting elements.

Iron responsive element The *iron responsive element* (IRE) is a 30nt sequence forming a stable hairpin structure that is located in the 5'UTR of ferritin and in the 3'UTR of the transferrin receptor (*TfR*) [18, 153]. The IREs control the transcript stability in *TfR* and translation initiation in ferritin. Two closely related, transacting factors Iron Regulatory Protein (*IRP* 1 and 2) recognize the IRE [296, 305, 144]. Cellular iron homeostasis is maintained (*i*) controlling iron uptake into the cell by limiting transcript stability of *TfR* and (*ii*) modulating the intracellular iron sequestration by controlling the translation of ferritin, an iron storage protein. If iron concentration is low, *IRP1* and *IRP2* protect *TfR* mRNA from degradation [32] and repress the translation of ferritin [139]. When iron levels in the cell rise, *IRP1* is inactivated and *IRP2* is degraded, ferritin translation is increased and transferrin mRNA stability is downregulated.

Histone stem-loop motif Replication-dependent histone mRNAs are the only metazoan mRNAs that are not poly-adenylated. Instead they form a 3' terminal stem-loop structure [271]. Histone-expression is tightly regulated during cell cycle and stabilization by the stem-loop structure depends on DNA synthesis [137]. The stem-loop is recognized by the stem-loop binding protein (*SLBP*), which accompanies histone mRNA during nuclear export and in the cytoplasm and may be responsible for the stability regulation of replication-dependent histone mRNA [365].

Adenyl-uridyl-rich elements AU-rich elements are probably the most investigated and best understood *cis*-acting elements in mRNA stability. Because they are particularly relevant for the work presented, we have devoted a separate section for their introduction, section 2.4.

Additional 3'UTR elements involved in mRNA stability are CA repeats in *Bcl-2* [207], the K box in *Drosophila* E(spl)-C genes [194], a UC-rich

region in androgen receptor mRNA [377] and elements in GLUT1 [242, 284], *alphaCP* bound regions in alpha1(I) collagen mRNA [228, 229, 219].

Regulation of mRNA stability

Recent efforts aimed to detect regulation at the level of transcript stability using microarrays indicate, that stability regulation is a widely used process in mammalian cells [290, 140]. Only a fraction of the identified transcripts contain AU-rich elements. However, studies on the pathways regulating mRNA stability have been performed primarily on for AU-rich elements.

Mitogen activated decay of *c-myc* seems to depend on the nuclear localization of an endonuclease (*G3BP*), which is dependent on phosphorylation [343]. This finding seems to link mRNA decay and classic signal transduction pathways. An other example for a direct link between intracellular signaling cascades and mRNA stability is the regulation of exonucleases [216].

2.1.6 Translation initiation and post-translational regulation

Translation initiation is controlled globally affecting the translation of all mRNAs in the cell and at an mRNA specific level often in dependence of localization. Efficient initiation requires either a capped mRNA or the presence of an internal ribosomal entry site (IRES) and is a complex process which requires the assembly of a complex consisting of more than 25 factors. Cap dependent initiation requires the association of the poly(A) tail via poly(A) binding protein (*PABP*), *eIF4G* and *eIF4E* with the cap of the mRNA and the 43S pre-initiation complex of the ribosome. Initiation is further influenced by secondary structures in the mRNA and binding of proteins and ribonucleoproteins to the mRNA. Another class of *trans*-factors which control translation is constituted by miRNAs. The mechanism how miRNAs inhibit translation is largely unknown. As they do not alter mRNA association with polysomes, it seems they do not block initiation but elongation or termination of translation. We recommend [245, 128] as recent reviews on translation initiation.

Post-translational regulation of gene expression is complex and variegated and exceeds the scope of our work. It includes protein e.g. localization, modification and export, see e.g. [233, 318] for recent reviews.

Table 2.1: Major RNA binding motifs listed in *InterPro*. Protein counts, indicated by "#", as of 2004/9/23. Motifs where no common fold has been identified are marked as "ncd".

Domain name	Fold	#	InterproID
CCHC Zink finger domain	ncd	4582	IPR001878
RNA recognition motif	$\beta1\alpha\beta2\beta3\alpha\beta4$	3120	IPR003954
K homology domain	$\beta1\alpha1\alpha2\beta2\beta3\alpha3?$	947	IPR004087
double stranded RNA binding	$\alpha1\beta1\beta2\beta3\alpha2$	445	IPR001159
S1/IF1 type	OB	230	IPR006196
Rho termination factors	ncd	132	IPR011113
eIF-4G, middle domain	all α	128	IPR003890
Pumilo/Puf RNA binding	$(\alpha1\alpha2\alpha3)_8$	121	IPR001313

2.2 RNA-protein interactions

All the above described post transcriptionally regulating processes involve the specific interaction between RNA and proteins. Beyond post transcriptional regulation, RNA-protein interactions are particularly important for the formation of various snRNPs (which themselves play an important role in post transcriptional regulation), in viral life-cycle regulation [27, 54, 195, 295] and RNA-enzyme interactions (e.g. aminoacyl-tRNA synthetases [82, 14, 13, 92]). This section will review the major protein motifs involved in RNA recognition, common binding mechanisms of RNA protein interactions.

2.2.1 Common RNA binding protein motifs

RNA-protein interactions involve the highly specific recognition [146] of sequence and/or structure features. This is reflected by the set of known RNA recognition motifs, which include motifs recognizing single stranded RNA, double stranded RNA and both.

RNA recognition motif

The *RNA recognition motif* (RRM) – also called *RNA binding domain* (RBD) is one the most frequently found motifs that bind single-stranded RNA. Also, a few single stranded DNA binding proteins contain RRMs. The motif is part of many RNA binding proteins involved in post transcriptional regulation. The RRM is about 90 amino acids in length and acquires a compact, globular fold of a four stranded anti-parallel β-sheet interspersed by two α-helices

– $\beta1\alpha1\beta2\beta3\alpha2\beta4$. A single exception of the fold has only been found in polypyrimidin tract binding protein, where the second RRM folds into a beta sheet of five strands [74]. Characteristic for RRMs are two conserved hexameric respectively octameric sequence elements in $\beta2$ and $\beta3$, termed *RNP1* and *RNP2* respectively. Another characteristic trait are the two or three solvent exposed aromatic amino acids on the β-sheet surface [33].

Several proteins containing only one RRM are known, however, the majority of RRM proteins contains more than one of these domains. In such proteins, the binding affinity between an isolated RRM and a target RNA tends to be small. Also, the sequence specificity of isolated domains can be different from the specificity of the intact protein [47]. In many RRMs the residues in the loop between $\beta2$ and $\beta3$ are involved in RNA recognition. In proteins containing multiple RRMs, also the hinge region between individual RRMs is very important for specific RNA interaction [146, 6]. In several of these proteins, specific binding to the RNA induces the formation of a stable αhelix in the linker region that interacts with the RNA [95, 148, 6].

K-homology domain

The K-homology domain (KH domain) is another RNA recognition unit found frequently in proteins with various cellular functions. The domain is constituted by approximately 70 amino acids and folds also into an α/β fold: $\beta1\alpha1\alpha2\beta2\beta3\alpha3$ [212, 277]. The loop connecting helices $\alpha1$ and $\alpha2$ exposes a conserved Gly-X-X-Gly element (X represents glycine, arginine or lysin) which together with a more variable region in the loop linking $\beta2$ and $\beta3$ is thought to interact with the RNA. The binding mode of the KH domain is different from RRM and dsRBDs. In *Nova-2*, a single-stranded tetranucleotide contacts a hydrophobic platform built from helices $\alpha1$, $\alpha2$ and strand $\beta1$ and is gripped by the two loop elements mentioned above. Apparently, interaction between the KH domain and the RNA does not involve intermolecular stacking interactions (like in the RRM), nor specific 2'-OH contacts (like in dsRBDs)[213].

Double-stranded RNA binding motif

One motif is currently known that binds exclusively to dsRNA, the *double-stranded RNA binding domain* (dsRBD). dsRBD proteins are involved in diverse functions and are found in virtually all organisms [116]. The domain is best characterized in the adenosine deaminases (section 2.1.2), in *Xenopus laevis* RNA biding protein A (*Xlrbpa*)[300] in the *Drosophila melanogaster* staufen protein [48] and in *E. coli* RNAse III [186]. The dsRBM is an approx-

imately 70 amino acid, globular domain with an $\alpha 1 \beta 1 \beta 2 \beta 3 \alpha 2$ fold. In *staufen* and *Xlrbpa-2*, the second dsRBD of *Xlrbpa*, the two helices are positioned at one side of the three stranded antiparallel β-sheet.

Xlrbpa-2 interacts with two successive minor grooves and the intervening major groove on one side of the dsRNA helix over a length of 16bp. The interaction is primarily mediated by direct and water mediated hydrogen-bonds with 2'–OH groups of both RNA strands. The bound RNA forms a conformation close to the ideal A-form. dsRBD-RNA interactions are presumably sequence independent [300]. This is corroborated by the structure data, as most RNA-protein interactions involve RNA backbone 2'–OH groups and interactions with the minor grooves are often water mediated. However, some specificity may be caused by an indirect readout of the sequence information, e.g by the ability of the RNA to form the slight deviations form the A-form observed in the *Xlrbpa-2* structure.

Pumilo family RNA binding repeats

The pumilo protein family (PUF or Pum-HD family) is widespread among eukaryotes, but missing so far in prokaryotes. PUF proteins analyzed so far are involved in diverse processes, but all proteins share a common function in the maintenance of germline stem cells [77] and seem to bind to the 3' UTRs of mRNAs [366]. PUF proteins have been found to accelerate the de-adenylation of unstable mRNAs [345].

PUF proteins are characterized by the presence of eight consecutive Puf repeats, each approximately 40 amino acids in length. Each Puf repeat folds into a three helix domain, individual domains stack on one another to form a crescent, which covers one third of a 42Å radius circle [107, 361]. The conserved core amino acids of each repeat form helices which are arranged on the inner, concave side of the crescent, and maintain interactions with the RNA. The outer face of the crescents provides protein-protein interaction sites, e.g with *Nanos*, another protein involved in post transcriptional regulation.

Zinc fingers

Zinc finger (ZF) proteins constitute a large and very diverse set of nucleic acid binding proteins. Though mainly known as DNA binding proteins, e.g as transcription factors, ZF proteins do also bind RNA and play a substantial role in post transcriptional regulation. A zinc finger is a small peptide domain with a particular secondary structure stabilized by a zinc ion bound to the Cis and His residues of the finger. The plethora of ZF proteins can be classified by the residues used differentially for zinc complexation, where prominent

classes are C_2H_2, C_2HC, C_2C_2 or CCCH [171]. In the classical C_2H_2 ZF, the finger contains two to three β-strands followed by an α-helix.

Depending on the number of fingers, zinc fingers bind to DNA, RNA, protein or lipids, or to combinations of them [198]. In *multiple-adjacent-C_2H_2 zinc fingers*, like *TFIIIA* or *WT1* only a fraction of the fingers is occupied with DNA binding and the remaining fingers participate in RNA interactions [53, 223, 342]. This led to the speculation that such proteins regulate gene expression both on transcriptional and post transcriptional levels. Another C_2H_2 ZF protein, *dsRBP-ZFa* binds exclusively to dsRNA and RNA-DNA hybrids [287]. Other ZF classes which bind to RNA are the CCHC class [274] and most importantly the CCCH proteins which are involved in mRNA stability regulation [196, 34].

2.2.2 Binding mechanisms

The foundations for understanding molecular recognition have been laid long ago, by Emil Fischer's proposition of lock and key mechanisms [119]. However, the perception of molecules as rigid bodies is of limited use for understanding biomolecules. The concept of *induced fit* has been developed to understand enzyme substrate interactions, where the catalytic site of the enzyme forms an active conformation only when the substrate is bound. In the case of RNA protein interactions, both interaction partners have a high conformational flexibility.

The concept of a folding funnel can be adopted for RNA-protein interactions [225]. A smooth funnel with a deep minimum corresponds to a rigid interface in contrast to a rather wide funnel with a rugged bottom and several minima. In the latter case, binding might occur by "conformational capture": one interaction partner binds conformational subpopulations, which are removed from the conformational equilibrium. Thus the occupancy of the bound conformations is progressively increasing until the pseudo-ternary equilibrium between protein bound RNA, RNA in accessible conformation and RNA in inaccessible conformation. Depending on whether the bound conformation pre-exists in a significant extent in absence of the binding partner, the process can be perceived as conformational capture or as induced fit.

Both, RNA and proteins are flexible caused by fundamentally different energetic principles [289]. In RNA the free energy contributions of the secondary structure are much bigger than of the tertiary structure. Consequently, RNAs are very flexible at the tertiary structure level. Refolding at the secondary structure level is mainly restricted to individual structural elements. Thus, induced fit does usually not include disruption of major

secondary structures, rather it involves the reorganization of local secondary structure elements, the fixation of unstructured single stranded elements in a defined conformation and the stabilization of a particular tertiary structure [209]. In some protein complexes with dsRNA, the formation of non-Watson-Crick base pairs has been shown to play an important role for specific recognition [156]. Also, RNA secondary structures are energetically very degenerate, which corresponds to a very rugged energy landscape with many local minima. Consequently, many different structures exist concomitantly and the concept of conformational capture is well applicable to RNAs.

On the contrary, formation of secondary and tertiary structure is tightly linked in proteins and the energy landscape is a combination of the contributions of both structure levels. Therefore, a classical induced fit or co-folding concept is more applicable for proteins in interactions.

Induced fit in RNA-protein interactions

In the case of RNA-protein interactions *induced fit* can be observed in the protein, in the RNA or in both [369]. A well understood example of protein folding induced by binding to an RNA is the ribosomal *L11* protein. It interacts with a multiloop in 23S rRNA and the complex is part of the site where the ribosome interacts with elongation factors. Though not directly shown, it is likely that the RNA structure remains largely unaltered upon binding [37]. Also, the *L11* structure is the same in either bound and free state, but a loop flexible in the free protein becomes ordered in the bound state [73, 370]. Such an RNA induced ordering of loop structures which participate in RNA binding has been observed in other RNA binding proteins as well [95, 148, 6].

The *S15*-rRNA complex is exemplaric for reorganization of RNA tertiary structure induced by protein binding. *S15* binds to a three helix junction in 16S ribosomal RNA. The structure of the *S15* protein is similar in bound and free state [4, 263, 286, 29]. However, the arrangement of the three RNA helices changes significantly. In absence of protein and Mg^{2+} the angle between the three helices is nearly equal, whereas in the complex two helices stack co-axially, the third forms an acute angle with the main *S15* binding site.

The complex between *U1A* and *U1A* mRNA 3'UTR has been studied extensively and demonstrates mutually induced fit in RNA-protein interactions. Structures are available of the free *U1A* and RNA [142, 17] as well as the complex [269, 351]. In free *U1A* the C-terminal helix is tightly packed against the β-sheet involved in RNA binding. In the complex, this helix is displaced allowing an intimate contact between RNA and protein. The RNA

exhibits stacking interactions in the free form, that are not present in the complex.

Induced fit might be solely an energetic by-product of the binding process. However, induced fit comes at the cost of reduced binding affinity as the binding free energy is used to overcome energy costs for refolding. These thermodynamic issues raise the question why interaction systems did not evolve to avoid induced fit. One explanation is that it might be difficult to preform the perfect interaction surface in the absence of the binding partner. A more intriguing argument is that several examples of RNA-protein interactions are known where the conformational re-organization of the protein is tightly coupled to a biological function. Upon binding to the poly-adenylation inhibition element in its own mRNA, the human *U1A* protein exposes its effector region in a conformation suitable for binding to the regulated enzyme, the poly(A) polymerase. This mechanism ensures that poly(A) polymerase, which is essential to the cell, is only downregulated when U1A is bound to U1A mRNA [351]. Another example is provided by the interaction between the poly(A) binding protein *PABP* and the translation initiation factor *eIF-4G*. The large interaction surface spans both RRM domains in *PABP* and is only created by a conformational re-organization upon binding of *PABP* to a target RNA [95, 301, 288].

2.3 Non-protein coding RNAs

About 98% the translational output in human cells is non-protein coding RNA [237]. Some species of non-protein coding RNAs (ncRNAs) have been known for long, like the tRNAs. Rather recently, ncRNAs have been identified that specifically determine the stability and / or translatability of mRNAs. Meanwhile, several diverse cellular mechanisms have been discovered which involve non-coding RNA and the number of identified non-coding RNAs is rapidly increasing. ncRNAs are subsequently perceived as an important if not dominant layer in post transcriptional regulation of gene expression [238]. We briefly introduce the ncRNA class of miRNAs not only because they are relevant in post transcriptional regulation, but because we will make use of similar, artificial ncRNAs in this work and will speculate about the existence of a new class of such ncRNAs. These and other classes of ncRNAs like RNAseP, MRP RNA, spliceosomal RNA, signal recognition particle RNAs, and microRNAs all perform their function as part of RNA-protein complexes. For general reviews on non-coding RNA see e.g. [334, 238, 237, 159, 104]

micro RNAs

micro RNAs (miRNAs) have been originally identified in *Caenorhabdidtis elegans* and currently known members of this ncRNA class bind elements in the 3'UTRs of mRNAs and suppress translation and promote transcript degradation [9, 262]. As miRNAs do not hinder polysome association, it is believed that they act on translation elongation or termination but not on the initiation of the translation machinery [128]. miRNAs originate from about 70 nt long precursor transcripts with a characteristic hairpin secondary structure. Cleavage by the endonuclease *Dicer* gives rise to the 22nt mature miRNA. The evolution of miRNAs has been recently investigated in [338].

The miRNA pathway has some intersection with RNA interference, a mechanism which probably serves to protect cells from invading double stranded RNA and to control transposon activity. RNAi also involves the processing of the double stranded RNA by *Dicer* and leads to the degradation of RNAs which are reverse complementary to the mature cleavage product.

2.4 AU-rich elements

The AU-rich elements (AREs) belong to the best studied *cis*-acting elements of mRNA stability regulation. AREs are found particularly frequently in the 3'UTR of genes that demand a very tight regulation to ensure proper cell function and are regulated on a very short time-scale. Approximately 3000 genes are currently believed to be regulated by the ARE pathway [20] and typical ARE genes are early response genes, the genes encoding growth factors and hormones, stress proteins, proteins involved in cell cycle regulation like the cyclins and many proto-oncogenes and cell surface receptors. An ARE was first identified in *fos*, by comparing the mRNAs of the cellular proto-oncogene *c-fos* with its viral oncogene counterpart *v-fos* from the FBJ murine osteosarcoma virus [81].

Sequence properties of AREs

Despite the numerous studies on ARE containing mRNAs, it is not possible to discriminate ARE from non-ARE sequences by a simple sequence profile. This is maybe partly explained by the fact that the term ARE is more a collection of sequence motifs than a motif itself. This will become particularly obvious when the various sequence binding motifs of ARE *trans*-acting factors are discussed (section 2.4.1).

AREs represent a combination of `AUUUA` and `UUAUUUA(U/A)(U/A)` motifs, stretches of U and U-rich regions. In [61] AREs are classified by correlating

sequence features with the ARE induced degradation kinetics. Type I AREs, like the *c-fos* ARE, contain one to three scattered copies of `AUUUA` coupled to a nearby U-rich stretch. This type of AREs induces a biphasic mRNA degradation, initiated by a rapid and synchronous de-adenylation, which allows to isolate intermediates with poly(A) tails shortened to 30 to 60 nucleotides. In the second phase the RNA body itself is degraded with first order kinetics. Type II AREs, like the *GM-CSF* ARE, are characterized by the presence of at least two overlapping copies of `UUAUUUA(U/A)(U/A)`. The induced degradation is biphasic as well, however, de-adenylation occurs asynchronously, with only poly(A) lacking degradation intermediates. Finally, type III AREs do contain none of the characteristic motifs of type I or II AREs. Like the *c-jun* ARE they are generally U-rich, lead to a biphasic degradation with synchronous de-adenylation like the class I AREs. In contrast to both class I and II AREs, class III ARE-induced degradation is insensitive to the drug Actinomycin d.

A different, purely sequence based approach of ARE classification has been pursued by the creators of the *ARED* database [20, 21]. Starting from a set of known ARE sequences, a 13nt consensus pattern was derived, `WWWUAUUUAUWW`. This pattern was used to mine sequence databases for potential ARE genes. The resulting sequence set was then clustered, allowing 10% of the pattern to be mismatched, for matching `AUUUAUUUAUUUAUUUAUUUA` (cluster I), `AUUUAUUUAUUUAUUUA` (cluster II), `WAUUUAUUUAUUUAW` (cluster III), `WWAUUUAUUUAWW` (cluster IV) and `WWWWAUUUAWWWW` (cluster V). In contrast to both approaches, an ideal clustering of ARE sequences should reflect the presence of binding motifs of the various ARE binding proteins.

2.4.1 ARE binding proteins

In the current understanding, AREs exert their stabilizing or destabilizing function not on their own, but together with *trans*-acting factors. Several ARE binding proteins have been identified; we will review those best understood and where a direct effect on transcript stability has been shown.

AUF1

AU-rich binding factor 1 (AUF1) is a two RRM protein existing in four alternatively spliced isoforms, p37, p40 , p42 and p45. The four isoforms differ greatly in their binding affinity four ARE RNAs, with p37 having the highest and p40 the lowest affinity for *c-fos* ARE [354]. Isoforms lacking exon 7 in (p37 and p40) are targeted for the ubiquitin-proteasome pathway, which may explain the reported destabilizing function of AUF1 [201, 202].

Additionally, presence or absence of exon 7 influences the nucleo-cytoplasmic distribution of AUF1 [307]. While *AUF1* has been found to bind many ARE mRNAs [31], its role in ARE dependent mRNA stability control is confusing. In a recent study, siRNAs were used to dissect the role of various ARE binding proteins among them *AUF1* [292]. Knockdown of *AUF1* only led to upregulation of ARE mRNAs if p40 and p45 were downregulated selectively. Though generally understood to be a destabilizing protein, *AUF1* has been fount to be part of the α-globin stabilizing complex [187]. Also, *AUF1* has been reported to bind DNA [93].

TIAR and *TIA-1*

TIA-1 (T-cell internal antigen 1) and *TIAR* (*TIA-1* related protein) are two closely related proteins, both encompassing three RRM domains [24]. Both bind to ARE sequences and act as translational silencers. Involvement in mRNA stability regulation has been largely excluded for TIA-1 [280].

Tristetraprolin and *BRF1*

The Tristetraprolin protein family consists of the CCCH tandem zinc finger proteins *Tristetraprolin* (*TTP*), two related proteins discovered in mammals (*TIS11d* and *cMG1*) and one protein cloned from frog and fish [34]. *TTP* is known to bind to class II AREs and promote their de-adenylation by poly(A) ribonuclease [197]. However, *TTP* is also capable of promoting the degradation of poly(A)$^-$ mRNAs [196]. TTP is localized in nucleus and cytoplasm and its nuclear export is dependent on interaction with *Nup214* [55]. The RNA sequence binding motif has been identified using SELEX. It is the class II ARE core element, `UUAUUUAUU` [373]. Structure data is available for the first domain of *TTP* [8] and for the Tristetraprolin family member *TIS11d* [163].

