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**A Y-BOX PROTEIN/RNA HELICASE COMPLEX
LINKS mRNP ASSEMBLY ON THE GENE TO
mRNA TRANSLATION**

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Cover picture: *Chironomus tentans* salivary gland cells stained with a ctYB-1 antibody and DAPI. Green: ctYb-1, red: DNA. Pseudocolors. (Illustration by D. Nashchekin)

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**“In this world nothing occurs sooner or later.
All happens in time”**

Confucius

Abstract

Immediately upon transcription, messenger RNA molecules become associated with proteins to form ribonucleoprotein (RNP) complexes, usually called mRNP particles. The mRNPs are the actual targets for regulation and dictate the fate of an mRNA in the gene expression pathway. To understand the molecular basis of mRNP formation on the gene, export and translation the behaviour of the mRNP proteins have to be investigated. In the salivary glands of the dipteran *Chironomus tentans*, it is possible to visualize the co-transcriptional assembly of a specific giant mRNP (Balbiani ring mRNP, BR mRNP) on the gene and follow it during the nucleocytoplasmic transport and the formation of polysomes in the cytoplasm. We have now used this system to characterize two evolutionarily conserved RNA-binding proteins: a Y-box protein and a DEAD box RNA helicase.

In vertebrates, cytoplasmic mRNPs contain an abundant Y-box protein called YB-1, which regulates translation, presumably by affecting the packaging of the mRNA. We have identified the *C. tentans* YB-1 (ctYB-1) homologue and revealed that ctYB-1 is added along the nascent transcripts and is present in the released BR RNP particles in the nucleoplasm as well as in the polysomes. Thus, ctYB-1 accompanies mRNA from the site of transcription in the nucleus to the place for translation in the cytoplasm.

We have shown that the ctYB-1 appears in two splice isoforms, p40 and p50, which differ only in the region close to the C-terminus. Except for tissue culture cells, both variants are present in all tested tissues but in different proportions. Protein composition analysis of isolated BR RNPs revealed that both isoforms are associated with nuclear and cytoplasmic BR mRNA and could be located on the same mRNA transcript.

Ded1 is a DEAD box RNA helicase essential for the initiation of mRNA translation in yeast. We have identified the *C.tentans* Ded1 homologue, hrp84, and shown that it binds co-transcriptionally to the nascent pre-mRNA transcript and remains associated with mRNA from the gene to the polysome. Injection of an hrp84 antibody into the nucleus did not affect mRNP assembly or transport, while injection into the cytoplasm blocked mRNA translation. Thus, hrp84 accompanies mRNA from the nucleus to the cytoplasm, where it exerts its function during translation.

In studies of *C. tentans* cultured cells, we have revealed by immunoprecipitation that hrp84 is associated with ctYB-1 both in the nucleus and cytoplasm, and the two proteins also appear together in polysomes. The interaction is likely to be direct as shown by *in vitro* binding of purified components. We conclude that the mRNA-bound hrp84/ctYB-1 complex is formed in the nucleus and is translocated with mRNA into the cytoplasm and further into polysomes.

In general, we conclude that two evolutionarily conserved translational regulators become bound to mRNA already during transcription, which indicates that translational competence of the mRNA in cytoplasm can be decided already co-transcriptionally during mRNP assembly.

LIST OF PUBLICATIONS

This thesis is based on the following papers that will be referred to in the text by their Roman numerals:

- I. Teresa Soop, **Dmitri Nashchekin**, Jian Zhao, Xin Sun, Alla T. Alzhanova-Ericsson, Birgitta Björkroth, Lev Ovchinnikov and Bertil Daneholt (2003). A p50-like Y-box protein with a putative translational role becomes associated with RNA concomitant with transcription. *Journal of Cell Science* 116:1493-1503.
- II. **Dmitri Nashchekin**, Teresa Soop, Elizaveta Kovrigina, Sergey Masich, Oxana Nashchekina and Bertil Daneholt. Two splicing isoforms of ctYB-1 appear on the same Balbiani ring mRNA molecule. *Manuscript*.
- III. Jian Zhao, **Dmitri Nashchekin**, Nathalie Fomproix, Birgitta Ivarsson and Bertil Daneholt. A Ded1-like RNA helicase links RNP assembly on the gene with mRNA translation. *Manuscript*.
- IV. **Dmitri Nashchekin**, Jian Zhao, Neus Visa and Bertil Daneholt (2006). A novel Ded1-like RNA helicase interacts with the Y-box protein ctYB-1 in nuclear mRNP particles and polysomes. *Journal of Biological Chemistry* 281:14263-14272.

ABBREVIATIONS

BR	Balbiani ring
CBC	Nuclear cap binding complex
C-terminal	Carboxy-terminal
CSD	Cold shock domain
CTD	Carboxy-terminal domain
DEAD-box	Asp-Glu-Ala-Asp motif
DNA	Deoxyribonucleic acid
eIF	Eukaryotic initiation factor
EJC	Exon-exon junction complex
ER	Endoplasmic reticulum
EM	Electron microscopy
HIV	Human immunodeficiency virus
hnRNP	Heterogeneous nuclear ribonucleoprotein
kb	Kilobase
kDa	Kilodalton
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
N-terminal	Amino-terminal
NES	Nuclear export signal
NLS	Nuclear localization signal
NMD	Nonsense mediated decay
NPC	Nuclear pore complex
PABP	Poly(A)-binding protein
PABPN1	Nuclear poly(A)-binding protein
Pol II	RNA polymerase II
pre-mRNA	Precursor mRNA
PTC	Premature termination codon
RNA	Ribonucleic acid
RBD	RNA binding domain
S	Svedberg
SR	Serine/arginine-rich
UTR	Untranslated region

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INTRODUCTION

The human genome contains about 30 000 genes. To express its information, each protein-coding gene must be transcribed in the nucleus by RNA polymerase to a messenger RNA (mRNA), which will be translated to a protein in the cytoplasm. However, before protein synthesis, the mRNA has to be processed (capped at the 5' end, polyadenylated at the 3' end, and introns removed), transported to cytoplasm and sometimes also directed to a specific region of the cytoplasm. From the site of transcription in the nucleus to the site of translation in the cytoplasm, mRNA never exists as a naked molecule. It is always associated with different types of RNA-binding proteins that determine the fate of mRNA (*Omnia mea mecum porto / I carry with me all my things*) (Spirin, 1978). mRNA and associated proteins form the messenger ribonucleoprotein (mRNP) complex. The protein composition of mRNP, the “mRNP code”, is unique for each transcript in a given cell type and changes as the primary nuclear transcript undergoes processing, export and translation (Singh and Valcarcel, 2005). Some components of mRNP are stably bound to mRNA, while others are subjected to dynamic exchange (Daneshmandi, 2001; Moore, 2005).

1. TRANSCRIPTION, PROCESSING AND mRNP ASSEMBLY

1.1 RNA polymerase II

The first step in the gene expression process is the synthesis of messenger RNA (mRNA), so-called transcription (Fig. 1). Transcription of protein coding genes is accomplished by DNA dependent RNA polymerase II (Pol II). Pol II is a large multiple subunit enzyme. The largest subunit of Pol II harbours the catalytic domain and the regulatory C-terminal domain (CTD). In humans, CTD consists of 52 heptapeptide repeats, and dynamic site-specific phosphorylation and dephosphorylation of these repeats are critical mechanisms for CTD function. Several different CTD kinases and at least one phosphatase have been implicated in this mechanism (Meinhart et al., 2005). CTD functions both as an assembly platform for and a regulator of transcription and pre-mRNA processing (Maniatis and Reed, 2002 and ref. therein). The three-dimensional structure of yeast Pol II revealed that the CTD is located close to the exit

groove of the polymerase and can load different factors onto the pre-mRNA as it emerges from the polymerase (Cramer et al., 2001).

1.2 Transcription initiation and elongation

Transcription consists of several steps, which include preinitiation, initiation, promoter clearance, elongation, and termination. The transcription starts with preinitiation complex assembly at the promoter. The preinitiation complex includes Pol II and the general transcriptional factors TFIID, TFIIB, TFIIE, and TFIIH. The assembly of transcription factors at the promoter starts with binding of the TATA-binding protein (TFIID) to the TATA box. Gene-specific transcription factors, activators or repressors, also bind to the promoter. Furthermore, transcription preinitiation involves the interaction of the unphosphorylated CTD with the Mediator complex, the central multiprotein coactivator that transmits signals from activators and repressors to Pol II (Kornberg, 2005). Once the complex has been established, transcription initiation occurs upon addition of the two initiating nucleotides. During transcription initiation, serine 5 of the CTD heptapeptide repeats becomes phosphorylated. This phosphorylation leads to dissociation of transcription factors from the CTD and promoter clearance. Once the promoter is cleared, the next round of transcription can be reinitiated.

Newly initiated Pol II is considered to be in a mode of abortive initiation (Pal and Luse, 2002). The formation of the stable elongation complex takes place only when the RNA-DNA hybrid reaches 10-20 nucleotides (Sims et al., 2004). Simultaneously with the beginning of elongation, the phosphorylation state of the CTD is changing: serine 5 becomes stepwise dephosphorylated while serine 2 gets phosphorylated. Since phosphorylation/dephosphorylation of the serines in various heptarepeats is not going on at the same time, CTD gains a phosphorylation “code” which specifies the position of Pol II in the transcription cycle (Buratowski, 2003). Such a “code” can be read by factors involved in the subsequent steps of RNA processing.

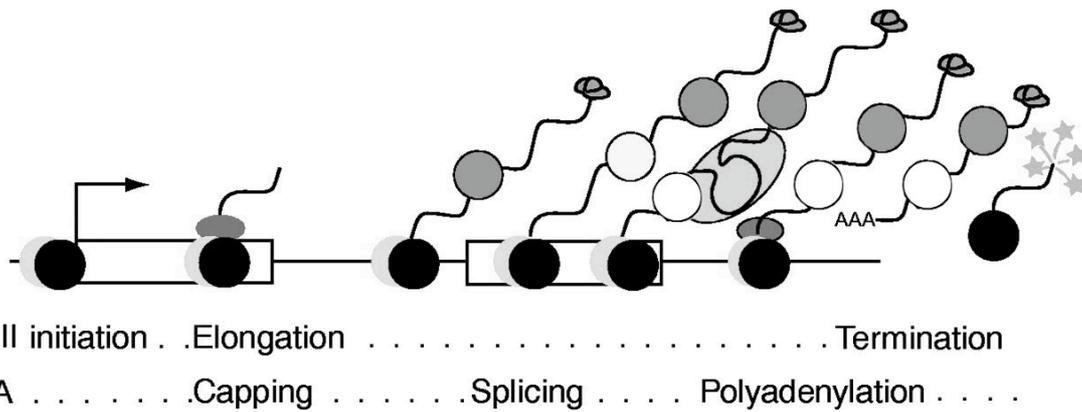


Figure 1. Overview of transcription and processing. Pol II (black ball) initiates transcription at the promoter (arrow) and proceeds along DNA during elongation and termination phases. Several polyadenylation factors bind directly to Pol II (light ball next to Pol II). Capping enzymes (gray oval) bind Pol II as it enters the elongation phase. RNA-binding proteins (gray and white balls) and the spliceosome (big gray oval) bind pre-mRNA co-transcriptionally. For polyadenylation, additional factors are recruited to pre-mRNA (dark gray oval). At termination, Pol II and the mRNP are released from the template, and the mRNP is exported. Exons are shown as boxes. Modified from Neugebauer, 2002.

