Chapter 1

Introduction – Post-transcriptional gene expression regulation

Post-transcriptional gene expression regulation

This chapter gives an overview of post-transcriptional gene expression regulation, with a strong focus on regulation at the level of mRNA translation. Translation initiation mechanisms are described and examples of translational regulation introduced. Furthermore, novel microarray-based techniques are discussed, which allow the study of post-transcriptional gene expression regulation on a genome-wide scale.

An overview

The phenotype of an organism is largely determined by the sum of functional proteins in the cell, the sequence of which are encoded as genes in its DNA. The control of gene expression is a fundamental process to bring the genome to life and mis-regulation at any level is usually associated with disease. Today, it is well established that gene expression is regulated at various levels and there is increasing evidence that the diverse processes involved in this regulation are integrated with each other (Maniatis and Reed 2002; Orphanides and Reinberg 2002; Proudfoot et al. 2002; Hieronymus and Silver 2004; Mata et al. 2005; Moore 2005; McKee and Silver 2007). Gene expression regulation can be divided into 2 main categories of (1) transcriptional control and (2) post-transcriptional control (Figure 1.1). Furthermore, downstream of these 2 processes, expressed proteins can still be regulated by post-translational modifications and protein degradation (post-translational control).

Transcriptional control has received much attention, through both traditional single gene studies (Kadonaga 2004) as well as through genome-wide approaches such as expression profiling (Lockhart and Winzeler 2000; Bertone et al. 2005), transcription factor binding studies and identification of regulatory sequence elements (Hanlon and Lieb 2004; Sandelin et al. 2007), and chromatin remodelling and epigenetics (Bernstein et al. 2007; Kouzarides 2007; Li et al. 2007). Post-transcriptional control has been less extensively studied. This discrepancy is apparent, when searching for these 2 terms within the scientific literature: roughly 55,000 articles are found on PubMed (www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed) for the search query "transcriptional regulation", whereas "post-transcriptional regulation" only returns roughly 5700 hits. This bias is partially based on historical as well as technical reasons: it is clear that transcription is one of the fundamental and intuitively

important steps within the cascade of gene expression regulation and techniques to study transcription and transcriptional control are well established in the scientific community.

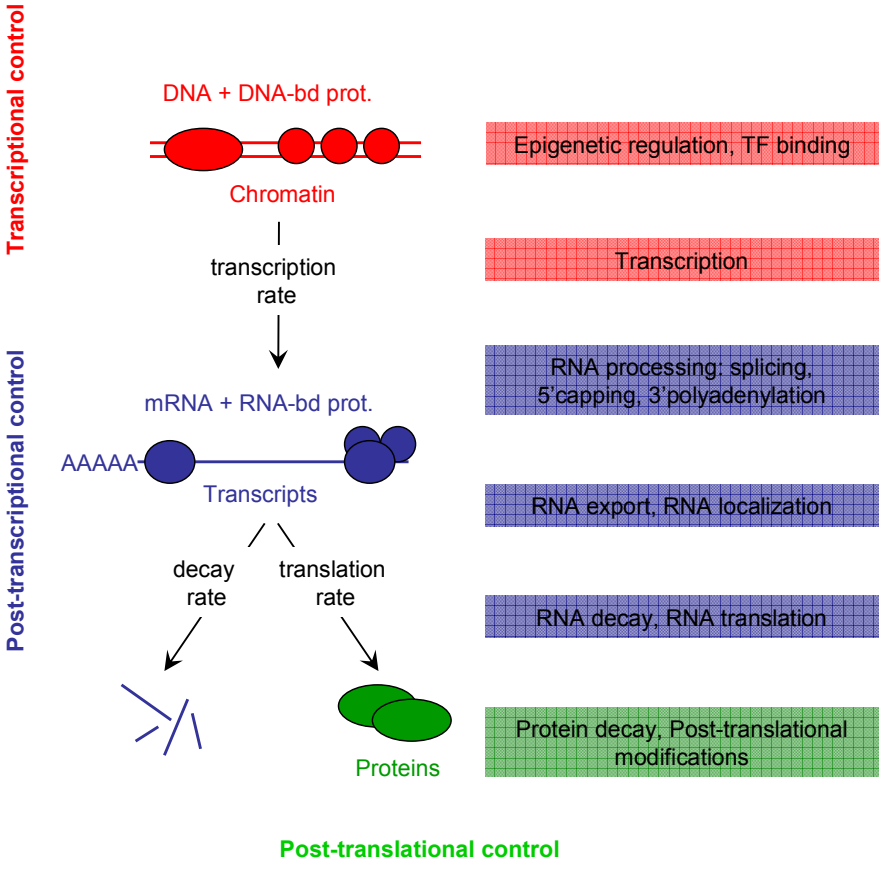


Figure 1.1 Layers of gene expression regulation
Shown is a schema of the various layers of gene expression regulation. The various regulatory processes are colour-coded according to their involvement in transcriptional control (red), post-transcriptional control (blue) or post-translational control (green). This figure is adapted from Mata et al. (2005).

However, recently there has been increasing appreciation of the necessity and importance of post-transcriptional gene expression regulation. Post-transcriptional regulation mechanisms comprise various processes such as mRNA processing (polyadenylation, capping and splicing), mRNA export and localization, mRNA decay, and mRNA translation (Figure 1.1). Despite this variety of regulatory mechanisms, there is one thing in common for all of them: they ultimately control if and where a given mRNA is translated into a protein. Consequently, translation and translational control are central to post-transcriptional gene expression regulation. Therefore, first translational initiation mechanisms and translational regulation will be

discussed in detail and then an overview of recent efforts to study post-transcriptional regulation on a genome-wide scale will be given.

Translational regulation

After transcription, before translation: RNA processing and export

Before an mRNA can be transported out of the nucleus into the cytoplasm in order to be available for the translational machinery, it has to undergo a series of processing steps: the mRNA acquires a cap structure at the 5' terminus, introns are spliced out from the pre-mRNA, and a specialized 3' end of the mRNA is generated, usually by polyadenylation. All these steps happen co-transcriptionally and can influence each other (Proudfoot et al. 2002). Only a brief overview of these processes will be given, especially as far as they are relevant to translational regulation, and some of the many reviews, which give a more detailed view of these specific RNA processing steps, will be pointed out.

The first processing step is the addition of the m^7G cap structure to the 5' end of the nascent mRNA and happens after 20-30 nucleotides have been synthesized (for reviews see Shatkin and Manley 2000; Gu and Lima 2005): In a three-step reaction, the GMP moiety from GTP is added to the first nucleotide of the pre-mRNA, and GMP is methylated at position N7. The m^7G cap is important for mRNA stability and translation (see below). In the nucleus, the m^7G cap gets bound by the cap binding complex (CBC), which consists of 2 subunits and after shuttling to the cytoplasm, it gets bound by translation initiation factor 4E, which is an essential step in translation initiation.

As the coding sequences (exons) of most mRNAs in higher eukaryotes are interrupted by introns, these introns must be spliced out of the pre-mRNA in order to generate a functional mRNA. Splicing requires consensus sequences on the mRNA, which mark the exon-intron boundaries, and the spliceosome, the catalytic complex which carries out the enzymatic reactions to remove the introns and ligate the flanking exons (for reviews see Kramer 1996; Collins and Guthrie 2000; Jurica and Moore 2003; Patel and Steitz 2003). The spliceosome consists of 5 small ribonucleoprotein particles (snRNPs: U1, U2, U4, U5 and U6), each of them made out of a small nuclear RNA (snRNA) and associated proteins, and many accesory proteins. In fact, well over a

hundred proteins are thought to be splicing factors (Jurica and Moore 2003). The catalysis of the splicing reaction itself is dependent on RNA-protein, RNA-RNA, and protein-protein interactions. Furthermore, the alternative use of exons (alternative splicing) can contribute to the creation of protein variety by allowing one gene to produce multiple isoforms (Matlin et al. 2005).

Most mRNAs also bear a specific structure in the form of a poly(A) tail at their 3' end. The only known protein-coding genes lacking poly(A) tails are histone mRNAs in most higher eukaryotes, but not in yeast (Fahrner et al. 1980). Polyadenylation is achieved in two steps: the nascent mRNA is cleaved at the site where polyadenylation is meant to begin, which is followed by poly(A) synthesis (for reviews see Zhao et al. 1999; Shatkin and Manley 2000; Proudfoot and O'Sullivan 2002). In analogy to splicing, formation of the poly(A) tail requires a multi-subunit polyadenylation complex and specific sequence-elements on the pre-mRNA. In mammalian cells, the site of cleavage lies mostly between an AAUAAA hexamer motif and a GU-rich downstream element (DSE) (McLauchlan et al. 1985). The AAUAAA hexamer is bound by the cleavage and polyadenylation specificity factor (CPSF), and the DSE interacts with the cleavage stimulatory factor (CstF). Cleavage factor I and II (CF I; CF II) are also required. Whereas both poly(A) polymerase (PAP) and CPSF are required for cleavage of the pre-mRNA and poly(A) addition, CstF is necessary for the endonucleolytic cleavage and – together with CPSF – for the recruitment of CF I and CF II (Takagaki et al. 1989; MacDonald et al. 1994; Murthy and Manley 1995). The principles of poly(A) tail formation are the same in yeast and mammalian cells and the protein complexes involved have orthologous components, but also specific accessory factors that are only found in one of the species (Shatkin and Manley 2000; Proudfoot and O'Sullivan 2002; Stevenson and Norbury 2006). Furthermore, in yeast, a variable A-rich element substitutes for the AAUAAA hexamer motif and there are 3 polyadenylation complexes: cleavage polyadenylation factor (CPF), which contains several factors homologous to CPSF and also the poly(A) polymerase, cleavage factor IA (CF IA) and cleavage factor IB (CF IB).

The emerging poly(A) tail is bound by the poly(A)-binding protein (PAPB). PABP is thought to influence the final length of the poly(A) tail on the one hand positively by stimulating the processivity of PAP, on the other hand negatively, by interacting with the poly(A) nuclease PAN (Mangus et al. 2003). Furthermore, PABPs are involved in nuclear export and are also important for the initiation of translation (see below). The

poly(A) tail is also crucial for several other post-transcriptional regulatory mechanisms in the cytoplasm and cytoplasmic polyadenylases can regulate the translational state and stability of various target mRNAs via modifying the length of the respective poly(A) tails (Read and Norbury 2002; Stevenson and Norbury 2006). The best studied example is probably that of translational regulation of maternal mRNAs in *Xenopus* oocytes, which are stock-piled in a translationally-repressed state with very short poly(A) tails, which become polyadenylated upon activation and as a consequence translated (see below and Mendez and Richter 2001; Richter 2007). mRNA decay by exonucleolytic mechanisms is also usually preceded by a shortening of the poly(A) tail (Wilusz et al. 2001; Parker and Song 2004), and recently deadenylation of poly(A) tails has also been shown to happen in microRNA (miRNA)-mediated expression regulation (Giraldez et al. 2006; Wu et al. 2006).

The last part in the journey from the site of transcription into the cytoplasm is the nuclear export of the mature mRNA. Export through the nuclear pore complex (NPC) happens in the context of messenger ribonuleoprotein particles (mRNPs) (for reviews see Daneholt 1997; Cole and Scarcelli 2006; Stewart 2007). mRNPs comprise the mRNA and associated RNA-binding proteins, which bind to the mRNA during the processing steps (Aguilera 2005; Moore 2005). Apart from the aforementioned CBC or PABP, such RNA-binding proteins include SR (serine/arginine rich) and hnRNP (heterogeneous nuclear RNP) proteins, or the exon junction complex (EJC), which is a set of proteins loaded onto the mRNA upstream of exon-exon junctions as a consequence of pre-mRNA splicing. These factors are important for the association of the mRNP with the NPC and the shuttling into the cytoplasm, and some of them stay associated with the mRNA as it is exported, whereas others are restricted to the nucleus. Furthermore, nuclear export is an important step in quality control, as faulty or un-processed mRNAs are not only useless, but potentially harmful, if translated in the cytoplasm. Only functional mRNAs are exported into the cytoplasm and this quality control step is closely coupled to RNA processing and the mRNP composition.

Again, it needs to be emphasized, that despite the introduction of mRNA transcription, capping, splicing, polyadenylation and nuclear export as sequential events, these events occur in the cell integrated with each other and not at all independently in spatial and temporal context (Proudfoot et al. 2002; Aguilera 2005; Moore 2005).

