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TRANSCRIPTION AND TRANSPORT OF A MESSENGER RNP PARTICLE: NOVEL REGULATORY MECHANISMS

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Cover picture: Electron micrograph of a salivary gland cell nucleus microinjected with wheat germ agglutinin. The Balbiani ring granules accumulate along the nuclear envelope, but do not translocate through the nuclear pore complexes.

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To my family ♥

ABSTRACT

In all living cells gene expression is a central process that involves several steps, including transcription, processing, transport and translation. A fine-tuning of these events is a pre-requisite for proper cellular function. Many proteins are involved in this regulation and they are added to the nascent transcript concomitant with transcription, although they may exert their function much later. The messenger pre-mRNP particle formed needs accurate processing before exiting the cell nucleus through the nuclear pore complexes.

The midge *Chironomus tentans* provides a unique experimental system for the structural analysis of the mRNP assembly and transport, due to the abundance and size of the RNP particles derived from the giant Balbiani ring (BR) genes. These granules can readily be identified in the electron microscope from early transcription in the nucleus to polysome formation in the cytoplasm. In this thesis, several different aspects of the BR RNP biogenesis have been investigated.

We revealed that the RNA-binding protein hrp65-2 interacts directly with nuclear actin, and is present all along the active gene associated with nascent pre-mRNP. Disruption of this interaction leads to transcriptional inhibition. We propose that actin is a part of a chromatin remodeling complex, coupled to pre-mRNP via hrp65-2.

We have identified and characterized a transcript-specific hnRNP protein, hrp130, present along the Balbiani ring 3 gene. The domain structure of hrp130 is similar to the human protein CA150 that works as a repressor of transcription elongation. We propose that hrp130 enables proper splicing of the intron-rich BR3 gene transcripts by regulating the elongation rate.

We discovered a link between transcription and export of the BR particle, by inhibiting transcription and looking for transport defects over the nuclear envelope. Upon transcriptional inhibition, the binding of BR particles to the nuclear pore complexes was drastically reduced and the export was almost abolished.

The passage of mRNPs and ribosomal particles through the nuclear pore complex was inhibited by the nucleoporin-binding agents wheat germ agglutinin and the monoclonal antibody mAb 414. There was an accumulation of BR and ribosomal particles in the nucleoplasm and remarkably also a zone free from rRNPs beneath the nuclear envelope. We suggest that normally the basket fibrils move freely and repel molecules not binding to nucleoporins.

In summary, we have identified novel mechanisms for the regulation of BR RNP particle transcription and transport. We found an actin-hrp65-2 interaction essential for the transcription elongation process and a transcription elongation repressor, hrp130, enabling cotranscriptional splicing of an intron-rich gene. A close coupling between ongoing transcription and RNA export was detected, possibly taking place via regulation of the transport of the mRNPs to the nuclear pore complex. Finally, we identified an exclusion zone free from rRNPs beneath the nuclear envelope, the clearing probably accomplished by constantly moving basket fibrils.

LIST OF PUBLICATIONS

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

I. Piergiorgio Percipalle, Nathalie Fomproix, **Karin Kylberg**, Francesc Miralles, Birgitta Björkroth, Bertil Daneholt, and Neus Visa. (2003)
An actin-ribonucleoprotein interaction is involved in transcription by RNA polymerase II.
Proceedings of the National Academy of Sciences USA, 100:6475-6480.

II. Xin Sun, Jian Zhao, **Karin Kylberg**, Teresa Soop, Kevin Palka, Erik Sonnhammer, Neus Visa, Alla T. Alzhanova-Ericsson, and Bertil Daneholt. (2004)
Conspicuous accumulation of transcription elongation repressor hrp130/CA150 on the intron-rich Balbiani ring 3 gene.
Chromosoma, 113:244-257.

III. **Karin Kylberg**, Birgitta Björkroth, Birgitta Ivarsson, and Bertil Daneholt.
Close coupling between transcription and exit of mRNP from the cell nucleus.
Experimental Cell Research, under revision.

IV. **Karin Kylberg**, Nathalie Fomproix, Birgitta Ivarsson, and Bertil Daneholt.
Exclusion of mRNPs and ribosomal particles from a thin zone beneath the nuclear envelope upon inhibition of transport.
Submitted.

CONTENTS

LIST OF ABBREVIATIONS

BR	Balbiani ring
C-terminal	Carboxy terminal
CTD	Carboxy terminal repeat domain
DNA	Deoxyribonucleic acid
DRB	5, 6-dichloro-1-β-D-ribofuranosyl-benzimidazole
EJC	Exon-exon junction complex
ER	Endoplasmic reticulum
EM	Electron microscopy
hnRNP	Heterogeneous nuclear ribonucleoprotein
IEM	Immunoelectron microscopy
kb	Kilobase
kDa	Kilodalton
MDa	Megadalton
mRNA	Messenger RNA
mRNP	Messenger RNP
N-terminal	Amino terminal
NE	Nuclear envelope
NES	Nuclear export signal
NLS	Nuclear localization signal
nm	Nanometer
NPC	Nuclear pore complex
nt	Nucleotide
Nup	Nucleoporin
Pre-mRNA	Precursor messenger RNA
RNA	Ribonucleic acid
RNA pol	RNA polymerase
RNP	Ribonucleoprotein
rRNA	Ribosomal RNA
S	Svedberg unit
TREX	Transcription and export complex
WGA	Wheat germ agglutinin
Å	Ångström

1 INTRODUCTION

The compartmentalization of the eukaryotic cell requires that the genetic information (DNA) in the nucleus is transferred to the translation machinery in the cytoplasm. This information transfer is accomplished by a messenger molecule, the ribonucleic acid (RNA). There are several types of RNA molecules produced by one of the three RNA polymerases: messenger RNA (mRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and transfer RNA (tRNA), with specific functions in gene expression. The protein-encoding mRNA molecules are produced by RNA polymerase II in an immature form, precursor mRNA (pre-mRNA), that is processed with the help of snRNAs in the spliceosomes to mature mRNA. In the cytoplasm the mRNAs are translated into an amino acid sequence in the ribosomes by tRNAs delivering the amino acids. The rRNA constitutes the ribosomal backbone. Thus, only the mRNAs are translated into protein. The pre-mRNA undergoes several processing steps before allowed admittance to the cytoplasm: capping, splicing and polyadenylation. In addition, it becomes associated with a large set of RNA-binding proteins for proper function, regulation and stability. Furthermore, a surveillance machinery monitors the transcription and the formation of mRNPs, which respond to malformed or non-processed transcripts by retention and degradation.

All transport between the nucleus and cytoplasm is accommodated by large protein assemblies spanning the nuclear envelope, the nuclear pore complexes (NPCs). Through this gateway proteins, RNAs and other molecules are translocated at high speed. The transport must be directional and ensure that only properly processed molecules leave or enter the nucleus. Distinct proteins and molecules are involved in the export of the different RNA species and the export and import of proteins.

The different steps in mRNP biogenesis and transport are not separated, but rather there is a close interplay of factors playing different roles during the course of this complex pathway. Everything is there to guarantee an efficient and accurate information transfer from the nucleus to the cytoplasm.

1.1 mRNP SYNTHESIS

1.1.1 Transcription

RNA polymerase II is a large protein complex of 12 subunits that catalyzes the mRNA synthesis. The transcription cycle includes preinitiation, initiation, promoter clearance, elongation, and termination. A preinitiation complex of RNA pol II associated with a set of general transcription factors (GTFs) is formed at the promoter by the recognition of the TATA-box by transcription factor TFIID. Subsequent binding of TFIIB leads to recruitment of the TFIIF/RNA pol II complex. Addition of TFIIE and TFIIH completes the preinitiation complex. The double-stranded DNA is melted into a single-stranded bubble and the transcription is initiated by the joining of the first two nucleotides (Orphanides et al., 1996; Thomas and Chiang, 2006). The transition between the

initiation and the elongation phase is called promoter clearance and represents the stage when the unstable initiation complex transforms into the stable elongation complex. However, the details of how this occurs have not yet been elucidated. The elongation phase involves a wide range of elongation factors to enable proper transcription. The template is highly compacted with proteins to form chromatin, i.e. DNA strands wrapped around the core histones forming nucleosomes. The accessibility to the DNA is enhanced by the action of chromatin remodeling and histone acetylation (Sims et al., 2004). Finally, the termination process is intimately linked to the 3' end processing and results in release of RNA pol II from the template (Buratowski, 2005).

The C-terminal domain (CTD) of the RNA pol II plays a key role in the transcription process. This protruding domain of the largest subunit comprises 52 heptad repeats (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) in humans and 26 in yeast that can be hyperphosphorylated by the action of several different CTD kinases. The CTD is hypophosphorylated in the preinitiation complex, when binding to promoter-associated proteins, and becomes hyperphosphorylated in the active elongating state after polymerizing 25-30 nucleotides. In the latter state, the CTD adopts a more extended shape to facilitate the interaction with the growing transcript for delivery of processing factors. Capping, splicing and polyadenylation depend on the inflow of proteins to the mRNA via the CTD-tail (Hirose and Manley, 2000; Howe, 2002; Phatnani and Greenleaf, 2006).

Nuclear actin

In the past years, a focus on cytoskeletal components in the nucleus has emerged rapidly. Both nuclear actin and myosin have been implicated in the transcription process. Actin has been shown to be involved in the transcription by all three RNA polymerases, although no common factor has been identified (reviewed in Percipalle and Visa, 2006; Grummt, 2006; Obrdlik et al., 2007). For instance, actin together with hnRNP U can interact with the phosphorylated CTD-tail of RNA pol II to ensure productive transcription (Kukalev et al., 2005).

Actin involved in transcription appears to be in a monomeric, globular state or in a short non-conventional, oligomeric form. This could be the reason for absence of phalloidin staining in the nucleus, which only recognizes filamentous actin (F-actin) (Percipalle and Visa, 2006). In contrast, actin has been localized to a network of nuclear filaments attaching to the NPCs and extending into the *Xenopus* nucleus (Kiseleva et al., 2004). These pore-linked filaments are made of actin and protein 4.1 and depend on polymerized actin, although most likely not in an F-actin form.