Early response genes are not only regulated by *TTP*, but *TTP* is an immediate early response gene itself: *TTP* is rapidly upregulated in fibroblasts in response to insulin, serum, growth factors and phorbol esters. Regulation of *TTP* occurs transcriptionally and post transcriptionally and is dependent on protein kinase p38. *TTP* downregulates its own expression by binding to a class I ARE in the 3'UTR of its mRNA [44, 339]. Pro-inflammatory stimuli which lead e.g. to the expression of *TNFα* upregulate also *TTP* which promotes degradation of *TNFα* mRNA [291]. Thus co-upregulation of *TTP* seems to limit the inflammatory response [51].

TIS11b or *BRF1* is closely related to *TIS11d* and has been identified by functional genetic screening [329]. It binds to various cytokine mRNAs and

has recently been found to be a major destabilizing counter-player of *HuR* [292].

The Hu proteins

The Hu protein family consisting of *HuR* (*HuA*), *HuB*, *HuC*, *HuD* is special in that it constitutes the only set of ARE binding proteins which are known to stabilize ARE mRNAs. While *HuC* and *HuD* expression is restricted to neuronal tissue, *HuB* to neuronal tissue and sex organs, *HuR* is ubiquitously expressed. The Hu proteins are highly conserved. However, the three neuronal homologues are clearly more related among each other than with HuR. The Hu proteins belong to the family of *ELAV* related proteins and share approximately 70% sequence identity with this *Drosophila melanogaster* protein. Other proteins involved in post transcriptional regulation and distantly related to the Hu protein are the poly(A) binding protein *PABP*, *UP1* (*hnRNP A1* – a heterogeneous nuclear ribonucleoprotein) and the sex-lethal (*Sxl*) protein from *Drosophila melanogaster*.

Hu proteins contain three RRMs (section 2.2.1) and residues critical for RNA recognition are identical for all family members. Consequently, it is justified to assume that the RNA binding properties of all Hu proteins are very similar. Like *ELAV*, the Hu proteins contain a hinge region between the second and third RRM, which is 50 to 80 amino acids in length an poorly conserved within the family. Structure data is available for the first two RRMs of *HuC* [167] and *HuD* [360].

NMR studies of the first two domains of *HuC* binding to ARE sequences revealed that individual RRMs bind weakly to `AUUUA`. Both domains together bind much stronger to longer ARE fragments [167], which is typical for RRM proteins (section 2.2.1).

The role of the third RRM domain remains enigmatic. In PC12 cells over-expression of *HuB*, *HuC* and *HuD* lead to a neuronal phenotype in the absence of nerve growth factor [5, 181]. Mutants of *HuB* and *HuC* lacking the third domain fail to produce this effect. Conversely, the isolated third domains of *HuB* and *HuC* act as dominant-negative proteins when co-transfected with wild-type *HuB* or *HuC* in PC12 cells [5]. The dominant-negative effect can probably not be explained by a competition for RNA binding as the third RRMs of *HuB* and *HuC* have largely lost their ability to bind RNA [5]. Probably, the third domains cause this effect by competing with their complete endogenous counterparts for interactions with other cellular factors, may be the same which are known to interact with *HuR* (section 2.4.2),[40]. This appears to be different in *HuD*, where deletion of the third domain was reported to increase on and off rates of *HuD*-RNA

complex formation [272].However, for *HuD* it has also been claimed that the third RRM binds poly(A) [227].

Hu proteins do not appear to block de-adenylation of mRNAs. Rather *HuB* has been shown to protect de-adenyated mRNAs generated from the turnover of ARE mRNAs *in vivo* [123].

The recognition of ARE mRNAs by *HuR* is the main model system used in this study, we have thus devoted a separate section to review *HuR*-RNA interaction (section 2.4.2).

2.4.2 HuR mRNA interactions

HuR is the most prominent member of the family of Hu proteins. It is ubiquitously expressed and has a rapidly increasing list of target mRNAs. A compilation of currently published *HuR* targets is given in Table A.1 in the appendix. Though this list of target mRNAs is impressive, the fact that known sequence constraints for *HuR* are rather loose – reflected by the finding that *HuR* binds both class I and II AREs – suggests that *HuR* is involved in the regulation of many ARE mRNAs and thus is a central regulatory node in the ARE pathway.

HuR was cloned as the last member of the Hu family identified so far [226]. Originally, *HuR* was believed to promote mRNA degradation, as cross-linking [112] and gel-shift [258] experiments revealed that *HuR* binds to ARE sequences known for their destabilizing function. Over-expression experiments, however, suggested a stabilizing function of the protein [114, 275]. As other Hu proteins, *HuR* appears to protect the body of the transcript rather than preventing de-adenylation [275], at least when it is over-expressed. Final evidence for *HuR*'s stabilizing function, excluding that the ascribed function is an artifact caused by over-expression, came from the findings that *HuR* knockdown using antisense techniques leads to an increase of mRNA decay [356, 358, 292].

Subcellular distribution of *HuR*

HuR's diversity in the pattern of nucleo-cytoplasmic distribution seems to be an important property of this protein's function. *HuR* is a predominantly nuclear protein. However, cytoplasmic concentrations of *HuR* vary throughout the cell cycle. In [16], *HuR* was found to localize in the cytoplasm during early G_0 phase. Contrariwise, levels of cytoplasmic *HuR* were found to peak during S and G_2 phase, when the stability of two *HuR* targets involved in cell cycle regulation, cyclin A and B1, were found to be highest [356].

In response to many different stimuli, *HuR* redistributes sub-cellularly which leads to *HuR* dependent stabilization of mRNAs like e.g. androgen in hepatoblastoma cells [320] or in response to UV light [358]. Remarkable is the response to heat shock, where *HuR* binding to mRNAs in the cytoplasm is suppressed and in the nucleus increased. Also *HuR* dependent export of the stress response protein *hsp70* is induced [127]. Under heat shock conditions, *HuR* switches in its nuclear export pathway to *CRM1*, manifested by the sensitivity of *HuR* function to leptomycin B, a *CRM1* inhibitor [126]. Under normal conditions, *HuR* shuttling is leptomycin B insensitive [39].

HuR shuttling is dependent on a 52 amino acid sequence located in the hinge region between RRM2 and RRM3, called HNS (for *HuR* nucleocytoplasmic shuttling) [113]. The HNS displays a weak similarity with the M9 shuttling sequence of *hnRNP A1* [40]. The role of *HuR* in mRNA stabilization and its ability to shuttle between nucleus and cytoplasm has led to the idea that *HuR* binds to its target RNAs in the nucleus, accompanies them to the cytoplasm, thereby possibly facilitating RNA export, and protects the target RNA from degradation in nucleus and cytoplasm.

Protein ligands of *HuR*

Several proteins have been identified that associate with *HuR*: *SETα*, *SETβ*, *pp32* and *APRIL* [40]. *SETα* and *SETβ* are identical in their C-terminal part and are probably splice variants of the same gene. *pp32* and *APRIL* are different but highly similar proteins. All four proteins contain acidic C-terminal tails of at least 50 amino acids. For *pp32* it has been shown that this tail is necessary for interaction with a region on *HuR* which contains parts of the hinge region and of RRM3 in *HuR* [126].

SETα and *SETβ* are found in nucleus and cytoplasm. *pp32* and *APRIL* are predominantly nuclear and exhibit a shuttling activity like *HuR*. Shuttling of both proteins depends on *CRM1* and can be inhibited with leptomycin B. Also, *CRM1* dependent export of *HuR* was found to be dependent on the same sites in the *HuR* structure which are necessary for interaction with *pp32* and *APRIL* [126].

Interestingly, *SETα*, *SETβ* and *pp32* have been identified previously as inhibitors of protein phosphatase 2A (*PP2A*) [215, 302]. *PP2A* de-phosphorylates targets of kinases and kinases themselves, affecting cellular functions like cell cycle progression, DNA replication, transcription, splicing development and morphogenesis [40]. The significance of the connection between *HuR* and *PP2A* is unclear, possibly *PP2A* is involved in signal cascades regulating mRNA decay.

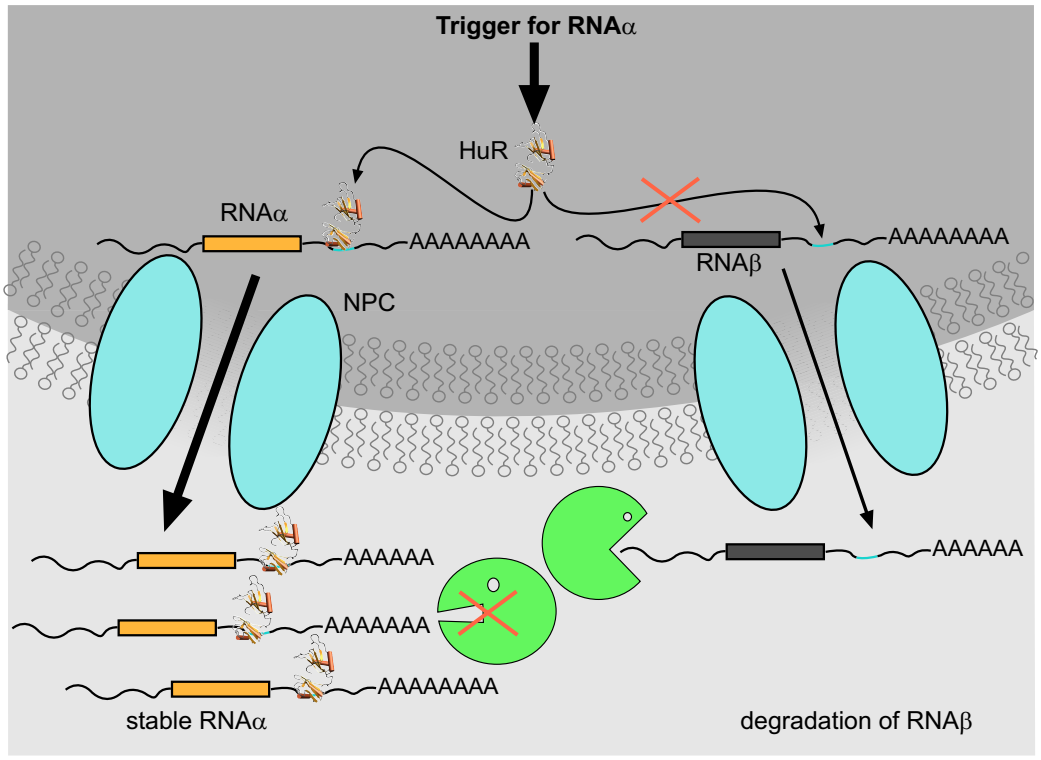


Figure 2.1: The specificity puzzle of *HuR* dependent mRNA stability regulation. Upon a particular trigger for the stabilization of mRNAα, *HuR* binds to this mRNA species, facilitates export and protects the mRNA from degradation. Other *HuR* target mRNAs not affected by the trigger are not bound by the protein though they are potentially present.

The specificity puzzle

HuR is capable of regulating the stability of various mRNAs coding for proteins of diverse function (Table A.1). Also, *HuR* performs this regulation in response to very different stimuli like hormones, cytokines, irradiation or heat shock. ARE mRNAs are often transcribed at a basal level, so that we can assume that *HuR*, when reacting to a stimulus, needs to be able to distinguish between the target mRNA to be bound and other *HuR* target mRNAs which remain unstable. It is fundamentally unclear, how *HuR* may achieve this. We consequently call this problem the *specificity puzzle in mRNA stability regulation*. A solution of the specificity puzzle is not only of great scientific interest, but is also a prerequisite for a potential exploitation of *HuR* in drug discovery.

Chapter 3

Theory, Algorithms, Implementations

Despite the many findings that RNA structure – and thus the thermodynamics of RNA folding – has a pivotal role in RNA ligand interactions, this topic has not yet been investigated systematically. In this chapter, we derive a quantitative model of the RNA secondary structure influence on RNA-protein affinities. We further develop a mechanism to manipulate RNA secondary structure in a controlled way by the hybridization of short oligonucleotides and describe a formalism to approximate the influence of this hybridization on RNA-protein affinities.

3.1 Quantitative Model of RNA-Ligand Binding

We consider here a (protein) ligand that binds to an RNA molecule in a simple two-state process with 1:1 stoichiometry. Multi-state processes involving a conformational rearrangement after ligand binding (induced fit, e.g [369]) are also described by this model provided that the free energy changes due to the structural rearrangement after binding are (nearly) independent of the RNA sequence. Furthermore we assume that only those RNA molecules can be bound that present the binding site(s) in a particular spatial conformation. We use the symbol RNA_* to denote this sub-population of RNA molecules.

$$\mathrm{Ligand} + \mathrm{RNA}_* \rightleftharpoons \mathrm{Ligand} \cdot \mathrm{RNA} \tag{3.1}$$

The law of mass action implies that the concentrations $[\mathrm{RNA}_*]$, [Ligand], and $[\mathrm{Ligand} \cdot \mathrm{RNA}]$ of free accessible RNA, free protein, and complex are related

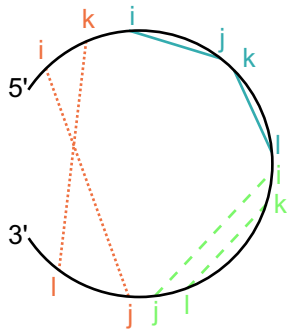


Figure 3.1: The non-pseudoknot condition. Each nucleotide s_i takes part in at most one base pair and base pairs do not cross (red, dotted line) , i.e., $(s_i, s_j) \in \Psi$ and $(s_k, s_l) \in \Psi$ with $i < j$, $k < l$, and $i < k$ implies either $j < k$ (blue, solid line) or $j > l$ (green, dashed line) .

through the dissociation constant

$$K_d = \frac{[\mathrm{RNA}_*]\ [\mathrm{Ligand}]}{[\mathrm{Ligand} \cdot \mathrm{RNA}]} \tag{3.2}$$

An RNA molecule with nucleotide sequence s may form many different structures. For our purposes it is sufficient to distinguish between secondary structures only. The set $\Sigma(s)$ consists of all secondary structures (i.e., lists of base pairs) Ψ satisfying the following conditions: (i) Each nucleotide s_i takes part in at most one base pair; (ii) base pairs do not cross, i.e., $(s_i, s_j) \in \Psi$ and $(s_k, s_l) \in \Psi$ with $i < j$, $k < l$, and $i < k$ implies either $j < k$ and $j > l$ (Figure 3.1); and (iii) each pair $(s_i, s_j) \in \Psi$ is one of the six canonical pairs GC, CG, AU, UA, GU, or UG. For each secondary structure Ψ of s one can compute a free energy $F(\Psi)$ by adding up energy contributions for stacked base pairs, hairpin loops, interior loops, bulges, and multi-branched loops. These energy contributions have been determined experimentally, see [236]. The frequency of a particular secondary structure Ψ in thermodynamic equilibrium ensemble can therefore be computed as

$$p(\Psi) = \frac{1}{Z} \exp\left(-\frac{F(\Psi)}{RT}\right) \tag{3.3}$$

where $Z = \sum_{\Upsilon \in \Sigma(s)} \exp(-F(\Upsilon)/RT)$ is the partition function of the RNA molecule s.

Writing $A(s) \subseteq \Sigma(s)$ for the accessible structures of our RNA molecule s we obtain

$$[\mathrm{RNA}_*] = p_* \, [\mathrm{RNA}] \tag{3.4}$$

where p_* is the fraction of accessible secondary structures:

$$p_* = \sum_{\Psi \in A(s)} p(\Psi) = \frac{1}{Z} \sum_{\Psi \in A(s)} \exp\left(-\frac{F(\Psi)}{RT}\right) = \frac{Z_*}{Z}\,. \tag{3.5}$$

We remark that we can of course describe the concentration of accessible RNA in terms of the law of mass action: The equilibrium constant for the refolding

$$\mathrm{RNA}_* \rightleftharpoons \mathrm{RNA}_- \tag{3.6}$$

between accessible and inaccessible conformations is given by

$$K_* = \frac{[\mathrm{RNA}_*]}{[\mathrm{RNA}_-]} = \frac{p_*}{1-p_*} \tag{3.7}$$

The problem thus reduces to computing the partition functions for the two sets of secondary structures $\Sigma(s)$ and $A(s)$. This can be achieved e.g., by means of dynamic programming [241] as we shall see below. Substituting equ.(3.4) into equ.(3.2) yields

$$\frac{[\mathrm{RNA}]\ [\mathrm{Ligand}]}{[\mathrm{Ligand}\cdot\mathrm{RNA}]} = \frac{K_d}{p_*} =: K_d^{\mathrm{app}} \tag{3.8}$$

Using conventional methods to measure RNA protein interactions, only the total concentration of unbound RNA, [RNA], can be measured. Hence, only the apparent dissociation constant $K_d^{\mathrm{app}} = K_d/p_*$ can be determined experimentally. As a consequence, we predict a structure dependence of the measured values of K_d^{app}. Under the assumption that the true value of K_d depends only on the ligand and the sequence-structure motif that binds the ligand and thus is independent of the structural context of the motif, we can predict sequence-dependent variations in RNA-ligand binding affinity by means of a computational analysis of the ensemble of RNA structures.

In the simplest case the sequences under consideration contain a single copy of the binding motif which must be present in a particular secondary structure conformation. Usually, the structural requirements will only be a few local base pairs at the binding site, or, conversely, it might be necessary that all or a part of the binding site remains unpaired. The number of accessible structures will therefore in general be too large to use equ.(3.5) directly. Instead, a modification of McCaskill's partition function algorithm [241] can be used to compute partition functions restricted to structures that contain a specified list of base pairs and/or a specified list of unpaired positions. We refer to [161] for a description of the algorithms, which are implemented as part of the `Vienna RNA Package` [162, 160]. Pseudoknotted

structures could be handled, albeit at much greater computational costs, by a generalized partition function algorithm [99]. The up-to-date collection of energy parameters contains both enthalpies and entropies and thus can be used to compute the ensemble of equilibrium secondary structures for a given temperature [236].

The computation of p_* becomes more complicated if the sequence motif is very degenerate and hence a single RNA sequence s can have more than one potential binding site. More precisely, we consider sequences with M binding sites B_i, $i = 1, \ldots, M$. We will restrict ourselves here to the case in which RNA-ligand complexes are always of 1:1 stoichiometry even if there are multiple protein binding sites on the RNA. In this case the set of accessible structures $A(s)$ consists of all those secondary structures in which *at least one* binding site B_i is accessible. We calculate the probability of structures $p(\mathcal{A})$ where a particular subset $\mathcal{A} \subseteq \{B_1, B_2, \ldots, B_M\}$ of binding sites is in accessible conformation, *irrespective* of the conformations at all other binding sites, again as a fraction of partition functions $p(\mathcal{A}) = Z(\mathcal{A})/Z$. In this notation $\mathcal{A} = \emptyset$ means that there is no constraint on the structure (and hence $p(\emptyset) = 1$), while $\mathcal{A} = \{B_1, B_2, \ldots, B_M\}$ means that all M binding sites are accessible simultaneously. Partition functions $Z(\mathcal{X})$ over all structures that satisfy a given structural constraint $\mathcal{X}$ can be computed using the `RNAfold` program from the `Vienna RNA Package`. Finally, p_* is obtained from the probability of its complement that all binding sites are inaccessible, which can be computed immediately using the inclusion-exclusion principle (which dates back at least from Bernoulli, see e.g. [336]):

$$1 - p_* = \sum_{\ell=0}^{M} (-1)^\ell \sum_{\substack{\mathcal{A} \\ |\mathcal{A}|=\ell}} p(\mathcal{A}) \tag{3.9}$$

For large numbers M of potential binding sites this becomes infeasible since equ.(3.9) requires 2^M evaluations of a partition function (one for the unconstrained molecule and $2^M - 1$ for the different combinations of binding sites). As an approximation the expansion can be truncated at order $\ell_{\max} < M$. The expansion in general converges quickly for long sequences, while for short sequences we need more or less all the terms, see Fig. 3.2. In practice, however, one will usually encounter binding motifs that are relatively rare since a ligand can fulfill its regulatory role only if it does not indiscriminately bind everywhere. We remark that p_* could alternatively be evaluated by using stochastic backtracking to obtain a Boltzmann-weighted sample of secondary structures instead of computing constrained partition functions [335, 97, 98, 161]. The sampling approach is computationally more

efficient, but it is less accurate for small probabilities p_* of the accessible structures.

3.2 A Statistical Test for the Influence of Secondary Structure

The theory outlined above predicts a linear dependence of the measured apparent K_d^{app} on $1/p_*$ if the RNA-ligand binding depends on particular secondary structure features of the binding site. This relationship can be turned into a statistical test for the influence of secondary structure given a set of binding data of RNA sequences that contain a known *sequence* motif required for ligand recognition.

Given the hypothesis that binding depends on a particular structural feature Ξ of the RNA, we may (*i*) compute the probability $p_*[\Xi]$ for all sequences in the data set that at least one binding sequence motif in the RNA sequence s satisfies the secondary structure constraints Ξ as outlined above, (*ii*) calculate the empirical correlation coefficient r between K_d^{app} and $1/p_*[\Xi]$ and (*iii*) test whether this correlation is significant. Applying a statistical test for correlation described in [75] we reject the null hypothesis of *no correlation* if and only if

$$\frac{(k-2)r^2}{1-r^2} \geq \{t_{(k-2)}(1-\alpha/2)\}^2 \tag{3.10}$$

is satisfied. Here k is the number of sequences, $t_{(k-2)}(y)$ is Student's t-distribution [331, 270] with $k-2$ degrees of freedom, α is the desired significance level and r is the empirical correlation coefficient between K_d^{app} and $1/p_*[\Xi]$.

In general there is a large number of different secondary structure elements Ξ that can be realized simultaneously by a set of related sequences [1]. Thus, it may not be feasible to find the optimal structure constraint *ab initio*. The test procedure above, however, allows to select or exclude a secondary structure element from a set of candidate elements.

This statistical test has been successfully applied to the *HuR*-RNA recognition mechanism and allowed to identify the secondary structure element required for *HuR* binding (section 4.1.2).

Once the importance of the secondary structure Ξ has been verified by the above test, one can use a simple least-squares fit to determine K_d from the $(K_d^{\text{app}}, 1/p_*[\Xi])$ pairs. Subsequent to the determination of K_d, apparent dissociation constants for any RNA molecule that contains the binding sequence motif can be predicted upon calculation of p_* using eq. (3.8). This

predictive model can be used to design RNA sequences with a predefined binding affinity. In section 4.1.2 we use this approach to predict K_d^{app} of four $TNF\alpha$ mutants, which were – in part – designed to meet preselected affinity requirements.

3.3 Modifier RNAs

The thermodynamics of an RNA molecule M changes when it hybridizes with a short oligonucleotide O. Since the nucleotides of M that bind the oligonucleotide O are no longer available for pairing in the intra-molecular secondary structure, the molecule M will typically refold. This can have drastic effects on the secondary structure of a binding sequence motif even if the oligonucleotide O binds far away from the binding site. Depending on the sequence of the oligonucleotide, the effect can be either an increase or a decrease in the fraction p_* of accessible secondary structures.

The thermodynamics of RNA-RNA hybridization is well understood [96]. At the time of writing this study, no implementation was available that considers all possible structures within each strand of two hybridized RNA molecules so that we use an approximate model here. An extension of the `Vienna RNA Package` that implements the complete folding model for two interacting RNAs is forthcoming [120]. We thus briefly describe the complete theory here and derive an approximation that can probably be used in most cases of practical interest, including the application to the HuR/ARE model system.