Molecular mechanism of translation initiation in eukaryotes

Translation can be divided in 3 major steps: initiation, elongation and termination. Translation initiation comprises the summary of events that lead to the positioning of an elongation-competent 80S ribosome over the AUG start codon of the mRNA. Polypeptide synthesis takes place during the elongation phase. The completed polypeptide is released after the ribosome encounters a stop codon during translation termination.

Much evidence points toward translation initiation being the rate limiting step in the process of translating an mRNA into a protein. When cells are treated with low doses of elongation inhibitors such as cycloheximide in a way that total protein synthesis is only minimally affected, most mRNAs are found to be resistant to low levels of elongation inhibitors and their translational efficiency is basically not altered (Lodish and Jacobsen 1972; Walden et al. 1981; Mathews et al. 2007). Furthermore, the average density of ribosomes along the mRNA is significantly lower than the maximum packing capacity of one ribosome per 30-40 nucleotides (Wolin and Walter 1988; Arava et al. 2003; Mathews et al. 2007). This maximum capacity can be obtained by treating mRNAs with drugs that slow down elongation. The complexity and importance of translation initiation compared to elongation and termination is further underscored by the fact that only few dedicated factors are needed for the latter two processes, whereas more than 25 proteins are needed to ensure proper translational initiation (Preiss and Hentze 2003; Pestova et al. 2007). Therefore, it is not surprising that most translational regulation is executed at the level of initiation (Preiss and Hentze 2003; Gebauer and Hentze 2004; Holcik and Sonenberg 2005; Mathews et al. 2007). An overview of the molecular mechanisms of translation initiation will be introduced here, as far as they are directly relevant to the regulation of translation and the examples presented below. For a more detailed view of the molecular events regulating translation initiation in mammalian cells and yeast, see references (Hinnebusch et al. 2007; Pestova et al. 2007).

43S pre-initiation complex formation. Translation initiation starts with the formation of the 43S pre-initiation complex (Figure 1.2). As physiological conditions favour the association of 40S ribosomal subunits and 60S ribosomal subunits to form 80S subunits (i.e. full ribosomes) but only free ribosomal subunits can initiate

translation, it is important that post-termination ribosomes dissociate (Pestova et al. 2001; Preiss and Hentze 2003). In prokaryotes this dissociation is achieved through a ribosome-recycling factor, but there is no known eukaryotic equivalent (Kisselev and Buckingham 2000). The eukaryotic initiation factors (eIFs) eIF3, eIF1 and eIF1A are thought to promote this dissociation in eukaryotes, but the mechanism for it is unknown and recent data suggest that the activity of these factors is not sufficient to prevent formation of 80S subunits (Preiss and Hentze 2003; Pestova et al. 2007), and it is thought that dissociation of empty 80S subunits is directly linked to 43S pre-initiation complex formation (Pestova et al. 2007).

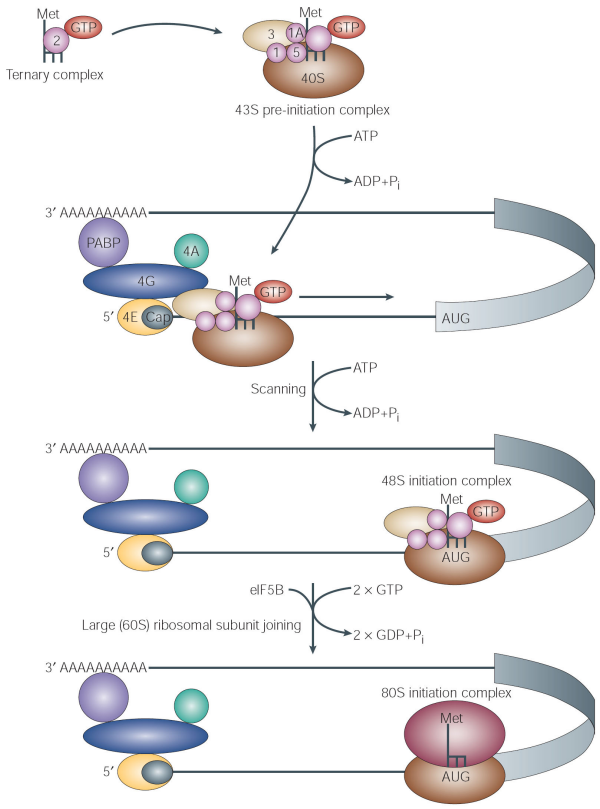
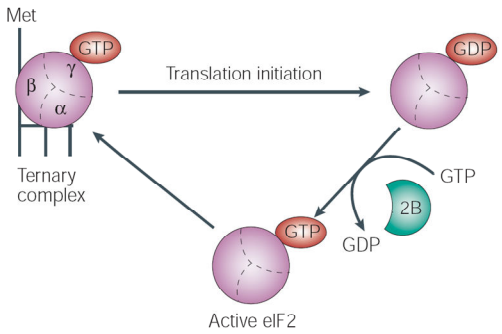


Figure 1.2 Molecular mechanisms of translation inititation
Shown are the major molecular events that lead to cap-dependent translation inititation. For a detailed description see main text. This figure is taken from Gebauer et al. (2004).

The first step in 43S pre-initiation complex formation is the formation of the ternary complex (Figure 1.3, Figure 1.2). The ternary complex consists of eIF2, a hetero-trimer of α, β and γ subunit, methionyl-initiator tRNA (Met-$tRNA_i^{Met}$) and GTP, and its assembly is regulated by the guanine nucleotide exchange factor (GEF) eIF2B (Figure 1.3): GTP is hydrolyzed after recognition of the AUG start codon producing

eIF2 bound to GDP, which has a 10-fold reduced affinity for Met-tRNA$_i^{Met}$ (Hinnebusch et al. 2007). eIF2B promotes the GDP-GTP exchange to re-generate active eIF2 (Figure 1.3) (Preiss and Hentze 2003; Hinnebusch et al. 2007; Pestova et al. 2007). Binding of the active ternary complex to the 40S ribosomal subunit is aided independently by eIF1, eIF1A and eIF3 in mammalian cells (Preiss and Hentze 2003; Pestova et al. 2007). In budding yeast eIF1, eIF3, eIF5 and the ternary complex can be isolated as a multifactor complex (MFC), which raises the possibility that this MFC is recruited to the 40S subunit as pre-formed unit (Hinnebusch et al. 2007). The 43S pre-initiation complex is then ready to bind to the 5' end of the mRNA.



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The ternary complex consists of eIF2, a hetero-trimer of α, β and γ subunit, methionyl-initiator tRNA (Met-tRNA$_i^{Met}$) and GTP, and its assembly is regulated by the guanine nucleotide exchange factor (GEF) eIF2B: GTP is hydrolyzed after recognition of the AUG start codon producing eIF2 bound to GDP, which has a 10-fold reduced affinity for Met-tRNA$_i^{Met}$. eIF2B promotes the GDP-GTP exchange to re-generate active eIF2. This figure is taken from Gebauer et al (2004).

Recruitment of the pre-initiation complex to the mRNA. Recognition of the m^{7}G cap structure at the 5' end of the mRNA is mediated by the cap-binding complex eIF4F, which comprises the 3 subunits eIF4E, eIF4G and eIF4A (Figure 1.2). eIF4E binds directly to the m^{7}G cap structure; eIF4A is a dead-box RNA helicase that is thought to unwind secondary structures in the 5' UTR (Un-Translated Region) so that the 43S complex can scan along the mRNA; and eIF4G is thought to act as scaffold protein (Preiss and Hentze 2003; Hinnebusch et al. 2007; Pestova et al. 2007). In mammalian cells, eIF3 from the pre-initiation complex interacts with the central domain of eIF4G (Lamphear et al. 1995). This interaction has not yet been found in budding yeast, where eIF4A is also not stably associated with eIF4E and eIF4G (Goyer et al. 1989; Hinnebusch et al. 2007). Altogether, the binding of the pre-

initiation complex to the mRNA involves the cooperative activities of eIF4F, eIF3, eIF4B and possibly the poly(A)-binding protein (PABP). PABP was initially identified as a protein that associates with the poly(A) tail at the 3' UTR of the mRNA. The concerted binding of PABP and eIF4E to eIF4G is thought to pseudo-circularize the mRNA (Figure 1.2) (Wells et al. 1998). Furthermore, in budding yeast poly(A)-binding protein PAB1 is essential for translation initiation (Sachs 2000). This circularization provides a possible framework by which 3' UTR-binding proteins can regulate translation initiation, as most known regulatory sequences are found in the 3' UTR, despite the fact that translation starts at 5' end of the mRNA (Gebauer and Hentze 2004).

Scanning of the mRNA and AUG recognition. After proper assembly at the 5' end of the mRNA, the pre-initiation complex needs to scan along the mRNA until the recognition of the AUG start codon (Kozak 1989; Kozak 2002). The model of scanning had originally been proposed by Kozak, and despite the fact that most biochemical and genetic data are consistent with the model (Kozak 1999), direct physical intermediates of the scanning process have not been identified to date. The 43S pre-initiation complex can bind to an mRNA having an unstructured 5' UTR independent of eIF4F, eIF4A and ATP, but needs eIF1 or eIF4G to scan to the start codon. However, an mRNA with a structured 5' UTR additionally requires eIF4F, eIF4B, ATP and eIF1A (Pestova et al. 1998; Pestova and Kolupaeva 2002). eIF4A helicase and eIF4F are thought to promote unwinding of the secondary structure of the mRNA, while eIF1 and eIF1A are thought to promote a structural conformation of the 43S pre-initiation complex, which allows scanning in 5'-3' direction.

Ready to go: formation of the translation competent 80S subunit. The 43S pre-initiation complex recognizes the start codon through formation of base-pairs between the anticodon loop of the initiator tRNA and the AUG start codon (Figure 1.2). This stable complex is referred to as 48S initiation complex. Selection of the correct start codon is dependent on eIF1 (Pestova et al. 1998; Pestova and Kolupaeva 2002). Then, several events take place in order for the 60S subunit to join the 48S complex and form the 80S subunit. eIF5 catalyzes the hydrolysis of eIF2-GTP, and as a consequence most of the initiation factors including eIF2-GDP disassociate from the small ribosomal subunit, leaving the initiator tRNA bound to the start codon

(Hinnebusch et al. 2007). Recently, it has been found that a second step of GTP hydrolysis is necessary for 60S joining and to render the resulting 80S subunit competent for polypeptide synthesis: GTPase activity of eIF5B is stimulated by 60S subunit and even stronger by 80S subunits. GTP-bound eIF5B stimulates 60S subunit joining and GTP hydrolysis occurs after 80S subunit formation has happened and is essential for the release of eIF5B (Pestova et al. 2000; Lee et al. 2002; Shin et al. 2002). Taken toghether, 2 steps of GTP-hydrolysis are required for 80S complex formation and also provide a checkpoint for proper start codon recognition.

Cap-independent translation initiation. The events of translational initiation described above are an account of cap-dependent translational initiation, which usually occurs for most cellular mRNAs. However, an alternative way of initiating translation can happen in a cap-independent way through internal ribosomal entry sites (IRES). IRES are heavily structured sequence elements in the 5' UTR of the mRNA with no obvious conserved consensus sequence (Baird et al. 2006). The structured IRES segment in the 5' UTR of the mRNA has an active role in the recruitment of the 40S subunit. IRES elements are found in viral mRNAs and also certain cellular mRNAs, which are involved in growth control, differentiation, apoptosis or oncogenesis (Doudna and Sarnow 2007; Elroy-Stein and Merrick 2007). These mRNAs are usually only weakly translated under normal conditions, but can be more efficiently translated upon down-regulation of cap-dependent translation. For further in-depth reviews on the topic of IRES see references (Hellen and Sarnow 2001; Stoneley and Willis 2004; Jackson 2005; Spriggs et al. 2005; Fraser and Doudna 2007).
For a detailed view of translation elongation and translation termination see references (Ehrenberg et al. 2007; Taylor et al. 2007).

Why translational regulation?