In addition, nuclear actin is involved in chromatin remodeling (Zhao et al., 1998; Olave et al., 2002) and is a part of the nascent RNP particle (Percipalle et al., 2001; 2002). In *Chironomus tentans*, actin becomes a part of the RNP concomitant with transcription and accompanies the mRNA to the cytoplasm and the polysomes (Percipalle et al., 2001).

1.1.2 mRNP assembly

No naked mRNA molecules are present in the cell; the RNA is associated with proteins at any given time, although the composition varies. The mRNA together with the proteins assemble into a messenger ribonucleoprotein particle (mRNP particle). Binding to RNA requires RNA-binding domains of which the most common ones are the RNP motif (also known as RNA recognition motif, RRM) and the K homology (KH) motif, but there are also less frequent domains such as the RGG box, the arginine-rich motif, and the double-stranded RNA-binding motif (Burd and Dreyfuss, 1994). Two families of ubiquitously expressed RNA-binding proteins, hnRNP and SR proteins, are important for most steps in mRNA biogenesis. The distribution of these proteins along the mRNA molecule is non-random, with high and low affinity binding sequences. Most likely, the proteins carry out their specific functions at the high affinity sites, whereas binding to low affinity sites, when at molar excess, provides general packaging of the mRNP particle (Singh and Valcarcel, 2005).

hnRNP proteins

The hnRNP proteins are a large group of RNA-binding proteins comprising at least 20 major species of 34 to 120 kDa (hnRNP A1 through U), and numerous less abundant proteins (Dreyfuss et al., 1993; 2002; Krecic and Swanson, 1999). They are as abundant as histones in the nucleus and are named after their ability of binding to heterogeneous nuclear RNA, i.e. a historical term for all transcripts produced by RNA pol II (Dreyfuss et al., 1993). Typically, an hnRNP protein has a modular structure with one to four RRMs and auxiliary domains mediating protein-protein interactions and the localization of the protein. The functions of the hnRNP proteins are diverse, including transcriptional regulation, splicing and 3’ end processing. HnRNP proteins participate also in later events such as nucleocytoplasmic transport of mRNA, mRNA localization, translation and stability (Dreyfuss et al., 2002, and references therein). Some hnRNP proteins are confined to the nucleus, while others shuttle between the nucleus and cytoplasm. HnRNP A1 is an example of a shuttling protein (Pinol-Roma and Dreyfuss, 1992), whereas hnRNP C is strictly nuclear because of a retention signal that overrides its nuclear export signal (Nakielny and Dreyfuss, 1996).

SR proteins

The other abundant group of RNA-binding proteins are the SR proteins, which were originally identified as essential splicing factors. However, the clear cut line between SR proteins and hnRNP proteins has been blurred. Up to date ten SR proteins (20 to 75 kDa) have been identified in mammals and they are conserved throughout evolution (Bourgeois et al., 2004). The N-terminal part contains one or two RNA-binding domains, and the C-terminal the characteristic RS domain. The RS domain constitutes a regular repetition of arginine and serine residues, often as dipeptides, for a stretch of 24 to 316 residues. The serines can be heavily phosphorylated by specific SR protein kinases, which modulate the binding partners of the SR protein (Bourgeois et al., 2004). For instance, the phosphorylation state of the protein determines whether the export receptor binds to the mRNP complex or not (see below, and Huang et al., 2004). The SR proteins are predominantly nuclear, but some have been shown to shuttle between the nucleus and cytoplasm (Caceres et al., 1998). In the nucleus they mainly

accumulate in speckles, until recruited to the site of pre-mRNA synthesis to assist in splicing (Bourgeois et al., 2004). The main function of the SR proteins involves splicing, both spliceosome assembly and regulation of alternative splicing (Singh and Valcarcel, 2005). They probably have general structural properties as well, since the *Chironomus tentans* SR protein hrp45 binds along the entire Balbiani ring transcript (Alzhanova-Ericsson et al., 1996).

1.1.3 Capping

To prevent instant degradation of the nascent RNA by 5'-3' exonucleases it is protected in the 5' end by a modified nucleotide, the cap. This is accomplished by the sequential action of three enzymes in yeast and two in metazoans, but the enzymatic activities are the same (Shuman, 2001). Initially, the γ-phosphate on the first transcribed nucleotide is removed by a 5'-phosphatase. Next, a guanylyltransferase attaches a guanosine via a 5'-5' triphosphate linkage. Finally, the terminal guanine is methylated at position 7 by a 7-methyltransferase. The enzyme performing the first two actions in metazoans is called the capping enzyme.

The capping enzymes are recruited to the RNA via the phosphorylated CTD of RNA pol II, and consequently only mRNAs are capped. *In vivo*, the cap is added as early as after 25-30 nucleotides have been synthesized (Howe, 2002). Two evolutionary conserved proteins are added shortly after cap formation, the cap binding proteins CBP20 and CBP80, forming the cap binding complex (CBC). The CBC remains associated with the cap until replacement by the cytoplasmic eukaryotic translation initiation factor 4E (eIF-4E) upon entry into the cytoplasm (Shatkin and Manley, 2000). The capping has several important tasks: stabilization of the mRNA, promoting splicing and 3' end processing, facilitation of mRNA cytoplasmic transport, and assistance in translation (Howe, 2002).

1.1.4 Splicing

The coding regions (exons) of most RNA pol II transcripts in metazoans are interrupted by non-coding sequences (introns). The introns are excised in a process called splicing, involving large molecular complexes, the spliceosomes. The number of introns per gene in higher eukaryotes varies between 0 to more than 50. A typical exon is rather short, 10-400 nt, whereas intron sizes can extend up to 200 000 nucleotides (Krämer, 1996). Thus, normally a very large portion of the pre-mRNA is excised during splicing. The introns have impact on several steps in gene expression, such as enhancing transcription and, when removed, promoting mRNA export (discussed in Le Hir et al., 2003). In addition, the presence of introns allows alternative splicing, which gives rise to a multitude of additional proteins from a single gene.

The spliceosomes comprise five snRNPs (U1, U2, U4, U5, and U6), each a complex of an snRNA molecule and several proteins. In addition, there are numerous associating splicing factors. Totally, the splicing process occupy up to 200 different proteins (Jurica and Moore, 2003). Splicing is a co-transcriptional process (Baurén and Wieslander, 1994), performed in two steps: 5' splice site cleavage and lariat formation, followed by 3' splice site cleavage and exon ligation. Upon splicing, a protein complex is deposited 20-24 nt upstream the exon-exon junction, the exon-exon junction complex (EJC) (Le

Hir et al., 2000). The known proteins of this complex are SRm160, RNPS1, ALY/REF, Y14, Magoh and Upf3 (Dreyfuss et al., 2002). The EJC enhances the nucleocytoplasmic transport of spliced mRNAs by binding to export factors (see below) (Le Hir et al., 2001).

1.1.5 Polyadenylation

The 3' end of the mRNA transcript is the last to be synthesized and processed. The processing, for all mRNAs except histones, involves endonucleolytic cleavage of the nascent transcript and addition of a poly(A)-tail. An AAUAAA polyadenylation signal in the mRNA sequence determines where the adenosines should be added. This element is located 10-30 nucleotides upstream of the cleavage site that is followed by a GU-rich sequence (Colgan and Manley, 1997; Shatkin and Manley, 2000). Initially, these sequences are recognized by two key proteins, the cleavage and polyadenylation stimulation factor (CPSF) and the cleavage stimulation factor (CstF). The actual cleavage is performed by cleavage factors I and II (CFI and CFII) and the polyadenylation is carried out by poly(A) polymerase bound to CPSF and the nuclear poly(A)-binding protein, PABPN1. The length of the poly(A) tail is about 200-300 in mammals and is regulated by PABPN1 and CPSF (Keller et al., 2000). PABPN1 remains associated to the transcript from the site of transcription to the translocation through the nuclear pores where the cytoplasmic PABP1 displaces PABPN1. Moreover, PABPN1 binds to the RNA pol II in the beginning of transcription, implying a coupling between transcription and the assembly of PABPN1 onto the poly(A)-tail (Bear et al., 2003). The functions of the poly(A)-tail include stability, nucleocytoplasmic transport and promoting translational efficiency (Colgan and Manley, 1997).

1.1.6 mRNA surveillance

The quality control of the produced mRNPs begins already at the gene and is carried out by the nuclear exosome, a complex of ten 3' to 5' riboexonucleases and accessory factors. The nuclear exosome degrades mRNAs when the splicing, polyadenylation, assembly or export is slow or defective (Butler, 2002; Vasudevan and Peltz, 2003; Saguez et al., 2005). In yeast, a strictly nuclear component of the exosome has been identified, Rrp6, which is important for keeping the defective mRNPs at the sites of transcription for subsequent degradation (Libri et al., 2002). However, it has not been elucidated how this retention is accomplished. If not hindered by the exosome, the mRNP is released from the gene for further processing and export. Interestingly, a physical interaction between the *Drosophila* exosome and the transcription elongation factors Spt5 and Spt6 has been established, implying a very early involvement of the exosome in metazoan transcription (Andrulis et al., 2002). Furthermore, the exosome component Rrp6 shows functional interactions with the mRNA export factors Yra1, Sub2 and Hpr1, all members of the TREX complex (see below) (Zenklusen et al., 2002). Hence, there seems to be a close interplay or competition between degradation and transcription, assembly and transport.

Another surveillance mechanism has been detected in yeast, involving the perinuclear proteins Mlp1 and Mlp2 attached to the nuclear pore complexes (similar to mammalian Tpr, see below) (Dimaano and Ullman, 2004). Mlp1 is responsible for the nuclear retention of unspliced mRNAs, although it does not affect the splicing process (Galy et al., 2004). Recently, it was proposed that the Mlp proteins could downregulate gene expression when the export receptor Yra1 was defective, serving as a feedback system for defective transport (Vinciguerra et al., 2005). In addition, interactions with the exosome component Rrp6 and Mlp proteins have been determined, suggesting that Mlps could participate in the exosomal degradation of malformed mRNPs (Dimaano and Ullman, 2004).