The mRNA molecule M and the oligonucleotide O together can form five molecular species[1]: the monomers M and O, the homodimers MM and OO and well as the heterodimer MO that we are primarily interested in. In thermodynamic equilibrium we have

$$[MM] = K_{MM}[M]^2 \qquad [OO] = K_{OO}[O]^2 \qquad [MO] = K_{MO}[M][O] \quad (3.11)$$

with equilibrium constants K_{MM}, K_{OO}, and K_{MO} that can be computed from partition functions by means of an extension of McCaskill's algorithm, see [96, 120]. For each of the monomer and dimer species, the probabilities $p_*(M)$, $p_*(MM)$, and $p_*(MO)$ that the binding motifs(s) are accessible can be computed by the same approach as in the previous section. We can therefore calculate the effective fraction p_* of mRNAs with accessible binding

[1] We neglect here multiple binding, i.e., species such as MO_2. These could be taken into account without conceptual difficulties at the expense of a more complicated set of equations.

sites as

$$p_* = p_*(M)\frac{[M]}{[M]_t} + p_*(MM)\frac{[MM]}{[M]_t} + p_*(MO)\frac{[MO]}{[M]_t}\,, \tag{3.12}$$

where $[M]_t = [M] + 2[MM] + [MO]$ is the total concentration of mRNA that is not bound to the ligand. The concentration $[M]_t$ is determined by the value of K_d, the three equilibrium constants K_{MM}, K_{MO}, and K_{OO}, and the initial concentrations of the mRNA, $[M]_0$ and the oligonucleotide, $[O]_0$.

Let us now make the following simplifying assumptions:

(*i*) The oligonucleotide O is (nearly) complementary to a unique target site on the mRNA M. This assumption is inspired by the small interfering RNAs [108] and their relatives, see e.g. [118] and the references therein.

(*ii*) Both the oligonucleotide O and the mRNA M are not significantly self-complementary.

(*iii*) The oligonucleotide O is present in excess.

Under these hypotheses we have $K_{MM}, K_{OO} \ll K_{MO}$, and $[M]_0 \ll [O]_0$, i.e., almost all mRNAs are hybridized with the oligonucleotide O. This allows us to use the approximation

$$p_* \approx p_*(MO)\frac{[MO]}{[M]_t} \approx p_*(MO)\,. \tag{3.13}$$

The set of possible secondary structures of the MO duplex can be approximated by those structures of the mRNA M in which the target site T of the oligonucleotide cannot pair with other nucleotides of M. The energy of such a secondary structure is $F(\Psi_{M\setminus T}) + F(TO)$ where $F(\Psi_{M\setminus T})$ is the energy of the secondary structure $\Psi_{M\setminus T}$ in which the target site for the oligonucleotide is unpaired and $F(TO)$ is the energy contribution for the hybridization of the oligonucleotide to its target site on M. While $F(TO)$ can in principle be computed, we can simply treat it as a constant independent of $\Psi_{M\setminus T}$ which therefore cancels in the partition function computations. Thus, we obtain

$$p_*^{MO}(\mathcal{A}) = Z(\mathcal{A}\cup\mathcal{T})/Z(\mathcal{T}) \tag{3.14}$$

directly from the constrained partition functions $Z(\,.\,)$ of the mRNA M using the additional constraint $\mathcal{T}$ that the target site T is unpaired. If a binding site $B_i \in \mathcal{A}$ and T overlap, then T takes precedence, i.e., we assume that B_i cannot be accessible when the oligonucleotide is bound at this position. We can now calculate p_*^{MO} using eq. (3.9) in the same way as for the mRNA alone. To this end we replace $p(\mathcal{A})$ by $p_*^{MO}(\mathcal{A})$ from eq.(3.14).

Equ.(3.14) describes the effect of a particular oligonucleotide O. Since O modifies the RNA-ligand binding we refer to O as a *modifier RNA*. It has been demonstrated experimentally in the *HuR*-mRNA system that modifier RNAs are functional [244]. Modifier RNAs can be designed by means of the following, generally applicable procedure: We fix a length N_0 of the modifier oligonucleotide O, say $N_0 = 20$ inspired by siRNAs and microRNAs, and compute the effect of the oligonucleotide when it binds the mRNA M starting from sequence position k. Examples of modifier-effect profiles $p_*^{MO}[k]$ are shown in section 4.2.1. Such modifier-effect profiles can be computed for moderate size mRNAs (e.g. TNFα) within about a day from equ.(3.14) using 30 Xeon CPUs. A sampling approach based on stochastic backtracking will be much more efficient provided one is only interested in oligonucleotides leading to large values of p_*^{MO}.

The modifier-effect profiles allow the specific design of RNA oligonucleotides that modulate the ligand binding affinity by *opening* ($p_*^{MO} \to 1$) or *closing* ($p_*^{MO} \to 0$) the binding sites to the ligand, Tab. 4.5. We demonstrate the feasibility of modifier RNA design for the *HuR*-RNA model system and the experimental validation of modifier RNAs for the manipulation of mRNA stability in section 4.2.

If we know the binding constant K_{MO} of the modifier O to the mRNA M we can calculate the dependence of the apparent dissociation constant

$$K_d^{\text{app}} := \frac{[\text{RNA}]\,[\text{Ligand}]}{[\text{RNA} \cdot \text{Ligand}]} = \frac{[M]\,[\text{Ligand}] + [MO]\,[\text{Ligand}]}{[M \cdot \text{Ligand}] + [MO \cdot \text{Ligand}]} \tag{3.15}$$

on the concentration of O using equ. (3.8) to substitute equ.(3.15) for both M and MO with their respective fractions p_*^M and p_*^{MO}, resp., of accessible structures. We obtain

$$K_d^{\text{app}} = K_d \, \frac{1 + K_{MO}[O]}{p_*^M + p_*^{MO} K_{MO}[O]} \tag{3.16}$$

which describes a hyperbolic transition from K_d/p_*^M to K_d/p_*^{MO} with increasing concentration $[O]$ of the modifier oligonucleotide . This behavior is indeed observed for some opener molecules (section 4.2.3, Figure 4.17). For other openers, such as $\mathsf{Op_1}$ from Table 4.5 in section 4.2.1, we find that very large opener concentrations lead again to an increase in K_d^{app}. This effect could be explained by opener oligos binding at multiple sites.

The computation of K_{MO} requires again a partition function calculation which could in principle be performed using the approach described in [96], the `RNAhybrid` approach [293], or `RNAcofold` [120].

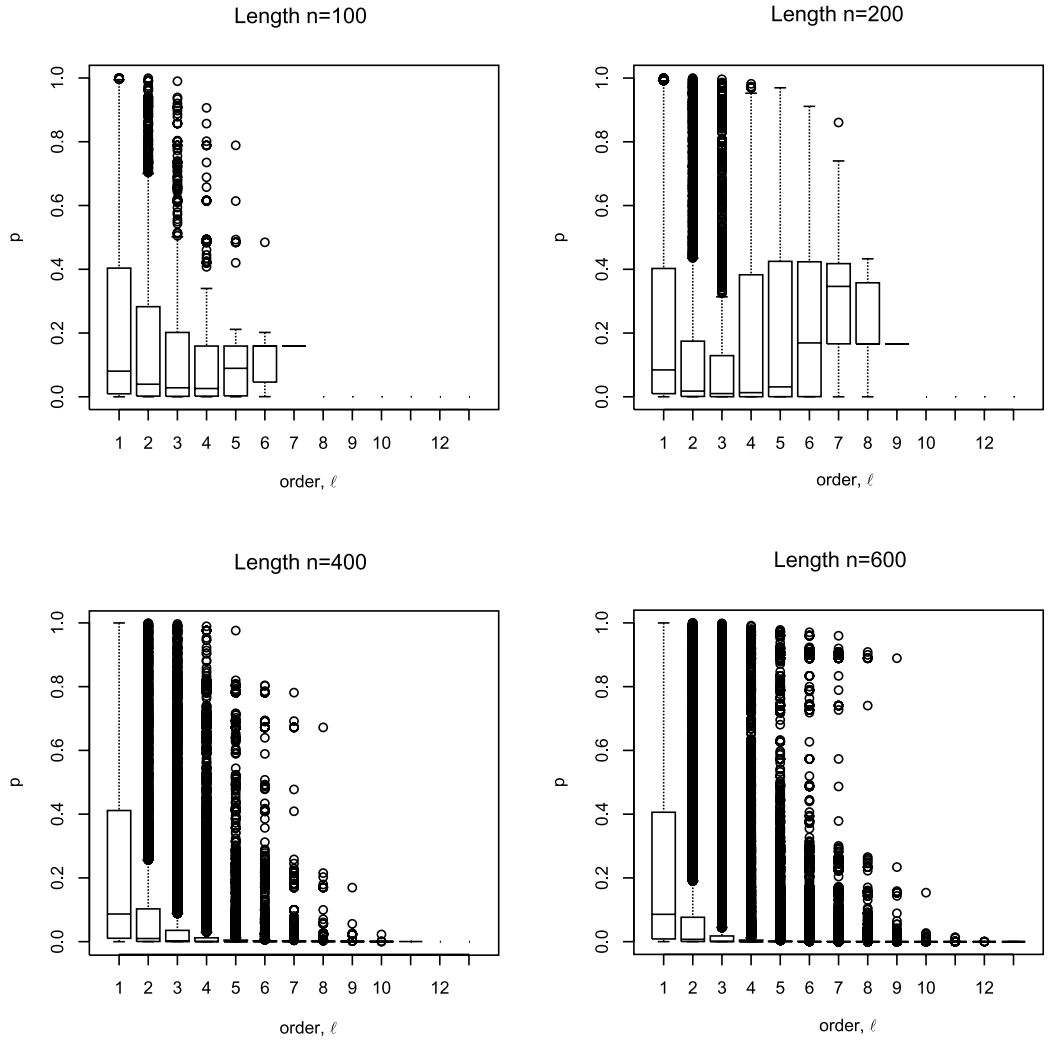


Figure 3.2: Convergence of Equ.(3.9) for the motif `NNUUNNUUU` in single-stranded conformation in random target sequences as a function of sequence length n. We plot the distributions of the absolute contribution of terms of order ℓ to p_* in eq. (3.9), for 10000 sequences. These contributions correspond to the probabilities that subsets of ℓ binding sites are simultaneously accessible. Boxes give the range from 1st to 3rd quartile, with median indicated by a line; whiskers indicate the position of the most extreme data point within 1.5 times the interquartile distance, outliers are shown as circles.

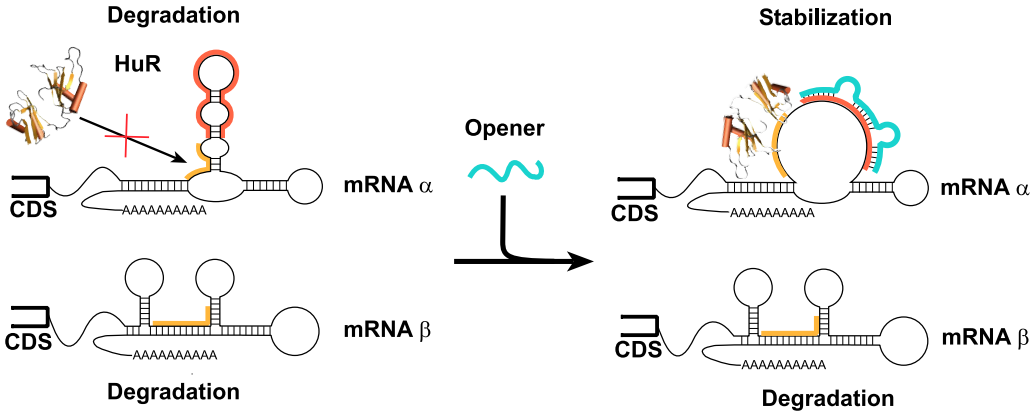


Figure 3.3: The modifier RNA principle illustrated here for the *HuR*-RNA system. As detailed in section 4.1.2, the binding site in the RNA has to be fully single stranded to enable *HuR* recognition. (**a**) The binding site is partly single stranded and *HuR* does not bind. (**b**) Added modifier RNA molecules which hybridize to the target RNA lad to a re-organization of the RNA structure and allow binding of the protein.

Chapter 4

Results

4.1 *HuR*-RNA binding mechanism

The computational methods described in the previous chapter have been validated on the *HuR*-RNA recognition system, which we have described in detail in section 2.4.1. Briefly, *HuR* is a key factor in post transcriptional regulation at the level of mRNA stability. *HuR* stabilizes potentially several thousand genes in a stimulus and target specific manner by binding to the 3'UTR of mRNAs. The basic question for our work on *HuR* was, how specificity can be maintained in this system or said more plastically, which mechanism ensures that the protein binds a particular RNA species in response to a stimulus, leaving other mRNA species untouched.

The *HuR*-RNA complexation was analyzed quantitatively using confocal fluorescence fluctuation analysis (2-dimensional Fluorescence Intensity Distribution Analysis , 2D-FIDA anisotropy, [183], see also appendix A.2). This method is advantageous to conventional techniques for measuring interactions between species of low solubility, high affinity or variable stoichiometry; at least the first two properties are relevant for the *HuR*-RNA system. The main reason for the advantages of the selected technique is that affinities are measured in homogeneous solution based on the determination of true particle concentrations. *HuR* was obtained in soluble form as native protein without a hydrophilic fusion tag using the IMPACT^{TM}-CN purification strategy (New England Biolabs). *HuR* bound to its native ARE target sequences with a high variation in affinities, with K_d^{app} values ranging from 130 pM to 13.6 nM (Table 4.1).

Table 4.1: *HuR*-mRNA interaction data and motif accessibilities from [244].
Positions are the respective start positions of the subsequence in the given RefSeq sequence. Measurements were performed at 23.5°C, computations were performed for this temperature using the `-T` option of `RNAfold` to rescale the energy parameters accordingly.

Gene	AccNo	Pos.	ARE Sequence	K_d^{app}[nM]	p_*
Cox-2	NM_000963	1991	UAUUAAUUUAAUUAUUUAAUAAUAUUUAUAUUAAA	13.63 ± 1.07	0.006
IL-1β	NM_000576	1242	UAUUUAUUUAUUUAUUUGUUUGUUUGUUUUAUU	0.12 ± 0.02	0.519
IL-2	NM_000586	795	UAUUUAUUUAAAUAUUUAAAUUUUAUAUUUAUU	9.50 ± 1.34	0.062
IL-4	NM_000589	833	AUAUUUUAAUUUAUGAGUUUUUGAUAGCUUUAUUUUUUAAGU-AUUUAUAUAUUUAUAA	3.21 ± 0.35	0.049
IL-8	NM_000584	1050	UAUUUAUUAUUUAUGUAUUUAUUUAA	1.09 ± 0.16	0.164
TNFα	NM_000594	1333	AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA	0.35 ± 0.06	0.360
IL-2 3'UTR	NM_000589	757-1035	(see database)	32.77 ± 4.48	0.004
TNFα 3'UTR	NM_000594	872-1568	(see database)	3.87 ± 0.42	0.200
			$(\text{AUUU})_3\text{A}$	1.40 ± 0.39	0.973
			$(\text{AUUU})_4\text{A}$	2.09 ± 0.16	0.906
			$(\text{AUUU})_5\text{A}$	0.40 ± 0.05	0.771
			$(\text{CUUU})_4\text{C}$	0.96 ± 0.02	1.000

4.1.1 RNA sequence binding motif of *HuR*

As described in the previous section, our theoretical methods on the secondary structure dependence of RNA-protein interactions require the knowledge of the exact protein binding motif in the RNA sequence. For *HuR*, such a motif was not available at the timepoint this work was done.

Predicting the HuR sequence binding motif from binding data

The *HuR*-RNA interaction data given in Table 4.1 contained sequences bound with varying affinity and sequences bound with an affinity too low to be detected by the methods used. Consequently, there was some chance to isolate a prototype sequence binding motif for HuR from the data, by identifying those motifs which are common to sequences bound by HuR, but are not present in the set of sequences not bound by HuR. Without further knowledge about the biological role and mechanism of *HuR* it is impossible to determine which apparent affinity discriminates between "*HuR* stabilized" and "not stabilized". Certainly it is unlikely that this affinity is the same as the detection limit of our assay.

String pattern regression (SPR) [22] is an approach which avoids the problem of selecting a numerical discriminant for classes in such a problem of pattern identification. SPR aims to identify a pattern which clusters the sequences into a set matching and another not matching the pattern so that some measure of clustering quality based on the numerical value of interest is optimal. A simple measure of clustering quality is e.g to calculate a t-statistics [331, 270] between the mean Kd^{app} values of the two clusters. The critical step in SPR is to come up with a useful set of candidate patterns. Clearly, enumerating all patterns which are compatible with subsets of the sequences in the test set quickly becomes computationally infeasible. Bannai suggested a Branch-and-Bound approach for this problem. We restricted our approach to selecting the most appropriate motif for further experimental testing from a series of candidate motifs from literature. As *HuR* was mainly known as an ARE binding protein, clear candidate patterns where the ARE core motifs and combinations of them. Also, a consensus motif for *HuD*, `N-U/C-U-N-N-U/C-U-U/C` had been identified previously and as detailed in section 2.4.1 there is some support that Hu proteins bind very similar sequences. As detailed in Table 4.2, the *HuD* consensus sequence binding motif was clearly superior to other candidate motifs in explaining the observed affinity distribution.

Table 4.2: A string pattern regression approach to identify potential *HuR* sequence binding motifs. Of all candidate patterns, the simplified *HuD* binding motif NUUNNUUU performed best. I.e., the pattern separating the set of sequences so that the distribution of apparent dissociation constants in the set lacking and in the set matching the pattern gave a maximal T-value. The later experimentally identified *HuR* motif NNUUNNUUU was not included in the set of candidate motifs. It would, however, have outperformed all other motifs from the candidate set in string pattern regression. This demonstrates the major weakness of the string pattern regression approach we have used. The table displays the T- and W-statistics for the distribution of K_d^{app} values in the group of sequences matching respectively not matching the pattern. In contrast to the t-test [331], the Wilcoxon test has less power but does not assume normal distribution of the data [83].

Motif	t-test		Wilcoxon test	
	T-value	p-value	W-value	p-value
NUUNNUUU	14.000	1.263e-09	29.000	4.365e-02
AUUUA	1.746	2.121e-01	31.000	2.302e-01
UUAUUUAUU	2.001	8.045e-02	62.000	1.390e-02
AUUUAUUUA	1.224	2.760e-01	44.000	1.537e-01
UUAUUUAUUUAUU	1.964	8.106e-02	59.000	2.150e-02
NNUUNNUUU	42468.190	2.200e-16	42.000	9.622e-03

Experimental deduction of the HuR motif

Despite the fact that the *HuD* consensus motif N-U/C-U-N-N-U/C-U-U/C explained the observed sets of bound and non-bound RNA sequences well, no binding [1] of *HuR* to 8mer variants of this motif (U_8 as well as AUUAAUUU, CUUCCUUU, GUUGGUUU) was observed in homogeneous solution assays. In previous experiments we had found that *HuR* binds to U_{30} with high affinity. We therefore determined the minimal required length of oligoU for *HuR* binding. Remarkably, a one nucleotide elongation from U_8 to U_9 was sufficient for high affinity binding of *HuR* ($K_d^{app} = 0.97 \pm 0.19$nM). Hence, *HuR* requires a minimum of nine nucleotides for recognition. As detailed in Figure 4.1, we deduced that the *HuR* binding site is the 9mer N-N-U-U-N-N-U-U-U in a series of binding experiments with strategically designed RNA fragments.

[1] see footnote 2 on page 42

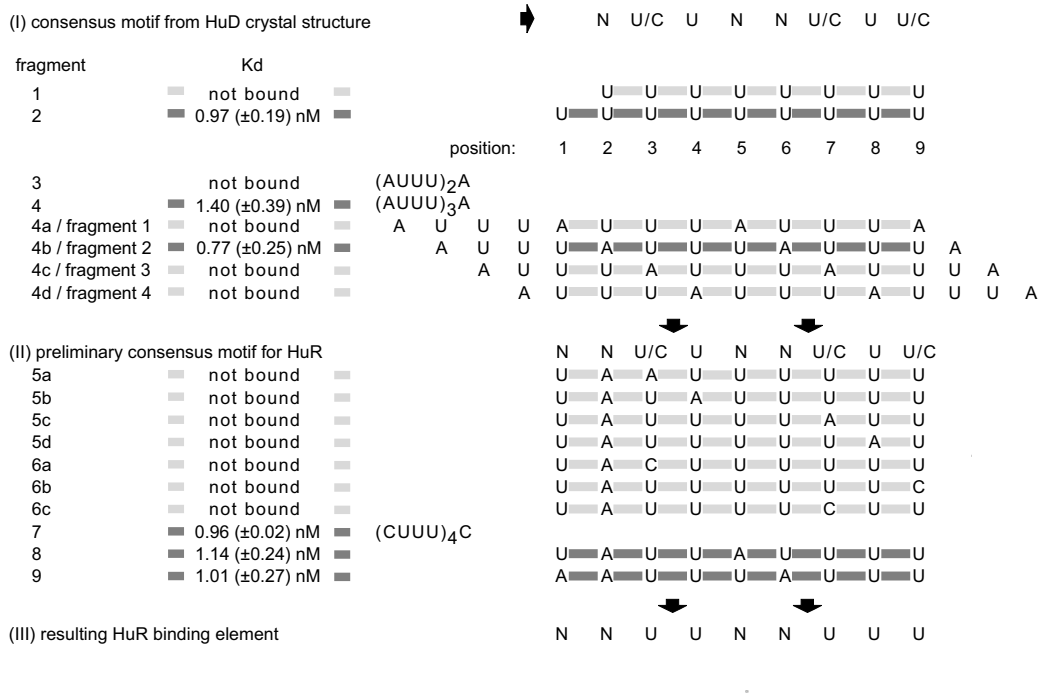


Figure 4.1: Experimental deduction of the *HuR* binding site. The experimentally determined affinity (K_d^{app}) of full length *HuR* to the individual synthetic RNA fragments (nucleotides connected with grey bars) is shown. The proposed and tested consensus motifs are given in bold letters. While the simplest variant of the consensus motif for *HuD*, $\mathtt{U}_8$ (frag. 1) was not recognized by *HuR*, an elongation by one nucleotide to $\mathtt{U}_9$ (frag. 2) was necessary and sufficient for high affinity binding. An influence of the fluorescent dye was excluded by competition experiments with unlabeled RNA fragments (data not shown). A 3'-terminally elongated *HuD* motif (9mer frag. 3) was not bound by *HuR*. However the high affinity binding to frag. 4 indicates that non-U nucleotides are tolerated within the *HuR* binding motif at certain positions. There are four possible "frames" of nine nucleotide motifs in $(\mathtt{AUUU})_3\mathtt{A}$, corresponding to fragments 4a to 4d. As only fragm. 4b is recognized, it sees that *HuR* binds to frame 2 within $(\mathtt{AUUU})_3\mathtt{A}$. This frame is consistent with the *HuD* motif, but 5'-terminally elongated by one uracil residue, suggesting the preliminary binding motif N-N-U/C-U-N-N-U/C-U-U/C. Fragments 5a-5d, 6a-6c, 7, 8 and 9 served to test the tolerance for non-U (exemplified by A) and C, respectively, at the depicted (bold) positions. In consequence, we propose that the *HuR* sequence binding motif is N-N-U-U-N-N-U-U-U. This interaction appears to follow an "all-or-nothing" mechanism (footnote 2 on the next page): While sequences with single mismatches are not recognized sequences fulfilling this motif are bound with high affinity and an invariable K_d of $0.96(\pm 0.48)$nM.