Why do cells need translational regulation and how do they benefit from it? There are several possible answers to this question, which are also addressed in reference (Mathews et al. 2007): first, regulation at the translational level can happen as a quick response without the necessity of going through all the upstream processes of gene expression such as transcription, mRNA processing or mRNA export. Furthermore,

translational regulation is usually reversible, as it is often mediated through reversible protein modifications such as the phosphorylation of initiation factors. The need for translational control is also apparent for systems, where transcriptional control is not possible. Examples for such systems are reticulocytes, which lack a nucleus, oocytes or RNA viruses. Another reason for the regulation of translation is spatial control of gene expression within the cell (St Johnston 2005; Schuman et al. 2006). The requirement for localized protein production in neurons or during development can only be met by translational regulation, as regulation of transcription is restricted to the nucleus of the cell. Another reason, which makes translational regulation a good option for the cell to regulate gene expression, is its flexibility. As can be seen by the complex mechanisms of translation initiation outlined above, there are many molecular targets for translational regulation, which consequently can change translational efficiencies for many or only a few mRNAs. A last but important point, why cells regulate translation, is fine tuning of gene expression, as there are numerous examples of genes that are regulated at both the transcriptional and translational level (e.g. GADD45α or TNF-α; Saklatvala et al. 2003; Lal et al. 2006).

Targets for translational regulation: initiation factors, mRNA and the ribosome

Translational control can in principle be divided into global regulation of translation and mRNA-specific translational regulation (Gebauer and Hentze 2004). Global regulation affects the translational efficiency of most mRNAs through a general switch-on or switch-off of translation. mRNA-specific regulation only affects the translation of a subset of mRNA. However, in some cases, this simple distinction cannot be made; for example, the general down-regulation of cap-dependent translation enhances translation of a subset of IRES-bearing mRNAs (see above).

What are the targets for translational control at the initiation step and what are the basic priciples? A simple answer to this question would be that most translational regulation prohibits or allows the association of the mRNA with the translational apparatus. Given the plethora of translation initiation factors, it is not surprising that many of them are targets for translational regulation and many have been shown to be modified post-translationally, which affects translational efficiency (Dever 2002; Raught and Gingras 2007). A key target in many regulatory mechanisms is the cap-

binding protein eIF4E that can be bound by inhibitory proteins, which subsequently hinders binding of the mRNA (see below for more details). Global regulation of translation is generally mediated through such modifications of translation initiation factors.

Another target for translational regulation is the mRNA itself, through cis-regulatory elements, which can be bound by trans-acting factors. The cis-regulatory elements on the mRNA could be found anywhere along the mRNA, but for most well characterized examples of translational regulation these elements are found in the 3' UTR or 5' UTR (Figure 1.4). mRNA-specific translational regulation happens mostly via regulatory proteins, that bind to the cis-regulatory elements of a given mRNA.

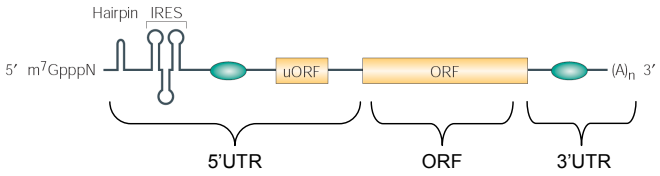

Figure 1.4 Cis-acting sequence elements that influence translation initiation of specific mRNAs
The m^7G cap structure at the 5' end and the poly(A) tail at the 3' end of the mRNA are both essential elements for cap-dependent translation initiation. Additionally, specific sequence elements in the 5' or 3' UTR (green ovals) can influence translation initiation in combination with bound trans-acting factors. Structured elements such as hairpins can inhibit translation initiation and structured internal ribosomal entry sites (IRES) can mediate cap-independent translation initiation. Upstream open reading frames (uORFs) usually inhibit translation initiation for the downstream start codon. This figure is taken from Gebauer et al. (2004).

The ribosome itself can also be targeted to exert translational regulation and several of its protein constituents can undergo post-translational modifications; a well studied example is the phosphorylation of ribosomal protein S6 (RPS6) by ribosomal S6 kinase (S6K), which was first shown more than 30 years ago (Gressner and Wool 1974). A correlation of RPS6 phosphorylation with an increase in translation initiation, especially of mRNAs posessing a 5'-terminal oligopyrimidine sequence (TOP mRNAs), prompted the hypothesis that translation of TOP mRNAs is actually regulated through this phosphorylation (Jefferies et al. 1994). However, recent data contradict this model and a simple causal relationship between S6 phosphorylation and translational efficiency: a double knock-out of both S6K homologues in mouse cells (Pende et al. 2004) or a knock-in of un-phosphorylatable RPS6 (Ruvinsky et al. 2005) does not affect translational regulation of TOP mRNAs. The elucidation of the

exact mechanism of RPS6 phosphorylation on translation is further aggravated by the discovery of various alternative substrates of S6K, which also include factors involved in translational initiation (Ruvinsky and Meyuhas 2006). Ribosomal proteins can also be modified through ubiquitination (Spence et al. 2000) or methylation (Bachand and Silver 2004; Swiercz et al. 2005).

In budding yeast, due to an ancient duplication event (Kellis et al. 2004), most genes encoding ribosomal proteins are duplicated. The open reading frame (ORF) and the protein sequence of the paralogues are very similar, but the UTRs and intron sequences can differ. Ribsomal gene pairs were generally considered to be functionally equivalent. However, recent genome-wide screens for genes required for various cellular processes such as telomere length homeostasis (Askree et al. 2004), centromeric cohesion (Marston et al. 2004), or for genes that exhibit deleterious haploinsufficient interactions with actin (Haarer et al. 2007), identified in several cases a specific effect for only one of the paralogues of the ribosomal protein, whereas deletion of the other paralogue would not affect the studied biological process. To date, the biological reason for this specialization is not clear. One possibility could be that specific ribosomal proteins are involved in cellular processes other than translation. Another intriguing hypothesis is heterogeneity of ribosomes: the cell could construct various kinds of ribosomes, which differ in terms of paralogue composition and post-translational modifications, and "specialized" ribosomes could play a role in the regulation of translation of specific subsets of mRNAs.

Classic examples of translational regulation

Translational regulation is crucial for diverse physiological processes. It is involved in the response to cellular stress (Holcik and Sonenberg 2005), in the mis-regulation of gene expression during cancer (Schneider and Sonenberg 2007), in apoptosis (Morley and Coldwell 2007), during development (Thompson et al. 2007), or in the establishment of synaptic plasticity and consequently in learning and memory (Klann and Richter 2007). Many examples of translational regulation have been reported within and also outside these areas. Instead of giving a broad overview of these regulatory mechanisms, I will focus below on several well-studied examples, for which the underlying molecular mechanisms have been reasonably well identified. Most of the regulatory mechanisms presented here - such as the regulation of ternary

complex formation, the regulation of translation via eIF4E-binding proteins, or the post-transcriptional regulation via ARE-elements - are probably conserved for most eukaryotes, despite the fact that these processes have mostly been studied in budding yeast and mammlian cells. Other regulatory mechanisms – such as the translational regulation of gene expression in *Drosophila* or *Xenopus* development – are probably specialized mechanisms to meet the specific requirements of gene expression regulation in the corresponding organism. However, the underlying principles for these regulatory mechanisms can be found in diverse variations in many eukaryotic cells.

Regulation of ternary complex formation. Exposure of cells to stress conditions (e.g. oxidative stress, nutrient limitation, hypoxia, temperature stress) results often, if not always, in a global down-regulation of translation (Holcik and Sonenberg 2005). One of the best studied examples of how this down-regulation is achieved, is regulation of the availability of active ternary complexes (Figure 1.5). Binding of the Met-tRNA$_i^{Met}$ to the 40S subunit through the ternary complex is an essential step in translation inititation, as described above (Figure 1.2; Figure 1.3). After the exposure to stress, the α-subunit of eIF2 (eIF2α) is phosphorylated and thereby inhibits the exchange of GDP for GTP by eIF2B and as a consequence formation of active ternary complexes is strongly reduced, and translation is down-regulated globally (Dever et al. 1992; Gebauer and Hentze 2004; Holcik and Sonenberg 2005; Ron and Harding 2007). The molecular mechanism for this inhibition is based on the fact that phosphorylated eIF2α-GDP turns into a competitive inhibitor of eIF2B, as eIF2B has a much higher affinity towards phosphorylated eIF2α-GDP than towards un-phosphorylated eIF2α-GDP (Rowlands et al. 1988). There are at least 4 kinases that have been identified to phosphorylate eIF2α at Ser51 in the response to various stresses (Figure 1.4; Dever et al. 2007): the haem-regulated inhibitor (HRI) is induced by haem depletion; general control non-depressible 2 (GCN2) is mainly activated by amino acid starvation; protein kinase activated by double-stranded RNA (PKR) is stimulated in response to viral infection; PKR-like endoplasmic reticulum kinase (PERK) is activated during endoplasmatic reticulum (ER) stress and the unfolded protein response (UPR).

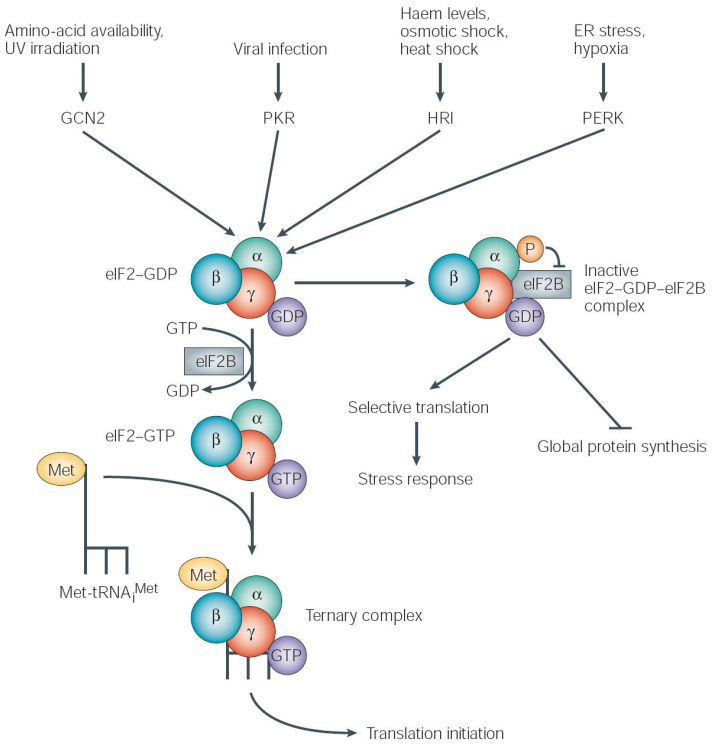

Figure 1.5 Inhibition of global protein synthesis in response to various stress stimuli through phosphorylation of eukaryotic initiation factor-2α
Several protein kinases (GCN2, PKR, HRI, or PERK) can phosphorylate the α-subunit of eIF2 in response to a variety of stress conditions. This phosphorylation inhibits the necessary GTP-GDP exchange on eIF2 by reducing the dissociation rate of the guanine nucleotide exchange factor eIF2B and active ternary complex formation is inhibited. As a consequence, translation initiation and global translation is down-regulated. This figure is taken from Holcik et al. (2005).