The best characterized mRNA surveillance system is the nonsense mediated decay (NMD), that takes place later during mRNP biogenesis, i.e. mainly in the cytoplasm. Transcripts harboring premature translational termination codons (nonsense) are degraded to prevent production of potentially devastating truncated proteins (Maquat, 2004; Conti and Izaurralde, 2005; Behm-Ansmant and Izaurralde, 2006). This mechanism identifies premature termination codons that are followed by an exon-exon junction located more than 50-55 nt downstream. Normally, there are no exon-exon junctions following the termination codon, and if there are they reside closer. Apart for the NMD specific factors, SR proteins have been implicated in the NMD pathway. Overexpression of SR proteins enhanced the NMD, by efficiently targeting mRNAs bearing a premature nonsense codon to the NMD pathway (Zhang and Krainer, 2004).

1.2 NUCLEOCYTOPLASMIC TRANSPORT

Before reaching the NPCs the mRNPs need to travel from the sites of transcription to the nuclear periphery. They move freely by Brownian diffusion in the interchromatin space, but can transiently interact with fibers in the nucleoplasm (Daneholt, 1999). Dissociation from fibers or dense chromatin cavities demands energy, because an ATP-dependent route of mRNP transport has also been discovered (Calapez et al., 2002; Shav-Tal et al., 2004; Vargas et al., 2005). The mRNPs spread randomly throughout the nucleus, before binding to the nuclear pore complexes for export.

The exchange between the nucleoplasmic and cytoplasmic compartment through the NPCs requires different import/export receptors, adaptors and signals depending on the cargo involved. Molecules transported include proteins, mRNPs, rRNPs and other small RNPs. The protein import and export is an active process depending on the RanGTP gradient across the nuclear envelope, whereas mRNP export is not directly dependent on the RanGTPase cycle (Clouse et al., 2001).

1.2.1 Protein export and import

The import and export of most proteins are mediated by a large family of transport factors, the karyopherins. They are conserved throughout evolution and mediate either nuclear import (also called importins) or nuclear export (also called exportins) (Nakielny and Dreyfuss, 1999; Pemberton and Paschal, 2006). The karyopherins interact either directly with its cargo or via adaptor proteins, e.g. importin-α. The karyopherins or adaptors interact with amino acid sequences in the proteins to be

transported, namely a nuclear localization signal (NLS) or a nuclear export signal (NES). The best-characterized NLS is the monopartite basic NLS, binding to the importin-β receptor via the adaptor importin-α. Furthermore, the leucine-rich and hydrophobic NES is recognized by the karyopherin Crm1 that mediates protein export.

The RanGTPase cycle is responsible for the directionality of the transport and provides the essential energy (Pemberton and Paschal, 2006). Ran has low nucleotide hydrolysis and exchange activities on its own, and thus in need of the GTPase-activating protein (RanGAP) and the guanine nucleotide exchange factor (RanGEF). These Ran regulators have restricted subcellular localizations. RanGAP and the coactivator Ran-binding protein 1 (RanBP1) are largely cytoplasmic, residing partially at the cytoplasmic filaments of the NPC, where they bind to the nucleoporin Nup358/RanBP2. On the other hand, RanGEF, is a nuclear protein associated with chromatin. This asymmetry results in that nuclear Ran is mainly in the energy-rich GTP-form, whereas the cytoplasmic Ran is hydrolyzed to RanGDP (Figure 1). The differential localization of RanGTP/GDP informs the transport receptors of their subcellular location, triggering release or binding of cargo depending on the receptor type. Import receptors dissociate from its cargo in the presence of RanGTP, whereas export receptors bind cargo with higher affinity (Nakielny and Dreyfuss, 1999; Kuersten et al., 2001).

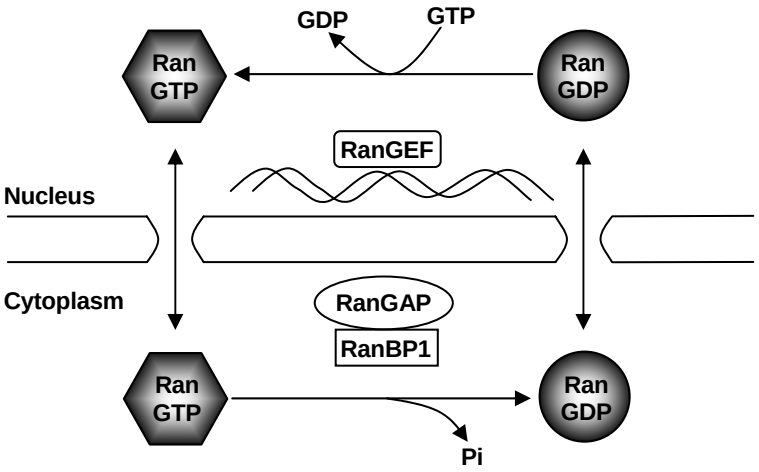

Figure 1. The RanGTPase cycle. The proteins responsible for the hydrolysis or nucleotide exchange of Ran are asymmetrically localized in the cell. The hydrolysis by RanGAP and RanBP1 takes place in the cytoplasm, whereas RanGEF replaces GDP with GTP in the nucleus. This is a prerequisite for the directionality of protein import and export.

1.2.2 RNA export factors

Messenger RNA

The export of mRNPs is complicated due to the heterogeneity in length, sequence and structure of the different species. The recruitment of the adequate export receptors to this group cannot rely on those parameters, but rather through coordination with the processing and assembly. The export of mRNAs is coupled to either transcription

(yeast) or splicing (metazoa) (see further Chapter 1.3) (Nakielny and Dreyfuss, 1999; Rodriguez et al., 2004; Köhler and Hurt, 2007). The bulk mRNP transport is mediated by the export receptor complex TAP-p15 (also known as NXF1-NXT1) in metazoans (Gruter et al., 1998), and Mex67-Mtr2 in yeast (Segref et al., 1997) (Figure 2). These export receptors are structurally unrelated to karyopherins, but can likewise interact with the phenylalanine-glycine (FG) repeats of the nucleoporins (see below). TAP was initially identified as the factor responsible for the export of unspliced viral RNAs, by directly interacting with the constitutive transport element (CTE) (Braun et al., 1999). However, the cellular mRNAs associate with TAP-p15 via the adaptor proteins ALY (also known as REF) in metazoans and Yra1 in yeast (Reed and Hurt, 2002). ALY is a component of the EJC (see above) indicating that the export receptor is only recruited to mature spliced transcripts (Le Hir et al., 2001). However, splicing is not an absolute requirement for RNA export, because many of the export factors can interact with mRNA independently of splicing (Rodrigues et al., 2001).

Another conserved export factor interacting with ALY is the RNA helicase UAP56 (Sub2 in yeast), which is a component of the transcription and export complex (TREX). The TREX complex links the steps in early mRNA biogenesis with export, and is present in both yeast and higher eukaryotes (Strässer et al., 2002). The yeast TREX complex harbors a subcomplex of four proteins, the THO complex (Tho2, Hpr1, Mft1 and Thp2) involved in transcription, the protein Tex1, and the two export components, Yra1 and Sub2 (Reed and Cheng, 2005). The TREX complex is a well conserved complex with identified counterparts in both *Drosophila* and human. In addition, another complex important for mRNA export has been identified in yeast, denoted the TREX-2 mRNA export complex comprising four proteins (Sac3, Thp1, Sus1 and Cdc31) (Köhler and Hurt, 2007). TREX-2 is located to the inner side of the NPC via the nucleoporins Nup1 and Nup60 (Fischer et al., 2002). Sac3 works as an additional export adaptor with ability to dock the Mex67-Mtr2 export receptor to the nuclear pore complex (Fischer et al., 2002; Lei et al., 2003).

Depletion of *Drosophila* ALY showed that it is dispensable for export of bulk mRNA; only a partial nuclear accumulation of mRNA was observed, implying that there are alternative adaptors (Gatfield and Izaurralde, 2002). Moreover, the vast majority of *Drosophila* mRNAs are transcribed and exported, although the THO complex is knocked down by RNA interference. Only heat-shock mRNA export requires the THO complex (Rehwinkel et al., 2004). SR proteins are candidates for serving as alternative adaptors (Huang and Steitz, 2001; 2005). The SR proteins bind to the same N-terminal domain of TAP that interacts with the ALY adaptor (Huang et al., 2003). The interaction with the export receptor depends on the phosphorylation status of the serines in the RS domain. During splicing the hyperphosphorylated SR protein becomes dephosphorylated and the interaction with TAP increases, and subsequently only spliced transcripts are selected for export (Huang et al., 2004). In yeast, an SR-like shuttling protein, Npl3, has been identified. Dephosphorylation of Npl3 by a nuclear phosphatase, Glc7, is required for mRNA export, demonstrating that the involvement of SR proteins in export may be conserved from yeast to higher eukaryotes (Gilbert and Guthrie, 2004).

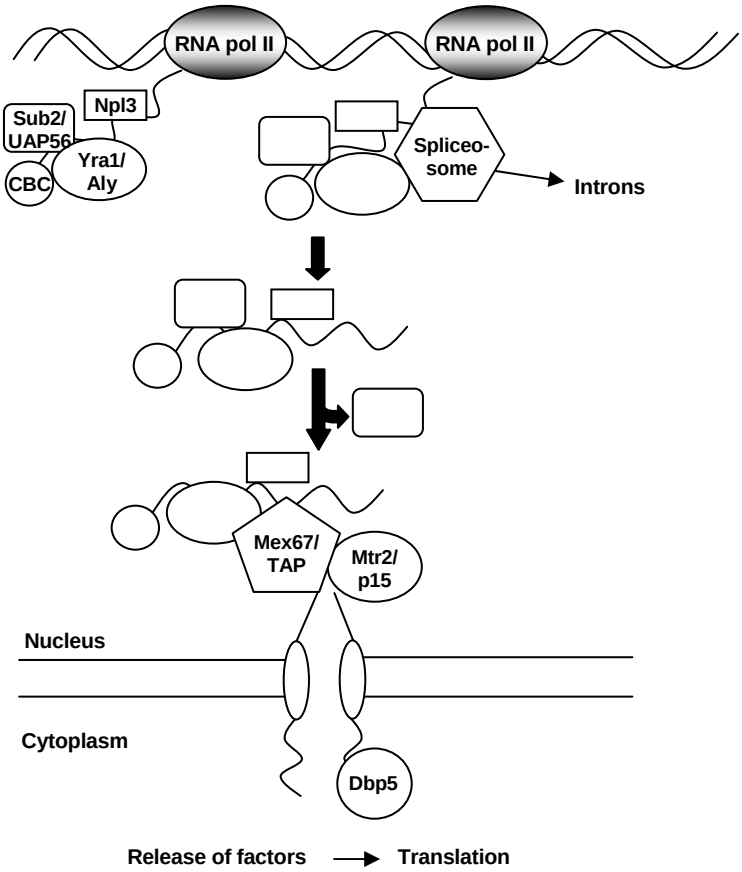

Figure 2. Messenger RNP assembly and export (simplified). Several proteins are added to the growing transcript, including export factors. The spliceosome removes the introns and export receptors are recruited. Upon translocation to the cytoplasm, with the help of the RNA helicase Dbp5, the majority of the factors are released and the mRNA becomes engaged in translation. See text for details.

