# Analysis of AU-rich Elements in the Yeast *Pichia pastoris*

vorgelegt von
Diplom-Biologe
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Von der Fakultät III - Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften
- Dr. rer. nat. -

genehmigte Dissertation

Promotionsausschuss:
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Berichter: Prof. Dr. rer. nat. Christine Lang
Tag der wissenschaftlichen Aussprache: 11.12.2007

Berlin 2008
D83

**Aufgeben ist das Letzte, was man sich erlauben darf.**

# Acknowledgements - Danksagung

Herrn Prof. Dr. Ulf Stahl möchte ich für die langjährige Unterstützung und die Möglichkeit danken, diese Arbeit am Institut für Biotechnologie, Fachgebiet Mikrobiologie und Genetik, der Technischen Universität Berlin anzufertigen.

Ganz besonderer Dank gilt Prof. Dr. Christine Lang, in deren Arbeitsgruppe diese Arbeit angefertigt wurde. Ich bedanke mich herzlich für die finanzielle Unterstützung, die Betreuung dieser Arbeit, die Hilfe bei der Antragsstellung des Stipendiums und die Korrektur der Dissertation. Außerdem möchte ich mich für die konstruktive Kritik und ständige Diskussionsbereitschaft bedanken.

Meiner Arbeitsgruppe danke ich herzlich für die nette Aufnahme und das tolle Arbeitsklima. Insbesondere bedanke ich mich bei Herrn Dr. Sean-Patrick Riechers, Andreas Raab und Frau Dr. Kristina Martin für die zahllosen wissenschaftlichen Diskussionen und bei Birgit Baumann für den netten Laboralltag sowie der Unterstützung bei diversen Versuchen und technischen Fragen. Meinen Praktikanten Kongla Sopachai, Sandra George, Caroline Heunemann, Heidi Löwa und Diana Güttner möchte ich für ihre Mitarbeit an meinem Projekt danken. Spezieller Dank gilt Heidi für ihr monatelanges Engagement bei der Reproduktion der Daten zur Proteinexpression. Außerdem bedanke ich mich bei Dr. Mewes Böttner für die theoretische und Natalia Bolotina für die praktische Unterstützung am Anfang meiner Doktorarbeit.

Desweiteren bedanke ich mich bei Dr. Udo Schmidt, der speziell für den Bereich RNA, aber auch für Fußball, immer ein sehr guter Diskussionspartner war. Susanne Engelhardt, Laura Funk und Anja Spielvogel danke ich für ihre geduldige Hilfe im Radioaktivlabor.

Isabell Schwieger (FMP Berlin-Buch), Roslin Bensmann und meiner Frau Katja Lautz (FMP Berlin-Buch) danke ich für die zusätzliche Durchsicht und Korrektur der Dissertation.

Außerdem bedanke ich mich bei allen anderen (auch ehemaligen) Mitarbeitern des Instituts, die mich bei dieser Arbeit durch ihr Zutun oder durch ihren Humor unterstützt haben.

Meiner Frau Katja Lautz danke ich für die ständige und unaufhörliche Unterstützung in Wissenschaft und Alltagsleben. Es tut so gut, jemanden an seiner Seite zu haben, der einen immer versteht und bedingungslose Rücksicht übt. Meinen Eltern, meiner Schwester und meinem Schwager, meinen Groß- und Schwiegereltern danke ich für die Unterstützung und das Verständnis während der Doktorarbeit, aber auch der letzten zehn Jahre. Es betrübt mich, dass meine verstorbenen Großväter den Tag der Verteidigung nicht mehr erleben durften.

Schließlich will ich noch meinen besten Freunden danken, die mir nicht nur in schwierigen Stunden immer zur Seite standen. Großer Dank gilt meinem langjährigen Freund Matthias Melchers, der mich mit seiner Art von Humor immer aufheitern konnte, obwohl ihm das sicher nicht immer bewusst war.

# Content

# List of Abbreviations

| | |
|---|---|
| A | Adenine |
| AmpR | Ampicillin resistance |
| AOX1 | Alcohol oxidase 1 |
| APS | Ammoniumpersulfate |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| C | Cytosine |
| ColE | Origin of replication for *E. coli* |
| C-Source | Carbon source |
| Da | Dalton |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide |
| DTT | Dithiotreitol |
| EDTA | Ethylenediamine-tetra-acetic acid |
| Fig. | Figure |
| g | Gramm |
| h | Hour |
| l | Liter |
| m | Meter |
| M | Molar |
| MOPS | Morpholinopropane-sulfuric acid |
| mRNA | messenger RNA |
| n/a | Not available / not analyzed |
| OD | Optical density |
| ORF | Open reading frame |
| Ori | Origin of replication |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PMSF | Phenylmethylsulfonylfluoride |
| RNA | Ribonucleic acid |
| RT | Room temperature |

| | |
|---|---|
| RV | Relative value |
| SDS | Sodium-dodecyl sulfate |
| SGD | Saccharomyces Genome Database |
| SSC | Saline buffered sodium citrate |
| T | Thymine |
| TAE | Tris-acetate-EDTA |
| TEMED | N, N, N´, N´-Tetra-methylethylethylendiamine |
| U | Uracile |
| UTR | Untranslated region |
| UV | Ultraviolet radiation |
| V | Volt |
| X g | Multiple of acceleration of gravity |
| YE | Yeast extract |
| YNB | Yeast nitrogen base |

# Index of Figures and Tables

## Figures

## Tables

# 1 RNA interference (RNAi) – Basics and Applications

In 1998, Andy Fire and Craig Mello initially characterized the potential of double-stranded RNA (dsRNA) to specifically silence genes in the nematode *Caenorhabditis elegans*. Although the silencing effects of complementary antisense RNA had been previously investigated (Guo and Kemphues, 1995), Fire and Mello were the first to show that small amounts of dsRNA were able to specifically and very potently silence its complementary target - a phenomenon termed RNA interference or RNAi (Fire et al., 1998). These experiments could also explain the observation that the introduction of a dsRNA encoding the purple colour of petunias in plant cells did not make the plant turn purple, but rather white (Jorgensen, 1990). In the following years, more and more scientists studied RNAi (Gerstein and Douglas, 2007) with the result that it is an important and widely spread cellular tool to control gene expression in such diverse kingdoms such as fungi, plants and animals (Ding et al., 2004). It has become obvious that the greatest potential of RNAi is based on the specific and potent knockdown of genes whose sequence is known and which cause disease (history of uncovering the mechanism of RNAi; (Sen and Blau, 2006)). Fire and Mello received the Nobel Prize in Physiology or Medicine in 2006 for their revolutionary discovery (Paulson and Gonzalez-Alegre, 2006).

## 1.1 Double-stranded RNA – Triggers of RNAi

RNAi pathways are triggered by small dsRNA molecules (~19 – 23 nt in length), which can either be subjected exogenously to the cell (small interfering RNAs – siRNAs), or processed endogenously from larger dsRNA molecules (micro RNAs – miRNA, miR). These small RNAs mediate sequence-specific mRNA cleavage or translational silencing, depending on the rate of complementarity to their target (post-transcriptional gene silencing – PTGS). Additionally, RNAi pathways can also regulate heterochromatin formation and transcriptional gene silencing in the nucleus (TGS; nuclear RNAi) through DNA methylation (Lippman and Martienssen, 2004; Wassenegger, 2005; Weinberg et al., 2006).

### 1.1.1 Small interfering RNAs (siRNAs)

Expression vectors encoding hairpin or linear long dsRNA molecules can be introduced into cells using common transfection methods. Transcribed small hairpin RNAs (shRNAs) are transported into the cytoplasm by the dsRNA-binding karyopherin exportin-5 (Yi et al., 2003) (see also fig. 1). Alternatively, cells can be transfected with synthetic double-stranded siRNAs, whose 5' ends are phosphorylated by endogenous kinases in the cytoplasm. The RNA duplexes are then processed by the RISC loading complex (RLC) consisting of the ribonuclease type III named Dicer (Bernstein et al., 2001; Carmell and Hannon, 2004), TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) (Lee et al., 2006). The resulting short DNA fragments are termed siRNAs and are 21 nt to 23 nt in length. SiRNAs are characterized by a 2 nt overhang at both 3' ends (usually deoxythymidine) and a phosphate-group at their 5' ends (Elbashir et al., 2001). Afterwards, TRBP transfers the RLC to Argonaute 2 (Ago2). This newly formed protein complex is termed the RNA-induced silencing complex (RISC) (Liu et al., 2004; Chendrimada et al., 2005; Haase et al., 2005). Currently it is proposed that the double-stranded siRNA is loaded onto Ago2 (Matranga et al., 2005). As it is a characteristic for members of the Argonaute family, Ago2 contains an amino-terminal PAZ (PIWI-Argonaute-Zwille) and a carboxy-terminal RNase H-like PIWI domain which bind the 3' end (Lingel et al., 2004) or the phosphorylated 5' end (Ma et al., 2004; Parker et al., 2005) of the "guide strand" (anti-sense strand), respectively. The specific incorporation of the guide strand is determined by the less thermodynamically stable 5' end of the siRNA (Khvorova et al., 2003). Ago2 can cleave the "passenger strand" (sense strand), which then dissociates from the guide strand and is consequently degraded. Alternatively, it is possible that the siRNA duplex is unwound by an RNA helicase and the passenger strand subsequently degraded (Matranga et al., 2005; Preall and Sontheimer, 2005; Rand et al., 2005). Recently, RNA helicase A has been identified to be a RISC-associated factor and to be involved in RISC loading and unwinding the siRNA duplex (Robb and Rana, 2007). Once the single stranded guide strand is incorporated (active RISC), the guide strand leads the RISC to its complementary target RNA, which is afterwards cleaved 10 nt to 11 nt from the guide strand's 5' end through the "slicing activity" of the Ago2 PIWI domain (Liu et al., 2004; Meister et al., 2004; Song et al., 2004). Degradation of the cleaved target mRNA most likely occurs in cytoplasmic processing bodies (P-

bodies), which are associated with Ago2 and RISC (Jakymiw et al., 2005; Liu et al., 2005). In addition, it has also been shown that RNA helicase rck/p54 is a further part of human RISC (Gregory et al., 2005; Chu and Rana, 2006) and is involved in mRNA degradation in P-bodies as well (Cougot et al., 2004).

### 1.1.2 MicroRNAs (miRNAs)

In contrast to the siRNA pathway, the miRNA pathway is initiated in the nucleus (fig. 1). MiRNAs are encoded either on the sense, or on the anti-sense strand of introns and intergenic regions (Lagos-Quintana et al., 2001; Lee and Ambros, 2001; Rodriguez et al., 2004). The majority of known miRNAs are transcribed by RNA polymerase II (Lee et al., 2004), although miRNAs transcribed by RNA polymerase III have been reported (Borchert et al., 2006). Primary miRNAs (pri-miRNAs) can be mono- or polycistronic transcripts and have a varying length of a few hundreds to thousand nucleotides. As is usual for RNA polymerase II transcripts, they possess a 5'-end cap and a 3' poly(A)-tail (Cai et al., 2004). Pri-miRNAs form a stem-loop structure that is flanked by single-stranded RNA sequences (Lee et al., 2002). Subsequently, pri-miRNAs are processed by the microprocessor complex consisting of the RNase III Drosha which displays affinity to the hairpin and the overhanging single strands of the pri-miRNA (Zeng and Cullen, 2005), and its dsRNA binding co-factor DGCR8 (DiGeorge syndrome critical region gene-8) (Gregory et al., 2004; Han et al., 2006). Processing products (precursor miRNAs or pre-miRNAs) are hairpin RNAs of ~70 nt up to 100 nt in length (Lee et al., 2003), containing a 5' phosphate and a 2 nt 3' overhanging end (Kim, 2005). Afterwards, the pre-miRNAs are transported into the cytoplasm by exportin-5 through a RanGTP-dependent mechanism (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004). They are cut by the Dicer-TRBP-PACT-complex (RISC loading complex, RLC) in the cytoplasm into ~22 nt mature microRNAs (miRNAs). Afterwards, RLC transfers the miRNA to RISC-associated Ago2. The miRNA duplex is unwound and the passenger strand discarded (Preall and Sontheimer, 2005). The guide strand can now direct the active RISC to its target mRNA. An important determinant for miRNA targeting are positions 2 to 8 of the guide strand's 5' end called the "seed" region (Lewis et al., 2005). Bulges that can form in the middle of the guide strand due to imperfect complementarity to its target mRNA are not susceptible to Ago2-mediated cleavage. As a consequence, RISC-miRNA-complexes accumulate in P-bodies where the

target mRNA is translationally repressed and/or degraded afterwards (Liu et al., 2005; Chan and Slack, 2006; Chu and Rana, 2006). However, the mechanisms underlying miRNA-mediated translational silencing and mRNA degradation are not fully characterized.

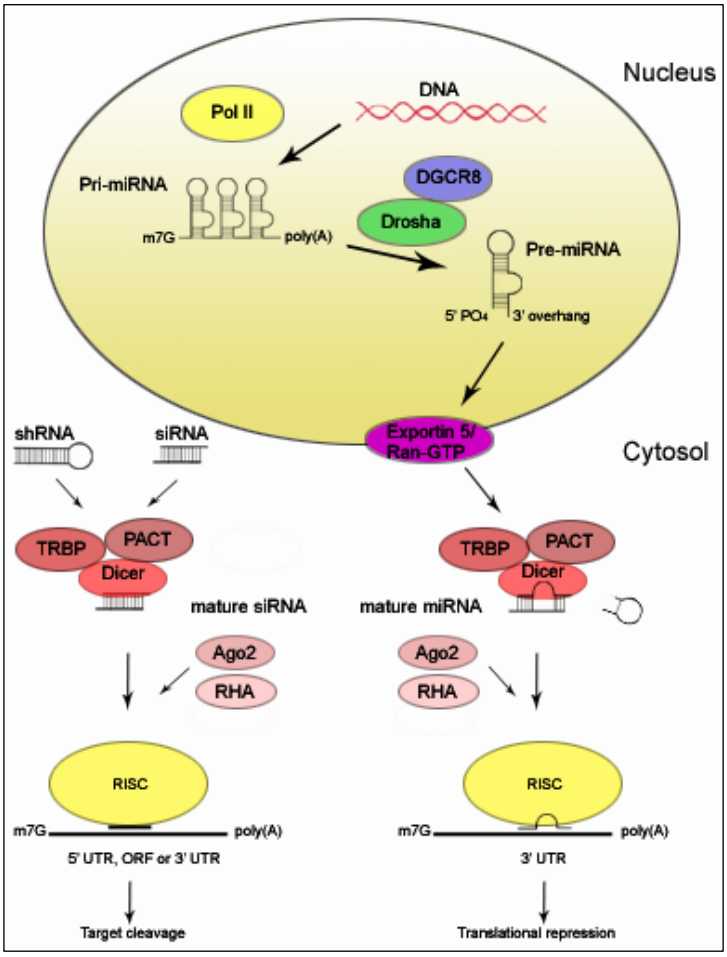


**Figure 1: Mammalian Mechanisms of RNAi.** Transfected siRNAs, or expressed shRNAs, are processed by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) termed the RISC loading complex (RLC) in the cytosol into mature siRNAs. SiRNAs are loaded onto Ago2 and subsequently unwound by RNA helicase A (RHA). Unwinding of the siRNA duplex and discarding of the passenger strand leads to the activation of the RNA induced silencing complex (RISC). Due to perfect complementarity of the guide strand, Ago2 is able to cleave the mRNA target, which is rapidly degraded. Endogenously encoded pri-miRNAs are transcribed by RNA polymerese II (Pol II) in the nucleus. Several miRNAs can be encoded on a polycistronic pri-miRNA, which is processed by a complex of Drosha and DiGeorge syndrome critical region gene-8 (DGCR8) named microprocessor into precursor miRNAs (pre-miRNAs). These are exported into the cytoplasm by exportin 5, where they are substrates of Dicer-TRBP-PACT-complex and processed into mature miRNAs. Unwinding and separation of the duplex activates RISC. Due to imperfect binding of the guide strand Ago2 cannot cleave the target RNA, which is then translationally repressed (figure adapted from (Kim and Rossi, 2007)).

### 1.1.3 Identification of miRNAs

Hundreds of miRNAs have been identified to date (Bentwich et al., 2005; Zamore and Haley, 2005; Berezikov et al., 2006) (http://microrna.sanger.ac.uk) and the number is rapidly growing. In *C. elegans* the first miRNAs (lin-4 and let-7) were identified genetically by chromosomal walking (Lee et al., 1993; Reinhart et al., 2000; Lee and Ambros, 2001). The application of *in silico* tools accelerates the bioinformatic identification of new miRNA coding sequences. Computer programs used for bioinformatic miRNA identification are miRseeker, miRScan, PalGrade, ProMiR, and miRAlign as examples that have been compared recently (Chaudhuri and Chatterjee, 2007; Doran and Strauss, 2007). The predictive function of these algorithms relies on the conservation of miRNAs among related species (conservation criterion), the stem-loop of pre-miRNAs and the coding sequence of the miRNA in one arm of that stem-loop (structure criterion). Once a miRNA has been predicted, its expression (expression criterion) has to be analysed by Northern blotting, RT-PCR, RNase protection assay (RNP) or microarray analysis (Chaudhuri and Chatterjee, 2007; Lindow and Gorodkin, 2007).

### 1.1.4 Principles of microRNA targeting

In order to understand the functionality of certain miRNA species, it is essential to characterize their specific mRNA targets. Each miRNA can target hundreds of transcripts due to its imperfect binding (Bartel and Chen, 2004; Lim et al., 2005). It is also possible that a single mRNA can be a target of several miRNAs (Lewis et al., 2003; John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005). This agrees with the finding that multiple target sites in the same target RNA can increase the rate of translational repression (Doench et al., 2003), whereas the optimal distance between miRNA seeds in the target has been elucidated to be from 13 up to 35 nt (Saetrom et al., 2007). Combinatorial binding of different miRNAs to one target is proposed to result in the normally detectable expression, suggesting a regulatory “fine-tuning” based on miRNA binding.

In general, miR targeting relies on nucleotides 2 to 8 from the 5’ end of the guide strand. Currently, this minimal “seed” region is regarded to be the most important factor in terms of efficiently silencing genes (Doench and Sharp, 2004; Kim and Nam, 2006). Consequently, mismatches at positions 1, 9 or 10 and/or the 3’ region do not

significantly affect the target repression. However, seed regions can be as small as 4 nt due to truncations by G:U wobble base pairs. Such interruptions require compensatory pairing of the 3' end in order to efficiently repress the target. In contrast, a perfect 3' end pairing is not sufficient for target repression. Thus, target sites are classified into 5' dominant and 3' compensatory sites, reflecting the asymmetry of a functional miRNA (Brennecke et al., 2005). It has also been reported that the seed sequence alone is a non-sufficient marker for targeting (Didiano and Hobert, 2006). When searching for further factors that regulate miRNA targeting, an additional aspect attracts attention - target accessibility. Indeed, it has been demonstrated that target accessibility and siRNA duplex asymmetry can alter knockdown efficiency by 26% to almost 40% (Shao et al., 2007). This is further supported by the finding that the transfer of a freely accessible miRNA target site into a thermodynamically stable hairpin adversely affects siRNA-mediated silencing (Kurreck, 2006). To access its target, it has been hypothesised that at least three tandem single-stranded nucleotides are required for hybridization to the 5' seed region of a miRNA (Robins et al., 2005). Recently, a two-step model for miRNA-target-hybridization was proposed. In the first step, the miRNA nucleates hybridization with four consecutive nucleotides. In the second step, base-pairing is driven forward resulting in the abolishment of the target secondary structure (Long et al., 2007). Although natural targets of miRNAs are located in the 3' UTR of their target transcripts, silencing is also efficient when the complementary target site is located at the 5' UTR. It has been suggested that the target site itself may be located at any position in order to maintain knockdown (Lytle et al., 2007).

Several computational algorithms have been developed in order to predict miRNA targets. Examples such as TargetScanS, PicTar, DIANAmicroT, miRanda, and RNAhybrid rely on complementarity, conservation and thermodynamics in order to find matching target sites (Chaudhuri and Chatterjee, 2007; Doran and Strauss, 2007; Lindow and Gorodkin, 2007). Interestingly, it has been shown that the non-protein coding gene IPS1 (Induced by Phosphate Starvation1) from *Arabidopsis thaliana* displays complementarity to miR-399. The expected cleavage site is covered by a non-matching loop, avoiding cleavage and sequestering miR-399. This process is termed "target mimicry" (Franco-Zorrilla et al., 2007). Furthermore, it is known that endogenous miRNAs can be targeted by synthetic siRNAs named "antagomirs" and it is also likely to occur naturally (Mattes et al.,

2007). Current computer programmes used for target prediction do not consider target mimicry or other non-coding RNAs (such as miRNAs) as potential targets.

## 1.2 MiRNA targets – Regulators of essential cellular processes

In order to verify miRNA targets, the most commonly used tools are reporter gene systems as well as miRNA gene knockdown techniques and target point mutations. Most miRNAs targets are currently undefined. Experimentally validated targets can be found in TarBase (http://www.diana.pcbi.upenn.edu/tarbase.html).

### 1.2.1 MiRNAs in serious diseases

MiRNAs regulate such diverse processes such as anti-viral defense (Ding et al., 2004; Lecellier et al., 2005), cell development, proliferation, survival, apoptosis and signal transduction (Yeung et al., 2005).

MiRNAs in vertebrates have also been functionally implicated in cardiogenesis, myogenesis, neurogenesis, and hematopoiesis (Alvarez-Garcia and Miska, 2005; Zhao and Srivastava, 2007). According to these observations, many miRNAs display a spatial expression in a tissue-specific manner (Lagos-Quintana et al., 2002; Wienholds and Plasterk, 2005). The involvement of miRNAs in these cellular processes and in organogenesis demonstrates specific expression patterns. Deregulation of such patterns strongly increases the likelihood of several serious diseases. Thus, miRNAs and their targets or proteins involved in miRNA biogenesis are suspected, or have even been demonstrated, to contribute to diverse diseases if they are de-regulated. Examples are neurodegenerative diseases such as spinal muscular atrophy (SMA) (Dostie et al., 2003) or fragile X mental retardation (FXMR). FXMR is caused by down-regulation of the fragile X mental retardation protein, which has otherwise been shown to be a likely part of RISC in *Drosophila melanogaster* (Ishizuka et al., 2002). Moreover, deletion of DGCR8, a component of the Microprocessor complex that is involved in miRNA biogenesis, is associated with the DiGeorge syndrome (heart defects and schizophrenia) (Shiohama et al., 2003).

### 1.2.2 Expression analysis of miRNAs in cancer

In order to analyse miRNA expression patterns, the use of custom micro arrays made large miRNA profiling studies possible. Based on large miRNA profiling studies, several distinct expression signatures of different cancer types could be set up and certain miRNAs determined as biomarkers for cancer. As the number of identified miRNAs is continuously growing and can be queried in databases as well as their targets, only a small subset of miRNAs is considered here in order to give a short overview of the broad range of involvements, targets and functions.

In order to differentiate lung cancer and healthy cells, a set of 42 miRNAs could be identified, including miR-21 (Yanaihara et al., 2006). Another study revealed a signature of 15 miRNAs in order to characterize breast cancer cells. Among these were miR-145, miR-21 and miR-155 (Iorio et al., 2005). For chronic lymphocytic leukemia (CLL), a profiling set of 13 miRNAs were identified including miR-15a, miR-155 and miR-16-1/2 (Calin et al., 2007). Another genome-wide analysis revealed a solid-cancer signature including miR-155, miR-17–5p, miR- 20a, miR-21, miR-92 and miR-106a (Volinia et al., 2006). Further studies include profiles for head and neck cancer (Tran et al., 2007), prostate cancer (Porkka et al., 2007), colon cancer (Akao et al., 2007), pancreatic cancer (Bloomston et al., 2007) and additional profiles for other types of cancer (Gaur et al., 2007). These profiles serve to characterize poorly understood human cancer in addition to mRNA expression profiles (Lu et al., 2005; Waldman and Terzic, 2007). These studies have demonstrated that miRNAs can either be down- or upregulated in cancer, compared to non-cancer tissues (Jay et al., 2007).

The expression of miR-15/16 is deleted, or at least down-regulated, in approximately 68% of B cell chronic lymphocytic leukaemia (B-CLL) (Calin et al., 2002; Calin et al., 2004a). The first identified miRNAs involved in solid tumorigenesis were miR-143/145, which display repressed expression in colon cancer (Michael et al., 2003; Akao et al., 2007) and lung cancer (Yanaihara et al., 2006). Let-7 has also been reported to be down-regulated in human lung cancer (Takamizawa et al., 2004; Yanaihara et al., 2006). MiR-125a and miR-125b are also down-regulated in breast cancer (Iorio et al., 2005). Recently, the down-regulation of miR-16, miR-143/145, MiR-125a and miR-125b and Let-7-family members was additionally shown in prostate cancer (Porkka et al., 2007).

In contrast to miR-15/16, the expression of the miR-17-92 was increased in B-CLL (He et al., 2005). This six-stem-loop polycistron encodes seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and mir-92-1. Increased expression can be observed in lymphomagenesis (Wienholds and Plasterk, 2005) as well as in lung cancers (Hayashita et al., 2005). It is also known as “OncomiR-1” for oncogenic miRNA-1 (He et al., 2005). Another miRNA that is upregulated in different kinds of cancer is miR-155 in correlation with its host gene B-cell integration cluster (BIC). Upregulation has been reported in childhood Burkitt lymphoma (Metzler et al., 2004), lung cancer (Yanaihara et al., 2006) and breast cancer (Iorio et al., 2005). Recent studies revealed the high over-expression of miR-21 in breast tumors (Si et al., 2007) and head and neck cancer cell lines (Tran et al., 2007). There are a lot more specific miRNAs that are de-regulated in other types of cancer and cannot be discussed here (Kusenda et al., 2006; Szafranska et al., 2007; Tran et al., 2007).

### 1.2.3 MiRNAs and their targets – a potential role in human cancer

The majority of known miRNAs is encoded in intergenic regions or within introns of either non-protein-coding or protein-coding sequences. They can be encoded on the sense or on the anti-sense strand (Lagos-Quintana et al., 2001; Mourelatos et al., 2002). However, miRNAs can also be located in exons or have their own promoters for transcription (Rodriguez et al., 2004; Zhao and Srivastava, 2007). A further feature of similar miRNAs is their clustered organization in polycistronic transcripts (Houbaviy et al., 2003). MiRNAs have been shown to be involved in cell growth, proliferation and apoptosis. In general, cancer is characterized by a deregulation of these processes (Lu et al., 2005; Sood et al., 2006; Zhang et al., 2006). Interestingly, a large number of human miRNAs are indeed located at cancer-related genomic regions (Calin et al., 2004b).

MiR-15/16 down-regulate the expression of the antiapoptotic factor B cell lymphoma 2 (Bcl2) (Cimmino et al., 2005). Bcl2 is over-expressed in different kinds of cancers like lymphomas, carcinomas and leukemias (Sanchez-Beato et al., 2003). The Let-7 miRNA family (lethal-7) are negative regulators of the onco-gene RAS in *C. elegans* (Johnson et al., 2005). Members of the guanine nucleotide binding *ras* gene family (K-, H- and N-RAS), which are localized at the inner plasma membrane, additionally often carry mutations in human cancer (Ishimura et al., 2003). The expression of the polycistronic cluster miR-17-92 is induced by the proto-oncogene *c-myc* that binds

upstream of the miR-17 locus. Recently, a synergistic co-operation between *c-myc* and miR-17-92 was demonstrated to contribute to aggressive cancer development (Tagawa et al., 2007). Furthermore, miR-17-5-p and miR-20a from this cluster down-regulate expression of E2F1 (O'Donnell et al., 2005). The oncogenes ERBB2 and ERBB3 are suppressed by miR-125a/b (Scott et al., 2007). miR-372 and miR-373 are two oncogenes identified in testicular germ cell tumors that slow down the p53 pathway (Voorhoeve et al., 2006). It is known that miR-181 is involved in hematopoietic differentiation (Chen et al., 2004). A very recent study could provide evidence for the involvement of miR-181a in acute myeloid leukaemia (AML), modulating the expression of 28 target genes that had been predicted earlier. A recent finding is that the deletion or inhibition of miR-21 expression in human hepatocellular cancer (HCC) increased the expression of the ubiquitous phosphatase and tensin homolog tumor suppressor PTEN being a direct target of miR-21 (Meng et al., 2007a). PTEN is a regulator of the PI3KAkt signalling pathway (phosphatidylinositide kinase), whose activation is associated with the formation of many tumors (Altomare and Testa, 2005). There is further evidence that miRNAs are involved in the in PI3KAkt pathway, which can be activated by the oncogene TCL1. Expression of TCL1 is regulated by miR-29 and miR-181in B-CLL (Calin et al., 2007). A direct target of miR-155 is currently uncertain. However, its host gene is known to interact with c-myc in avian lymphoma (Tam et al., 2002).

Predicted targets of miR-155, miR-17–5p, miR- 20a, miR-21, miR-92 and miR-106a turned out to be tumor suppressors and oncogenes (Volinia et al., 2006). Recently, a set of seven miRNAs has been shown to be involved in head and neck cancer (let7-a, miR-16, miR-21, miR-205, miR-342, miR-346, and miR-373) (Tran et al., 2007). A significantly high number of their predicted targets are likely to play a role in carcinogenesis. However, predicted targets and the extent of regulatory influence still have to be confirmed experimentally.

A specific class of genes frequently related to cancer are cytokines, proto-oncogenes and transcription factors, whose expression is regulated by an AU rich element (ARE) in the 3' UTR of their mRNAs. An example for this class is the proto-oncogene c-myc, whose over-expression down-regulates the PI3K pathway (Bellmann et al., 2006). PI3K inactivates the ARE-binding protein (ARE-BP) KSRP (K homology-type splicing regulatory protein), which in turn promotes decay of β-catenin (Gherzi et al., 2006). β-catenin has been implicated in human colon cancer (Verma et al., 2003). Other

targets of KSRP are *hnRNP* A/B/F mRNAs (Ruggiero et al., 2007) which encode different ARE-BPs and can play a role in chronic myelogenous leukaemia (Perrotti and Neviani, 2007). Furthermore, hnRNP A1 binds miR-18 pri-miRNA and regulates its processing (Grosshans and Svoboda, 2007). A similar type of interaction has been shown between miR-370 and the ARE-regulated interleukin-6 (IL-6). IL-6 is over-expressed in malignant human cholangiocytes and reduces miR-370 expression (Meng et al., 2007b). Other ARE-regulated genes are colony-stimulating factor-1 (CSF-1), whose over-expression promotes ovarian cancer progression, the proto-oncogene *c-fos*, which is involved in cervical cancer (van Riggelen et al., 2005) and vascular endothelial growth factor (VEGF). VEGF is involved in age-related macular degeneration and different kinds of cancer (Aigner, 2007; Shen et al., 2007).

Although ARE-regulated gene products and miRNAs are involved in the regulation of the same cellular pathways such as PI3K and proliferation, direct interactions between miRNAs or compoments of the miRNA pathway have only rarely been reported. The *D. melanogaster* VIG protein, a homologue of the human ARE-BP PAI-RBP-1 (plasminogen activator inhibitor-RBP-1), was shown to be included in RISC. Furthermore, microRNAs could be localized to identical cytoplasmic bodies (Liu et al., 2005) as ARE-BPs (Stoecklin et al., 2004). Finally, the regulation of a tumor necrosis factor $\alpha$-3' UTR-containing reporter (TNF$\alpha$) by miR16-1 and the ARE-BP Tristetraprolin (TTP) was shown in *D. melanogaster* (Jing et al., 2005). More recently, the ARE-mediated up-regulation of translation of a TNF$\alpha$ ARE-bearing reporter by FXR1 and Argonaute 2 has been demonstrated. Both proteins can interact with each other and are involved in the assembly of RISC (Vasudevan and Steitz, 2007). Another report revealed that human microRNAs target genes with AT-rich/AU-rich 3' UTRs implicated in transcription and translation processes, which supports a possible regulatory role depending on miRNAs (Robins and Press, 2005).

In summary, this data provides direct and indirect evidence for the involvement of specific miRNA deregulation in human cancer. MiRNAs can function as tumor suppressors or oncogenes or at least induce/inhibit expression of those. This suggests the possibility that miRNA can be exploited for clinical applications such as a therapeutic for human diseases, especially for cancer diagnosis, prognosis and treatment.

## 1.3 RNAi as a tool in Gene Therapy

The potential of RNAi-based gene therapy relies on the capacity of siRNAs to specifically and potently silence complementary disease-causing targets, which either can be mRNAs or miRNAs acting as oncogenes. In order to establish successful therapies a number of major issues need to be solved in siRNA applications: Unspecific and off-target effects (OTEs), stability and simultaneous effectiveness of an siRNA and efficient tissue-specific as well as human dose-feasible delivery *in vivo*.

### 1.3.1 The cellular interferon (IFN) response and off-targets effects (OTEs)

Highly abundant siRNAs (21 nt) or long dsRNA molecules (30 nt or longer) are able to induce an endogenous type I interferon (IFN) response (Sledz et al., 2003; de Veer et al., 2005; Kim and Rossi, 2007) through the activation of dsRNA-dependent protein kinase, which starts to phosphorylate the $\alpha$-subunit of eukaryotic initiation factor 2 (eIF2a). As a consequence, protein synthesis is inhibited unspecifically (Stark et al., 1998). In addition, dsRNA can activate 2',5'-OligoA synthetase. The synthesis of 2',5'-polyadenylic acid induces the non-specific RNase L, which degrades ssRNA (de Veer et al., 2005). Both processes are lethal for the cell and lead to apoptosis (Gil and Esteban, 2000). A recent study examined the effect of non-targeting, unspecific siRNA molecules in tumor cells. Although there were no sequence complementary targets an IFN response could be detected affecting cell viability, proliferation and apoptosis in human tumor cells. In order to monitor unspecific cellular activities the expression of IFN-related genes such as diverse interferons, tumour necrosis factor $\alpha$ or interleukin-6 need to be analysed frequently (Tschaharganeh et al., 2007), something which has already been considered before (Grimm et al., 2006).

Moreover, it has been reported that siRNAs can also act as miRNAs (Doench et al., 2003). As mentioned before, a perfect sequence match is not required for miRNA targeting. This might result in unwanted OTEs due to translational silencing or mRNA degradation of non-desired targets (Jackson et al., 2006). A set of 15 consecutive nucleotides was shown to be sufficient for silencing non-targeted transcripts. Interestingly, a silencing effect of the passenger strand was observed in the case of an IGF1R (insulin-like growth factor receptor) targeting siRNA (Jackson et al., 2003). It was recently shown that OTEs can be mediated by cooperation of transfected

siRNAs with endogenous miRNAs. Optimal spacing of target seed regions for gene silencing could be determined as 13 nt up to 35 nt. This optimal spacing was detected more frequently in off-target genes than in non-off target genes, despite having seed sites (Saetrom et al., 2007).

While designing siRNAs it is important to utilize bioinformatics sequence tools to reduce the possibility of OTE. To yield appropriate gene silencing and maximum reduced OTEs, several rules for siRNA generation have been established. Parameters concerning sequence, size and structure are linked to effective RNAi. Optimal siRNA length of 21-23 nt, a GC content between 36% and 52%, 2 nt 3' overhanging ends with 5' blunt ends, targeting the open reading frame and some more criteria have successfully been applied to mediate efficient gene silencing and to avoid OTEs (Elbashir et al., 2002; Izquierdo, 2005; De Paula et al., 2007). In addition, it is known that siRNA modifications, particularly O-methyl-groups at the ribose 2' position within the seed region can also contribute to a reduction of OTEs (Fedorov et al., 2006; Jackson et al., 2006). Nevertheless, any *in silico* created siRNA requires an experimental validation and analysis.

### 1.3.2 miRNA pathway saturation

Another problematic limitation in RNAi based gene therapy is the fact that ectopically expressed shRNAs/siRNAs (small hairpin RNA, see 1.3.3) can compete with endogenous miRNAs for components of the RNAi pathway. As a consequence, miRNAs can be inhibited resulting in alterations of its expression. This was recently demonstrated by a combinatorial approach using synthetic siRNAs and expressed shRNAs. It turned out that competition can occur between synthetic siRNAs, expressed shRNAs and endogenous miRNAs. This competition partially depends on the saturation of exportin 5, which is responsible for the transport of miRNAs and shRNAs from the nucleus to the cytoplasm (Yi et al., 2003). Another bottleneck identified is the incorporation of shRNAs and siRNAs into RISC and an involvement of TRBP in this issue. Interestingly, siRNAs expressed from a microRNA backbone do not compete with each other and the RNAi machinery (Koller et al., 2006; Castanotto et al., 2007). Furthermore, it was shown that a high expression of shRNA induced the saturation of exportin 5 in an *in vivo* mouse model, resulting in down-regulation of miRNA export from the nucleus and following tissue-damage and death (Grimm et al., 2006).

### 1.3.3 Chemical siRNA-modifications and the application of small hairpin RNAs (shRNAs) can prolong RNAi-mediated effects

Specific gene inhibition by siRNAs in mammalian culture cells is only transient in contrast to worms or plants, where these effects are stable. The RNA-dependent RNA polymerase (RdRp) in these organisms can use the siRNA antisense strand as a primer in order to produce a dsRNA precursor from the target mRNA. This precursor can again serve as a substrate for Dicer (secondary siRNA) and silence the same target. Till date, no homologue of RdRp has yet been found in vertebrates (Ahlquist, 2002; Chiu and Rana, 2002).

Transient siRNA-mediated effects are unsuitable for long-term experiments or long-lasting therapeutic applications. In order to avoid nucleolytic degradation and to maintain activity at the same time, several modifications of the siRNA have been tested. It was found that the ribose 2'-OH is not required for gene silencing activity and that chemical modifications at this specific position increased siRNA stability and prolonged the RNAi effect. However, modifications leading to alterations of the RNA A-form major groove of the guide strand-mRNA helix resulted in a complete loss of siRNA activity (Chiu and Rana, 2003; Czauderna et al., 2003a). The most common chemical siRNA modifications are phosphodiester modifications at the nucleic acid backbone like phosphorothioate (PS) or boranophosphonate (-$BH_3$) and 2'-ribose modifications like 2'-O-methyl (2'-OMe), 2'- deoxy-2'-fluoro modifications as well as locked nucleic acids. Backbone modifications have been shown to increase siRNA stability, whereas 2'-modifications have an additional potential to reduce interferon response activation (De Paula et al., 2007).

The application of chemically synthesized shRNAs was shown to elevate RNAi-mediated effects. ShRNAs form a stem of 19-29 bp flanked by a loop structure which is processed by Dicer into a mature siRNA. Best results were obtained with a 29 bp stem of the shRNA hairpin (Siolas et al., 2005). Additional prolongation of silencing effects could be demonstrated by endogenous vector-based expression of shRNAs (Brummelkamp et al., 2002b; Paddison et al., 2002; Sui et al., 2002). However, it is known that vector-based shRNA expression is a possible activator of the interferon response (Bridge et al., 2003). *In vivo* shRNA expression can be driven by RNA polymerase II promoters. The resulting transcripts contain a poly(A)tail and a 5'-cap in order to mimic pri-miRNAs. Alternatively, two RNA polymerase III (pol III) promoters like H1 or U6 can be used to transcribe either sense and anti-sense strand

of an siRNA or the complete shRNA as a single transcript (Miyagishi and Taira, 2002; De Paula et al., 2007). A disadvantage of the constitutive activity of these promoters is their disability to adjust to specific cellular conditions in order to regulate growth and survival. Importantly, the development of inducible pol III promoters by inserting the target sequence for transcription inhibitors can largely avoid this problem (Czauderna et al., 2003b; Wiznerowicz and Trono, 2003; Gupta et al., 2004).