Regulation through uORFs. Interestingly, whereas translation of most mRNAs is down-regulated by eIF2α phosphorylation, translation of several specific mRNAs can be up-regulated in response to reduced availability of ternary complex. In response to various starvation conditions and amino acid deprivation in budding yeast, Gcn2p kinase is up-regulated through a mechanism that recognizes lack of amino acids; this is mediated through binding of un-charged tRNAs to the kinase (Dong et al. 2000). Ternary complex formation and global translation are down-regulated as a consequence. However, Gcn4p, a master transcriptional regulator, which activates transcription of amino acid-biosynthesis genes, is translationally up-regulated under these conditions (Hinnebusch and Natarajan 2002). This up-regulation is achieved by regulatory upstream open reading frames (uORFs). Four of these uORFs can be found in the 5' UTR of the *GCN4* mRNA (Hinnebusch and Natarajan 2002; Hinnebusch

2005): In optimal growth conditions and availability of ternary complex, translation usually starts at uORF1 and ribosomes can resume scanning afterwards to resume translation at uORF2, uORF3 and uORF4 (Figure 1.6). However, ribosomes cannot re-initiate translation after termination at these latter uORFs and as a consequence, *GCN4* mRNA is not translated. After eIF2α phosphorylation, when ternary complexes become limited, ribosomes are more likely to resume scanning without re-initiating at the downstream uORFs and translation is initiated at the actual start codon of *GCN4* (Figure 1.6). The response to amino acid starvation via the GCN2 kinase seems to be an evolutionarily conserved mechanism, as was shown by two recent reports that GCN2 activity in the mouse brain is essential for the restriction of intake of diets lacking essential amino acids (Hao et al. 2005; Maurin et al. 2005): in these studies it was show that the GCN2 pathway is used to recognize depressions in serum amino acid levels that occur during consumption of food with an imbalanced composition of amino acids, which results in a behavioral response that limits the consumption of imbalanced foods and favours the intake of a balanced diet

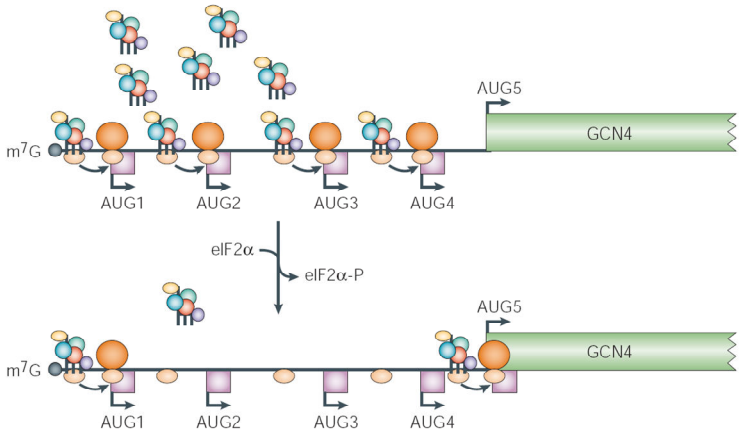

Figure 1.6 Translational regulation of *GCN4* by upstream open reading frames (uORFs) With low levels of eIF2α-phosphorylation and abundant active ternary complex, ribosomes initiate translation at uORF1, resume scanning, and re-initiate translation at uORF2, uORF3 or uORF4. However, they do not resume scanning to re-initiate translation at the start codon of *GCN4*. When cells are starved for amino acids, eIF2α is phosphorylated and as a consequence the number of active ternary complexes is down-regulated. In these conditions, re-initiation at uORF2-uORF4 happens less frequently and scanning can resume to the actual start codon of *GCN4*, which is then translated. This figure is taken from Holcik et al. (2005).

In fission yeast eIF2α phosphorylation has also been reported to be mediated by the kinases Gcn2p, Hri1p and Hri1p (Zhan et al. 2002; Dunand-Sauthier et al. 2005). However, no homologue of Gcn4p exists in fission yeast.

The mammalian transcription factor ATF4 is regulated in a similar way in response to ER stress or amino acid starvation by uORFs (Harding et al. 2000; Scheuner et al. 2001), and there is evidence that GCN2 also regulates synaptic plasticity through modulation of ATF4 translation (Costa-Mattioli et al. 2005 and references therein).

There are numerous other examples of mRNAs whose translation is regulated by uORFs (Dever 2002). Recent genome-wide bioinformatics approaches in yeast and mammals suggest that the occurrence of functional uORFs is widespread and might be a common regulatory mechanism of translation (Iacono et al. 2005; Cvijovic et al. 2007).

Regulation by eIF4E inhibitory proteins. An important step during translation initiation is the binding of the m^7G cap by eIF4F (Figure 1.2). The backbone of this complex is eIF4G, which interacts with the cap-binding complex eIF4E and the helicase eIF4A. Translational initiation can be regulated by the disruption of eIF4E-eIF4G binding through inhibitory proteins, which were originally called 4E-BP (for 4E binding proteins) (Richter and Sonenberg 2005). These inhibitory proteins have been reported to control a variety of biological processes such as development, cell growth, and may repress tumour formation (Richter and Sonenberg 2005). 4E-BPs compete with eIF4G for the binding to eIF4E, and the binding affinity is regulated through phosphorylation of 4E-BPs (Gingras et al. 1999): in the hypo-phosphorylated state, 4E-BPs bind to eIF4E and prevent translation initiation; in the hyper-phosphorylated state, 4E-BPs binding to eIF4E is blocked.

In addition to 4E-BPs, several other proteins can bind eIF4E in an mRNA-specific manner and inhibit translation initiation. The mRNA specificity for these proteins comes through interactions with sequence-specific elements within the mRNA or through the interaction with RNA-binding proteins.

In *Xenopus* oocytes, many mRNAs remain dormant with short 3' poly(A) tails. When the oocytes are stimulated, these mRNAs become polyadenylated and translationally active. A cytoplasmic polyadenylation element (CPE) in the 3' UTR of the mRNA is important for both masking and translational activation of the mRNA and is bound by

the cytoplasmic polyadenylation element binding protein (CPEB) (Mendez and Richter 2001; Richter 2007). When dormant, CPEB is bound by Maskin, which inhibts the binding between eIF4E and eIF4G (Figure 1.7), acting as a mRNA specific 4E-BP (Cao and Richter 2002). After stimulation of the oocyte to complete meiosis, CPEB stimulates polyadenylation of the mRNA; the poly(A) tail is then bound by PABP, which then can bind eIF4G and displace Maskin (Figure 1.7; Cao and Richter 2002). During translational repression, the CPEB-containing complex also inculdes PARN, a poly(A)-specific ribonuclease, which overrides the polyadenylating activity of the poly(A) polymerase GLD2, which contributes to the short poly(A) tail of target mRNAs during translational repression (Kim and Richter 2006).

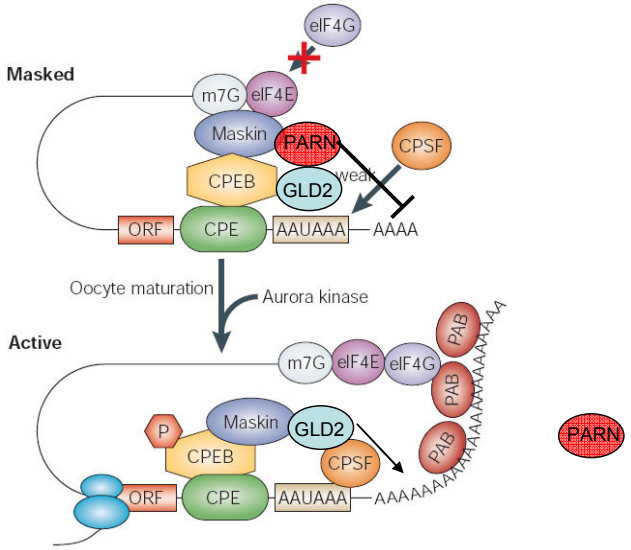

Figure 1.7 Regulation of translation by the cytoplasmic polyadenylation element (CPE) mRNAs that bear a CPE in their 3' UTR are translationally repressed in developing oocytes by the binding of the cytoplasmic polyadenylation element binding protein (CPEB) and Maskin. Maskin interacts directly with the cap-binding protein eIF4E and prevents its association with eIF4G, which is crucial for translation initiation. The short poly(A) tail is maintained by blocking access of cleavage and polyadenylation specificity factor (CPSF) for the AAUAAA sequence and by the poly(A)-specific ribonuclease PARN, which counteracts the polyadenylating activity of GLD2. Induction of oocyte maturation results in phosphorylation of CPEB. Consequently, Maskin and PARN dissociate from the complex and CPSF binds to the AAUAAA sequence. Binding of CPSF activates the poly(A) polymerase GLD2 that extends the poly(A) tail. These events lead to successful translation initiation and translation of the previously translationally repressed mRNA. This figure is adapted from Kuersten et al. (2003).

Another example of an mRNA-specific 4E-BPs is the homeodomain transcription factor Bicoid, which apart from its activity as transcription factor inhibits translation of Caudal mRNA in *Drosophila* (Dubnau and Struhl 1996; Rivera-Pomar et al. 1996). Similar to Maskin, Bicoid has an eIF4E-binding motif, and it was initially thought that Bicoid directly binds to eIF4E (Niessing et al. 2002). However, recent work

showed that Bicoid interacts with d4EHP (Drosophila 4E-homologous protein), an eIF4E-like protein that can interact with the m^7G cap but not with eIF4G (Cho et al. 2005).

Recent studies have also identified Cup as a translational regulator in *Drosophila*, which interacts with eIF4E and prevents eIF4F complex formation and translational initiation (Wilhelm et al. 2003; Nakamura et al. 2004; Nelson et al. 2004). Nanos and Oskar are examples of mRNAs regulated by Cup.

Other mechanisms of mRNA-specific translation regulation. AU-rich elements (AREs) are present in the 3' UTR of many mRNAs and are potent sequence elements for post-transcriptional regulation of gene expression. AREs influence the stability or translation of a given mRNA usually through binding of ARE-specific RNA-binding proteins (Barreau et al. 2005). AUF1 was the first ARE-binding protein to be identified and was shown to exist in 4 isoforms (Wilson et al. 1999). Binding of ARE-binding proteins of the AUF1 family to AREs have been shown to promote degradation of mRNAs encoding cytokines (IL-3, GM-CSF) or cell cycle regulators ($p16I^{NK4a}$, $p21^{WAF1/CIP1}$, cyclin D1) (Lal et al. 2004; Raineri et al. 2004; Wang et al. 2005), and AUF1 has been shown to interact with the heat shock proteins hsc70-hsp70, eIF4G and PABP (Laroia et al. 2002). Despite its role in promoting mRNA decay, recent work showed that AUF1 can induce translation of MYC proto-oncogene mRNA (Liao et al. 2007): down-regulation of AUF1 abundance by RNA-interference (RNAi) *in vivo* did not result in altered MYC mRNA levels, as expected based on earlier *in vitro* studies (Brewer 1991), but significantly reduced MYC mRNA translation. In contrast, TIAR, another ARE-binding protein, was shown to suppress translation of MYC mRNA. Despite competitive binding of AUF1 and TIAR to the MYC ARE, translational up-regulation through AUF1 was not simply achieved by suppression of TIAR binding, as shown in double knockdown experiments (Liao et al. 2007).

Repression of translation through the ARE-binding protein TIAR has been shown for several mRNAs such as GADD45α (Lal et al. 2006), the translation initiation factors eIF4A and eIF4E, especially in response to UV radiation (Mazan-Mamczarz et al. 2006), and TNFα (Gueydan et al. 1999).

To date, many more ARE-binding proteins have been identified (e.g. HuR (Myer et al. 1997); TTP (Carballo et al. 1998); or KSRP (Gherzi et al. 2004)), and it is well recognized that AREs in conjunction with their ARE-binding proteins can influence gene expression through the modulation of mRNA turnover and translation. However, despite the identification of a large number of ARE bearing mRNAs and ARE-binding proteins, the full complexity of this regulatory mechanism is far from understood.

Multistep mechanisms of translational regulation. As is already evident from some of the examples given above, translational regulation can also be exerted as a multistep mechanism, which means that more than one mechanism is used to ensure tight translational regulation for critical proteins, whose mis-expression would be deleterious for the cell. One good example for this kind of control is the translational regulation of male-specific-lethal (*msl-2*) mRNA in *Drosophila*. Expression of MSL-2 in females causes inappropriate assembly of dosage compensation regulators on the X chromosomes and female lethality in *Drosophila* (Kelley et al. 1995). MSL-2 expression is inhibited by Sex-lethal (SXL), a female specific RNA binding protein, which also regulates sex determination via alternative splicing (Forch and Valcarcel 2003). First, SXL promotes retention of a facultative intron in the 5' UTR of *msl-2* and then represses its translation (Bashaw and Baker 1997; Kelley et al. 1997; Gebauer et al. 1998). SXL binds to sites in the 3' UTR and the intronic 5' UTR of *msl-2* (Figure 1.8) and represses translation in a dual way: SXL bound to the 3' UTR inhibits recruitment of the 43S pre-initiation complex, and SXL bound to the 5' UTR can inhibit scanning of the 43S pre-initiation complex, in case of escape from the first inhibitory mechanism (Beckmann et al. 2005). Furthermore, to exert its function via the 3' UTR, SXL requires the RNA-binding protein UNR (upstream of N-*ras*) as corepressor (Grskovic et al. 2003; Abaza et al. 2006; Duncan et al. 2006).