Ribosomal RNA

The ribosomal RNA is transcribed as a 45S precursor rRNA by RNA polymerase I in the nucleolus. The pre-rRNA is processed to three smaller components of 28S, 18S and 5.8S, which together with more than 70 ribosomal proteins and the 5S rRNA (produced elsewhere) form the large (60S) and small (40S) ribosomal subunits. These subunits follow separate export routes, of which the export pathway of the small subunit is poorly understood. To date, only the involvement of the RanGTPase cycle and the export receptor Crm1 is known (Moy and Silver, 1999). On the other hand, the mechanism for the export of the large subunit have been revealed in both yeast and metazoa (Cullen, 2003; Köhler and Hurt, 2007). It is a conserved pathway, depending on the Ran system and the export receptor Crm1. The adaptor protein Nmd3 contains an NES, and serves as the link between the 60S subunit and the export receptor. However, it was recently reported in yeast that the general mRNP receptor complex, Mex67-Mtr2, has a distinct binding surface for the 60S subunit, mediating export (Yao et al., 2007). Moreover, the auxiliary shuttling export factor Arx1, with ability to interact with FG nucleoporins, is recruited to the large subunit in parallel with Nmd3 and Mex67-Mtr2 (Bradatsch et al., 2007). Thus, the large molecular complex may require more than one export receptor to facilitate its translocation.

1.2.3 Nuclear pore complex

The NPC accommodates all transport between the nucleus and cytoplasm, allowing passive diffusion of ions and small molecules as well as facilitated transport of receptor-associated proteins and RNPs (Davis, 1995; Fahrenkrog and Aebi, 2003; Suntharalingam and Wente, 2003). The amount of NPCs varies depending on the cell type and species, typically in the range of 200 NPCs per yeast cell and 2000 in a vertebrate nucleus. The nuclear pore complex is a large macromolecular assembly of 30 different proteins forming a tripartite architecture with eight-fold symmetry (Rout et al., 2000; Cronshaw et al., 2002). The central framework consists of a spoke assembly sandwiched between the nuclear and cytoplasmic ring. In the center, there is a channel containing fibrous material, often referred to as the central plug or transporter (Hinshaw et al., 1992; Stoffler et al., 1999; Vasu and Forbes, 2001). From the nuclear ring eight fibers emanate out, with ability to connect at their distal ends forming the nuclear basket structure. The cytoplasmic ring anchors eight short, kinky filaments. The overall structure of the NPC is conserved, although the yeast NPC is smaller (60 MDa) than the vertebrate NPC (125 MDa). The central channel has a functional diameter of 39 nm, i.e. the upper limit of the diameter of cargo translocating through the pore (Panté and Kann, 2002). The native three dimensional structure of the NPCs is currently being revealed by cryoelectron tomography. Two studies provide more information about the nature of the central channel (Stoffler et al., 2003; Beck et al., 2004). The variability and temperature dependence of the central plug might indicate that it to some extent represents cargo in transit.

The proteins of the nuclear pore complex can be divided into two main groups, the pore membrane proteins (POMs) and the nucleoporins (Nups) (Davis, 1995). The POMs are integral membrane proteins, and therefore anchored in the nuclear envelope. On the other hand, the Nups are peripheral proteins residing outside of the nuclear envelope. The distribution of several nucleoporins has been determined by immunoelectron microscopy. Most of the nucleoporins are symmetrically distributed, but some are localized to either the nuclear or the cytoplasmic side (Rout et al., 2000) (Figure 3). At least ten of the nucleoporins are modified by addition of *N*-acetylglucosamine (GlcNAc) (Miller et al., 1999). This O-linked glycosylation is carried out in the cytoplasm by a cytosolic glycosyltransferase, a pathway distinct from the N- and O-linked glycosylation in the ER and Golgi complex (Davis and Blobel, 1987). The glycosylation is highly dynamic, but the biological significance is not completely understood (Miller et al., 1999).

A common feature of about half the nucleoporins are the FG-repeats (phenylalanine-glycine) that mediate the main interaction between nucleoporins and soluble transport receptors. The FG-repeats comprise the sequences FxFG, GLFG or FG (where x represents any amino acid), which are separated by spacers of variable length. These 150-700 amino acids domains lack secondary structure and are thus unfolded and very flexible (Denning et al., 2003). Three of the FG-repeat containing nucleoporins and their involvement in nucleocytoplasmic transport are described below.

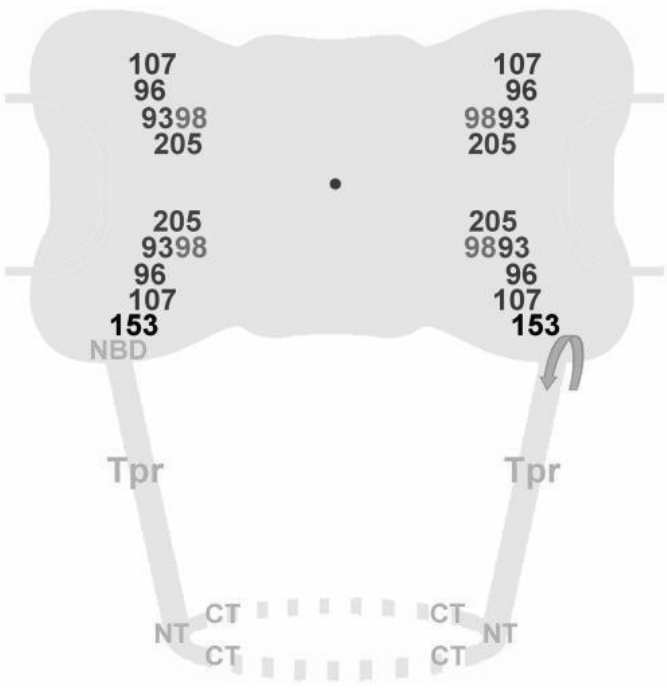

Figure 3. A schematic representation of the nuclear pore complex and the localization of nucleoporins. The N-terminal of Nup153 anchors Tpr, which is the architectural element of the nuclear basket. CT, Tpr C-terminal, NT; Tpr N-terminal; NBD, Tpr NPC binding domain. Modified from Krull et al., 2004.

Nup98

Nup98 is a O-linked glycoprotein, and the only known vertebrate nucleoporin to contain the GLFG repeat. Nup98 was shown to be involved in export of RNAs, because antibody injections into the nucleus inhibited the export of all RNAs except tRNAs. However, the import of proteins and snRNPs was not affected (Powers et al., 1997). Multiple subcellular localizations have been suggested for Nup98, i.e. within the nucleus and bound to both the nuclear and cytoplasmic side of the NPC (Griffis et al., 2002). In the nucleoplasm Nup98 is targeted to the so called GLFG bodies through its GLFG domain. The mobility of Nup98 between the different locations is dependent on ongoing transcription by RNA polymerases I and II. Upon DRB or actinomycin D treatment the mobility was reduced dramatically, indicating a potential role for Nup98 in the direction of export substrates to the pores (Griffis et al., 2002).

Nup153

Nup153 is a large nucleoporin of 1476 amino acids, comprising a tripartite structure of a zinc-finger domain flanked by N- and C-terminal domains (Sukegawa and Blobel, 1993). The zinc-finger domain interacts with RanGTP (Nakielny et al., 1999), the N-terminal domain is responsible for the NPC-binding (Enarson et al., 1998) and the C-terminal domain contains numerous FG-repeats. Nup153 is located mainly at the nuclear side of the NPC, although there is an exchange on and off the pore. Interestingly, this exchange is, as for Nup98, dependent on ongoing transcription (Griffis et al., 2004). Moreover, the C-terminal domain is highly flexible and can be mapped to the nuclear ring, distal ring or even on the cytoplasmic side (Fahrenkrog et al., 2002). In addition, the N-terminal domain of Nup153 anchors the architectural

element of the nuclear basket, Tpr, to the nuclear ring of the NPC (Hase and Cordes, 2003) (Figure 3).

Nup153 is important for the initial steps in the nucleocytoplasmic transport. Injection of antibodies against Nup153 into *C. tentans* salivary gland nuclei resulted in a complete inhibition of export of both mRNPs and ribosomal subunits (Soop et al., 2005). The entry into the basket structure is dependent on a functional Nup153. In agreement with this, Lim et al. proposes that the FG-repeats of Nup153 serve as an entropic barrier "trapping" exclusively transport receptor-associated cargo to facilitate transport (Lim et al, 2006).

Nup214

In contrast to Nup153, Nup214 (or CAN) is located to the cytoplasmic ring of the NPC via its N-terminal domain (Kraemer et al., 1994). Nup214 is another glycosylated nucleoporin containing C-terminal FG-repeats, a highly flexible structure localized to both the cytoplasmic and the nucleoplasmic side. Recently, a domain topology study of Nup214 revealed a transcription-dependent localization of this domain. When the transcription was inhibited over 75% of the C-terminal domains resided on the nuclear side of the NPC, implying a role of this domain in RNA export. This arm might bind to the cargo-receptor complex and guide it through the central channel (Paulillo et al., 2005).

1.2.4 Translocation through the NPC

The translocation through the nuclear pore complex is a rapid process; a single NPC can *in vitro* accommodate the transport of 1000 macromolecules or 100 MDa per second (Ribbeck and Görlich, 2001). For proper translocation to occur, there is a need of directionality in the system. Previously, it was thought that the asymmetric nucleoporins contributed to the directionality. However, it has been shown that all asymmetric FG-domains are dispensable for transport. In fact, half of the total mass of FG-repeats could be deleted without loss of viability or affecting the permeability barrier of the NPC (Strawn et al, 2004). In contrast, inversion of the RanGTP-gradient *in vitro* reverses the direction of traffic, suggesting that this gradient is responsible for the directionality (Nachury and Weis, 1999). The maintenance of the RanGTP-gradient over the nuclear envelope is the energy-requiring process, not the translocation *per se*.