### 1.3.4 Delivery of siRNA can be enhanced by chemical siRNA-modifications and the application of viral vectors

Furthermore, chemical modifications of siRNAs can be exploited to perform cell-specific delivery of an siRNA. As siRNAs possess a negatively charged backbone, they are not easily able to pass through cellular membranes. They can be modified with cholesterol, cell-penetrating peptides (CPPs) for this purpose, or incorporated into stable nucleic acid lipid particles (SNALPs) and cationic lipids/polymers. More target-specific approaches for delivering siRNAs are modifications with receptor-specific aptamers, heavy-chain antibody fragments (Fabs) or protamine and incapsulation into nanoparticles (De Paula et al., 2007; Kim and Rossi, 2007).

Despite great progress in this field, the use of siRNAs and expression vectors is limited. Bringing siRNAs or plasmids into cells requires techniques such as transfection, electroporation or microinjection. Mammalian cell lines (or easily accessible organs) are susceptible to these methods in contrast to non-dividing, primary or stem cells (Clayton, 2004; Hannon and Rossi, 2004). Furthermore, *in vivo* intravenous or hydrodynamic applications of siRNA often use high amounts of siRNA in order to achieve appropriate effects. As described before, high siRNA concentrations can result in an unspecific interferon response (Aigner, 2007).

In order to resolve these difficulties, viral vectors have also been established for siRNA-mediated gene therapy, including retroviral, lentiviral, adenoviral and adeno-associated viral (AAV) vectors. In general, retroviral vectors are derived from murine stem cell virus Moloney murine leukaemia virus and can integrate in replicating cells, whereas lentiviral vectors are derived from human immuno-deficiency virus (HIV)-1 and can integrate in (non-)dividing cells. Chromosomal integration guarantees long-term and stable expression, but carries the risk of insertional mutations. Adenoviral vectors and AAVs are applied when only a moderately stable expression is needed. These vectors do not integrate into the genome and thus prevent the risk of disease-

causing and/or lethal mutations (De Paula et al., 2007; Kim and Rossi, 2007; Masiero et al., 2007). In contrast to their advantages, viral vectors also lack sufficient target specificity. Even more importantly, the putative toxicity effects will limit or even prevent its application in humans (Reid et al., 2002).

### 1.3.5 Therapeutic targets in RNAi-based applications

There are several studies that address the question of siRNA approaches in cancer therapy in order to silence over-expressed onco-genes.

As mentioned before, the *ras* gene family often carries mutations in different cancer types. When mutated, K-RAS was silenced in human pancreatic cells by applying a retroviral expression vector and tumorigenicity was abolished (Brummelkamp et al., 2002a). Silencing of mutated K-RAS by synthetic siRNAs also reduced the angiogenic potential in these cell lines (Brummelkamp et al., 2002a; Fleming et al., 2005). Both observations make the *ras* gene family a potential target for *in vivo* siRNA-based therapies. Furthermore, mutations in *ras* genes induce constitutive activation of the serine/threonine kinase Raf-1. SiRNA-mediated knockdown of Raf-1 in cerebral microvascular endothelial cells demonstrated inhibition of tumor growth and identified Raf-1 as a potential therapeutic target (Culmsee et al., 2006). Interestingly, Raf-1 activity can also be triggered by vascular endothelial growth factor VEGF (Alavi et al., 2003). ARE-regulated VEGF has already successfully been targeted by siRNAs in clinical trials as well as its cellular receptor VEGFR1 (Aigner, 2007; Kim and Rossi, 2007). Further examples of ARE-regulated genes that are targeted in RNAi gene therapy are β-catenin in human colon cancer (Verma et al., 2003) and murine melanoma growth inhibition (Takahashi et al., 2006), c-myc in human breast cancer (Wang et al., 2005) and TNFα in collagen-induced arthritis (Schiffelers et al., 2005; Khoury et al., 2006). There are numerous further examples and larger overviews of siRNA therapeutic applications and can be found in the literature (Aigner, 2007; Masiero et al., 2007).

### 1.3.6 Concluding remarks

The discovery of dsRNA silencing effects and the following elucidation of the RNA silencing pathways has revolutionized biological research. Science is fast moving forward in the RNAi field, but there are still a lot of questions to be answered. There

is still a need to understand and unravel the detailed enzymatic characteristics of the RNAi pathways, including siRNA function, miRNA biogenesis, chromatin remodelling and DNA methylation. It is clear that not all components of RISC have been identified. This is absolutely essential in order to understand how RNAi really works in detail and might lead to the answer of the question why functional microRNAs or siRNAs have a length of 21 nt and (only) the seed region is an important factor for targeting. Elucidating the secrets of miRNA targeting is a further essential aspect that will require attention. Although interesting and progressive consolidated findings have been made concerning miRNA and target structure, it will be necessary to understand why certain nucleotides of a miRNA are less important than others. It has been shown that many endogenous miRNAs and their targets are involved in different types of cancer. The identification of these targets will definitely contribute to a better understanding of how cancer evolves and reveal targets for gene therapy. A putative target class are ARE-regulated gene targets, whose deregulation has already been shown to be responsible for cancer in various tissues. Furthermore, there is emerging evidence for their regulation by miRNAs and/or their involvement in miRNA pathways. Although AREs are located in 3' UTRs of mRNAs, ARE-regulated genes are involved in the same cellular processes such as miRNAs and even a few connections have been demonstrated between ARE- and miRNA-regulation, although a direct breakthrough has not yet been reported.

The therapeutic potential of siRNAs in diverse diseases has been shown. Non-viral delivery systems provide several advantages versus viral delivery. Although even viral therapeutic approaches can be applied safely, the most important advantage of non-viral delivery is its much safer application. Currently, there have been attempts at overcoming hurdles such as low transfection efficiency through generating appropriate lipid or polymeric vehicles for transportation. This will likely lead to the discovery of techniques that can be exploited to distribute RNAi effectors in human dose feasible amounts. However, great success has already been achieved by applying RNA-based therapies to AMD and viral diseases (Kim and Rossi, 2007). In order to only target *in vivo* cancer cells, different possibilities have been examined such as targeting specific receptors that are primarily expressed on the surface of cancer cells – this research has been very promising.

# 2 Subject Description

The tight regulation of mRNA stability and translation by specific *cis*-acting sequences and *trans*-acting factors is an essential means for the control of gene expression. These processes allow cells to rapidly adjust the expression pattern of regulatory factors and response transiently to internal and external signals including cell proliferation, signal transduction, inflammatory stimuli and radiation. A today well known important *cis*-acting sequence element that controls mRNA stability is the AU-rich element (ARE) found in the 3' untranslated region (3' UTR) of many, but not all, unstable mRNAs of various growth factors, cytokines, proto-oncogenes and transcription factors.

## 2.1 ARE-mediated gene regulation

More than 20 years ago Gray Shaw and Robert Kamen (Shaw and Kamen, 1986) demonstrated that a 51 nt AU rich sequence (ARE) from the human lymphokine gene granulocyte-monocyte colony stimulating factor (GM-CSF) caused the otherwise stable β-globin mRNA to become highly unstable *in vivo*. Since then extensive afford has been made to identify the mechanism that AREs undergo.

### 2.1.1 AREs as *cis*-acting elements

AREs can have a varying sequence length of 50 nt up to 150 nt. Their location within the 3' UTR of their host mRNAs can vary widely as well. In normal resting cells AREs mediate the rapid deadenylation-dependent degradation of its host mRNA, but depending on the cellular context and precise stimuli the same AREs are also capable to stabilize their host mRNA. Such stimuli can be hypoxia (Levy et al., 1998), UV light (Wang et al., 2000b) or treatment with phorbol ester (Wilson et al., 2003b).

The first identified ARE sequence motif was the pentamer AUUUA. Although the presence of the sequence pentamer does not necessarily mean a destabilizing function, it plays a critical role in determining the effect of an ARE. By use of a synthetic ARE the nonamer UUAUUUA(U/A)(U/A) was demonstrated to have a modest destabilizing effect. Based on the pentamer AUUUA, AREs have been grouped into three classes (Chen and Shyu, 1995; Barreau et al., 2005) (see table 1). Class I AREs contain 1 to 5 dispersed AUUUA motifs in a U-rich context. Class II

AREs contain at least two overlapping UUAUUUA(U/A)(U/A) nonamers. Class III AREs are less well defined and do not contain an AUUUA pentamer. These classes represent functionally and structurally independent distinct sequence motifs.

**Table 1: Classification of AREs based on the presence of the pentamer AUUUA and the nonamer UUAUUUA(U/A)(U/A).** Data taken from (Barreau et al., 2005).

| Class | Definition | Example gene |
|---|---|---|
| Class I | 1 to 5 dispersed AUUUA motifs in a U-rich context | c-myc, c-fos, Cyclin A, B1 & D1 |
| Class IIA | $(AUUU)_5A$ | GM-CSF, TNF$\alpha$ |
| Class IIB | $(AUUUA)_4A$ | Interferon$\alpha$ |
| Class IIC | $(A/U)(AUUU)_3(A/U)$ | Cox-2, IL-2/3, VEGF |
| Class III | No AUUUA, U-rich region | c-jun, GLUT1, p53, hsp 70 |

The ARE of the human proto-oncogene *c-fos* is a well studied example of class I. The *c-fos* gene encodes a transcription factor that plays a role in cell growth and differentiation. Rapid decay of the *c-fos* transcript is maintained by both the protein-coding region (Chen et al., 1992; Schiavi et al., 1994), which is structurally unrelated to the ARE, and the ARE itself present in the 3' UTR. The *c-fos* ARE consists of two structurally distinct and functionally independent domains shown in fig. 2. An interaction between both domains specifies the destabilizing function of the ARE (Chen et al., 1994).

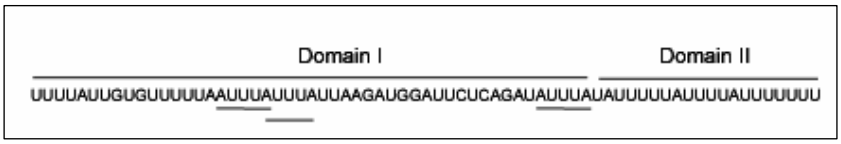


**Figure 2: The 69 nt *c-fos* ARE consists of two structurally and functionally distinct domains.** The two domains are indicated and the AUUUA motifs are underlined. The complete sequence has been used for analysis in mammalian (Chen et al., 1995) and *S. cerevisiae* (Vasudevan and Peltz, 2001) reporter systems.

Domain I contains the AUUUA motifs and a high general AU content, both representing the basic features for the destabilizing ability. Domain II is not able to destabilize a message on its own. However, its presence enhances the ability of domain I to accelerate deadenylation and its deletion does not affect the decay rate

of the mRNA body. Both domains have been studied extensively in a mammalian reporter system (Chen et al., 1995) and in the yeast *S. cerevisiae* (Vasudevan and Peltz, 2001).

The ARE of the cytokine TNFα (class II) is a very well studied example of class II. TNFα is an important proinflammatory cytokine produced by activated macrophages and lymphocytes and is involved in the inflammatory response such as leukocyte migration, tissue resorption and fever (Beutler and Cerami, 1989). Its expression is regulated by a constitutive decay element (CDE), which is located directly downstream of the ARE (Stoecklin et al., 2003). The TNFα ARE can act both as an RNA instability and a translational modulator (Zhang et al., 2002; Stoecklin et al., 2003). The ARE sequence is shown in fig. 3. The overlapping nonamers UUAUUUA(U/A)(U/A) are underlined as well as the partial sequence that has been used for analysis in mammalian (Xu et al., 1997) and yeast (Vasudevan and Peltz, 2001) reporter systems.

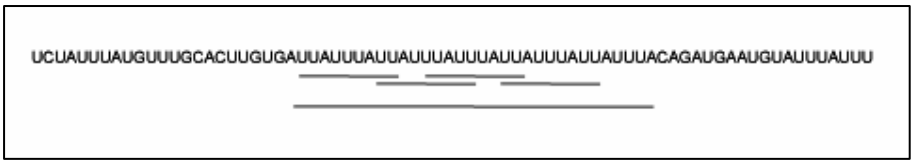


**Figure 3: The human 74 nt TNFα ARE.** The overlapping UUAUUUA(U/A)(U/A) are underlined as well as the core sequence that has been used extensively for analysis in mammalian reporter system (Xu et al., 1997) and in the yeast *S. cereviasiae* (Vasudevan and Peltz, 2001).

### 2.1.2 ARE mRNA binding proteins (ARE-BPs) as *trans*-acting factors

In order to understand how various AREs function mainly *in vitro* RNA electrophoretic mobility shift assays (EMSAs) aimed at the identification of ARE-BPs. Only a few studies have focussed on interactions between endogenous transcripts and binding proteins. Binding of these proteins can have either negative or positive effects on diverse processes as mRNA stability, translation and location. In order to mediate these effects ARE-BPs in general can be nuclear or cytoplasmic, or can shuttle between both compartments and their binding activity can correlate inversely or proportionally with the stability of ARE bearing mRNAs. The fact that several ARE-BPs can bind simultaneously to an mRNA suggests that their ultimate function is determined by the complexes in which they are integrated. This leads to the assumption that their presence in multimeric RNA-protein complexes does not

necessarily rely on their RNA-binding activity (reviewed in Chen & Shyu, 1995, Barreau et al. 2006).

Well studied examples of ARE-BPs are listed in table 2. AUF1 (also hnRNPD, heterogenous nuclear ribonucleoprotein D) binds class I and class II AREs and can have four different isoforms, which are named $p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$ and $p45^{AUF1}$. The large isoforms p42 and p45 are located in the nucleus, whereas p37 and p40 lack the sequence determinant and are also found in the cytoplasm (Wilson et al., 2003b). HuR (also called HuA) has a nuclear/cytoplasmic shuttling ability (Fan and Steitz, 1998a; Keene, 1999) and binds to class I, II and III AREs. It is a member of the Hu protein family, which exhibits strong homology to those of the *Drosophila* RNA binding proteins Elav (embryonic lethal, abnormal vision) (Ma et al., 1996). TIAR (TIA-1-related protein) and TIA-1 (T-cell intracellular antigen-1) are related U-rich RNA-BPs, can be located in the nucleus or cytoplasm as well and bind to class II AREs. Their RNA-binding activity is mediated by a different number of RNA recognitions motifs (RRM). KSRP (K homology-type splicing regulatory protein) contains four RNA-binding K homology (KH) domains and is located in the cytoplasm. TTP (Tristetraprolin) is predominantly cytoplasmic and is a member of a family of three tandem CCCH zinc finger proteins (Cys-Cys-Cys-His) that can bind class II AREs in single-stranded mRNA (Lai et al., 2005). Whereas TIAR/TIA-1, KSRP and TTP are known to decrease mRNA stability, AUF1 can have stabilizing or destabilizing effects. HuR seems to be a stabilizing ARE-BP at all.

**Table 2: A small subset of known ARE-BPs.** AUF1 (also hnRNPD, heterogenous nuclear ribonucleoprotein D) is essentially nuclear and binds to class I and II AREs. HuR (also called HuA) HuR has a nuclear/cytoplasmic shuttling ability (Keene, 1999) and binds to class I, II and III AREs. TIAR (TIA-1-related protein) and TIA-1 (T-cell intracellular antigen-1) are related U-rich RNA-BPs can be located in the nucleus or cytoplasm as well and binds to class II AREs. Their RNA-binding activity is mediated by a different number of RNA recognitions motifs (RRM). KSRP (K homology-type splicing regulatory protein) contains four RNA-binding K homology (KH) domains is located in the cytoplasm. TTP (Tristetraprolin) only binds class II AREs, is predominantly cytoplasmic and uses a zinc finger motif for RNA binding.

| Protein | Molecular Mass (kDa) | RNA-binding motif | ARE |
|---|---|---|---|
| AUF1 | 37, 40, 42, 45 | RRM | c-myc, c-fos, GM-CSF |
| HuR | 36 | RRM | c-myc, c-fos, TNF$\alpha$, GM-CSF |
| TIAR/TIA-1 | 40, 42 | RRM | TNF$\alpha$, GM-CSF |
| KSRP | 78 | KH | c-fos, TNF$\alpha$ |
| TTP | 44 | Cys-Cys-Cys-His, zinc finger | TNF$\alpha$, interleukin-3, GM-CSF |

It has been shown that ARE bearing mRNAs can be stabilized in response to external or internal cellular stress signals like heat shock (Laroia et al., 1999), hypoxia (Levy et al., 1998), UV light (Wang et al., 2000b) and cell proliferation (Wang et al., 2000a). Such stress signals can initiate a signalling cascade, which activates the mitogen-activated protein kinase (MAPK) p38. TTP is a target of MAPK-acitvated protein kinase 2 (MK2) and can be phosphorylated at multiple sites (Mahtani et al., 2001). Phosphorylated TTP is excluded from stress granules (SG) leading to the stabilization of ARE-containing mRNAs. It is hypothesized that exclusion of TTP allows the binding of the stabilizing ARE-BP HuR (Stoecklin et al., 2004). Another example for an ARE-BP that can be phosphorylated is $p40^{AUF1}$. Its phosphorylation influences ARE-binding affinity, RNA conformational dynamics and the global structure of the $p40^{AUF1}$-ribonucleoprtein complex (Wilson et al., 2003a). Additional signal cascading pathways (Widmann et al., 1999) were shown to influence stability, translation and/or localization of ARE-containing messages (Espel, 2005) demonstrating the great variety of the ARE-regulated network.

### 2.1.3 ARE-mediated mRNA degradation (AMD)

In mammalian cells degradation of mRNAs in the cytoplasm always starts with deadenylation, which can be catalyzed by several enzymes like CAF1, CCR4 and PARN. Deadenylation is followed by exonucleolytic decay in the 3'-5' or the 5'-3' direction. The 3' end is degraded by the exosome, a multimeric complex of 3'-5' exonucleases (Bevilacqua et al., 2003; Buttner et al., 2006), whereas the 7-methyl guanosine cap at the 5' end is removed by the decapping enzymes Dcp1 and Dcp2 The mRNA body is then degraded by the 5'-3' exonuclease Xrn1. The remaining cap-structure is finally degraded by the scavenger decapping protein DcpS (see fig. 4) (Wilusz and Wilusz, 2004). Dcp1/Dcp2 and Xrn1 form a complex with the Lsm1-7 proteins. These proteins are localized at cytoplasmic foci named processing bodies (P-bodies) (Anderson and Kedersha, 2006). In yeast and mammalian cells decay factors involved in the 5'-3' mRNA degradation are concentrated in P-bodies (Sheth and Parker, 2003).

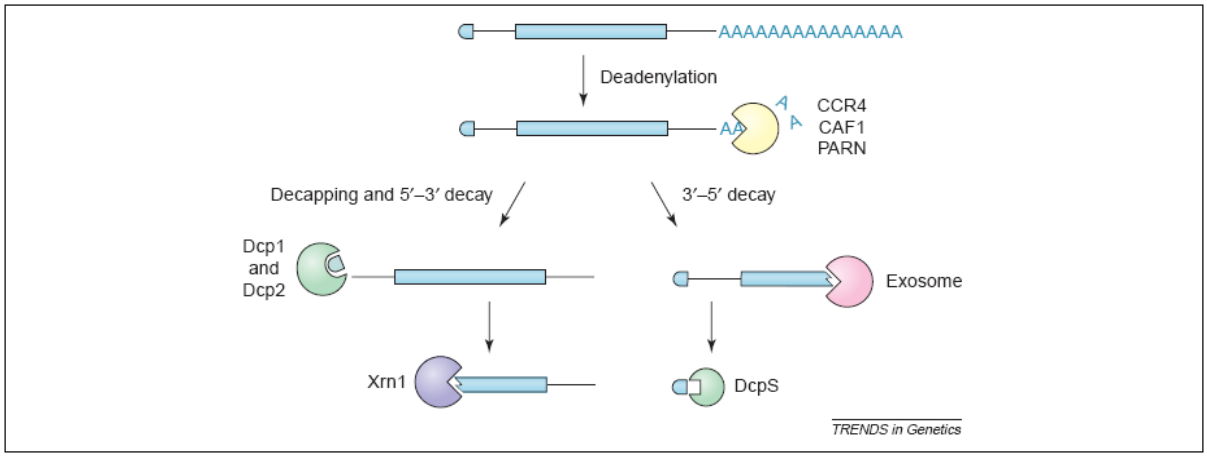


**Figure 4: Degradation of mRNA in mammalian cells.** Degradation is initiated by deadenylation, which can be catalysed by several enzymes like CAF1, CCR4 and PARN. Afterwards the mRNA body can be degraded in 5'-3' direction by the decapping complex (Dcp1/Dcp2) and the exoribonuclease XRN1. Alternatively, the transcript can be degraded in 3'-5' direction by the exosome, a large complex of exonucleases. The remaining m7G cap is degraded by the scavenger decapping protein DcpS. These basic processes can be dramatically altered by a large number of mRNA-binding proteins (permission for use of this figure granted by Elsevier) (Wilusz and Wilusz, 2004).

It was demonstrated that the mammalian exosome mediates the degradation of ARE bearing mRNAs *in vitro* (Mukherjee et al., 2002). Furthermore, it was shown that TTP is recruited to stress granules (SGs; reviewed in Anderson and Kedersha, 2006). SGs are dynamic cytoplasmic foci that are assembled in response to environmental stress and where stalled translation initiation complexes accumulate. Typical SG-associated proteins are TIA-1, TIAR and HuR (Stoecklin et al., 2004). Additionally, AMD was shown to be inhibited by the knockdown of Xrn1 and as a consequence uses primarily the 5'-3' decay pathway. It was hypothesized that P-bodies may participate in AMD (Stoecklin et al., 2006). Recently it was demonstrated that the degradation of a GM-CSF ARE-containing reporter transcript needs poly(A)ribonuclease PARN, enzymes involved in 5'-3' as well as 3'-5' (exosome) decay. The reporter accumulates in discrete cytoplasmic granular structures, which could be differentiated from P-bodies and stress granules (Lin et al., 2007). These observations are complementary to the finding that ARE-mRNA localized to P-bodies by using DCP1a-GFP as a marker (Franks and Lykke-Andersen, 2007). In conclusion these findings demonstrate complexity of AMD and that it has not been fully understood till today.

### 2.1.4 AREs in yeast

ARE-mediated mRNA decay is a highly conserved mechanism among eukaryotes (Fan and Steitz, 1998b). The AU-rich 3' UTR of the yeast transcript *TIF51A* (encodes eIF5A protein) and MFA2 (mating pheromone 2) as well as the human *c-fos* and TNFα AREs were demonstrated to regulate mRNA stability in the yeast *S. cerevisiae* (Vasudevan and Peltz, 2001). Deadenylation-dependent decapping that leads to the decay of the mRNA body was demonstrated as well. Moreover, they were able to identify the 51 kDa yeast ELAV homologue Pub1p (Poly(U)-binding protein, also RNP1) (Anderson et al., 1993; Cusick, 1994) as a regulator of TNFα ARE-mediated stability, but not of MFA2 transcript stability. They could further show the dependency on the Hog1p/p38 MAP kinase pathway, which was examined in more detail for the translational regulation of the MFA2 transcript (Vasudevan et al., 2005), and the regulation of MFA2 mRNA turnover by the yeast homologue of Hsp70, Ssa1p (Duttagupta et al., 2003). Pub1p has also been shown to selectively bind to a stabilizer element (STE) located in the 5' UTR of the upstream open reading frame (uORF) containing transcripts YAP1 and GCN4 and to prevent their turnover through the NMD pathway (nonsense-mediated mRNA decay) (Ruiz-Echevarria and Peltz, 2000). These results demonstrate that Pub1p can bind to at least two classes of stability elements and modulate decay. Additionally the global mRNA turnover in isogenic *PUB1* and Δ*pub1* strains was examined (Duttagupta et al., 2005) involving the identification of 573 transcripts that are destabilized and 78 that are stabilized by the loss of Pub1p. Direct interaction targets of Pub1p regulate such diverse processes and components like protein biosynthesis, ribosomal biogenesis and ribonucleoprotein complexes. In agreement with findings in mammalian cells the ARE-regulatory network consisting of ARE-sequences and ARE-BPs displays a high complexity as well. However, the ARE network has been analysed in yeast in a far less extent than in mammalian cells. It could be shown that yeast is a well-established system for the analysis of AREs and can contribute to the understanding of how AREs regulate gene expression.

## 2.2 Aim of the thesis

The baker's yeast *Saccharomyces cerevisiae* as well as the methylotrophic yeast *Pichia pastoris* are suitable hosts for the production of proteins of higher organisms. This process is called heterologous protein expression. Previous work demonstrated that not all selected (human) proteins could be expressed in the yeast *P. pastoris* and that all analysed transcripts so far are characterized by a low transcript stability (Boettner, 2004). Low transcript stability can be a relevant parameter for low recombinant protein expression (Mikaelian et al., 1996). In order to modulate, preferably increase, stability of selected transcripts and possibly as a consequence from that increase the yield of the heterologous protein expression in the yeast *P. pastoris,* ARE-mediated effects on mRNA stability and translation are to be exploited. Based on this hypothesis this work aimed at the following aspects.

1. Is there a functional ARE network in the yeast *P. pastoris* that controls transcription and translation as it is described in other eukaryotes?
It is not known whether there are ARE-mediated effects in the yeast *P. pastoris* or not until today. Thus no endogenous ARE-containing mRNA or ARE-BP can be selected for analysis so far. Consequently a heterologous system has to be established like it has successfully been used as a reporter system in mammalian cell lines (Xu et al., 1997) and in the yeast *S. cerevisiae* (Vasudevan and Peltz, 2001). For this purpose the well described human AREs of the proto-oncogene *c-fos* and the cytokine TNF$\alpha$ will be cloned into the 3' UTR of *P. pastoris* expression vectors. Their effects will be analysed on the mRNA and protein level in order to determine possible ARE-mediated effects.

2. If AREs have an effect in the yeast *P. pastoris*, is it possible to increase transcript stabilities and/or yields of heterologous protein expression?
If the cloned AREs have a (general) stabilizing effect on transcript stability and/or an increasing effect on translation, it would be of great importance for the biotechnological production of proteins that could be expressed only in low amounts so far. Independently of the observed effects the molecular background of the ARE-regulation will be interesting to explore.

3. If AREs have an effect in the yeast *P. pastoris*, which factors influence their activity?

ARE-mediated regulation of transcript stability and translation is based on a large variety of mRNA sequences and specific ARE-BPs as it is described above. Establishing a system for the analysis of AREs in the yeast *P. pastoris* will provide an alternative system for ARE-analysis which can help to identify novel regulatory factors in this yeast and thus give a more detailed look into the cellular ARE network, which will be of general interest.

# 3 Materials and Methods

## 3.1 Materials

### 3.1.1 Primers

Primers were purchased from Metabion GmbH, Martinsried, Germany.

Primers for cloning the *c-fos* ARE:
3:NSXc-fos-for:
5'-
ATAAGAAT*GCGGCCGC*TTGACTCGAGTTTTATTGTGTTTTTAATTTATTTATTAAG
ATGGATTCTCAGATATTTA-3'

4:Ec-fos-rev:
5'-
CG*GAATTC*GGTAGAAAAAAATAAAATAAAAATATAAATATCTGAGAATCCATCTT-
3'

Primers for cloning the *TNF$\alpha$* ARE:
5:NSXtnf-for:
5'-GGCCGCTTGACTCGAG*ATTATTTATTATTTATTTATTATTTATTTATTTA*G-3'

6: Etnf-rev:
5'-AATTC*TAAATAAATAAATAATAAATAAATAATAAATAAT*CTCGAGTCAAGC-3'

Primers for removing the STREP-II-Tag from pPICHS / creation of vector pPICH:
16: NotIStopAvrIIw/oStrep-for: 5'-GGCCGCTTGAATTCC-3'
17: NotIStopAvrIIw/oStrep-rev: 5'-CTAGGGAATTCAAGC-3'

Primers for amplification of cDNA 29:
5'Primer: 5'-G*AAGATCT*ATGACTCTGGAAGAAGTCCG-3'
3'Primer: 5'-ATAGTTA*GCGGCCGC*TCACTCGGGGAGGGTGATGC-3'
3'-Primer (w/o stopcodon): 5'-ATAGTTA*GCGGCCGC*CTCGGGGAGGGTGATGC-3'

Primers for universal *E. coli* and *P. pastoris* colony PCR (a kind gift from Mewes Böttner):

5´-AOX1-f: 5´-GACTGGTTCCAATTGACAAGC-3´

3´-AOX1-r: 5´-CAAATGGCATTCTGACATCC-3´

Primers for constructs without STREP-II-Tag for *P. pastoris* colony PCR:

5´-AOX1-f and 18:w/oStrep-rev: 5'-GCCCTAGGGAATTCAAGCGGCCGC-3'

Primers for AOX1 probe generation against the 5' UTR from pPICHS (a kind gift from Mewes Böttner):

AOX1UTRkurz5´: 5´-ATCATCATTATTAGCTTACTTTCA-3´

AOX1UTR3´II: 5´-CGTGATGGTGATGGTGATGAGAC-3´

Primers for generating a probe against endogenous Actin of *P. pastoris*:

PpAct-f: 5'-CAATGGTTCCGGTATGTGTAA-3'

pPAct-r: 5'-TCGAACATGATTTGGGTCATCTT-3'

Primers for creation of *in vitro* transcription templates:

T7-cfos-for: 5'-

TAATACGACTCACTATAGGTTTTATTGTGTTTTTAATTTATTTATTAAGATG-3'

T7-cfos-rev: 5'- AAAAAAATAAAATAAAAATATAAATATCTGAGAATC-3'

T7-tnf-for: 5'-TAATACGACTCACTATAGG-3'

T7-tnf-rev: 5'-

TAAATAAATAAATAATAAATAAATAATAAATAATCCTATAGTGAGTCGTATTA -3'

Primers for insertion of single point mutations in the pPICHcfos29+S+S construct:

NotImutI-for: 5'-GGCCACTTGAC-3'

NotImutI-rev: 5'-TCGAGTCAAGT-3'

NotImutII-for: 5'-GGCCTCTTGAC-3'

NotImutII-rev: 5'-TCGAGTCAAGA-3'

NotImutIII-for: 5'-GGCCGGTTGAC-3'
NotImutIII-rev: 5'-TCGAGTCAACC-3'

### 3.1.2 Plasmids

| | |
|---|---|
| pPICHS | (a kind gift from Mewes Böttner; Böttner et al. 2002) Derived from pPIC3.5 (Invitrogen); contains an expression cassette with a 5' coding sequence for a $HIS_6$-Tag and a 3' coding sequence for a STREP-II-Tag. cDNAs can be cloned between these tags using *Bam*HI and *Not*I. |

### 3.1.3 Strains

| | |
|---|---|
| *E. coli* JM109 | (Stratagene, CA) *e14$^-$(McrA$^-$), recA1, endA1, gyrA96, thi-1, hsdR17($r_{K-}m_{K+}$),supE44,relA1,Δ(lac-proAB), [F'traD36proABlaq$^q$ZΔM15]* |
| *E. coli* XL-1 blue | (Stratagene, CA) *recA1, endA1, gyrA96, thi-1, hsdR17, supE44 relA1 lac [F'proAB lacI$^q$ZΔM15 Tn10 (Tet$^r$)]* |
| *P. pastoris* GS115 | (*his*4; Invitrogen™, CA) |

### 3.1.4 Media

| | |
|---|---|
| LB medium | 1 % tryptone; 0.5 % yeast extract; 0.5 % NaCl; pH 7.2. |
| LB agar plates | 1.5 % Agar (Serva, Heidelberg, Germany) added to the medium |
| Antibiotic concentration | Ampicillin (Boehringer, Mannheim, Germany), 100 µg/ml |
| Trace elements (1000x, 100 ml) | 175 mg $ZnSO_4x7H_2O$; 50 mg $FeSO_4x7H_2O$; 10 mg $CuSO_4x5H_2O$; 10 mg $MnCl_2x4H_2O$; 10 mg $Na_2MoO_4x2H_2O$;<br>Dissolve in 10 mM EDTA and filtrate sterilely; storage at 4°C |
| Vitamine solution (250x, | 0.0625 g biotin; 1.25 g Ca-pantothenate; 0.25 g |

| | |
|---|---|
| 100 ml) | nicotinic acid; 0.625 g pyridoxine; 0.25 g thiamine; filtrate sterilely; storage at 4°C |
| WMIX | 1 litre: 10 g Na-glutamate; 75 mg meso-inosite; 250 mg $MgCl_2x6H_2O$; 100 mg $CaCl_2$ $x2H_2O$; 550 mg $MgSO_4$ $x7H_2O$; dissolved in 860 ml $H_2O$ and autoclaved. Afterwards addition of 40 ml 0.5 M Ka-phosphate buffer pH 6.5; 100 ml 20 % glucose; 1 ml 1000x trace elements; 4 ml 250x vitamine solution; |
| WMIX$NH_4Cl$ | 1 litre: 3.16 g $NH_4Cl$; 75 mg meso-inosite; 250 mg $MgCl_2x6$ $H_2O$;100 mg $CaCl_2x2H_2O$; 550 mg $MgSO_4x7H_2O$; dissolved in 500 ml $H_2O$ and autoclaved. Afterwards addition of 400 ml 0.5 M Ka-phosphate buffer pH 6.5; 100 ml 20 % glucose; 1 ml 1000x trace elements; 4 ml 250x vitamine solution; |
| YEPD medium | 0.5 % yeast extract; 2 % glucose; 2 % tryptone; pH 6.3 |
| YNB medium | 0.67 % YNB ("yeast nitrogen base", Difco, Augsburg, Germany); 2 % glucose |
| YNB agar plates | 2 % Agar (Serva, Heidelberg, Germany) added to the medium |

### 3.1.5 Buffers, solutions, reagents

| | |
|---|---|
| Acrylamide solution | 40 % Acrylamide; 29:1 Acrylamide: Bis-Acrylamide (Biometra, Göttingen, Germany) |
| [$\gamma$-$P^{32}$]CTP / ATP | Hartmann Analytic GmbH, Braunschweig, Germany |
| [$\alpha$-$P^{32}$]UTP | Hartmann Analytic GmbH, Braunschweig, Germany |
| 100 bp-marker | MBI Fermentas, St. Leon-Rot, Germany |
| Agarose | SeaKem ME Biozym, Hess. Oldendorf, Germany |
| Agarose gel loading buffer (stopper solution) | 60 % Saccharose; 20 mM EDTA; 0.025 % Bromephenol blue |
| APS, Ammoniumpersulfate | APS-solution 10 % in $H_2O$ (Serva, Heidelberg, Germany) |
| Binding buffer (5 x) | 50 mM HEPES pH 7.0, 200 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 25% glycerol; aliquotted and stored at -80°C. |

| | |
|---|---|
| Bio-Rad Protein Assay Kit | BioRad, München, Germany |
| BSA, Bovine serum albumin | Sigma, Deisenhofen, Germany; Fraction V, 96-99 % |
| Complete, Mini (protease inhibitor cocktail) | Roche Applied Science, Mannheim, Germany |
| Coomassie gel staining solution for SDS polyacrylamidegels | 2 g/l Coomassie Brilliant Blue R250; 30 % methanol; 60 % distilled $H_2O$; 10 % acetic acid. |
| Ethidiumbromide (10 mg/ml stocksolution) | BioRad, München, Germany |
| Gel destaining solution for Coomassie blue stained SDS polyacrylamidegels | 45 % methanol; 45 % distilled water; 10 % acetic acid |
| Gel drying solution for SDS polyacrylamidegels | 10 % glycerine; 30 % methanol; 60 % distilled water |
| Gene Ruler$^{TM}$ 100bp DNA Ladder Plus | MBI Fermentas, St. Leon-Rot, Germany |
| Gene Ruler$^{TM}$ DNA Ladder Mix | MBI Fermentas, St. Leon-Rot, Germany |
| glass beads | 0.25-0.5 µn (Roth, Karlsruhe) |
| Heparin sodium (177,000 IU/g) | Serva, Heidelberg, Germany |
| HRP substrate | Western Lightning$^{TM}$ Chemiluminescent Reagent Plus (Perkin Elmer, MA) |
| Laemmli buffer (4 x) | 8 % SDS; 20 % glycerol; 20 % ß-mercaptoethanol; Tris-HCl pH 6.8; 0.05 % bromephenol blue. |
| Lysis buffer for total RNA extraction | 10 mM Tris, pH 8.0, 1 mM EDTA, 1% SDS, 100 mM NaCl, 2 % Triton X-100 |
| MOPS buffer (10 x) | 250 mM MOPS; 50 mM Na-acetate; 20 mM EDTA; pH 7.0 (with NaOH) |
| MOPS buffer (10 x) | 250 mM MOPS, 50 mM NaOAc, 20 mM EDTA (pH 7.0, adjusted with NaOH) in 0.1 % DEPC treated $H_2O$ |
| PBS (phosphate buffered saline) | 150 mM NaCl; 8.4 mM $Na_2HPO_4$; 1.6 mM $KH_2PO_4$, pH 7.4 |

| | |
|---|---|
| PBST | 1x PBS pH 7.4; 0.05 % Tween 20 |
| PMSF | Fluka, Biochemika, Sigma-Aldrich, Germany |
| Rainbow marker | Amersham, Braunschweig, Germany |
| Rapid Hyb Buffer | GE Healthcare, Amersham, Braunschweig, Germany |
| RNA Ladder, Low Range, ready-to-use | MBI Fermentas, St. Leon-Rot, Germany |
| RNA sample buffer | 250 µl formamide, 83 µl 35 % formaldehyde, 50 µl 10*MOPS, 100 µl 50 % (v/v) glycerol, 10 µl 2,5 % (w/v) bromephenoleblue, 7,5 µl EtBr (10mg/ml) |
| RNA stop buffer | For a final volume of 5 ml: 4 ml formamide, 1 ml 50 mM Tris/HCl, pH 7.6 (in 0.1% DEPC $H_2O$), 0.0125 g bromphenolblue, 0.0125 g xylenecyanol; aliquotted and stored at -80°C. |
| SDS PAGE buffer for separating gel (4x) | 1.5 M Tris; 0.4 % SDS; pH 8.8 |
| SDS PAGE buffer for stacking gel (4x) | 0.5 M Tris; 0.4 % SDS; pH 6.8 |
| SDS PAGE running buffer (10x) | 144.1 g glycine; 30.2 g Tris; 10 g SDS ad 1 litre with distilled water |
| SSC buffer (20 x) | 3 M NaCl; 0.3 % Na-citrate; pH 7.0 |
| TAE buffer | 20 mM sodium acetate; 40 mM Tris; 2 mM EDTA; with glacial acetic acid pH 8.3 |
| TBE buffer (10 x) | 1 M Tris-HCl, 1 M boric acid, 10 mM EDTA (pH 8.3) |
| TE buffer (10 x) | 0.1 M Tris-HCl; 0.01 M EDTA pH 8.0 |
| TEMED | N, N, N´, N´-tetramethyl ethylene diamine (Serva, Heidelberg, Germany) |
| Transfer Buffer for Western Blotting | 3.03 g Tris, 14.4 g glycine, 100 ml methanol ad 1 l with distilled water |
| Urea | Serva, Heidelberg, Germany |

### 3.1.6 Enzymes

| | |
|---|---|
| Restriction enzymes | MBI Fermentas, St. Leon-Rot, Germany |
| GenTherm DNA- | Rapidozym, Berlin, Germany |

| | |
|---|---|
| Polymerase (5U/µl) | |
| Klenow polymerase | MBI Fermentas, St. Leon-Rot, Germany |
| Ribonuclease A (RNase A) | MBI Fermentas, St. Leon-Rot, Germany |
| RNasin ® Plus RNase Inhibitor | Promega, Madison (WI), USA |
| $T_4$ DNA ligase | MBI Fermentas, St. Leon-Rot, Germany |
| T7 RNA Polymerase | MBI Fermentas, St. Leon-Rot, Germany |
| Takara *Ex Taq*™ (5U/µl) | Takara Bio Inc., Shiga, Japan |

### 3.1.7 Antibodies

| | |
|---|---|
| Horse radish peroxidase coupled rabbit anti mouse immunoglobulin | DAKO, Denmark |
| Penta His® antibody | Qiagen, Hilden, Germany |

### 3.1.8 Programmes and databases

| | |
|---|---|
| BioEdit Sequence Alignment Editor V7.0.4.1 | Hall, T. A. 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html |
| Saccharomyces Genome Database | Dolinksi et al. 1998 http://www.yeastgenome.org/ |
| Tina V2.09f | Raytest Isotopenmeßgeräte GmbH |
| mFold v2.3 | http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1-2.3.cgi (Zuker, 2003) |

## 3.2 Cultivation techniques

### 3.2.1 *E. coli* culture preparation and growth conditions

For *E. coli* freezing cultures 300 µl 50% glycerol (v/v) were added to 1 ml of an exponentially growing culture. Cells were stored in a Cryo.s tube Cellstar® (Greiner GmbH, Frickenhausen, Germany) at -80°C.