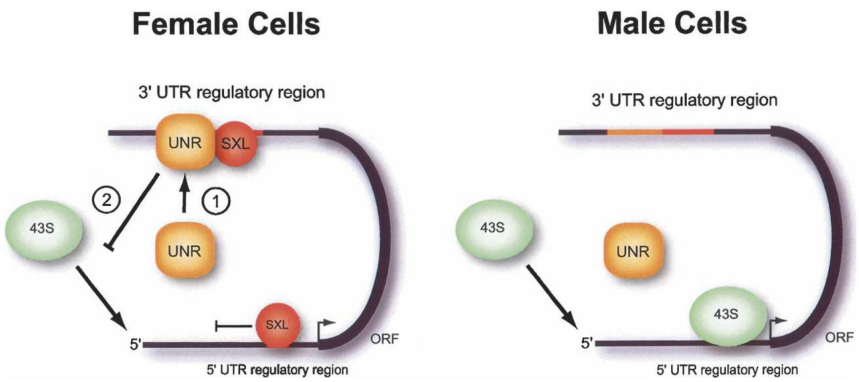

Figure 1.8 Translational regulation of male-specific-lethal (*msl-2*) mRNA in *Drosophila melanogaster* through a multi-step mechanism
msl-2 translation is inhibited by Sex-lethal (SXL), a female specific RNA binding protein. First, SXL promotes retention of a facultative intron in the 5' UTR of *msl-2* and then represses its translation. SXL binds to sites in the 3' UTR and the intronic 5' UTR of *msl-2* and represses translation in a dual way: SXL bound to the 3' UTR inhibits recruitment of the 43S pre-initiation complex, and SXL bound to the 5' UTR can inhibit scanning of the 43S pre-initiation complex, in the case that it escaped the first inhibitory mechanism. Furthermore, to exert its function via the 3' UTR, SXL requires the RNA-binding protein UNR (upstream of N-*ras*) as corepressor. In male cells, *msl-2* translation can be initiated, as SXL is not expressed. This figure is taken from Duncan et al. (2006).

Novel concepts in translational control: P-bodies and microRNAs

In the past few years, two new ways to modulate mRNA fate at the post-transcriptional level have attracted a great deal of attention. One is the discovery of cytoplasmic processing bodies (P-bodies), initially described as foci within the cell with a high concentration of mRNA decay enzymes (Bashkirov et al. 1997; Ingelfinger et al. 2002; Lykke-Andersen 2002; van Dijk et al. 2002; Sheth and Parker 2003; Cougot et al. 2004). The other discovery is that of small RNAs, which can regulate stability and translation of target mRNAs (Bartel 2004; Filipowicz 2005; Valencia-Sanchez et al. 2006). Interestingly, recent work suggests that there is also a connection between P-bodies and microRNA (miRNA)-mediated gene silencing (Liu et al. 2005; Liu et al. 2005; Sen and Blau 2005). These novel concepts will be introduced here, with a focus on their involvement in translational regulation.

P-bodies and polysomes. P-bodies were first visualized by various groups using microscopy of factors involved in mRNA decay and accessory factors such as DCP1, DCP2, XRN1 and LSM (Bashkirov et al. 1997; Ingelfinger et al. 2002; Lykke-Andersen 2002; van Dijk et al. 2002; Sheth and Parker 2003; Cougot et al. 2004). In

mammalian cells, GW182 protein is another marker of P-bodies and they are therefore sometimes also referred to as GW bodies (Eystathioy et al. 2002; Eystathioy et al. 2003).

mRNA decay in eukaryotes can be controlled in different ways via endonuleolytic or exonucleolytic pathways (for reviews see Wilusz et al. 2001; Parker and Song 2004). Exonucleolytic degradation is usually initiated by deadenylation of the poly(A) tail of the mRNA. Transcripts will then be degraded from their 5′ ends by the exonuclease XRN1, following removal of the 5' cap (decapping). Alternatively, the exosome complex can degrade transcripts from their 3′ ends before decapping.

P-bodies are probably a site of mRNA decay, as intermediates in the 5'-3' degradation pathway can be found localized to P-bodies (Sheth and Parker 2003). Furthermore, mutations in the decapping enzymes (DCP1, DCP2) or in the 5'-3' exonuclease XRN1 increase the size and number of P-bodies, which corresponds to a clogging of the system (Sheth and Parker 2003). Factors of the nonsense-mediated decay (NMD) pathway, which is responsible for the rapid degradation of mRNAs with a premature stop codon (Conti and Izaurralde 2005), can also be found in mammalian P-bodies (Unterholzner and Izaurralde 2004). However, it is not clear whether P-bodies are the only site of 5'-3' decay, as enzymes involved in this process can also be found elsewhere in the cytoplasm of yeast (Heyer et al. 1995) or mammalian cells (Bashkirov et al. 1997). It is also unclear whether mRNAs need to be deadenylated in order to enter P-bodies. In yeast, the deadenylase Ccr4p does not visibly localize to P-bodies (Sheth and Parker 2003), but the mammalian homolog does (Cougot et al. 2004). In mammalian and yeast cells, depletion of Ccr4p results in a reduction of P-bodies (Sheth and Parker 2003; Andrei et al. 2005), which is in favour of a model that mRNAs need to be deadenylated before entering P-bodies.

What are the connections between P-bodies and translation? Several lines of evidence indicate that mRNAs exist in 2 states: actively translated and associated with polysomes or in a translationally repressed state associated with P-bodies. When yeast cells are exposed to stress, such as glucose deprivation, translation is inhibited at the level of initiation, which is reflected by a strong decrease of polysomes, which corresponds to less mRNAs being associated with many ribosomes (Coller and Parker 2005). While translation gets down-regulated, P-bodies increase in size (Coller and Parker 2005). After removal of the stress, P-bodies decrease in size and polysomes reform, even in the absence of new transcription (Figure 1.9; Brengues et al. 2005).

Therefore, P-bodies in yeast seem to serve as sites of mRNA storage, which can then be released back into the translating pool without actually undergoing decay. The idea that the recruitment of mRNAs to P-bodies interferes with translation initiation and that only mRNAs not yet associated with ribosomes can be localized to P-bodies is strengthened by the finding that inhibition of translation elongation causes P-bodies to disappear, whereas inhibition of translation initiation increases P-bodies in size and number (Sheth and Parker 2003; Cougot et al. 2004; Andrei et al. 2005; Brengues et al. 2005; Teixeira et al. 2005). In budding yeast, the decapping activators Dhh1p and Pat1p are required for translational repression (Coller and Parker 2005). In mammalian cells, several proteins with established roles in translational repression localize to P-bodies: RCK/p54, CPEB and the eIF4E inhibitory protein eIF4E-T (Andrei et al. 2005; Ferraiuolo et al. 2005; Kedersha et al. 2005; Wilczynska et al. 2005; Chu and Rana 2006). However, the exact mechanism how mRNAs shuttle into P-bodies and become translationally repressed is not clear at the moment.

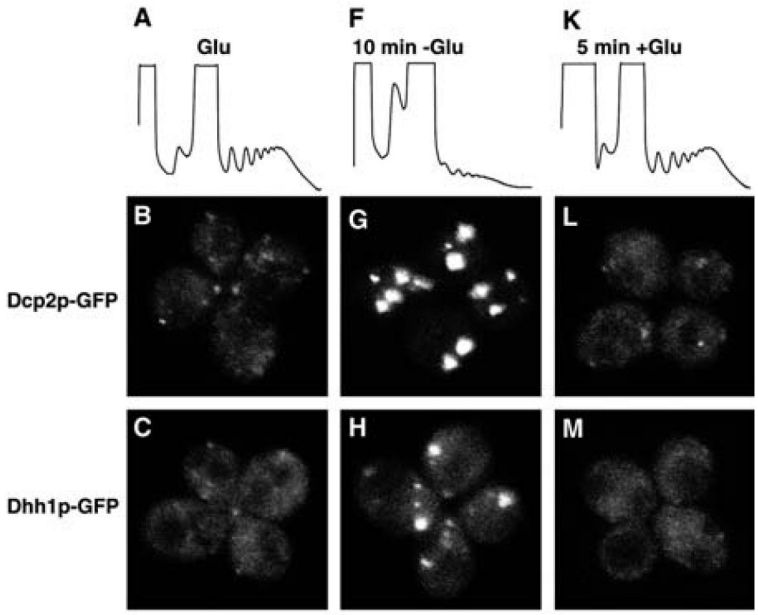

Figure 1.9 Movement of mRNAs between polysomes and P-bodies
Deprivation of glucose leads to repression of translation, which can be seen by diminished polysomes (**A**, **F**). This translation inhibition also results in increased number and size of P-bodies, which were visualized using the GFP-tagged reporters Dcp2p (**G**) and Dhh1p (**H**), whose presence in P-bodies is dependent on mRNA. After the re-addition of glucose, polysomes re-appear (**K**) and P-bodies basically disappear (**L**, **M**). These data are consistent with a move of mRNAs from polysomes to P-bodies after the inhibition of translation, and re-entering of mRNAs into the translation pool after translation is restored. This figure is taken from Brengues et al. (2005).

Another kind of cytoplasmic foci linked to translational repression can be observed in mammalian cells after the exposure to stress: stress granules (SGs) contain translationally silent mRNAs. These mRNAs are associated with pre-initiation complexes lacking the ternary complex and can also be shuttled back into polysomes after the removal of the stress (Kedersha and Anderson 2002). Despite the analogy to P-bodies and some shared components, SGs are distinct subcellular entities, as they also contain SG specific components such as 40S ribosomal subunits and translation initiation factors, which apart from eIF4E are not found in P-bodies, or ARE-binding proteins (Kedersha et al. 2005). However, fusion events and close association between SG and P-bodies could be observed in cells (Kedersha et al. 2005; Wilczynska et al. 2005).

Foci resembling stress granules have also been described in fission yeast (Dunand-Sauthier et al. 2002): Sum1p, a component of the translation initiation factor eIF3 complex, relocalizes to multiple cytoplasmic foci after the exposure to osmotic stress. In response to heat stress Sum1p is additionally localized to the inner nuclear periphery and furthermore colocalizes with eIF4E. All these data point to a spatial re-organization of the translational machinery to specific foci in these conditions. Furthermore, Sum1p interacts with components of the 26S proteasome and Sum1p relocalization in response to heat stress is dependent on an intact 26S proteasome.

Post-transcriptional gene expression regulation by small RNAs. Two types of small RNA molecules have emerged as regulators of mRNA stability and translation in the last decade: microRNAs (miRNAs) and short interfering RNAs (siRNAs). Current estimates from bioinformatic analysis suggest that the human genome encodes hundreds of different miRNAs and that they potentially regulate up to 30% of all genes (Lewis et al. 2005). However, only a few miRNAs and their targets have been validated to date.

