CHAPTER FIVE

Translational Control of Gene Expression: From Transcripts to Transcriptomes

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Contents

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Abstract

The regulation of gene expression is fundamental to diverse biological processes, including cell growth and division, adaptation to environmental stress, as well as differentiation and development. Gene expression is controlled at multiple levels from transcription to protein degradation. The regulation at the level of translation, from specific transcripts to entire transcriptomes, adds considerable richness and sophistication to gene regulation. The past decade has provided much insight into the diversity of mechanisms and strategies to regulate translation in response to external or internal factors. Moreover, the increased application of different global approaches now provides a wealth of information on gene expression control from a genome-wide perspective. Here, we will (1) describe aspects of mRNA processing and translation that are most relevant to translational regulation, (2) review both well-known and emerging concepts of translational regulation, and (3) survey recent approaches to analyze translational and related posttranscriptional regulation at genome-wide levels.

Key Words: Translation, Posttranscriptional control, mRNA processing, P-bodies, microRNA, microarray, ribosome, RNA-binding protein.

1. Introduction

The control of gene expression is a fundamental process to bring the genome to life, and misregulation is usually associated with disease. It is now well established that gene expression is regulated at multiple levels, and emerging data suggest that the diverse processes involved in this regulation are integrated with each other (Hieronymus and Silver, 2004; Maniatis and Reed, 2002; Mata *et al.*, 2005; McKee and Silver, 2007; Moore, 2005; Orphanides and Reinberg, 2002; Proudfoot *et al.*, 2002). Gene regulation can be divided into transcriptional and posttranscriptional control (Fig. 5.1). Furthermore, proteins themselves can be regulated by posttranslational modifications and protein degradation.

Transcriptional control has received much attention, through both traditional single-gene studies (Kadonaga, 2004) and genome-wide approaches, including expression profiling (Bertone *et al.*, 2005; Lockhart and Winzeler, 2000), transcription factor binding studies, and identification of regulatory sequence elements (Hanlon and Lieb, 2004; Sandelin *et al.*, 2007), as well as chromatin remodeling and epigenetic analyses (Bernstein *et al.*, 2007; Kouzarides, 2007; Li *et al.*, 2007). In comparison, posttranscriptional control has been less extensively studied. This discrepancy is apparent when searching within the scientific literature: approximately 55,000 articles are found in PubMed for the query ''transcriptional regulation,''

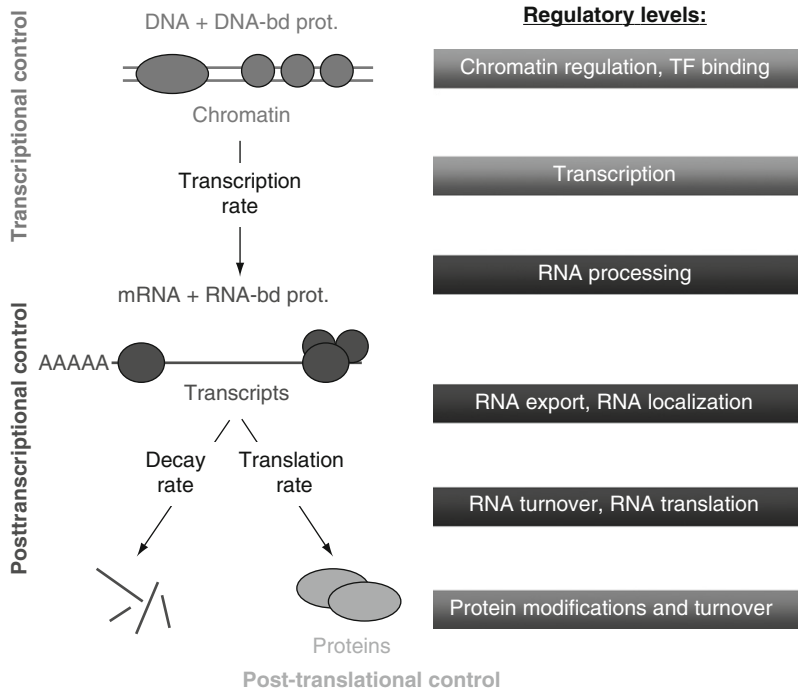

Figure 5.1 Scheme of different layers of gene regulation. The regulatory processes are listed according to their involvement in transcriptional, posttranscriptional, or posttranslational control. Adapted with permission from Mata *et al.* (2005).

whereas "posttranscriptional regulation" only returns about 5700 hits. This bias reflects historical and technical reasons: it is clear that transcription is one of the fundamental and intuitively important steps for gene regulation, and techniques to study transcription and transcriptional control are well established in the scientific community.