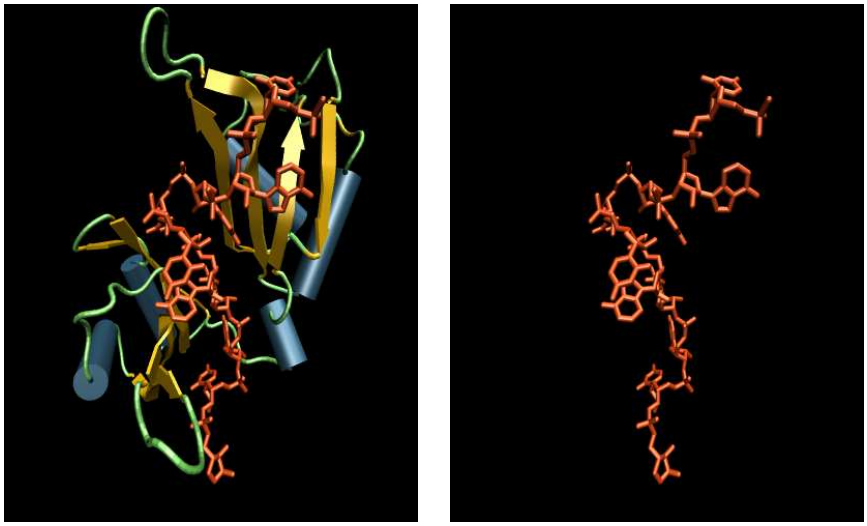
Validation of the sequence binding motif

This *HuR* binding motif is further supported by an *HuR* homology model (section 4.1.4) based on the structures of HuD (1FXL,1G2E) [360] and Sxl (1B7F) [148]. The motif is present in all validated *HuR* target mRNAs currently described in the literature (see Table A.1) and in 98.7 % of all 896 sequences in *ARED 1.0*, a database of *in silico* identified ARE mRNAs [20] (100 % in clusters I - IV, 97.9 % in cluster V). Additionally, the frequency of `NNUUNNUUU` containing sequences is significantly higher in the set of *HuR* targets than in the transcriptome (χ^2-test p-value < 0.00001). Importantly, *HuR* binding to 9mers follows an "all-or-nothing"[2] mechanism: `NNUUNNUUU` sequences are bound with an almost invariable K_d of 0.96($\pm$0.48)nM, while a single mismatch in this motif leads to a complete loss in the recognition.

4.1.2 RNA secondary structure dependence of HuR recognition

As stated above, *HuR* binds to its native target ARE sequences with an unexpectedly high variation in affinities. While the presence of the motif `NNUUNNUUU` allows to discriminate between bound and non-bound sequences, this variation in K_d^{app} values cannot be explained at the primary sequence level. In section 3.1 we have derived a quantitative model for RNA-ligand interactions which depend on the formation of a particular secondary structure element. This model predicts a dependence of the experimentally determined K_d^{app} values on the probability p_* of the required secondary structure element in the secondary structure ensemble of the RNA sequence. Also, we have presented a statistical test to judge whether a particular element is required for binding. Thus, we (*i*) might be able to explain the variation in *HuR*-RNA affinities by the dependence on a the formation of secondary structure element and (*ii*) might select the required element using the statistical test procedure if we were able to provide a reasonable set of candidate secondary structure elements.

[2] no binding refers to the detection limit of our assay: the complex formation becomes in-detectable at less than three standard deviations change in the anisotropy signal at the maximum *HuR* concentration of 5 - 10 μM in the assay, which is determined by the solubility limit of *HuR*. This corresponds to a K_d^{app} detection limit of approximately 100μM. In relation to the K_d of 1nM for binding of *HuR* to nine nucleotide `NNUUNNUUU` sequences we refer to this $>$ 100000 fold difference in the affinity as "all-or nothing"



(a) (b)

Figure 4.2: *HuD* bound to the AU-rich element of *TNF*α mRNA (1G2E) [360]. (a) shows the entire complex and (b) the RNA in the complex only. α-helices are colored blue, β-strands yellow, loops and coils green and the RNA is displayed in red. The conformation of the RNA suggests that HuD recognizes single stranded RNA. No intramolecular stacking is detectable in the RNA, rather RNA nucleotides stack with protein residues, which is typical for RRM-RNA interactions.

Candidate secondary structure elements

We have discussed in section 2.4.1 that Hu-proteins bind to RNA with three *RRM* domains (section 2.2.1). Current examples of RRM proteins are predominantly single stranded RNA (ssRNA) binding. Also, the structural organization of multi RRM proteins suggests that these proteins are restricted to ssRNA interaction. Finally, RRM-RNA interactions are stabilized predominantly by stacking interactions between nucleotides and amino acids, a mechanism which is fundamentally different from currently known double-stranded RNA-protein complexes.

Expectedly, the RNA structure is clearly single stranded in the co-crystals of *HuD* and AU-rich RNA fragments of 11nt length (Fig. 4.2). However, these RNA fragments are anyway too short to form stable secondary structures. It remains, thus, enigmatic whether Hu-proteins require fully single stranded RNA (fully single stranded in the region that interacts with the protein) or whether partially double stranded RNAs are bound as well.

Candidate secondary structure elements are, consequently, fully single

stranded NNUUNNUUU and partially double stranded variants of this sequence / structure motif. We may reduce the set of sensitive candidates even further, if we assume that HuR as an RRM protein will require at least a single stranded core motif. Considering, that hairpin loops of less than four unpaired bases are energetically highly unfavorable, we may restrict ourselves to elements, where only the terminal bases of NNUUNNUUU are base paired.

Because no data on the secondary structure preferences of *HuR* was available, we have shown experimentally that fully double stranded NNUUNNUUU motifs are not recognized[3] by determining the affinity between *HuR* and a target ARE RNA complexed with its exact reverse complementary sequence. However, unraveling experimentally whether partially double stranded RNA is bound is intricate if not impossible, which nicely demonstrates the power of the presented methods for the analysis of RNA-ligand binding mechanisms.

HuR binds to fully single stranded NNUUNNUUU

Following the procedure described in section 3.2, we stored the K_d^{app} values in the vector $\mathbf{K}_d^{app}$. For any candidate secondary structure Ξ, we computed the corresponding vector of probabilities $\mathbf{p}_*[\Xi]$ of structures in the ensemble where at least one NNUUNNUUU is in conformation Ξ. Subsequently, the empirical correlation coefficient r between $\mathbf{K}_d^{app}$ and $\mathbf{p}_*[\Xi]$ was calculated and the statistical significance of the correlation tested. Table 4.3 lists r, the test property and the corresponding probability that under the assumption of the null hypothesis, *no correlation*, an even bigger value of Student's t-distribution would have been observed (p-value).

Clearly, NNUUNNUUU is bound in all single stranded conformation. The detailed p_* data for single stranded NNUUNNUUU are given in Table 4.1. Figure 4.3 displays the data from Tab. 4.1 on a double-logarithmic scale. The dashed line is a regression of equ.(3.8) to the data with K_d as the only fitting parameter.

Prediction of K_d^{app} for *TNF*α ARE mutants

For any sequence matching the sequence motif, apparent dissociation constants may be predicted based on the knowledge of K_d and p_*, as detailed in section 3.2 . For NNUUNNUUU in single stranded conformation, $K_d = 0.118$nM (Figure 4.3). We have predicted K_d^{app} for three sequence variants derived from the *TNF*α ARE and for one sequence specifically designed to reveal the lowest possible *HuR* affinity by inducing not more than two point mutations in the native *TNF*α ARE sequence. The respective sequences are given in

[3] see footnote 2 on page 42

Table 4.3:]
Empirical correlation coefficient r of $\mathbf{K}_d^{app}$ and $\mathbf{1}/\mathbf{p}_*(\Xi)$, test property $\sqrt{(k-2)r^2/(1-r^2)}$ and the p-value of the test property for a two-tailed test and $k = 12$, given for selected candidate secondary structures Ξ for `NNUUNNUUU`. The critical value of Student's t-distribution for the given data and $\alpha = 0.01$ is 3.1639 [331, 270]. Candidate secondary structures are encoded using the constraining symbols of *RNAfold* [162] , where '`x`' stands for unpaired, '`|`' for paired, '`.`' for any, '`(`' for an opening base pair, '`)`' for a closing basepair. Secondary structure constraints which include a mandatory basepair are so rare in the secondary structure ensemble that $p_*(\Xi)$ is too close to zero to calculate $1/p_*(\Xi)$ and r is not defined. However, those candidate structures can be readily excluded without using the statistical test.

Ξ (`NNUUNNUUU`)	r	$\sqrt{(k-2)r^2/(1-r^2)}$	p-value
`xxxxxxxxx`	0.953	9.957	1.65e-06
`..xxxxx..`	0.366	1.245	2.42e-01
`.xxxxxxx.`	0.617	2.480	3.25e-02
`||.....||`	NA	NA	NA
`|||||||||`	NA	NA	NA
`((.....))`	NA	NA	NA
`(xxxxxxx)`	NA	NA	NA

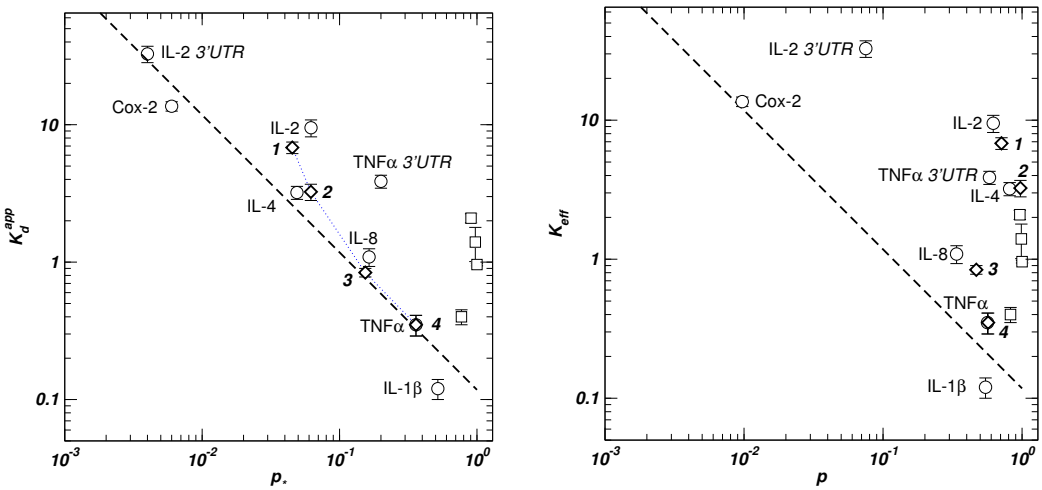


Figure 4.3: (a) Apparent dissociation constants for *HuR*-mRNA complexes at $23.5°C$ for natural ARE and UTR sequences (○), four artificial molecules (□), see Table 4.1 for details, and four designed mutants of the TNFα ARE (◊) plotted versus p_* of `NNUUNNUUU` in conformation `xxxxxxxxx` (all nucleotides single stranded). The dashed line marks $K_d^{\rm app} = K_d/p_*$ with $K_d = 0.118$. The value of K_d is obtained by non-linear regression with a correlation coefficient of 0.946 and $\chi^2 = 122.6$. The artificial repetitive sequences might be more regularly structured than expected from the secondary structure calculation. (b) The same K_d^{app} data plotted versus one of the test conformations given in Table 4.3, `..xxxxx..` (only the inner five nucleotides are constrained to single stranded conformation). The obvious dependence of K_d^{app} on p_* is lost, reflected by a correlation coefficient of 0.366.

Table 4.4: Variants of the *TNFα* ARE sequence used to compare predicted with experimentally measured affinities.

Name	Sequence
$TNF\alpha_{42}$	UGUGAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUACAGA
$TNF\alpha_{45}$	UGUGAUGAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUACAGA
$TNF\alpha_{mut}$	AUUAUUUAUUAUUUAUUUAUUAAAUAUUUAUUUA

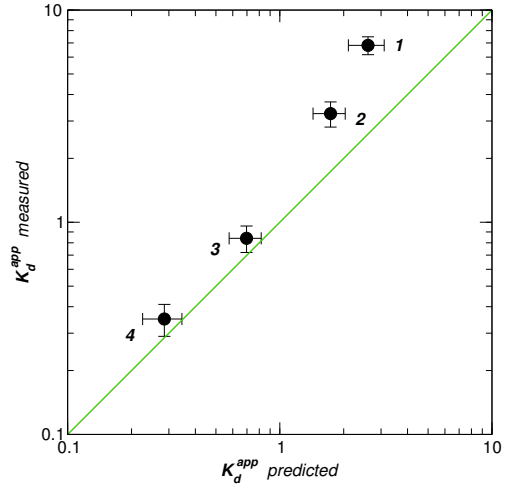


Figure 4.4: Comparison of predicted and measured values of K_d^{app} for 3 mutants of TNFα. Calculations have been performed for $T = 23.5°C$ at which the measurements were performed in ref. [244]. (1) *TNFα*$_{mut}$, (2) *TNFα*$_{45}$, (3) *TNFα*$_{42}$, (4) *TNFα* ARE.

Table 4.4, predicted versus experimentally measured affinities are compared in Figure 4.4.

Temperature dependence

For the analysis of the secondary structure dependence of *HuR*-RNA interaction we have so far always calculated p_* for the ambient temperature at which *in vitro* measurements were performed, 23.5°C. Eventually, we want to apply our model to *in vivo* systems and it is thus of interest how p_* changes in dependence of the temperature, particularly between the "*in vitro* temperature" and *in vivo* temperatures around 37°C. Free energies of RNA secondary structures are, of course, temperature dependent, in both their enthalpic and entropic contributions. p_* is moreover a property of the secondary structure

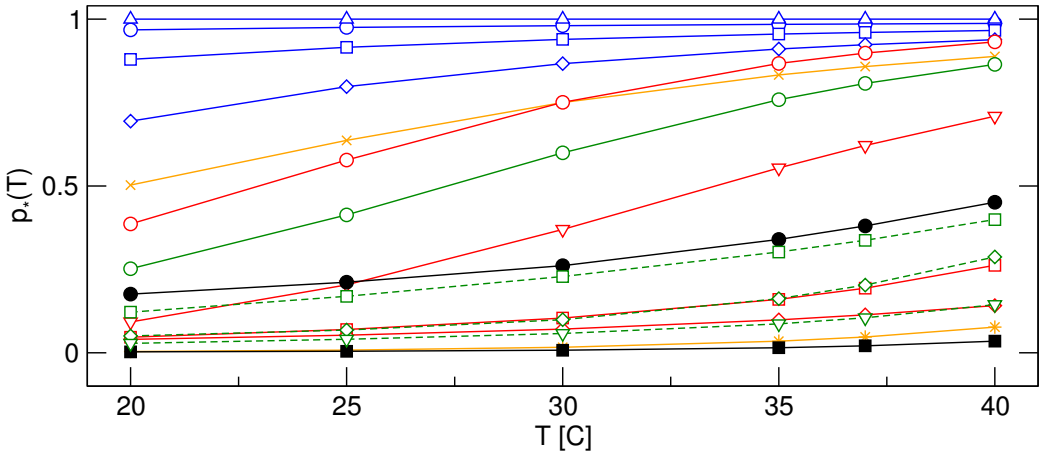


Figure 4.5: Dependence of p_* on temperature. p_* has been calculated for sequences in Table 4.1 for temperatures T between 20°C and 42°C. (AUUU)$_3$A (○)(AUUU)$_4$A(□),(AUUU)$_5$A(◇), (CUUU)$_4$C(▽), *IL1β* ARE (○), *IL2* ARE (□), *IL4* ARE (◇), *IL8* ARE (▽), *COX-2* ARE 1 (×), *COX-2* ARE 2 (∗), *TNFα* ARE (○), *TNFα* $_{42}$ (□), *TNFα* $_{45}$ (◇),*TNFα* $_{mut}$ (▽), *TNFα* 3'UTR (•), *IL2* 3'UTR (■).

ensemble and thus dependent on state occupancy, which is in itself temperature dependent as any Boltzmann distribution flattens with increasing temperature. We have calculated $p_*(T)$ for the set of sequences given in Table 4.1 for temperatures between 20°C and 42°C; the resulting curves are given in Figure 4.5.

4.1.3 *In vivo* relevance of secondary structure control

So far, we have developed a model for RNA-protein interactions, which is based on – and explains well – experimentally observed data *in vitro*. However, in the *HuR* study, we set out to explain the specificity puzzle of mRNA stability regulation *in vivo*. It is a valid question, whether the dependence of *HuR*-RNA recognition on secondary structure *in vitro* is of any relevance in a cellular environment, where physicochemical properties (ion concentrations, viscosity of solvent, more than one protein ligand for the RNA, *etc.*) are certainly different from the *in vitro* system we have used.

A challenging *in vivo* test case for our model is to explain the phenotype of *New Zealand white (NZW) mice*. NZW mice suffer from a systemic lupus erythematosus like phenotype caused by a deficiency in *TNFα*. Previous studies linked the defect to a trinucleotide insertion in the *TNFα* 3'UTR, in proximity to, but outside of the ARE [174]. It was possible to show that the low levels of *TNFα* are due to an aberrant regulation at the post tran-

scriptional level of gene expression [235]. Complex formation between *TNFα* mRNA and *HuR* is reduced and leads to reduced *TNFα* mRNA stability.

With the understanding of *HuR*-RNA recognition at that time it was not possible to explain why *HuR* binding would be affected by an insertion of nucleotides outside of its binding site. If we apply our model of *HuR* binding, we observe that the insertion leads to a decrease in p_* of `NNUUNNUUU` in the ARE sequence from 0.33 for the wild type (WT) to 0.19 for the NZW mouse (p_* calculated for 37°C). The ratio of $p_{*,NZW}/p_{*,WT} = 0.59$ corresponds excellently to the ratio of *HuR* complex formation between NZW phenotype and wild type of 0.65.

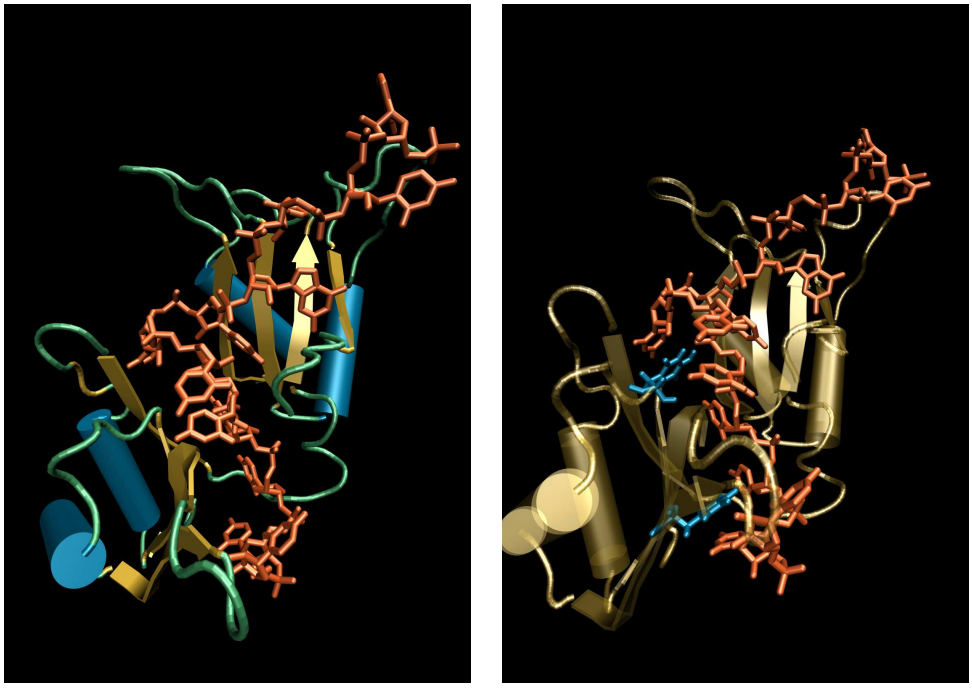
A similar effect has been observed for porcine *hsp70.2* [315], where the mutant mRNA is approximately 2.5 fold more stable than the wild type. However, in this case it is not clear whether the stabilization can be attributed to *HuR*. If this was the case, our model would again quantitatively explain the observed effect ($p_{*,WT}/p_{*,MUT} = 0.33$).

Consequently, we have some evidence that our *in vitro* derived model of *HuR*-RNA recognition is relevant *in vivo* as well. More evidence for the *in vivo* relevance of our model will be provided in chapter 4.2.2, where we show that predicted modifier RNAs are functional in cellular lysates.

4.1.4 On the 3D structure of *HuR*

So far, no high-resolution three-dimensional structure data is available for *HuR*. However, such data is available for RNA complexes with shortened variants of the closely related Hu family members *HuC* [167] and *HuD* [360] and for other ELAV family members like *sex lethal* [205, 206]. Based on these data we constructed homology models for *HuR* binding to the *TNFα*-ARE RNA. As expected for RRM proteins these models exhibit a single stranded RNA conformation with characteristic stacking interactions between RNA bases and protein amino acid residues (Figure 4.6).

Unfortunately, no structure data is available for full length Hu proteins, including the third RRM domain, which role is unclear and seems to be different for the individual Hu proteins (section 2.4.1 on page 23). We may make some inferences on the third domain by comparison of RNA interaction data for full length *HuR* and a variant including only the first two RRMs (data not shown). It seems that the third domain increases affinity and specificity of *HuR*-RNA interactions. We have no indications that the third domain binds to poly(A), which has been reported for *HuD* [227]. Though we have identified a nonamer as the binding motif of full length *HuR*, we cannot fully exclude that the third domain interacts with RNA nucleotides outside of the nonamer. However, from the homology model data, there is



(a) (b)

Figure 4.6: Homology model of *HuR* based on the structures of *HuD* and *Sxl*. (**a**) Cartoon model of the protein, colored by secondary structure. The RNA displays a typical conformation for RRM–RNA interactions and is clearly single stranded. (**b**) Another characteristic feature of RRM-RNA binding are stacking interactions between amino acid sidechains (highlighted in blue) and RNA nucleotides.

also no reason to exclude that all three RRM domains interact with only nine consecutive RNA nucleotides.

4.2 *HuR*-RNA modifier RNAs

We have derived a model for the change in RNA-ligand affinities when the RNA is hybridized to small "modifier RNAs" in section 3.3. Here we describe the application of the computational methods to the *HuR*-ARE system for the design of modifier RNAs and their experimental validation *in vitro* and in cellular lysates.

The potential value of *HuR* modifier RNAs goes beyond the validation of our methods. Any means to specifically up- or downregulate *HuR* mRNA association may provide a mechanism which solves the specificity puzzle in mRNA stability regulation. Moreover, given the impressive list of disease associated *HuR* target mRNAs (Table A.1), such a means is a starting point for mRNA stability based therapeutic intervention.

4.2.1 Modifier RNA design

Modifier RNAs (modRNAs) are designed based on equation (3.14). Inspired by siRNAs and miRNAs we selected a modifier RNA length of 20nt. As detailed in section 3.3, the effect of hybridization on p_* of the target RNA is evaluated for any possible exactly reverse complementary modRNA of the given length, resulting in a *modifier profile*, (e.g. Fig. 4.7).

The effect of hybridization is evaluated using either the exact partition function, the truncated partition function or a sampling approach. If not stated differently, the data given below have been produced using the exact partition function approach. Modifier profile calculations have been performed for 23.5°C, the ambient temperature for *in vitro* testing. We present here modRNA design for the cytokine mRNAs of interleukin 2 (*IL2*) and tumor necrosis factor alpha ($TNF\alpha$).