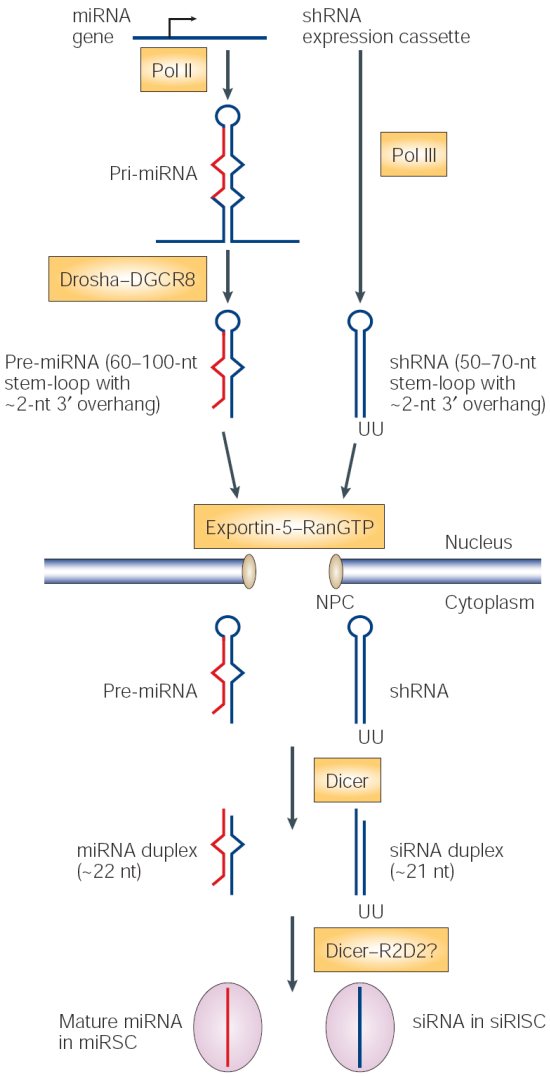

Figure 1.10 Biogenesis of miRNAs and siRNAs
Shown are the different ways of biogenesis for miRNAs and siRNAs. miRNAs are derived from longer precursors that include a ~70 nt imperfectly based hairpin segment and are usually transcribed by RNA polymerase II; siRNAs are of similar length but are derived from perfectly complementary RNA precursors, which are usually transcribed by RNA polymerase III. Despite the different mode of biogenesis, processing for both siRNAs and miRNAs is dependent on Dicer, and the regulatory function for both RNAs is exerted through proteins of the Argonaute (Ago) family. This figure is taken from Kim et al. (2005).

miRNAs and siRNAs are short RNAs of 21-26 nucleotides (nt) and are distinguished based on their biogenesis (Kim 2005; Jackson and Standart 2007): miRNAs are derived from longer precursors that include a ~70 nt imperfectly based hairpin segment; siRNAs are of similar length but are derived from perfectly complementary RNA precursors (Figure 1.10). Despite the different mode of biogenesis, processing for both siRNAs and miRNAs is dependent on Dicer, and the regulatory function for

both RNAs is exerted through proteins of the Argonaute (Ago) family: miRNAs and siRNAs associate with Ago proteins to form RNA-induced silencing complexes (RISCs), through which they modulate gene expression. During RNA-interference (RNAi), exogenously introduced siRNAs target mRNAs for endonucleolytic cleavage (Tomari and Zamore 2005). Such endonucleolytic cleavage has now also been described for plant (Llave et al. 2002; Allen et al. 2005) and mammalian (Yekta et al. 2004) miRNAs. Initially it was thought that perfect base-pairing between the miRNA/siRNA and the target mRNA favours endonucleolytic cleavage, whereas imperfect base-pairing results in the repression of the target by alternative mechanisms. However, it was shown that endonucleolytic cleavage still can occur when there are mismatches between the miRNA and the target mRNA (Mallory et al. 2004; Yekta et al. 2004).

In animal cells, most miRNAs are only partially complementary to their target mRNAs and the down-regulation of protein levels of the target is usually greater than the down-regulation of its mRNA abundance, which suggests regulation at the level of translation in these cases (Jackson and Standart 2007). The classic example is that of *lin-4* miRNA regulating *lin-14* protein levels in *Caenorhabditis elegans* through interactions with the 3' UTR of the mRNA (Arasu et al. 1991; Wightman et al. 1991). Regulation of *lin-14* through *lin-4* does not involve changes in mRNA levels, but protein levels are dramatically altered. As *lin-14* mRNA could be found associated with polysomes in both the active and the repressed state, it was suggested that translation of the mRNA is repressed at a point after initiation (Olsen and Ambros 1999). A recent study using an artificial CXCR4 siRNA directed against a luciferase reporter with six bulged target sites in its 3' UTR reported a similar result as described for *C. elegans lin-14* repression (Petersen et al. 2006): luciferase expression is down-regulated by 95% without large changes in mRNA abundance and repressed mRNAs were still associated with polysomes. Furthermore, repression is also seen for IRES-initiated translation, which further suggests a repressive mechanism that acts after translation initiation (Petersen et al. 2006). The authors suggest a drop-off of ribosomes at various points along the ORF resulting from miRNA repression (Petersen et al. 2006). However, it is unclear how this mechanism works, and it is hard to imagine how the polysomal distribution under repressed conditions would be similar to the distribution in a un-repressed state if ribosomal drop-off would occur continuously (Jackson and Standart 2007).

In contrast to the idea that miRNAs regulate mRNAs at a step after translation initiation, two reports point towards initiation as the regulated step (Humphreys et al. 2005; Pillai et al. 2005). Using the same CXCR4 system, Humphreys et al. (2005) could show a similar strong down-regulation at the protein-level of a luciferase reporter mRNA bearing four partially complementary binding sites for the CXCR4 siRNA. However, this down-regulation is not seen with IRES-containing mRNAs. Furthermore, the down-regulation is dependent on the 5' cap and 3' poly(A) sequences. Pillai et al. (2005) also used luciferase reporters, which contained either one perfectly complementary or three imperfectly complementary target sites for *let-7* miRNA. Expression of the reporter is down-regulated and reporter mRNA containing imperfect let-7 target sites is found in lighter polysomal fractions upon expression of let-7 miRNA, but not in a control reaction, when let-7 is bound by an antisense 2'-O-Me oligonucleotide (Pillai et al. 2005). Furthermore, using in vitro synthesized mRNAs, it could be shown that the 5' cap is necessary for miRNA-mediated repression (Pillai et al. 2005). However, in contrast to the study by Humphreys et al. (2005), repression is not markedly relieved when the poly(A) tail is absent (Pillai et al. 2005). Taken together, the two latter studies strongly support miRNA-mediated respression at the level of translation initiation.

What could be the explanation for the discrepancies in miRNA-mediated translational repression reported by these various groups? First, in their study, Petersen at al. (2006) used a reporter mRNA that was transcribed in the nucleus by RNA polymerase II, whereas in the other two studies by Humphreys et al. (2005) and Pillai et al. (2005) the reporter mRNAs were co-transfected with the miRNA. Secondly, the number, origin, specificity and location of target sites on the reporter might influence the observed effect. Furthermore, in a recent paper, Thermann et al. (2007) describe the formation of heavy miRNPs after repression by the miRNA miR2 in *Drosophila*. These miRNA-mRNA assemblies, which the authors call "pseudo-polysomes" show the same sedimentation characteristics as polysomes, but even form under conditions of effectively blocked 60S subunit joining (Thermann and Hentze 2007). One could speculate that the association with polysomes described for miRNA-repressed mRNAs could actually be an association with such "pseudo-polysomes".

However, it is also plausible that miRNAs exert their repression on translation through various mechanisms, and as a consequence it may be necessary to validate the regulatory mechanism for each miRNA-target pair individually. Furthermore,

translation could also be influenced by miRNAs indirectly, as recent studies have shown that miRNAs can accelerate deadenylation of their target mRNAs (Giraldez et al. 2006; Wu et al. 2006).

Apart from Drosha, fission yeast has homologues of all important genes involved in the RNAi machinery such as Dicer (Dcr1p), Argonaute (Ago1p) and RNA-dependent RNA polymerase (Rdp1p). However, post-transcriptional gene expression silencing for protein coding genes has not been described in fission yeast yet, but the RNAi machinery is involved in heterochromatin silencing (Zofall and Grewal 2006). In this case, RNAi-mediated silencing involves the processing of repeat transcripts from *dg*/*dh*/*cenH* repeat elements into an RNAi-induced initiation of transcriptional silencing (RITS) complex, which then targets proteins such as the histone methyl-transferase Clr4p, or the heterochromatin binding protein Swi6p, to these repeat regions, which ultimately leads to heterochromatin formation and limited accessibility for Pol II.

The connection between miRNAs and P-bodies. Several recent reports have found connections between the gene silencing pathway via miRNAs/siRNAs and P-bodies. Pillai at al. (2005) show that mRNAs, which are translationally repressed by *let-7* miRNA, localize to P-bodies or to cytoplasmic foci adjacent to P-bodies. Apart from the localization of translationally repressed mRNAs to P-bodies, Ago proteins, the effector molecules of miRNA-mediated silencing, have also been found to localize to P-bodies (Liu et al. 2005; Sen and Blau 2005). Argonaute proteins also interact with GW182, a key P-body subunit in mammalian cells, and depletion of GW182 impairs the repression of miRNA-reporters (Jakymiw et al. 2005; Liu et al. 2005).

A recent report also shows the reversibility of miRNA-mediated repression and the involvement of P-bodies: Bhattacharyya et al. (2006) used the cationic amino acid transporter (CAT-1) mRNA or reporter mRNAs bearing its 3' UTR, which is negatively regulated by miRNA miR-122. In Huh7 cells, miR-122 is endogenously expressed and CAT-1 protein levels are significantly down-regulated and both CAT-1 and miR-122 can be found in P-bodies (Bhattacharyya et al. 2006). However, after exposure to stress, CAT-1 mRNA can escape the translational repression, and the de-repression and the exit from P-bodies is dependent on ARE elements in the 3' UTR. Bhattacharyya et al. (2006) could further show that the ARE-binding protein HuR is necessary for the release from translational repression and P-body entrapment.

All the above examples make it clear that P-body components are important for gene silencing via miRNA/siRNA-mediated repression. However, it is not clear if the spatial environment of the P-body itself or P-body components are important for this interaction. Recent work suggests that disruption of P-bodies does not necessarily affect siRNA-mediated silencing (Chu and Rana 2006). Therefore, concentration of miRNAs and miRNA-targets in P-bodies could be a consequence rather than a prerequisite of miRNA/siRNA-mediated gene silencing.

Taken together, regulation of gene expression via small RNAs and sequestration to P-bodies and its interplay between mRNA translation and decay adds further complexity to the control of post-transcriptional mRNA fate. As mentioned above, 30% of human genes are potential miRNA targets (Lewis et al. 2005), and it is entirely possible that miRNAs exert their function in a combinatorial mode: a given mRNA might be regulated by several miRNAs and a given miRNA might target several mRNAs. However, further research will be needed to elucidate the exact molecular events behind these regulatory mechanisms.

Functional genomics of post-transcriptional gene expression

Genome-wide approaches to identify targets of post-transcriptional gene expression regulation

The advent of microarray technology allowed the genome-wide study of gene expression at the level of steady-state mRNA abundance. Furthermore, microarray technology combined with chromatin immunopreciptitations is an invaluable tool to identify transcription factor binding sites and chromatin modifications on a global scale. Together, these studies revealed global networks of transcriptional control in a variety of organisms and physiological conditions (Babu et al. 2004; Luscombe et al. 2004; Barrera and Ren 2006; Walhout 2006).

However, as gene expression is often regulated at the post-transcriptional level, it is important to also gain an understanding of these regulatory processes and their targets on a genome-wide scale. In the same way as DNA and its interaction with transcription factors and chromatin modifiers is integral to transcriptional regulation, mRNA and its association with RNA-binding proteins is essential for the regulation of gene expression at the post-transcriptional level. Consequently, recent work of many groups has focused on the large-scale systems analysis of mRNA-protein interactions and mRNA dynamics. Many of these studies employ microarray-based approaches to study a variety of processes on a genome-wide scale such as (1) the association of mRNAs with specific RNA-binding proteins, (2) mRNA stability, or (3) the association of mRNAs with ribosomes and thus the efficiency with which these mRNAs are translated. These large-scale approaches are especially useful to identify potential targets for each of the myriads of possible post-transcriptional regulatory mechanisms, and building on this knowledge can in turn be useful to examine the underlying molecular mechanisms of the regulatory process at the molecular level. Here, some of these techniques and the interesting findings obtained from them will be introduced.

Translation

Translational efficiency can be measured on a genome-wide scale by assessing the number of ribosomes that are bound to a given mRNA. This can be achieved by

combining the traditional method of polysome profiling with microarray technology, which is referred to as translational profiling (Figure 1.11): Usually, cells are treated with the elongation-inhibitor cycloheximide, which "traps" ribosomes on the mRNA they are translating. Cellular lysates are then resolved according to their density on a sucrose gradient by ultracentifugation. As the ribosome is a huge macromolecular complex with a molecular mass above 3 megadalton (Taylor et al. 2007), the density of the mRNA-ribosome particles is determined by the amount of ribosomes bound to the mRNA. The sucrose gradient is then fractionated and a polysome profile is obtained by measuring the RNA abundance (Figure 1.11; right panel). Going along from the light to the heavy density fractions, free mRNAs are obtained, followed by the ribosomal 40S and 60S subunits, the monosome or 80S subunit, and the polysome fractions corresponding to mRNAs with increasing numbers of bound ribosomes. mRNAs from diverse fractions can then be extracted and quantified using microarrays.