Transcription takes place on chromatin, a complex between DNA and histones. The basic unit of chromatin is the nucleosome. The nucleosome core particle consists of a histone octamer made up of a tetramer of histone H3/H4 and two histone H2A/H2B dimers. A lot of different factors help Pol II to get through chromatin during elongation (reviewed in Sims et al., 2004). Some of them, e.g. the SWI/SNF complex, remodel chromatin in an ATP-dependent manner. Others, such as FACT, work as histone chaperones destabilizing nucleosome structure by removing one H2A/H2B dimer (Sims et al., 2004). Histone acetylation destabilizes chromatin structure, and active genes are typically enriched in acetylated histones. Thus, recruitment of histone acetyltransferases to the elongating Pol II facilitates transcription.

1.3 mRNP assembly

Concomitant with transcription, mRNA becomes associated with various RNA-binding proteins forming an mRNP complex. The protein composition of the mRNP is very dynamic and it changes at every step of the mRNA pathway (Fig. 2). Some proteins stay with mRNA all the way from genes to polysomes, while others only appear bound to mRNA during specific stages of gene expression.

hnRNP proteins

The predominant proteins bound to mRNA already co-transcriptionally are hnRNP proteins. In human cells, at least 20 major hnRNP proteins have been identified, and are designated from A1 (34kDa) to U (120 kDa) (reviewed in Dreyfuss et al., 2002; Dreyfuss et al., 1993). Many of them comprise several isoforms, and numerous less abundant proteins are also present. The name hnRNP proteins comes from the early observation that the proteins bind heterogeneous nuclear RNA (hnRNA or pre-mRNA).

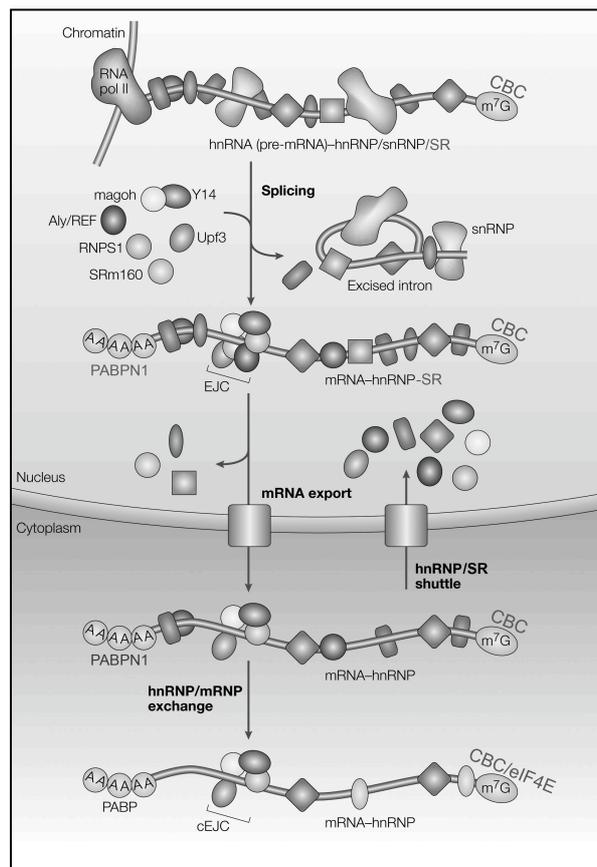


Figure 2. mRNP dynamic. The protein composition of the mRNP changes during transcription, processing and nucleocytoplasmic transport. CBC, nuclear cap binding complex; EJC, exon-exon junction complex; hnRNP, heterogeneous nuclear ribonucleoprotein; mRNP, mRNA-protein complex; PABP, poly(A)-binding protein; PABPN1, nuclear poly(A)-binding protein; SR, SR protein; m⁷G, 5' 7-methylguanosine cap. Modified from Dreyfuss et al., 2002

Many hnRNP proteins are abundant nuclear proteins that shuttle between the nucleus and cytoplasm (Pinol-Roma and Dreyfuss, 1992) and can accompany mRNA from gene to polysome (Visa et al., 1996a). The non-shuttling hnRNP proteins, such as hnRNP C, contain nuclear retention signals that can override nuclear export signals (Nakielny

and Dreyfuss, 1996). hnRNP proteins interact with themselves and other factors and can influence diverse cellular events (reviewed in Krecic and Swanson, 1999; Dreyfuss et al., 2002). They possess RNA strand displacement and annealing activities providing them with ability to organize RNP structure. hnRNP proteins consist of one or several RNA binding domains: usually they have one or more RNA Binding Domain (RBD) and an auxiliary domain containing RGG boxes involved in RNA binding and protein-protein interactions.

The hnRNP proteins are arranged on nascent transcripts in such a way that pre-mRNA becomes coated with hnRNP proteins throughout its length forming an RNP fibril (Daneshmandi, 1997; Dreyfuss et al., 1993). The binding of hnRNP proteins is not random with respect to RNA sequence, and each protein has its own sequence preferences (Dreyfuss et al., 1993; Singh and Valcarcel, 2005). It means that each mRNA species is associated with a unique combination of the proteins. However, an hnRNP protein might not be bound exclusively to high-affinity sites if it is present in high molar excess (as is the case for many hnRNP proteins). In this case, it may be bound “nonspecifically” (with low affinity) to other RNA sequences (Dreyfuss et al., 2002).

SR proteins

Other proteins that are involved in the formation of the mRNP fibril are SR proteins. SR (serine/arginine-rich) proteins are evolutionarily conserved phosphoproteins. They contain one or two N-terminal RBD domains and a C-terminal domain rich in repeating arginine-serine dipeptides (the RS domain) (Fu, 1995). The RS domain of SR proteins can be phosphorylated at multiple positions by several specific cellular kinases. SR proteins can inhibit or promote splicing, and in some cases the same factor can have positive or negative effects, depending on where it is recruited to the transcript or on its level of phosphorylation (Singh and Valcarcel, 2005). SR proteins can affect early or late events in spliceosome assembly, they can directly recognize the pre-mRNA or act as co-factors for other pre-mRNA-binding proteins, and they can affect a specific

subset of transcripts or have global effects on processing (Singh and Valcarcel).

Although SR proteins were originally described as essential splicing factors, they can probably also function as structural components of pre-mRNP particles. It has been shown that the *Chironomus* SR protein hrp45 is present uniformly along the entire length of BR transcripts (Alzhanova-Ericsson et al., 1996). The fact that recruitment of the mammalian SR protein ASF/SF2 to nascent transcripts prevents the formation of an R loop structure (an RNA:DNA hybrid between nascent transcript and the template strand of DNA) supports the proposition for a structural function of SR proteins in mRNP formation (Li and Manley, 2005).

In the nucleus SR proteins are accumulated in nuclear bodies called speckles and recruited from there to the site of transcription. Some SR proteins shuttle between nucleus and cytoplasm together with mRNA (Caceres et al., 1998; Huang et al., 2003) and were found in polysomes (Sanford et al., 2004; Windgassen et al., 2004). The activity and localization of SR proteins are regulated by the phosphorylation state of the RS domain. Initially, SR proteins are recruited to pre-mRNA in their hyperphosphorylated form (Huang and Steitz, 2005) and then become hypophosphorylated during splicing by specific phosphatases (Gilbert and Guthrie, 2004). Interestingly, only shuttling SR proteins seem to be dephosphorylated (Lin et al., 2005). Following mRNA export, cytoplasmic rephosphorylation by specific kinases facilitates release of SR proteins from mRNA and re-import to the nucleus (Huang and Steitz, 2005).

The RNA-binding proteins recruited to the pre-mRNA during transcription are not only involved in RNP assembly, but can also influence transcription. It has been shown recently that components of the growing RNP fibril recruit chromatin mod-modifying factors to transcriptionally active genes (Percipalle and Visa, 2006). In particular, *Chironomus* hrp65 associated with nascent pre-mRNP interacts with a histone acetyltransferase (HAT) and allow the HAT to travel along the transcribing gene and

acetylate histone H3 that maintains the chromatin in a transcription-competent conformation (Sjolinder et al., 2005).

1.4 Capping

Capping of the 5'-end of the mRNA with a 7-methylguanosine takes place during the transition from transcription initiation to elongation when the nascent pre-mRNA is only 20-40 nucleotides long. Two proteins in humans and three in yeast are responsible for the triphosphatase, guanylyltransferase and methyltransferase activities. The recruitment of capping enzymes is stimulated by the Ser 5 phosphorylation of the CTD. In turn, completion of capping triggers re-activation of Pol II after a promoter-proximal pause required for capping. This provides a possible capping checkpoint ensuring that only capped transcripts are further elongated (Saguez et al., 2005). Binding of the capping enzyme to the CTD not only localizes the enzyme but also regulates it allosterically (Ho and Shuman, 1999). Removal of the Ser 5 phosphate by phosphatases early during elongation is coupled to release of the capping machinery (Schroeder et al., 2000). Only RNAs synthesized by Pol II are capped, presumably due to the binding of the capping enzyme to the CTD (Neugebauer, 2002). The 5' cap modification protects pre-mRNA and mRNA from 5' to 3' exonucleases. In addition, the cap serves as a binding site for the cap-binding complex (CBC) in the nucleus and the translation initiation factor eIF4E in the cytoplasm (Lewis and Izaurralde, 1997). Like capping, CBC binding is co-transcriptional (Visa et al., 1996b). CBC is composed of two subunits, CBP80 and CBP20, and plays a role in splicing of the first intron (Lewis et al., 1996) and protects transcripts from degradation.

1.5 Splicing

Pre-mRNAs contain both coding sequences (exons) and non-coding sequences (introns). To create mature mRNAs with intact open reading frames introns must be excised and flanking exons must be spliced. The whole process is called splicing. Introns are removed by a macromolecular machine termed the spliceosome. The spliceosome consists of five small nuclear ribonucleoprotein particles (snRNPs), U1,

U2, U4, U5 and U6 snRNPs, and a large number of protein splicing factors (Kramer, 1996). Many components of the spliceosome have recently been identified by proteomic approaches, and more than 300 putative spliceosomal proteins were revealed (Jurica and Moore, 2003).