IL2

Interleukin-2 (*IL2*), also known as T-cell growth factor, is a powerful immunoregulatory lymphokine. It is produced upon stimulation by mature T-cells and constitutively by certain T-cell lymphoma cell lines. *IL2* acts as a growth hormone for both B and T lymphocytes..

Modifier design for the 280nt *IL2* mRNA 3'UTR with two `NNUUNNUUU` motifs takes about 30min on a single desktop CPU. The modifier profile is given in Figure 4.7. modRNAs which maximize $p_*[k]$ – we will call them *openers* for the *HuR* system as they open the binding site for the protein – are restricted to clusters, positioned around the *HuR* binding motifs. Due to the low basis p_* there is no potential for the design of modRNAs which minimize

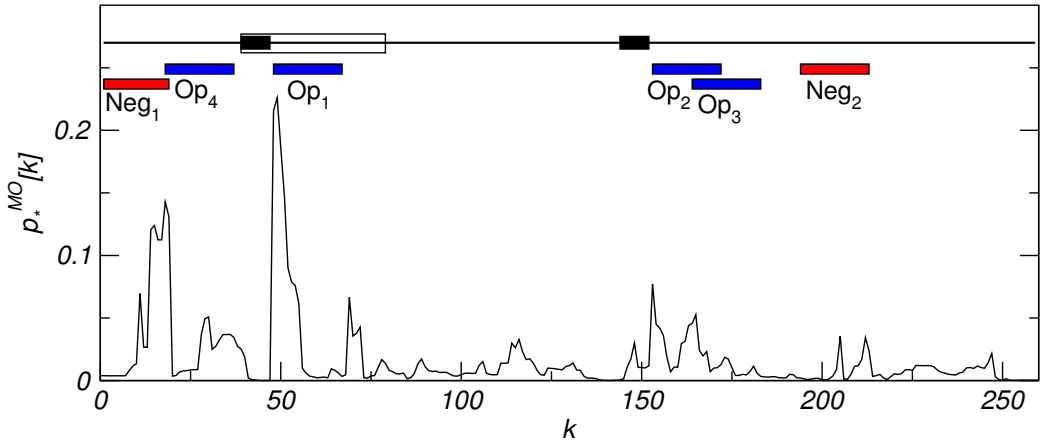


Figure 4.7: Modifier profile for the 3'UTR sequence of human *IL2* mRNA for oligonucleotides of length $N_0 = 20$. The binding motif for *HuR* is the sequence `NNUUNNUUU` in an open conformation, $\Xi =$ '. . . . . '. The position k is the start position of the modifier bound region in the target RNA, $p_*^{MO}[k]$ is the probability of *HuR* accessible sequences in the secondary structure ensemble if a modifier is hybridized to position k. The ARE is marked as an open box, *HuR* binding motifs are indicated by black filled boxes. Modifiers of significant impact on p_*^{MO} are restricted to few positions mainly in proximity of the *HuR* binding sites. At several positions, hybridization of an oligonucleotide does not influence the accessibility of *HuR* motifs, which allows to design negative controls. Four openers (Op_1, Op_2, Op_3, Op_4) and two negative controls Neg_1,Neg_2), which were selected for further experimental analysis, are indicated by blue and red boxes, respectively.

Table 4.5: Modifier oligonucleotides for IL2 mRNA (NM_000586) selected for further experimental analysis.

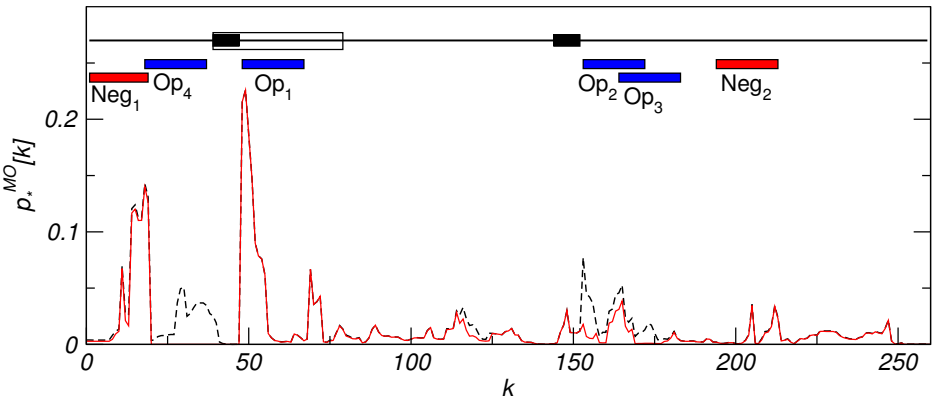
Name	Position	Sequence
Op_1	804-823	AATATAAAATTTAAATATTT
Op_2	909-928	TAGAGCCCCTAGGGCTTACA
Op_3	920-939	TGAAACCATTTTAGAGCCCC
Op_4	774-793	AAGGCCTGATATGTTTTAAG
Neg_1	757-775	AGTGGGAAGCACTTAATTAC
Neg_2	950-969	CATAATAATAAATATTTTGG

$p_*[k]$ – we will call those modRNAs *closers* in the *HuR* system as they close the binding site. At several positions k, hybridization of a modRNA does not change $p_*[k]$ significantly, which allows the design of negative control modRNAs.

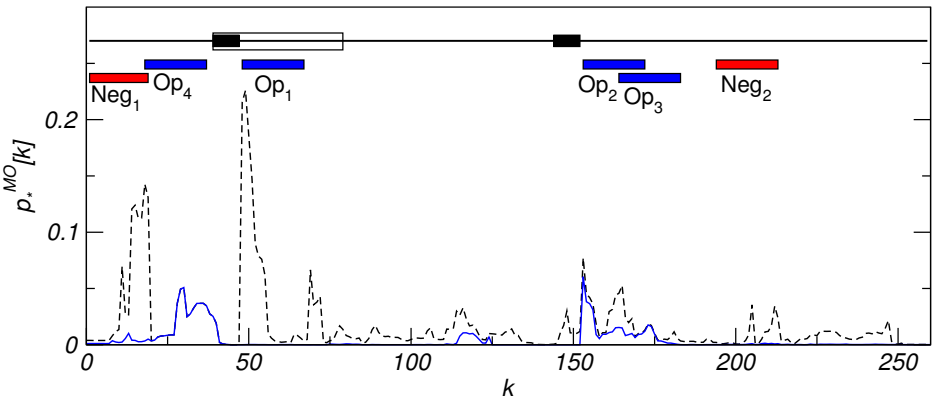
As the openers cluster around the *HuR* binding sites it can be assumed that they act predominantly on local secondary structures. This may be a prerequisite for a later application in cellular systems, as the global secondary structure may be fundamentally different when the mRNA forms a ribo-nucleoprotein. Local structures, however, are less influenced by the plethora of *trans*-acting factors. If openers act on local secondary structures they should open individual HuR binding sites specifically. To test this, we repeated the modifier profile calculation, however, for each of the binding sites separately. Figure 4.8 demonstrates that opener action is confined to opening the adjacent biding site for Op_1, Op_2 and Op_4. Only Op_3 acts primarily on the accessibility of the far NNUUNNUUU match.

The fact that modRNAs act predominantly locally has another important consequence for computation. Global secondary structures change with sequence elongation, whereas this is usually only true for local secondary structures involving terminal nucleotides. Thus, approximating a modifier effect profile for an mRNA by the modifier effect profile of a subsequence, e.g. the 3'UTR is valid if local secondary structures are of interest. Prediction of modRNAs which act on long range base pairs may be erroneous when approximated by a subsequence. Figure 4.9 displays an aligned overlay of the modifier profile for *IL2* 3'UTR and the modifier effect profile for the whole *IL2* mRNA. Obviously, openers – except Op_3 – identified for the UTR are also valid for the entire mRNA.

Openers selected for further experimental testing are listed in Table 4.5.



(a) binding site 1



(b) binding site 2

Figure 4.8: Modifier profile calculated for for the first (**a**) and second (**b**) binding site of *HuR* individually in the human *IL2* mRNA 3'UTR for $23.5°$C and an opener length of 20 nucleotides. Openers Op_1 and Op_4 act locally on the accessibility of binding site one, Op_2 locally on binding site two. Op_3 – though hybridizing to a region in proximity of binding site two – acts on the accessibility of the distant first binding site, thus interfering with long range base pairs. Hybridization of negative control oligonucleotides Neg_1,Neg_2 is expected not to influence the accessibility of either binding site. Please refer to the caption of Figure 4.7 for a description of the opener and closer symbols in the figure.

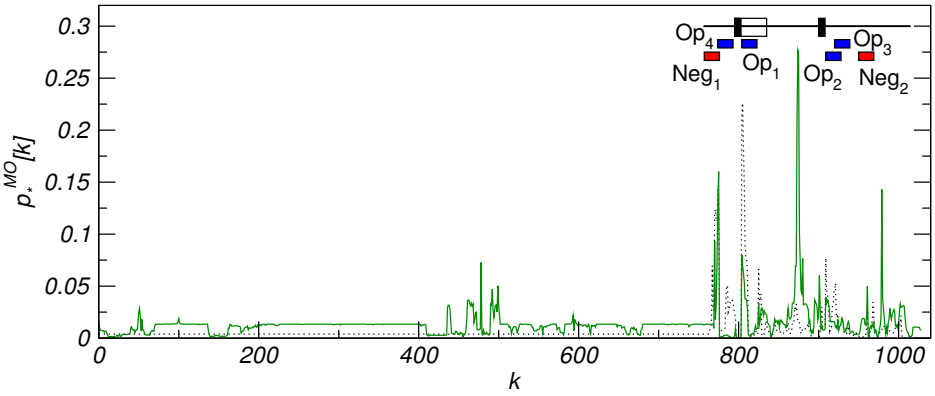


Figure 4.9: Alignment of *IL2* mRNA and 3'UTR modifier profiles. The modifier profile for the *IL2* 3'UT is plotted as a dotted black line, the mRNA profile as a solid green line. Both profiles were calculated for 23.5°C. Please refer to the caption of Figure 4.7 for a description of the opener and closer symbols in the figure.

TNFα

Tumor necrosis factor α (*TNFα*) is a multi-functional pro-inflammatory cytokine that belongs to the tumor necrosis factor superfamily. It is mainly secreted by macrophages. The cytokine can binds to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. *TNFα* is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer.

We performed modifier RNA prediction for the *TNFα* mRNA at 23.5°C and 37°C (Figure 4.10). With approximately 1600 nucleotides in length and five *HuR* binding motif matches, computation was significantly more intensive than for the *IL2* 3'UTR or mRNA and took approximately one day on 30 Xeon CPUs. The modifier effect profile allows to design openers and closers in distance and proximity to the `NNUUNNUUU` matches. Openers and closers selected for synthesis and further experimentally analysis are listed in Table 4.6.

We have again performed opener effect calculation for individual `NNUU-NNUUU` matches to further investigate the modRNA mode of action (Figure 4.11). $\mathsf{Op_A}$, $\mathsf{Op_B}$ and $\mathsf{Op_E}$ act distantly on the last *HuR* binding site. Closer $\mathsf{Cl_C}$ acts remotely on the second third and fourth binding site. $\mathsf{Op_F}$ acts locally on first, third fourth and last binding site, $\mathsf{Op_H}$ locally on first second and third. Finally, closer $\mathsf{Cl_G}$ reduces the accessibility of first second and third binding site by local interactions.

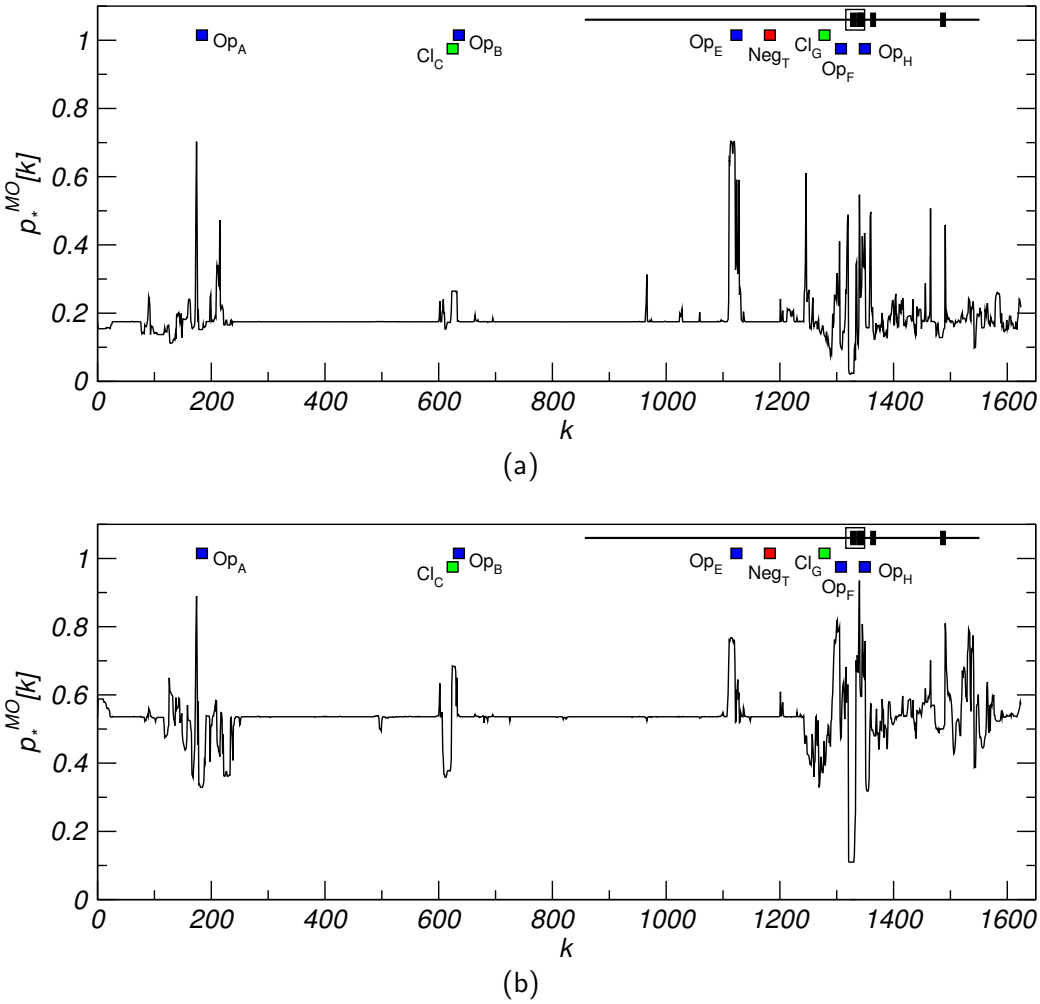


Figure 4.10: Modifier profile for *TNF*α mRNA, for the binding sequence motif of *HuR*, NNUUNNUUU, in fully single stranded conformation, for modifier oligonucleotides of length $N_0 = 20$ and a temperature of (**a**) $23.5°$C and (**b**) $37°$C. The position k is the start position of the modifier bound region in the target RNA, $p_*^{MO}[k]$ is the probability of *HuR* accessible sequences in the secondary structure ensemble if a modifier is hybridized to position k. The 3'UTR is marked as a black line, the ARE as an open box, *HuR* binding motifs are indicated by black filled boxes. Modifiers of significant impact on p_*^{MO} are spread over the whole mRNA, but accumulate in proximity of the *HuR* binding motifs. At many positions, hybridization of an oligonucleotide does not influence the accessibility of *HuR* motifs, which allowed to design a negative control oligonucleotide ($\mathrm{Neg_T}$, indicated by a red box). Five openers ($\mathrm{Op_A}$, $\mathrm{Op_B}$, $\mathrm{Op_E}$, $\mathrm{Op_F}$ and $\mathrm{Op_H}$) and two closers ($\mathrm{Cl_C}$ and $\mathrm{Cl_G}$), were selected for synthesis and experimental analysis and are indicated by blue and green boxes, respectively.

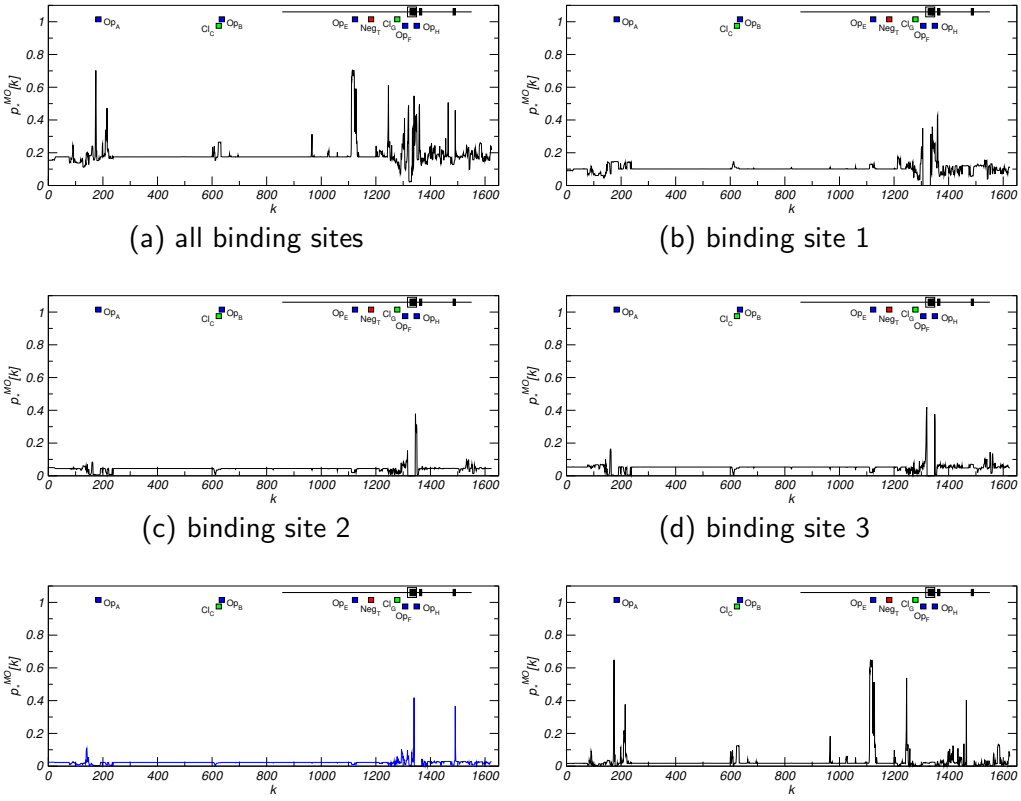


Figure 4.11: Modifier effect profile for *TNF*α mRNA for individual binding sites, $23.5°$C and a modifier length of 20 nucleotides. Op_A, Op_B and Op_E act distantly on the last *HuR* binding site. Closer Cl_C acts remotely on the second, third and fourth binding site. Op_F acts locally on first, third, fourth and last binding site, Op_H locally on first second and third. Finally, closer Cl_G reduces the accessibility of first, second and third binding site by local interactions. Please refer to the caption of Figure 4.10 for a description of opener and closer symbols in the plot.

Table 4.6: Modifier oligonucleotides for *TNF*α mRNA (NM_000594) selected for further experimental analysis.

Name	Position	Sequence
Op_A	174-193	TCGGCCAGCTCCACGTCCCG
Op_B	626-645	TCTGGTAGGAGACGGCGATG
Cl_C	615-634	ACGGCGATGCGGCTGATGGT
Op_E	1114-1133	ATTCCAGATGTCAGGGATCA
Op_F	1298-1317	ATCACAAGTGCAAACATAAA
Cl_G	1269-1288	CTGGCTCCATGGGGAGGGCT
Op_H	1340-1359	CATTCATCTGTAAATAAATA
Neg_T	1173-1192	TGAGGTCTTCTCAAGTCCTG

4.2.2 Experimental validation of modifier RNAs

Selected modifier RNAs were validated experimentally *in vitro* and in cellular lysates. For the cellular testing we have selected a biological system with the rare property that transcriptional and post transcriptional regulation are largely functionally separated. We will initially briefly describe this system.

IL2 regulation in T-cell activation

Regulation of T-cell activity is a process of major importance for an efficient cellular immune response. When a T-cell recognizes an antigen on an antigen presenting cell (APC) with its T-cell receptor (TCR)it is only activated if a second co-stimulatory signal is presented by the APC as well. This signal is transduced by a receptor called *CD28*. If the T-cell recognizes antigen from an APC lacking the co-stimulatory signal it is not activated, conversely, it falls into a state of hypo-responsiveness called *T-cell anergy*.

The expression of *IL2* is diagnostic for T-cell activation. *IL2* mRNA expression is triggered transcriptionally by T-cell receptor signaling (inducible by anti *CD3* antibody). However, this does not lead to a remarkable increase of *IL2* at the protein level. Only the presence of the co-stimulatory signal leads to a dramatic rise in *IL2* protein expression. This signal can be mimicked by anti *CD28* antibody. In contrast to the TCR signal, the *CD28* signal acts predominantly via post-transcriptional mechanisms, mainly on *IL2* mRNA stability. The *CD28* signal promotes *IL2* mRNA stabilization via several pathways, including an AU-rich element located in the 3'UTR.

All-together, this makes *IL2* regulation in T-cell activation a perfect model system for modifier RNAs. Anti CD3 stimulation allows to trigger transcription of *IL2* thus providing sufficient concentrations of *IL2* mRNA to study stability. Modifier oligonucleotides can be tested in this environment for their potential to mimic the *CD28* response on *IL2* mRNA stability.

Validation of *IL2* modifier RNAs *in vitro*

IL2 specific opener oligonucleotides were validated by measuring the apparent dissociation constant K_d^{app} between *IL2* mRNA 3'UTR and *HuR* using a 1D-FIDA assay (appendix A.2). The tested openers $\mathsf{Op_1}$,$\mathsf{Op_2}$ and $\mathsf{Op_3}$ decreased K_d^{app} as predicted, i.e. increased the apparent affinity between *HuR* and *Il2* 3'UTR. An *IL2* specific negative control oligonucleotide Neg, predicted to hybridize to the mRNA without changing p_*, did not change K_d^{app} significantly (Figure 4.12). All tested openers act in a concentration dependent manner, which is discussed in more detail in section 4.2.3.