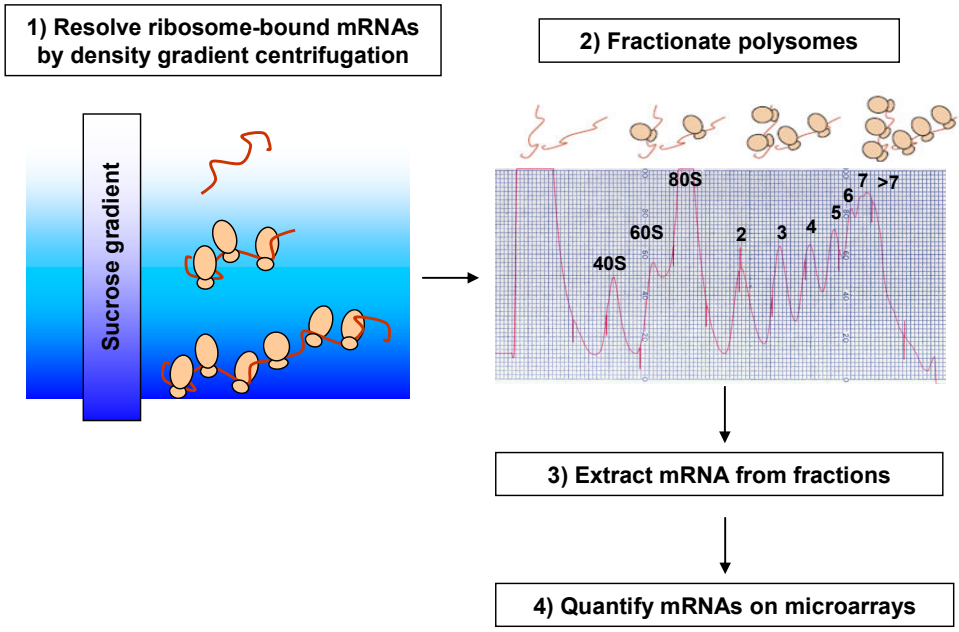

Figure 1.11 Translational profiling
Genome-wide measurements of translation can be achieved by combining polysome profiling with microarray technology, which is referred to as translational profiling. mRNAs are resolved on a sucrose gradient by ultracentifugation according to their density, which is determined by the number of associated ribosomes. After fractionation, mRNA from diverse fractions can then be extracted and quantified using microarrays.

In most studies where this technology is used to study translational regulation, the pool of mRNAs associated with polysomes is compared to the pool of un-translated

mRNAs or total mRNA preparations in order to define translationally regulated transcripts (Johannes et al. 1999; Kuhn et al. 2001; Kash et al. 2002; Rajasekhar et al. 2003; Qin and Sarnow 2004; Dinkova et al. 2005; Bushell et al. 2006; Iguchi et al. 2006; Spence et al. 2006; Thomas and Johannes 2007). Some studies, however, have used more than 10 fractions spaced along the polysome profile, which are then probed with microarrays to obtain higher-resolution data of changes in ribosome association for given mRNAs (Arava et al. 2003; Preiss et al. 2003; MacKay et al. 2004; Qin et al. 2007).

Using translational profiling, the effect on global and mRNA specific translational regulation has been examined in a variety of conditions. Examples are the exposure of cells to stress or changing environmental conditions such as hypoxia, treatment with rapamycin, heat shock, or change in carbon-source (Kuhn et al. 2001; Grolleau et al. 2002; Preiss et al. 2003; Thomas and Johannes 2007); the translational regulation during the mitotic cell cycle, meiosis, or during recovery from cell cycle arrest (Serikawa et al. 2003; Qin and Sarnow 2004; Iguchi et al. 2006); the dependence of mRNAs on specific translation initiation factors (Johannes et al. 1999; Dinkova et al. 2005); or translational regulation in response to oncogenic signaling or in transformed cells (Rajasekhar et al. 2003; Spence et al. 2006).

One of the first studies using translational profiling was conducted by Johannes et al. (1999): the requirement for cap-dependent translation initiation was examined by studying the association of mRNAs with polysomes in cells with reduced eIF4G concentrations, which was achieved by infecting the cells with poliovirus. Most of the examined mRNAs show the expected down-regulation in translation, whereas a small percentage remains associated with polysomes or even exhibits increased polysome association. These mRNAs are probably translated via IRES-mediated translational initiation and included mRNAs encoding immediate-early transcription factors and mitogen-acitvated regulators (Johannes et al. 1999). Another study conducted in *C. elegans* investigated the effect of the selective knock-out of one isoform of the cap-binding translation initiation factor eIF4E (Dinkova et al. 2005). Mutant worms show a mixture of phenotypic effects, reproduce more slowly and exhibit an egg laying defect. Using translational profiling, several mRNAs could be identified that show changes in their polysomal association without altered total mRNA levels. Interestingly, these mRNAs are enriched for genes with functions related to egg

laying, providing a possible explanation for the observed phenotype (Dinkova et al. 2005).

Kuhn et al. (2001) used translational profiling to measure the translational response in budding yeast cells to the transfer from a fermentable (glucose) to a non-fermentable (glycerol) carbon source. This shift results in a global down-regulation of translation. mRNAs encoding ribosomal proteins are strongly down-regulated in terms of total mRNA abundance as well as in their translational status, indicated by a diminished association with polysomal fractions. However, a few mRNAs show increased association with polysomes and most of these mRNAs also show increased abundances in their total mRNA levels. A similar connection between changes in total mRNA levels and polysome association was described in another study, which examined translational regulation in response to treatment with rapamycin and heat shock (Preiss et al. 2003). They found that mRNAs that show increased abundance in response to the treatment often also show increased translational efficiency. The same was true for mRNAs with decreased abundance. Furthermore, such a correlation between changes in total mRNA levels and translational efficiency has been observed in budding yeast in response to treatment with mating pheromone (MacKay et al. 2004). This coordination between changes in transcript levels and translation has been termed "potentiation" (Preiss et al. 2003). However, further studies will be required to determine whether potentiation happens through coordinated yet independent regulation of transcription and translation, or whether increased translation is a mere consequence of *de novo* transcription – for example *de novo* transcription could influence mRNP composition or could simply provide "new" and "intact" messages, which are then more efficiently translated.

Translational profiling has recently been used to study translational changes in the response to hypoxia (Thomas and Johannes 2007). When PC-3 cells are grown under hypoxic conditions, translation is globally down-regulated, concomitant with mTOR inactivation and phosphorylation of eIF2α (see above), and mRNAs encoding ribosomal proteins are found to be most sensitive to the global translational down-regulation. Again, several mRNAs were identified, which escape the translational down-regulation and still are associated with polysomal fractions under hypoxic conditions (Thomas and Johannes 2007). The authors suggest that translational regulation of these mRNAs might be initiated via cap-independent mechanisms. This

is another example of how certain mRNAs can be selectively translated in response to a specific stimulus, while most other cellular mRNAs are translationally down-regulated in this condition. Such sets of mRNAs could only be identified using genome-wide, unbiased approaches such as translational profiling, as their involvement in certain biological processes is unexpected and could not have been anticipated by traditional biological studies.

Another use of translational profiling was made by Arava et al. (2003): in this study, translational profiling was not used to look into translational regulation in response to changing conditions, but the authors give a comprehensive picture of translational efficiency in vegetatively growing budding yeast cells. mRNA extracted from 14 fractions across the polysomal profile are analyzed on microarrays, and the peak of the distribution for each mRNA along the profile is used to determine the average number of ribosomes associated with a given mRNA on a genome-wide scale. Several interesting findings could be made using this approach. For most mRNAs, 70-80% of the transcript was associated with polysomal fractions. Among the few mRNAs not associated with polysomal fractions several mRNAs were known to be translationally regulated. Furthermore, the authors could show that ribosomes are spaced well below the maximum packing capacity on most mRNAs, which corroborates the fact that translation initiation is the rate-limiting step in translation. The density of associated ribosomes varied strongly between transcripts and showed an inverse correlation to the length of the transcript.

Recently, Qin et al. (2007) also used a similar high-resolution translational profiling approach to study the extent of translational control during early *Drosophila* embryogenesis. One of their findings is that mRNAs that were known to be regionally translationally repressed in the early fly embryo such as Nanos, Hunchback or Caudal mRNA indeed only show a small portion of their transcript associated with polysomal fractions.

Alternative proteomic approaches to study translational regulation

At the moment, translational profiling should be the method of choice when examining translational regulation on a genome-wide scale, mainly due to the fact that the read-out of the assay is based on microarray measurements. Microarray technology has become robust, reliable and also affordable, and combined with proper

and careful analysis, translational profiling is a powerful tool to screen for translationally regulated mRNAs. However, recent advances in proteomic approaches will also be useful to study translational regulation. In 2 recent studies, the authors combined the measurement of absolute protein levels using proteomics and total mRNA levels using microarrays (Newman et al. 2006; Lu et al. 2007). Newman et al. (2006) used a collection of yeast strains, in which each protein is fused to green fluorescent protein (GFP) under the control of its own promoter. Using a flow cytometry approach, GFP abundance was measured for each strain grown in full medium or minimal medium and mRNA levels were measured using DNA microarrays in both conditions. Lu et al. (2007) used a mass spectometry approach together with a novel algorithm to make absolute measurement of protein levels (APEX, absolute protein expression measurements) in the same conditions. Both studies came to the conclusion that changes in protein levels between the conditions examined are largely due to changes in the abundance of the corresponding mRNAs, but certain mRNAs were identified, where the change in protein level could not be attributed solely to a change in mRNA level. These mRNAs are prime candidates for regulation at the translational level or at the level of protein stability.

There is a downside to these proteomic approaches: in the case of the GFP-tagged strain collection, the tag might interfere with translational regulation, which might be executed via sequence elements in the UTR, and mass spectometry approaches do not yet manage to identify every expressed protein in the cell and are biased towards highly abundant proteins. However, as these techniques improve, they will become an important tool for the genome-wide study of translational control.

mRNA decay

As mentioned above, mRNA turnover in the cell is regulated by multiple mechanisms (Wilusz et al. 2001; Parker and Song 2004). Deadenylation of the transcript is a key step in these regulatory mechanisms, and mRNAs are then decapped and degraded via the XRN1 exonuclease or, alternatively, mRNAs can be degraded without decapping by the exosome complex. In certain cases, mRNAs can be degraded via endonucleolytic mechanisms, such as degradation via the RNAi machinery (Tomari and Zamore 2005). Furthermore, nonsense-mediated decay (NMD) serves as a mRNA-quality control mechanism to degrade faulty mRNAs with a premature stop

codon. These mRNAs are decapped and directly degraded without prior deadenylation (Fasken and Corbett 2005). mRNAs that are lacking proper stop codons are degraded without decapping by the exosome in a process called non-stop decay (Vasudevan et al. 2002).

Global mRNA stability is often measured by blocking transcription with drugs or by using mutants of RNA polymerase II. At several times after the transcription block, mRNA is isolated and probed on a microarray (Figure 1.12; Mata et al. 2005). Using this approach, genome-wide mRNA stability has been determined in various organisms such as yeast (Wang et al. 2002; Grigull et al. 2004), plants (Gutierrez et al. 2002) and human cell lines (Raghavan et al. 2002; Yang et al. 2003).

The picture emerging from these studies is that mRNA decay is a controlled process and that decay rates vary substantially between different transcripts. mRNA decay rates often also correlate among mRNAs that encode functionally related proteins or proteins of the same macromolecular complex (Wang et al. 2002). mRNAs encoding transcription factors, parts of the transcriptional machinery, proteins involved in ribosome-biogenesis and the translational machinery have in general fast decay rates, whereas mRNAs encoding central metabolism proteins have slower decay rates (Wang et al. 2002; Yang et al. 2003; Grigull et al. 2004; McCarroll et al. 2004). It was suggested that the fast decay rates for mRNAs involved in the transcriptional and the translational process might be advantageous for fast regulation of these central gene expression processes in response to changing environmental conditions. However, it has to been mentioned that the transcriptional shut-down itself, and the use of drugs or mutants of RNA polymerase II in these experiments can also trigger a general stress response in the cell (Grigull et al. 2004). Thus, the fast decay of mRNAs involved in transcription and translation might happen rather as a response to the stress, and decay rates for the same mRNAs might actually be much slower in un-stressed vegetatively growing cells.