*E. coli* cultures were grown on a reciprocal shaker at 120 rpm and 37°C. The culture volume did not exceed 1/5 of the flask used for cultivation.

### 3.2.2 *P. pastoris* culture preparation and growth conditions

For *P. pastoris* freezing cultures 500 µl 50% glycerol (v/v) were added to 1 ml of a 3 days pre-culture. Cells were stored in a Cryo.s tube Cellstar® (Greiner GmbH, Frickenhausen, Germany) at -80°C.

20 ml WMIX pre-cultures were inoculated with 100 µl of a freezing culture and grown in 250 ml baffled flasks for 3 days on a circular shaker at 160 rpm and 28°C. After measuring the optical density $OD_{600}$ (approx. 20-25) cells were centrifuged at 8000 x g for 2 minutes in 50 ml Greiner centrifugation tubes. The supernatant was decanted and cells were resuspended in 45 ml WMIX, 4.25 ml sterile $Aqua_{dest.}$, 250 µl 20% glucose and 500 µl methanol (total 50 ml). After measuring the $OD_{600}$ again cultures were further incubated in 250 ml baffled flasks on a circular shaker at 160 rpm and 28°C. For isolation of total RNA samples were taken after 2 hours. For the analysis of protein expression cells were grown for 24 hours, feeding again with 250 µl 20% glucose and 500 µl methanol.

## 3.3 DNA Techniques

### 3.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a Minigel device (BioRad, Sub-Cell GT Mini, München, Germany) using a 1% (w/v) TAE gel. Samples were mixed with 1/2 (v/v) stopper solution and applied to the gel. 1 µg of λ-DNA restricted with *Hind* III (BioLab, Swalbach, Germany), 100 bp ruler (MBI Fermentas, St.Leon-Rot, Germany) or 100 bp Plus DNA Ladder (Bioneer, Deajeon, Korea) were used as markers. DNA was separated at 75 V for 30 to 90 minutes. Gels were stained for 15 minutes in an ethidium bromide solution (0.4 mg/l TAE buffer) and for detection exposed to UV-light ($\lambda$=254 nm). Gels were documented using a Video documentation system (Photointegrator V4.66, INTAS, Göttingen, Germany).

### 3.3.2 Plasmidisolation from *E. coli*

For the isolation of small amounts of plasmid DNA (1 up to 5 µg) from *E. coli* a 1.5 ml overnight $LB_{Amp}$ culture (100 µg/ml) was incubated on a reciprocal shaker (120 U/min) at 37°C. Plasmid isolation was performed using the GFX$^{TM}$Micro Plasmid Kit (Amersham, Braunschweig, Germany) following manufacturer's instructions. Alternatively, isolation was carried out following the standard protocol (Ausubel et al., 1994). Isolated DNA was solved in 100 µl TE buffer. For restriction analysis 10 µl of this mini preparation was applied.

For the isolation of larger amounts of plasmid DNA from *E. coli* a 25 ml culture was incubated as described above. Plasmid isolation was performed using the Plasmid Midi Kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

### 3.3.3 Restriction of DNA

All restrictions were arranged in a final volume of 30 µl, containing the DNA (1 up to 5 µg), 3 µl of the manufacturer's 10 x restriction buffer and 10 to 20 units of the restriction enzyme. The samples were incubated for at least two hours at 37°C in a water quench.

### 3.3.4 PCR Amplification

For amplification of cDNA 29 a 50 µl approach was set up, containing 5 µl of 10 x Takara Ex Taq buffer, 0.3 µl of Takara Ex Taq (3.75 units), 1 µl of the primers (10 pmol/µl), 5 µl of the dNTP stock solution (12.5 mM) and 200 ng template DNA. The PCR programme was 94°C for 4 minutes, then 30 cycles 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 1 minute 20 seconds.

All other PCRs were arranged in a final volume of 20 µl, containing 5 µl of 4 x PCR buffer, 0.3 µl of GenTherm DNA-polymerase (1,6 units), 0.4 µl of each primer (10 pmol/µl), 1.6 µl of the dNTP stock solution (12.5 mM) and approx. 100 ng template DNA, if not indicated.

### 3.3.5 Purification of DNA fragments

DNA fragments like PCR or restriction products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA was eluted in 30 µl TE buffer

### 3.3.6 Hybridisation of oligonucleotides

Oligos were designed to have already compatible overhanging ends for their specific clonings. For Hybridisation 100 pmol of each oligonucleotide in Annealing buffer AB (10mM Tris*HCl, pH 8,0 ) were denatured for 10 minutes in a water quench. The temperature was decreased to 80°C through addition of ice. The samples were cooled down to room temperature for 90 minutes and were finally stored at -80°C. Hybridised oligonucleotides were:

| | |
|---|---|
| 3:NSXc-fos-for and 4:Ec-fos-rev | *c-fos* ARE |
| 5:NSXtnf-for and 6: Etnf-rev | TNF$\alpha$ ARE |
| 16:NotIStopAvrIIw/oStrep-for and 17:NotIStopAvrIIw/oStrep-rev | For removal of the STREP II tag |
| NotImutI-for and NotImutI-rev | For single point mutagenesis |
| NotImutII-for and NotImutII-rev | |
| NotImutII-for and NotImutII-rev | |

### 3.3.7 Klenow fill up reaction

After hybridization overlapping oligonucleotides, especially the *c-fos* ARE hybrid, were filled up with klenow polymerase. A 30 µl sample contained 20 µl hybridized oligonucleotides, 3 µl 10 x klenow buffer, 2 µl dNTPs (12.5 mM), 1 µl klenow polymerase and 4 µl $Aqua_{dest.}$ The samples were incubated for 30 minutes at 37°C in a water quench. The enzyme was then inactivated for 10 minutes at at 75°C and the samples were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany).

### 3.3.8 Ligation of DNA fragments

Ligation reactions were arranged in a final volume of 20 µl, containing 2 µl 10 x $T_4$ ligation buffer, 1 µl $T_4$ ligase (1U) and vector / insert in a 1:3 proportion. The samples were incubated for two hours at room temperature or over night at 16°C.

### 3.3.9 Transformation of *E. coli*

A 20 ml LB pre-culture (w/o ampicillin) was inoculated with 50 µl of a JM109 / XL1 Blue freezing culture and incubated on a reciprocal shaker (120 U/min) over night at 37°C. The 100 ml LB main-culture (w/o ampicillin) was inoculated with 1% of the pre-culture (v/v) and incubated up to on $OD_{600}$ of 0.4 (approximately 2 hours and 20 minutes). Cells were cooled down on ice and afterwards centrifuged for 10 minutes at 4,000 rpm and 4°C in a GSA centrifugation tube (Centrifuge RC50 Sorvall Du Pont de Nemours, Bad Homburg, Germany, GSA rotor). The pellet was washed in an ice cold TFBI solution (0.1 M $MgCl_2$; 0.01 M Tris-HCl, pH 7.6), centrifuged again and then washed with 50 ml of an ice cold TFBII solution (0.1 M $CaCl_2$; 0.01 M Tris-HCl, pH 7.0). After a next centrifugation step the cells were resuspended in 4 ml of TFBII solution and incubated for 30 minutes on ice. After addition of 800 µl glycerol aliquots of 240 µl were frozen at -80°C.

The transformation mixture contained 100 µl PEG-solution (1 mM EDTA;0.2 M NaCl; 8 % PEG, 2,000;10 mM Tris-HCl, pH 7.6), the ligation sample (20 µl) or a control plasmid (1 µg) and the 240 µl aliquot of competent cells. The samples were placed on ice for 30 minutes and then subjected to a 42°C water quench for one minute (heat shock). 1 ml LB medium was added immediately and cells were incubated for 60 up to 90 minutes at 37°C on a reciprocal shaker at 120 rpm. Afterwards 100 µl of the culture were spread onto $LB_{Amp}$ plates (100 µg/µl). The remaining cells were centrifuged for 10 seconds at 12,000 rpm and the supernatant was discarded. Cells were then resuspended in the remaining medium and spread onto $LB_{Amp}$ plates, too. The plates were incubated over night at 37°C.

### 3.3.10 Colony-PCR of *E. coli*

Single *E. coli* colonies were dissolved in 10 µl of $Aqua_{dest.,}$ which 1 µl was used of as the PCR template. 0.6 µl DMSO were added per sample. The PCR reaction was set up as described under PCR amplification (see 3.3.4). If the primers have been 5´-

AOX1-f and 3´-AOX1-r the PCR programme was 94°C for 4 minutes, then 24 cycles 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 1 minute 20 seconds. If the primers have been 5´-AOX1-f and Ec-fos-rev or Etnf-rev the PCR programme was 94°C for 4 minutes, then 30 cycles 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 1 minute 20 seconds.

### 3.3.11 Transformation of *P. pastoris*

Electroporation was carried out after a modified standard protocol (Invitrogen 1997).
A 20 ml YEPD pre-culture was inoculated with 50 µl of a *P. pastoris* GS115 freezing culture (*his*4; Invitrogen™, CA) and incubated on a reciprocal shaker (120 rpm) at 28°C for three days. Then a 500 ml YEPD main-culture was inoculated with 0.2 ml of the pre-culture and incubated over night for approximately 16 hours till the culture reached an optical density of $OD_{600} \approx 1.3$ to 1.5. Cells were then centrifuged in a GS3 tube at 4000 rpm and 4°C for 10 minutes. Afterwards cells were washed twice with 500 ml ice cold $Aqua_{dest.}$ and once with ice cold 1 M sorbitol. Finally cells were resuspended in 1 ml ice cold 1 M sorbitol frozen at -80°C in 80 µl aliquots. Cells were competent up to 8 days.
For electroporation 10 µg of a plasmid were linearized using 30 units of the restriction enzymes *Sal*I or *Stu*I, depending on the sequence of the cloned cDNA. Restriction was done as described above (see 3.3.3). The samples were purified during dialysis against $Aqua_{dest.}$ for 1 hour using a nitrocellulose filter (0.025 µm, Millipore, MA) afterwards mixed with an 80 µl aliquot of competent *Pichia* cells and incubated on ice for 5 minutes. The cells were then transferred to an ice cold 0.2 cm electroporation cuvette. Electroporation parameters were 1500 V, 200 Ω and 25 µF. Immediately after the electric pulse 1 ml ice cold 1 M sorbitol was added to the cuvettes. Twice 500 µl of the cell suspension were finally spread on YNB plates and incubated for 3 days at 28°C.

### 3.3.12 Colony-PCR of *P. pastoris*

Stable integration of the expression cassette was checked by colony-PCR. Selected $His^+$-colonies were transferred to 100 µl YNB medium and incubated at 28°C over night. This was necessary to avoid false positives. Reactions were set up as described above (3.3.4). 2 µl of the over night culture were applied to the PCR

mixture, primers were 5´-AOX1-f and 3´-AOX1-r and 0.6 µl DMSO were added. The programme was 94°C for 4 minutes, then 30 cycles 94°C for 15 seconds, 55°C for 45 seconds, 72°C for 2 minutes 30 seconds.

### 3.3.13 Isolation of total/genomic DNA from *P. pastoris*

10 ml of a 3 days culture were harvested in 15 ml Greiner falcon tubes by centrifugation at 8.000 rpm for 3 minutes at 4°C. The medium was decanted, cells were resuspended in 500 µl distilled water and transferred to an eppi tube. Cells were centrifuged again (5 minutes, 5.000 rpm, 4°C) and then resuspended in 200 µl lysisbuffer. After addition of 300 ml glass beads, 100 µl phenol and 100 µl chloroform/isoamylalcohol (24:1) the samples were vortexed for 3 to 4 minutes. Then 600 µl TE buffer were added and the samples were centrifuged for 5 minutes at 10.000 rpm. The upper aqueous phase was transferred to a new 2 ml eppi tube and 1 ml ethanol$_{abs.}$ (96%) was added. The samples were centrifuged again at 10.000 rpm for 10 minutes, the pellet was resuspended in 400 µl TE buffer and transferred again to a new eppi tube. 3 µl RNase A (10 µg/µl) were added and the samples were incubated for 5 minutes at 37°C in a water quench. The DNA was precipitated by addition of 10 µl 5 M ammonium acetate and 1 ml ethanol$_{abs.}$ (96%) and centrifugation for 10.000 rpm for 10 minutes. The pellet was washed with 70% ethanol und afterwards dried at room temperature. Finally the pellet was solved in 50 µl TE buffer.

## 3.4 RNA Techniques

### 3.4.1 PCR based probe synthesis

The AOX1 probe (146 bp) was generated from the 5' UTR of the vector pPICHS (see appendix, fig. 1 for vector map) through PCR. 2.5 ng of the plasmid, 20 pmol of the primers AOX1UTR3´II and AOX1UTRkurz5´ and 2 µl of the dNTP stock solution (12.5 mM) were used for the PCR in a 20 µl volume. The PCR programme was 94°C for 2 minutes, then 30 cycles 94°C for 15 seconds, 40°C for 15 seconds, 72°C for 30 seconds and a final step of 72°C for 10 minutes. The result of the PCR reaction was checked by agarose gel electrophoresis.

The Actin probe (341 bp) was generated from *P. pastoris* genomic DNA (see 3.3.13) via PCR. 1 µl of a genomic DNA preparation and the primers PpAct-f and pPAct-r were used for the PCR reaction. The PCR reaction was performed in a 20 µl volume as described above (see 3.3.4). The programme was 94°C for 4 minutes, then 30 cycles 94°C for 15 seconds, 55°C for 45 seconds, 72°C for 2 minutes 30 seconds. The result of the PCR reaction was checked by agarose gel electrophoresis.

### 3.4.2 Radioactive labelling of PCR generated probes

Approximately 50 ng of a PCR generated probe were labelled radioactively using the Megaprime DNA Labelling System (GE Healthcare, Amersham, Braunschweig, Germany). Normally 10 ng probe per 1 ml hybridization buffer are applied. The PCR product was mixed with 5 µl random primer solution and filled up to 30 µl with distilled water. The sample was incubated for 3 minutes at 100 °C and then directly put into liquid nitrogen. The frozen sample was put on ice. Then 4 µl of each dNTP (except the radioactive dNTP), 5 µl 10 x reaction buffer and 2 µl Klenow enzyme (all provided with the kit) were added. Finally 1 µl radioactively labelled $\alpha[P^{32}]$ATP or $\alpha[P^{32}]$CTP (Hartmann Analytic GmbH, Braunschweig, Germany) was added. If the radioactivity has already passed the first date of its half life 2 µl were added. The samples were incubated for 30 minutes at 37 °C and could then be stored at -20°C. Normally the labelled probes were used directly for hybridization.

### 3.4.3 Isolation of total RNA from *P. pastoris*

Cultures were incubated as described (see 3.2.2). For isolation of total RNA all steps were performed on ice, all solutions were treated with 0.1 % DEPC and pipette tips as well as all tubes were RNase free. After incubation cells were directly put on ice and an amount of 100 $OD_{600}$ was harvested by centrifugation in a pre-cooled 50 ml falcon tube for 2 minutes at 8.000 rpm and 4°C. The medium was discarded, cells were resuspended in 1 ml ice cold 10 mM Tris/HCl pH 8.0 and transferred to a 2 ml tube (RNase free) and centrifuged again for 30 seconds at 12.000 rpm and 4 °C. Afterwards, cells could be stored at -80°C.

The pellet was resuspended in 500 µl ice cold 0.5 M NaCl/200 mM Tris/HCl pH 7.5/10 mM EDTA and 500 µl glass beads (RNase free, sterilized for 4 – 6 hours at 160°C) were added. The suspension was mixed with 250 µl at 60°C pre-warmed

phenol (liquified and Tris saturated, Biomol, Hamburg), 25 µl 10 % SDS and 30 µl 3 M NaOAc, pH 4.8. The samples were vortexed with full speed for 3 minutes and then incubated for 5 minutes at 60°C. After cooling down to room temperature for approximately 5 minutes 250 µl chloroform/isoamylalcohol (24:1 mixture) were added. The samples were heavily shaken, centrifuged for 3 minutes at 12.000 rpm and the upper aqueous phase was transferred to a new tube. After adding 500 µl pre-warmed phenol and 50 µl 3 M NaOAc, pH 4.8, samples were heavily shaken again, centrifuged for 3 minutes at 12.000 rpm and the upper aqueous phase was transferred to a new tube. Then 500 µl chloroform/isoamylalcohol (24:1 mixture) were added. Sample were heavily shaken again and centrifuged for 3 minutes at 12.000 rpm. The upper aqueous phase (approximately 400 µl) was transferred to a new tube. Total RNA was precipitated by addition of 40 µl 3 M NaOAc, pH 4.8 and 1.2 ml absolute ethanol at -80°C for 1 hour and by centrifugation for 20 minutes at 12.000 rpm and 4°C. The supernatant was wasted and samples were dried for 5 to 10 minutes at room temperature. Finally, RNA was solved in 35 µl RNA sample buffer. 5 µl of the sample were used to measure the concentration and purity with a photometer at 260 (1 $OD_{260} \approx 40$ µg/ml), 280 and 310 nm. Samples could be stored at -80°C, but were normally directly prepared for gel electrophoresis.

### 3.4.4 Denaturing RNA gel electrophoresis

RNA solved in RNA sample buffer was denatured at 65 °C for 10 minutes and directly put on ice. For the gel electrophoresis 20 µg of each sample were loaded onto the lanes.

For preparation of denaturing RNA gels 1.3 % agarose was boiled in 1 x MOPS. The liquid was cooled down at room temperature and 2% formaldehyde (37%) was added under the extractor hood. The gel was then poured into the electrophoresis chamber. Total RNA was then separated at 75 V for 2.5 hours till the bromphenol band reached 2/3 of the gel size. The running buffer was 1 x MOPS. Gels were documented using a Video documentation system (Photointegrator V4.66, INTAS, Göttingen, Germany).

### 3.4.5 Northern Blotting

The gel was washed twice in 20 x SSC buffer. RNA was transferred on a nylon membrane (Hybond N+, Amersham, Braunschweig, Germany) by capillary blotting. Therefore 6 sheets of 3 MM whatman paper of the same size like the gel were soaked in 20 x SSC buffer and put on a cling film. The gel was positioned on the whatman paper. The cling film was turned down covering all edges of the gel to put the membrane on the top avoiding air bubbles. The two left 3 MM whatman paper sheets were put onto the membrane and a layer of 15 cm pulp on the top was covered by a glass plate, which was weighted down with a weight of 300 g (like a half filled 500 ml water bottle). RNA was blotted over night for at least 12 hours.

The gel lanes were then marked and the RNA was cross-linked from both sides to the membrane using a Stratalinker 1800 in auto-cross-link-mode (Stratagene, CA). The successful transfer to the membrane was then checked under UV light. The bands of the ribosomal RNA were marked, too. The membrane could be stored covered in pulp and aluminium foil at 4°C.

### 3.4.6 Probe hybridisation and detection

The nylon membrane was put into a glass hybridization tube and attached to the inner surface of the tube avoiding air bubbles by using 9 ml rapid hyb buffer. Normally 1 ml rapid hyb buffer is applied for 1 $cm^2$ membrane surface. The membrane was incubated for pre-hybridization for 20 minutes at 38°C for the AOX1 probe in a hybridization oven. The radioactively labelled probes (see 3.4.2) were denatured for 5 minutes at 100 °C and then put on ice for 5 minutes and then transferred into the hybridization buffer. Hybridization was done for 4 hours at 38°C. Afterwards the probe containing hybridization buffer could be store at -20°C and reused 3 times. The membrane was then firstly washed with 2 x SSC + 0.1 % SDS at room temperature, and then secondly washed twice with 1 x SSC + 0.1 % SDS at 38°C. Finally the membrane was covered by thermoplastic foil and shrink-wrapped. Detection was done by exposition to an x-ray film (SupeRX, Fuji, Japan) for 3 to 5 days.

For detection of actin mRNA the membrane was again subjected to pre-hybridization without incubation in stripping solution. Pre-hybridization for 20 minutes and

hybridization itself for 1 hour were performed at 65°C as well as the two final washing steps. Exposition was done for only 1 day.

## 3.5 RNA-protein interaction techniques

### 3.5.1 Template matrices for *in vitro* transcription

For creation of the TNFα-ARE template for an *in vitro* transcription 2.2 nmol of each of the T7-tnf-for and T7-tnf-rev oligonucleotetides were hybridized as described above (see 3.3.6). The sample was directly used for *in vitro* transcription and could be stored at -20°C.

The *c-fos* ARE template for an *in vitro* transcription was created by PCR in a 20 µl PCR reaction mix as described above (see 3.3.4). 0.1 µl Takara Taq polymerase (0.5 U), the primers T7-cfos-for and T7-cfos-rev and 100 ng of the plasmid pPICHcfosARE4 were added. The PCR programme was 94°C for 4 minutes, then 30 cycles 94°C for 15 seconds, 55°C for 45 seconds, 72°C for 25 seconds. The sample was directly used for *in vitro* transcription without purification and could be stored at -20°C.

### 3.5.2 *In vitro* transcription

For *in vitro* transcription reactions were set up in a final volume of 20 µl. 3 µl up to 7 µl DNA template, 4 µl 5 x transcription buffer, 1 µl $T_7$-RNA polymerase (20 units; both from MBI Fermentas, St. Leon-Rot, Germany, delivered together), 0.75 µl RNasin (30 units, Promega, Madison (WI), USA), 4µl 2.5 mM rNTPs (Ambion, Applied Biosystems, Darmstadt, Germany) were mixed and filled up to 19 µl with distilled water (0.1 % DEPC). Afterwards 1 µl radioactively labelled α[$^{32}$P]UTP (Hartmann Analytic GmbH, Braunschweig) was added to the reaction mix. For non-radioactively transcripts the mix was directly filled up to a volume of 20 µl. The samples were incubated for 2 hours at 37°C. The samples were then mixed with 10 µl RNA stop buffer and could be stored at -20°C, but were normally directly subjected to a denaturing polyacrylamide gel electrophoresis for purification.

### 3.5.3 End-labelling of oligonucleotides

For controlling the size of *in vitro* transcripts random oligonucleotides (34 nt and 70 nt) were radioactively labelled. For this purpose a 20 µl was set up containing 2 µl oligonucleotide (200 pmol), 14 µl Aqua $_{dest.}$, 2 µl 10 x labelling buffer (delivered with kinase), 1 µl $\gamma[^{32}P]ATP$ and 1 µl $T_4$-Kinase (10 U, MBI Fermentas, St. Leon-Rot, Germany), which labels the 5' end. The samples were incubated for 30 minutes at 37°C. Afterwards the kinase was inactivated by an incubation for 4 minutes at 65 °C. 1 µl was applied to the gel (see purification of *in vitro* transcripts, 3.5.4).

### 3.5.4 Purification of *in vitro* transcripts

*In vitro* transcripts were denatured for 10 minutes at 65°C in a water quench and separated on a denaturing 5% 1 mm polyacrylamide gel. A 25 ml gel volume contained 12 g Urea (8 M) to avoid secondary structures, 2.5 ml 10 x TBE buffer, 14 ml distilled water (0.1 % DEPC) and 3.125 ml PAA stocksolution (40%, 29:1). Polymerisation was started by simultaneous addition of 500µl 10% APS and 250 µl TEMED. The gel was quickly poured between the glassplates (18 x 20 cm), which were sealed before with agarose, the gel comb was inserted and removed again after 3 minutes, when the gel was polymerized. The remaining gel parts covering the lanes were removed carefully. Before loading the samples into the lanes, the lanes were again carefully cleaned by washing them with running buffer (1 x TBE). The gel was run in a Zabona vertical chamber (Zabona AG, Basel, Schweiz) for 2 hours at 100 V. After the gel run the running buffer was carefully removed with a plastic bottle and then wasted. The upper glass plate was removed and the bottom plate together with the gel covered with cling film. A 5 minute exposition to an x-ray film (SupeRX, Fuji, Japan) revealed the radioactively labelled transcripts. The position was marked on the gel for the radioactive transcripts and for the non radioactive ones on the same height as well. Now the transcripts could be cut out of the gel and be eluted in 400 µl elutionbuffer (0.5 M ammoniumacetate, 0.1 mM EDTA, 0.1% SDS) over night at 4°C. The buffer was transferred to a new tube and RNA was precipitated with 1.2 ml 96% ethanol for 1 hour at -20°C. Then the samples were centrifuged for 15 minutes at 12.000 rpm and 4°C. The supernatant was wasted and the precipitated RNA was washed with 70% ethanol (set up with DEPC-treated $Aqua_{dest.}$). After a next centrifugation the pellet was dried for 5 minutes at room temperature. Radioactive

transcripts were solved in 50 µl distilled water (0.1 % DEPC), non-radioactive samples were solved in 5 µl distilled water (0.1 % DEPC), which were pooled afterwards.
The success of purification and yield were controlled on a denaturing formaldehyde gel (2 µl). Transcripts could be stored at -20°C.

### 3.5.5 Binding reaction

The crude protein extract applied to the binding reaction was created as described under 3.6.1. Samples were taken after 2 hours of induction.
A 20 µl binding sample was pipetted as follows: x µl DEPC $Aqua_{dest.}$, 4 µl 5 x binding buffer, 3 µl heparin solution (10 µg/µl), x µl crude protein extract (0, 5, 25 and 50 µg) and 2.5 µl labelled transcript (100 ng).
Proteinase K digested samples were prepared as follows: 7.5 µl proteinase K (150 µg; Merck, KGaA, Darmstadt, Germany) were mixed with 5, 25 and 50 µg protein extract, if necessary filled up to 10.5 µl with DEPC $Aqua_{dest.}$ and incubated over night at 37°C. These samples were mixed with 4 µl 5 x binding buffer, 3 µl heparin solution (10 µg/µl) and 2.5 µl labelled transcript (100 ng).
The samples were then incubated for 30 minutes on ice at 4°C. Afterwards samples were cross-linked in a stratalinker 1800 (Stratagene, CA) to bind proteins to the transcripts. Then 5 µg RNase A (10 mg/ml) were added and the samples incubated for 15 minutes at 37°C. Samples were then mixed with 10 µl 4 x Laemmli buffer, denatured for 3 minutes at 100°C and directly put on ice. After cooling down samples were centrifuged shortly.
Samples were separated on an 8% PAA-Gel. Glass plates were prepared as described under 3.5.4. The 30 ml gel mixture contained 6 ml PAA (40%, 29:1), 7.5 ml 4 x SDS PAGE buffer for separating gels and 16.5 ml $Aqua_{dest.}$. Polymerisation was started by addition of 500 µl 10% APS and 250 µl TEMED. The gel pre-run at 100 V and 4°C was done for 30 minutes. Samples were loaded onto the gel and the gel run was done for 3 hours at 100 V and 4°C. The gel was transferred to 3 MM Whatman paper (Whatman Int., Maidstone, England), covered with a cling film and dried for 45 minutes on gel dryer (Zabona AG, Basel, Schweiz) at 80°C. The gel was exposed to an x-ray film (SupeRX, Fuji, Japan) for 3 to 7 days.

## 3.6 Protein Techniques

### 3.6.1 Preparation of crude cell extracts

A pre-culture was inoculated and incubated as described above (3.2.2). An optical density of 20 units was harvested by a 10 second centrifugation at 12,000 rpm and 4°C. The pellet was washed in 500 µl 1 x PBS and stored at -80°C. The same amount of cells of the main-culture was harvested, too.

All following steps were performed on ice. Cells were resuspended in 100 µl PBS and 1 mM PMSF. Disruption of the cells was done by addition of 100 µl glass beads (0.25 – 0.5 mm) and vortexing for 10 x 1 minutes with breaks of 1 minute incubation on ice. Afterwards cells were centrifuged for 5 minutes at 12,000 rpm and 4°C. The supernatant was transferred to a new eppi tube.

### 3.6.2 Bradford protein determination

For the quantitative determination of the total protein amount a Bradford assay was done. Therefore cell extracts were diluted 1:2500 and 800 µl samples were measured in triplicate. The standard γ-Globulin solution (0,1 mg/ml) was prepared by adding 12,7 ml distilled water to 1 ml of 1.37/ mg/ml γ-Globulin stock solution. 800 µl aliquots were stored at -20°C. Samples were prepared in cuvettes as following:

**Table 3: Scheme for Bradford calibration**

| Sample No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Water [µl] | 800 | 780 | 760 | 740 | 720 | 680 | 640 | 600 |
| 0.1 mg/ml γ-Globulin solution | 0 | 20 | 40 | 60 | 80 | 120 | 160 | 200 |
| Protein/800µlmixture | 0 | 2 | 4 | 6 | 8 | 12 | 16 | 20 |

After adding 200 µl BioRad Bradford solution (supplied with the kit) the samples were incubated for 15 minutes. Then the absorption at 595 nm was measured and the total protein amount calculated. Waste was collected in a flask, applied to an activated carbon-filter and finally neutralized with NaOH.

### 3.6.3 SDS PAGE

For separation of proteins a 1 mm 15 % polyacrylamide separating gel (8.5 x 5.5 cm) and a 4.5 % stacking (8.5 x 1.5 cm) gel were used. For 7.5 ml separation gel 2.75 ml $Aqua_{dest.}$, 1.9 ml SDS PAGE buffer for separating gel and 2.8 ml acrylamide solution were mixed. Then 50 µl 10% APS solution and 15 µ TEMED were added simultaneously to start polymerisation. The gel was poured between the fixed glass plates in the mini gel device (mini protean® 3, BioRad, München, Germany) and covered with isopropanol/water (1:1). After polymerisation the isopropanol/water was decanted. For 5 ml stacking gel 3.14 ml distilled water, 1.25 ml SDS PAGE buffer for stacking gel and 0.55 ml acrylamide solution were mixed. Then 50 µl 10% APS solution and 10 µ TEMED were added simultaneously to start polymerisation. The stacking gel was poured onto the separating gel and the gel comb was inserted carefully. 1/4 Laemmli buffer (4x) of the final volume was added to 25 µg of protein extract of cDNA 205 - and 45-constructs. For cDNA 29 protein extracts an amount of 1,5 µg was used. Proteins were denatured for 3 minutes at 95°C, centrifuged at 13,000 rpm at 4°C and then placed on ice. The gel was prepared in the gel apparatus and the chamber was filled with 1 x SDS PAGE running buffer. The comb was removed carefully, lanes were washed and samples loaded onto the gel. Samples were separated at 100 V for 2 hours.

The upper gel part above the 46 kDa band (yellow) of the rainbow marker was applied to Coomassie blue-staining (see 3.6.5) and used for later quantification. The bottom part of the gel containing the proteins of interest was subjected to Western Blotting.

### 3.6.4 Western Blotting

SDS polyacrylamide gels were electroblotted onto PVDF membranes (Millipore, MA) in a semi-dry Blotter (Hölzel, Wörth, Germany). For this purpose 4 layers of 3 MM Whatman$^{TM}$ paper (Whatman Int., Maidstone, England) previously cut to the same size of the gel and soaked in transfer-buffer are placed below the PVDF membrane, which has been previously activated with $ethanol_{abs}$. The gel is then placed on top of the filter and covered with 4 additional layers of 3 MM Whatman$^{TM}$ paper (Whatman Int., Maidstone, England). Gels were electroblotted at 500 mA per gel for 20 min.

### 3.6.5 Coomassie blue staining

The upper parts of the SDS polyacrylamide gels were incubated in Coomassie gel staining solution for SDS polyacrylamidegels over night at room temperature. Then gels were applied three times to gel destaining solution for Coomassie blue stained SDS polyacrylamidegel for 1 hour. Next the gel was placed into gel drying solution for SDS polyacrylamidegels for 30 minutes. During that incubation two gel drying films (Promega, Madison (WI), USA) were placed into water for 30 minutes. Then the gel was placed between the two layers of gel drying films and dried for 3 days.

### 3.6.6 Immunodetection of proteins

Membranes were placed into 20 ml blocking solution (3 % BSA, 0.1 % Tween20 in 1 x PBS) and incubated over night at 4°C. All following incubations were done at room temperature and gentle shaking unless indicated. For detection of the $His_6$ tag membranes were incubated for 1 hour in antibody solution I (10 ml blocking solution and 5 µl Penta-His antibody (1:4000; BSA-free, mouse monoclonal $IgG_1$; Qiagen, Hilden, Germany). The antibody solution was stable up to four weeks and could be reused (storage at 4°C). Then the membranes were washed three times for 10 minutes in 20 ml PBST (1 x PBS and 0.05% (v/v) Tween 20). The incubation with the second antibody was done in antibody solution II (20 ml 1 x PBS, 5 % milk powder and 5 µl secondary rabbit $\alpha$-mouse HRP antibody (Dako, Denmark)). Then the membranes were washed again three times for 10 minutes in 20 ml PBST (1 x PBS and 0.05% (v/v) Tween 20). Bands were visualized with NEN TM System (Perkin Elmer, Life Science, Boston, USA), which contains luminol as HRP substrate.

## 3.7 Bioinformatics

### 3.7.1 RNA secondary structure prediction

For mRNA secondary structure prediction the mFold webserver v2.3 (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1-2.3.cgi) was used applying the following parameters: linear sequence, folding temperature 28°C, percent suboptimality 5, upper bound 50, window parameter default, maximum interior/bulge loop size 30, maximum asymmetry of an interior/bulge loop 30, maxium

distance between paired bases no limit, image width huge, structure format bases, energy dot plot on, structure draw mode default, base numbering frequency 20, sequence numbering offset 0, regularization angle (in degrees) 0, structure rotation angle (in degrees) auto, structure annotation high-light. The region to be high-lighted was inserted.

### 3.7.2 Calculation of relative protein expression and mRNA stability

Quantification was performed with the programme Tina V2.09f. For calculation of the relative protein expression, firstly, a selected region of the Coomassie stained SDS-PAA gel was quantified. The quantified pPICH value was divided by each quantified value of the detected proteins resulting in value A. Secondly, detected protein signals were quantified. Each signal was divided by the pPICH value resulting in value B. To get relative values ($RV_{protein}$), which are consequently normalized the pPICH value, values A and B were multiplied. Each experiment was repeated at least twice. From these experiments the average values and standard deviation were calculated.
For calculation procedure of the relative mRNA stability is similar. Instead of total protein and protein signals, the endogenous *AOX*1 mRNA and mRNA of constructs to be analysed was detected.

### 3.7.3 Calculation of translational effects

For calculation of translational effects, the average values $RV_{protein}$ were divided by $RV_{mRNA}$ for each experiment and construct (see 3.7.2). From this data the average value ($RV_{TE}$) and standard deviation was calculated.

# 4 Results

In this thesis, the effect of human AU rich elements (AREs) was analysed in the yeast *P. pastoris*. AREs are mRNA *cis*-acting elements, which can regulate mRNA turnover and translation through the interplay with ARE binding proteins (ARE-BPs). These ARE-BPs or *trans*-acting factors can either promote or prevent degradation of the ARE-containing transcript as well as translation by recruiting proteins or protein complexes involved in these processes.

In contrast to other model organisms such as *D. melanogaster*, *Homo sapiens, Xenopus sp. and S. cerevisiae,* a possible ARE-mediated regulatory system has to date, not been examined in *P. pastoris*.

## 4.1 Construction of *P. pastoris* expression clones

There is no knowledge about the effects of AREs, ARE-BPs or other factors involved in *P. pastoris*. Thus, no endogenous ARE-containing mRNA or ARE-BP could be selected for analysis to date. However, to find out if this highly conserved mechanism in eukaryotes is also present in *P. pastoris,* it is possible to use a heterologous reporter mRNA or protein. Testing the effect of a known ARE from a different organism than *P. pastoris* on transcript stability and translation can help to prove the existence of an ARE-regulated mRNA turnover pathway. Such heterologous systems are commonly used to demonstrate principle effects in an organism and to identify components involved. In order to establish a heterologous ARE-regulated system for *P. pastoris,* two human AREs that are well described in the literature and three human cDNAs as repoters were selected for analysis.

### 4.1.1 Selection of AREs – the human *c-fos* and TNFα AREs

ARE regulatory systems are described for many eukaryotic model organisms. Since the time that ARE function was first described in human cells (Shaw and Kamen, 1986), research in this field has mainly focussed on mammalian systems. As a consequence, a large number of AREs and associated factors could be identified in these systems. However, there are only a few AREs whose regulatory effects and interactions have been analysed to a greater extent till date. Two examples are the AREs of the proto-oncogene *c-fos* (class I ARE) and of the cytokine TNFα

(class II ARE) (Barreau et al., 2005). Since these AREs have additionally been shown to trigger ARE-mediated mRNA decay in *S. cerevisiae* (Vasudevan and Peltz, 2001), they were selected for analysis in *P. pastoris* (fig. 6).

### 4.1.2 Construction of the basic expression vectors – Human AREs are cloned into the 3' UTR

Before the ARE sequences could be cloned into *P. pastoris* expression vectors, the question of where exactly the AREs are located in the 3' UTR had to be answered. It could not be excluded that the distance to the stop codon might have had an effect on regulation. As a consequence, the original locations of the *c-fos* and TNFα AREs in their native mRNAs and in eukaryotic reporter systems were examined (fig. 5). It turned out that their position can vary widely. They are located more than 400 nucleotides downstream of the stop codon in their native mRNAs, whereas in the *S. cerevisiae* reporter system they were cloned directly next to the stop codon and in the mammalian reporter system they were cloned near the stop codon, respectively. Obviously, the position of these AREs (or of AREs in general) only plays a subordinate role in the ARE-mediated regulatory system. This suggests that the basic ARE function does not depend on distance to the stop codon.

For general analysis (detection and purification), cDNAs were cloned into the vectors pPICHS (Boettner et al., 2002) (see appendix, fig. 1 for vector map) and pPICH (see appendix, fig. 2 for vector map), both containing a 6 x His- at the N-terminal and in the case of pPICHS an additional a Strep-II-tag at the C-terminal end of the mature protein. The AREs were cloned into the 3' UTR directly downstream of the stopcodon of the vector pPICH (fig. 6; see appendix, fig. 3 and 4 for vector map).

The vector pPICHS was restricted with *Not*I and *Avr*II to create the pPICH construct. The oligonucleotides 16:NotIStopAvrIIw/oStrep-for and 17:NotIStopAvrIIw/oStrep-rev were hybridized. The resulting double-stranded DNA fragment already possessed the corresponding overhanging ends needed for ligation. The ARE-containing vectors were established derived from this newly generated construct. To clone the *c-fos* ARE into pPICH, the two oligonucleotides 3:NSXc-fos-for and 4:Ec-fos-rev were hybridized. The overhanging ends were filled up with dNTPs using klenow enzyme. The two oligonucleotides 5:NSXtnf-for and 6: Etnf-rev were hybridized for the TNFα ARE. The resulting hybrid already contained the correct overhanging ends. Subsequently, the *c-fos* dsDNA fragment was restricted with *Not*I and *Eco*RI as well

as the vector pPICHS. The *c-fos* and TNFα DNA fragments were ligated with the restricted vector pPICHS resulting in the ARE-containing expression vectors pPICHcfos and pPICHtnf.