The splicing machinery marks the exon junction site with a multiprotein complex termed the exon-exon junction complex (EJC). The EJC deposition site is about 20-24 nucleotides upstream of the junction point (Le Hir et al., 2000). The EJC is loaded in a sequence-independent, position-dependent manner and as a consequence of the splicing reaction. The EJC serves as a binding platform for factors involved in mRNA export and nonsense-mediated decay (NMD) (Le Hir, et al., 2001; Lykke-Andersen, et al., 2001). It is also involved in RNA localization in *Drosophila* oocytes (see below). The components of EJC include splicing-associated factors SRm160 and RNPS1, a DEAD-box RNA helicase eIF4AIII, Y14, Magoh, MLN51 and Upf3 (Dreyfuss et al., 2002). Several experiments have indicated that RNPS1 and SRm160 are peripheral EJC components and weakly bound to mRNA while Magoh, Y14 and eIF4AIII form a core complex (Ballut et al., 2005). The eIF4AIII binds to spliced mRNA at the EJC deposition site and marks on mRNA the former positions of excised introns (Chan et al., 2004; Shibuya et al., 2004). The stable association of the eIF4AIII with mRNA is maintained by inhibition of the eIF4AIII ATPase activity by Magoh-Y14 (Ballut et al., 2005).

Pre-mRNA splicing begins co-transcriptionally and may continue post-transcriptionally depending on the excision efficiency of the various introns (Bauren and Wieslander, 1994; Wetterberg et al., 1996). There is interplay between transcription and splicing. On one hand, splicing components can stimulate elongation by recruiting transcription-stimulating factors (reviewed in Neugebauer, 2002; Le Hir et al., 2003). On the other hand, the processivity of Pol II affects splice site selection (Sanford and Caceres, 2004). For instance, a slow Pol II, and/or the presence of internal transcriptional pause sites, results in inclusion of an alternative exon harboring a weak 3' splice site. By contrast, when the same pre-mRNA is transcribed by highly processive Pol II, the weak

alternative 3' splice site is unable to compete with the stronger downstream 3' splice site, which results in skipping of the alternative exon (Sanford and Caceres, 2004).

The strength of a splice site is controlled by two types of sequence elements, the enhancer and silencer elements. These elements can be located within introns or exons. Intronic and exonic splicing enhancers (ISEs and ESEs) promote splicing while intronic and exonic splicing silencers (ISSs and ESSs) inhibit splicing. The balance between these elements determines the overall strength of a splice site in promoting splicing (Singh and Valcarcel, 2005). Two families of RNA-binding proteins, hnRNP proteins and SR proteins, interact with enhancer and silencer elements and play crucial roles in splicing regulation. In general, SR proteins interact with ESEs and facilitate recruitment of U1 and U2 snRNPs to pre-mRNA, while hnRNP proteins recognize ESSs and inhibit early steps of spliceosome assembly antagonizing the splicing activity of SR proteins (Singh and Valcarcel, 2005).

1.6 Polyadenylation

The final step in the formation of a mature mRNA is polyadenylation of the 3' end, which protects the mRNA from degradation. The first step of polyadenylation is the cleavage of the pre-mRNA at a site located between the canonical AAUAAA sequence – where cleavage and polyadenylation specificity factor (CPSF) binds – and a downstream G/U-rich region – where cleavage stimulatory factor (CstF) binds. Cleavage is accomplished by cleavage factors I and II (CFI and CFII), and nuclear polyadenylation is performed by poly(A) polymerase (PAP) bound to CPSF and the nuclear poly(A)-binding protein (PABPN1) (Neugebauer, 2002). CPSF has been shown to be a component of TFIID and to be bound to the CTD since the start of elongation (Dantonel et al., 1997). PABPN1 is associated with Pol II during transcription and remains associated with the poly(A) tail of mRNA until the translocation of mRNP through the nuclear pore, where PABPN1 is exchanged with cytoplasmic poly(A)-binding protein (PABP) (Bear et al., 2003). According to the current model, the poly(A) tail works both as a signal for release of mRNA from the site of transcription to

nucleoplasm and as a quality control of mRNP (reviewed in Jensen et al., 2003). The mRNP is retained close to its site of transcription by an exosome-dependent mechanism. When PAP adds poly(A) to the transcript, it creates a potential substrate for the exosome. The efficiency of polyadenylation and addition of poly(A)-binding proteins to the poly(A) tail can be affected by mRNP components. Failure to create a proper tail leads to mRNA degradation. Successful tail formation, on the other hand, results in mRNP release (Jensen et al., 2003).

2. mRNA EXPORT

Essentially all traffic of molecules between nucleus and cytoplasm occurs through the nuclear pore complexes (NPCs) in the nuclear envelope. Each NPC is a huge structure with an estimated total mass of 90-120 MDa (reviewed in Fahrenkrog and Aebi, 2003). The active, energy dependent, export or import of protein and RNA cargos through the NPC is mediated by soluble proteins, the nuclear transport receptors, termed exportins/importins or karyopherins (Nakielny and Dreyfuss, 1997). Cargos identify themselves to the nucleocytoplasmic transport machinery by signals (nuclear localization signals, NLSs, and nuclear export signals, NESs). The signal can be part of a protein or RNA, or both at the same time. Often, the transport signals are recognized by adaptor proteins, which interact with transport receptors bringing the cargos to the NPC. The directionality of transport is accomplished by two factors: the nuclear pore proteins specific to one or the other face of the pore and a Ran-GTP/Ran-GDP gradient across the nuclear envelope (Vasu and Forbes, 2001). Ran is a small GTPase associated with GTP or GDP. Ran-GTP is found only in the nucleus as a result of the exclusive localization of Ran GTP exchange factor (RCC1) on chromosomes. In contrast, Ran-GDP is found in the cytoplasm, due to the presence of Ran GTPase activating factor (Ran-GAP) on the cytoplasmic filaments of the NPC and in the cytoplasm (Vasu and Forbes, 2001).

While the adaptors and receptors for most RNA cargos have been identified and characterized, the mechanism of mRNA export still remains unclear. The export of most

mRNAs is independent of the Ran system and karyopherins (Clouse et al., 2001). The key mediator of bulk mRNA export is instead a heterodimer consisting of TAP (Mex67p in yeast) and p15 (Mtr2p in yeast), the latter being required for nucleocytoplasmic shuttling of the dimer (reviewed in Cullen, 2003; Erkmann and Kutay, 2004). Genome-wide analysis in *Drosophila* showed that TAP is involved in the export of 75% of the 6000 mRNAs analyzed (Herold et al., 2003). TAP was originally discovered as a cellular factor engaged in the export of the incompletely spliced viral mRNA of MPM Virus. TAP specifically binds to the constitutive transport element (CTE) of the viral mRNA, and an excess of CTE RNA blocks cellular mRNA export. Since TAP binds cellular mRNA in a non-sequence-specific manner and its RNA-binding domain is not essential for mRNA export *in vivo*, TAP requires an adaptor to be recruited to mRNA. One of the first discovered candidates to be such an adaptor was REF or Aly (Yra1p in yeast). REF binds both RNA and TAP, and can stimulate mRNA export (Fig. 3). The identification of REF as a component of EJC suggests the interesting possibility that TAP can be recruited only to properly spliced mRNAs. The idea that pre-mRNA splicing is linked to mRNA export was strengthened by the observation that REF binds to UAP56 (Sub2 in yeast), a DEAD box RNA helicase involved in pre-mRNA splicing and also essential for mRNA export.

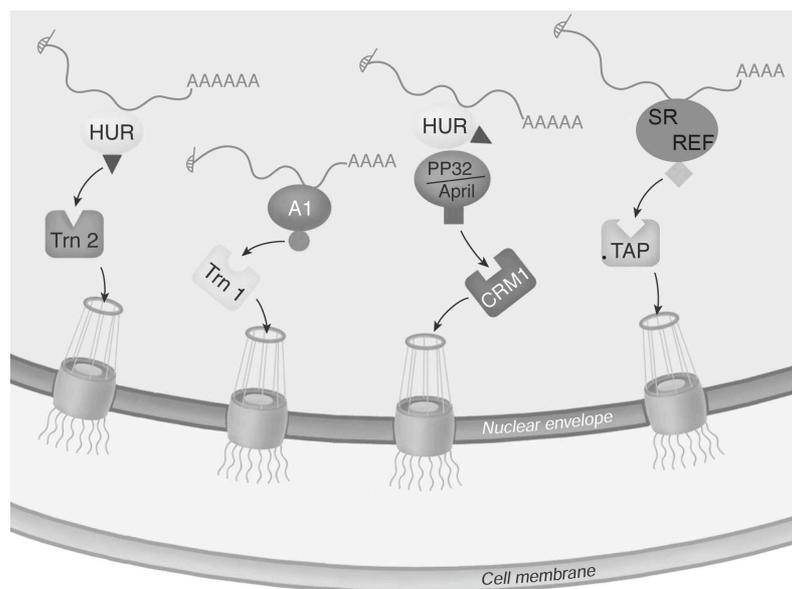


Figure 3. mRNA export pathways. Most mRNAs use TAP as an export receptor and REF and/or SR proteins as adaptors. Alternative pathways involve the export receptors CRM1 and transportin 2 (Trn 2) with the adaptor protein HUR and the export receptor transportin 1 (Trn 1) with hnRNP A1 as adaptor. Modified from Moore and Rosbash, 2001.

In yeast, association of Yra1p with mRNA occurs co-transcriptionally via interaction between Sub2p and the transcription elongation factor Hpr1p, a component of the THO complex, involved in transcriptional elongation (Erkman and Kutay, 2004). Consequently, the THO complex has been suggested to help loading Yra1p onto nascent mRNAs. Because THO associates with Sub2p and Yra1p and links transcription to export, this assembly is called the transcription-export (TREX) complex (Strasser et al., 2002). In human cells, REF and UAP56 have been found in a complex with several proteins homologous to yeast THO complex components (Strasser et al., 2002). In mammalian cells, deposition of the export factors UAP56 and REF onto mRNA is dependent on prior splicing, and recruitment of these factors together with the THO complex is not co-transcriptional *per se*. Although splicing occurs co-transcriptionally, TREX in mammals may be recruited by the splicing machinery rather than by the elongating Pol II (Guthrie and Steitz, 2005; Reed and Cheng, 2005).