For many mRNAs, the fast decay rate correlates with the presence of ARE elements in their 3' UTR, but not all fast decaying mRNAs have ARE elements (Raghavan et al. 2002; Yang et al. 2003). However, no strong correlation between mRNA stability and other mRNA features such as ORF length, mRNA abundance or ribosome density seems to exist (Wang et al. 2002).

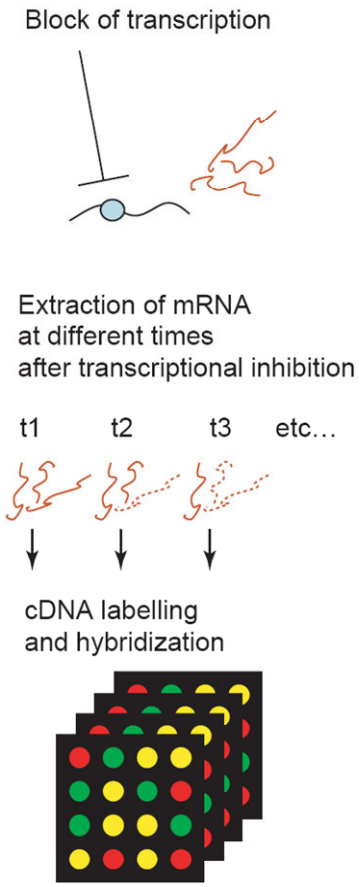

Figure 1.12 Genome-wide measurements of mRNA half-lives
Transcription is blocked using using drugs or mutants of RNA polymerase II. At different times after the transcriptional block, transcripts are isolated and quantified using DNA microarrays. mRNA half-lives can then be deduced from these data. This figure is taken from Mata et al. (2005).

In a recent study, Shock et al. (2007) determined the global decay rates of mRNAs in various stages during the intra-erythrocytic development cycle of *Plasmodium falciparum*, the pathogen causing human malaria. Interestingly, as the parasite passes through the examined intra-erythrocytic developmental stages, decay rates decrease globally for essentially all examined mRNAs, which suggests that post-transcriptional regulation might be the main mechanism of gene regulation in *Plasmodium falciparum*. Such genome-wide regulation of mRNA decay rates has not yet been described for any other organism.

Insights into the global regulation of mRNA decay also comes from measuring total mRNA levels in cells deleted for factors involved in mRNA degradation. An example is the measurement of global effects in yeast or mammalian cells compromised for

NMD function (He et al. 2003; Mendell et al. 2004). Apart from the involvement in quality control of mRNAs, a new aspect of this pathway could be detected through these global studies: several hundred mRNAs were found to be induced as a consequence to NMD switch-off, and they were enriched for mRNAs with specific functions. In mammalian cells, many of the enriched mRNAs are involved in amino acid metabolism (Mendell et al. 2004). As NMD requires translation and amino acid depletion inhibits translation, the authors suggest that the abundance of these transcripts is regulated by NMD to couple their mRNA levels to amino acid availability. Inhibition of translation and NMD might increase the abundance of these transcripts in order to turn on amino acid biosynthesis (Mendell et al. 2004). Thus, these genome-wide studies revealed that NMD not only functions in ensuring quality control of mRNAs but also acts as a more general regulator of gene expression.

In another recent genome-wide approach, Hollien et al. (2006) could show that the inositol-requiring enzyme 1 (IRE-1), which is involved in activating the unfolded protein response (UPR) as a consequence of accumulation of mis-folded proteins in the endoplasmatic reticulum (ER), is involved in the specific and immediate degradation of a subset of mRNAs during the UPR. IRE-1 is involved in the detection of unfolded proteins in the ER and subsequently activates a transcription factor, X-box–binding protein 1 (XBP-1), through endonucleolytic cleavage of its mRNA. In this study, IRE-1 or XBP-1 were depleted by RNAi in *Drosophila* S2 cells, in which the UPR has been induced. Global mRNA levels from these cells were then measured using DNA microarrays. A subset of mRNAs could be identified, whose repression is solely dependent on IRE-1, and not on XBP-1, and IRE-1 mediates the degradation of these mRNAs, based both on their localization to the ER membrane and on the amino acid sequence they encode (Hollien and Weissman 2006).

RNA-binding proteins and their target mRNAs

Central to virtually all aspects of post-transcriptional gene expression regulation – from mRNA processing and export to mRNA decay and translation – is the interplay between mRNAs and RNA-binding proteins (RBPs). Some RBPs bind most of the transcripts in the cell (e.g. PABP), whereas others bind only to a small set of specific mRNAs in order to exert a specialized function in determining these mRNAs' post-transcriptional fate (Hieronymus and Silver 2004; Mata et al. 2005; Moore 2005;

Keene 2007). Furthermore, RBPs most likely can also act in a combinatorial way, as each mRNA can be bound by several RBPs. In budding yeast, there are about 600 proteins estimated to have RNA-binding capacity, and this number is probably even higher in mammalian cells (Maris et al. 2005; Moore 2005).

Much insight into gene expression regulation via RBPs has come from the genome-wide identification of their targets via "RBP Immunoprecipitation followed by chip analysis" (RIP-chip, Figure 1.13): RBPs are immunopurified together with their associated RNAs, via an epitope-tag or via an antibody against the RBP of interest. The RNAs are then isolated from the immunoprecipitate, purified, labelled and then hybridized onto microarrays. In one of the first studies to employ this technology, Tenenbaum et al. (2006) used cDNA-filter arrays containing ~600 murine genes to identify mRNAs associated with the RBPs HuB, PABP and eIF4E, which all are involved in the regulation of translation. Even though only a few mRNAs were analyzed, each RBP bound a different subset of mRNAs, with PABP being associated with many mRNAs and HuB only associated with a few mRNAs. Furthermore, the authors found that the pattern of association of mRNAs with HuB is significantly altered after cells were induced to differentiate by treatment with retinoic acid.

One of the most comprehensive studies using RIP-chip was conducted by Gerber et al. (2004), who identified targets of all 5 members of the Pumilio family of RBPs in budding yeast (Puf1p-Puf5p). Forty to 220 mRNAs were found to be associated with each of the five Puf proteins, and the subset of mRNAs bound to each of the RBPs were enriched for common functional groups or subcellular localization. Puf1p and Puf2p associate with mRNAs encoding membrane-associated proteins; Puf3p nearly exclusively binds mRNAs that encode mitochondrial proteins; Puf4p associates with nucleolar ribosomal RNA-processing factors; and Puf5p associates with mRNAs encoding chromatin modifiers and components of the spindle pole body. Furthermore, distinct sequence elements in the 3' UTR of mRNAs bound by Puf3p, Puf4p and Puf5p could be identified (Gerber et al. 2004). A similar sequence motif was identified in mRNAs that co-immunoprecipitate with the *Drosophila* Pumilio protein (Gerber et al. 2006). Many of the mRNAs associated with Pumilio in *Drosophila* also encode functionally related proteins; however, these mRNAs are not related to the mRNAs associated with Puf3p in budding yeast (Gerber et al. 2006).

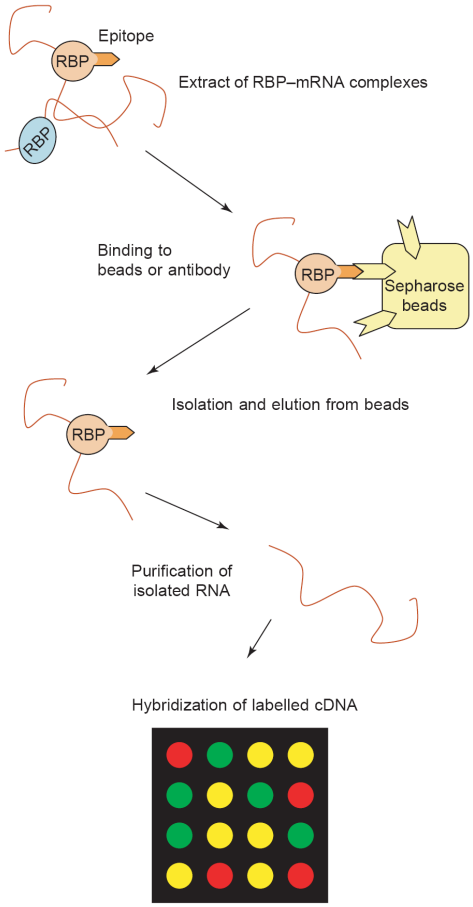

Figure 1.13 Genome-wide determination of mRNA targets of RNA-binding proteins (RBPs)
Targets of RBPs can be determined globally by "RBP Immunoprecipitation followed by chip analysis" (RIP-chip). RBPs are immunopurified together with their associated mRNAs, via an epitope-tag or via an antibody against the RBP of interest. The mRNAs are then isolated from the immunoprecipitate, purified, labelled and then hybridized onto microarrays. This figure is taken from Mata et al. (2005).

RIP-chip approaches have also been used to identify global targets of RBPs involved at other levels of post-transcriptional gene expression regulation such as splicing (Gama-Carvalho et al. 2006), nuclear mRNA export (Hieronymus and Silver 2003; Kim Guisbert et al. 2005), mRNA decay (Duttagupta et al. 2005), and poly(A) tail length control (Beilharz and Preiss 2007). Common to these studies is the finding that RBPs involved in a common process often share mRNA targets, but on top of that, each RBP seems to have unique targets; and mRNAs targeted by a certain group of RBPs often share functional specificity. Furthermore, RIP-chip studies also provided clues to unexpected functions of RPBs. An example is the identification of mRNAs

associated with the yeast La protein (Lhp1p). Lhp1p is involved in the biogenesis of non-coding RNAs transcribed by RNA polymerase III, and thus many non-coding mRNAs were identified as targets of this RBP (Inada and Guthrie 2004). However, Lhp1p was also found to bind a subset of coding mRNAs such as *HAC1* mRNA, which encodes a transcription factor required for the UPR. Follow-up experiments indicate that Lhp1p might play a role in the translational regulation of *HAC1* mRNA (Inada and Guthrie 2004 387).

Recently, RIP-chip approaches are also employed to measure translation on a global scale. In this case, the RBP is an epitope-tagged ribosomal subunit and polyribosomal complexes are immunopurified, which correspond to mRNAs bound to ribosomes. The feasibility of these approaches was first shown in budding yeast (Inada et al. 2002). The ribosomal protein Rpl25p was epitope-tagged and immunopurification via the epitope-tag yielded intact polysomal fractions. Zanetti et al. (2005) used a similar approach with epitope-tagged ribosomal protein RPL18 in *Arabidopsis* to isolate polyribosomes. The authors furthermore probed the mRNA from these immunopurified complexes with DNA microarrys and compared the data to total cellular mRNA samples. Their data show that for most genes the mRNAs are associated with polysomal complexes with an average level of association of 62%, which is slightly below the number of ribosome association determined for yeast mRNAs by translational profiling (Arava et al. 2003). This technology could become a powerful tool to study translational regulation in varying conditions or different cellular subtypes.

Aim of this thesis

The work of this thesis is based on a simple question: "What are the global patterns of translational regulation in fission yeast?"

Underlying this question was the fact that in recent years a wealth of genome-wide data was generated in fission yeast to describe changes in mRNA levels in a variety of conditions such as the response so stress (Chen et al. 2003), during the mitotic cell cycle (Rustici et al. 2004), and during meiosis and sporulation (Mata et al. 2002; Mata and Bähler 2006). Work from these studies gave a comprehensive overview of transcriptional regulation in the conditions examined. However, no genome-wide approaches had been conducted in fission yeast to examine gene expression regulation at the post-transcriptional levels – such as at the level of translation.

Therefore, the first aim of this work was to establish translational profiling in fission yeast and to measure translational rates on a global scale in vegetatively growing cells. These data could then be compared to genome-wide data sets of mRNA features and to genome-wide data sets of other levels of gene expression regulation.

A second aim of this study was to identify mRNAs that are specifically regulated at the level of translation in response to environmental and genetic perturbations such as cellular stress or in mutant yeast strains. Using translational profiling in combination with measurements of changes in total mRNA levels with microarrays, mRNAs can be identified that are regulated solely at the level of mRNA abundance, solely at the level of translation, or regulated at both levels. These data should provide a global view of the extent of translational regulation and identify candidates for translational regulation, which can be examined in follow-up studies.