The exact mechanism whereby the cargo-receptor complex complete the journey through the NPC in either direction is not known. A clear role of the FG-repeat containing nucleoporins throughout the process has been established. The highly flexible long stretches of FG-repeats interacts with the transport receptors to facilitate the translocation. Two main models for the translocation have been proposed, in which the FG-repeats are regarded primarily as either a physical or an energetic barrier for selective translocation. In the physical barrier model, or the selective phase model, the FG domains are thought to form a meshwork or even a hydrophobic gel through interactions between them (Ribbeck and Görlich, 2002; Frey and Görlich, 2007). Only molecules or molecular complexes that are able dissolve the FG mesh, like export receptor complexes, could enter into the NPC channel. Inert molecules are efficiently excluded. In contrast, the energetic barrier model, or the Brownian affinity

gate model, is based on the fact that the interaction between the export receptor and the FG-containing nucleoporins would allow the export complex to overcome an entropic threshold for further diffusion through the NPC (Rout et al., 2003).

Directionality in mRNP transport is achieved by a RanGTP-independent mechanism. The export pathway for mRNPs begins at the nuclear side with the basket fibers, whose main constituent is the coiled-coil protein Tpr (Frosst et al., 2002; Krull et al., 2004). After translocation through the central channel (see above), the mRNP encounters the fibrils on the cytoplasmic side where a remodeling process begins to prevent the immediate return of the mRNP to the nucleus, and thereby accomplish directionality. This process involves the RNA helicase Dbp5 that removes the export receptor Mex67 from the mRNPs with the input of ATP (Lund and Guthrie, 2005). The ATPase activity of the Dbp5 helicase is activated by the mRNA export factor Gle1 that is located on the fibers at the cytoplasmic side of the NPC. Removal of export factors leads to a restriction in motion in one direction, also called ratcheting (Stewart, 2007).

1.3 COUPLING OF STEPS IN GENE EXPRESSION

Above, the distinct steps in gene expression have been described, from transcription to export through the nuclear pore complexes. All processes can be performed separately in various *in vitro* assays, but the *in vivo* situation is completely different. To make the gene expression more efficient and accurate, there is an extensive coupling among the steps and the molecular machines involved (Figure 4). The coupling is not limited to sequential steps; there is a connection between very early and late steps as well. The advantages are many, all ensuring that only stable and fully processed mRNPs are targeted for export to the cytoplasm (Maniatis and Reed, 2002; Aguilera, 2005; Bentley, 2005).

The CTD of RNA pol II is important for the coupling between transcription and pre-mRNA processing. Certain CTD deletions result in reduction of capping, splicing and polyadenylation efficiencies. The addition of processing factors to the CTD drastically increase the local concentration close to the exit groove for the pre-mRNA. In this way the cap can be added early and efficiently to the nascent RNA, to prevent premature degradation. Productive transcription is not initiated until the capping has occurred to avoid wasteful transcription (Maniatis and Reed, 2002, and references therein).

Similarly, the splicing process is coupled to transcription, although the mechanism is not completely understood. The recognition of the small exons between the usually very long exons is a difficult task, which would be facilitated by tethering the splicing factors directly adjacent to where the pre-mRNA emerges from the polymerase (Maniatis and Reed, 2002). Indeed, in yeast, the splicing factor Prp40 binds to the CTD and brings the 5’ and 3’ splice sites together (Morris and Greenleaf, 2000). Moreover, SR proteins together with U1 snRNP are cotranscriptionally recruited to the 5’ splice sites on the growing transcript through RNA pol II interaction. This loading increases the efficiency of spliceosome assembly and thus the splicing process (Das et al., 2007).

RNA pol II also contributes to polyadenylation efficiency. Some polyadenylation factors bind to the CTD, whereas other components associate with the Pol II holoenzyme (Neugebauer, 2002). Thus, the polyadenylation machinery is brought close to the nascent mRNA and at least part of the process can occur cotranscriptionally.

Coordination between transcription and export works differently in yeast and metazoans due to the intron content. In yeast, 95% of the genes lack introns and the TREX complex is loaded onto the emerging mRNA cotranscriptionally (Strässer et al., 2002). In contrast, the metazoan TREX complex and subsequently the export receptors are recruited to the mRNP during splicing (Masuda et al., 2005), or more specifically to the 5' end of the transcript (Cheng et al., 2006). This is in good agreement with the fact that the Balbiani ring mRNP particle traverses the NPC with the 5' end in the lead (Mehlin et al., 1992). In addition, the fact that the protein Sus1 links the transcription initiation complex SAGA to the export factors Sac3-Thp1 was revealed in yeast (Rodriguez-Navarro et al., 2004). This indicates that there are presumably more coupling pathways, linking transcription and export, yet to be discovered.

Finally, there is an important coupling between mRNP assembly and degradation. It would be potentially harmful for the cell to allow export of malformed mRNPs. Therefore, the nuclear exosome monitors the mRNP formation on the gene, and sequesters aberrant mRNPs for degradation (Libri et al., 2002; Zenklusen et al., 2002).

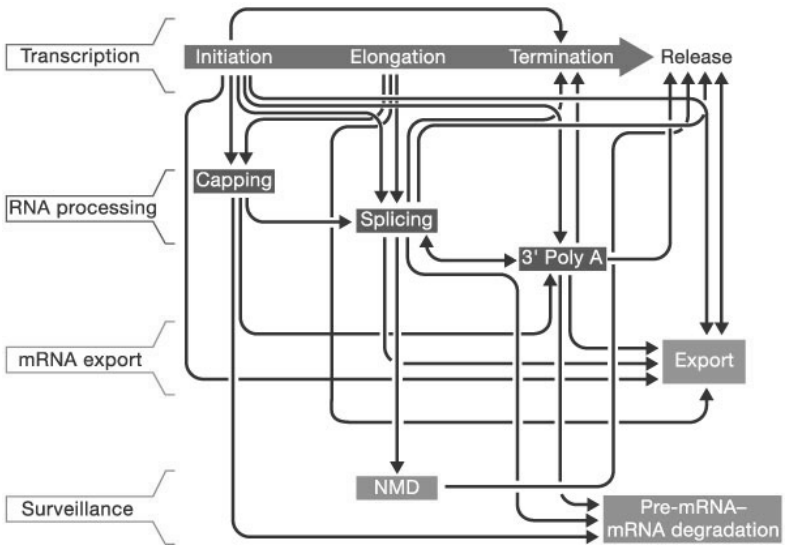

Figure 4. There is a complex network of physical and/or functional interactions between the molecular machines in gene expression. The coupling is not limited to sequential steps, but also between the earliest and latest steps. Modified from Maniatis and Reed, 2002.

2 PRESENT INVESTIGATION

2.1 AIM OF THE THESIS

The overall aim of the current thesis was to investigate different aspects and regulatory mechanisms of the transcription and transport of a specific messenger RNP particle, mainly by electron microscopy *in situ* analysis.

Specific aims for:

Paper I: To determine the function of nuclear actin in transcription by identifying a novel binding partner and disrupting the specific interaction.

Paper II: To characterize a novel transcript-specific hnRNP protein and reveal its potential function.

Paper III: To investigate a possible link between the transcription and transport of an RNP particle by inhibiting the transcription with two specific drugs.

Paper IV: To study the initial stage of RNA translocation through the nuclear pore complex by blocking the peripheral nucleoporins with either wheat germ agglutinin or a nucleoporin antibody.

2.2 EXPERIMENTAL SYSTEM

2.2.1 *Chironomus tentans*

The dipteran *Chironomus tentans* is a widely spread and non-biting midge that can be reared in the laboratory (Figure 5). The life cycle, including egg, larva, pupa, and adult, varies from 4 to 8 weeks of which only the adult stage is nonaquatic. Most of this time is spent in the larval stage that is divided into four instars, depending on the development and size (0.6-2 cm). Our experimental material is the salivary glands of the fourth instar larvae. Each larva has a pair of salivary glands (1-2 mm in length) that produce huge amounts of secretory proteins. These silk proteins are used by the larva to build a housing and feeding tube, as well as for pupation later on (Case and Wieslander, 1992).

The salivary glands consist of a lumen surrounded by 30-40 secretory cells with giant nuclei harboring four polytene chromosomes. The chromosomes are exceptionally large with a diameter of 10 µm and lengths between 45 and 100 µm, allowing manual isolation of separate chromosomes (Wieslander, 1994). The polyteny arises from numerous replications without intervening cell divisions, resulting in chromosomes with 8 000-16 000 perfectly aligned individual chromatids. Polytene chromosomes have a striped pattern due to different compaction of the chromatin along the chromosomes. Expanded, transcriptionally active areas are referred to as puffs. There are polytene chromosomes in other larval tissues, like the malphigian tubules and colon, but the advantage of the salivary glands stems from the discovery of a few

remarkably large puffs on chromosome IV by E.G. Balbiani in 1881 (Balbiani, 1881). These puffs, referred to as Balbiani rings (BRs), correspond to heavily transcribed and tissue-specifically expressed genes. The size and abundance of the BR gene products permit unique electron microscopy studies of the assembly and transport of a specific mRNP particle (see below and Daneholt, 2001b). The compaction of the interphase chromosomes facilitates these studies, because of the large areas of nucleoplasm free from chromatin.