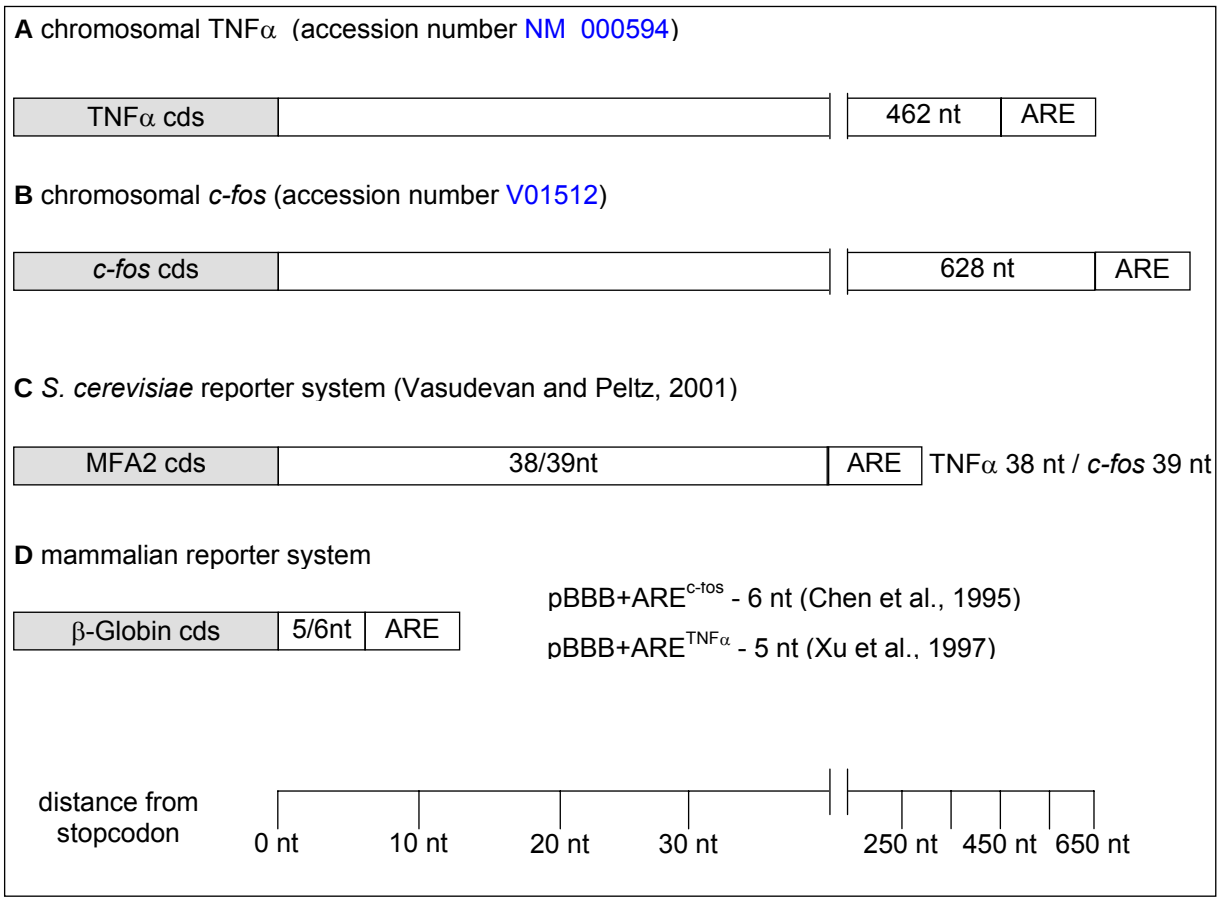


**Figure 5: Schematic overview of *c-fos* and TNFα ARE locations from the literature.** In their native transcripts, the AREs are located more than 400 nucleotides downstream of the stop codon **(A, B)**. The AREs in the *S. cerevisiae* and mammalian reporter systems are located near the stop codon **(C, D)**. This suggests that the basic function of these AREs or AREs in general does not depend on the distance to the stop codon.

### 4.1.3 Cloning of human cDNAs – example cDNAs for analysis

In the following, three different human cDNAs were selected (Tab. 1) and cloned from the existing expression vector pPICHS into the expression vectors pPICH, pPICHcfos and pPICHtnf. The new constructs were transformed into *P. pastoris*. Afterwards, selected transformants were checked by colony PCR (fig. 7).

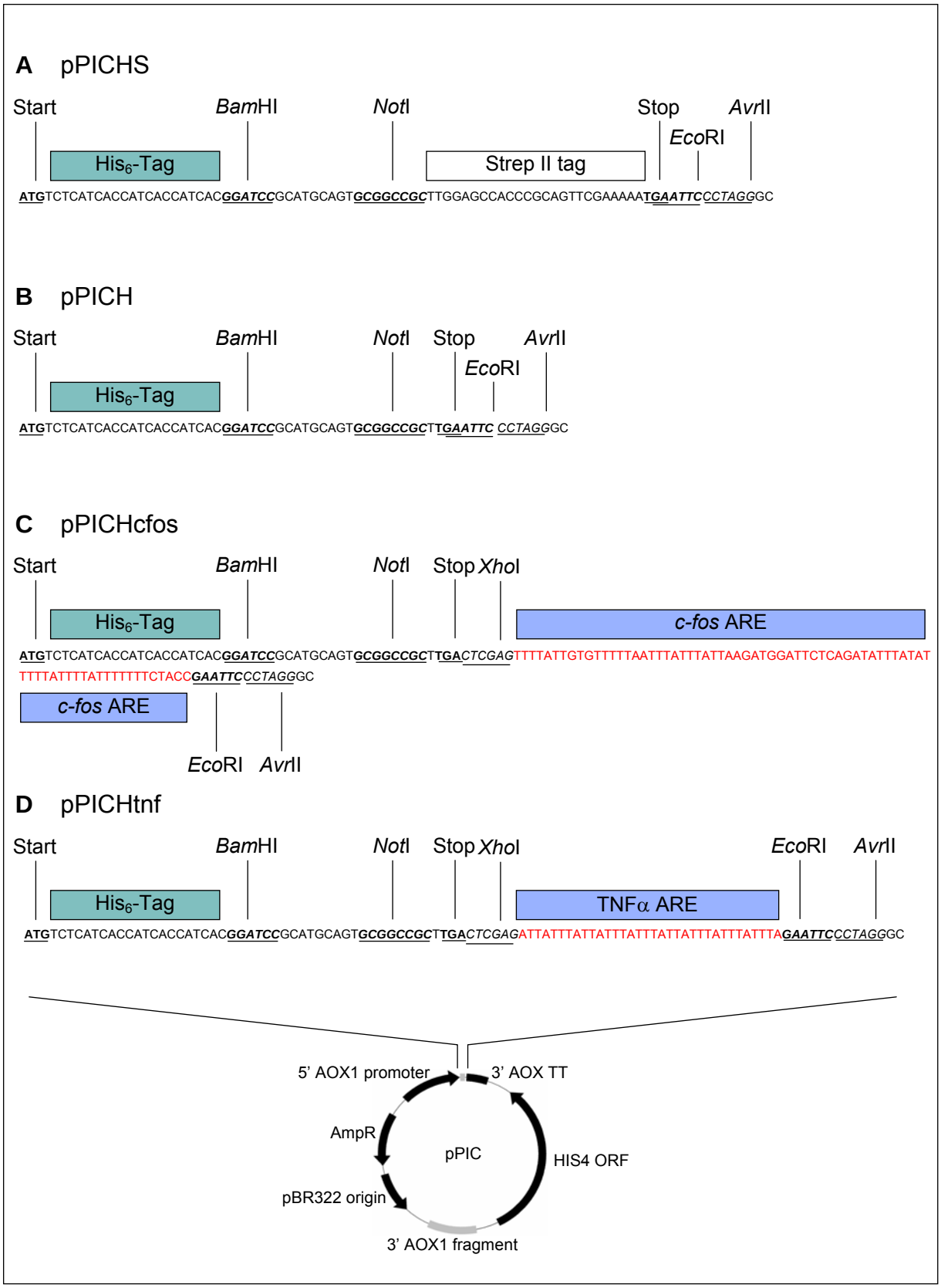


**Figure 6: Basic expression vectors used for creation of *Pichia* expression clones.** For general analysis, cDNAs were cloned in the vectors pPICHS (Böttner et al. 2002) and pPICH, both containing a 6 x His at the N-terminal and and in the case of pPICHS an additional a STREP II tag at the C-terminal end of the mature protein. AREs were cloned into the 3' UTR directly downstream of the stop codon.

### 4.1.4 Selection of cDNAs – AT-rich sequences in the coding region are a critical factor

A set of human cDNAs whose expression could be detected by Western Blot (Boettner, 2004), was selected as reporter constructs from the database from the Protein Structure Factory (Heinemann et al., 2000) (www.proteinstrukturfabrik.de). Previous work revealed a statistically significant correlation between the expression and the existence of AT-rich clusters in the coding region turned out (Boettner, 2004). Such AT-rich clusters can trigger premature transcription termination. It is known that fortuitous basepair sequences from higher eukaryotes can be recognized as transcription and polyadenylation signals in yeast. These signals are less conserved and more variable in yeast than in other organisms, especially mammals. However, they consist of AT-rich clusters as found in mammals (Zhao et al., 1999). As a result, AT-rich clusters can cause the formation of an aberrant transcript, which is rapidly degraded. To assure the complete and correct transcription of the newly generated constructs and to avoid the influence of such AT-rich clusters, only cDNAs missing AT-rich clusters in their coding region were selected for analysis (Table 1). In the following, these cDNAs are referred to as, for example, cDNA 29, 45 and 205.

cDNAs 45 and 205 were cut out of the already present vector pPICHS using the restriction enzymes *Bam*HI and *Not*I. The backbone of the pPICHS vector was digested with *Pst*I. Using this strategy, a PCR for amplification of the cDNAs was not necessary. As cDNA 29 contains an internal *Bam*HI restriction site, this cDNA had to be amplified by PCR with cDNA specific primers including a *Bgl*II site. The resulting PCR products included either an additional start and stop codon or only an additional start codon depending on the primers (fig. 14). These PCR fragments were restricted with *Not*I and *Bgl*II, the target vectors pPICHcfos and pPICHtnf were restricted with *Bam*HI and *Not*I. The restricted PCR products of cDNA 29 and the restricted cDNAs 45 and 205 were afterwards cloned into the target vectors pPICHcfos and pPICHtnf. The final constructs were sequenced.

**Table 1: Selected cDNAs from the protein structure cDNA database.** The criteria for selection were a detectable expression and the absence of AT-rich clusters in the coding region, which can trigger premature transcription terminations in yeast.

| clone | cDNA | function |
|---|---|---|
| 500000362 | AAC83329 | Growth arrest and DNA-damage inducible protein GADD45-gamma |
| 500001103 | AAC27445 | SH3 domain binding glutamatic acid-rich-like protein |
| 50000385 | AAB81205 | Sorting nexin-like protein, SNX15 |

### 4.1.5 Transformation of the new constructs in *P. pastoris* – creation of expression clones

For integration into the *P. pastoris* genome, the resulting expression vectors were linearized in the *HIS4* ORF with *Sal*I or *Stu*I depending on the sequence of the cloned cDNA. Linearization in the *HIS4* ORF leads to the integration into the chromosomal *HIS4* locus through a single recombination event. Transformation was carried out by electroporation. At least four resulting histidin prototrophic transformants were checked by colony PCR for successful integration of the expression cassette (fig. 8). The primer pair AOX1-f and AOX1-r were used for *P. pastoris* colony PCRs. These primers hybridize in the *AOX*1 promoter (forward) and in the *AOX*1 transcription terminator region (reverse). As both promoter and terminator are endogenous sequences of the *P. patoris AOX*1 gene and additionally present on the integrated plasmid two PCR products are possible (fig. 7).

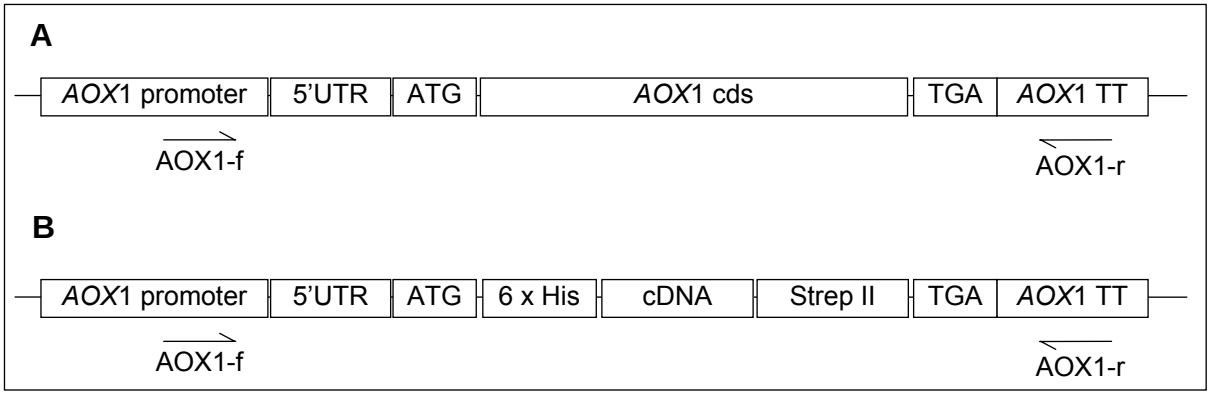


**Figure 7: Schematic overview of the *P. pastoris* endogenous *AOX*1 gene (A) and the expression cassette used in the vector pPICHS (B).** Positive transformants show two distinct bands (endogenous *AOX*1 and integrated vector) after colony PCR using the AOX1 primer pair.

A typical PCR sample shows the band of the endogenous *AOX*1 gene at approximately 2.2 kb and the successfully integrated AOX expression cassette depending on the size of the cloned cDNA (fig. 8).

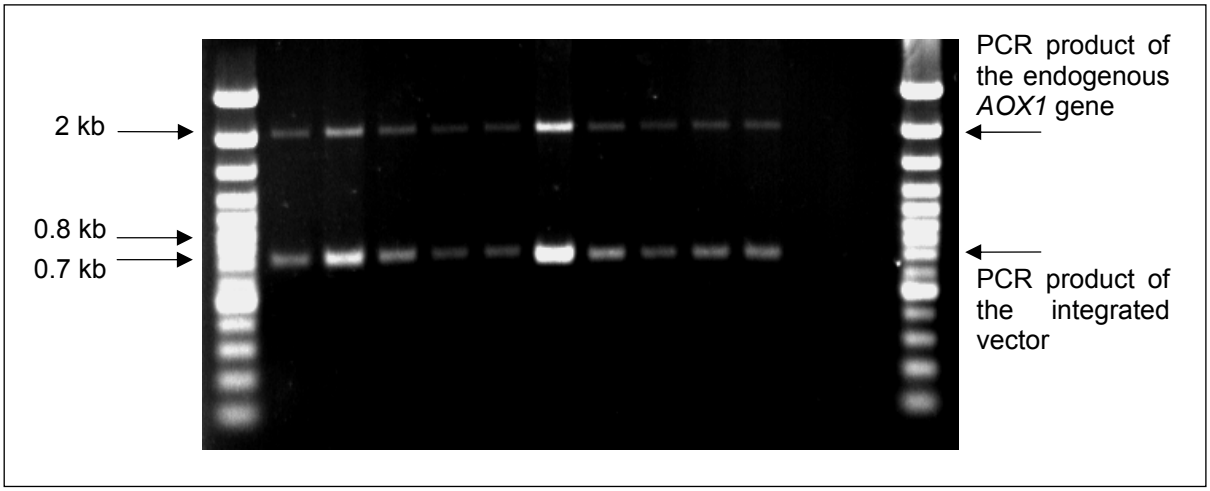


**Figure 8: Representative gel of a *P. pastoris* colony PCR.** Up to five colonies were tested for the integration of the expression cassette. Primers (AOX1-f and AOX1-r) hybridize in the *AOX*1 promoter and terminator of the integrated vector and the endogenous *AOX1* gene resulting in two bands.

## *4.2* Analysis of *P. pastoris* expression clones

To analyse the C-terminal protein, modifications and introduced AREs in *P. pastoris* possible effects on transcript stability and translation were tested by Northern and Western Blot under standardized conditions. Experiments were performed in 50 ml cultures and carried out according to the Invitrogen protocols (Invitrogen, 1997). Each experiment was repeated at least twice for the total set of clones for each cDNA.

### 4.2.1 Standardized comparison of the protein level – AREs can influence translation in *P. pastoris*

Yeast pre-cultures were cultivated for three days in WMIXNH$_4$Cl / 2.0% (w/v) glucose. Cells were then resuspended in fresh WMIXNH$_4$Cl with 0.1% glucose (w/v) and 1% methanol (v/v). This main culture was fed again with 0.1% glucose (w/v) and 1% methanol (v/v) and cultivated for 24 hours. As a control, a strain transformed with the empty vector pPICHcfos was always treated like the other cultures. Cells were mechanically disrupted with glass beads and the total protein amount was determined *via* Bradford assay. For experiments with cDNA 45 and 205, constructs

25 µg and for cDNA 29 constructs 1.5 µg total protein were applied to the SDS gel. After SDS PAGE (15 % PAA gel) and Western Blot proteins were detected with the PentaHis® antibody. As a reference, either a certain band or a defined area (both can be related to the total protein amount) in the Coomassie blue stained SDS gel was used to normalize the values for quantification. The standard protein marker was the Rainbow Marker (Amersham, Braunschweig, Germany).

The His/Strep-II-tagged protein of cDNA 45 (pPICHS) shows a distinct band at approximately 14.2 kDa (fig. 9, A). The His-tagged protein (pPICH) does not show a difference in size, although the coding sequence for the Strep II tag is deleted and expression differs significantly (fig. 9, B). In all other experiments (data not shown), the bands show at least a slight difference in size. Thus, it can be concluded that the removal of the Strep II tag results in a lower protein expression. The addition of the *c-fos* and TNF$\alpha$ AREs (pPICHcfos/tnfARE) triggers a dramatic down-regulation of translation (fig. 9, A and B).

The His/Strep-II-tagged protein of cDNA 205 (pPICHS) shows a distinct band at approximately 22.5 kDa (fig. 9, C). The removal of the Strep-II-tag (pPICH) results in a faster migrating band and a significantly reduced protein level (fig. 9, D). As already observed, the introduction of *c-fos* and TNF$\alpha$ AREs (pPICHcfos/tnfARE) can trigger a dramatic down-regulation of translation (fig. 9, C and D).

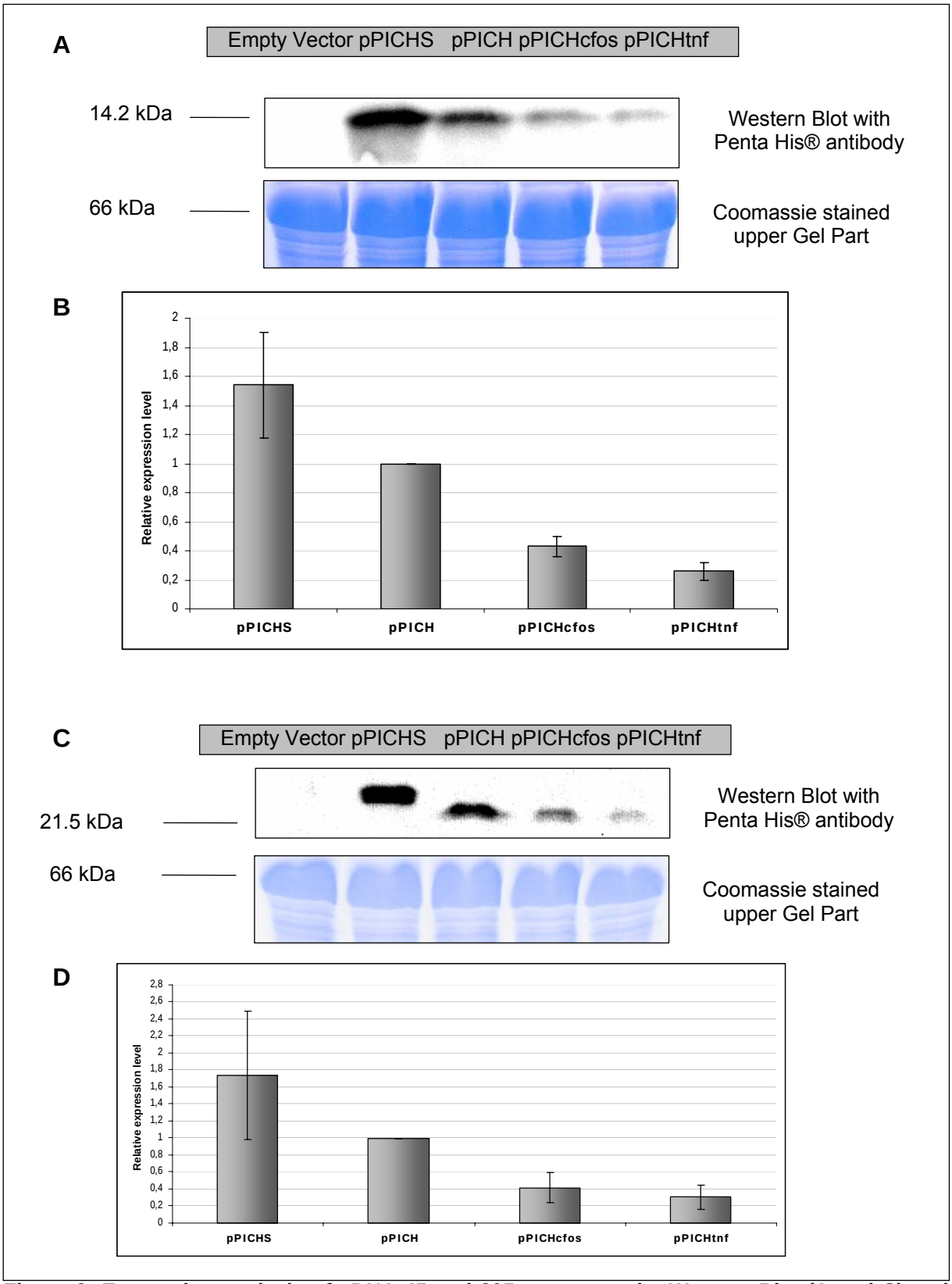


**Figure 9: Expression analysis of cDNA 45 and 205 constructs by Western Blot (A and C) and quantified expression analysis normalized to the total protein amount and the pPICH value (B and D).** As a control, a strain transformed with the empty vector pPICHcfos was used. An amount of 25 µg total protein was applied to the gel. Detection was carried out with a Penta His® antibody (Qiagen). The standard marker for protein gels was the rainbow marker (Amersham, Braunschweig, Germany).

The His/Strep-II-tagged protein of cDNA 29 (pPICHS) shows a distinct band at approximately 23.0 kDa (fig. 10, A). The removal of the Strep-II-tag (pPICH) reduces the protein size and significantly reduces the protein level (fig. 10, B). The addition of a start and stop codon by PCR leads to a highly increased protein expression level (pPICH+S+S). Interestingly, the introduction of the *c-fos* ARE into pPICH+S+S does not cause such a dramatic down-regulation of translation by far (pPICHcfos+S+S) as it could be observed for cDNAs 45 and 205 (fig. 9), whereas the TNFα ARE (pPICHtnf+S+S) results with a similarly lowered expression level. To test the influence of the additional stop codon in both ARE-constructs, the stop codon was deleted by PCR with a corresponding primer (pPICHcfos/tnf+S). In both cases a further down-regulation of translation can be observed.

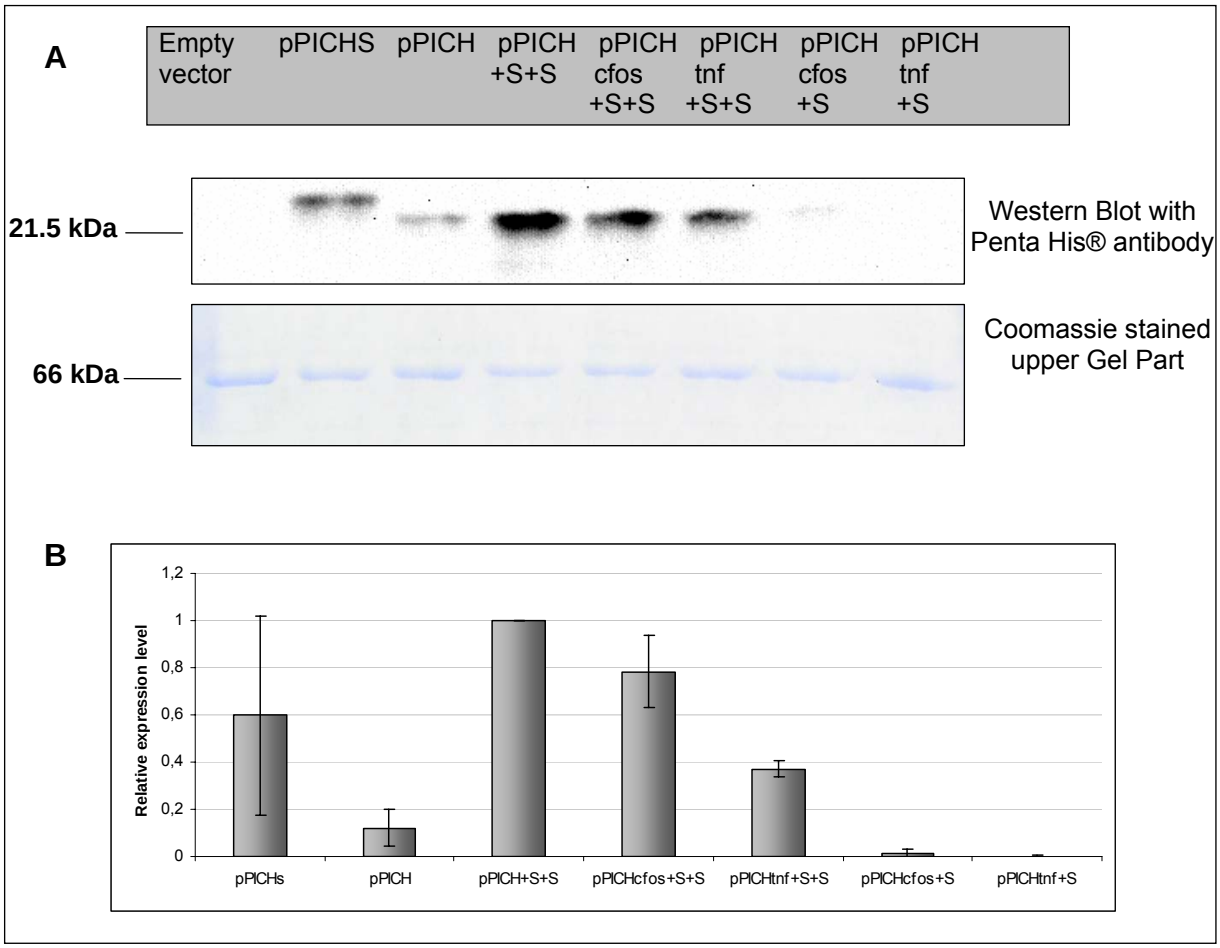


**Figure 10: Expression analysis of cDNA 29 constructs by Western Blot (A) and quantified expression analysis of cDNA 29 constructs normalized to the total protein amount and the pPICH value (B).** As a control, a strain transformed with the empty vector pPICHcfos was used. An amount of 1.5 µg total protein was applied to the gel. Detection was carried out with a Penta His® antibody (Qiagen, Hilden, Germany). The standard marker for protein gels was the rainbow marker (Amersham, Braunschweig, Germany). The constructs marked with +S+S have an additional start and stop codon, constructs marked with only +S have an additional start codon.

### 4.2.2 Standardized comparison of the mRNA level – AREs can influence transcript stability in *P. pastoris*

To gain further insight into the regulation of the ARE-mediated effects found on translation, the transcriptional level was also analysed. Transcript half-lives were not measured in this study. A reduced rate of *AOX*1 promoter activity in the ARE-containing constructs is unlikely and no effects like that have ever been reported between any ARE and promoter. As a result, in the following mRNA abundance is equated with the transcript stability itself.

Yeast cultivation procedures were the same as for the protein expression analysis. Samples were taken two hours after resuspension in fresh WMIX$NH_4Cl$ with 0.1% glucose (w/v) and 1% methanol (v/v). As a control, a strain transformed with the empty vector pPICHcfos was always treated like the other cultures. Cells were disrupted mechanically with glass beads. Total RNA was isolated and the yield was determined photometrically. 20 µg of total RNA were used for denaturing agarose gel electrophoresis and Northern Blot. To obtain comparable results, all transcripts were detected with the same radioactively labelled probe against the 5' UTR (fig. 11, A).

This 5' UTR is not only present in the integrated expression vectors, but also in the original *AOX1* gene. Therefore, two mRNAs can be detected, the endogenous *AOX*1 mRNA as a loading control and the transcripts of interest encoded on the integrated expression vectors (fig. 11, B). As a further loading control, a PCR generated probe against endogenous actin mRNA was applied to the membranes (data not shown). In order to avoid experimental variations, the membranes were not treated with stripping solution which can lead to a loss of signal intensity. The standard RNA marker was the Low Range RNA Ladder (MBI Fermentas, St. Leon-Rot, Germany).

Due to the additional Strep-II-tag coding sequence, the cDNA 45 mRNA of construct pPICHS is slightly bigger than pPICH (fig. 12, A). However, the amount of the transcript is nearly the same (fig. 12, B). The introduction of the *c-fos* ARE leads to two distinct bands which differ approximately 200 nt in size (pPICHcfos). It is possible that the human *c-fos* ARE is recognized as a poly(A) signal in *P. pastoris.* However, it can be assumed that both transcripts are translated. Thus, in the case of the *c-fos* ARE a strong down-regulation of transcript stability can be observed as well as in the case of the TNF$\alpha$ ARE (pPICHtnf).

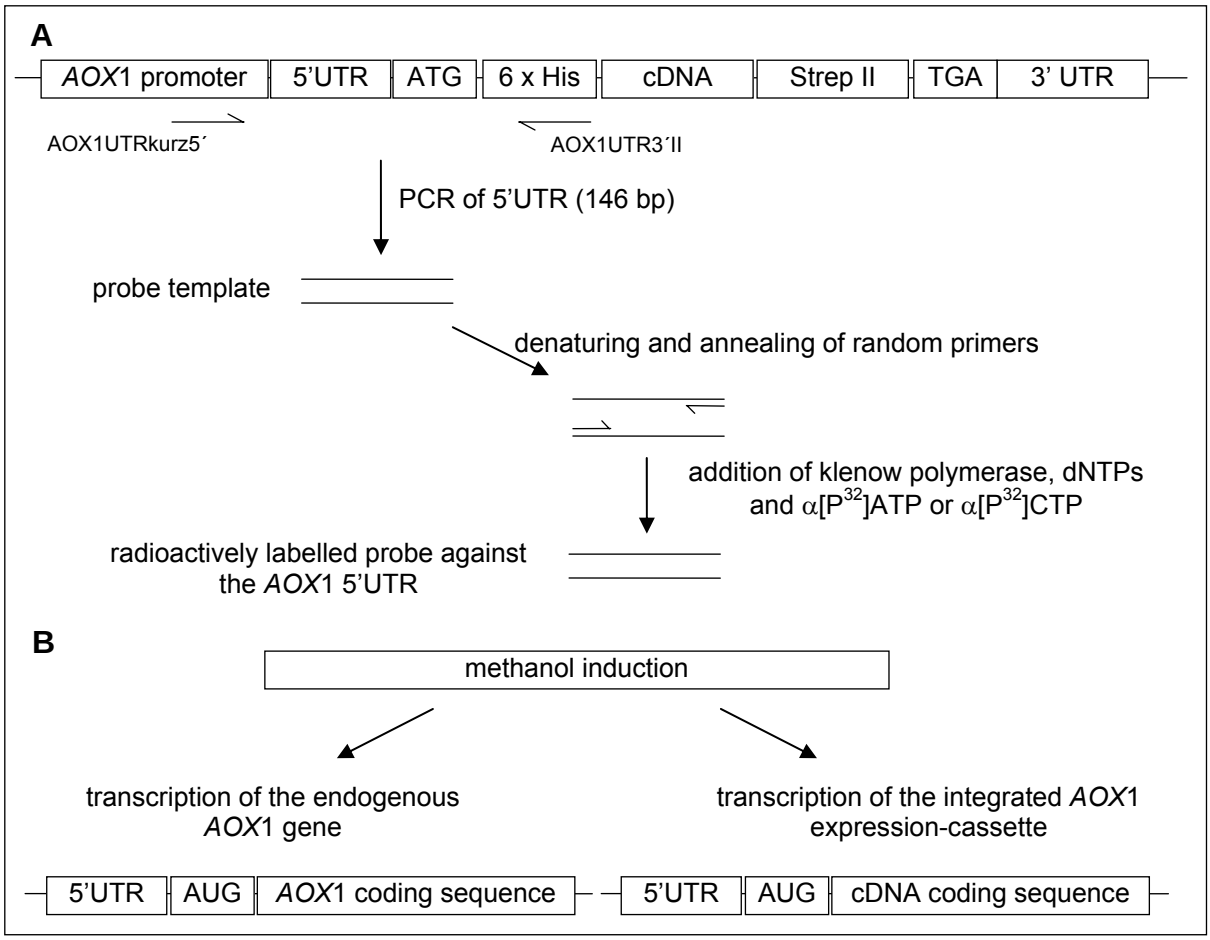


**Figure 11: (A) Creation of the radioactively labelled probe against the 5' UTR of the *AOX*1 gene in the vector pPICHS and (B) its targets under inducing conditions.** The probe template is generated by PCR using specific primers for the 5' UTR of the *AOX*1 gene present in all applied *P. pastoris* expression vectors. This template is used for radioactive labelling. The radioactive probe can be used to specifically detect the two indicated transcript types under inducing conditions.

Interestingly, the situation for the cDNA 205 transcripts is partially different. The transcript of the pPICH construct is slightly smaller than the transcript of the pPICHS construct (fig. 12, C). The amount of both transcripts is also nearly the same (fig. 12, D). However, the introduction of the human AREs has no obvious influence on mRNA stability. The amount of the mRNA is not reduced at all. Again. the two transcripts of the *c-fos* ARE construct which differ in size of approximately 200 nt can be detected.

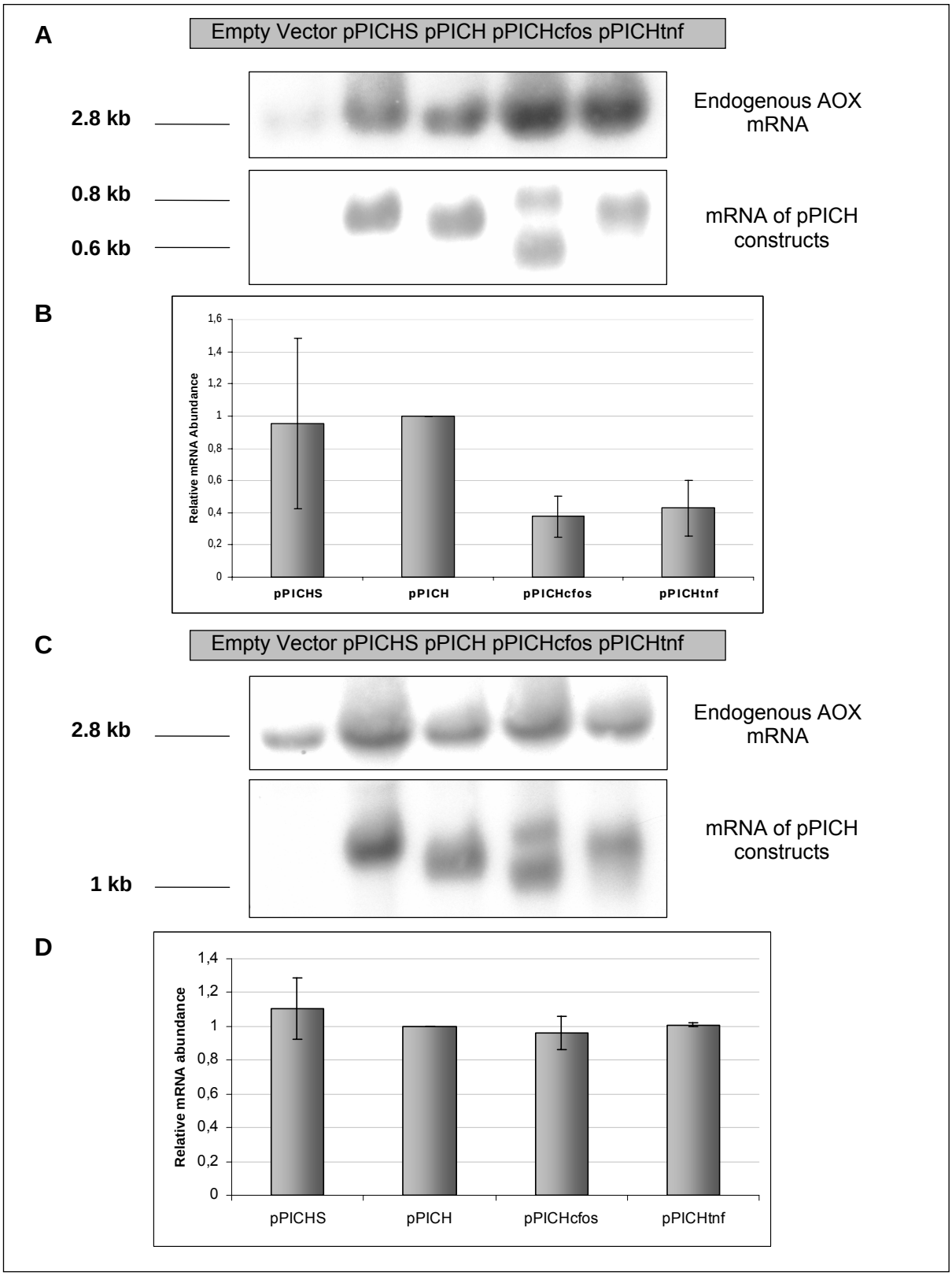


**Figure 12: mRNA stability analysis of cDNA 45 and 205 constructs by Northern Blot (A and C) and quantified mRNA stability analysis normalized to the engodenous AOX mRNA and the pPICH value (B and D).** As a control, a strain transformed with an empty vector was used. An amount of 20 µg total RNA was applied to the gel. Detection was carried with an AOX-5' UTR specific radiolabelled probe. The standard marker for RNA gels was the low range RNA ladder (MBI Fermentas, St. Leon-Rot, Germany).

The mRNA stability of cDNA 29 pPICHS, pPICH and pPICH+S+S constructs do not differ significantly (fig. 13, A and B), although the protein expression does (fig. 10). The introduction of the human *c-fos* ARE into the pPICH+S+S construct does not lower the rate of transcript stability, it is even slightly increased (pPICHcfos+S+S). In contrast, the introduction of the TNFα ARE leads to the known result, a strong down-regulation of transcript stability (pPICHtnf+S+S). The removal of the additional stop codon causes down-regulation of transcript stability in the case of the *c-fos* ARE (pPICHcfos+S), whereas the rate of transcript stability in the case of the TNFα is not significantly influenced (pPICHtnf+S).