Apparently, splicing could be involved in recruiting mRNA export factors to establish competence for export. However, the vast majority of yeast genes as well as several metazoan genes lack introns, and still the corresponding transcripts are efficiently exported. What is then the mechanism for splicing-independent recruitment of the export machinery? In metazoans and yeast it has been shown that the key mRNA export factors UAP56/REF/TAP also affect the export of mRNAs derived from genes lacking introns. On the other hand, depletion of REF and other components of the EJC is dispensable for nuclear export of bulk mRNA (Gatfield and Izaurralde, 2002). Also, microarray analysis of mRNAs associated with Yra1p and Mex67p revealed that these factors associated with distinct and not all populations of mRNAs (Hieronymus and Silver, 2003). Moreover, the vast majority of mRNAs in *Drosophila* are transcribed and exported independently of the THO complex. Only heat shock mRNA export does required this complex (Rehwinkel et al., 2004). Thus, a series of observations suggest that other adaptors, than REF/Aly and Yra1p, must exist that mediate the interaction between TAP and mRNA. The only general candidates so far reported are SR proteins (Huang et al., 2003; Huang and Steitz, 2001). The N-terminal domains of SR proteins

interact with the same N-terminal portion of TAP that associates with REF (Huang and Steitz, 2005). TAP interacts preferentially with shuttling hypophosphorylated SR proteins (Huang et al., 2004). Thus, whereas SR proteins are initially recruited to pre-mRNAs for splicing in their hyperphosphorylated forms, they become partially dephosphorylated during the splicing reaction and gain higher affinity for TAP (Huang and Steitz, 2005). These results suggest that the phosphorylation state of mRNA-bound SR proteins contributes to the ability of the export machinery to discriminate between spliced and unspliced mRNPs (Huang and Steitz, 2005). Cycles of nuclear dephosphorylation and cytoplasmic phosphorylation of SR proteins may be used to control export factor loading and dissociation, and thus contribute to the gain of directionality in mRNA export (Erkmann and Kutay, 2004).

Some specific mRNAs can use export pathways distinct from the TAP-mediated pathway (Dreyfuss et al., 2002). For example, there are two pathways for export of *c-fos* mRNA, CRM1-dependent and transportin-dependent. In both cases the RNA-binding protein HuR is used as an adaptor (Gallouzi and Steitz, 2001). For some mRNAs the RNA-binding protein, hnRNP A1, could be used as adaptor (Gallouzi and Steitz, 2001; Izaurralde et al., 1997). Another interesting possibility would be the involvement of cap and CBC in mRNA export. Such a proposition is based on the observation that giant mRNPs in larval salivary glands in *Chironomus tentans* is always exported through the NPCs with their 5' end in the lead (Daneshmandi, 2001).

Upon delivery of the transcript to cytoplasm, export receptors and adaptors should be removed from the mRNA. One of the crucial factors required for this process seems to be a DEAD-box RNA helicase, Dbp5. Dbp5 is a highly conserved protein among eukaryotes and is required for mRNA export in yeast and animal cells (Schmitt et al., 1999; Snay-Hodge et al., 1998; Tseng et al., 1998). It was identified initially by both a genetic screen to isolate yeast mutants defective in mRNA export and an analysis of the localization and possible roles of a number of yeast DEAD-box proteins (Cole and Scarcelli, 2006). Dbp5 is concentrated on the cytoplasmic fibrils of the NPC via

interaction with two nucleoporins, CAN/Nup159 and GLE1 (Hodge et al., 1999; Schmitt et al., 1999; Strahm et al., 1999). Point mutations in Nup159, that disrupt the Dbp5-Nup159 interaction, lead to release of Dbp5 from NPC and an mRNA export defect (Weirich et al., 2004). The non-NPC-associated fraction of Dbp5 is mainly located in cytoplasm, although Dbp5 shuttles between nucleus and cytoplasm and it accumulates in the nucleus when synthesis and/or export of mRNA is inhibited (Schmitt et al., 1999; Strahm et al., 1999; Zhao et al., 2002). Dbp5 binds to pre-mRNA co-transcriptionally and accompanies mRNPs from the transcription site to cytoplasm (Zhao et al., 2002). Dbp5 interacts with transcription factor IIH and the interaction could play a role in loading Dbp5 onto nascent mRNP (Cole and Scarcelli, 2006).

The localization of Dbp5 on the cytoplasmic side of NPC and its involvement in mRNA export suggested that Dbp5 may act in a terminal step of mRNA export by unloading export factors from the mRNP or remodeling the mRNP when entering the cytoplasm. The first possibility was recently demonstrated to occur in yeast, as it was shown that Dbp5 is required for dissociation of the mRNA export receptor Mex67 from the mRNP following its translocation through the nuclear pore (Lund and Guthrie, 2005). Still another model that has been proposed, is that NPC-bound Dbp5 may work as a molecular motor, and the Dbp5-coupled ATP hydrolysis may be used to pull the mRNP through the nuclear pore into the cytoplasm (Cole and Scarcelli, 2006).

3. mRNA LOCALIZATION

After export from the nucleus some mRNAs are transported to specific places in a cell before translation. For example, 10% of randomly selected mRNAs localize to the anterior of the *Drosophila melanogaster* oocyte, whereas an estimated 400 mRNAs are targeted to the dendrites of mammalian neurons (St Johnston, 2005). The main goal of mRNA localization is the accumulation of gene products only at specific sites in the cell at a certain time. To achieve this, the localizing mRNA has to be silent until it reaches the final destination and/or until it should be translationally activated. Many localized mRNAs contain cis-acting elements (or zipcodes), usually in the 3'-

untranslated region (3'-UTR), that govern their subcellular localization. A "model" localizing mRNP would include an adaptor that recognizes the zipcode, a motor that interacts with cytoskeleton and directs the mRNP to the appropriate location and a repressor that maintains the mRNA translationally inactive (Mendez and Wells, 2002).

Not all proteins which are required for cytoplasmic mRNA localization bind to the target in the cytoplasm. Identification of adaptor proteins for several localizing mRNAs have shown that many of the adaptors are shuttling hnRNP proteins and bind to the mRNA already in the nucleus (reviewed in Farina and Singer, 2002; Van de Bor and Davis, 2004). For example, localization of the myelin basic protein (MBP) mRNA to the myelin-forming processes in mammalian oligodendrocytes requires hnRNP A2. This protein binds to an "RNA trafficking sequence" (RTS) in the 3'-UTR of MBP mRNA, and mutations in RTS that disrupt hnRNP A2 binding also inhibit RNA transport. The *Drosophila* protein Squid (Sqd) is a homolog to human hnRNP A1. Sqd binds to the 3'-UTR of *gurken* (*grk*) mRNA and is needed for the proper localization of *grk* during oogenesis (Norvell et al., 1999). In *Xenopus laevis* oocytes, a homolog to human hnRNP I and the RNA-binding protein Vg1RBP/vera forms a complex with Vg1 mRNA already in the nucleus and is involved in Vg1 localization to the vegetal pole. Upon export to the cytoplasm, the RNP complex is remodeled such that hnRNP I and Vg1RBP/vera no longer directly interact. In the cytoplasm, extra proteins associate with the RNP, creating the cytoplasmic localizing RNP (Kress et al., 2004). These and other findings lead to the suggestion that some part of the localization process occurs in the nucleus, and nuclear proteins could either mark specific transcripts for localization and/or escort them to their ultimate destination in the cytoplasm (Farina and Singer, 2002).

Recently, it has been shown that splicing is coupled to mRNA localization. In *Drosophila* oocytes, components of EJC (homologues to human Magoh, Y14 and eIF4AIII) are required for *oskar* mRNA localization, and they co-localize with *oskar* mRNA at the posterior pole of the oocyte (St Johnston, 2005). Moreover, splicing at the

first exon-exon junction of *oskar* RNA is essential for *oskar* mRNA localization (Hachet and Ephrussi, 2004). It seems that the position of EJC is specifying the architecture of the *oskar* mRNA localization complex, since only the first EJC had effect on *oskar* localization and the transport of other mRNAs produced from intron-containing genes, e.g. *gurken*, are independent of the EJC (Hachet and Ephrussi, 2004). This also lead to the suggestion that alternatively spliced mRNAs might be directed to different cytoplasmic locations depending on the formation of alternative mRNP complex architectures (Hachet and Ephrussi, 2004).

4. mRNA TRANSLATION

Upon delivery to cytoplasm, the information encoded in the mRNA will be translated to a protein, the final product of gene expression. Some mRNAs are engaged in translation directly upon export, other mRNAs have to be transported to a specific place in the cell prior to translation, while still others are stored in the cytoplasm to be used later on.

Upon entering cytoplasm before and/or during mRNA translation, the mRNP composition is subjected to major changes (reviewed in Moore, 2005). Export receptors (such as TAP) and adaptors (such as REF or SR proteins), shuttling hnRNP proteins and other nuclear factors (such as EJC) are removed from the mRNA as the mRNA is transiting the nuclear pore, soon after reaching the cytoplasm, or during mRNA translation. Nuclear cap and poly(A) binding proteins have to be exchanged with their cytoplasmic counterparts, eukaryotic initiation factor 4E (eIF4E) and cytoplasmic poly(A) binding proteins (PABPs), respectively.

The factors can be removed from mRNP by several means. It can be a special mechanism, such as the phosphorylation that promotes dissociation of SR proteins from mRNA (see above). Another possibility would be that a specific protein, such as the DEAD box RNA helicase Dbp5, actively removes proteins from mRNA. Factors can also leave mRNA because of competition with other factors. For example, low

concentration of CBP20/80 and PABPN1 in cytoplasm coupled with the high concentration of eIF4E and PABPs could naturally lead to the latter set replacing the former with a reasonable dissociation rate (Moore, 2005). Finally, ribosomes can remove nuclear factors from mRNA during the so called “pioneering round” of translation when 80S ribosomes passes along mRNA for the first time (Ishigaki et al., 2001).

For translation, mRNA associates with the necessary initiation factors and binds to a 40S ribosomal subunit associated with additional factors and transfer RNA. The 40S subunit scans the 5' end of the mRNA for an initiator codon. The recognition of the initiator codon (generally an AUG codon) and the subsequent joining of a 60S ribosomal subunit complete the initiation phase of protein synthesis and create an 80S ribosome that decodes each nucleotide triplet and elongate a polypeptide chain until it meets a nonsense or stop codon (UAA, UAG or UGA). Recognition of a stop codon promotes the release of the polypeptide and ribosome (Amrani et al., 2006).

Two classes of proteins affect translation by modulating the mRNP structure, viz. DEAD-box RNA helicases and Y-box proteins (Fig. 4).

DEAD-box RNA helicases

RNA helicases are enzymes that are thought to unwind double stranded RNA (dsRNA) molecules in an energy-dependent fashion by hydrolysis of NTP, preferentially ATP. The DEAD-box and the related DEAH, DExH and DExD families are commonly referred to as the DExD/H helicase family. DEAD box proteins are identified by the presence of nine conserved motifs (Rocak and Linder, 2004). The motifs appear at similar positions in all members of the family, defining a highly conserved helicase core. The core is involved in the binding of RNA and ATP. It is usually flanked by divergent amino- and carboxy-terminal sequences that can be responsible for substrate specificity and/or might direct the protein to a specific subcellular location (de la Cruz et al., 1999; Silverman et al., 2003). Today, over 500 different DEAD-box-protein sequences are present in protein databases (Rocak and Linder, 2004).