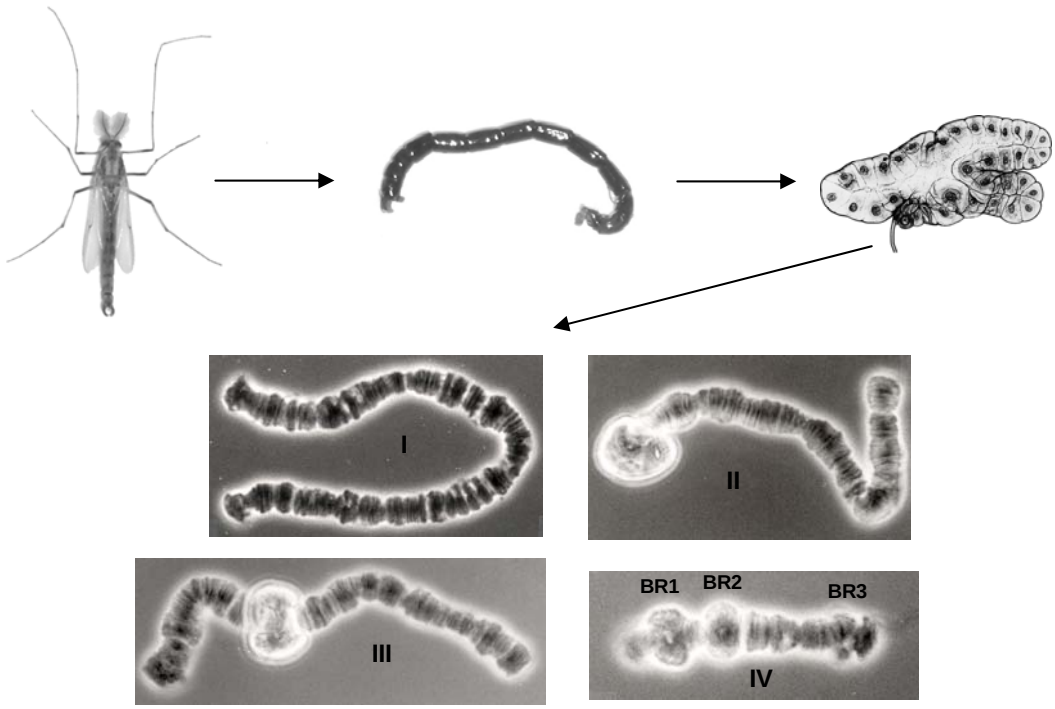

Figure 5. The *Chironomus tentans* experimental system. The larval stage of the non-biting midge *C. tentans*, houses a pair of salivary glands with giant nuclei. The nuclei contain four polytene chromosomes, designated I-IV. On chromosome IV three conspicuous transcriptional puffs are visible, the Balbiani rings (BR1-3).

2.2.2 Balbiani ring genes

In *C. tentans* there are five Balbiani ring genes: BR1, BR2.1, BR2.2, BR3 and BR6. They all encode secretory proteins and are specifically expressed in the salivary glands. The related BR1, BR2 and BR6 genes are very large, 35-40 kilobases (kb), encoding proteins of 1-1.4 MDa. BR6, on chromosome III, has normally very low activity but can be induced by the addition of galactose, glycerol or ethanol to the growth medium (Wieslander, 1994). The BR1 and BR2 genes are highly repetitive and contain only four short introns; three in the 5' end and one in the 3' end (Figure 6). Consequently, the mature mRNA is only slightly shorter than the 35-40 kb pre-mRNA, and forms a granule of 50 nm in diameter easily detectable by electron microscopy in the nucleoplasm (Skoglund et al., 1983).

Due to the polyteny the BR1 and BR2 puffs comprise a large number of active transcription units, which loop out from the chromosome. The gene loci are exceptionally active, corresponding to 85% of the RNA synthesis on chromosome IV (Daneholt et al., 1969). Each transcription unit corresponds to one gene with on average 123 growing RNPs per unit (Lamb and Daneholt, 1979), and 20 minutes are needed for the polymerase to traverse the entire gene at 18°C (Egyhazi, 1976). The RNA polymerases keep the loops in an active state; upon block of transcription there is a rapid condensation into dense chromatin. The active 5 nm chromosome fiber forms a 10 nm fiber, and later on a 30 nm thick chromatin fiber (Andersson et al., 1982; 1984).

From the appearance in the electron microscope the loops can be divided into three regions, from 5' to 3' end, i.e. proximal, middle and distal portion (Andersson et al., 1980). In the proximal region, closest to the promoter, the RNA is fibrillar, in the middle part the 10 nm RNP fibril is starting to fold, and in the distal region the stalked RNP granule is growing until the release of the BR particle. This morphology makes it possible to map when in the transcriptional process e.g. different proteins become associated with the nascent transcript, even though a complete transcription unit is rarely seen in a single electron microscopic section.

The BR3 gene is 10.5 kb long and intron-rich with its 38 introns (Paulsson et al., 1990). The introns are spliced cotranscriptionally (Wetterberg et al., 1996) and the final mature BR3 mRNA is 5.5 kb long corresponding to a 185 kDa secretory protein (Dignam and Case, 1990; Paulsson et al., 1990). Because of the considerable smaller size, the same detailed structural analysis as for BR1 and BR2 is not possible with the BR3 gene transcription and transport.

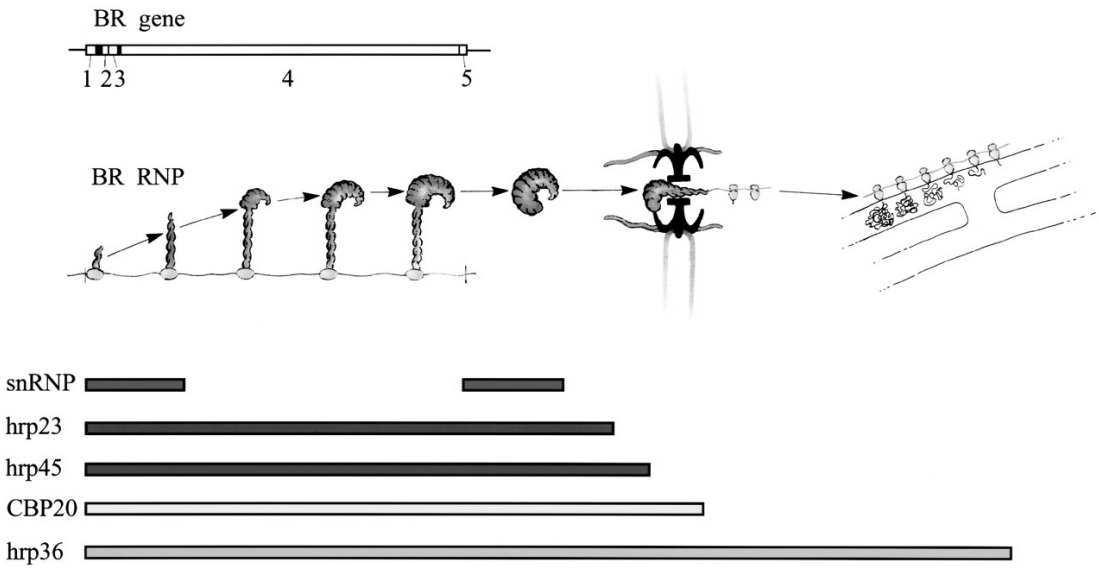

Figure 6. BR RNP particle transcription, assembly and transport. The BR gene comprises five exons with four short intervening introns (top). During ongoing transcription the nascent pre-mRNA associates with hnRNP proteins and assembles into a ring-like structure before unwinding during translocation through the nuclear pore complex (middle). The flow patterns of six proteins that interact with the BR RNA (bottom). Modified from Daneholt, 2001a.

2.2.3 BR RNP particle assembly

Concomitant with transcription many proteins bind to the nascent BR transcript and assemble into an RNP particle. They all have distinct functions and the association with the BR RNP particle is accordingly; some proteins are only transiently a part of the RNP, whereas other proteins follow the transcript from early transcription all the way to the polysomes (Figure 6). Several BR RNP components have been identified and characterized during the last ten years (reviewed in Daneholt, 2001a; Kiesler and Visa, 2004).

An abundant hnRNP A1-like protein, hrp36, is added to the nascent RNA during ongoing transcription and remains associated throughout the processing and translocation steps and even in the polysomes (Visa et al., 1996a). Similarly, the RNA helicase Dbp5 is added early, but is probably not exerting its function until the exit from the nuclear pore complexes when the RNP is unwound to enter into polysomes (Zhao et al., 2002). Simultaneously, a number of factors are released in favor of cytoplasmic proteins. The cap binding protein CBP20 is added shortly after transcription initiation, and is shed before polysome formation (Visa et al., 1996b). There are also examples of non-shuttling proteins that never reach the cytoplasm, hrp45 (Alzhanova-Ericsson et al., 1996) and hrp23 (Sun et al., 1998). The snRNP protein Y12 has an ever more temporary association to the BR RNP; it is only present during early and very late transcription when the splicing of the few introns occurs (Kiseleva et al., 1994). Thus, the flow patterns for the different proteins are specific and most likely closely related to the particular function of the protein.

Hrp65

Another hnRNP protein, hrp65, was first identified as a component of the nucleoplasmic fibers, which occasionally connects with the BR particles in the nucleoplasm and slows down the diffusion rate (Miralles et al., 2000). These fibers were investigated in detail with electron tomography to visualize the interaction in three dimensions. A monoclonal antibody, mAb 1B7, was shown to interact with the fibers and was used to identify and clone the protein component, namely hrp65 (Miralles et al., 2000). Database searches revealed similarity to proteins in other organisms: human PSF (Patton et al., 1993), human $p54^{nrb}$ (Dong et al., 1993), human PSP1 (Fox et al., 2002) and *Drosophila* NonA/BJ6 (Jones and Rubin, 1990; von Besser et al., 1990). These multi-functional proteins are involved in several nuclear processes including transcriptional regulation, pre-mRNA splicing, and retention of defective RNAs (reviewed in Shav-Tal and Zipori, 2002). Like the other similar proteins, hrp65 contains a highly conserved DHBS (*Drosophila* behavior and human splicing) domain consisting of two RNA binding domains and 100 additional amino acids involved in oligomerization. Three splicing variants of hrp65 have been identified: hrp65-1, hrp65-2 and hrp65-3. The isoforms are identical from amino acid residues 1 to 499, but differ in the C-terminal end. In transient transfection assays the localization of the proteins were determined; hrp65-1 is nuclear, whereas hrp65-2 and hrp65-3 are distributed all over the cells (Miralles and Visa, 2001). This indicates that the C-terminal end is important for the localization and presumably the function of the protein (see Paper I).

2.2.4 BR mRNP transport

Investigation of the movement of most RNA species is not possible, because they are not distinguishable in the nucleoplasm. However, the BR particles are clearly visible by electron microscopy. After the release from the gene, the BR RNPs begin their journey to the nuclear envelope. The residence time in the nucleoplasm has been estimated to one hour (Edström et al., 1978). By labeling newly synthesized BR RNA with BrUTP the transport of the BR particles was examined by IEM (Singh et al., 1999). From these studies it was concluded that the BR granules move randomly in the nucleoplasm and appear in all regions of the nucleus, not only in the gene neighboring areas. Kinetic analysis revealed diffusion coefficients compatible with free diffusion, although transient binding to nuclear structures could not be excluded (reviewed in Daneholt, 1999). In agreement with this, BR particles are occasionally associated with connecting fibers in the nucleoplasm (Miralles et al., 2000).