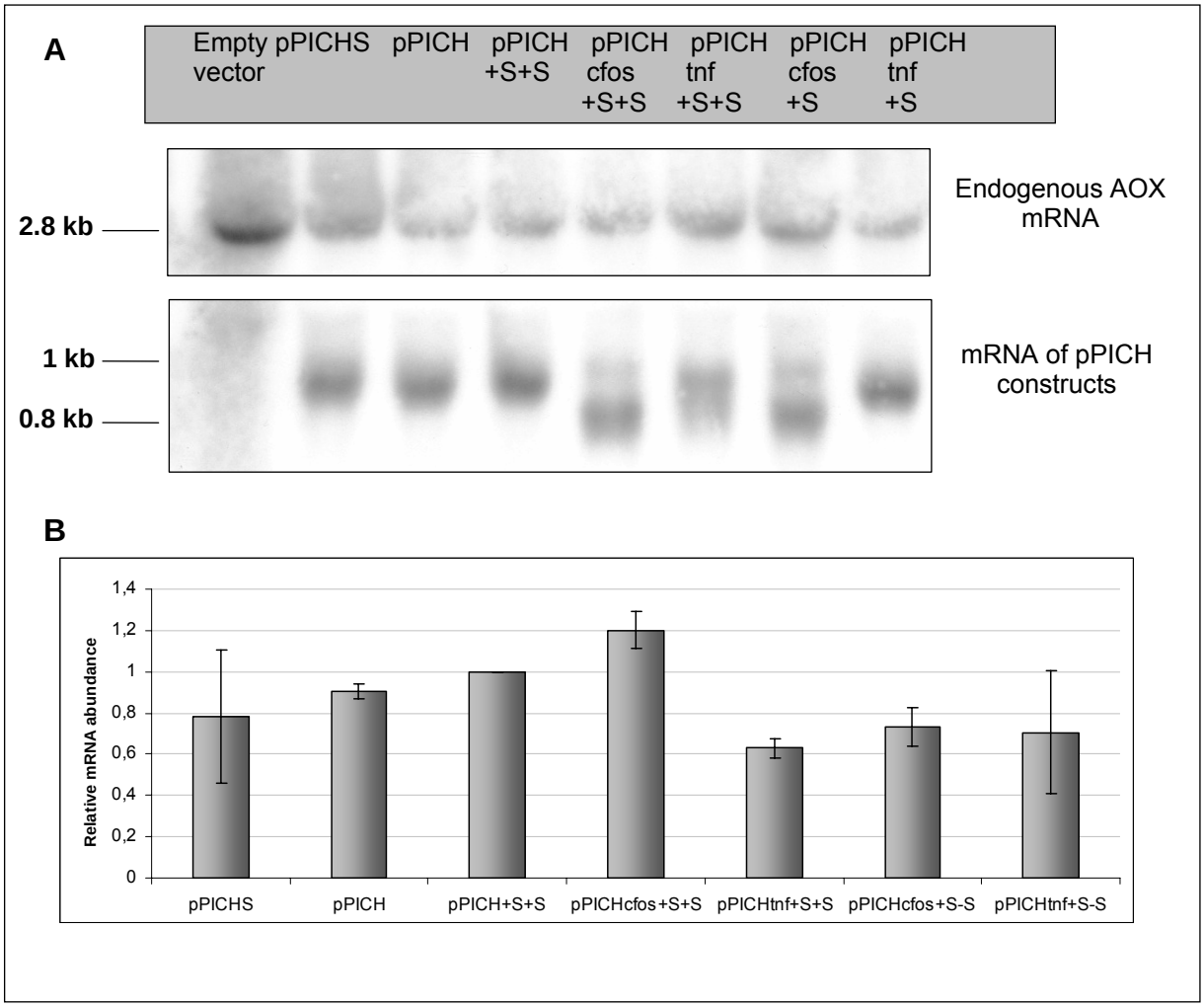


**Figure 13: mRNA stability analysis of cDNA 29 constructs by Northern Blot (A) and quantified mRNA stability analysis of cDNA 29 constructs normalized to the engodenous AOX mRNA and the pPICH value (B).** As a control, a strain transformed with an empty vector was used. An amount of 20 µg total RNA was applied to the gel. Detection was carried out with an AOX-5' UTR specific radiolabelled probe. The standard marker for RNA gels was the low range RNA ladder (MBI Fermentas, St. Leon-Rot, Germany).

It can be summarized that evidence is provided for an ARE-based regulatory system of transcript stability and translation as known for mammals is also found in the yeast *P. pastoris* On the one hand, it is known that the *c-fos* ARE as a class I ARE and class II AREs can have a destabilizing influence on a stable reporter in mammalian cells. This effect can be compensated, at least partially, by over-expression of a stabilizing ARE-BP-like HuR (Fan and Steitz, 1998b). Destabilizing functions of the TNFα ARE have also been reported for a cell-fee system (Chen et al., 2001). On the other hand, it was demonstrated that the TNFα AREs is also able to repress translation (Zhang et al., 2002). These effects ARE-mediated by distinct ARE-BPs under certain conditions. The same ARE-effects on transcript stability and translation can be observed in the yeast *P. pastoris* and thus the system found has similar or even the same characteristics as in mammals. However, all these effects can be observed under the same reproducible cultivation conditions. Therefore, it is unlikely that external stimuli induce the expression of (de)stabilizing ARE-BPs.

## 4.3 Identifying factors that influence ARE-based regulation in *P. pastoris*

It is assumed in general that ARE-flanking sequences can influence ARE-based regulation itself. For example in the case of the TNFα, ARE phylogenetic analyses of flanking sequences revealed a high conservation among different species (Fialcowitz et al., 2005). These flanking sequences might be proximal targets for ARE-BPs and involved in ARE-mediated regulation (Wilson et al., 2001; Duttagupta et al., 2003). To test this hypothesis and to further investigate the found ARE-regulated system, single point mutations were introduced upstream of the *c-fos* ARE within the 3' UTR of the pPICHcfos29+S+S construct. New *P. pastoris* expression clones were then created and transcript stability and translation were tested.

### 4.3.1 Mutational analysis – exchange of one nucleotide upstream of the *c-fos* ARE alters mRNA and protein level

To test the influence of ARE-flanking sequences single point mutations were introduced into the *Not*I restriction site of construct pPICHcfos29+S+S (fig. 14, A and B). The additional stop codon moves the *Not*I restriction site into the 3' UTR. The

three alanine-aminoacid-residues are not translated. Thus mutational changes of the DNA sequence at this location do not alter the protein itself. They only result in a change of the mRNA.

To introduce the single point mutations, the vector pPICHcfos29+S+S was restricted with *Not*I and *Xho*I. The samples were then purified from the small DNA fragment that was cut out. To introduce the single point mutations, three pairs of oligonucleotides (NotImutI, NotImutII and NotImutIII) carrying three different mutations were hybridized (fig. 14). These hybrids possessed the corresponding overhanging ends for ligation. The new constructs were sequenced and used for creation of new *P. pastoris* expression clones. To test the influence of these mutations, Western and Northern Blot analyses were performed.

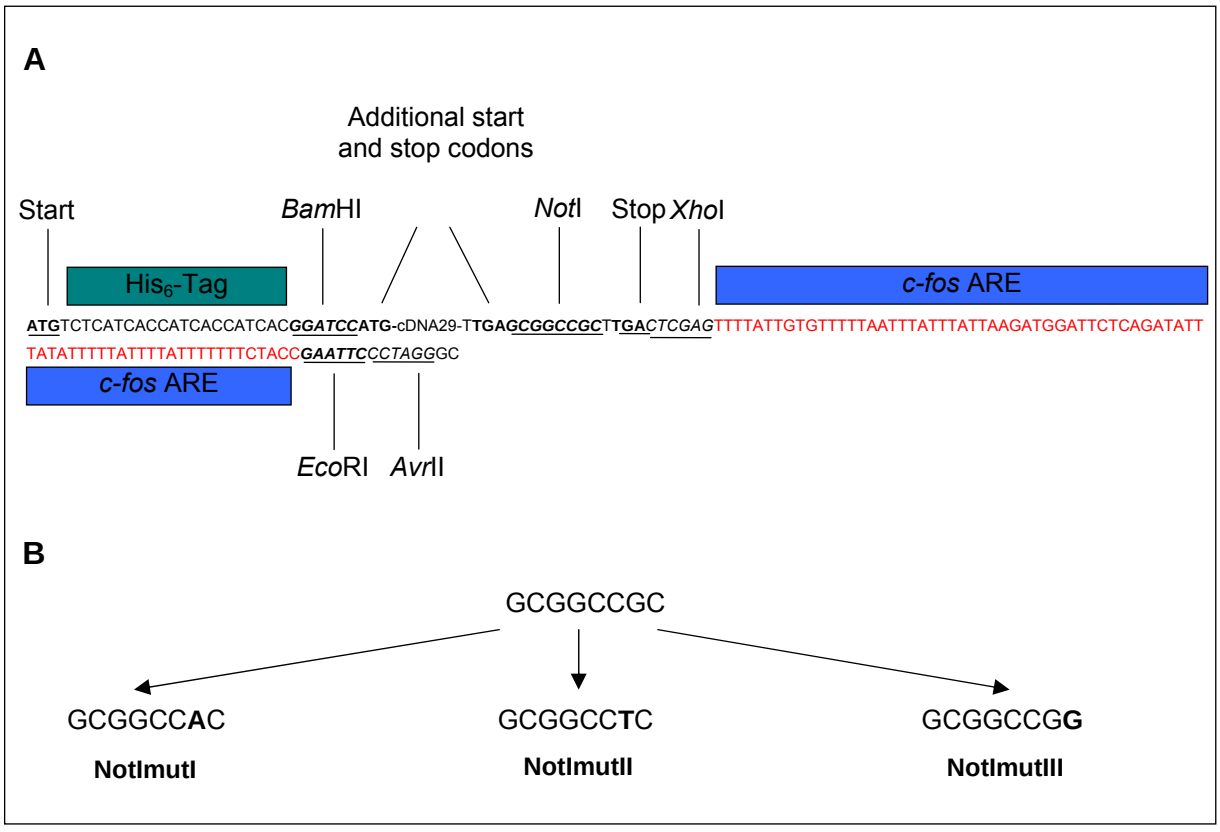


**Figure 14: (A) Schematic overview of construct pPICHcfos29+S+S. (B) Single point mutations introduced into the NotI restriction site resulting in an altered secondary structure prediction.** (A) The presence of the additional stop codon moves the NotI restriction site into the 3' UTR. (B) Oligonucleotides carrying the indicated mutations were hybridized and ligated to the vector pPICHcfos29+S+S, which was restricted with *Not*I and *Xho*I.

The experiments were performed as described above. For the analysis of the newly generated strains, the non-mutated strain containing the construct pPICHcfos29+S+S was set as a reference (cfos29+S+S in Fig. 15, A and B). Interestingly, all three

single point mutations in the *Not*I restriction site lead to a strong decrease of translation. Although the level of translation varies slightly, there is no significant difference. The reduction of translation can be set from 45 % to 65 % in average. This finding supports the assumption that ARE-flanking sequences can influence ARE-mediated regulatory pathways.

To find out at which stage the regulation is influenced (transcriptional or translational control), the rate of transcript stability was also investigated. For this purpose experiments were performed as described above. As shown in fig. 16, the rate of transcript stability of all three mutants is dramatically decreased compared to the

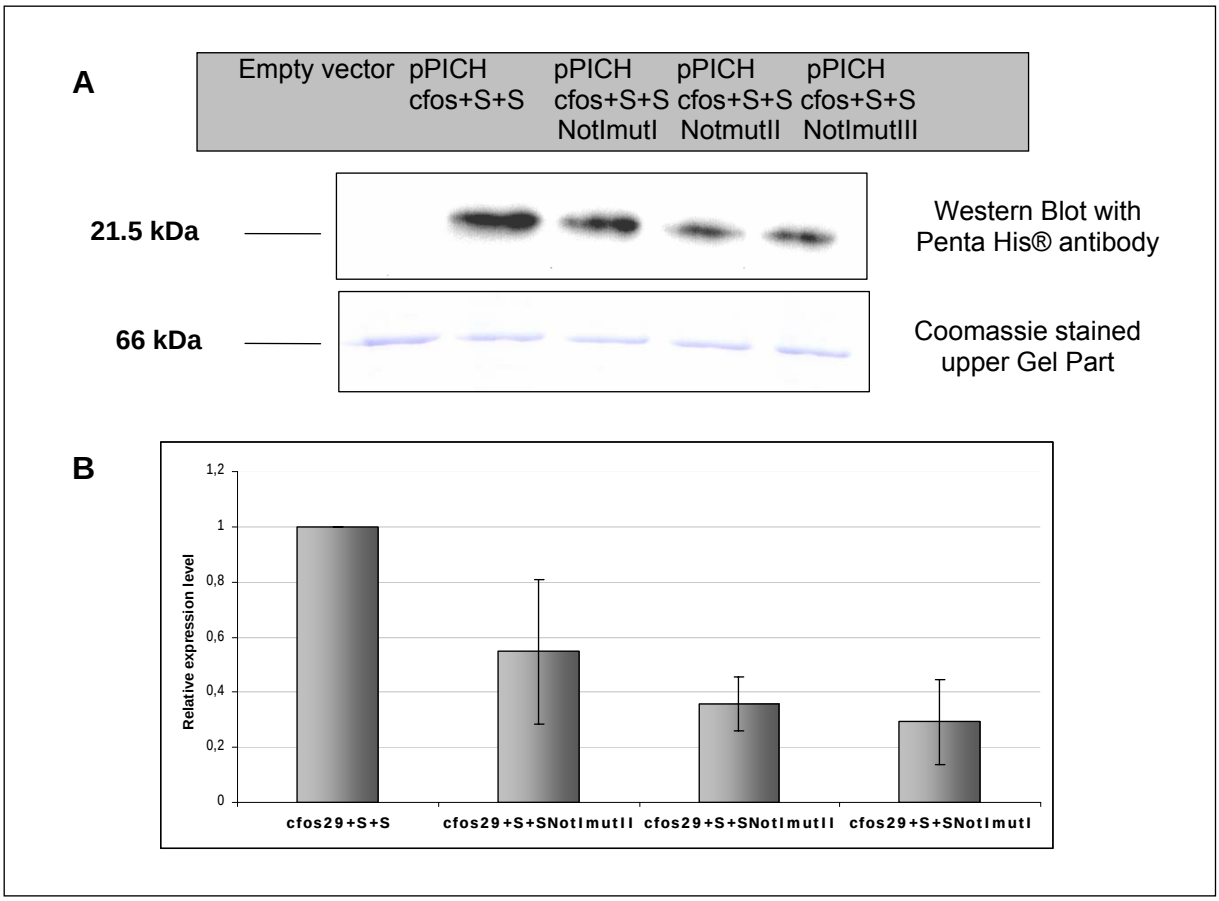


**Figure 15: (A) Expression analysis of NotImut constructs derived from pPICHcfos29+S+S by Western Blot. (B) Quantified expression analysis of NotImut constructs derived from pPICHcfos29+S+S normalized to the total protein amount and the pPICH value.** As a control a strain transformed with an empty vector was used. An amount of 1.5 µg total protein was applied to the gel. Detection was carried with a Penta His® antibody (Qiagen, Hilden, Germany). The standard marker for protein gels was the rainbow marker (Amersham, Braunschweig, Germany). The constructs marked with +S+S have an additional start and stop codon, constructs marked with only +S have an additional start codon.

non-mutated construct pPICHcfos29+S+S. As observed for translational analysis, the rate of transcript stability also varies slightly. However, there is no significant difference. As observed for all *c-fos* ARE-containing constructs, the second band

already known can be detected in this experiment. The size of these two transcripts is not influenced by the single point mutations.

To summarize the findings it can be said that ARE-flanking sequences can in fact influence ARE-mediated regulation on the transcriptional level and possibly as a consequence, the level of translation.

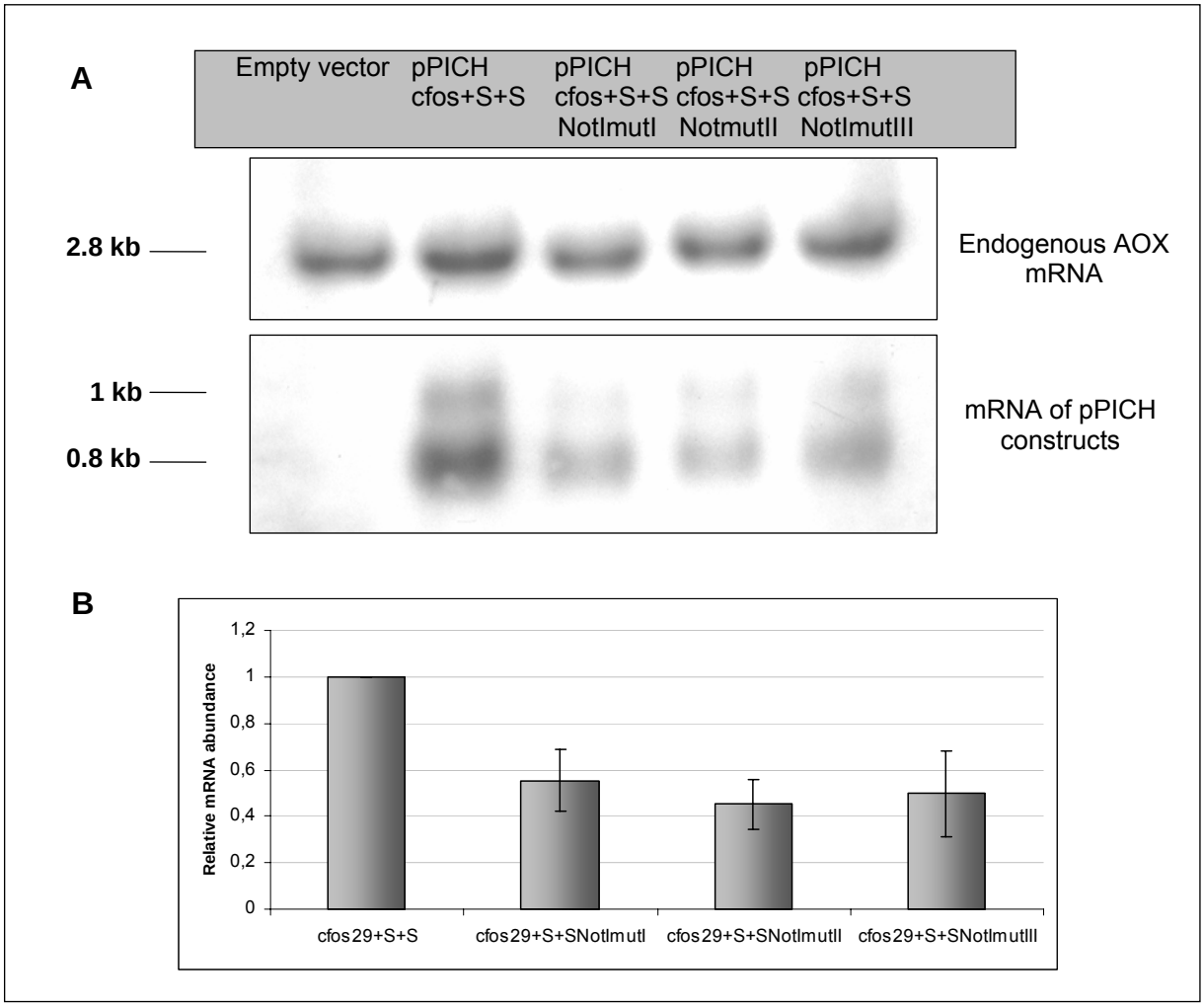


**Figure 16: (A) mRNA stability analysis of NotImut constructs derived from pPICHcfos29+S+S by Northern Blot. (B) Quantified mRNA stability analysis of NotImut constructs derived from pPICHcfos29+S+S normalized to the engodenous AOX mRNA and the pPICHcfos29+S+S value.** As a control, a strain transformed with an empty vector was used. An amount of 20 µg total RNA was applied to the gel. Detection was carried out with an AOX-5' UTR specific radiolabelled probe. The standard marker for RNA gels was the low range RNA ladder (MBI Fermentas, St. Leon-Rot, Germany).

### 4.3.2 Analysis of RNA-protein-interactions *in vitro* – both AREs bind a protein of the same size

In general, an ARE regulatory system consists of *cis*- and *trans*-acting factors. *Cis*-acting factors are sequences on the mRNA (AREs) and *trans*-acting factors are those which bind them (ARE-BPs). The ARE-BPs can recruit other proteins or protein complexes which regulate the turnover of the ARE-containing mRNA. To obtain more insight into the ARE system found in *P. pastoris,* it is necessary to identify such ARE-BPs.

In order to find endogenous *trans*-acting factors that bind the human AREs and are possibly involved in the *P. pastoris* endogenous ARE-mediated regulatory system, a number of gel retardations was performed. These types of experiments serve to identify RNA binding proteins. In principle, a radioactively labelled transcript is subjected to a cell protein extract. Interacting proteins are cross-linked to the RNA and the samples are separated on an SDS gel. Slower migrating bands show assembled complexes.

### 4.3.3 Creation of RNA transcripts – *in vitro* transcription

*In vitro* transcription requires a purified linear DNA template containing a promoter, ribonucleotide triphosphates (rNTPs), a buffer including DTT and magnesium ions, and an appropriate phage RNA polymerase.

The T7 phage polymerase / promoter system was applied for the experiments described below. The consensus sequence for the T7 promoter is shown in fig. 17.

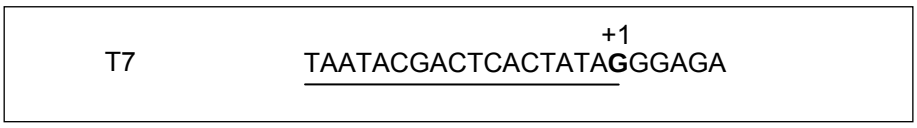


**Figure 17: Consensus sequence of the promoter T7.** The +1 base (bold) is the first base incorporated into the transcript. The underlined sequence marks the minimum promoter sequence.

The DNA template must contain a double-stranded promoter region where the polymerase can bind and initiate transcription. It is important to notice that the base composition of +1 and +2 are critical and must be a G and purine, respectively, to yield an efficient transcription. Template DNAs can be linearized plasmids, PCR products or oligonucleotide hybrids.

For creation of the *c-fos* ARE RNA, the transcription template was generated by PCR. The PCR DNA template was the vector pPICHcfos and primers used for this PCR were T7-cfos-for and T7-cfos-rev. The T7 promoter is added to the PCR product by including the promoter sequence at the 5' end of the forward primer.

As the TNFα ARE is relatively short, the template could be created by hybridization of the two complementary oligonucleotides T7-tnf-for and T7-tnf-rev. As only part of the DNA template (-17 to +1 of the promoter) needs to be double-stranded, it was more economical to hybridize one short (forward) and one long oligonucleotide (reverse), generating an asymmetric hybrid.

I*n vitro* transcription reaction samples, including the DNA template, transcription buffer, T7 RNA polymerase, RNasin (RNase inhibitor), rNTPs and the radioactively labelled α[$^{32}$P]UTP (Hartmann Analytic GmbH, Braunschweig) were incubated for 2 hours at 37°C. The samples were then mixed with RNA stop buffer and subjected to denaturing polyacrylamide gel electrophoresis. After the run, the gel was exposed to an x-ray film. A typical result of *in vitro* transcription is displayed in fig. 18.

As there is no RNA marker available that could be used for this kind of experiment, randomly selected oligonucleotides of 34 nt and 75 nt were labelled at their 5' ends with γ[$^{32}$P]ATP through a T4 kinase reaction. The water control for this kinase reaction did not contain an oligonucleotide (see fig. 18, lanes 1-3).

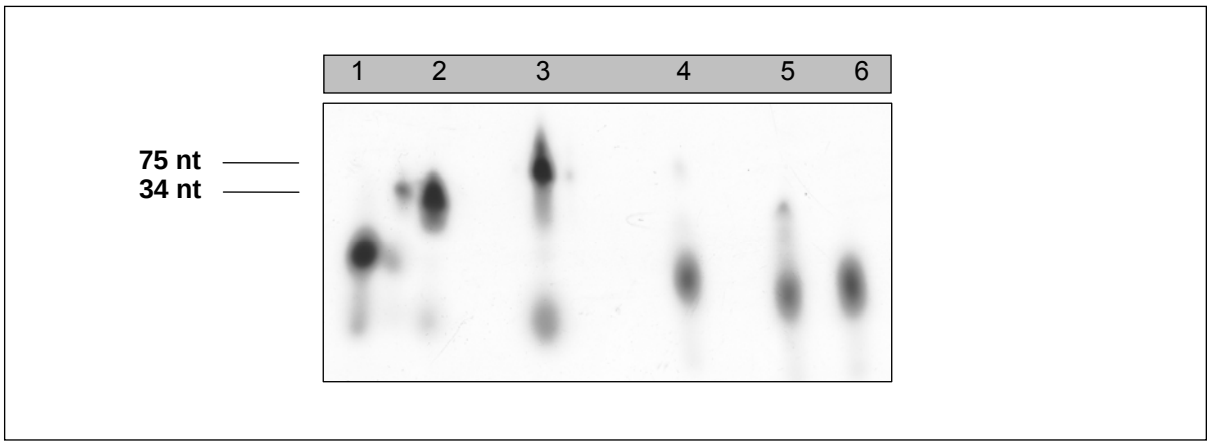


**Figure 18: Typical result of an *in vitro* transcription.** To mark the size of transcripts, random single stranded oligonucleotides of 34 nt and 75 nt in size were labelled with [γ$^{32}$P]ATP (lanes 2 and 3, lane 1 is the water control of the reaction showing free radiolabelled nucleotides). The *c-fos* ARE transcript has a size of 70 nt (lane 4) and the TNFα ARE has a size of 35 nt (lane 5). Lane 6 shows the water control of the *in vitro* transcription. The spots were cut out of the gel and transcripts were purified.

The size of the *c-fos* ARE transcript is 70 nt (lane 4) and the size of the TNFα ARE is 35 nt (lane 5). This can be checked by the marker oligonucleotides. It is striking that the yield of the *c-fos* ARE transcript is always lower than the one of the TNFα ARE transcript. There are two possible explanations for this. The PCR template of the *c-fos* reaction cannot be purified due to its small size. This means that there are a lot of ions left in the sample which can disturb the *in vitro* transcription, whereas the TNFα template is free from disturbing reagents. The other possible explanation is that the partial double stranded DNA template of the TNFα is an advantage for an *in vitro* transcription. For the transcription process itself, the template has to be unwound and single stranded at the location of transcription. This is already given for the TNFα template, whereas the *c-fos* template must be completely unwound. The T7 polymerase has this unwinding activity, but possibly it is more efficient for *in vitro* transcription if this activity is not needed This might make no difference inside a cell as certain co-factors could support the polymerase in this situation. The radioactively labelled *c-fos* and TNFα ARE transcript spots are cut out and the RNA is eluted overnight. The purification and yield could be checked by denaturing formaldehyde agarose gel electrophoresis.

### 4.3.4 Gel retardation experiments – the TNFα and *c-fos* ARE bind a 14 kDa protein

The purified radioactively labelled transcript was incubated with a protein extract from *P. pastoris* under binding conditions (Ruiz-Echevarria and Peltz, 2000) which were chosen because the binding of the *S. cerevisiae* ARE-BP Pub1p to RNA was demonstrated under these conditions. Furthermore, even earlier, the binding of cytoplasmic proteins to RNA was shown using similar conditions (Leibold and Munro, 1988). During incubation an endogenous protein, protein complexes or even other molecules can bind to the transcript. Assembled RNA-protein-complexes are cross-linked by UV light. Afterwards, free RNA is digested by RNAse A that specifcally degrades single stranded RNA at C and U residues. RNA target sites that are bound by a protein or form double-helices are protected from RNase A degradation. The samples are separated on an 8 % denaturing SDS PAA. After the run the gel is dryed on 3 MM Whatman paper and exposed to an x-ray film. Slower migrating bands show the assembly of ribonucleo-particle (RNP).

The result of this analysis indicated that addition of increasing amounts of protein extracts to the TNFα ARE RNA resulted in the appearance of a slower migrating band with an apparent molecular mass of approximately 14 kDa (fig. 19, A, lanes 1 – 4). This complex is not detected after the addition of proteinase K (fig. 19, A, lanes 5 – 7) to the protein extract and a following incubation for 24 hours at 37°C before the binding reaction. This suggests the presence of a protein component in the complex. However, it might be possible that the chosen amount and incubation time is not sufficient to digest all proteins in the 50 µg protein sample. This is indicated by the remaining band in lane 10. The thick faster migrating bands in all lanes are assumed to be double-stranded RNA helices that are not digested by RNAse A. In lanes 5 to 7 (fig. 19, A) these bands migrate more slowly than the bands in lanes 1 to 4. This might be due to a different salt concentration. When proteins were digested with proteinase K, approximately one third of the reaction volume is used by the addition of proteinase K which is missing in the other samples.

The same experiments were performed for the *c-fos* ARE RNA. The addition of increasing amounts of protein extract resulted in the appearance of band with an apparent molecular mass of 14 kDa as well as (fig. 19, B, lanes 1 – 4). However, the formation of this complex cannot be completely abolished by the addition of proteinase K to the protein extract before the binding reaction (fig. 19, B, lanes 5 - 7).

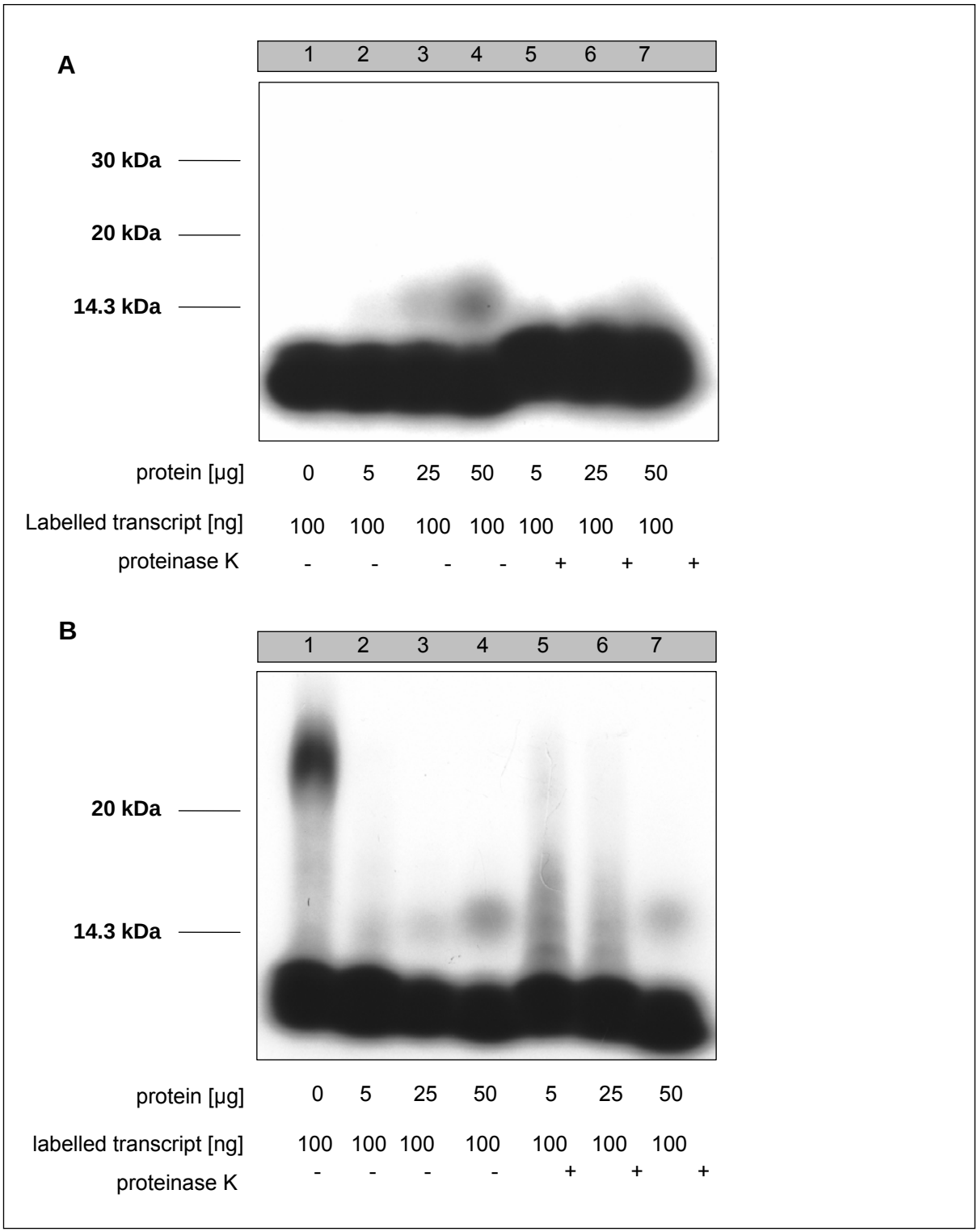


**Figure 19: Gel retardation of the (A) TNFα ARE transcript and (B) *c-fos* ARE transcript with a *P. pastoris* endogenous protein of approximately 14 kDa.** As a control, an experiment without protein extract was performed (lane 1). Increasing amounts of protein lead to the appearance of a slower migrating band with an apparent molecular mass of approximately 14 kDa (lanes 2 – 4). The assembled complex is not detected after addition of proteinase K (lanes 5 - 7) to the protein extract and incubation for 24 hours at 37°C before binding reaction.

Interestingly, the control sample without protein extract shows a broad smear (fig. 19, B, lane 1) with a stronger band above the 20 kDa marker band. The smear and especially the strong band might be the result of higher order structures of the *c-fos* ARE transcript. Such structures would not even be digested after the addition of RNAse A. However, the smear and the band cannot be detected in the protein extract containing samples (fig. 19, B, lanes 2 – 4). Thus, it is possible that the protein extract contains proteins that can unwind such higher order structures to make the RNA sensible to digestion. This assumption is supported by the results of the proteinase K digested samples. It is likely that the proteinase K added can digest 5 µg and 25 µg total protein (fig. 19, B, lanes 5 and 6). This leads to the same or similar situation as in the sample without protein (fig. 19, B, lane 1). Assuming 50 µg total protein cannot be digested completely by the added proteinase K (as this is the same interpretation for the result in fig. 19, A, lane 7), the smear disappears and the remaining protein results in the assembly of the ribonucleoparticle (fig. 19, B, lane 7).
Taken together, the binding of a protein of approximately 14 kDa to both transcripts was detected. It is possible that it is the same protein for both transcripts. Interestingly, the TNFα and *c-fos* ARE transcripts demonstrate different binding behaviours *in vitro*, which could be an indication of their differential regulatory function *in vivo*.

### 4.3.5 Gel retardation experiments – demonstrating the specific binding to both transcripts

To demonstrate specificity of the assembled RNA-protein-complex, *in vitro* transcribed radioactively labelled RNA was incubated with a defined amount of total protein extract and a specific competitor. As a specific competitor, the same *in vitro* transcribed RNA, but not radioactively labelled was applied with increasing amounts. In the case of specific binding, non-radioactively labelled RNA is able to compete with radioactively labelled RNA and replaces it. As an unspecific competitor, yeast tRNA was applied in increasing amounts. If the binding is specific, yeast tRNA cannot compete with the target RNA and cannot replace it.
For the experiments, 100 ng radioactively labelled RNA and 50 µg or 20 µg (only in fig. 20, B) total protein amount were applied. The specific competitor was added in 4 and 12 times amount, whereas the unspecific competitor (yeast tRNA) was added in

10, 100 and 1000 times of the amount. The binding reaction was performed as described above.

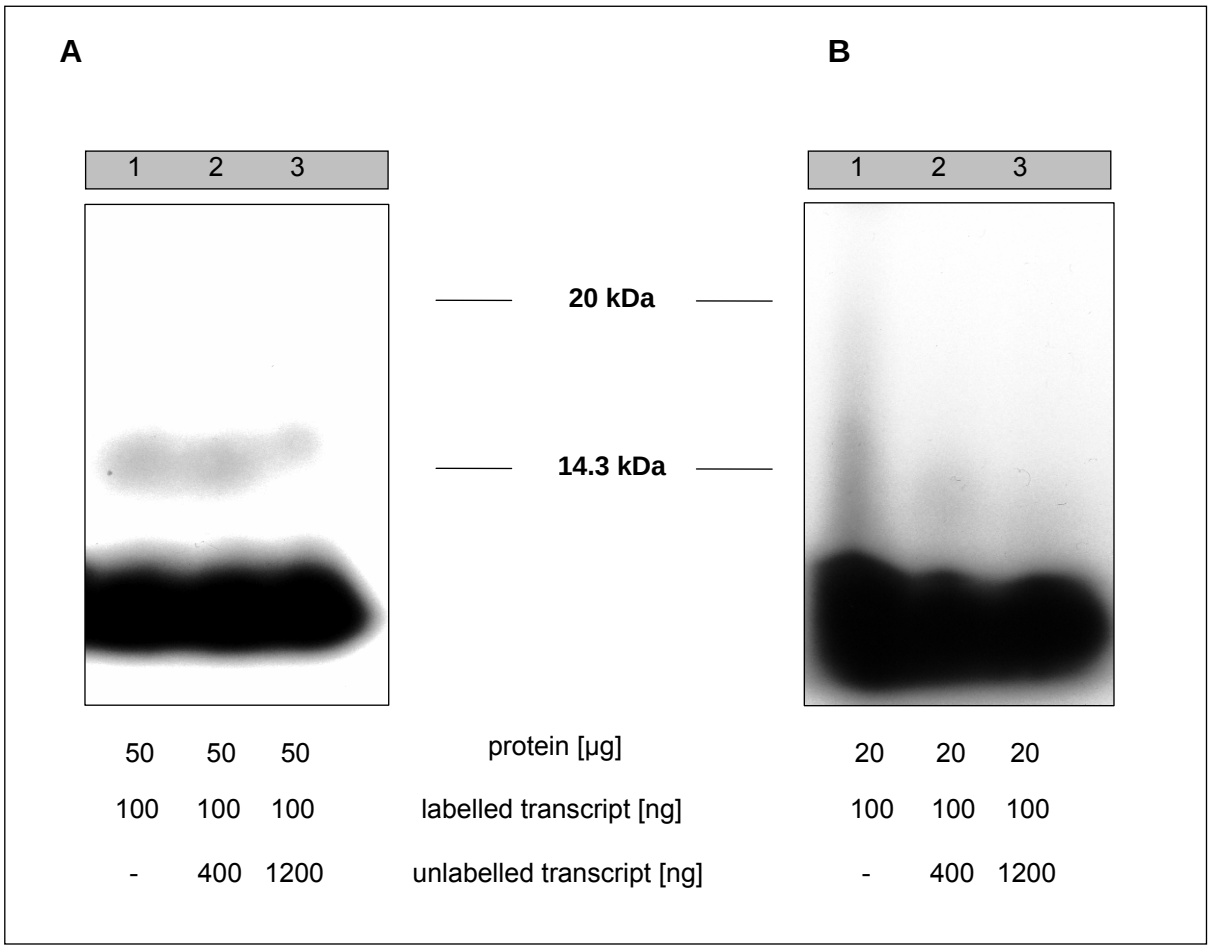


**Figure 20: Competition assay of the TNFα ARE transcript (A) and the *c-fos* ARE transcript (B) with unlabelled transcript as a specific competitor.** As a positive control, a binding experiment without competitor was performed (lane 1). With the same protein amount for each sample, the amount of the specific inhibitor (unlabelled transcript) was increased to replace the labelled transcript specifically.

The intensity of the distinct band of lower mobility at 14 kDa (fig. 20, lane 1) could be successfully reduced with an increasing amount of specific competitor. The intensity is weakened, as the unlabeled transcript replaces the labelled transcript from the protein-complex (fig. 20, lanes 2 and 3).

With an increasing amount of the unspecific competitor, the intensity of the 14 kDa band remains constant (fig. 21).