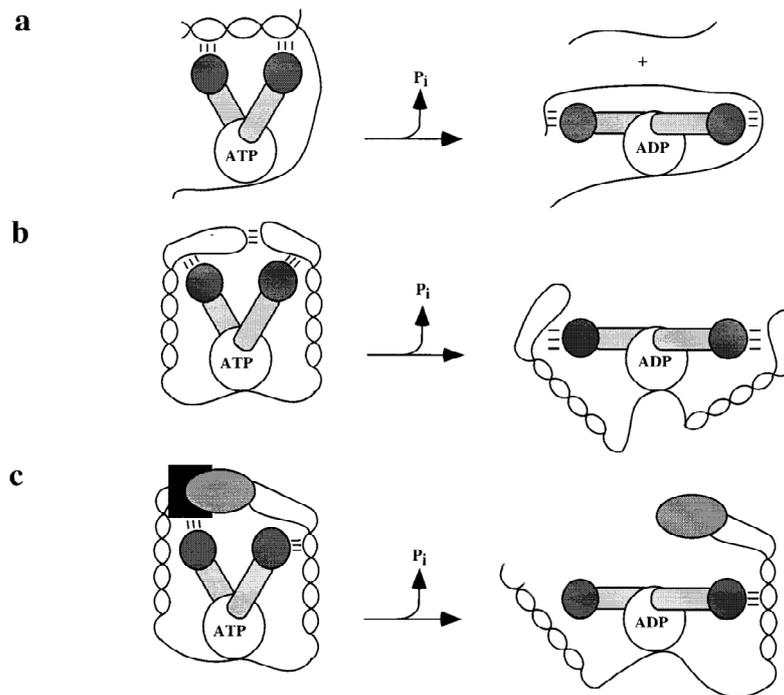


Figure 5. Proposed functions for DEAD-box proteins: (a) unwinding of dsRNA; (b) rearrangement of RNA tertiary structure; (C et al.) alteration of RNA-protein or protein-protein interactions. Modified from Lorsch and Herschlag, 1998.

DEAD-box proteins are involved in splicing, ribosome biogenesis and mRNA export. However, only a few are directly related to the translation process (Linder, 2003): the initiation factor eIF4A, yeast Ded1p, *Drosophila melanogaster* Vasa (Carrera et al., 2000), *Xenopus* Xp54 and its homologues (Weston and Sommerville, 2006) and RNA helicase A (Hartman et al., 2006). Vasa and Xp54 play a role in mRNA translation during embryogenesis, and RNA helicase A is necessary for translation of selected unspliced mRNAs, while eIF4A and Ded1 seem to be more general translation factors. As these two latter proteins are especially relevant for the present thesis, they will be presented in some detail.

The eIF4A protein is the prototype of the DEAD box family and the most extensively described RNA helicase (Rogers et al., 2002). The eIF4A is an important factor for cap-dependent translation initiation. According to the current model, eIF4A unwinds the local secondary structure of the 5'-UTR and maintains the mRNA in a single stranded

form that is appropriate for scanning of the 40S ribosomal subunit to find the initiation codon (Sonenberg, 1988). The requirement for eIF4A in translation is in direct proportion to the degree of secondary structure in the 5' of mRNA (Svitkin et al., 2001). eIF4A works as a component of the eIF4F, and the unwinding activity of eIF4F is approximately 20-fold more efficient than the activity of free eIF4A (Ray et al., 1985). The activity of eIF4A can be stimulated by addition of eIF4B or eIF4H (Richter-Cook et al., 1998; Rogers et al., 2001; Rozen et al., 1990). The relatively low helicase activity of eIF4A could be compensated in the cell by a large excess of eIF4A. Unlike many of the initiation factors that are present in 0.2-1.5 copies per ribosome, eIF4A is appears as 3-4 copies per ribosome (Hershey and Merrick, 2000).

There are three isoforms of eIF4A (I-III), which possess similar RNA-dependent ATPase activities and ATP-dependent RNA helicase activities *in vitro*. Only eIF4AI and eIF4AII appear to function in protein synthesis, whereas eIF4AIII inhibits translation (Li et al., 1999). Recently, it was shown that eIF4AIII is localized in the nucleus. It is a component of EJC and required for mRNA localization and nonsense-mediated mRNA decay (Chan et al., 2004; Palacios et al., 2004; Shibuya et al., 2004).

For many years eIF4A was the only ATPase identified in the translation initiation pathway, and it was considered to be the “motor” for scanning (Hershey and Merrick, 2000). However, when another member of the DEAD-box family, Ded1, was shown to exhibit RNA helicase activity (Iost et al., 1999) and proven to be essential for translation initiation in yeast (Chuang et al., 1997; de la Cruz et al., 1997), the picture became more complex (Fig. 6). The function of Ded1 and eIF4A are not redundant: deletion of the Ded1 gene or the gene encoding the eIF4A yeast homologues (Tif1 and Tif2) is lethal. Although involvement of Ded1 in mammalian translation remains unknown, yeast Ded1 can be substituted by mouse, human and *Drosophila* homologues (Chuang et al., 1997; Johnstone et al., 2005; Mamiya and Worman, 1999) 2005). This result suggests that the role of Ded1 in translation is conserved in mammals. A recent report showed that the capability of 40S ribosomal subunits to maintain scanning competence

on a long, relatively unstructured 5' UTR is more dependent on Ded1 than on eIF4A (Berthelot et al., 2004). Based on this observation, it was proposed that Ded1 facilitates the scanning process not by unwinding secondary structures but by another, still unknown mechanism. It might be that Ded1 works in translation not only as an RNA helicase but also as an RNase, facilitating translation by removing proteins (e.g. shuttling SR and hnRNP proteins or EJC components) from 5'-UTR during the 40S scanning. It is interesting to note in this context that Ded1 is able to remove EJC from mRNA *in vitro* (Fairman et al., 2004).

Strikingly, Ded1-like proteins seem to play a crucial role in germ cells. The murine homolog of Ded1, PL10, was originally described as a male germ specific RNA helicase (Leroy et al., 1989). It was also shown that the genes encoding DBX and DBY, the human homologues of Ded1, are located on the X and Y chromosomes, respectively (Lahn and Page, 1997). Deletions of DBY were found in men with most severe testicular pathology (Foresta et al., 2000). Analysis of the expression of the DBX and DBY genes showed that while both genes are transcribed in many different human tissues, the DBY protein is synthesized only in testis (Ditton et al., 2004). In *Xenopus*, the Ded1 homologue is called An3. The An3 mRNA is recorded in oocytes and located in the animal hemisphere (Gururajan et al., 1991; Rebagliati et al., 1985). In *Drosophila*,

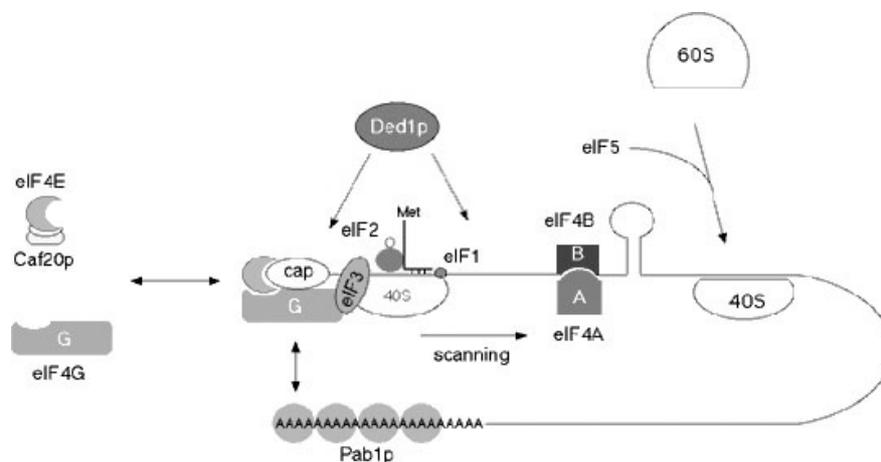


Figure 6. Possible roles for yeast Ded1 in translation initiation. Ded1 might influence cap recognition by initiation factors or the scanning of the 5'-UTR by 40S ribosomal subunit. eIF, initiation factor; 40S and 60S, ribosomal subunits; Pab1; cytoplasmic poly(A)-binding protein. From Patrick Linder group webpage.

the Ded1 homologue Bel is accumulated in the posterior part of the oocyte, and hypomorphic *bel* mutants are male-sterile (Johnstone et al., 2005). Thus, available data suggest that the Ded1-like family of DEAD-box proteins has an evolutionarily conserved role in fertility and during development. Since Ded1-like proteins are likely to work as translation factors, one can assume that their role in development might be coupled to the regulation of translation of specific mRNAs required at certain stages of development. It should, however, be emphasized that in the species mentioned the Ded1-like proteins are also expressed in the somatic tissues, indicating a general role in translation.

Y-box proteins

The Y-box binding (YB) proteins belong to a family of conservative multifunctional nucleic acid binding proteins (Graumann and Marahiel, 1998; Wolffe, 1994; Wolffe et al., 1992). They consist of three domains: variable N and C- terminal parts and a very conserved, 80 amino acid long, cold shock domain (CSD). The CSD exhibits more than 45% identity with bacterial cold shock proteins (Wistow, 1990). It is a five-stranded β -barrel containing RNP1- and RNP2-like consensus motifs that recognize both DNA and RNA (Bouvet et al., 1995; Schindelin et al., 1993; Wolffe, 1994). Comparison of the three-dimensional structures of RBD and CSD revealed that YB and hnRNP proteins might use a common RNA-binding strategy (Manival et al., 2001). The N-terminal domain of YB proteins is rich in alanine and proline and has an actin-binding motif (Ruzanov et al., 1999). In vertebrates, the C-terminal domain contains alternating clusters of basic and acidic amino acids (Murray et al., 1992), binds DNA and RNA in a sequence-independent manner, and mediates protein-protein interactions (Sommerville and Ladomery, 1996b; Wolffe, 1994).

YB proteins are named after the binding specificity originally shown for the human YB-1. This protein was shown to bind to the Y-box sequence present in the promoter region of the MHC class II genes (Didier et al., 1988). Further investigations revealed that YB proteins are ssDNA binding transcriptional factors involved in the activation and repression of many genes (reviewed in (Swamynathan et al., 1998).