The translocation of the BR RNP through the nuclear pore complexes has been studied in detail (Daneholt, 1997). The step-wise process begins with the binding of the BR particle to the basket fibers of the NPC (Figure 7). Upon binding a terminal ring is formed and a basket structure appears. The particle proceeds into the basket and docks in front of the central channel. The large diameter of the particle prevents translocation without unfolding, and therefore the RNP starts to unfold before entering the transport channel. Further unpacking of the tightly packed ribbon structure starts concomitantly with the translocation, and the RNP fibril is almost fully unfolded when reaching the cytoplasm. Instead of particle reformation in the cytoplasm, the RNP immediately becomes involved in polysome formation and protein synthesis. The translocation always begins with the 5’ end in the lead, permitting translation to start although the 3’ end of the transcript remains in the nucleus (Mehlin et al., 1992).

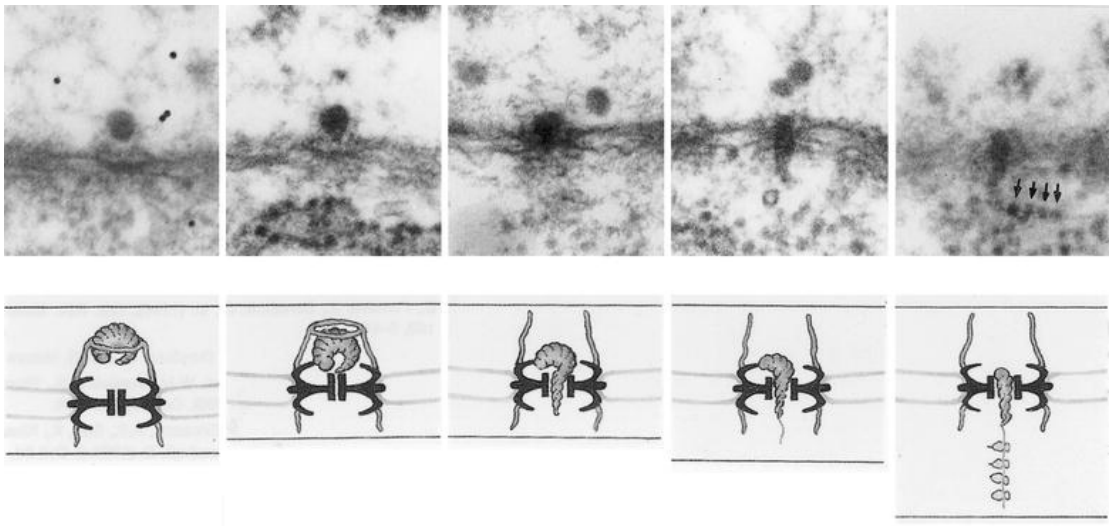

Figure 7. Translocation of the BR particle through the nuclear pore complex. The consecutive steps of the translocation process from binding to the NPC to polysome formation are shown from left to right with both electron micrographs (top) and schematic images (bottom). Modified from Daneholt 2001b.

2.3 RESULTS AND DISCUSSION

2.3.1 The role of the actin/hrp65-2 interaction in transcription (Paper I)

Previously, nuclear actin has been shown to be associated with nascent pre-mRNP particles in *C. tentans* in complex with the hnRNP protein hrp36 (Percipalle et al., 2001). To further explore the function of actin in the cell nucleus we sought to identify novel binding partners of actin. Monomeric G-actin is specifically binding to DNase I, and in a DNase I affinity chromatography experiment with a *C. tentans* nuclear extract several proteins were present in the DNase I bound fraction. The interaction with hrp36 was confirmed, but the presence of another previously characterized protein was established, namely hrp65 (Miralles et al., 2000). The interaction between hrp65 and actin was strictly nuclear, because hrp65 was not present when repeating the experiment with a cytoplasmic extract.

There are three identified splicing variants of hrp65, denoted hrp65-1, hrp65-2 and hrp65-3. The isoforms are identical from amino acid 1-499, but with separate C-terminal ends (Miralles and Visa, 2001). To determine if there was a direct interaction between actin and hrp65, an *in vitro* pull-down assay was performed with purified actin and each of the three isoforms of hrp65. Only hrp65-2 showed a specific interaction with actin, and this specificity was confirmed by competition studies where the C-terminal domain of hrp65-2 (65-2CTS) could disrupt the interaction between actin and the full-length protein. Mutation of the 65-2CTS peptide impaired actin interaction, providing further evidence for the sequence containing an actin-binding motif. A direct interaction of hrp65-2 and actin was confirmed by *in vivo* crosslinking experiments with DSP (dithiobis-succinimidylpropionate). DSP is a reversible crosslinker connecting proteins not further apart than 11 Å. Actin was coimmunoprecipitated with hrp65-2 even under denaturing conditions, indicating a direct interaction *in vivo*. This interaction was disrupted by adding the 65-2CTS peptide.

The role of the actin-hrp65-2 interaction was explored by *in vivo* fluorescence experiments in the salivary glands of *C. tentans*. Under normal conditions the Balbiani rings are highly transcriptionally active and readily visible by immunostaining. Upon microinjection of the 65-2CTS peptide the BRs reduced in size dramatically and mimicked the scenario when the transcription was blocked by actinomycin D. The effect on RNA polymerase II transcription was confirmed by microinjection of BrUTP that becomes incorporated into newly synthesized transcripts; the BrUTP incorporation was drastically reduced when injected together with 65-2CTS peptide. This implies that the actin-hrp65-2 complex is involved in the transcription of most RNA pol II genes.

That hrp65-2 is associated with nascent transcripts on actively transcribing chromosomes was determined by immunocytology on isolated chromosomes. The Balbiani rings were stained together with several other gene loci, and the staining disappeared after RNase treatment. To investigate the localization of hrp65-2 in more detail immunoelectron microscopy was performed. The unique structure of the BR genes allowed mapping of hrp65-2 along the entire BR gene, either bound to the RNA itself, associated to the RNA polymerase or the chromatin axis. This localization suggested a role for the actin-hrp65-2 complex in transcription elongation, and a

nuclear run-on transcription assay confirmed this. Anti-actin antibodies and hrp65-2CTS peptide perturbed the incorporation of radiolabeled UTP in the growing transcripts.

In summary, we have found a novel actin binding partner, hrp65-2, that in direct contact with actin is required for the RNA polymerase II activity. The hrp65-2 protein is distributed all along the active gene and the interaction with actin is essential for transcription elongation.

2.3.2 Coupling between transcription rate and splicing (Paper II)

Most of the proteins that assemble into the mRNP particle are ubiquitous factors that bind several or most gene loci. When screening for novel nuclear RNA binding proteins in *C. tentans*, we found a transcript-specific hnRNP protein. The monoclonal antibody specifically decorated the BR3 gene on isolated chromosome IV and only occasionally two other loci on chromosome I and II, respectively. Immunoelectron microscopy revealed a distribution all along the active BR3 gene. The antibody recognized a protein of 130 kDa and consequently the protein was called hrp130. Upon RNase A treatment of isolated chromosomes the hrp130 immunostaining vanished, whereas RNA pol II staining remained. Thus, hrp130 is associated to the nascent RNA rather than to the polymerase or the chromatin.

The hrp130 cDNA was isolated by screening *C. tentans* expression libraries with the monoclonal antibody and a sequence corresponding to 1028 amino acids was identified. A polyclonal antibody was raised against a part of the predicted protein, and in biochemical and immunocytological experiments the two antibodies provided identical results, confirming that the isolated cDNA encoded hrp130.

Two types of domain structures were found in the predicted sequence, namely three N-terminal WW domains and six C-terminal FF domains. Both domains comprise 40-50 amino acids and are characterized by two tryptophan (W) and two phenylalanine (F) residues, respectively. Both domains are known to mediate protein-protein interactions (Sudol et al., 1995; Bedford and Leder, 1999). The same or similar conserved domain organization is present in several other proteins, e.g. the human elongation factor CA150 and the yeast splicing factor Prp40. CA150 can interact with the phosphorylated CTD of the RNA pol II and works as a transcription elongation repressor (Sune and Garcia-Blanco, 1999). Moreover, CA150 binds to splicing factor SF-1 (Goldstrohm et al., 2001) implying a connection between transcription rate and splicing. That Prp40 has similar binding partners (Morris and Greenleaf, 2000), further strengthens this notion.

We have identified and characterized a novel hnRNP protein, hrp130. The very sparse localization of hrp130 implies a very specific role for one or a few gene loci. We suggest that hrp130 is involved in regulating the transcriptional elongation rate, to permit cotranscriptional splicing of the intron-rich BR3 gene.

2.3.3 A link between transcription and RNA-export (Paper III)

Traditionally, the steps of gene expression and transport have been regarded as sequential but separate events. Growing evidence points to an opposite situation where most processes are intimately coupled in different ways (see Introduction). In this study we explored the possibility of a signaling system between ongoing transcription and transport to and through the nuclear pores. This is feasible on a morphological level in our model system, due to the possibility of following the BR particle from the transcription site to the different steps of translocation through the NPC.

Salivary glands were isolated and treated with either of the transcription inhibitors DRB or actinomycin D. The sister gland from the same animal was left untreated as control. The effects of the two drugs on the Balbiani ring gene transcription and transport were studied by both immunofluorescence and electron microscopy.

Previously, DRB-treated Balbiani rings have been investigated in detail (Andersson et al., 1982; 1984). The interruption of transcription leads to a rapid reformation of the thick chromosome fiber and a packing into dense chromatin. Our results are in good agreement with these data; after 90 min of incubation the conspicuous BR puffs had regressed and were no longer apparent. In contrast, actinomycin D has a different working mechanism and as predicted the regression of the actinomycin D treated BRs was not as prominent. On the other hand, the effect of actinomycin D on the BRs on the electron microscopic level was evident. Thus, the expected transcriptional inhibition was accomplished for both drugs.