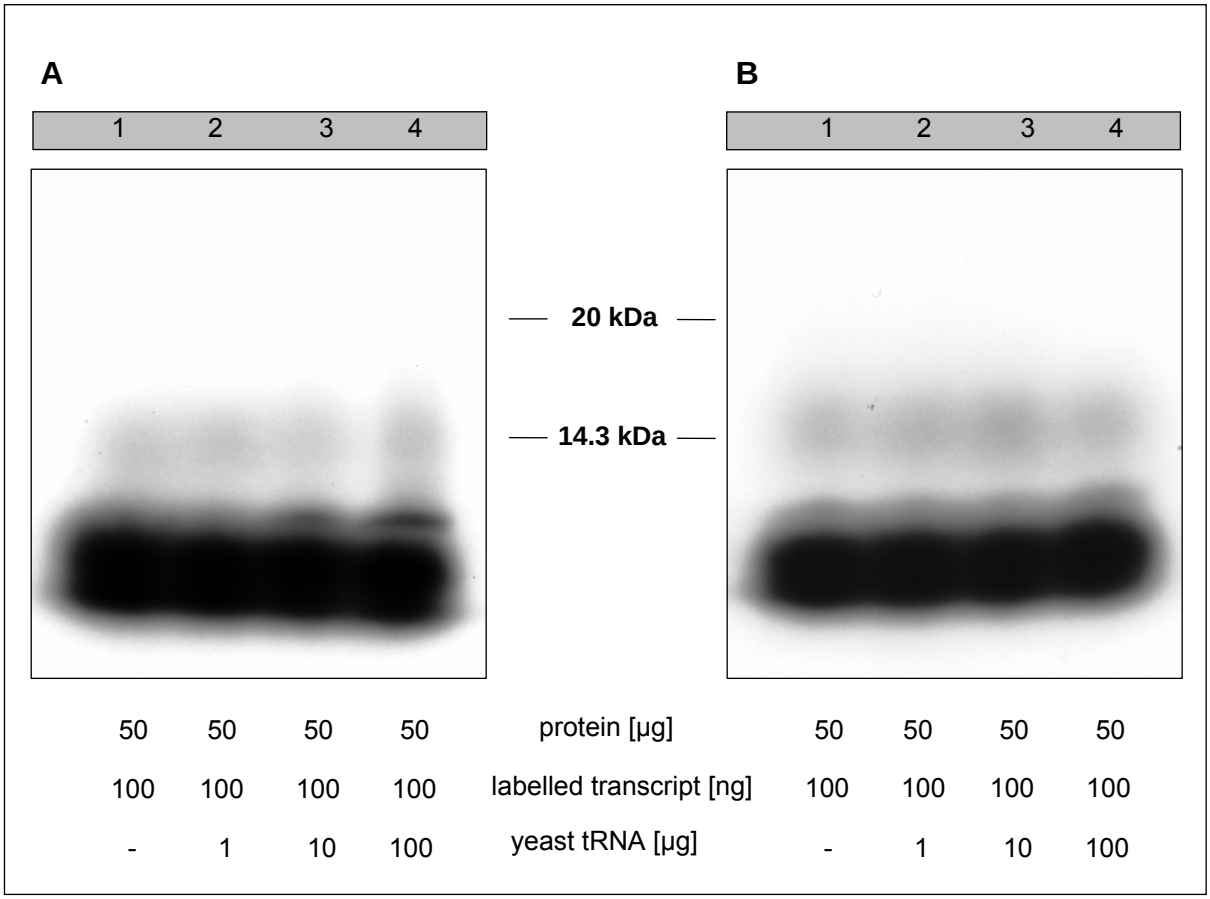


**Figure 21: Competition assay of the TNFα ARE transcript (A) and the *c-fos* ARE transcript (B) with yeast tRNA as an unspecific competitor.** As a positive control, a binding experiment without tRNA was performed (lane 1).

In summary, the complex assembly is very specific for the tested RNAs, also for both the TNFα and the *c-fos* ARE transcript as the signal can be reduced by a specific competitor and as it fails to assemble on the non-specific yeast tRNA. These results demonstrate that the TNFα ARE and *c-fos* RNA are able to interact with at least one cellular protein of *P. pastoris* and form an RNP.

# 5 Discussion

The subject of this thesis is the analysis of AU rich elements in the yeast *P. pastoris* and is divided into two major parts. In the first part, the effects of AREs in the yeast *P. pastoris* were determined. A heterologous system for analysis was developed for this purpose. In the second part, the ARE-mediated regulatory effects on transcript stability and translation found were further characterized. It is shown that ARE-flanking sequences and structures can have a strong effect on the regulation of an ARE-containing transcript. In addition, an endogenous *P. pastoris* ARE-BP, which binds specifically to ARE-RNA, could be detected.

## 5.1 The ARE-based regulatory pathway is conserved among eukaryotes – also in *P. pastoris*

The tight regulation of transcription and translation is essential for the control of gene expression (Mitchell and Tollervey, 2000; Barreau et al., 2005). A large number of mRNAs encoding cytokines, growth factors and proto-oncogenes display regulation depending on AREs located in the 3' UTR of their transcripts. Such transcripts are targeted for degradation in normal resting or unstimulated cells leading to a low level of translation. In response to intra- or extracellular stimuli, transcripts containing AREs can be rapidly stabilized and the cell can adapt its gene expression pattern to the new conditions (Wodnar-Filipowicz and Moroni, 1990; Levy et al., 1998; Bevilacqua et al., 2003). Destabilization and stabilization ARE-mediated through the interplay with specific ARE-BPs, of which a large number has been identified till date (Barreau et al., 2005). However, despite efforts in this field the mechanical basis of ARE-mediated mRNA instability remains unclear. The regulation based on AREs and ARE-BPs is widespread among eukaryotes (Fan and Steitz, 1998b), it is even also present in the yeast *S. cerevisiae* (Vasudevan and Peltz, 2001). Evidence for an ARE system in the yeast *P. pastoris* is provided in this thesis. The unravelled characteristics of this system demonstrate strong similarities to consolidated findings in other organisms.

### 5.1.1 Identical AREs can have different effects – like in mammals

In the literature AREs are described as destabilizing elements located in the mRNA 3' UTR of so called early response genes. Cloning AREs into the 3' UTR of a reporter transcript triggers rapid degradation (Shaw and Kamen, 1986; Fan and Steitz, 1998b; Cok et al., 2003). In addition, AREs can play a role in translational efficiency of target mRNAs (Piecyk et al., 2000; Zhang et al., 2002). Once cells are exposed to specific extracellular signals, the same AREs are required for transcript stabilization and/or increased translational efficiency.

In order to analyse AREs in the yeast *P. pastoris* the human *c-fos* and TNFα AREs were cloned into the 3' UTR of different reporters. These reporter constructs were used to generate *P. pastoris* strains. Analysis of these newly generated strains demonstrated the functionality of AREs in the yeast *P. pastoris*. Stabilizing and destabilizing effects as well as translational repression were observed. These effects could be distinguished from simple coding sequence modifications, which only result in a change of translational efficiency. The data is characterized by considerable similarities to the ARE effects already known from other eukaryotes, and thus an ARE-mediated regulation of transcript stability and translation in the yeast *P. pastoris* is evident.

When analysing reporter cDNA 45 strains it became obvious that the cloned AREs have the capacity to significantly decrease mRNA stability by approximately 60% to 70% compared to the reference without an ARE in the cDNA's 3' UTR. Most probably as a consequence of this, translation is also decreased in the same range as well (fig. 21, A). This data suggests that the reduced accumulation levels of cDNA 45 mRNA is due to a posttranscriptional event. Reduced transcript stability is characteristic for AREs. This is triggered by the binding of a destabilizing ARE-BP, which can recruit a set of mRNA degrading enzymes, which is called the exosome, in the cytosol. The exosome is a 300-400 kDa complex consisting of 11 proteins which degrade ARE-containing transcripts in 3'-to-5' direction and thus lead to a lower transcript half life (Chen et al., 2001). The Strep II protein modification alters protein expression. In general it is accepted that protein tags change protein charge and/or solubility (Braun et al., 2002), which results in different protein accumulation. A positive or negative influence on protein expression cannot be predicted. To clarify influence on translation of both, protein modification and AREs, translational repression/activation was additionally calculated. For this purpose protein expression

was normalized to the corresponding mRNA stability values. A factor of 1 displays no change on the translational level, whereas a factor 1.5 (or higher) and 0.5 (or lower) was defined to be significant for translational activation or repression, respectively. The data demonstrates that there is no influence on translation in the case of the AREs for this reporter. In contrast, the addition of the Strep II tag modification results in an approximately 1.5 times protein expression level.

The results of reporter cDNA 205 strains are different. The insertion of the AREs does not affect mRNA stability of the reporter at all. Both ARE-modifications only lead to a significantly reduced protein expression by 60% to 70% compared to the reference without an ARE in its 3' UTR (fig. 21, C) suggesting a regulatory effect on translation. A significant translational repression of more than 50% can be calculated (fig. 21, D). It is known that a direct correlation between translation and transcription cannot always be observed. HuR, for example, can bind to TNF$\alpha$ or *Cox*-2 mRNA. While increasing stability of the ARE-containing transcript, translation is decreased (Katsanou et al., 2005). However, it is interesting that the inserted ARE sequences have different effects on different reporters. The Strep II tag modification only results in a change of translation as it could be observed before for cDNA 45 previously.

Surprisingly, insertion of the *c-fos* ARE does not result in a rapid change of mRNA stability or protein expression for reporter cDNA 29 (fig. 22, A, pPICHcfos+S+S) as could be observed for the two other reporters (fig. 21). In every performed experiment protein expression is slightly lower and mRNA stability is slightly higher than the reference without an ARE (fig. 22, A, pPICH+S+S). This finding suggests another method of *c-fos* ARE regulation for this reporter.

Insertion of the TNF$\alpha$ ARE element results in a strong down regulation of transcript stability (pPICHtnf+S+S) and as a result, expression from that protein is also decreased. Transcript stability and translational rates are not reduced in the same range. However, the difference is defined not to be significant (fig. 22, B).

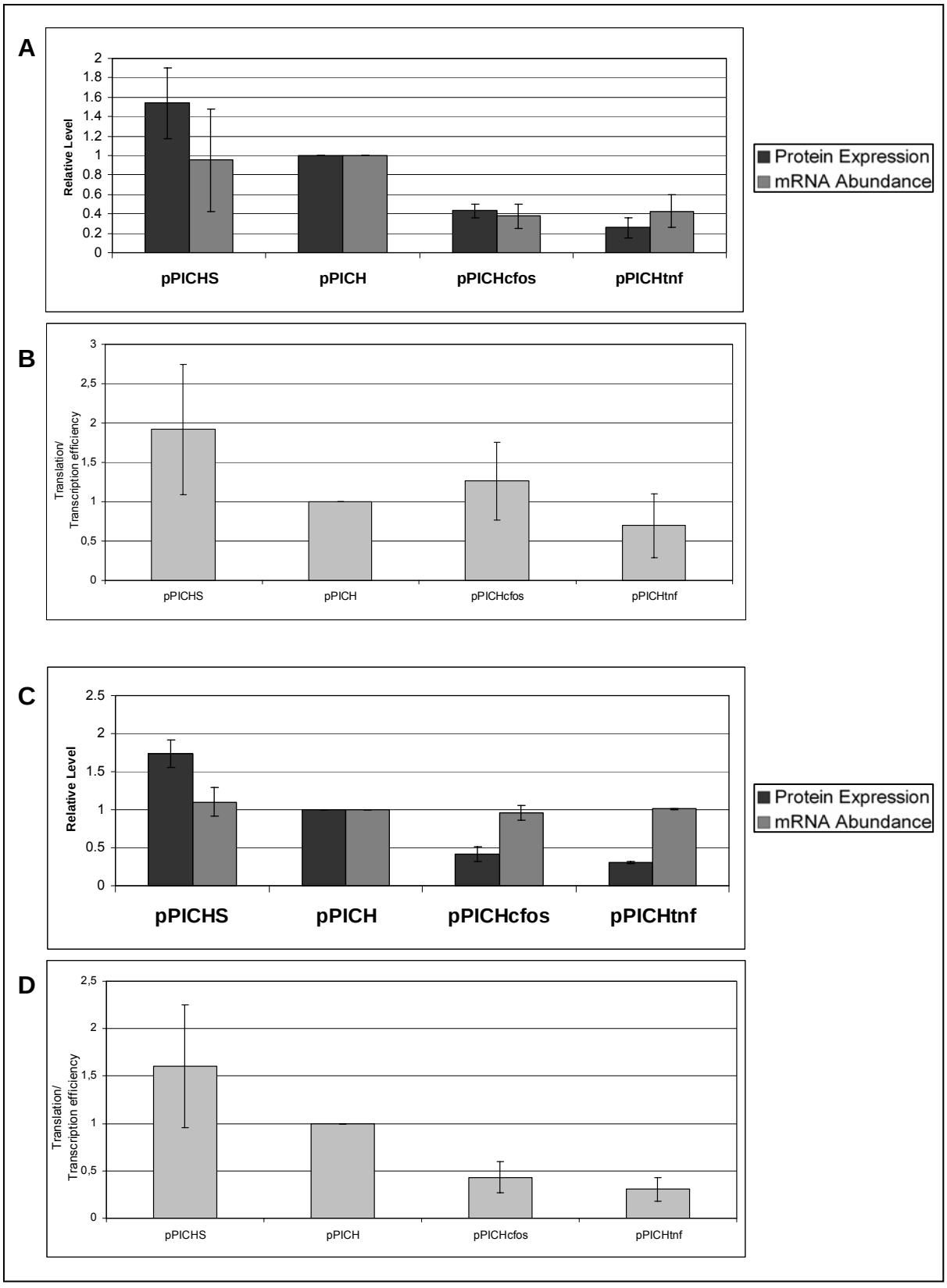


**Figure 21: Transcript stability and translational rates of cDNA 45 (A) and 205 (C) and translational repression/activation triggered by introduced modifications (B and D).** The protein modifications (Strep-II-tag) can only regulate translation, whereas the AREs can also regulate transcript stability. To test if the effects are due to transcript stability or translational repression all translation values were normalized to mRNA stability and compared to the pP translation rate. A factor of 1.5 (or higher) and 0.5 (or lower) was defined to be significant for translational activation and repression, respectively. Experiments were performed at least three times.

The additional stop codon was removed (fig. 22, A, pPICHcfos/tnf+S) to test if the three additional amino acid residues encoded by the *Not*I restriction site (see fig. 14) have any detectable influence on ARE-dependent regulation (fig. 22, A, pPICHcfos/tnf+S). The modification led to a further translational repression and to a non-detectable level of protein for both constructs, which means a far stronger reduction in the case of the *c-fos* ARE-containing reporter. Interestingly, mRNA stability of the *c-fos* reporter is reduced by half in comparison to the *c-fos* reporter including the additional stop, whereas mRNA stability of the TNFα construct without the additional stop codon does not show a significant change. Comparing the non-ARE construct containing strains, the major change observed is in the protein expression level. The removal of the additional start and stop codons (fig. 22, pPICH) leads to a strong decrease of translation, whereas mRNA stability remains at the same level. Although this step includes two modifications, it can be assumed that the additional stop codon can play an important role in the rapid change in translation, whereas the additional start codon only has a subordinated role. This assumption is consistent with the observation that the removal of the additional stop codon in the case of the ARE-containing constructs leads to a strong down regulation of translation. It is likely that similar, or even the same effects, are observed. The Strep II tag modification does not lead to a significant change in transcript stability, although it shows a high variation (pPICHS). This variation has also been reported for protein expression. However, the additional Strep II tag increases protein expression as has been observed for all other reporters. To determine whether translational activation or repression plays a role in all of these observations protein expression was normalized to the corresponding mRNA stability values (fig. 22, B) as described above. The insertion of the *c-fos* and TNFα ARE results in a slight translational repression, which is defined not to be significant (pPICHcfos/tnf+S+S), whereas the removal of the additional stop codon results in a significant repression (pPICHcfos/tnf+S).

For historical reasons AREs are known as destabilizing elements that provoke degradation of the host mRNA. However, depending on a precise stimulus, the presence of an ARE can also lead to the stabilization of an mRNA. In this context attention was drawn to ARE-BPs which are mediators of these effects. ARE-BPs such as TTP, AUF1 and HuR are expressed at a certain stage in the cell cycle or due to a certain stimulus. In this way they are able to act on different species of ARE-

containing mRNAs, which are then stabilized or degraded. Interestingly, stabilizing and destabilizing effects can be observed in the yeast *P. pastoris* under the same cultivation conditions and at the same time.

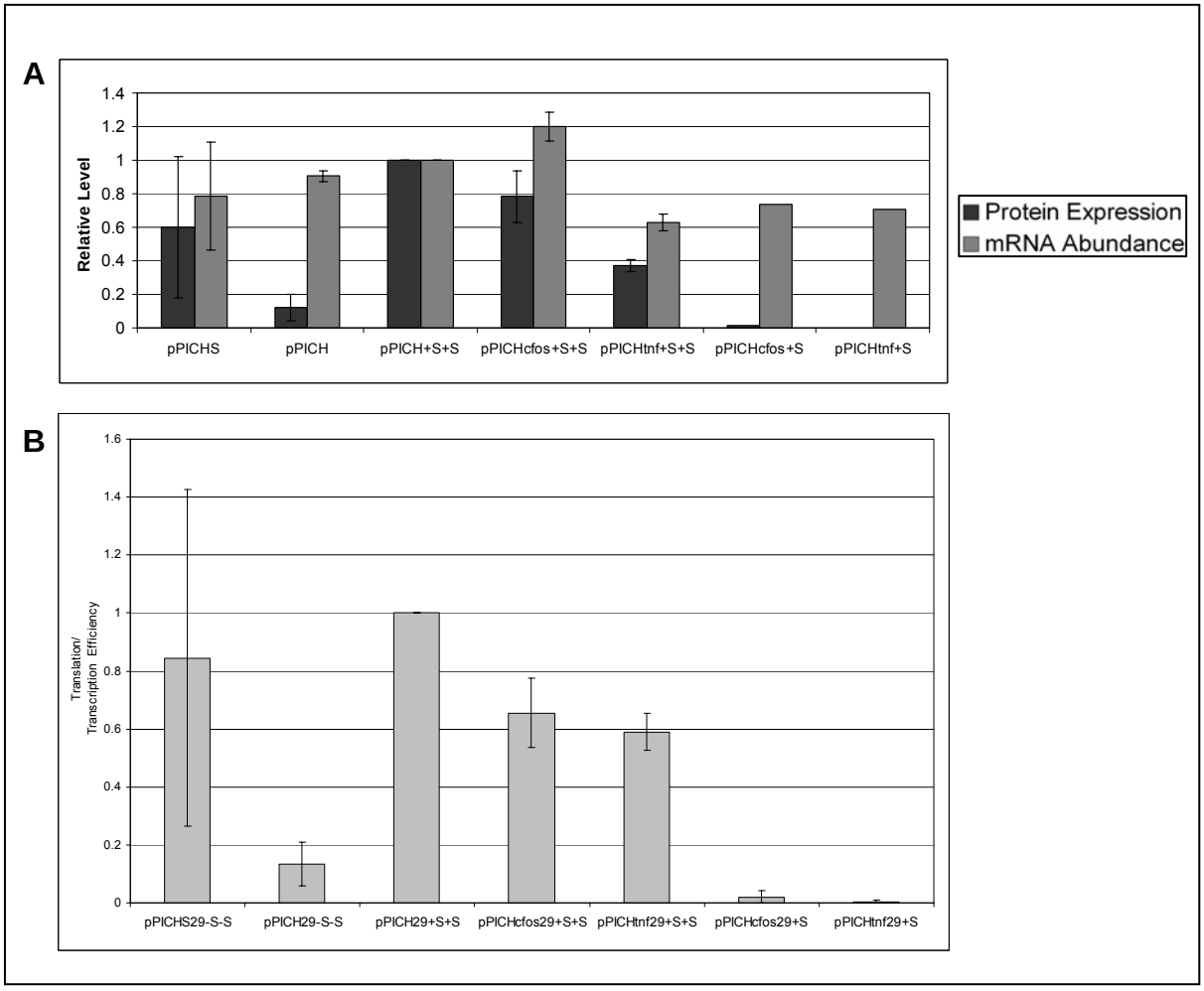


**Figure 22: Transcript stability and translational rates of cDNA 29 (A) and translational repression/activation triggered by introduced modifications (B).** The protein modifications (Strep-II-tag) can only regulate translation, whereas the AREs can also regulate transcription. To test if the effects are due to transcript stability or translational repression all translation values were normalized to mRNA stability and compared to the pPItransorfactor of 1.5 (or higher) and 0.5 was defined to be significant for translational activation and repression, respectively. Experiments were performed at least three times.

Consequently, different internal or external stimuli that induce the activity of ARE-BPs (Levy et al., 1998; Wang et al., 2000b; Wilson et al., 2003b) cannot be the reason for the observations and can be excluded. The protein expression pattern in the cell is the same and thus the selectivity of ARE-BPs must be based on another regulatory factor. Furthermore, many of the known ARE-BPs have been shown to have overlapping specificities crossing the consensus ARE classifications (Barreau et al. 2006). In addition many studies depend on *in vitro* experiments such as UV-

crosslinking assays. Only a relatively small number of experiments have studied the associations between endogenous ARE-mRNAs and ARE-BPs. Some of these studies mention or discuss the involvement of ARE-flanking sequences in the binding of ARE-BPs to their target mRNAs. For example, in the case of the TNF$\alpha$ ARE phylogenetic analyses of flanking sequences revealed a high conservation among different species (Fialcowitz et al., 2005). It is hypothesized that such flanking sequences might be proximal targets for ARE-BPs and involved in ARE-mediated regulation (Wilson et al., 2001; Duttagupta et al., 2003). Furthermore, it is known that the ARE secondary structure can also be a criterion for ARE-BP selectivity.

## 5.2 Stem-loop structures are critical factors for transcript stability and translation

RNA molecules can form different structures of high complexity. The most important structural element of RNA is the A-form double helix, which is dominant in RNA stems. However, a simple A-form RNA stem is not a RNA motif. Motifs change their conformation if residues are added or deleted, whereas A-form stems can only differ in length (Svoboda and Di Cara, 2006).

Hairpins or stem-loops consist of an A-form helix with not only internal loops or bulges but also terminal loops. The structure of a RNA hairpin depends on several parameters such as loop size, stem length and the number of bulges among others (fig. 23).

It has been reported that RNA hairpins can regulate stability, translation and localization of its target mRNA, as was shown for the ARE-containing interleukin-6 and c-myc transcripts (Chabanon et al., 2005; Paschoud et al., 2006). As a consequence, it is generally accepted that the functionality of AREs depends on different ARE-BPs, and additionally, also on the sequence accessibility as well.

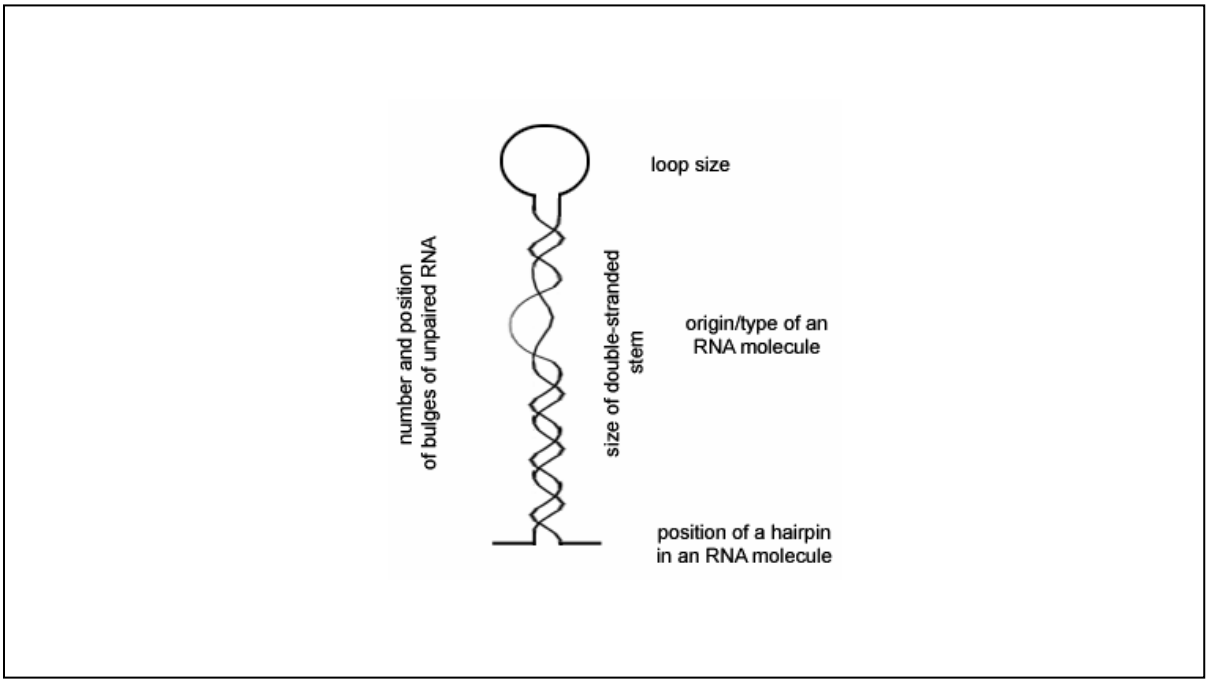


**Figure 23: Schematic overview of an RNA stem-loop.** Important parameters defining its specific structure are indicated (Svoboda and Di Cara, 2006).

### 5.2.1 The different observed effects of the *c-fos* ARE can be correlated to the local ARE structure

In order to find a logical explanation for the different observed ARE effects on transcript stability and translation it was tested if RNA secondary structure, particularly in the local area of the ARE, could be an important factor in that context. Therefore mRNA structure predictions were calculated with mFold v2.3 (Zuker, 2003) for the *c-fos* ARE-containing constructs of reporter cDNAs 45, 205, 29+S+S and 29+S. The local ARE sections of these predictions are shown in fig. 25, A. Interestingly, in agreement with the four different effects of the *c-fos* ARE on transcript stability and/or translation exactly four different structure predictions have been calculated. This indicates an involvement of the local ARE structure in the regulatory effects. All predictions display hairpin structures within the ARE sequence. These hairpins are probably putative RNA motifs, where RNA-BPs or more specific ARE-BPs are able to bind. However, the details of the hairpins differ significantly from each other. The highlighted ARE region (green) of the cfos45 is predicted to have the same structure as the ARE region of the cfos29+S+S and cfos29+S reporters, whereas the prediction of cfos205 is completely different. The cfos45, cfos29+S+S and cfos29+S even have a small stem-loop directly downstream of the highlighted ARE in common (in the pictures on the right of the ARE). If the structure is a critical

factor in this system the binding of a different protein to cfos205 ARE and to the *c-fos* ARE structure in cfos45, cfos29+S+S and cfos29+S is a logical consequence. However, this cannot explain all the effects observed. The involvement in binding selectivity of ARE-BPs of neighbouring hairpins is very likely. Cfos45 has no additional stem-loops whereas the prediction of cfos29+S+S shows a large hairpin directly upstream of the ARE (hairpin B), a second one even further upstream (hairpin C) and a third one (hairpin D) downstream of the hairpin the three *c-fos* constructs have in common. This high number of additional hairpins most probably strongly influences the accessibility of the ARE itself, and thus can possibly modulate the binding of an ARE-BP or of an RNA-BP proximal to the ARE. Examining the hairpins of cfos29+S, it turns out that hairpin C is also present. This hairpin very likely does not play a role in the regulation in contrast to the newly predicted hairpin A of cfos29+S. This one possibly influences the assembly of the same protein complex to the ARE like in cfos29+S+S and resulting from that the formation of hairpin A seems to alter transcript stability and translation as well in an ARE-dependent manner.

In summary the results suggest a direct involvement of the RNA secondary structure flanking the ARE resulting in different effects on transcript stability and translation. These effects are most likely mediated by different ARE-BPs, whose binding is selected by the discovered secondary structure binding motifs. Focussing on the mRNA secondary structure as the only critical regulatory aspect, it must not be disregarded that there may also be observed influences due to the protein modification from construct cfos29+S+S to cfos29+S. In addition, the global structure of each special mRNA molecule may have an influence on its turnover or regulation. In order to rule out such influences attention in the next experiments was drawn to cDNA 29 reporter constructs.

### 5.2.2 Exchange of one nucleotide upstream of the *c-fos* ARE alters structure, mRNA and protein level of the pPICHcfos29+S+S reporter

In order to test if the predicted structures are really involved in ARE-BP selectivity and thus in the regulation of transcript stability and/or translation, a number of single-point mutations within the *Not*I restriction site of reporter pPICHcfos29+S+S was introduced. Changing nucleotides within this sequence does not change the protein primary structure as it is located in the 3' UTR (see fig. 14 for details). Interestingly, all three single-point mutations lead to a rapid downregulation of transcript stability

and as a consequence to a reduced protein expression. A direct correlation can be observed (fig. 24, A). An additional significant translational activation or repression cannot be detected (fig. 24, B), although mutation NotImutIII shows slight differences. The first mutation (NoImutI) leads to structural reorganization of the ARE secondary structure (fig. 25, B; highlighted), which is the same for cfos45 and cfos29+S. However, strikingly regulation differs, which further supports the involvement of neighbouring hairpins. In addition, the single-point mutation introduced results in the formation of hairpins C and A. This suggests that the existence of hairpin A supports down-regulation of transcript stability. In this context hairpin C can be excluded as it is present in different constructs like cfos29+S+S and cfos29+S. These constrcuts display regulatory differences. Consequently, these differences cannot be caused by hairpin C. Furthermore, when comparing both stem-loops to cfos29+S a change of the internal and external loops of hairpin A becomes obvious. In addition, this suggests that an increased size of hairpin A itself and/or the two loops can up-regulate translation and vice versa.

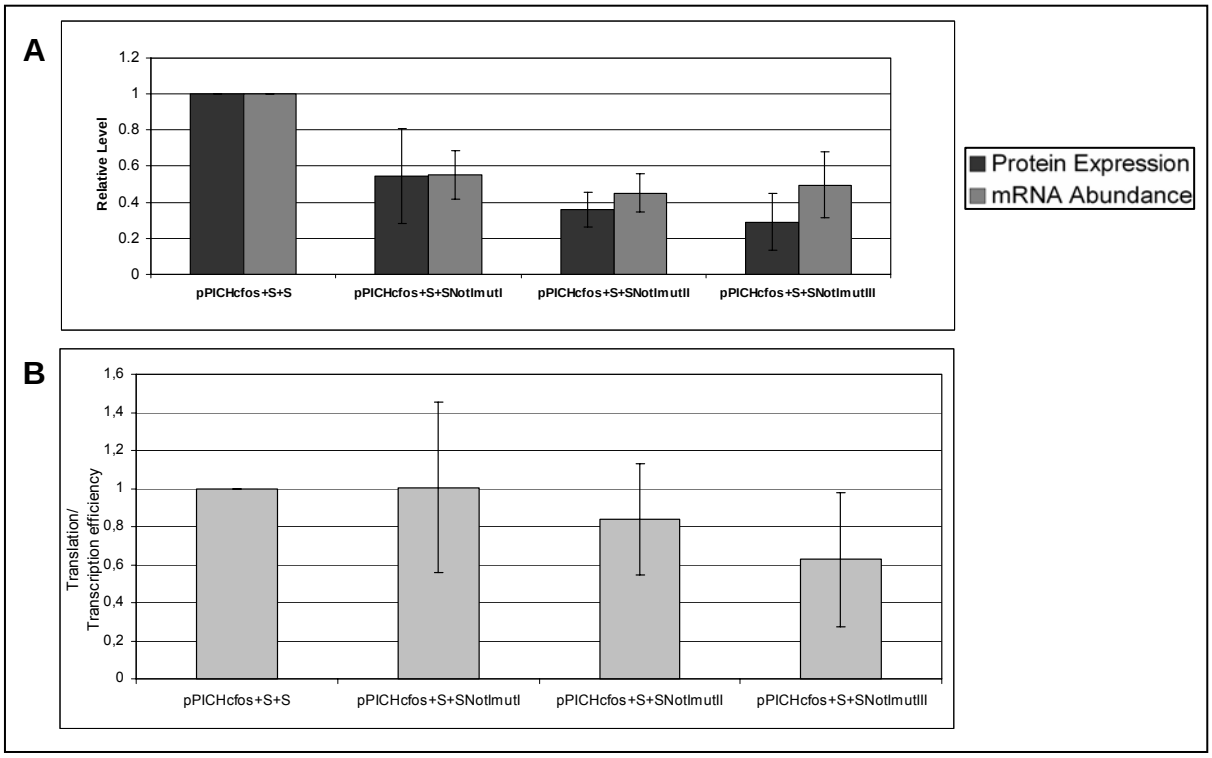


**Figure 24: Transcript stability and translational rates of cDNA 29 NotI-site mutants (A) and translational repression/activation triggered by introduced modifications (B).** All three single-point mutations lead to a rapid down-regulation of transcript stability an as a consequence to a reduced protein expression. A direct correlation can be observed. An additional significant translational activation or repression cannot be detected, although mutation NotImutIII shows slight differences.

This assumption is further supported by the introduction of the second mutation (NotImutII). Hairpins C and A are fused to a large stem-loop structure at the same location as hairpin A before. In this construct hairpin A is even larger than in mutant NotImutI. Most probably this specifically enlarged hairpin inhibits the binding of a RNA-BP that can repress translation as observed for construct cfos29+S. This perfectly agrees with the structure and the regulatory effects of cfos45. If a large hairpin A inhibits the formation or binding of a protein complex and a small one promotes binding at or near the ARE, this protein complex will not bind to the cfos45 ARE structure because hairpin A is not present. This means the same effect for a large hairpin A or its absence, leading to the inhibition of the binding of a translational repressor.

The third mutation (NotImutIII) does not reorganize the ARE structure when compared to the two previous mutations. In this case, the size of both loops of hairpin B is altered, particularly the size of the terminal loop. Interestingly, mRNA stability is decreased in the same range as in NotImutI and NotImutII. However, translation differs slightly from the rate of transcript stability. This data suggests that hairpin B plays a putative role in the *c-fos* ARE regulation. It is likely that the different structures lead to similar results, but are bound by different RNA-BPs. This completely agrees with the observations and explanations made for the *c-fos* ARE constructs in fig. 25, A.

It can be summarized that binding selectivity of RNA-BPs of the analysed *c-fos* ARE constructs can be directly related to the ARE or ARE-flanking structures. It is most likely that the cDNA 205 *c-fos* ARE is bound by a completely different protein or protein complex. The cDNA 45 and cDNA 29 reporter constructs exhibit different regulatory backgrounds, which can be traced back to hairpins A and B, whose existence and size is critical for the ARE-mediated effects.

### 5.2.3 The identified stem-loop (hairpin A) can influence translation of non-ARE, TNFα ARE and *c-fos* ARE-containing transcripts

Compared to the *c-fos* ARE structure the TNFα ARE forms a predicted hairpin with one or two internal and one terminal loop (fig. 25, C and D) in all constructs. However, different regulatory effects are observed. This also suggests neighbouring structures are in involved, as shown for the *c-fos* ARE. Interestingly, a particular

TNFα ARE-containing constructs of reporter cDNA 29 (tnf29+S) displays the predicted hairpin A that has already been shown to be essential for *c-fos* ARE regulation. Interestingly, in this TNFα construct hairpin A only has also an effect on translation. Additionally, the large (fused) terminal loop of hairpin A in tnf29+S+S does not lead to a significant translational repression (fig. 22, B), whereas the small terminal loop of hairpin A in tnf29+S does as could be observed for the *c-fos* ARE constructs. Nevertheless, it is noteworthy that the removal of the additional stop codon also changes the protein primary structure, as in the case of the cfos29+S reporter. It is much more likely that the structural mRNA motifs in this system play an essential role, however. This is supported by the structure predictions (the start of the 3' UTR is highlighted in green, there is no ARE present) of the non-ARE-containing constructs of reporter cDNA 29 (fig. 25, C), which also display hairpin A. In the case of cDNA 29-S-S, translation is almost completely repressed. This is an additional indication of what the function of hairpin A is. If it is small, translation is repressed in non-ARE-containing constructs, whereas a large hairpin A increases translation (cDNA 29+S+S). For the remaining two constructs tnf45 and tnf205 (fig. 25, D) a different type of binding is likely for the same reason. The neighbouring hairpins are completely different suggesting a different set of bound ARE-BPs.

To summarize the data, the identification of hairpin A has to be spotlighted. This stem-loop structure regulates translation of non-ARE and TNFα ARE-containing mRNAs depending on its size. A large hairpin A increases translation, whereas a small one leads to a decrease. The size of hairpin A is also a critical factor for the translational regulation of the cDNA 29cfos constructs. If hairpin A is present as in construct pPICHcfos+S (fig. 22, A and D5, A), translation is inhibited as well. Interestingly, *c-fos* ARE regulation is different, which is demonstrated by an influence of hairpin B on transcript stability.

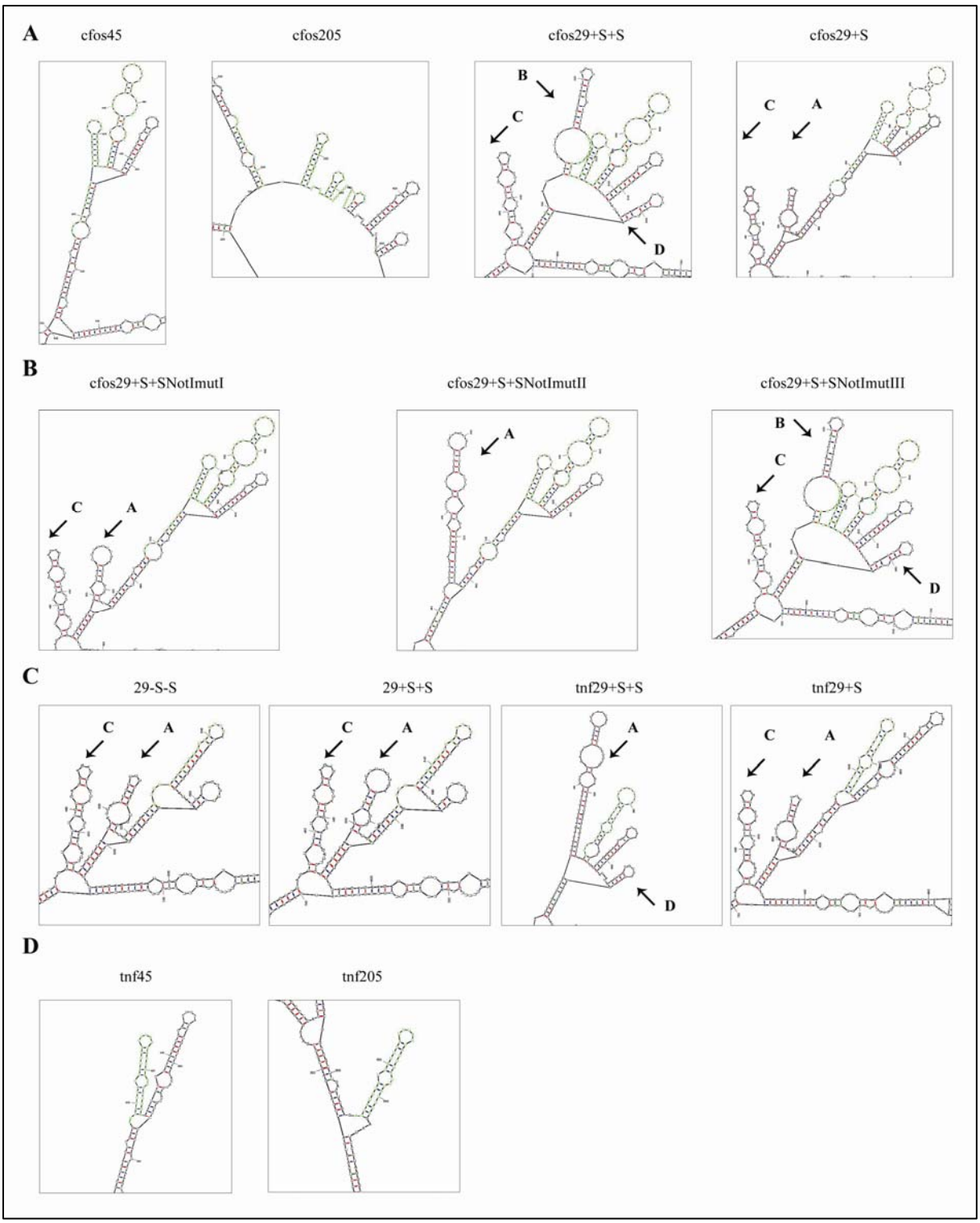


**Figure 25: Sections of the mRNA secondary structure predictions of analysed constructs.** The *c-fos* ARE (highlighted in green) of reporter constructs cDNA 29+S+S, cDNA 29+S, 45 and 205 show four different structures **(A)** resulting in four different regulatory effects. Structure predictions of the *c-fos* ARE (highlighted in green) in the pPICHcfosNotImut constructs of cDNA 29+S+S **(B)** lead to the identification of hairpin A, whose presence and size is involved in translation and transcript stability. The importance of hairpin A and its different effects on non-ARE, TNF$\alpha$ and *c-fos* ARE-containing reporters is supported by the corresponding structure predictions **(C and D)**. mRNA secondary structure predictions were calculated with mFold v2.3 (Zuker, 2003). Complete structure predictions can be found in the appendix, figures 5 to 17.