YB proteins are also involved in pre-mRNA splicing. YB-1 binds A/C-rich exon enhancer elements and thereby directs alternative splicing (Stickeler et al., 2001). Moreover, YB-1 was found in prespliceosomes (Hartmuth et al., 2002), and it also has been shown that YB-1 interacts with the SR protein SRp30 and affects splice site selection (Raffetseder et al., 2003).

Apart from its function in transcription and splicing, YB proteins are RNA binding proteins and act as regulators of mRNA translation. Analysis of proteins from stored maternal mRNPs in *Xenopus* oocytes revealed that the YB protein FRGY2 is an abundant component in the mRNPs and prevent the mRNA from being translated (masking role) (Richter and Smith, 1984; Murray et al, 1992; Sommerville and Ladomery, 1996a). Later on, it was shown that proteins homologous to FRGY2 (MSY1, MSY2 and MSY3) are expressed during murine spermatogenesis and oogenesis and form complexes with stored mRNAs (Gu et al., 1998; Kwon et al., 1993; Mastrangelo and Kleene, 2000; Tafuri et al., 1993). Absence of MSY2 results in male and female infertility (Yang et al., 2005a). A *Drosophila* YB protein, Yps, was recorded as a component of *oskar* mRNA localization granules in oocytes (Wilhelm et al., 2000), and it seems to be involved in the regulation of localization and translation of *oskar* mRNA (Mansfield et al., 2002).

While YB proteins mask mRNA in germ cells, the YB protein YB-1 is a major component of polysome-associated and free mRNPs in mammalian somatic cells (Evdokimova et al., 1995). In these cells, YB-1 is required for initiation of mRNA translation (Evdokimova et al., 1998) and protects mRNA from 5' end degradation (Evdokimova et al., 2001). Like several other RNA-binding proteins, YB-1 contributes to the cap dependence of translation preventing spontaneous initiation at noncanonical initiation sites (Svitkin et al., 1996). On the other hand, an excess of YB-1 on mRNA inhibits protein synthesis both *in vitro* and *in vivo* (Davydova et al., 1997; Nekrasov et al., 2003; Pisarev et al., 2002). It has been shown *in vitro* that YB-1 can melt secondary structure of RNA and promote annealing of complementary single stranded RNA

molecules (Evdokimova et al., 1995; Skabkin et al., 2001). YB-1 and its germ cell counterparts are considered to be structural proteins, working as RNA “histones” responsible for mRNA packaging and formation of the mRNP structure, and in this way affecting the translation of mRNA (Evdokimova et al., 1995; Tafuri and Wolffe, 1993).

The fact that inactive mRNPs contain about two times more YB proteins than active polysome-associated mRNPs suggested that the inhibitory and stimulatory effect of YB proteins on mRNA translation depends on the YB protein/mRNA ratio (Fig. 7). Binding of a few YB protein molecules to mRNA makes the mRNA accessible for binding of translation factors and/or facilitates the scanning of the 5'-UTR by 40S ribosomal subunits. Additional binding of YB proteins leads to condensation of the mRNP structure and inhibition of translation (Evdokimova and Ovchinnikov, 1999; Minich and Ovchinnikov, 1992).

The precise molecular mechanism that regulates the amount of YB proteins on mRNA is still unknown. FRGY2 and YB-1 were found to be phosphorylated by casein kinase II (ckII). This enzyme is also present in stored mRNPs and its activity correlates with the degree of mRNA masking in *Xenopus* oocytes. These results led to the suggestion that phosphorylation can regulate the YB protein–RNA interaction (Sommerville and Ladomery, 1996a). However, the direct effect of ckII phosphorylation on the binding of the YB proteins to RNA is controversial (Evdokimova and Ovchinnikov, 1999). Recently, it was shown that YB-1 can be phosphorylated by Akt kinase and that such a phosphorylation facilitates translational activation of silenced mRNAs (Evdokimova et al., 2006). An alternative mechanism is based on the fact that a Y-box protein-associated protein, YBAP1/p32, interacts with YB-1. YBAP1 is an acidic protein and its interaction with YB-1 results in the release of YB-1 from reconstituted YB-1-mRNA complexes, thereby reducing the translation repression caused by YB-1 in the *in vitro* system (Matsumoto et al., 2005).

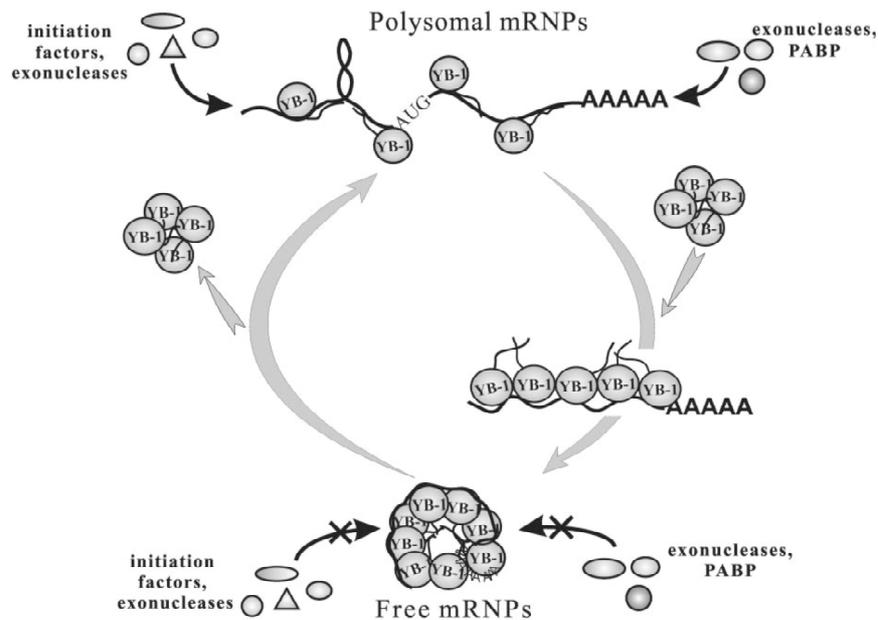


Figure 7. The relative amount of YB-1 in the mRNP affects translational activity of mRNA. A high YB-1/mRNA ratio silences mRNA, while a lower ratio facilitates translation. From Skabkin et al., 2004.

5. mRNA DEGRADATION

The poly(A) tail of the mRNA is continually shortened during translation until a critical minimum length has been reached, usually 10-25 adenylate residues (reviewed in Amrani et al., 2006). This shortening minimizes the available binding sites for PABP, thereby altering the structure of the translated mRNP and the ability of additional proteins to bind to the tail. In particular, the poly(A) tail loses its connection with the initiation complex at the cap region, which promotes mRNA decay. Decapping and deadenylating enzymes bind to the 5' and 3' ends of the mRNA, respectively. Once decapped and/or deadenylated, the transcript is degraded by exonucleases. mRNA is degraded in a cytoplasmic processing body, the P body.

If mRNA contains a premature termination codon (PTC), it can be degraded by a process called nonsense-mediated mRNA decay (NMD). There are at least two models describing how a cell distinguishes between a PTC and a normal termination codon. In mammalian cells, recognition of a PTC takes place during the "pioneer round" of translation and depends on the presence of an EJC (reviewed in (Maquat, 2004). For proper recognition, the PTC should reside >50-55 nucleotides upstream from the EJC. When a ribosome stops at a PTC and recruits translation termination factors, it interacts

with the downstream EJC, NMD proteins are recruited to the transcript, and the mRNA is rapidly degraded (Behm-Ansmant and Izaurralde, 2006; Lejeune and Maquat, 2005). Mammalian NMD only takes place on CBP80/20-bound mRNA and not on eIF4E-bound mRNA. This implies that the pioneer translation initiation complex differs from the steady-state translation initiation complex (Chiu et al., 2004; Lejeune et al., 2002; Lejeune et al., 2004).

The alternative model is pertained to NMD in yeast and *Drosophila*. There PTC recognition occurs independently of the EJC. It is proposed that premature translation termination is intrinsically aberrant, as the PTC is not in the appropriate context (Amrani et al., 2006; Conti and Izaurralde, 2005). According to this model (the false UTR model), the 3'-UTRs would be marked by a specific set of proteins. If a terminating ribosome is able to interact with these 3'-UTR-bound proteins, proper termination can occur. However, if the terminating ribosome cannot establish these interactions, the termination process is impaired or too slow. The NMD complex will then be assembled, and the mRNA is rapidly degraded (Conti and Izaurralde, 2005). One of the 3'-UTR bound factors that can stimulate the termination process is a cytoplasmic poly(A)-binding protein (Amrani et al., 2006).

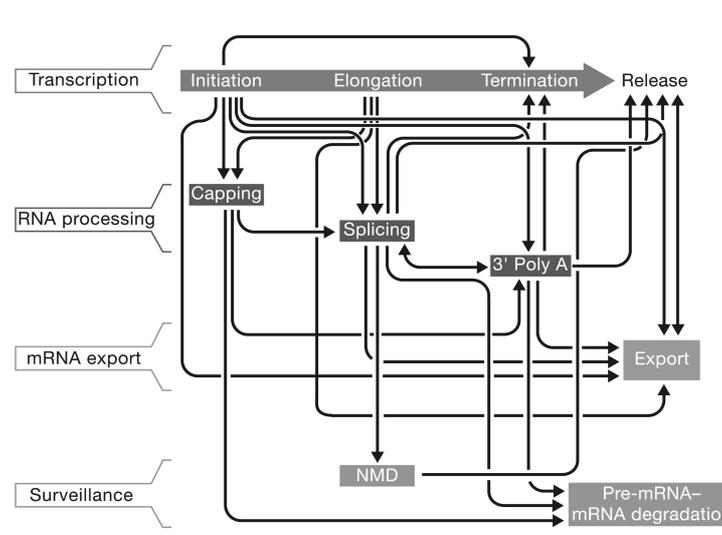


Figure 8. Interconnections of various steps in gene expression. From Maniatis and Reed, 2002.

6. COUPLING OF THE GENE EXPRESSION STEPS

The many steps of gene expression are not simply following each other but rather intimately coupled to each other (Fig. 8). For example, nuclear processes such as transcription, processing, mRNP assembly and mRNA export are interconnected by influencing each other and often share protein components. Indeed, coupling occurs not only between sequential steps but also between the earliest and latest steps (Maniatis and Reed, 2002). It has been shown that nuclear events, in particular splicing, influence cytoplasmic processes such as mRNA localization, mRNA masking and mRNA degradation (reviewed in Farina and Singer, 2002; Matsumoto and Wolffe, 1998; Wilkinson, 2005). Moreover, recent data have revealed that splicing could even enhance mRNA translation (Nott et al., 2004).

PRESENT INVESTIGATION

Aims of the study

The main objective of this thesis was to clarify the connection between mRNP assembly on the gene and mRNA translation in the cytoplasm using *Chironomus tentans* as experimental system.