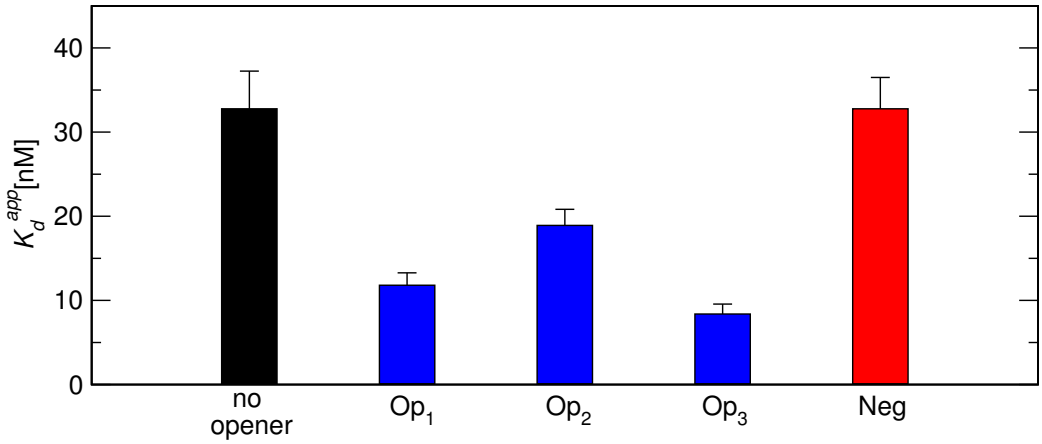


Figure 4.12: Validation of *IL2* specific openers *in vitro*. All three tested opener oligonucleotides enhance the *HuR* association with *IL2* mRNA 3'UTR, reflected by a decrease in the apparent dissociation constant (Op_1: $K_d^{app} = 11.80 \pm 1.48$nM, Op_2: $K_d^{app} = 18.91 \pm 1.91$nM, Op_3: $K_d^{app} = 8.38 \pm 1.18$nM, without opener $K_d^{app} = 32.77 \pm 4.48$nM, *IL2* 3'UTR at 0.5nM, Op_1 and Op_3 at the concentration optima of 1.56 and 5nM respectively, Op_2 at 25nM). Hybridization of the negative control oligonucleotide Neg leaves K_d^{app} unaffected ($K_d^{app} = 32.77 \pm 3.72$nM, Neg at 25nM).

Validation of the opener effect on endogenous *HuR*-mRNA complex formation

RNA secondary structure formation is strongly dependent on physicochemical properties of the environment, like pH, temperature, ion concentrations, particularly of bivalent cations or protein occupancy. Thus, having positively validated openers *in vitro*, does not necessarily allow to expect that openers are functional in the cell. E.g. might secondary structures form differently, or might openers have to compete with cellular RNA ligands for the same binding sites. Also, all other mRNA ligands may have an opening or closing effect on *HuR* biding sites. Consequently, an obvious next step is to test whether openers increase the complex formation between endogenous *HuR* and the target mRNA under cellular conditions. We were able to show that both tested openers Op_1 and Op_2 increase complex formation between *HuR* and *IL2* mRNA in lysates of human peripheral blood mononuclear cells (PBMC), Figure 4.13. The observed effect was dependent on the opener concentration and negative controls like the *IL2*-specific Neg and the *TNFα* specific Op_T did not change complex formation significantly. In lysates of PBMC stimulated with anti *CD3* or anti *CD3* and anti *CD28* antibodies, both openers again increase *IL2* mRNA association in dependence of opener

concentration. Both openers applied at 2.5μM to anti *CD3* treated cells, quantitatively mimic the effect of anti *CD28* stimulation without opener addition. This suggests that the cellular response to anti *CD28* stimulation may involve an modRNA like mechanism to promote *HuR* dependent *IL2* mRNA stimulation (see also Figure 5.1 on page 73).

Openers block the degradation of cytokine mRNAs

Finally, we investigated whether increased *HuR-IL2* mRNA complex formation leads to the expected increase in transcript stability. The concentration for *IL2* mRNA was quantified over time in presence and absence of openers or negative controls, Figure 4.14. *IL2* mRNA is rapidly degraded in absence of any opener ($\tau_{1/2} = 10.9 \pm 2.27$ min), while the control mRNA of a non-ARE gene is stable throughout the total observation time of 70 min. In presence of opener $\mathsf{Op_1}$ ($c = 10\mu$M) *IL2* mRNA degradation is completely halted over a period of 15 min, a time-point at which untreated *IL2* mRNA is already degraded to more than 80%. The subsequent decay after 15 min incubation time is also slowed down significantly compared to the untreated sample. At higher concentrations, opener $\mathsf{Op_1}$ blocks degradation over the entire time of observation. Opener $\mathsf{Op_2}$, which targets a different *HuR* binding site than $\mathsf{Op_1}$ exhibits a similar stabilizing effect. Hybridization of the negative control Neg did not change the degradation kinetics significantly ($\tau_{1/2} = 6.82 \pm 1.96$ min).

The stability of other ARE containing HuR targets was monitored to ensure that the observed opener effect is target specific. Neither *TNFα* nor *IL1β* mRNA degradation was affected by the presence of *IL2* specific openers 4.15. Additionally, a *TNFα* specific opener, $\mathsf{Op_T}$, displayed similarly specific stabilization of *TNFα* mRNA without influencing the stability of *IL2* mRNA 4.15a.

If openers exert their function as expected, the stabilizing effect has to be dependent on the presence of functional *HuR*. We therefore monitored opener induced *IL2* mRNA stabilization in the presence of a neutralizing monoclonal anti-*HuR* antibody. In consistence with the supposed mode of action of the opener oligonucleotides, *IL2* mRNA decay is not delayed by openers in presence of the *HuR* neutralizing antibody.

4.2.3 The concentration dependence of the modifier RNA effect

In section 3.3, we have derived an approximate expression for the dependence of K_d^{app} on the concentration of the modifier RNA (equation 3.16). This equa-

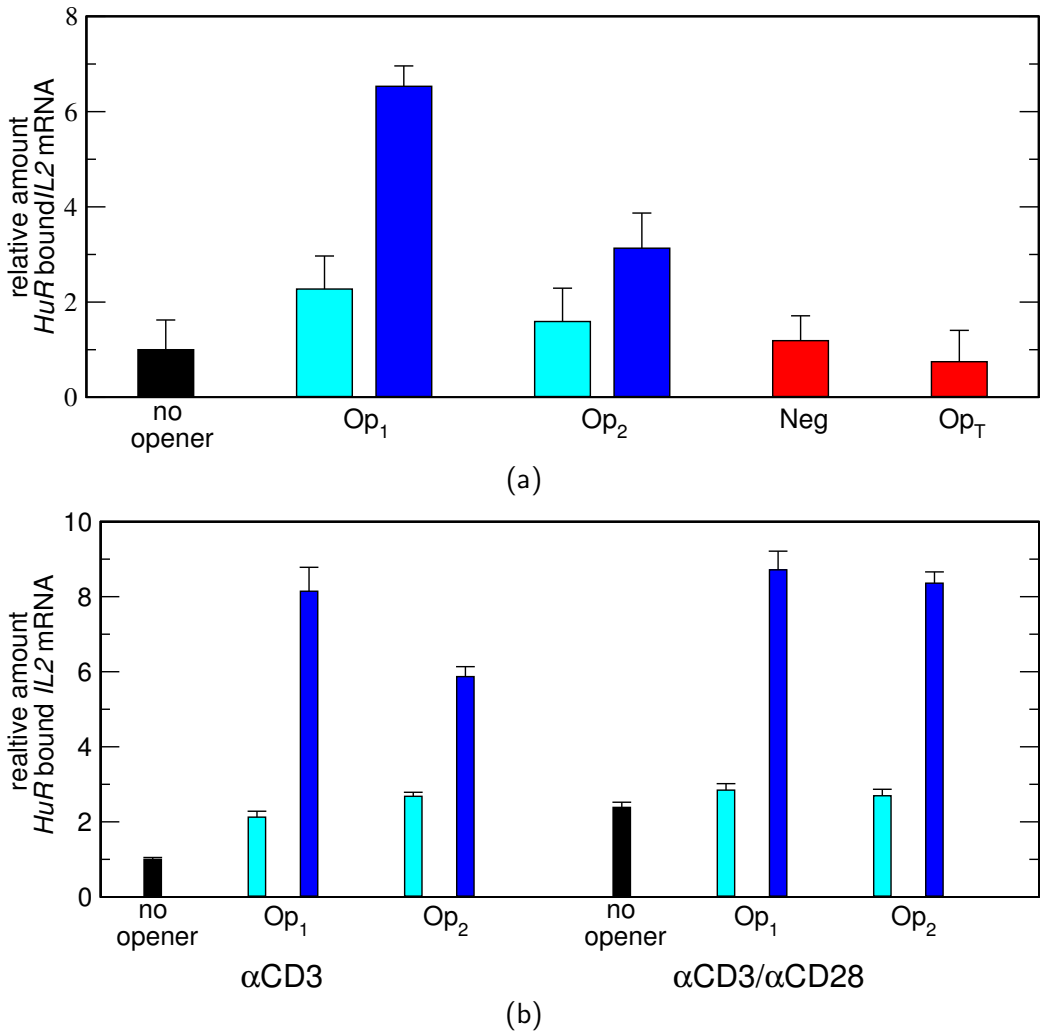


Figure 4.13: *IL2* mRNA openers increase endogenous *HuR-IL2* mRNA association. *HuR*-mRNA complexes were co-immunoprecipitated from lysates of human peripheral blood mononuclear cells.without or after treatment with opener or negative control oligonucleotides Op_1, Op_2, Neg and Op_T. *HuR* bound *IL2* mRNA was quantified by real-time RT-PCR. *IL2* mRNA amounts were normalized to levels in untreated cells (black bar). Openers were added to 2.5μM (cyan bars) or 10μM (blue bars), negative controls Neg and Op_T to 10μM concentration. (**a**) In lysates of otherwise untreated cells, Op_1 and Op_2 boost *HuR*-mRNA complexation to up to 6.5- or 3.1-fold higher levels, respectively, while the negative controls do not increase *HuR*-mRNA complex formation significantly. (**b**) In lysates of PBMC activated with anti *CD3* or anti *CD3* and anti *CD28* antibodies, both openers again increase *IL2* mRNA association. Interestingly, both openers applied at 2.5μM to anti *CD3* treated cells, quantitatively mimic the effect of additional anti *CD28* stimulation; potentially anti *CD28* stimulation involves mechanism similar to modRNA hybridization.

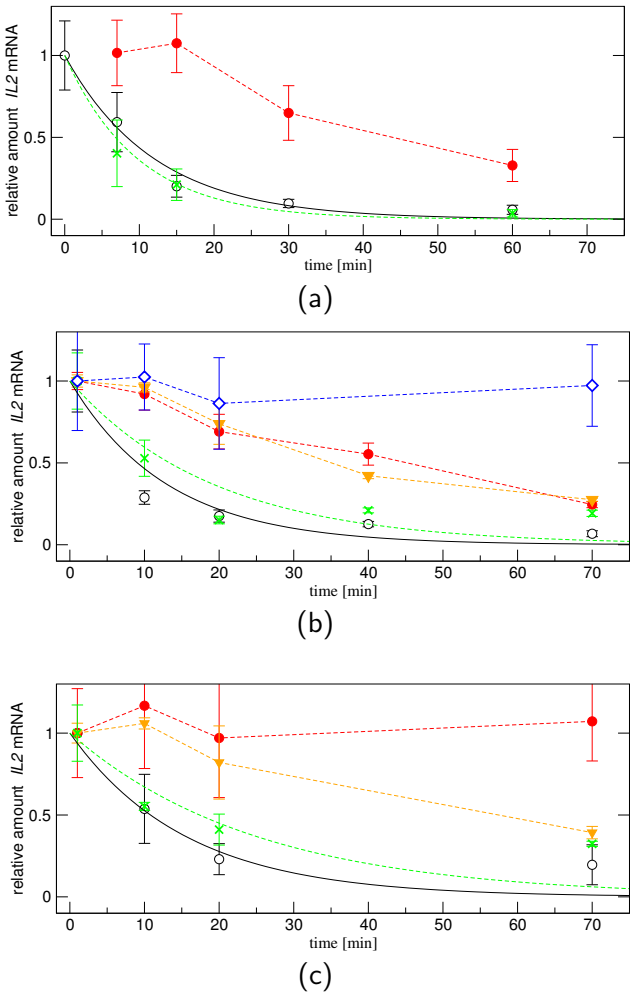


Figure 4.14: *IL2* openers inhibit *IL2* mRNA degradation in cellular lysates. Degradation of *IL2* mRNA was monitored in human PBMC lysates. Upon the addition of Mg^{2+} $(t = 0)$, the amount of remaining *IL2* mRNA was quantified over time ny quantitative real-time RT-PCR in the presence and absence of openers Op_1, Op_2 and or negative control Neg at **(a)** 10μM (i.e. $2f$mol per cell), **(b)** 25μM (i.e $5f$mol per cell) and **(c)** 40μM (i.e. $8f$mol per cell). All data represent averages of at least three independent samples and were normalized to the levels at the time point $t = 0$. The data were fitted to a single exponential decay (no opener displayed by a solid line, negative control by a dashed line). *IL2* mRNA is rapidly degraded with a half-life of $\tau_{1/2} = 10.9 \pm 2.27min$ without any opener (○), as well as in presence of 10μM negative control Neg (× , $\tau_{1/2} = 6.82 \pm 1.96min$).
Addition of the openers Op_1 (●) or Op_2 (▼) promotes a transient *IL2* mRNA stabilization in a concentration dependent manner. At 40μM, Op_1 blocks the degradation over the entire incubation time of $70min$, Op_2 shows a similar stabilizing effect, although it targets another HuR binding site. *EF-1*α, a non-ARE mRNA, remains stable over the entire observation time (◊).

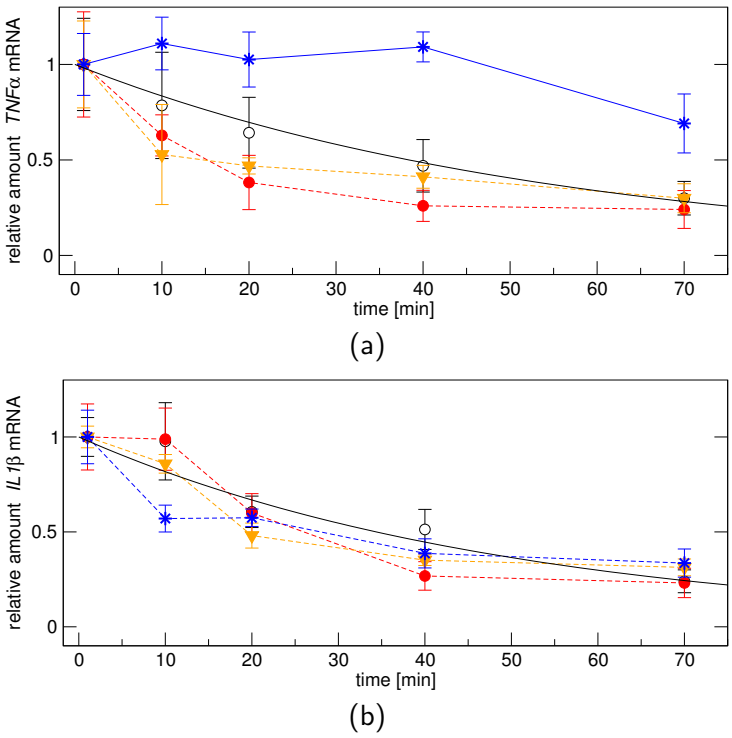


Figure 4.15: Opener oligonucleotides promote specific ARE mRNA stabilization. The specificity of the opener-induced mRNA stabilization was tested by monitoring the *IL2* openers' effect on the decay of other ARE-containing cytokine mRNAs, **(a)** *TNF*α and **(b)***IL1*β. *TNF*α and *IL1*β degradation are characterized by a half-life of $\tau_{1/2} = 36.0 \pm 2.2$ min (**a**, $\circ$) and $\tau_{1/2} = 37.6 \pm 5.6$ min (**b**, $\circ$) respectively. In presence of either of the *IL2* specific openers Op_1(●) or Op_2 (▼), both at a concentration of 25μM, neither *TNF*α nor *IL1*β mRNA decay is altered. Under the same conditions, an opener designed for *TNF*α (Op_T, $*$) specifically stabilizes the *TNF*α mRNA with affecting *IL1*β mRNA concentrations.

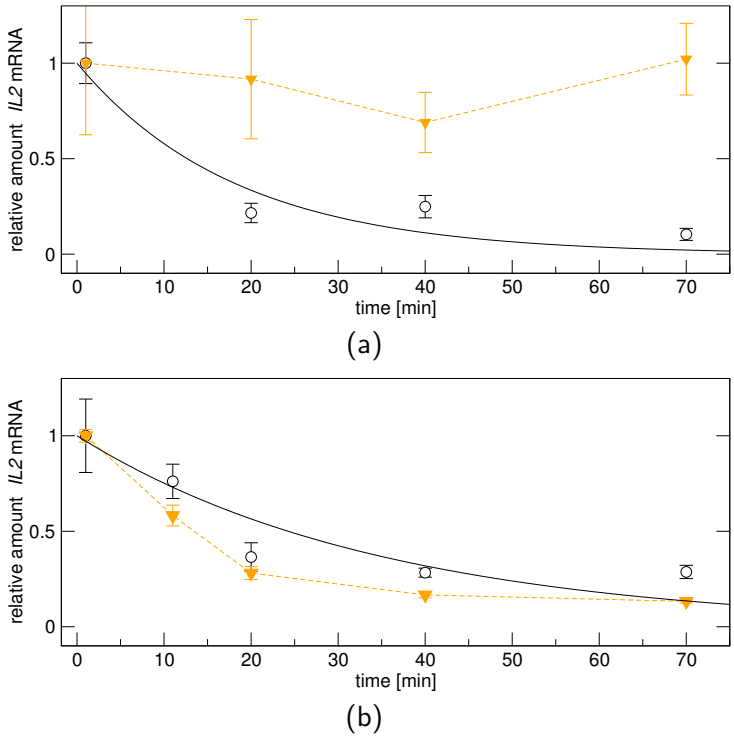


Figure 4.16: Dependence of the opener effect on *HuR*. Opener-mediated stabilization can be neutralized with anti-*HuR* antibody. *IL2* mRNA decay **(a)** in the presence of an *HuR* specific antibody, **(b)** control experiment without antibody. The stabilization of the *IL2* mRNA induced by opener Op_2 (○ without opener; ▼ opener Op_2 at 40μM) is neutralized in the presence of the monoclonal antibody.

tion describes the hyperbolic transition from K_d/p_*^M to K_d/p_*^{MO}. Figure 4.17 displays such an anticipated hyperbolic transition with increasing amounts of *IL2* opener $\mathsf{Op_3}$. For other openers, such as the *IL2* specific $\mathsf{Op_1}$ or the *TNFα* specific $\mathsf{Op_F}$, we find that large opener concentrations lead again to an increase in K_d^{app} (Figure 4.17). This effect could be explained by opener oligonucleotides binding at multiple sites. For a *TNFα* specific closer ($\mathsf{Cl_G}$) a concentration dependent effect on *TNFα* mRNA-*HuR* affinities, consistent with equation (3.16), was observed.

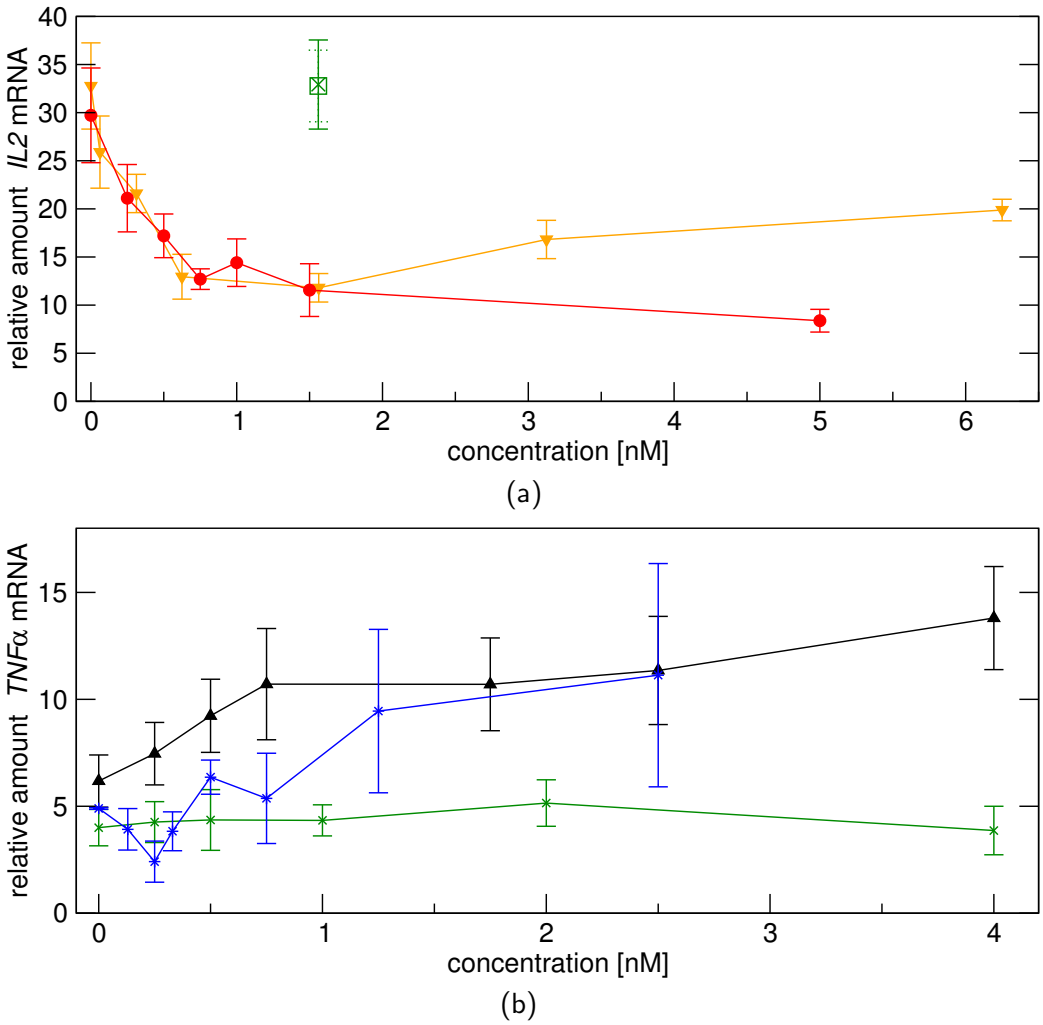


Figure 4.17: The concentration dependence of the effect of a complementary opener of length $N_0 = 20$ on *in vitro* *HuR*-RNA affinities. (**a**) The apparent affinity of recombinant HuR to IL-2 3'UTR was determined in presence and absence of the opener Op_3(●), of the opener Op_1 (▼) and of the negative controls Neg_1 (×) and Neg_2 (□, dotted error bars) with 1D-FIDA detection [244]. Opener Op_3 has a concentration dependent effect on the apparent affinity that is consistent with equ.(3.16). Opener Op_1 exhibits a similar behaviour at lower concentrations but decreases affinity at higher concentrations, which might be caused by binding to the target RNA at multiple sites. (**b**) *HuR*-*TNFα* mRNA affinities were determined in absence and presence of increasing concentrations of the *TNFα* specific opener Op_F (∗), a *TNFα* specific negative control Neg_T (×) and the *TNFα* specific closer Cl_G (▲). Opener Op_F increases affinity at very low concentrations, but decreases affinities rapidly with increasing concentrations. This is remarkable, as Op_F is very effective in stabilizing endogenous *TNFα* mRNA in cellular lysates (Figure 4.15 on page 64). Closer Cl_G decreases affinity in dependence of concentration as expected. The negative control Neg_T does not change affinities significantly, independent of concentration.

Chapter 5

Discussion

5.1 Methods for the analysis of RNA-protein interactions

In this contribution, we present methods for the quantitative analysis of RNA-protein interactions which depend on the formation of a particular RNA secondary structure. We have demonstrated in section 2.2 that RNA-protein interactions are of great importance for the control of many cellular processes, particularly in post transcriptional regulation of gene expression. We have further discussed that proteins specifically recognize a particular RNA sequence pattern alone or in combination with a particular secondary structure motif or a structure motif alone.