### 5.2.4 ARE-flanking sequences and structures play a role in ARE-mediated regulation of transcript stablity and translation

Currently literature discusses that ARE-flanking sequences can have an influence on the ARE-based regulation itself. ARE-flanking sequences are suspected to be proximal target sites for ARE-BPs or its interaction partners (Wilson et al., 2001). Experiments in *S. cerevisiae* also suggest that the surrounding context or secondary structure is important for the recognition of the ARE of MFA2 by regulatory factors (Duttagupta et al., 2003). Stem-loop structures downstream (Paschoud et al., 2006) or within an ARE (Chabanon et al., 2005; Fialcowitz et al., 2005) have already been identified and shown to be important for ARE-regulation.

Evidence is provided in this thesis which supports the involvement of ARE-flanking sequences. Data obtained shows that single-point mutations within the 3' UTR directly upstream of the *c-fos* ARE (flanking sequence) play a crucial role in the examined system. Furthermore, mRNA secondary structure within or nearby the AREs is the key to understanding the different regulation. It could be demonstrated that surrounding hairpins, whose size and existence is dependent on the sequence, are involved in transcript stability and translation.

It can be summarized that the observed effects and most likely as a consequence the binding selectivity of ARE binding proteins of the analysed *c-fos* ARE constructs can be directly related to the ARE or flanking ARE structures. It is most likely that the cDNA 205 *c-fos* ARE is bound by a different protein or protein complex than the *c-fos* ARE construct of cDNA 29 and cDNA 45. This makes sense, as the structure is completely different and parts of the ARE sequence is unpaired. RNA binding proteins have different binding motifs, the best known of which are the RNA recognition motif (RRM) (Maris et al., 2005) and the double-stranded RNA binding motif (dsRBM) (Chang and Ramos, 2005). RRMs are often found in multiple copies in eukaryotic proteins and/or together with additional domains and can bind single stranded as well as double stranded RNA molecules. The human HuR ARE-BP has three RRMs, no additional ARE binding domains and can stabilize *c-fos* ARE-containing host mRNAs (Peng et al., 1998). The most abundant additional domains are the zinc fingers of the CCCH (Cys-Cys-Cys-His) and CCHC (Cys-Cys-His-Cys) type. While the well known CCHHs bind double-stranded DNA or RNA, the CCCH and CCHC domains bind single stranded RNA. Well studied CCCH domain containing ARE-BPs are the mammalian Tristetraprolin (TTP, *zfp*-36) and AUF1, both

involved in the degradation of *c-fos* and TNFα ARE host mRNAs (Barreau et al., 2005). This fact demonstrates the complexity of the ARE-mediated regulation and the overlapping specificity of ARE-BPs. Since the primary sequence of an existing RNA helix is an unlikely feature for binding specificity, the recognition of flanking non-helical structures by a RNA-BP within the duplex RNA have been suggested as such a feature (Chang and Ramos, 2005). This is in agreement with the results of the performed experiments and predicted mRNA secondary structures. Concerning theses structures, hairpins A and B play a crucial role. Hairpin A is present in the non-ARE-containing constructs of reporter cDNA 29. A small size, thereby meaning the terminal loop, results in a drastic translational repression compared to the large size. This suggests that the large loop promotes translation. The functionality of hairpin A in the TNFα ARE-containing reporter constructs of cDNA 29 is the same. The presence of a small hairpin A results in a strong translational repression which can be abolished by a large (fused) hairpin A. This fact is strongly supported by the data of the *c-fos* ARE reporter constructs. The highly complex hairpin structure of construct pPICHcfos29+S+S is an exceptional case. This structure does not lead to down-regulation of transcript stability or translation compared to the non-ARE construct. Thus, it can be concluded that the structure is a binding motif for a stabilizing ARE-BP. This clearly demonstrates a direct correlation between the ARE-mediated regulation of transcript stability and hairpin B. In contrast, creation of a small sized hairpin A in this construct leads to a moderate decrease of the mRNA stability and a rapid inhibition of translation (pPICHcfos29+S). Interestingly, a small hairpin A also triggers strong translational inhibition in the pPICHtnf29+S construct. However, it cannot be said if hairpin A mediates a direct or indirect (independent) effect. It seems to be an ARE-independent effect as hairpin A is also predicted for non-ARE constructs and its translational repressing activity can be observed for such constructs.Further studies are necessary to investigate the connection between these effects. However, the decreased mRNA stability underlines the different regulation of the *c-fos* and TNFα AREs. Although the presence of hairpin A in construct pPICHcfos29+S results in a lowered mRNA stability it cannot be completely excluded that the protein modification plays a role in this context. Nevertheless, this is pretty unlikely as other modifications such as addition or removal of the Strep-II-tag can be handled like controls, which do not change the mRNA level. An increased size of hairpin A (pPICHcfos29+S+SNotImutI/II) abolishes translational repression. It is likely

that a large hairpin A inhibits the formation or binding of a protein complex and a small hairpin A promotes binding at or near the ARE. This protein complex cannot bind to the cfos45 ARE structure because hairpin A is not present. This explains why the translational inhibition can be observed for pPICHcfos29+S and not for pPICHcfos29+S+SNotImutI/II and pPICHcfos45.

Importantly, it is striking that different reporters display different types of regulation in our system. The involvement of ARE-flanking sequences or structures, respectively, from the applied reporters are likely to influence the ARE-mRNA turnover, thereby affecting the selectivity of RNA-BPs. This could explain the binding of different ARE-BPs to the same ARE described in the literature. For example, *in vitro* studies showed that recombinant and purified AUF1 can bind the TNF$\alpha$-ARE-RNA (Wilson et al., 1999), whereas the same ARE is bound by TTP when cloned in the 3' UTR of a mammalian reporter construct (Lai et al., 2002). There are several (heterologous) systems available for analysis of AREs and described in the literature. Examples are mammalian GFP reporter (Raineri et al., 2004), $\beta$-globin reporter (Peng et al., 1998) and Luciferase/Renilla reporter systems (Barreau et al., 2006) as well as *S. cerevisiae* MFA2 reporter (Vasudevan and Peltz, 2001). With the background of this thesis it can be hypothesized that the application of such reporter systems can identifiy ARE-BPs and give an idea of how regulation can work in fact, but regulation of a wild type ARE mRNA might differ significantly. This strongly suggests the analysis of wild type ARE-regulation transcripts in order to determine their real regulation.

Finally it can be summarized that a functional ARE network that controls transcription and translation as it is known from other eukaryotes could be demonstrated in the yeast *P. pastoris* for the first time. However, although a slight mRNA stabilizing effect of the *c-fos* ARE can be observed in construct pPICHcfos29+S+S, it is evident that AREs are not suitable for mRNA stabilization or increasing protein expression in general. For this purpose it is likely that the application of a different 3' UTR, instead of the *AOX*1 3' UTR, would lead to a stabilization of the transcripts and probably to an increased protein expression. This project further shows the direct involvement of ARE-flanking structures in the turnover of its host transcript.

### 5.2.5 Identified stem-loops play a crucial role in ARE-mediated regulation – a model

One crucial finding in the presented work is the involvement of ARE-flanking sequences in ARE-mediated regulation of transcript stability and translation. In addition, all gathered data about these two processes in *P. pastoris* suggest ARE- and flanking structures play an essential role. In a broader sense, flanking sequences also include the coding sequence of the chosen reporter cDNAs. However, RNA binding motifs have not been described in the literature for the three analysed cDNAs. Furthermore their cellular function does not suggest the necessity of that kind of regulation. CDNA 29 encodes a DNA damage inducible protein (GADD45), whereas cDNA 45 encodes a SH3 domain binding glutamatic acid-rich-like protein and cDNA 205 the sorting nexin SNX15. Similar transcripts, proteins or family members have an ARE independent regulation. It is concluded that the structure of the mRNA itself is the sole factor for selectivity of RNA-BPs and consequently influences transcript stability and translation in the examined yeast system.

Setting the focus on the identified hairpin A, a model can be proposed that shows the relation between hairpin A and the effects on regulation (fig. 26). Firstly, this model describes the binding of ARE-BP I to the core sequence of the *c-fos* ARE. This interaction is likely as the target ARE structure remains the same while the surrounding context changes. Furthermore this explains the basic destabilizing function of AREs. Secondly, the surrounding context can be a factor for selectivity of RNA-BPs, possible modulator proteins. One example could be PSBP (proximal stabilizing binding protein) which binds to a hairpin directly upstream of the ARE. The activity of PSBP abolishes the destabilizing function of ARE-BP I and consequently leads to the stabilization of the ARE-containing transcript. It cannot be predicted if there is a direct interaction between PSBP and ARE-BP I and which effects such an interaction might have. However, the interaction which results in an inhibition of ARE-BP I binding motifs for mRNA degrading enzymes is likely. If the target site of PSBP is altered (change of size of internal and terminal loops), its binding is inhibited and the ARE-containing transcript is rapidly degraded due to the destabilizing function of ARE-BP I. If the flanking sequence/structure is changed and a small-sized hairpin A is present it is recognized by TLR (translational repressor). This protein has a negative influence on translation. Thus, an additive effect of TLR and ARE-BP I can be observed. Both direct and indirect interaction of TLR and ARE-BP I cannot be

excluded. If the size of hairpin A is increased or if hairpin A is not present, TLR cannot bind to its target and its translation repressing activity can no longer be detected. The ARE-containing transcript is rapidly degraded due to the binding of ARE-BP I. Furthermore it is likely that TLR also binds the non-ARE and TNFα ARE-containing transcript exhibiting a small-sized hairpin A and consistently does not bind a large hairpin A.

It has to be added that AREs have been implicated with the miRNA pathway and the involvement of miRNA16 in the degradation of a TNFα-ARE-bearing reporter is known (see RNAi – Basics and Applications). Although translational repression can observed due to structural reorganization and different (hairpin A), partially single-stranded RNA strands (tnf/cfos205 constructs) and a certain miRNA could thus gain access its target sequence, till date nothing is known about a RNAi pathway in *P. pastoris.* Even a proof of miRNA interaction with these special sequences would not provide evidence for a direct connection to the ARE-mediated posttranscriptional regulation. It has been reported that target site accessibility (Kertesz et al., 2007) and an AU-rich nucleotide composition near the miRNA target site can be a criterion for efficient silencing (Grimson et al., 2007). However, an involvement of hypothetic miRNAs in the examined yeast system is questionable. In other words an involvement of miRNAs can only be conjectured or speculated about. An involvement of ARE-BPs which inhibit the translational machinery is much more likely and is in large agreement with published data.

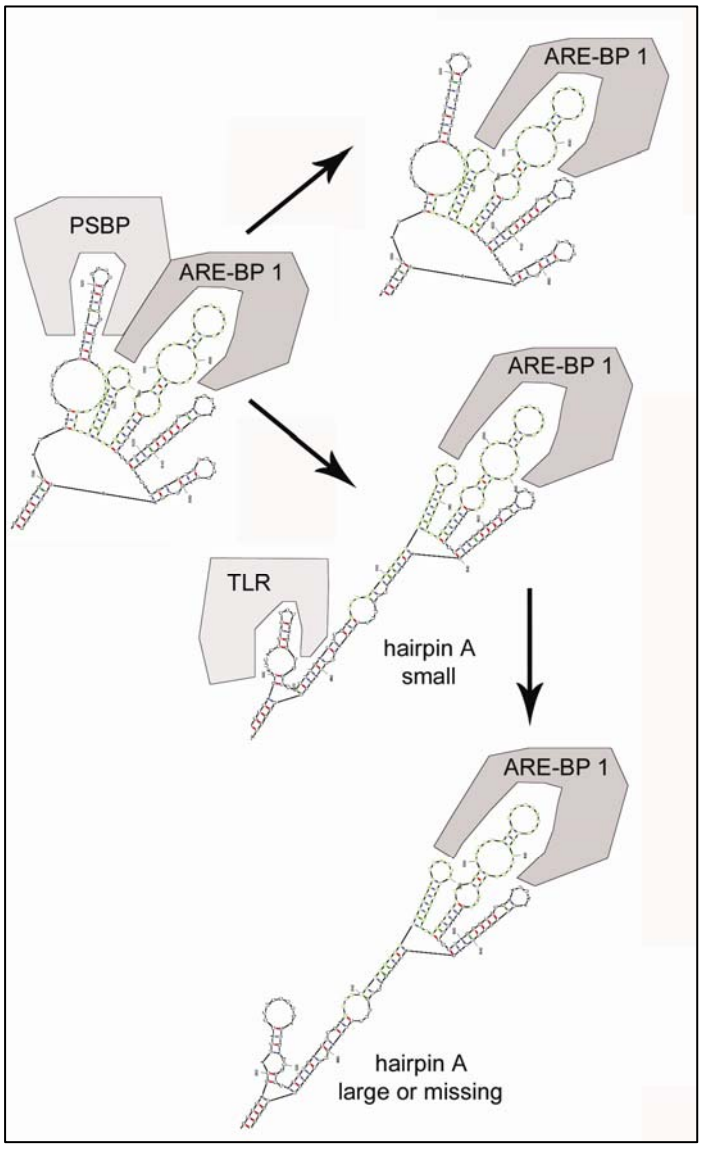


**Figure 26: A schematic model of the *c-fos* ARE regulation in *P. pastoris*.** The core sequence of the ARE is always bound by ARE-BP I, which destabilizes the ARE-containing transcript. Its effect can be abolished by PSBP (proximal stabilizing binding protein). If the structure of the PSBP target is altered the transcript is rapidly degraded. A small size hairpin A is the target for TLR (translational repressor) in the *c-fos* and non-ARE constructs. Additive effects of ARE-BP I and TLR can be observed. An increased size of hairpin A inhibits binding of TLR (same for a missing hairpin A), thus the additive effect can no longer be observed.

It is important to notice that all *c-fos* ARE-containing mRNAs show two distinct bands on a Northern Blot which differ in size by approximately 200 nt. This suggests that the *c-fos* ARE triggers polyadenylation directly within the ARE sequence, or nearby downstream. Due to the recognized effects, it is likely that the *c-fos* ARE remains untouched in the transcript, otherwise no stabilizing or destabilizing effects could be observed. Conclusively, polyadenylation is likely to occur downstream of the *c-fos* ARE. This poly(A)site is not recognized in 100% of all cases and thus quiet inefficient

as a second AU rich region approximately 200 nt downstream can be found in the transcript, it can be assumed that this sequence represents the original poly(A) site.
In order to analyse the found complex ARE regulation in *P. pastoris* UV-crosslinking assays were performed as a first step to identify ARE-BPs. The cloned sequences of the *c-fos* and TNFα AREs were used as target mRNAs for this purpose (fig. 6). These experiments demonstrated the specific binding of a 14 KDa to both transcripts. It is possible that either one single protein binds both RNAs or that two proteins of similar size are detected. The TNFα signal always seems to run slightly higher than the 14.3 kDa marker band, whereas the *c-fos* signal always seems to run a bit lower. The untreated *c-fos* ARE sample (fig. 19, B) shows a broad smear, which suggests the formation of internal hybridization products. In order to avoid this issue, denaturing of the transcript prior to the binding reaction could avoid this problem. It seems as if secondary structures persist in the presence of a low amount of crude cell extract (fig. 19, B, lanes 2, 3, 5 and 6). This suggests a helicase activity in the extract making the RNA accessible at higher amounts of the cell extract (fig. 19, B, lanes 4 and 7) to the RNase A treatment after cross-linking. Consequently only the bound protein species can be detected in these samples. In agreement with the TNFα gel retardation assay (fig. 19, A, lane 7) it is possible that proteinase K activity was not sufficient to digest 50 µg total protein resulting in the same detectable band at approximately 14 kDa as can be observed in the sample without proteinase K treatment (fig. 19, B, lanes 4 and 7). However the signal is weakened by treatment with RNAse A.
Until sequence data is available for this ARE-BP comparison to already known ARE-BPs can only be made by size. A comparison based on the size of the protein only does not lead to the identification of a possible homologue. ARE-BPs like AUF1, HuR and TTP are approximately 20 kDa to large in size. Purification and following sequencing will lead to a characterization of the found ARE-BP.

# 6 Outlook

To define the role of the identified 14 kDa ARE-BP *in vivo* will be an interesting step further. Therefore the protein has to be purified through affinity binding and sequenced. For this purpose protein species with RNA binding activity will have to be purified from a total protein extract. In the first step the crude total protein extract is subjected to commercially available heparin sepharose. In the second step the sample is subjected to DEAE-Sepharose. Binding is dependent on the buffer pH. If the protein has positive charge it will be in the flow through, while a negative charge makes it bind to the resin. The target transcript can be bound to commercially available adipic acid dihydrazide-agarose beads (Sigma) (Caputi et al., 1999). In the last step the purified extract is incubated with the RNA beads. Samples are finally separated on a denaturing SDS gel. The purified protein can be visualized with protein staining like Coomassie blue or silver staining. Protein sequencing by mass spectrometry will identify the primary structure of the protein, lead to the identification of homologues in other organisms and give information of the protein's characteristics. For *in vivo* analyses the construction of a *P. pastoris* deletion or over-expression mutant is interesting to further characterize the identified ARE system in detail.

Furthermore it will be interesting to transfer knowledge of translation inhibiting structures to other expression systems in order to increase or modify protein yields. If significant differences between mRNA abundance and protein expression level can be detected, it is possible to change local secondary structures in the 3' UTR without affecting the protein.

# 7 Summary

The tight regulation of mRNA stability and translation by specific *cis*-acting sequences and *trans*-acting factors is an essential means for the control of gene expression. These processes allow cells to rapidly adjust the expression pattern of regulatory factors and response transiently to internal and external signals including cell proliferation, signal transduction, inflammatory stimuli and radiation. A well known important *cis*-acting sequence element that controls mRNA stability is the AU-rich element (ARE) found in the 3' untranslated region (3'UTR) of many, but not all, unstable mRNAs of various growth factors, cytokines, proto-oncogenes and transcription factors.

A heterologous system for the analysis of AREs in the yeast *Pichia pastoris* was established. The AREs from the human proto-oncogene *c-fos* and the cytokine TNFα were separately cloned into the 3' UTR of *P. pastoris* expression vectors. It was demonstrated that both AREs are functional in this yeast and that they are regulated in different ways. This finding further stresses that the ARE-regulated system is conserved among eukaryotes and it underlines the importance of the system in the cellular context. A set of reporter constructs and mutational analysis were used to, analyse the importance of two special hairpins, which we hypothesize to have significant influence on transcript stability and translation. Thus, evidence is provided for the involvement of ARE-flanking sequences in ARE-mediated mRNA turnover and most likely in the selectivity of RNA-binding proteins. Finally, a model is proposed that explains the observed effects on protein and mRNA level in a RNA-structure-dependent manner. In order to find further support for the findings and to identify ARE-BPs gel retardation assays were performed. As a result an approximately 14 kDa protein could be identified as a binding partner of the core *c-fos* and TNFα AREs in *Pichia*.

# 8 Zusammenfassung

Die strenge Regulation von mRNA Stabilität und Translation durch spezifische "*cis*-acting"-Sequenzen und „*trans*-acting"-Faktoren ist für die Kontrolle der Genexpression ein essentielles Mittel. Diese Prozesse erlauben es der Zelle, das Expressionsmuster von regulatorischen Faktoren als transiente Antwort auf interne oder externe Signale wie Zellproliferation, Signaltransduktion, inflammatorische Stimuli oder (UV)-Strahlung einzustellen. Eine heute gut untersuchte „*cis*-acting"-Sequenz, die die mRNA-Stabilität kontrolliert, ist das „AU reiche Element" oder „AU rich element" (ARE), das in der 3' untranslatierten Region (3' UTR) von vielen, aber nicht allen, instabilen mRNAs von verschiedenen Wachstumsfaktoren, Cytokinen, Protooncogenen und Transkriptionsfakotren zu finden ist.

Es wurde ein heterologes System für die Analyse von AREs in der Hefe *P. pastoris* etabiliert. Die zwei AREs des humanen Protoonkogen *c-fos* und des Cytokins TNF$\alpha$ wurden in die 3' UTR von *P. pastoris* Expressionsvektoren kloniert. Es wurde gezeigt, dass beide AREs in dieser Hefe Funktionalität besitzen und verschiedenartig reguliert werden. Dieses Ergebnis betont zusätzlich die Konservierung des ARE-regulierten Systems unter Eukaryonten und unterstreicht seine Wichtigkeit im zellulären Zusammenhang. Eine Auswahl von verschiedenen Reporterkonstrukten sowie Mutationsanalysen zeigten die Wichtigkeit zweier spezieller mRNA Hairpins, die einen signifikanten Einfluss auf Transkriptstabilität und Translation haben können. Damit konnte die Involvierung von ARE-flankierenden Sequenzen und Strukturen bei dem ARE-regulierten mRNA-Umsatz und sehr wahrscheinlich bei der Selektion von ARE-Bindungsproteinen (ARE-BP). Schließlich konnte ein Modell aufgestellt werden, das die beobachteten Effekte abhängig von der mRNA Sekundärstruktur erklärt. Um weitere für dieses Modell unterstützende Daten zu sammeln und um endogene ARE-BPs zu identifizieren, wurden Gelretardationsassays durchgeführt. Es konnte ein ca. 14 kDa großes Protein als Bindungspartner für die *c-fos* als auch TNF$\alpha$ ARE Sequenzen identifizert werden.

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# 10 Appendix

**Figure 1: The *Pichia* expression vector pPICHS (Boettner et al., 2002)**

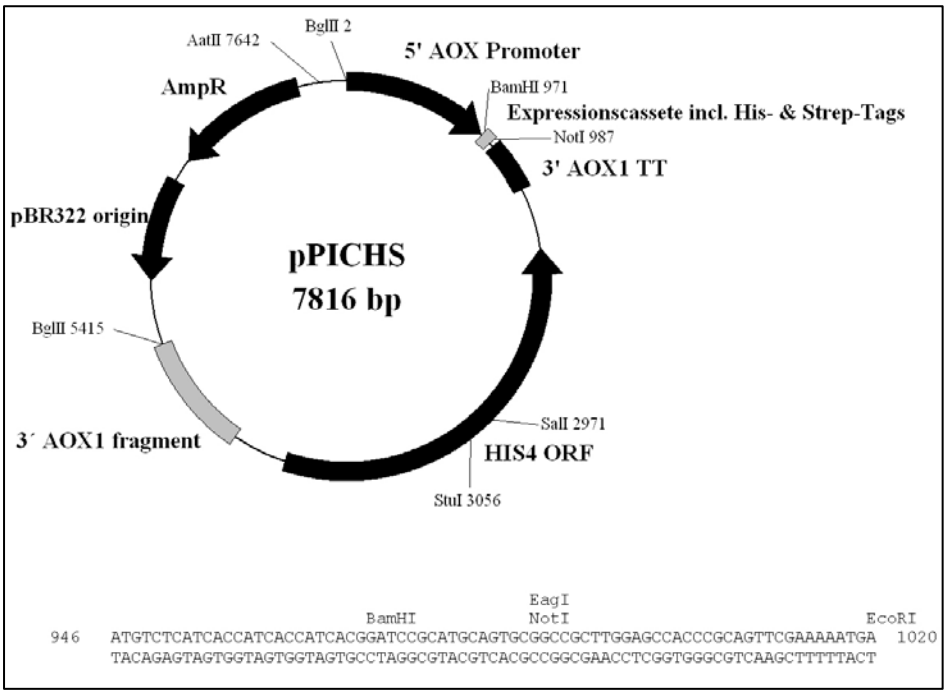



**Figure 2: The *Pichia* expression vector pPICH**

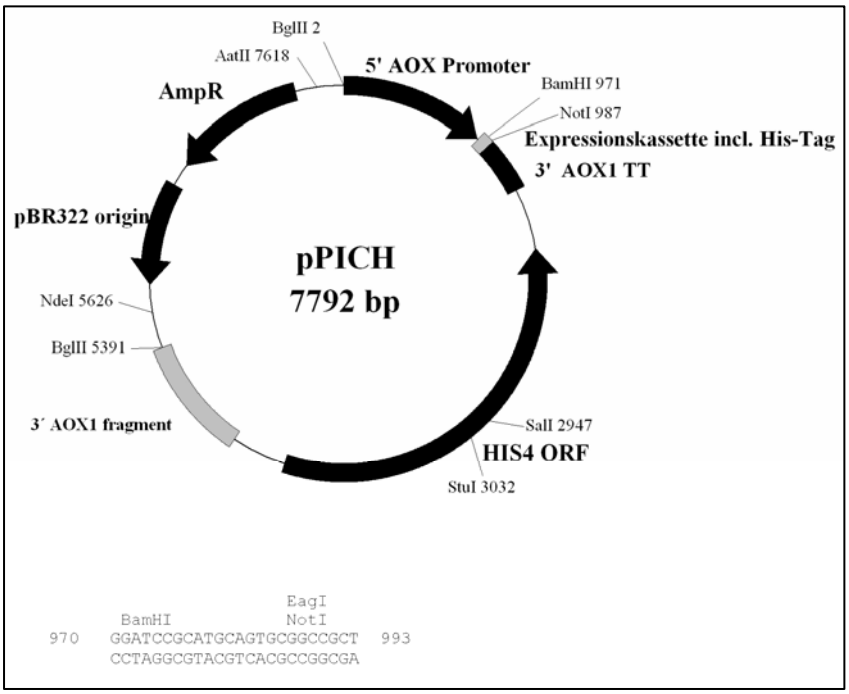


**Figure 3: The *Pichia* expression vector pPICHcfos**

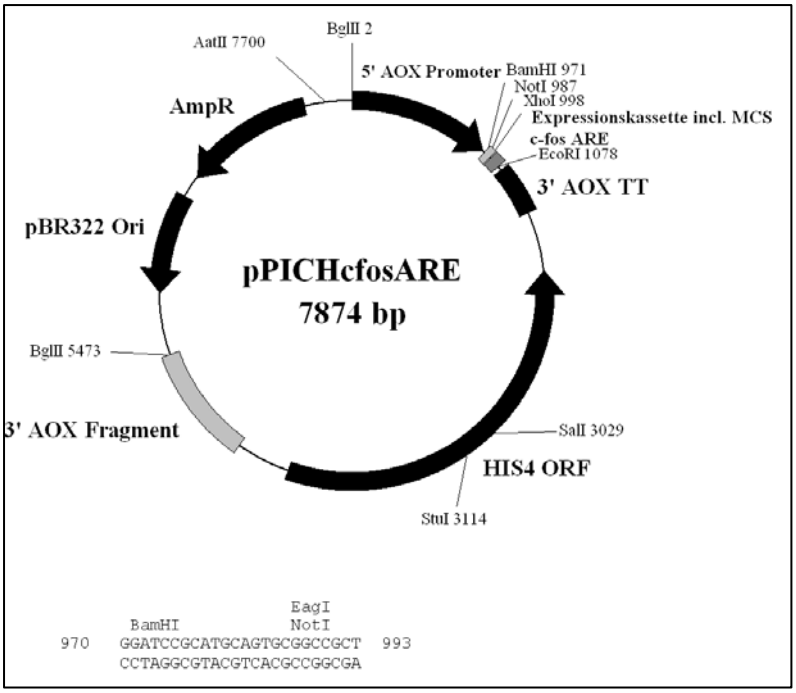



**Figure 4: The *Pichia* expression vector pPICHtnf**

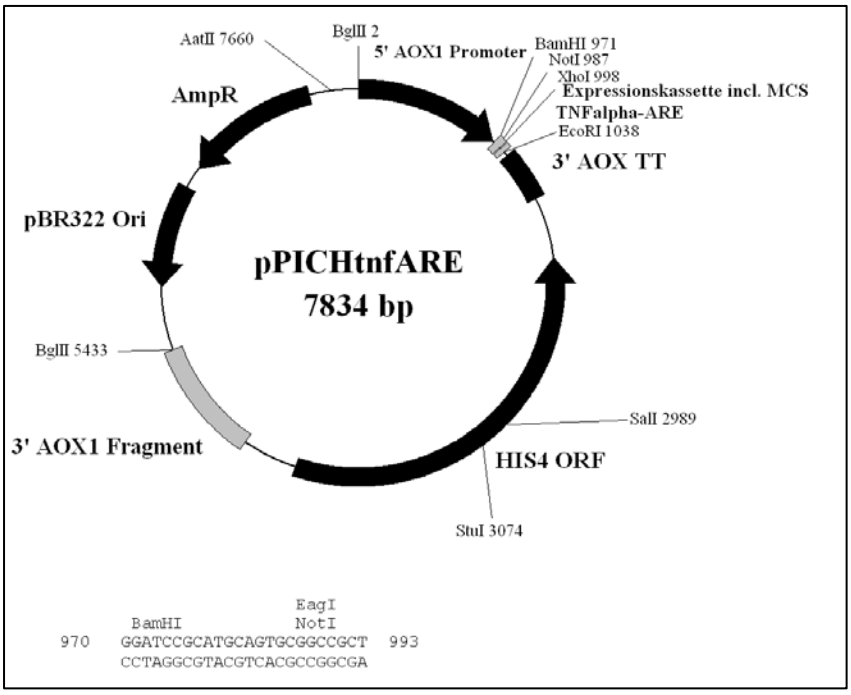


**Figure 5: mFold Structure Prediction of pPICHcfos45**

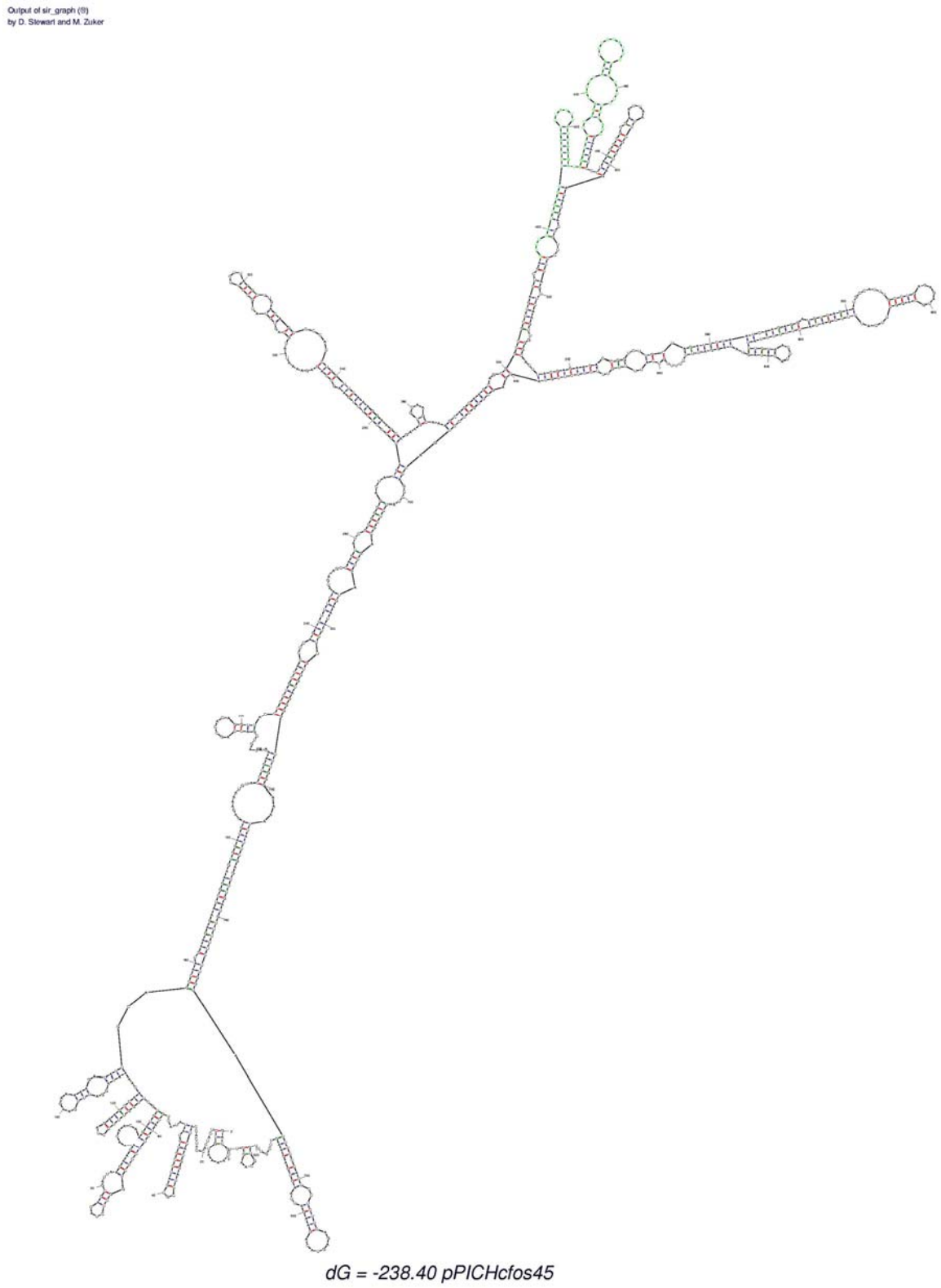
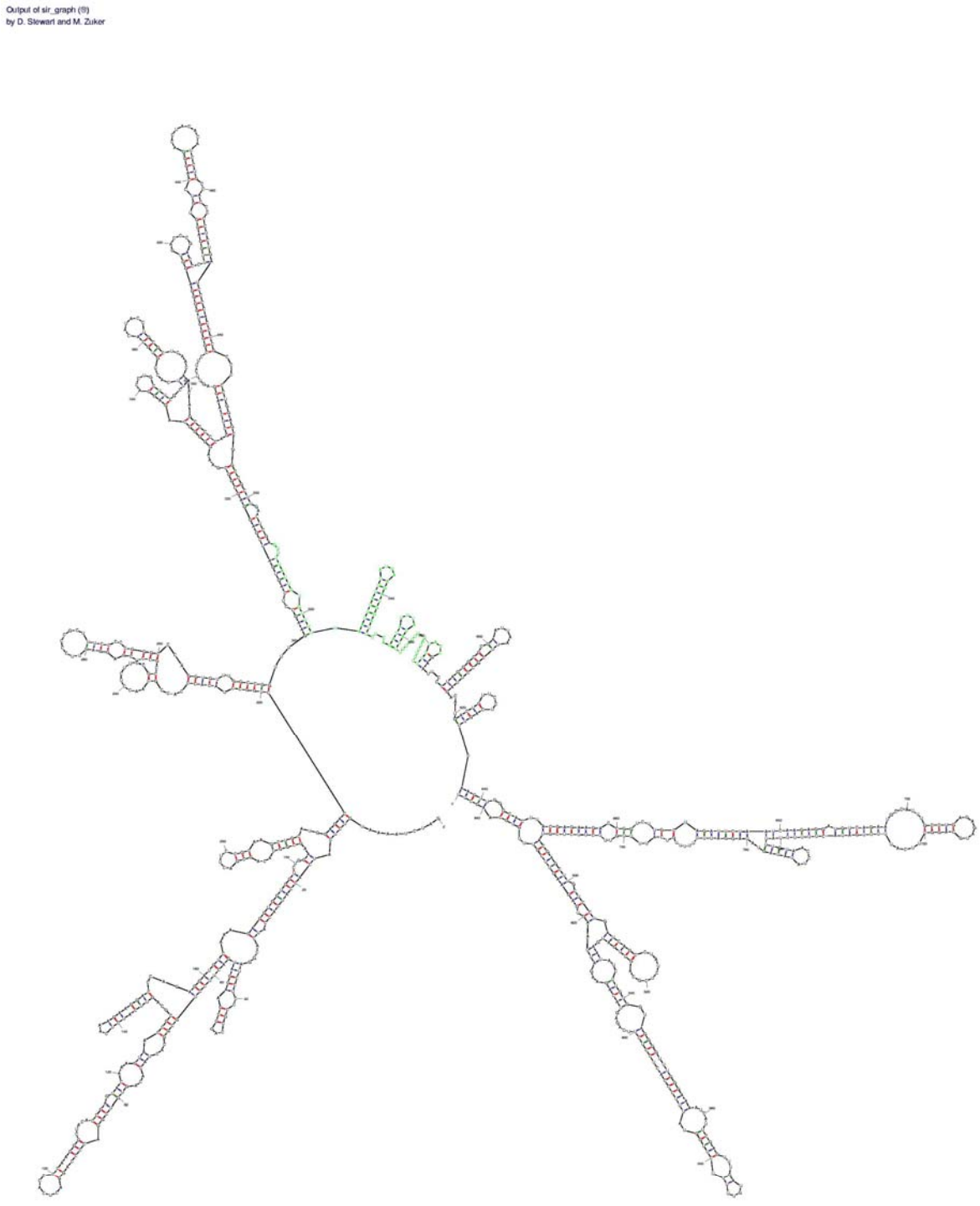
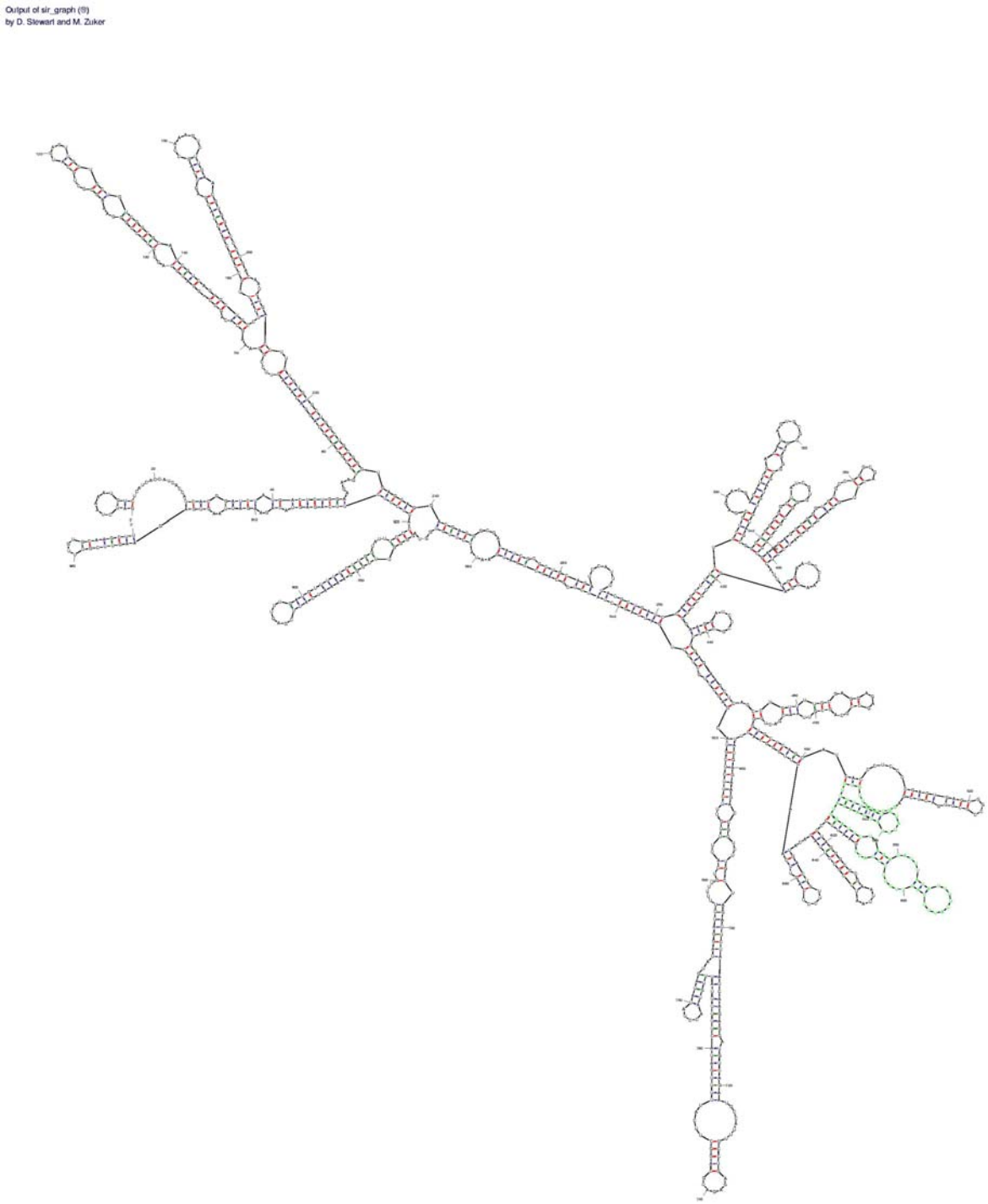