The specific aims of this thesis were:

- I To establish whether the putative translation regulator ctYB-1 is already loaded onto pre-mRNA co-transcriptionally.
- II To study two size variants of ctYB-1 in relation to a specific mRNA.
- III To investigate the behaviour of a Ded1-like RNA helicase during the transfer of mRNA from genes to polysomes and the possible function of the helicase.
- IV To identify a binding partner for the Ded1-like RNA helicase.

The Balbiani ring experimental system

The dipteran *Chironomus tentans* is a widely spread, non-biting midge. The larval stage of *C.tentans* is divided into four instars. The fourth instar larvae are 1-2 cm long, and their salivary glands produce massive amounts of secretory proteins. The proteins assemble into insoluble silken threads that the larvae use to build a burrow, later on also used for pupation (Case and Wieslander, 1992). A salivary gland is approximately 1-2 mm in length and consists of 30-40 secretory cells with huge nuclei. Each nucleus contains four large polytene chromosomes (I-IV).

The sites of intensive transcription on the chromosomes appear as expanded regions, puffs. The biggest puffs on chromosomes IV are referred to as Balbiani rings (BRs), named after E.G. Balbiani who described them in 1881 (Balbiani, 1881). BRs contain genes, which encode the main secretory proteins of the glands. The BR1 and BR2 genes on chromosome IV are closely related and belong to the same gene family. They are about 35-40 kilobases long, are internally repetitive, and encode secretory proteins of 850-1100 kDa in size (Case and Wieslander, 1992; Sümegi et al., 1982). The genes contain only four short introns, and hence the mature BR mRNA is only marginally reduced in size during processing and appears as a 75S molecule (Case and Daneholt, 1978).

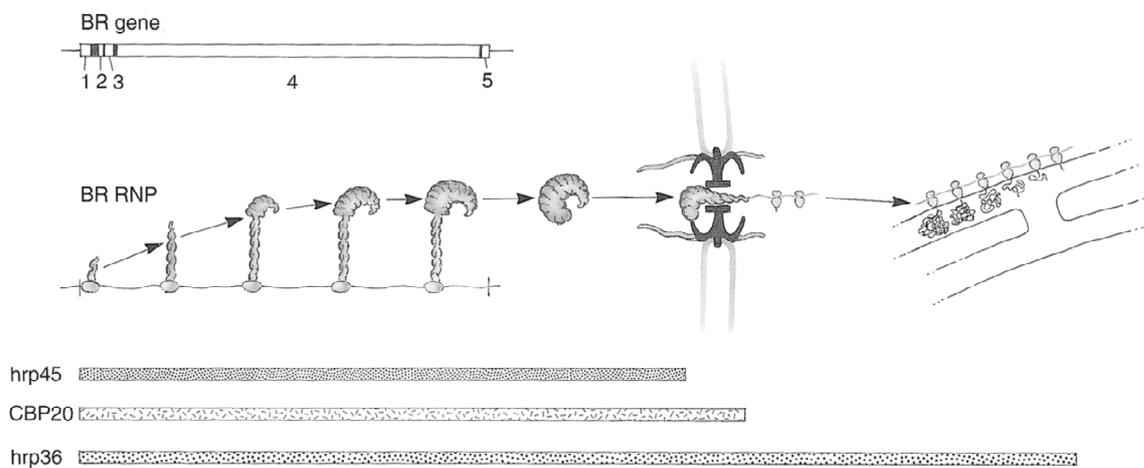


Figure 9. The Balbiani ring experimental system. The BR gene with its five exons is displayed on the top. Proteins and the BR RNA are assembled to the BR RNP particle on the gene. The BR particle is transported to the NPC, unfolds and translocates to the cytoplasm, where it associates with ribosomes. The flow of three RNA-binding proteins in the BR mRNP is outlined below. Modified from Daneholt, 1997.

The enormous size of the BR mRNA makes it possible, using electron microscopy, to visualize BR mRNP during both assembly on the gene and nucleocytoplasmic transport (Fig. 9). During transcription the BR RNP fibril is folded into a ribbon, which is gradually bent into a ring-like structure, 50 nm in diameter, that can be identified in the nucleoplasm as a dense particle (Skoglund et al., 1983; Skoglund et al., 1986). During translocation through the nuclear pore, the ring-like structure becomes elongated and the mRNP fibril unfolds when it reaches the cytoplasm (Kiseleva et al., 1996; Mehlin et al., 1992). The passage of the BR mRNP through the pore starts with the 5' end of the transcript in the lead. As soon as the 5' end reaches the cytoplasm proteins synthesis

is initiated, even though the 3' end of the mRNA still remains in the nucleus (Mehlin et al., 1992). Although BR mRNP particles are not recorded in the cytoplasm, 75S BR mRNA can be recovered from large-size polysomes on rough endoplasmic reticulum (Daneholt et al., 1977).

The fate of individual RNA-binding proteins associated with BR mRNP can be followed by immuno-EM (reviewed in Daneholt, 1997) (Fig. 9). It has been shown that some proteins, e.g. the hnRNP A1-like protein hrp36, bind BR mRNA co-transcriptionally and accompany it from the gene to the polysomes (Visa et al., 1996). The SR protein hrp45 associates with BR RNA on the gene and is released at the nuclear pore (Alzhanova-Ericsson et al., 1996). Other proteins, e.g. CBP20 and PABPN1, are loaded onto the nascent RNA and released from BR mRNP upon entry into the cytoplasm (Bear et al., 2003; Visa et al., 1996b).

RESULTS AND DISCUSSION

Paper I

In somatic cells of vertebrates, the Y-box protein YB-1 and its homologues regulate mRNA translation in cytoplasm and transcription in the nucleus. To explore the possibility that YB-1 may bind mRNA already concomitant with transcription influencing both the nuclear and cytoplasmic fate of the transcript, we studied the behaviour of the *C.tentans* equivalent of YB-1, ctYB-1.

An antibody to mammalian YB-1 was used to screen a cDNA expression library from *C.tentans* salivary glands. Two ctYB-1 size variants, p40 and p50, were found. The variants share the first 258 amino acids, while the C-termini differ; the p40-specific terminus comprises six amino acids and the p50-specific 59 amino acids. The longer variant of ctYB-1 has an amino acid sequence that is 47% identical to that of mammalian YB-1. ctYB-1 displays the three-domain organization characteristic of Y-box proteins.

An antibody, raised against the p40 variant, recognized two proteins in salivary gland cells, 40 kDa and 50 kDa, corresponding to the two variants of ctYB-1. Only the p40 variant was detected in tissue culture cells, originally derived from *C.tentans* embryonic epithelial cells. Using Western blot analysis and immunocytology we showed that ctYB-1 is a very abundant protein in cytoplasm and is also present in the nucleus. By an *in vivo* UV crosslinking technique we revealed that in both compartments ctYB-1 is bound to poly(A)⁺ RNA. On isolated polytene chromosomes, the ctYB-1 antibody labeled Balbiani rings (BRs) and a large number of smaller puffs. The labeling disappeared when the chromosomes were treated with RNase. We conclude that the occurrence of ctYB-1 in the puffs is dependent on RNA, not DNA, and that ctYB-1 binds to RNA (including BR RNA) already at the site of transcription.

To further investigate the behaviour of ctYB-1 during the assembly and transport of BR RNP, we studied isolated chromosomes and cryosections from salivary glands by immunoelectron microscopy (IEM). ctYB-1 was added to the BR pre-mRNA early

during transcription and remained on the transcript during the course of transcription. In the nucleoplasm ctYB-1 was found associated with released BR mRNPs, while in the cytoplasm ctYB-1 was localized at the tubular ER, where translation of BR mRNA occurs. Together the biochemical and IEM data demonstrate that ctYB-1 binds to mRNA co-transcriptionally and appears with mRNA during nucleocytoplasmic transport and translation (see also papers II and IV). We conclude that ctYB-1 accompanies mRNA from gene to polysomes.

The finding that ctYB-1 binds mRNA already co-transcriptionally suggests that YB-1 can be involved in the formation of mRNP already during transcription along with hnRNP and SR proteins. In fact, Y-box proteins and hnRNP proteins resemble each other as to structural organization, RNA-binding strategies and ability to change secondary structure of RNA (Manival et al., 2001). Moreover, YB-1 is known to interact with SR proteins and affect alternative splicing. However, hnRNP proteins are located mainly in the nucleus, and are major components of hnRNPs, but usually leave the mRNA upon its entry into cytoplasm or prior to initiation of translation. In contrast, YB-1 is mainly located in the cytoplasm, is a major component of cytoplasmic mRNPs and stays with mRNA during translation. Thus, although the main structural components in nuclear and cytoplasmic mRNPs are the same, the relative abundance of the proteins changes upon transit from nucleus to cytoplasm. In the nucleus, hnRNPs and mRNPs are packed mainly by hnRNP and SR proteins to ensure proper processing and nucleocytoplasmic transport. The mRNP structure is drastically altered upon entry of the mRNA into the cytoplasm: the majority of the hnRNP and SR proteins are released, and additional YB-1 is recruited to the mRNA forming an mRNP structure favourable for translation.

What does it imply that YB-1 is bound to mRNA concomitant with transcription? The answer is probably that despite the similarity to the hnRNP proteins, YB-1 has specific features necessary for proper organization of mRNP in the nucleus. Another possibility would be that YB-1 marks mRNA in the nucleus for proper translation in the cytoplasm.

Paper II

In the vertebrate germ and somatic cells, Y-box proteins exist in multiple variants. Whether a specific transcript harbours just one or many Y-box protein variants is still

unclear. We studied the appearance of two size variants of the *C.tentans* ctYB-1 (p40 and p50) in salivary gland cells in relation to the BR mRNA transcript.

We showed by Southern blot analysis of total DNA and *in situ* hybridization on polytene chromosomes that there is only one ctYB-1 gene, which implies that the two ctYB-1 size variants are splicing isoforms. Western blot analysis of extracts from different larval tissues revealed that p40 and p50 were present in all samples although in different proportions. In salivary glands, both splicing variants of ctYB-1 were recorded in the nucleus as well as cytoplasm. Isolation of nuclear BR RNPs and cytoplasmic giant-sized BR polysomes showed that the two splicing isoforms of ctYB-1 are associated with both nuclear and cytoplasmic BR mRNA. The ability of the p50-specific antibody to co-immunoprecipitate p40 from BR polysomes suggests that the two ctYB-1 variants appear on the same BR mRNA molecule.

Previous studies have revealed that Y-box proteins are ubiquitously expressed in all germ and somatic cells. We now show that the relative amounts of ctYB-1 isoforms vary in different tissues. A similar result has been obtained in murine somatic cells where the mRNA expression levels of Y-box isoforms are different (Mastrangelo and Kleene, 2000). These results suggest that the pattern of expression of Y-box proteins differ between tissues. We note that the ratio between the two ctYB-1 variants associated with BR mRNA corresponded to the ratio between the variants in the cell extract, suggesting that the appearance of the isoforms on mRNA could simply reflect the expression levels of the variants.