Our methods are based on a simple but powerful quantitative model of RNA-protein interactions. For simplicity and clarity of presentation, we restrict the model to interactions of $1:1$ stoichiometry. However, this is no principal restriction and at the cost of somewhat more complex expressions, the model can be extended to other binding modes.

We later-on confine the methods developed to the analysis of RNA secondary structures. This is done primarily because the thermodynamics of RNA secondary structures is well understood [236] and efficient algorithms for the calculation of partition functions of thermodynamic RNA secondary structure ensembles are available (e.g. [241, 162, 161]). Importantly however, the quantitative model for RNA-protein interactions is not restricted to secondary structures but would allow to deal with any type of RNA conformation. If algorithms for the calculation of partitions functions of RNA tertiary structure ensembles were available, they would seamlessly fit into our quantitative model and could be easily integrated into our computational methods. The partition function algorithms we employ in this study are all restricted

to the analysis of non-pseudoknot RNA secondary structures. While an algorithm for the calculation of partition functions able to deal with pseudoknots has been presented recently [99], it comes at the cost of higher computational complexity. As our algorithms are rather intensive themselves a combination with an expensive partition function algorithm appears not feasible. The use of an algorithm for the stochastic sampling of conformations including pseudoknots seems, however, highly interesting. Pseudoknots are known to be biologically important. However, our success in the analysis of the biologically very relevant *HuR*-ARE system suggests that there is ample room for the application of our methods in biologically important processes though we neglect pseudo-knotted RNA secondary structures.

In section 2.2.2 we have discussed that binding of RNA and protein often involves conformational rearrangements in RNA, protein or both. Though our quantitative model of RNA-ligand interaction is based on the assumption of a simple two state process, it is also compatible with multi state process like induced fit provided that the free energy changes due to the structural rearrangement after binding are (nearly) independent of the RNA sequence.

The methods we have presented enable – based on the analysis of RNA-protein affinity data – the selection of an RNA sequence-structure motif required for protein binding using a statistical test, the prediction of apparent affinities for experimentally untested RNA sequences if the required motif has been identified and the design of RNA sequences with a pre-selected affinity to the protein. The feasibility these steps has been demonstrated by application the *HuR*-ARE system.

5.2 Modifier RNAs

An important consequence of our quantitative model for RNA-protein interactions is that the manipulation of the RNA secondary structure allows to modulate apparent RNA-protein affinities. One possibility to modify RNA secondary structures in a controlled way is the hybridization of small reverse complementary RNAs, which we call modifier RNAs (modRNAs). The thermodynamics of RNA-RNA hybridization is well understood [96]. However, for the calculation of partition functions of RNA duplices, no implementation was available that considers all secondary structures in both RNA strands. We have therefore derived an approximate model. This approximate model of RNA-RNA hybridization can be incorporated in our model of RNA-protein interactions resulting in algorithms for the computational prediction of modifier RNAs.

Consequently, the same limitations enumerated above for the analysis

methods apply for the prediction of modifier RNAs. Pseudo-knotted structures are excluded and modRNA prediction is computationally intensive if the partition function approach is used. A sampling approach drastically reduces the computational effort for long sequences. It is, however, an approximate method and the optimal sample size, which determines the speed/precision tradeoff, is intricate to choose.

We have designed modRNAs for several cytokine RNAs bound by *HuR*. Successive experimental validation of the modRNAs *in vitro* and in cellular lysates indicates that out methods – despite the approximations made – allow the prediction of modRNAs with an impressive success rate.

Hybridization of short oligonucleotides or peptide nucleic acids (PNAs) have been used previously to influence the RNA secondary structure equilibrium in favor of a particular structural feature. Isaacs et al. [168] demonstrate that translation of mRNAs that are not translatable because their ribosome binding site is inaccessible can be activated by means of small artifical "transactivating RNAs". Small RNAs have been used to allosterically modulate ribozyme activity [189, 190], to drive one of two competing secondary structures of the spliced leader RNA of *Leptomonas collosoma* [204] or for oligonucleotide directed RNA misfolding [65, 66]. A related concept by Goodchild and coworkers uses "facilitator oligonucleotides" to enhance ribozyme substrate binding [134]. In contrast to our understanding of modRNA action, these facilitators were found to act by co-axial stacking with the ribozyme substrate [278].

5.3 *HuR*'s binding mechanism

HuR appears to be a central node in the ARE pathway, which controls the stability of potentially several thousand mRNAs. We applied the described methods to the *HuR*-ARE system to study the mechanism of *HuR*-RNA recognition with the aim to provide a solution to *the specificity puzzle in mRNA stability regulation* (section 2.4.2).

5.3.1 Binding RNA sequence

Initial efforts were aimed to the identification of the *HuR* binding RNA sequence motif, which is a necessary prerequisite for the application of the described methods. *HuR* binding has been mapped in previous studies to sequences containing multiple `AUUUA` repeats [258] and to `U`-rich sequences [377]. A precise binding motif for *HuR* was, however, not available.

We applied *string pattern regression* to a set of *HuR*-RNA binding data

to select the most plausible *HuR* motif from a set of candidate motifs (section 4.1.1). The top ranked motif was `NUUNNUUU` a simplified variant based on the motif of the Hu family member *HuD*. Later experimental analysis identified `NNUUNNUUU` as the true *HuR* motif, which was not included in the set of candidate motifs of the string pattern regression. `NNUUNNUUU` would have outperformed all other candidate motifs in string pattern regression if it was included in the initial set of candidates (Table 4.2).

We corroborated the identified motif by successfully matching `NNUUNNUUU` with the human orthologous sequences of all validated mammalian *HuR* targets found in the literature. Though the identified motif is very degenerate and thus frequently found in the genome, `NNUUNNUUU` matching sequences are significantly more frequent among *HuR* targets than in the overall transcriptome. However, given the imbalance between the size of the set of *HuR* targets and the set of sequences matching `NNUUNNUUU` where no information about *HuR* binding is available, it is currently not feasible to assume that all `NNUUNNUUU` containing mRNAs are targets of *HuR*.

In a recent study, DeSilanes et al. [89] observed that a short stem-loop without sequence constraints except one uracil position is predictive for HuR targets. No data has, however, been presented on the significance of these motif much more degenerate than `NNUUNNUUU`. This motif is not directly supported by previous studies on *HuR* binding mentioned above. Nevertheless, it will be interesting to see whether this stem-loop motif contains binding sites for proteins associated with the HuR pathway.

5.3.2 RNA Secondary structure dependence of *HuR*

Applying the presented statistical method allowed to identify that *HuR* requires a particular RNA secondary structure for binding: only `NNUUNNUUU` in single stranded conformation is recognized. This finding is well supported by the fact that *HuR* binds to the RNA with three RNA recognition motifs (RRMs). Most RRM proteins bind single stranded RNA exclusively. Also, an *HuR* homology model based on the Hu protein structures of *HuC* and *HuD* displays an RNA conformation stacking interactions between RNA and protein, which both are typical for the interaction between RRMs and single stranded RNA.

The RNA secondary structure dependence of *HuR* binding has been identified from a set of *in vitro* binding data. It may certainly be questioned whether the identified secondary structure constraints are of any relevance in a cellular environment, which differs from the *in vitro* system in many aspects. Two facts suggest strongly that the identified secondary structure dependence is relevant in a cellular system (i) our model quantitatively ex-

plains the *TNFα* deficient phenotype of the NZW mouse (section 4.1.3) and (*ii*) modifier RNAs designed to modulate the binding of *HuR* to cytokine mRNAs are effective in cellular lysates, where environmental conditions resemble largely an *in vivo* environment (section 4.2.2).

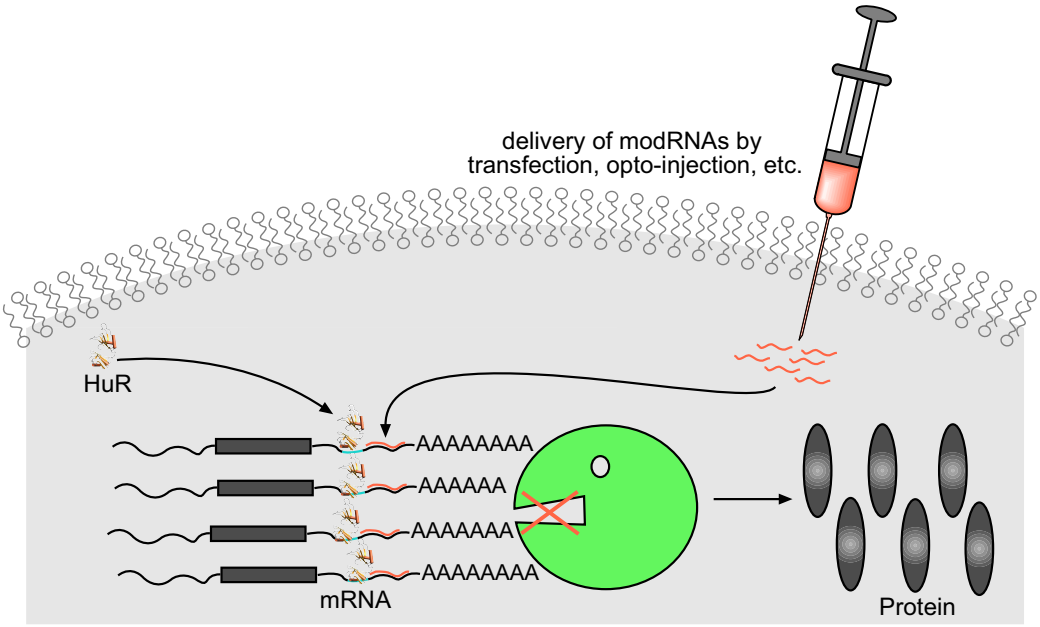
5.3.3 Modifier RNAs may solve the specificity puzzle

Modifier RNAs were designed for several *HuR* target cytokine mRNAs to maximize or minimize *HuR*-RNA affinities by increasing (opener RNAs) or decreasing (closer RNAs) the fraction of structures with single stranded `NNUUNNUUU` in the ensemble. All tested opener RNAs increased the *in vitro* *HuR*-RNA affinity as predicted; a tested closer decreases affinity as expected. Moreover, opener RNAs increased also the fraction of endogenous *IL2* mRNA associated with endogenous *HuR*. Finally, we tested whether openers allowed to manipulate the biological effect of *HuR* binding. *IL2* mRNA stability was quantified by monitoring mRNA concentrations in cellular lysates over time. In presence of opener RNAs, the degradation of cytokine mRNAs was delayed of entirely halted during the observation time depending on the opener RNA concentration. We were able to ensure that modifier RNAs act specifically, by monitoring *TNFα* and *IL1β* degradation kinetics in the presence of *IL2* specific openers and *IL2* mRNA degradation in the presence of a *TNFα* specific opener. Negative control oligonucleotides, designed to hybridize to the target mRNAs without a significant effect on the binding motif secondary structure, performed as predicted in all of the described experiments.

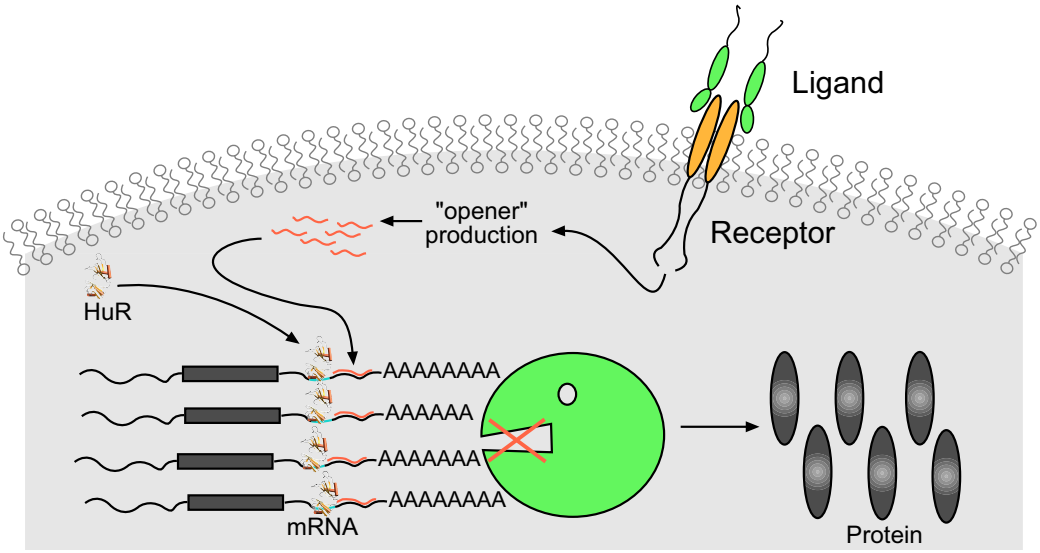
The successful demonstration that RNA secondary structure manipulation allows to specifically switch *HuR*-RNA binding on and off, nurtures speculations whether an analogous mechanism might ensure specificity in *HuR* dependent mRNA stabilization in the cell. An external stimulus might trigger the production or release of modifier RNAs which specifically modulate *HuR* target binding (Figure 5.1). Such a mechanism would share many properties with the endogenous system: fast responses to stimuli, high specificity in the presence of alternative not to be up-regulated *HuR* targets and independence of protein synthesis. We thus propose a modifier RNA dependent mechanism as a potential solution for the *specificity puzzle* in mRNA stability regulation.

5.4 Endogenous modifier RNAs?

If openers really promote *HuR* dependent up-regulation, there is no reason to assume that the action of small modifier RNAs should be restricted



(a) science



(b) fiction

Figure 5.1: (**a**) We have shown that regulation of mRNA stability by modRNAs in a cellular environment is feasible. (**b**) We propose that the specificity puzzle in mRNA stability regulation might be solved by a triggered release of target RNA specific modRNAs.

to the *HuR*/ARE system. This scenario extends David Bartel and Chang-Zheng Chen's proposal of microRNAs as "micro-managers of gene expression" [23] and follows John Mattick's argument for a dominating layer of RNA-mediated regulation [238].

There is indeed mounting evidence for a vast variety of regulatory active small RNAs [238, 334]: Some organisms, such as *Leishmania* and related kinetoplastids, have reduced transcriptional regulation of gene expression to a minimum, maybe to the point of having lost any specific polymerase II transcription initiation [69]. Instead, *Leishmania* uses an elaborate cleavage and trans-splicing mechanism based on the action of $\sim$ 40nt "spliced leader" RNA. *Tetrahymena* appears to use an RNA-based mechanism for directing its genome-wide DNA rearrangements [251, 375]. The *E. coli* genome encodes more than 50 small RNA genes at least some of which (e.g. *MicF*, *OxyS*, *DsrA*, *Spot42*, *RhyB*) act by basepairing to activate or repress translation [330]. A large fraction of the mouse transcriptome consists of non-coding RNAs, many of them anti-sense to known protein-coding transcripts [333]. Similarly, about half of the transcripts from Human chromosomes 21 and 22 are non-coding [57, 180]. The possible roles of anti-sense RNAs are discussed in [255]. Ambros and coworkers [10] reported more than 30 *tiny non-coding RNAs* in a recent survey of *C. elegans*. These "tncRNAs" are slightly shorter than microRNAs, are not processed from hairpin precursors, and are poorly conserved between related species.

Riboswitches, i.e., RNAs that drastically change their structure, are important regulatory elements. For instance, the terminator and anti-terminator, two alternative RNA hairpins, regulate gene expression in *E. coli* and *B. subtilis* by attenuation [19, 115, 283]. Riboswitches can provide exact temporal control as in the *hok/sok* system of plasmid R1 which triggers programmed cell death [261, 254]. Riboswitches also play a role in the spliced leader of trypanosomes and nematodes [204]. Artificial RNA switches have been designed as well, see e.g. [325]. For instance, in [324] an RNA is described whose conformation change is triggered by ligand binding using a switching mechanism similar to the one proposed for the ribosomal `A` site. An RNA controlled allosteric hammerhead ribozyme is presented in [189]. An RNA molecule that has two different ribozyme functions depending on its spatial conformation is described in [313]. A theoretical study shows that potential riboswitches, i.e., RNAs that have very different secondary structures with near-groundstate energy, are relatively frequent and easily accessible in evolution [121].

Riboswitches might be just the extreme cases of a regulatory mechanism that works more generally by modifying the relative concentrations of different RNA structures (or structural classes). The modifier RNA mechanism

outlined in this presentation would provide a general and gene specific way to both up- and down-regulate RNA-ligand binding affinities and thus, allow a fast and specific fine-tuning of the eventual expression level of a gene product. The mechanism is independent of an elaborate machinery of RNP complexes since the modifiers exert their function by directly binding to their target RNA. This reduces evolutionary constraints on the hypothetical modifier RNAs. Furthermore, mutations in modifier RNAs will often have small quantitative rather than qualitative effects on expression levels because the effect of point mutations on RNA helices is limited to a few kcal/mol. On the other hand, some mutations can lead to drastic changes in the preferred structures in the same way as for isolated RNA molecules [122]. The hypothetical modifier RNAs would therefore not be subject to strong multiple constraints, so that they would rapidly drift along neutral networks in sequence space as described in Ref. [314]. In particular, if we assume that the major source of the hypothetical modifier RNAs are antisense transcripts, they evolve without the need for compensatory mutations to maintain complementarity between the modifier and its target. In another scenario, trans-acting modifiers might avoid exact complementarity to their target in order to avoid triggering the RNAi pathways; in this case their binding patterns are essentially unconstrained so that compensatory mutations are also not necessary. It is thus entirely plausible that a regulatory level based on modifier RNAs evolves very fast and does not leave phylogenetic footprints or other easy-to-find signals in the genomic DNA.

5.5 modRNAs in drug discovery

Synthetic modRNAs as tools in biology and drug discovery

Synthetic modifier RNAs have a broad range of potential applications as tools in experimental biology and drug discovery. The downstream effects of virtually any RNA-protein interaction which is RNA secondary structure dependent might be modified using modRNAs. This is particularly interesting for RNA-protein interactions which are involved in post transcriptional regulation of gene expression. An example for such modRNAs is given by the *opener* and *closer* RNAs we have used to manipulate *HuR*-RNA associations. With these modRNAs we introduce a method for the controlled manipulation of ARE transcript stability, which is – for ARE genes – potentially complementary to RNAi [346, 380, 108]. While providing a comparable

level of target specificity[1] the opener (closer) methodology differs from RNAi in several aspects: (*i*) The artificially induced conformational reorganization allows both, to hide or present the recognition site of a regulatory factor such as *HuR* and can thereby be used to drive the associated regulatory process in both directions. Unlike RNAi or conventional antisense approaches, it therefore not only allows to potentially silence but also boost the expression of the target gene, a particular advantage for target validation in drug discovery. (*ii*) The high precision of the computational mRNA opener design reduces the effort which is often required to experimentally assess functional siRNA or antisense hybridization positions. (*iii*) The manipulation is further quantitatively tunable and correlates with the applied opener dose. (*iv*) Functional openers are not dependent on recognition and processing by host cell enzymes (i.e. the *Dicer/RISC* machinery [56]). This offers a higher flexibility with respect to the opener nucleic acid length and species, provided that the sequence specificity is not affected. Single stranded RNA, DNA or PNA oligonucleotides with virtually any 2'- or backbone modification might be usable, allowing to adjust the metabolic opener stability and its biochemical properties. Also, labeling with fluorescent tags appears feasible. (*v*) In addition, multiple *HuR* binding sites within one messenger RNA might be individually opened or closed. This would allow to successively study the biological role of individual *HuR* binding sites in the regulation of an mRNA.

While RNAi is applicable to virtually any target gene, the opener methodology remains confined to the set of *HuR* controlled genes. However, with an estimated number of 3,000 ARE genes [20] most of them being tightly controlled and ultimately related to disease relevant processes, there remains a wide field for potential applications. As this set encompasses functionally diverse genes, distinct pathways in the regulatory network can be studied by interfering at the node of mRNA stability control. As for RNAi, the main issue is the delivery of the opener oligonucleotides into the target cells. It has to be emphasized that so far, we have validated the opener effect in human PBMC lysates. Advances in effective but mild transfection methods like optoinjection, delivery by TAT-peptide chimera [264] or viral vectors for small RNA transcripts promise to make a final proof in vivo attainable.

[1]Recent findings indicate that RNAi may cause unspecific effects by partial hybridization with non-target mRNAs, possibly by triggering micro RNA pathways [173, 308]. If this is the case, opener and closer modRNAs offer higher specificity than RNAi: modRNA cross-hybridization to a non-target mRNA does only lead to an effect if it occurs at a position where hybridization leads to a conformational rearrangement at a protein binding site. We can infer from the conducted modRNA profile calculations that such positions are rare and consequently the risk of side effects by cross-hybridization is minimal.

Synthetic modRNAs as drugs?

modRNAs seem to act in a highly specific and dose dependent way thereby fulfilling two important prerequisites for a potential application as drugs. However, nucleic acids are in general not seen as potent pharmaceutical agents, mainly because of stability and general pharmacokinetic issues. Therefore, clinical use of RNA drugs is currently thought to be restricted to specific, topic applications. A unique example for such an application is *Cand5* of Acuity Pharmaceuticals. An siRNA directed against vascular endothelial growth factor (*VEGF*) which is injected directly into the eye to treat *wet age-related macular degeneration* and *diabetic retinopathy*, which are both caused by an excess production of *VEGF*.

Recent work on the therapeutic use of siRNAs shows, however, that RNAs have probably been underestimated in their pharmaceutical potential. Soutschek and colleagues were able to silence an endogenous gene (*apoB*) in mouse by intravenous injection of a chemically modified siRNA linked with cholesterol [326]. Other encouraging results originate from experiments on the *in vivo* protection from hepatitis using RNAi, reviewed in [218]. However in these experiments, hydrodynamic injections were used to deliver the siRNA which is not appropriate for therapeutic applications as it requires the rapid injection of 10–20% of the blood volume in mice. In summary, it seems very plausible to speculate about a potential application of modRNAs as drugs in combination with stabilizing RNA modifications (or the use of PNAs) and linked to a delivery enhancing moiety like cholesterol or TAT-peptide.

If endogenous modRNAs existed...

The drug discovery perspective of modRNAs is fundamentally different if our speculation that modRNAs might constitute another class of endogenous non-protein coding RNAs is true. In this case a pharmaceutical approach can concentrate on interference with modRNA – mRNA interaction. The history of drugs binding RNA specifically is long, particularly antibiotics act frequently by inhibitory interaction with RNAs. However, those interactions are mainly restricted to specific binding of ribosomal RNA, specific mRNA binders are the exception [155]. It is certainly a challenge to identify drug-like low molecular weight compounds that specifically bind mRNA with a sufficiently high affinity to prevent modRNA hybridization or to prevent conformational rearrangement of a protein binding site though the respective modRNA has hybridized with the target RNA. A possibility is to start from bio-macromolecules like peptides to identify an active substance which is

later replaced by means of (peptido-) mimetics.

An nucleic acid compound based approach is also feasible to interfere with endogenous modRNAs. One may use artificial modRNAs to counteract the effect of endogenous modRNAs. Alternatively, short nucleic acids resembling the modRNA binding site on the mRNA can be used as decoys, to compete with the target mRNA binding site for modRNA interaction.

Appendix A

Appendix

A.1 *HuR* target mRNAs described in the literature

Table A.1: NNUUNNUUU is present in validated mammalian *HuR* targets. The presence of NNUUNNUUU in human orthologous mRNAs of validated mammalian *HuR* targets has been tested applying the EMBOSS program *Fuzznuc* to the respective *Refseq* sequences. *ARED2.0* ([21]) cluster numbers are specified for sequences contained in this database. Renin mRNA, for which *HuR* associated mRNA stability regulation has been reported recently [3] contains the motif, but with a single U to C mismatch.