**Figure 6: mFold Structure Prediction of pPICHcfos205**



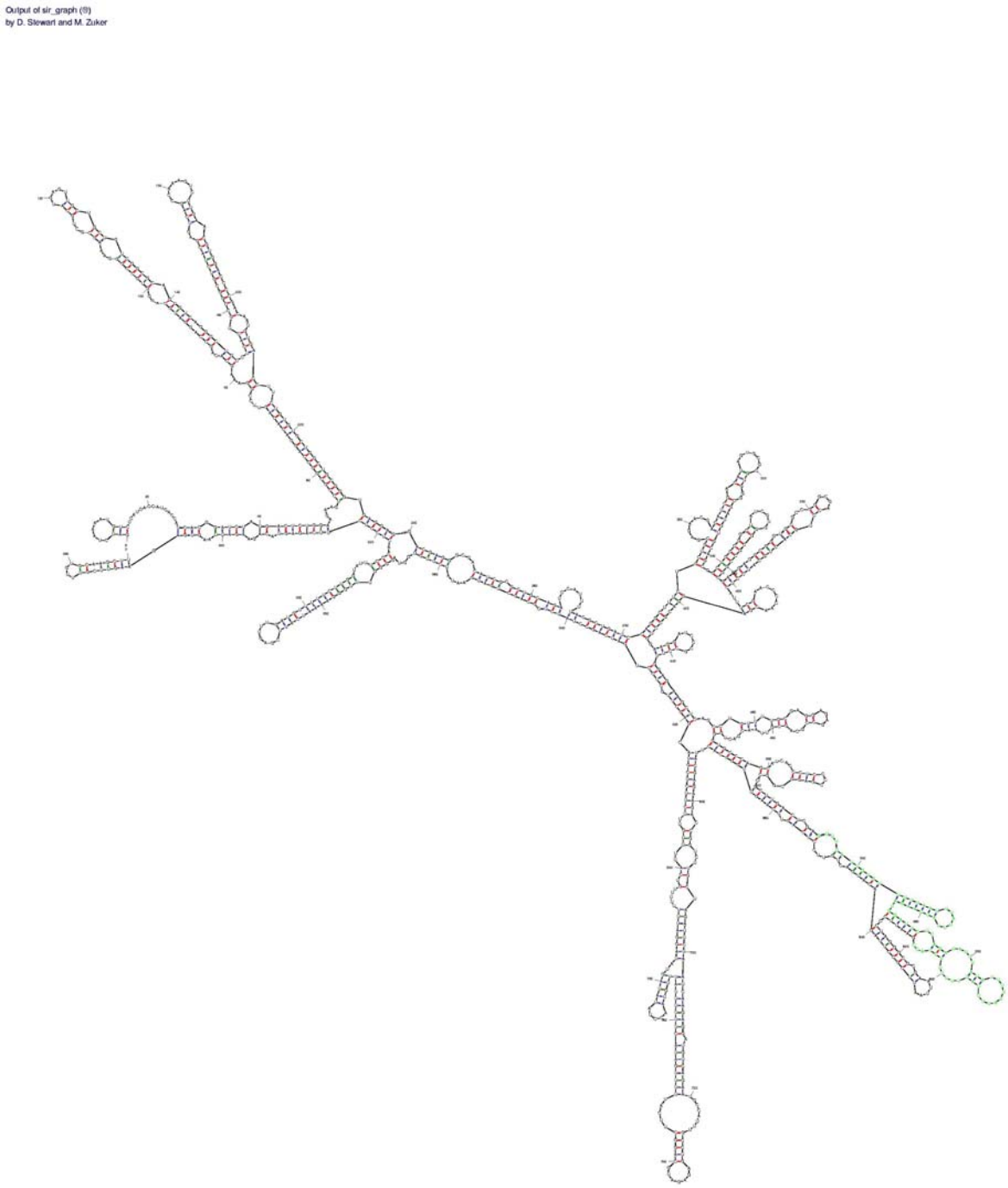

*dG = -313.10 pPICHcfos205*

**Figure 7: mFold Structure Prediction of pPICHcfos29+S+S**



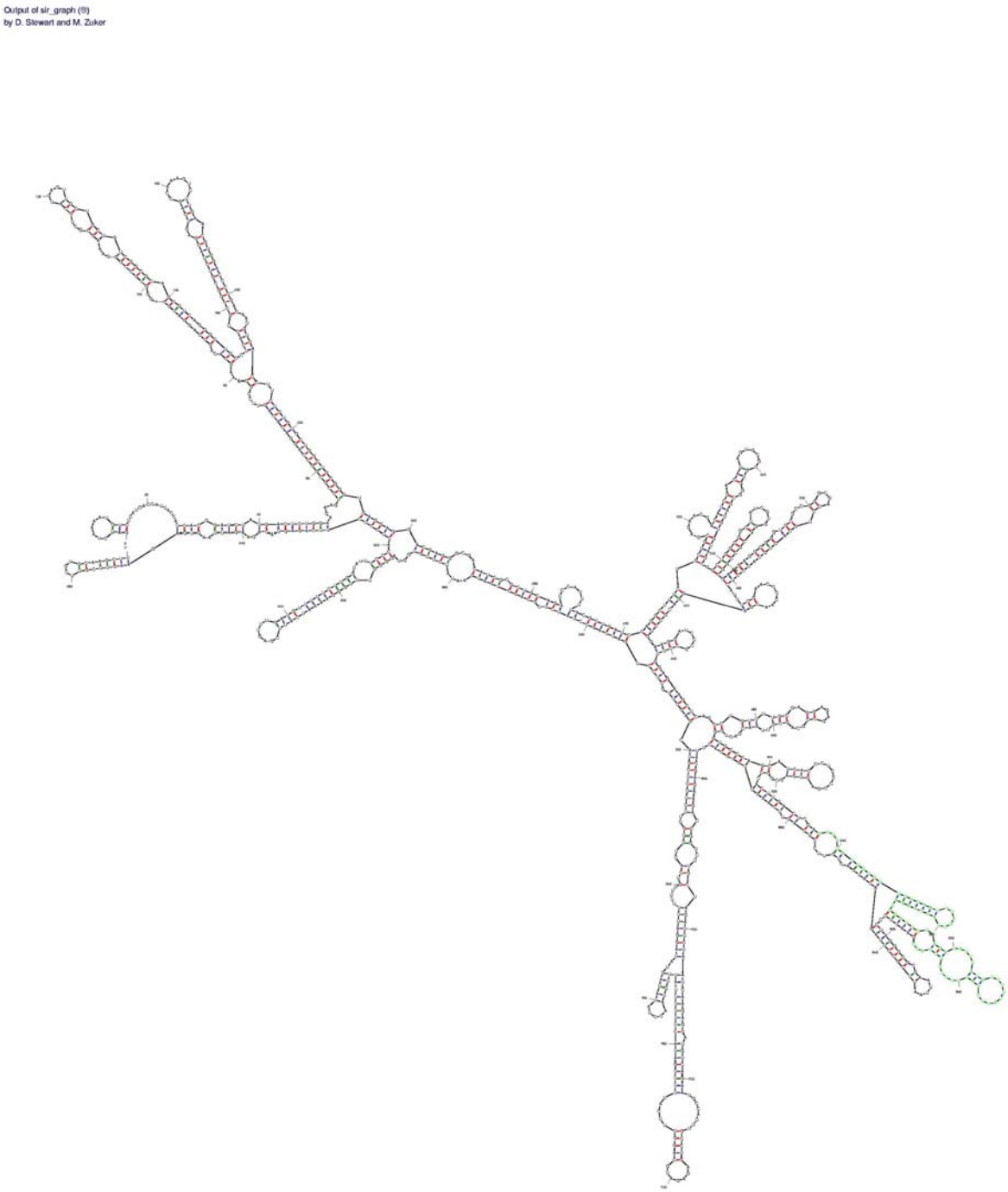

*dG = -361.20 pPICHcfos29+S+S*

**Figure 8: mFold Structure Prediction of pPICHcfos29+S**



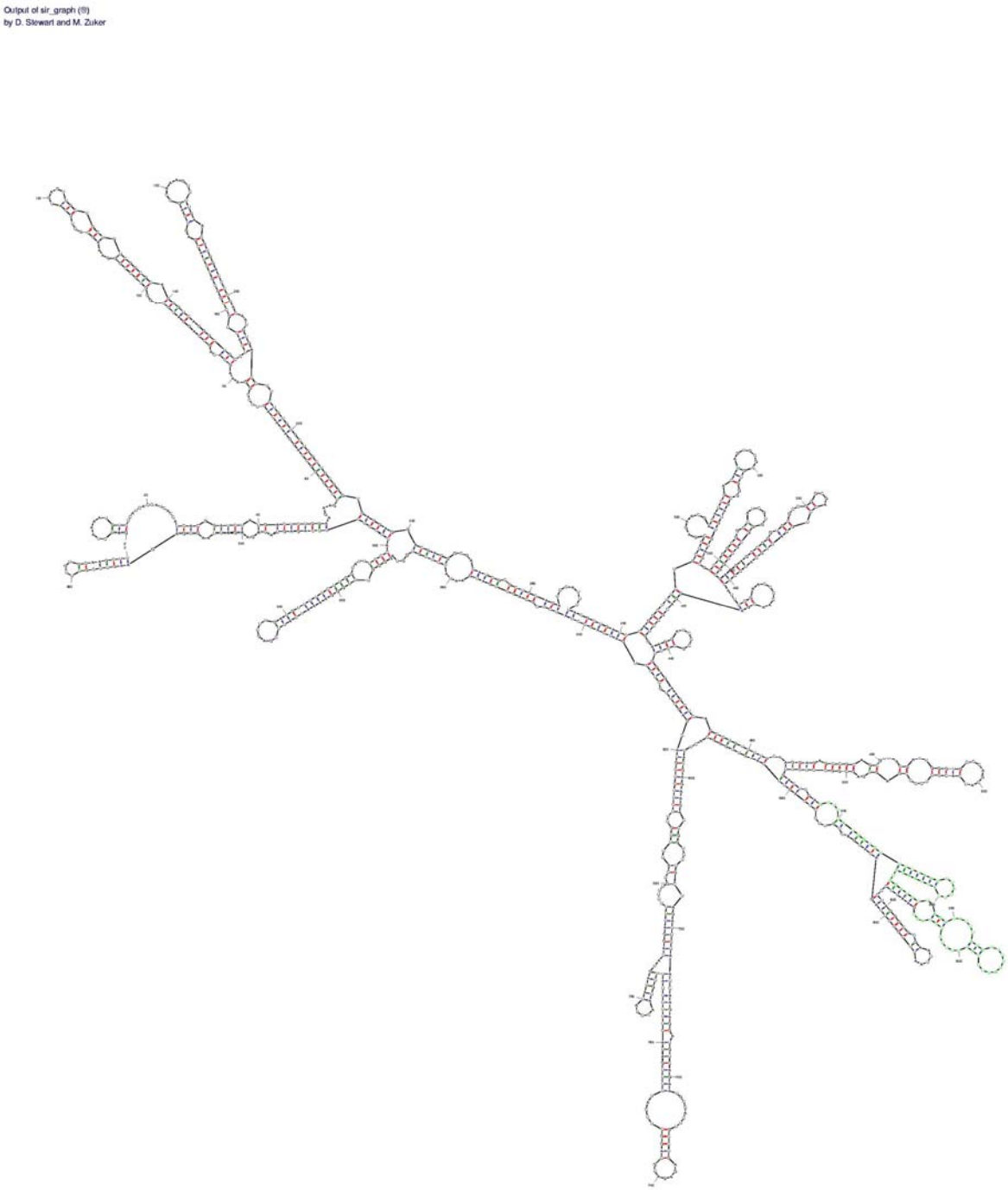

*dG = -359.60 pPICHcfos29+S*

**Figure 9: mFold Structure Prediction of pPICHcfos29+S+SNotImutI**



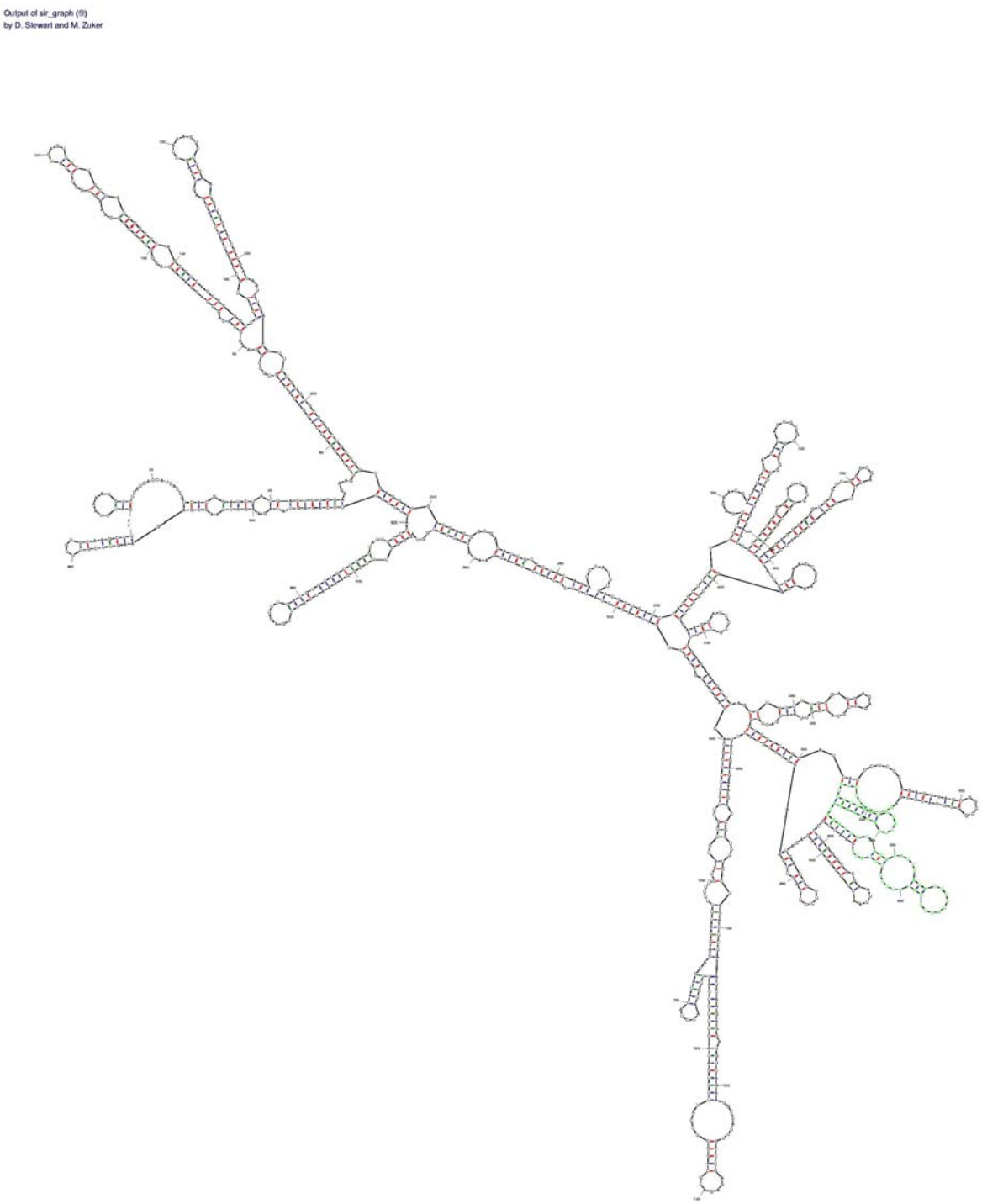

*dG = -360.70 pPICHcfosARE29+S+SDNotI*

**Figure 10: mFold Structure Prediction of pPICHcfos29+S+SNotImutII**



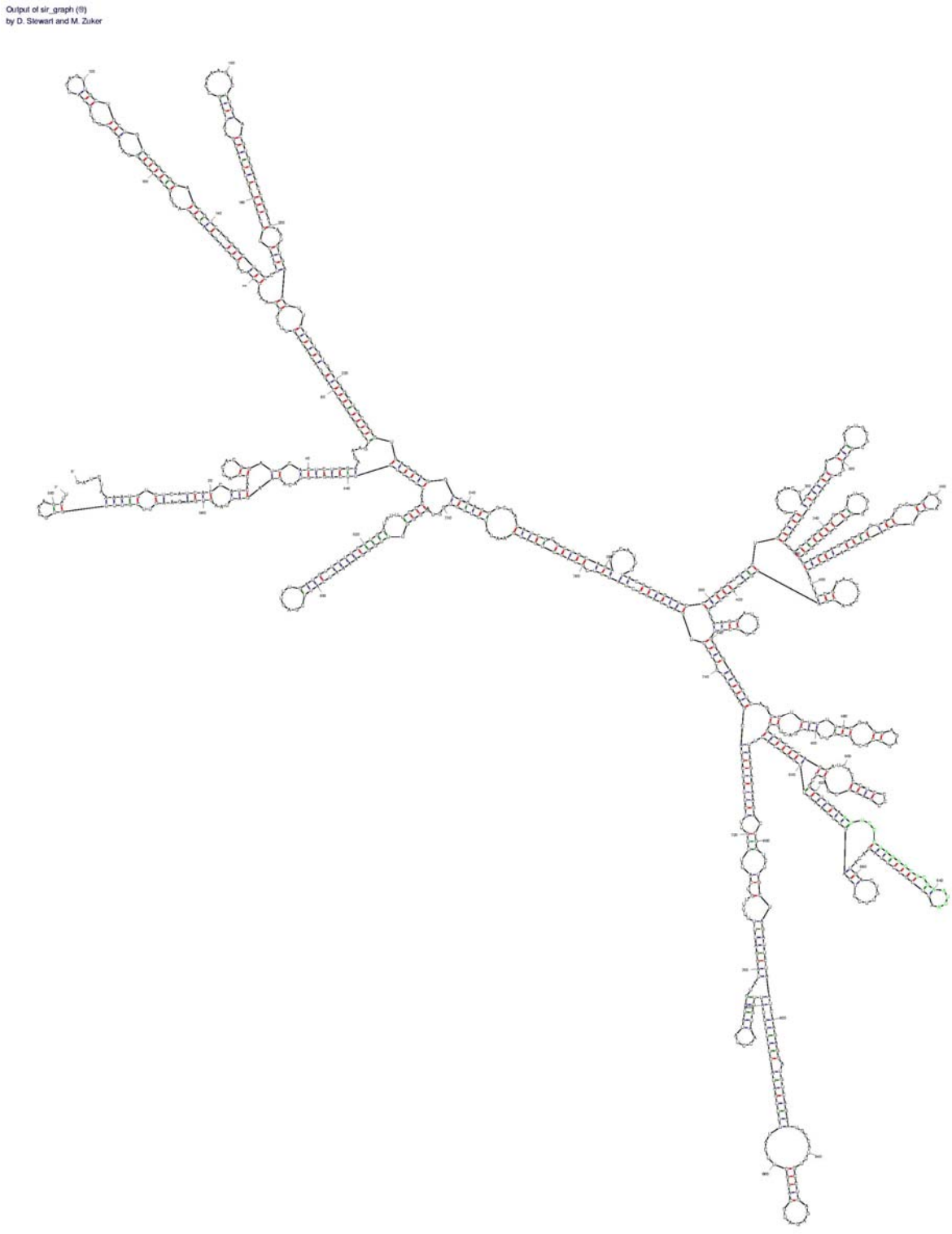

*dG = -360.00 pPICHcfosARE29+S+SDNotI2*

**Figure 11: mFold Structure Prediction of pPICHcfos29+S+SNotImutIII**



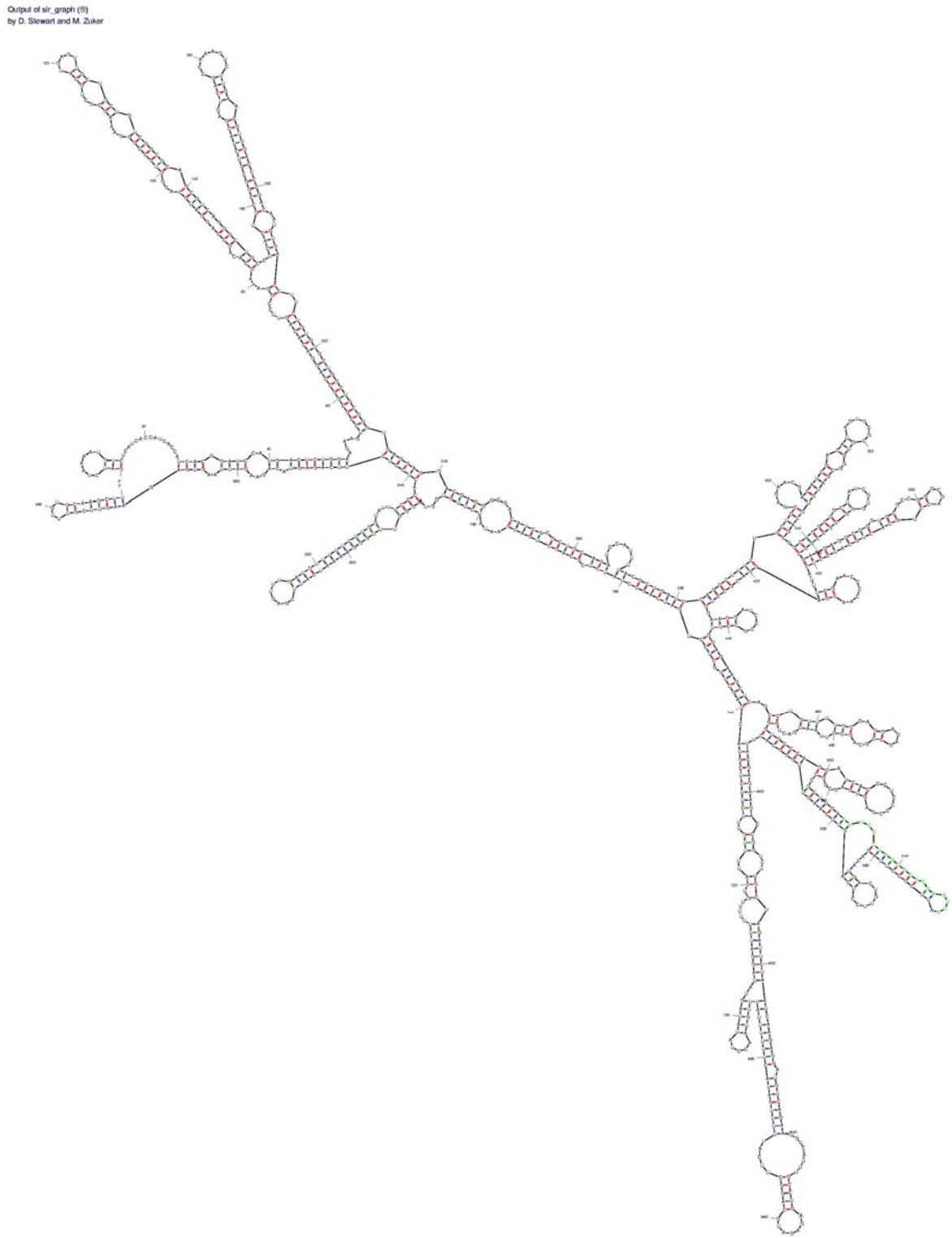

*dG = -359.40 pPICHcfosARE29+S+SDNotI3*

**Figure 12: mFold Structure Prediction of pPICH29**



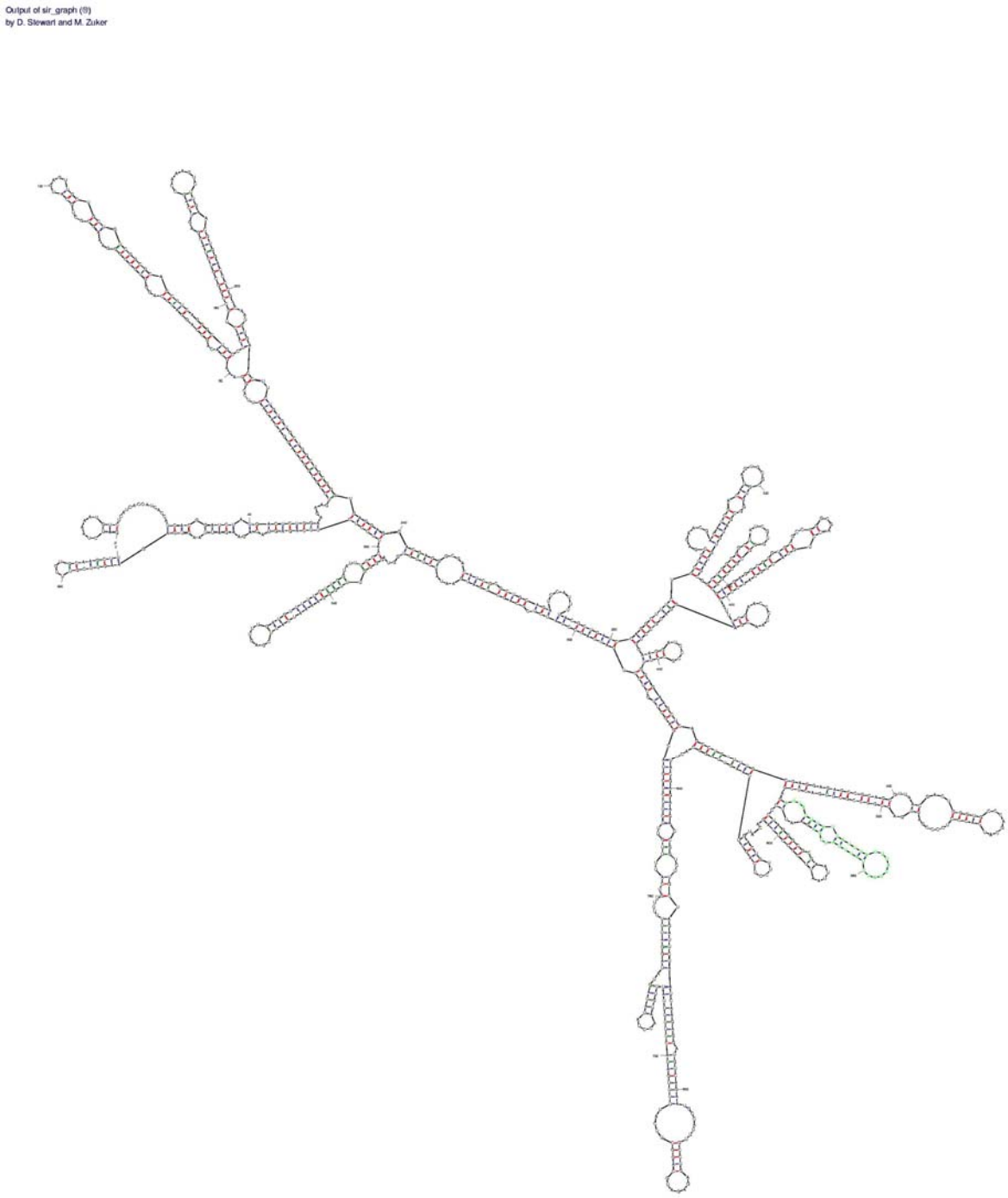

*dG = -344.40 pPICH29*

**Figure 13: mFold Structure Prediction of pPICH29+S+S**



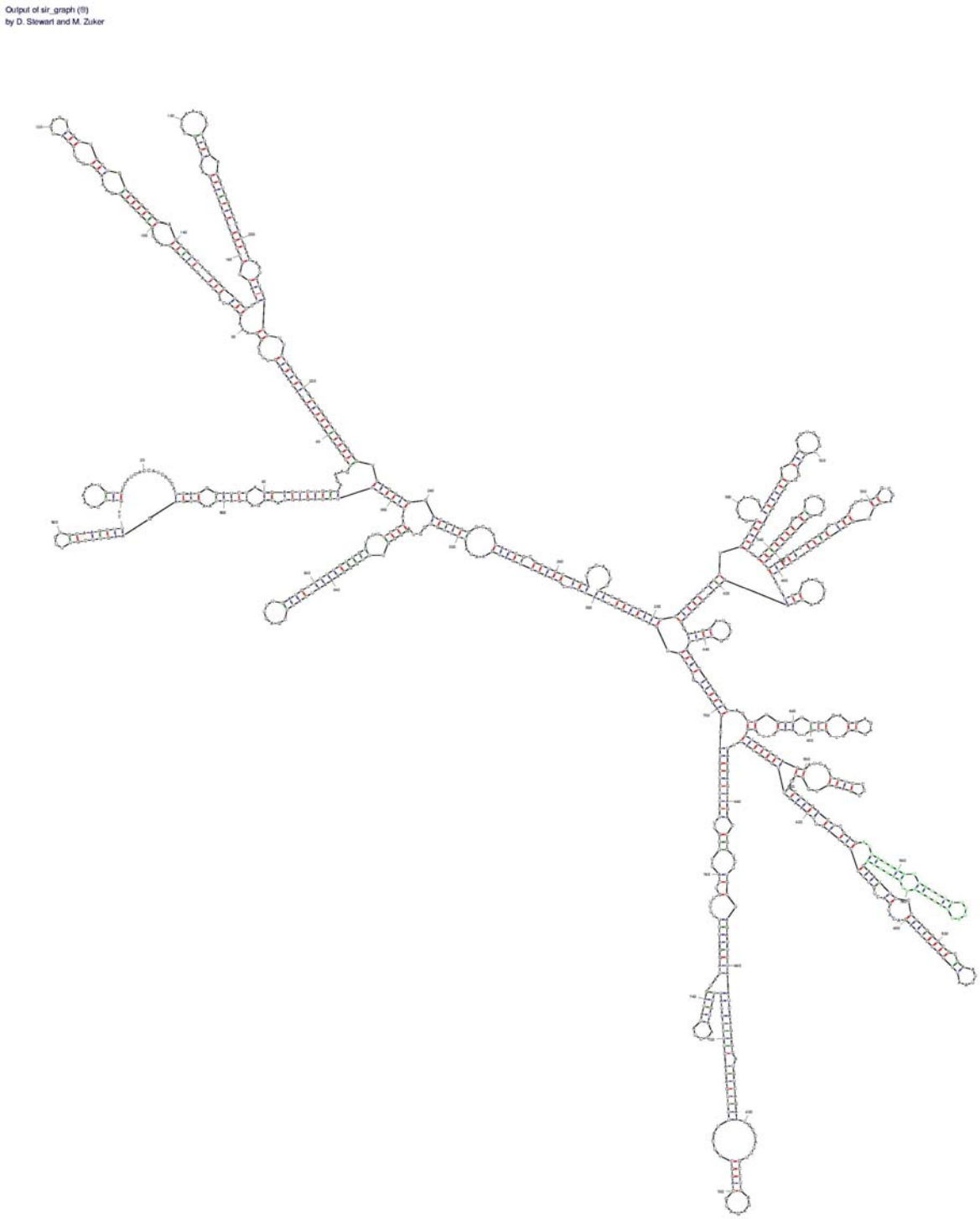

*dG = -347.30 pPICH29+S+S*

**Figure 14: mFold Structure Prediction of pPICHtnf29+S+S**



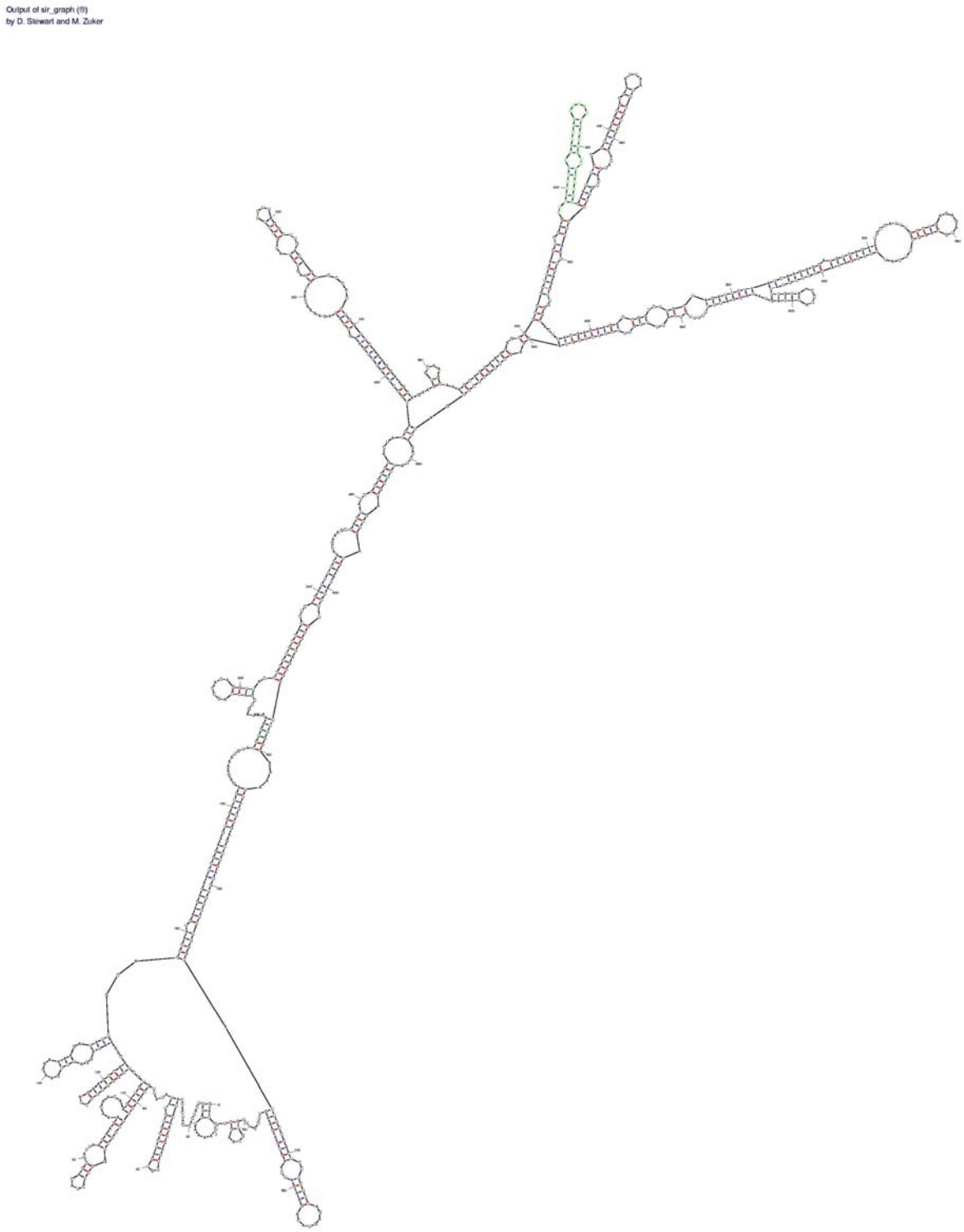

*dG = -350.70 pPICHtnf29+S+S*

**Figure 15: mFold Structure Prediction of pPICHtnf29+S**



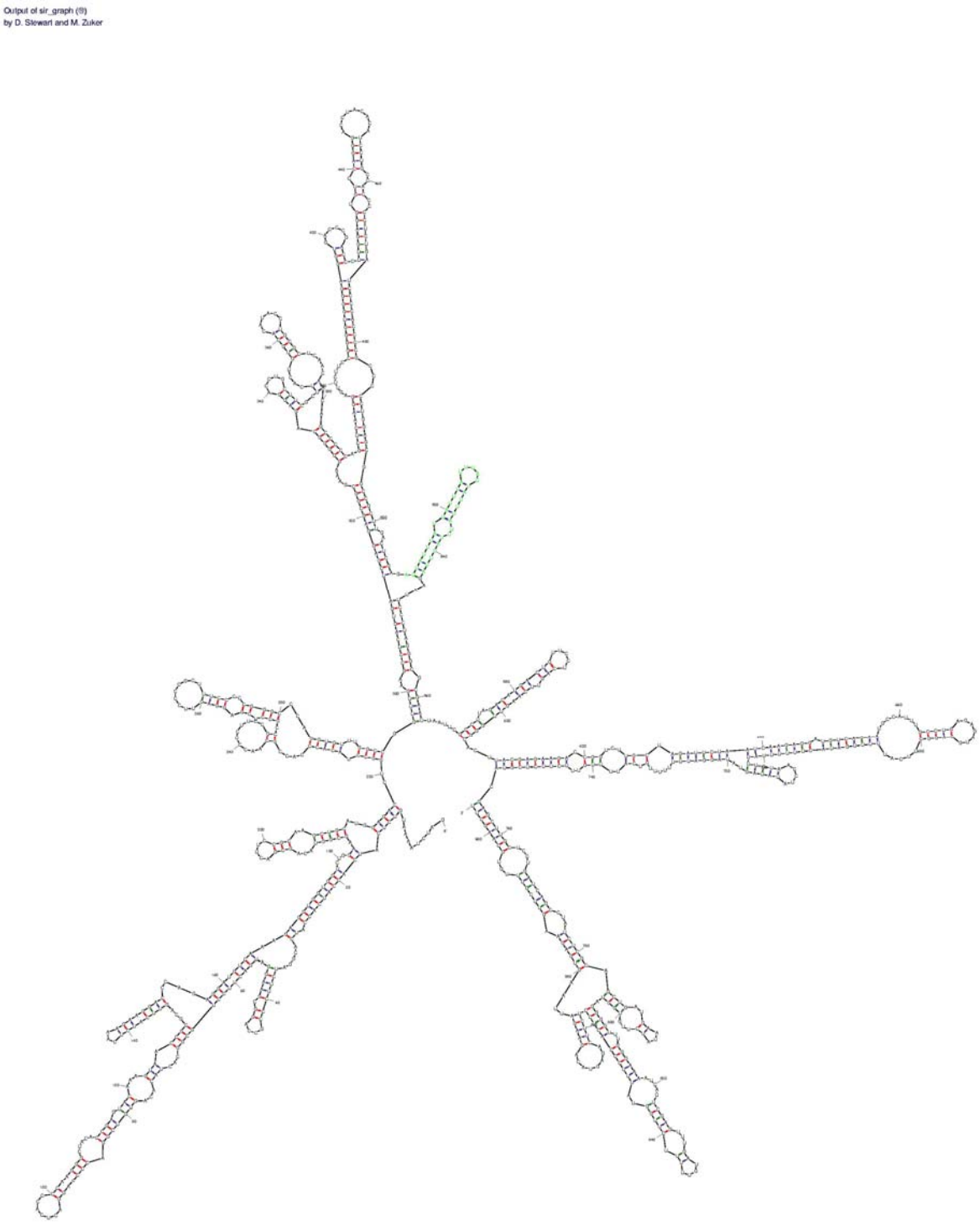

*dG = -349.50 pPICHtnf29+S*

**Figure 16: mFold Structure Prediction of pPICHtnf45**




*dG = -230.39 pPICHtnf45*

**Figure 17: mFold Structure Prediction of pPICHtnf205**




*dG = -304.60 pPICHtnf205*