We have shown that both ctYB-1 variants are associated with the same BR mRNA molecule. Similarly, it has been shown previously that in murine germ cells two Y-box proteins bind to the 3'-UTR of protamine 1 mRNA and co-immunoprecipitate in an RNA-dependent manner (Davies et al., 2000; Giorgini et al., 2001). Thus, also these experiments suggest that different Y-box proteins can appear on the same mRNA transcript. This is perhaps not unexpected as the main RNA-binding specificity is provided by the cold shock domain (CSD) (Evdokimova et al., 2006; Bouvet et al., 1995; Giorgini et al., 2002; Yang et al., 2005b). Since Y-box proteins have almost identical CSDs, the RNA binding specificity of different Y-box proteins isoforms should be similar or the same. Thus, one might expect that the pattern of Y-box proteins on a specific

message should depend on the sequence of the transcript and the expression levels of the Y-box isoforms.

YB-1 is a predominant protein in cytoplasmic mRNPs and regulates the translation of mRNA by modifying the structure of the mRNP template. It might also work as a binding platform for other proteins. The two ctYB-1 variants differ in their C-terminal domain, which is likely to be responsible for protein-protein interactions and unspecific RNA binding. Thus, it is possible that the p40 and p50 variants affect mRNA translation differently by recruiting, or not recruiting, certain factors to mRNA or by modulating the mRNP structure in a variant-specific manner.

Paper III

Ded1 is a DEAD box RNA helicase essential for the initiation of mRNA translation in yeast, and it is highly conserved from yeast (Ded1) to mammals (DDX3). However, the function of Ded1-like proteins in higher eukaryotes is still elusive. The Ded1 homologue in *Chironomus tentans* is hrp84. In this study we investigated the behavior of hrp84 during the transfer of mRNA from genes to polysomes and explored whether hrp84 exert its function in the nucleus and/or the cytoplasm.

We observed that hrp84 appears abundantly in the cytoplasm but is also present in the nucleus. Furthermore, hrp84 was located in the chromosomal puffs on the polytene chromosomes in an RNA dependent manner. Immunoelectron microscopy revealed that hrp84 is added co-transcriptionally to the nascent pre-mRNA transcript in the giant BR puffs and remains associated with the RNA during processing, nucleocytoplasmic transport, and translation. Injection of an hrp84 antibody into the nucleus did not affect mRNP assembly or transport, while injection into the cytoplasm blocked mRNA translation. We conclude that hrp84 gets bound to mRNA already in the nucleus concomitant with transcription, accompanies the mRNA to the cytoplasm, where it exerts its function during translation.

How does hrp84 affect mRNA translation? It seems likely that hrp84 functions in a similar way as the yeast Ded1, i.e. by activating translation initiation. More specifically, it might influence the recognition of cap by initiation factors and the 40S ribosomal

subunit or the scanning of the 5'-UTR by the 40S ribosomal subunit (see Introduction for details).

The fact that hrp84 is located in the nucleus and binds mRNA already co-transcriptionally suggests that the protein could also play a role in the nucleus. Indeed, earlier studies have indicated that members of the Ded1 subfamily could be present in the nucleus. Mammalian DDX3 is required for Rev-dependent export of HIV RNA, while the yeast Ded1 was originally described as a suppressor of the splicing factor Prp8 (Yedavalli et al., 2004; Jamieson et al., 1991). However, we could not find any effect of the hrp84 antibody on the assembly or transport of BR mRNP. These observations do not rule out a nuclear function of the protein, but we have not found any evidence for a role other than the cytoplasmic one.

Paper IV

The activity of DEAD-box helicases is often modulated by protein cofactors. Clearly, to fully understand the function of a helicase, specific interacting proteins have to be identified. In this study we identified and characterized a novel *C. tentans* DEAD-box protein, hrp84, and searched for interacting proteins.

Sequence analysis of a cDNA encoding a putative RNA-binding protein, hrp84, revealed that the protein contains a typical helicase core domain, a glycine rich C-terminal part and a putative nuclear export signal. The helicase core domain of hrp84 contains the nine highly conserved motifs characteristic of DEAD-box RNA helicases. The amino acid sequence of hrp84 has 55-60% sequence identity to yeast and vertebrate homologues of the DEAD-box RNA helicase Ded1. Thus, hrp84 is likely to be the *C.tentans* homologue of Ded1.

We have revealed by immunoprecipitation that hrp84 is associated with ctYB-1 both in the nucleus and cytoplasm, and the two proteins also appear together in polysomes. The interaction is likely to be direct as shown by *in vitro* binding of purified components. Using *in vivo* UV-crosslinking we demonstrated that hrp84 is associated with mRNA in the nucleus and in the cytoplasm. We conclude that the mRNA-bound complex between hrp84 and ctYB-1 is formed in the nucleus and seem to accompany the mRNA to cytoplasm and most likely exerts its function in polysomes during translation

of the mRNA. As both Ded1 and YB-1 are known to regulate the initiation of translation, we propose that the hrp84/ctYB-1 protein complex affects the efficiency of the initiation process.

How does the ctYB-1/hrp84 complex exert its role during translation? One possibility would be that the complex modulates the overall structure of the mRNP and in this manner controls translation. The proteins might cooperate in such a way that hrp84 melts RNA duplex regions and ctYB-1 maintains the mRNA in a single-stranded form (Sommerville, 1999). Notably, it has been revealed that both Ded1 and YB-1 are involved in the 5'-UTR scanning process (Berthelot et al., 2004; Evdokimova et al., 1998), suggesting that the ctYB-1/hrp84 complex may organize the 5'-UTR structure in a manner favourable for translation. It seems that the ability of CSD-containing proteins to interact with DEAD-box RNA helicases is an ancient phenomenon. As shown in *Bacillus subtilis*, a cold shock protein, CspB, interacts with two putative DEAD-box RNA helicases, CshA and CshB, to rescue misfolded mRNA molecules and maintain proper initiation of translation at low temperature in bacteria (Hunger et al., 2006).

In germ cells, Y-box protein/RNA helicase complexes repress rather than stimulate translation. In *Drosophila* and *Xenopus* oocytes, the DEAD-box RNA helicase DDX6 cooperates with YB-1 homologues to keep maternal mRNA silent (Weston and Sommerville, 2006). Thus, a second function of DEAD box proteins might be to control the amount of Y-box proteins on the mRNA. The effect of Y-box proteins on translation might then depend on what DEAD box RNA helicase it interacts with. In oocytes, an RNA helicase (e.g. DDX6) promotes the binding of the Y-box protein to the mRNA and in this way blocks translation, while in somatic cells another RNA helicase (hrp84 and other members of the Ded1 family) might remove an excess of ctYB-1 and facilitate the engagement of the mRNA in translation.

GENERAL DISCUSSION

In the present thesis, I show that two evolutionarily conserved translation regulators, the Y-box protein ctYB-1 and the DEAD box protein hrp84, are associated with pre-mRNA and mRNA in the cell nucleus. ctYB-1 and hrp84 form a complex, presumably already on the pre-mRNA, and accompany mRNA from the site of transcription to polysomes. These observations indicate that the nuclear history of the mRNA, especially the RNP assembly on the gene, can affect translation competence of the mRNA in the cytoplasm. How could this happen? There are several options that do not exclude each other.

One possibility would be that for proper translational initiation in the cytoplasm some of the factors involved in this process have to bind to mRNA already in the nucleus during mRNP formation. These factors, e.g. the ctYB-1/ hrp84 complex, may affect the organization of the transcript in the mRNP particle and poise mRNA for efficient use during subsequent translation.

A second possibility is suggested by the observation in *Drosophila* that an RNA helicase-Y-box protein complex is involved in silencing mRNAs during transport from nurse cells to a specific cytoplasmic position in oocytes (see Introduction). Thus, it is close at hand to propose that also in somatic cells a Y-box protein and an associated RNA helicase form a non-translatable mRNP complex in the nucleus and keep mRNA silent during nucleocytoplasmic transport.

A third possibility to explain a nuclear history would be that splicing is coupled to translation. Recently, studies from the Cullen, Moore and Wilkinson laboratories have shown that the presence of an intron in pre-mRNA increases the translation rate of the spliced mRNA product (Gudikote et al., 2005; Nott et al., 2004; Wiegand et al., 2003). At least in part, the ability of splicing to enhance translation is mediated by EJC components, mainly by RNPS1 and Y14 (Nott et al., 2004; Wiegand et al., 2003). However, it is not clear how these EJC proteins are able to promote steady state translation since they are removed from mRNA upon the first passage of ribosomes along mRNA (Dostie and Dreyfuss, 2002; Lejeune et al., 2002; Nott et al., 2004). A second possibility would be the nucleocytoplasmic shuttling proteins, the hnRNP and

SR proteins, that first bind mRNAs in the nucleus and then travel with them to the cytoplasm (Gudikote et al., 2005; Sanford et al., 2004; Visa et al., 1996a; Windgassen et al., 2004). These proteins influence splicing, and it is possible that they could stimulate translation in a splicing-dependent way. A third possibility would be that splicing recruits translation factors to mRNA. In this case, the best candidates for such factors would be YB-1 and Ded1, which are known to be translational regulators. It remains to be shown whether recruitment of YB-1 and Ded1 to mRNA can be affected by splicing. However, an early yeast genetic study suggested that Ded1 is involved in splicing (Jamieson et al., 1991). Moreover, Ded1 was recorded in spliceosomes (Stevens et al., 2002). Finally, it has been shown that YB-1 is involved in alternative splicing, binds SR proteins and is part of prespliceosomes (Hartmuth et al., 2002; Raffetseder et al., 2003; Stickeler et al., 2001). Thus, recruitment of YB-1 and Ded1 to mRNA could be mediated by spliceosomal components, SR proteins or perhaps EJC factors. The binding of translation regulators to mRNA already in the nucleus might mark properly spliced mRNA and provide an advantage in ribosome recruitment in cytoplasm.

A fourth possibility would be that the nuclear YB-1/Ded1 complex could affect the subsequent location of mRNA in cytoplasm. It has been proposed that EJC might enhance translation by targeting mRNPs to subcellular locations highly active in protein synthesis (Nott et al., 2004). The mRNA translation in eukaryotes is believed to take place on actin filaments, and the efficiency of mammalian protein synthesis depends on an intact F-actin system (Stapulionis et al., 1997). Interestingly, it has been shown that YB-1 has an actin-binding motif in the N-terminal domain and binds preferentially to F-actin (Ruzanov et al., 1999). Thus, upon export to cytoplasm, mRNA provided with YB-1 could be targeted to the actin cytoskeleton for translation.

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