Next our focus turned to the nuclear envelope and RNP export. BR granules located within 50 nm (one particle diameter) from the midline of the nuclear envelope, were classified as bound to the NPCs, whereas pear- or rod-shaped RNPs within the NPC were regarded as particles in transit. BR particles of the two groups were quantified for the two inhibitors at the different time points (5, 10, 30 and 90 min). For both DRB and actinomycin D the drop in translocations was rapid and almost complete. A drastic effect of DRB appeared as early as 5 min after drug addition. The number of NPC-bound BR particles also decreased.

However, the nucleoplasmic concentration of BR RNPs remained constant. This means that although there are numerous RNPs present they are either immobilized in the nucleoplasm or no longer able to bind to the NPC. Interestingly, the nucleoporins Nup98 and Nup153 have been shown to have transcription-dependent mobility (Griffis et al., 2002; 2004). When transcription is inhibited their mobility is blocked. Perhaps the nucleoporins, primarily Nup98, are components of the mRNP and mediate this immobilization. Another explanation could be the involvement of SR proteins. A hyperphosphorylation of the SR proteins leads to a release of export receptors and thus disability of binding to the NPCs and export failure. Nevertheless, the nature of the signaling pathway from the transcription machinery to the kinases and/or phosphatases is still an open question.

We conclude that two transcription inhibitors, with different working mechanisms, can influence the RNA export in a similar manner. The binding to the nuclear pore

complexes was decreased and there was an almost complete block of translocations through the NPC. Therefore, there is an evident link between ongoing transcription and RNA export.

2.3.4 Exclusion of RNPs from a zone beneath the nuclear envelope upon transport inhibition (Paper IV)

The initial phase in the RNA export pathway through the nuclear pore complexes is not entirely understood. To explore the role of the peripheral nucleoporins we used two separate agents that interfere with their function, namely wheat germ agglutinin (WGA) and the monoclonal antibody mAb 414. Both inhibitors bind to an overlapping set of nuclear pore complex components, including the basket structure. WGA is a plant derived lectin binding to *N*-acetylglucosamine in glycosylated proteins (Nagata and Burger, 1974), and mAb 414 recognizes a few glycosylated FG-repeat proteins (Davis and Blobel, 1987; Shah and Forbes, 1998; Drummond and Wilson, 2002).

WGA-associated proteins in a *C. tentans* nuclear extract were detected by Western blot analysis and several proteins of a broad size range were shown to bind to WGA, most likely including the glycosylated nucleoporins. Previously the reactivity of the mAb 414 was determined in *C. tentans* (Soop et al., 2005).

The intracellular distribution of the WGA-binding proteins was established by microinjection of fluorochrome-labeled WGA into the salivary gland cell nucleus. WGA-labeling appeared mainly around the nuclear envelope, presumably binding to the glycoproteins of the nuclear pore complexes. Previously, WGA was shown to block protein import (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988). The ability of WGA to block protein import in *C. tentans* was confirmed by a subsequent cytoplasmic microinjection of BSA conjugated to both a fluorescent marker and a nuclear localization signal. The import of the BSA substrate was completely inhibited after nuclear injection of WGA.

Electron microscopic analysis was used to investigate the nuclear envelope and possible RNP transport defects in detail. Cells in the same salivary gland were either microinjected with WGA, mock-injected with KCl buffer or not injected as internal controls. After 90 min incubation, a very clear effect of the WGA on the RNA export was seen, whereas the KCl-injected cells appeared normal. Both the export of mRNA and ribosomal particles was affected; there was an accumulation of both BR particles and putative ribosomal particles in the nucleoplasm. More BR RNPs were recorded on top or within the nuclear baskets, suggesting that initiation of translocation had been inhibited. Accordingly, the number of BR RNPs involved in translocation was decreased to half. Remarkably, the high density of ribosomal particles in the nucleoplasm revealed a thin exclusion zone close to the nuclear envelope at the level of the nuclear baskets. This zone was typically free from rRNPs, only rarely did the ribosomal particles appear therein. Microinjection of mAb 414 resulted in similar effects, although not as drastic.

The export defects were presumably not due to unspecific clogging of the pore, but rather an inhibition of a crucial step in the early translocation process. This was

previously demonstrated by unblocked diffusion of dextran molecules through the NPCs after WGA injection (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988).

We suggest that the exclusion zone is formed due to a constant movement of the nuclear fibrils of the NPC. When the normal function of the fibers is impaired by binding of WGA or mAb 414 the RNPs are kept away at a certain distance from the transport pathway, instead of being brought into it. An alternative explanation could be an additional lattice connecting the top of the nuclear baskets, the nuclear envelope lattice, that was proposed by Goldberg and Allen (Goldberg and Allen, 1992). However, we have no indications for the existence of such a fibrous sheet in *C. tentans*.

In conclusion, upon inhibition of nucleoporins with either a lectin or an antibody, the nuclear export of RNA was reduced. BR RNPs accumulated bound to the nuclear pores while the translocating BR RNPs decreased, suggesting that the initiation of translocation proper had been inhibited. Ribosomal particles accumulated in the nucleoplasm and did not enter into or between the nuclear baskets. Thus, the transport of rRNPs had been blocked prior to the entry into the NPCs. We propose that normally freely moving nuclear fibers repel the RNPs from the NPCs unless the particles can bind to nucleoporins within the basket and be further transported through the NPC.

2.4 SUMMARY AND PERSPECTIVES

We have studied a number of aspects of the gene expression in our model organism *C. tentans*, and we have identified novel players and regulatory mechanisms of the transcription and transport of the Balbiani ring mRNP.

In Paper I the hnRNP protein hrp65-2 was identified as a binding partner for nuclear actin. This interaction was direct and the complex was localized all along the growing BR transcript. Disruption of this interaction resulted in inhibition of transcription elongation. Interestingly, further light on the function of the actin-hrp65-2 complex in transcription elongation was shed by a recently published study regarding a protein with histone acetyltransferase activity, namely p2D10 (Sjölinder et al., 2005). This nucleoplasmic protein is structurally similar to the general transcription factor TFIIIC and is associated with hrp65 (Sabri et al., 2002). This interaction is direct, whereas the interaction with actin is indirect via hrp65. The model proposed links p2D10 to the active gene through the actin-hrp65-2 complex. Histone H3 becomes acetylated by p2D10 which results in a more open conformation of the chromatin leading to a facilitated transcription elongation (Sjölinder et al., 2005). Thus, hrp65-2 functions as an adaptor between actin and the histone acetyltransferase, and recruits p2D10 to the BR genes. When the interaction between actin and hrp65-2 was disrupted by the hrp65-2 peptide, the histones remained unacetylated and the transcription elongation was inhibited.

In Paper II, hrp130, a *C. tentans* ortholog of the mammalian transcriptional repressor CA150, was identified and characterized. Peculiarly, the localization of hrp130 was limited to the Balbiani ring 3 gene and a couple of additional loci. We suggest that this

restricted distribution is due to the large number of introns in the BR3 gene. The hrp130 protein could modulate the transcription rate to allow proper splicing. However, the intron content of the other hrp130-binding loci has not been investigated. The adjustment of the transcription rate is thought to be accomplished by recruiting or displacing positive and negative transcription elongation factors (Goldstrohm et al., 2001). Most probably hrp130, like CA150, exerts its function on the RNA pol II via the CTD-tail, although this interaction has not been elucidated. The interaction of hrp130 with splicing factors also remains to be established. Nevertheless, the conservation between hrp130 and CA150 points to very similar functions of the proteins.

In Paper III a striking connection between the transcription and transport was discovered. Inhibition of transcription led to an almost complete block of BR RNP translocation, and a clear reduction of binding to the nuclear pore complexes. Surprisingly, the nucleoplasmic concentration of BR particles remained constant, which suggested that the BR RNPs either were arrested in the nucleoplasm or could not bind to the nuclear pore complexes. Interestingly, the nucleoporin Nup98 has transcription-dependent mobility (Griffis et al., 2002). Perhaps, Nup98 as a component of the BR particle mediates the detention in the nucleoplasm. The flow pattern of Nup98 in *C. tentans*, with or without ongoing transcription, could be investigated by electron microscopy with an antibody directed against Nup98. In addition, the phosphorylation state of the SR proteins binding to the BR particle would be something to explore further. If the SR proteins become phosphorylated in the response to inhibited transcription the interaction with the export receptor would be lost, and consequently the binding to the nuclear pore complexes. In our system it is only possible to examine the transcription and transport of the BR gene product, but most likely this transport inhibition applies more globally because similar effects have been seen in other systems (e.g. Tokunaga et al., 2006).

Finally, in Paper IV we investigated the initial stages of the translocation through the nuclear pore complexes by inhibiting the nuclear export with wheat germ agglutinin or the mAb 414 antibody against a subset of the FG nucleoporins. Both agents are known to inhibit both protein import and RNA export, and consequently we found that BR particles and putative ribosomal particles accumulated in the nucleoplasm and that the protein import was inhibited when WGA or mAb 414 was microinjected into the nucleus. However, there was no effect on transport upon cytoplasmic injection indicating that only the FG nucleoporins on the nuclear side of the pore are crucial for nuclear export of RNA. A similar inhibition of transport was revealed when an antibody against the N-terminal part of the nucleoporin Nup153 was injected into the *C. tentans* cell nucleus (Soop et al., 2005). Although WGA and mAb 414 both bind to Nup153 the transport block was not as extensive as in that study, perhaps reflecting that the N-terminal domain is more important than the C-terminal part recognized by WGA and mAb 414. The main finding of Paper IV was a thin zone beneath the nuclear envelope essentially free from RNP particles. Especially the ribosomal particles were absent from this excluding zone. We suggest a model for the clearing of this zone by freely moving nuclear basket fibrils that repel RNPs not binding to nucleoporins in the basket. That would increase the selectivity of the NPC barrier.

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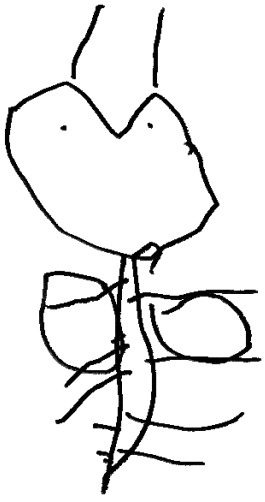

“Mygga”, Hilde 3 år

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