Gene symbol	Gene name, alternative names	Sequence ID	Reference	ARED cluster	Contains NNUUNNUUU
Cytokines, chemokines, growth factors					
BMP6	bone morphogenetic protein 6	NM_001718	[259]	V	x
CCL11	chemokine (C-C motif) ligand 11, eotaxin	NM_002986	[15]		x
CSF2	colony stimulating factor 2, GMCSF	NM_000758	[292, 110, 123, 16]	I	x
EGF	epidermal growth factor	NM_001963	[321]	V	x
FSHB	follicle stimulating hormone beta	AH003599	[232]		x
IL1b	interleukin 1 beta	NM_000576	[244]	II	x

Continued on next page

Table A.1: (continued) Presence NNUUNNUUU in validated *HuR* targets

Gene symbol	Gene name, alternative names	Sequence ID	Reference	ARED cluster	Contains NNUUNNUUU
IL2	interleukin 2	NM_000586	[2, 16, 316]	III	x
IL3	interleukin 3	NM_000588	[226, 291, 248]	V	x
IL4	interleukin 4	NM_000589	[244]	III	x
IL6	interleukin 6	NM_000600	[259]	IV	x
IL8	interleukin 8	NM_000584	[259, 371, 260]	II	x
MYOD1	myogenic factor 3	NM_002478	[117, 348]		x
MYOG	myogenin	NM_002479	[117, 348]		x
NF1	neurofibromin 1	NM_000267	[145]		x
PITX2	paired-like homeodomain transcription factor 2	NM_000325	[42]		x
TNFa	tumor necrosis factor alpha	NM_000594	[91, 235, 243, 152, 303]	III	x
VEGF	vascular endothelial growth factor	NM_003376	[133, 211, 337, 101]	IV	x
Tumor suppressor genes, proto-oncogenes, cell cycle regulators					
CCNA2	cyclin A	NM_001237	[356, 357]		x
CCNB1	cyclin B1	NM_031966	[356, 357]		x
CCND1	cyclin D1	NM_053056	[42, 356, 357]	V	x
CCND2	cyclin D2	NM_001759	[42]		x
CD83	CD83 antigen	NM_004233	[150].		x
CDKN1A	cyclin-dependent kinase inhibitor 1A, p21, Cip1	NM_000389	[117, 131, 356]		x
CDKN1B	cyclin-dependent kinase inhibitor 1B, p27,kip1	NM_004064	[192]		x
DEK	DEK oncogene	NM_003472	[89]		x
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog, c-fos	NM_005252	[226, 275, 359, 62]	IV	x

Continued on next page

Table A.1: (continued) Presence NNUUNNUUU in validated *HuR* targets

Gene symbol	Gene name, alternative names	Sequence ID	Reference	ARED cluster	Contains NNUUNNUUU
HIF-1α	hypoxia-inducible factor 1, alpha	NM_001530, NM_181054	[321]	III	x
HLF	hepatic leukemia factor	NM_002126	[89]		x
JUN	v-jun sarcoma virus 17 oncogene homolog (avian), c-jun	NM_002228	[42, 275]		x
MYC	v-myc myelocytomatosis viral oncogene homolog, c-myc	NM_002467	[193, 136]		x
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived, n-myc	NM_005378	[226, 234]		x
TP53	tumor protein p53	NM_000546	[240, 125]		x
Enzymes					
HDAC2	histone deacetylase 2	NM_001527	[89]		x
MMP9	matrix metalloproteinase 9	NM_004994	[102, 165]		x
NDUFB6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex	NM_002493	[89]		x
NOS2A	nitric oxide synthase 2A	NM_000625	[294]		x
PLAU	urokinase plasminogen activator	NM_002658	[344]	IV	x
PTGS2	prostaglandin-endoperoxide synthase 2, COX2	NM_000963	[259, 101, 70, 332, 317, 94]	III	x
SERPINB2	serine (or cysteine) proteinase inhibitor, PAI-2	NM_002575	[239]	V	x

Continued on next page

Table A.1: (continued) Presence `NNUUNNUUU` in validated *HuR* targets

Gene symbol	Gene name, alternative names	Sequence ID	Reference	ARED cluster	Contains `NNUUNNUUU`
UBE2N	ubiquitin-conjugating enzyme E2N	NM_003348	[89]		x
Receptors, membrane proteins					
ADRB1	beta-1-adrenergic receptor	NM_000684, U29690	[35, 36]		x
ADRB2	beta-2 adrenergic receptor	NM_000024	[35, 36]		x
AR	androgen receptor	NM_000044	[377, 367]		x
CALCR	calcitonin receptor	NM_001742	[376]		x
CDH2	cadherin 2, type 1, N-cadherin	NM_001792	[89]		x
GAP43	growth associated protein 43	NM_002045	[68]		x
SLC2A1	solute carrier family 2 member 1, GLUT1	NM_006516	[175]		x
PLAUR	urokinase plasminogen activator receptor	NM_002659	[344]	IV	x
SLC5A1	solute carrier family 5, SGLT1	NM_000343	[220]		x
TNFSF5	tumor necrosis factor (ligand) superfamily, member 5, CD154	NM_000074	[304]	IV	x
Miscellaneous					
ACTG1	actin, gamma 1	NM_001614	[89]		x
CTNNB1	catenin (cadherin-associated protein), beta 1	NM_001904	[88]		x
MARCKS	myristoylated alanine-rich protein kinase C substrate	NM_002356	[364]		x
MTA1	metastasis associated 1	NM_004689	[89]		x
PITX2	paired-like homeodomain transcription factor 2	NM_000325	[42]		x

Continued on next page

Table A.1: (continued) Presence `NNUUNNUUU` in validated *HuR* targets

Gene symbol	Gene name, alternative names	Sequence ID	Reference	ARED cluster	Contains `NNUUNNUUU`
SLC7A1	cationic amino acid transporter, CAT-1	NM_003045	[374]		x

A.2 Experimental procedures referred to in this manuscript

For the sake of completeness, we have included a description of the experimental methods referred to in the text, taken mainly from [244].

Fluorescently labeled RNA

5' amino−C6 modified RNA was synthesized on an 394A synthesizer (Applied Biosystems) using *5'*−O−dimethoxytrityl−*2'*O−triisopropyloxymethyl− protected β-cyanoethyl-(*N*,*N*-diisopropyl)nucleotide phosphoramidites (Glen Research) adopting published procedures [58, 309] and manufacturer's protocols. The oligoribonucleotides (ORNs) were cleaved from the support, base-, phosphate- and *2'*- de-protected and purified by denaturing polyacrylamide gel electrophoresis following standard protocols. RNA concentrations were calculated from UV-absorption at 260nm according to Beer's Law, using the exact molar extinction coefficient at 260nm as determined according to reference [138]. All ORNs were $>$ 99% pure according to analytical RP-HPLC (Reversed-Phase High Performance Liquid Chromatography) analysis (VYDAC C_{18} column, 5μm, 300$\AA$, 4.6mm x250mm, in triethylammonium acetate (0.1M, pH 7.0) with gradient elution, $0 - -50\%$ CH_3CN in 45min, UV-detection at 260nm). *5'*-carboxytetramethylrhodamine (TMR, Molecular Probes) was attached to the *5'* aminolinker in a standard reaction of the primary amine with a succinimidylester-activated fluorophore to form a stable carboxamide. Unreacted dye was hydrolyzed by addition of hydroxylamine-hydrochloride (1.5M). The labeled RNA was separated from the free dye by gel filtration, purified from unlabeled RNA by RP-HPLC and the concentration determined by UV absorption spectroscopy as described above but with correction for the dye absorption at 260nm.

3'UTRs were prepared by run-off transcription from dsDNA templates with T7 RNA polymerase (T7 MEGASCRIPT in vitro transcription kit, Ambion). The T7 promoter was incorporated into the transcription templates during PCR amplification, using primers encompassing the 3'UTRs of *IL2* and *TNFα* (*IL2*: nt 707−1035, *TNFα*: nt 872−1568, GenBank accession numbers NM_000589 and NM_000594, respectively). The transcript was 3' terminally oxidized with Na(m-)IO_4 and coupled to hydrazide activated Cy3 (AP Biotech), essentially as described in reference [285]. The product was subsequently purified by RP−HPLC as described for synthetic oligoribonucleotides, desalted and transferred into aqueous solution by gel filtration. A 1:1 labeling stoichiometry was controlled by determination of the Cy3 and RNA concentration by UV/VIS absorption spectroscopy with correction of

the dye absorbance at 260nm.

Recombinant *HuR*

The coding sequence for full−length HuR (amino acids 1−326, RefSeq accession: NP_001410) was amplified from cDNA prepared from activated human T-lymphocytes. The product was cloned directionally into the NdeI and SapI sites of the vector pTXB1 (IMPACTTM −CN system, New England Biolabs), allowing C−terminal fusion with an intein−chitin binding domain tag without additional amino acid insertion. The fusion protein was expressed in E.coli ER2566 (New England Biolabs) upon induction with IPTG ($1m$M, for 6 hours at 28°C). The bacterial cells were lysed by successive freezing/thawing cycles in a buffer of Tris/Cl (tris(hydroxymethyl)aminomethane, $20m$M pH 8.0), NaCl (800mM), EDTA (N,N,N',N'−ethylenediaminetetraacetic, 1mM) and Pluronic F-127 (0.2%w/v, Molecular Probes). After DNA digestion, the lysates were cleared by ultracentrifugation and the fusion protein was captured onto chitin agarose beads (New England Biolabs). After extensive washing with lysis buffer, the recombinant protein was recovered by thiol-induced on-column self-splicing of the intein tag with 2-mercaptoethanesulfonic acid (sodium salt, $50m$M) for 12 hours at 4°C [67]. Any co-eluted intein tag and uncleaved fusion protein were removed from the eluate in a second, subtractive affinity step. The protein was transferred into the storage buffer (Na_2HPO_4/NaH_2PO_4 (25mM) pH 7.2, NaCl (800mM), Pluronic F-127 (0.2%w/v)) by gel filtration (DG-10 columns, Bio-Rad), shock−frozen in small aliquots in liquid nitrogen and stored at −80°C. Under these conditions, full length HuR was soluble without presence of higher aggregation states (analytical size exclusion chromatography), and showed the characteristic CD-spectra for RRM domains, [231] (data not shown). The protein was > 99% pure according to Liquid Chromatography / Electrospray Ionization-Mass Spectrometry, RP-HPLC and SDS-PAGE analysis. N-terminal sequencing revealed a correct N-terminus quantitatively missing Met_1. For a precise determination of the concentration, purified HuR was lyophilized, dissolved in guanidinium hydrochloride (6M) and the concentration was determined by UV−spectroscopy according to reference [132]. This solution was used as external standard for determination of HuR concentrations by RP−HPLC quantification.

2D-FIDA-anisotropy *HuR*-RNA binding assay

The fluorescently labeled RNA was thermally denatured for $2min$ at 80°C in assay buffer (PBS, Pluronic-F-127 (0.1%w/v), $MgCl_2$ (5mM)), refolded

by cooling to room temperature ($-0.13°\mathrm{C}s^{-1}$) and diluted to 0.5nM, which ensures an average of < 1 fluorescent particles in the confocal volume in the described setup[111]. The accurate concentration in each sample was determined based on the particle number derived from a parallel Fluorescence Correlation Spectroscopy evaluation and the size of the confocal volume, as given by the adjustment parameters for the point spread function[111] . Fluorescently labeled RNA was titrated against increasing concentrations of recombinant HuR (at least 11 titration points). *HuR*-RNA samples were incubated for at least 15min at room temperature prior to each measurement.

HuR-RNA complex formation was monitored under true equilibrium conditions by determination of the fluorescence anisotropy with 2D-FIDA. Measurements were performed in 96 well glass bottom microtiter plates (Whatman) on an EvotecOAI PickoScreen 3 instrument at ambient temperature (constant at 23.5°C). The Olympus inverted microscope IX70 based instrument was equipped with two fluorescence detectors, a polarization beamsplitter in the fluorescence emission path and an additional linear polarization filter in the excitation path. A HeNe laser ($\lambda = 543$nm, laser power = 495W) was used for fluorescence excitation. The excitation laser light was blocked from the optical detection path by an interference barrier filter with optical density $OD = 5$. TMR in assay buffer (at 0.5nM) was used for the adjustment of the confocal pinhole (70μm) and for the determination of the G-factor of the instrument [199]. The molecular brightness q was extracted from the 2D-FIDA raw data for each polarization channel using the FIDA algorithm [183, 182]. The anisotropy was then calculated as described in reference [199]. The $2\mathrm{D}-\mathrm{FIDA}$ anisotropy signal was averaged from 10 consecutive measurements (10 s each). The G-factor (calculated using $P_{(true)}TMR = 0.034$) was determined after every 11 measurements.

The anisotropy data were fitted based on the exact algebraic solution of the binding equation describing the average steady-state anisotropy signal r in dependence of the degree of 1:1 complex formation derived from the law of mass action,[84] to extract t he equilibrium dissociation constant K_d^{app} (nonlinear least square regression, GraFit 5.0.3, Erithacus software, London):

$$r = \frac{r_{min} + (r_{max}Q - r_{min})A}{1 - (1 - Q)A} \tag{A.1}$$

where:

$$A = \frac{1}{2[RNA_0]}\left[B - -\sqrt{B^2 - 4[HuR_0][RNA_0]}\right] \tag{A.2}$$

$B = [RNA_0] + [HuR_0] + K_d^{app}$, $[RNA_0]$: total concentration of RNA, $[HuR_0]$: total concentration of *HuR*, r_{min}: anisotropy of free RNA, r_{max}: anisotropy

of *HuR*-RNA complex, r: average anisotropy for the steady-state equilibrium at the given $[HuR_0]$ and $[RNA_0]$ concentrations; $r = (q_{\parallel} - Gq_{\perp})/(q_{\parallel} + 2Gq_{\perp})$, $q_{\parallel}$,$q_{\perp}$: molecular brightnesses in parallel and perpendicular polarization channels, Q: quenching factor for 2D-FIDA-anisotropy measurements, $Q = q_{tot(min)}/q_{tot(max)}$; at $q_{tot} = q_{\parallel} + 2q_{\perp}$; All presented data are averages from at least three independent experiments.

1D-FIDA HuR mRNA binding assay

The relative size increase that a fluorescently labeled mRNA or 3'UTR subsides upon binding of the relatively small *HuR* does not provide a significant detection parameter for the interaction. For this reason, a one dimensional FIDA assay for HuR binding to 3'terminally Cy3-labeled mRNAs was established. The labeled mRNA was thermally denatured for $2min$ at 80°C in assay buffer (PBS, Pluronic-F-127 ($0.1\%w/v$), $MgCl_2$ (5mM)) and refolded by cooling to room temperature ($-0.13°Cs^{-1}$). Opener or negative control oligodeoxynucleotides were added to final concentrations between 0.5 and 100nM. The final concentration of Cy3-labeled mRNA was 0.5 nM, accurate particle numbers were determined as described for the 2D-FIDA anisotropy measurements.

The labeled mRNA was titrated against increasing concentrations of HuR in presence and absence of openers or negative control oligodeoxynucleotides. *HuR* mRNA complex formation was monitored under true equilibrium conditions by determination of the molecular brightness with 1D-FIDA [183]. A HeNe laser ($\lambda = 543$nm, laser power = 495W) was used for fluorescence excitation, the optical setup was analogous to the setup for 2D-FIDA anisotropy measurements,using one detection channel only and no polarization beam splitters in the optical paths. The molecular brightness q was extracted from the 1D-FIDA raw data using the FIDA algorithm [182] and averaged from 20 consecutive measurements (10s each). The molecular brightness data were fitted based on an equation analogous to Eq.A.1, adapted for fluorescence intensity measurements:

$$q = q_{min} + \frac{(q_{max} - q_{min})\left[B - \sqrt{B^2 - 4[RNA_0][HuR_0]}\right]}{2[RNA_0]} \tag{A.3}$$

where $B = [RNA_0] + [HuR_0] + K_d^{app}$, q_{min}: molecular brightness of free RNA, q_{max}: molecular brightness of RNA HuR complex, q: average molecular brightness for the steady-state equilibrium at the given $[HuR_0]$ and $[RNA_0]$ concentrations. All presented data are averages from at least three independent experiments.

Preparation and stimulation of cells

Human peripheral blood monocyte cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque centrifugation, washed with PBS containing bovine serum albumin (BSA, 15%w/v), resuspended at $2 \cdot 10^6$mL^{-1} in RPMI1640 (Gibco/BRL) supplemented with heat-inactivated fetal calf serum (10%v/v), L-glutamine (2mM), streptomycin (100μg mL^{-1}) and penicillin (100umL^{-1}) and incubated in a 37°C CO_2 incubator. PBMC were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA, 25ng mL^{-1}, Sigma-Aldrich) and anti-CD3 mAb (1gmL^{-1}, Pharmingen) in absence and presence of anti-CD28 mAb (1g mL^{-1}, Pharmingen).

Co-immuneprecipitation of HuR-mRNA complexes

For each immuneprecipitation, $5 \cdot 10^6$ nonstimulated cells were washed with PBS/BSA and lysed at 4°C in hypotonic buffer (100μL, Tris/Cl (10mM) pH 7.5, NaCl (10 mM), EDTA (10 mM), Protease Inhibitor (Complete Mini EDTA free Protease Inhibitor Cocktail, Roche; 3 tablets per 50mL lysis buffer) and Nonidet-P-40 (0.5%v/v)). RNAsin (0.4 u mL^{-1}, Promega) and SuperasIn (0.2 u mL^{-1}, Ambion) were added to inhibit unspecific RNA degradation. The lysates were centrifug ed at 4°C for 4 min at $15,000xg$ to pellet nuclei. The cleared lysates were incubated for 5 min with anti-*HuR* mAb (5g mL^{-1} , 19F12, Molecular Probes) at 4°C in presence and absence of opener or negative control oligonucleotides. After addition of biotinylated anti(mouse) IgG mAb (10g mL^{-1}, Amersham Pharmacia), the immunecomplexes were captured on streptavidin sepharose beads (Amersham Pharmacia). The beads were washed thoroughly with lysis buffer. *HuR* and the complexed mRNA were eluted under acidic conditions (Glycin/HCl (50mM, pH 2.5), NaCl (50mM), prewarmed to 95°C). The eluates were passed by centrifugation through BioSpin gel filtration columns (BioRad), pre-equilibrated with H_2O. Co−precipitated RNA was quantified by real-time RT-PCR.

mRNA decay

$5 \cdot 10^6$ stimulated PBMC were lysed in lysis buffer (250μL) as described above, in presence or absence of opener or negative control oligonucleotides. For neutralization studies, a monoclonal antibody specifically recognizing HuR (19F12, Molecular Probes) was added to the lysates to a final concentration of 30g mL^{-1}. mRNA degradation was initiated in the cleared lysates by addition of $MgCl_2$ (net concentration of 5mM free Mg^{2+}). The degradation reaction was proceeded at room temperature and stopped after various

timepoints between 2 and 70 min incubation (50μL aliquots for each timepoint) by addition of EDTA and guanidinium isothiocyanat containing buffer (Qiagen). RNA was isolated using the RNeasy Miniprep RNA isolation kit (Qiagen) according to the manufacturers protocol, with DNAse I treatment for elimination of residual DNA.

Quantitative real–time RT PCR

RNA was reverse transcribed to cDNA using the TaqMan RT PCR reagents (Applied Biosystems) and random hexamers for priming, following standard protocols. Control reactions for genomic DNA contamination were performed without addition of reverse transcriptase. Quantitative RT-PCR was performed with SYBR Green detection on an ABI7700 instrument (Applied Biosystems) with the following primers: *IL2* mRNA: forward: `5'-TCACCAGGATGCTCACATTTAAGTT-3'`; reverse: `5'-GGAGTTTGAGTTCTTCTTCTAGACACTGA-3'`; *TNFα* mRNA: forward: `5'-AGGCGGTGCTTGTTCCTC-3'`; reverse: `5'-GTTCGAGAAGATGATCTGACTGCC-3'`; *IL1β* mRNA: forward: `5'-GTACCTGAGCTCGCCAGTGA-3'`; reverse: `5'-TCGGAGATCGTAGCTGGATG-3'` (Primers were a gift from F. Kalthoff, Novartis Institute for Biomedical Research Vienna). *EF-1α* was used as endogenous control (primers: forward `5'-TTTGAGACCAGCAAGTACTATGTGACT-3'`, reverse `5'-TCAGCCTGAGATGTCCCTGTAA-3'`). The $\Delta\Delta$Ct method was used for relative quantification of *IL2* mRNA levels (as described eg. in [12]) using in vitro transcribed *IL2* mRNA for calibration. All presented data are averages from at least 5 identical independent samples and representative of at least two independent experiments using cells from independent donors.

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Curriculum Vitae

Jörg Hackermüller
joerg@tbi.univie.ac.at

Personal Details:

date of birth	January, 3rd, 1976
place of birth	Steyr, Austria
citizenship	Austria
marital status	single

Experience:

2002 — 2004	Novartis Institutes for Biomedical Research, Vienna, Austria; PhD student in computational biology
autumn 1998, winter 1998/1999	Swedish Institute for Infectious Disease Control, Stockholm, Sweden guest student in the group of Mikael Rhen (bacteriology), research activities on the expression of Salmonella virulence factors Microbiology and Tumorbiology Center, Karolinska Institute Stockholm, Sweden guest student in the group of Francesca Chiodi (virology), research activities on AIDS associated dementia
summer 1998	Institute for Biochemistry and Cellular Biology, University Vienna, Austria;co-worker in the group of G. Wiche (molecular cellular biology) fluorescence and electron microscopy of the mouse skeletal muscle
summer 1997	Swarco M. Swarovski GesmbH & Co.KG, Amstetten Austria; development of an optimal surface coating for drop-on reflective glass beads for road markings

Education:

2002 — date	PhD thesis on the computational detection of regulatory elements in eukaryotic mRNAs; supervisor Peter F. Stadler (Inst. for Theoretical Chemistry and Molecular Structural Biology, University Vienna, Austria)

1994 — 2001	MSc. Biochemistry (highest honors), University Vienna, Austria
1999 — 2001	Diploma thesis "Recombination and the structure of globular proteins" at the Institute for Theoretical Chemistry and Molecular Structural Biology, University Vienna, Austria, in the group of P.K. Schuster, under supervision of Peter Stadler
1994	Austrian Institute of East and Southeast European Studies summer school in Russian language
1986 — 1994	comprehensive secondary school with focus on natural sciences, Waidhofen/Ybbs, Austria

Skills:

Language	German (native), English, Russian, Swedish
Computer	C, C++, Perl, SQL, XML
Additional	project management (workshops for software project management, Vienna, 2003 2004)

Activities and Interests:

2001 — 2002	Arbeiter Samariter Bund sterreichs, Vienna Austria alternative civilian service as an ambulance officer
1999 — 2001	chair of the council of chemistry students
1999 — 2000	editor in chief of the journal of the council of students of the faculty of natural sciences
2000	Participation in Kapsch Future Team Management Competition 2000

Publications and Patents:

Hackermüller J, Meisner N, Auer M, Jaritz M and Stadler PF, The effect of RNA secondary structures on RNA-ligand binding and the modifier RNA mechanism: A quantitative model. *Gene*, in press.

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