An increasing appreciation of the importance of posttranscriptional gene regulation is emerging. Posttranscriptional regulation mechanisms comprise various processes such as mRNA processing (polyadenylation, capping, and splicing), mRNA export and localization, mRNA decay, and mRNA translation (Fig. 5.1). Despite this variety of regulatory mechanisms, they all have one thing in common: they ultimately control if, where, and how efficiently a given mRNA is translated into protein. Consequently, translation and translational control are central to posttranscriptional regulation of gene expression. We will therefore first discuss in some detail different mechanisms and strategies for the regulation of translation in eukaryotes and will then give an overview of recent efforts to study posttranscriptional regulation of translation and related mRNA processes on a genome-wide scale.

2. Preparation for Translation: RNA Processing and Export

Before a transcript can be exported from the cell nucleus to become available for the translation machinery in the cytoplasm, 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. These maturation steps are cotranscriptional and can influence each other's activities (Proudfoot *et al.*, 2002). Only a brief overview of these processes will be given, as far as they are relevant to translational regulation, while referring to key reviews that present more detailed views of these RNA processing steps.

The first processing step is the addition of the m^7G cap structure to the 5′ end of the nascent mRNA and takes place after 20–30 nucleotides (nt) have been synthesized (Gu and Lima, 2005; Shatkin and Manley, 2000). In a three-step reaction, the nascent transcript is hydrolyzed, the GMP moiety from GTP is added to the first nt 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 is bound by the two-subunit cap-binding complex (CBC), and, after export of the mRNA to the cytoplasm, is replaced by the translation initiation factor 4E, which represents an essential step in translation initiation.

As the coding sequences of most mRNAs in eukaryotes are interrupted by introns, these introns must be spliced out of the pre-mRNA to generate a functional mRNA. Splicing requires consensus sequences in the pre-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 (Collins and Guthrie, 2000; Jurica and Moore, 2003; Kramer, 1996; Patel and Steitz, 2003). The spliceosome consists of five small ribonucleoprotein particles (snRNPs: U1, U2, U4, U5, and U6), each of which is made of a small nuclear RNA (snRNA) and associated proteins, as well as numerous accesory proteins. In fact, well over a hundred different proteins are thought to function as 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 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 metazoan histone mRNAs (Marzluff, 2005). Polyadenylation is achieved in two steps: the nascent mRNA is cleaved near the site of polyadenylation, which is followed by poly(A) synthesis (Proudfoot and O'Sullivan, 2002;

Shatkin and Manley, 2000; Zhao *et al.*, 1999). In analogy to splicing, formation of the poly(A) tail requires multi-subunit polyadenylation complexes and specific sequence-elements in 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 factors 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 (MacDonald *et al.*, 1994; Murthy and Manley, 1995; Takagaki *et al.*, 1989). The principle of poly(A) tail formation is 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 (Proudfoot and O'Sullivan, 2002; Shatkin and Manley, 2000; Stevenson and Norbury, 2006). Furthermore, in yeast, a variable A-rich element substitutes for the AAUAAA hexamer motif, and there are three polyadenylation complexes: cleavage polyadenylation factor (CPF), which contains the PAP and several factors homologous to CPSF, cleavage factor IA (CF IA), and cleavage factor IB (CF IB).

The emerging poly(A) tail is bound by nuclear and cytoplasmic poly(A)-binding proteins (PABPs). PABPs are thought to influence the final length of the poly(A) tail positively by stimulating the processivity of PAP, as well as 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 (Section 3.1.2). The poly(A) tail is also crucial for several other posttranscriptional regulatory mechanisms in the cytoplasm, and cytoplasmic PAPs 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 the translational regulation of maternal mRNAs in *Xenopus* oocytes, which are stock-piled in a translationally repressed state with short poly(A) tails that become polyadenylated upon activation and, as a consequence, translated (Mendez and Richter, 2001; Richter, 2007). mRNA decay by exonucleolytic mechanisms is also usually preceded by a shortening of the poly(A) tail (Parker and Song, 2004; Wilusz *et al.*, 2001), and recently deadenylation of poly(A) tails has also been shown to occur in microRNA (miRNA)-mediated gene regulation (Giraldez *et al.*, 2006; Wu *et al.*, 2006).

Mature mRNAs need to be exported from the nucleus to the cytoplasm for translation. Export through the nuclear pore complex (NPC) occurs in the context of messenger ribonucleoprotein particles (mRNPs) that are assembled cotranscriptionally (Cole and Scarcelli, 2006; Stewart, 2007;

Strässer *et al.*, 2002). mRNPs contain the mRNA and associated RNA-binding proteins (RBPs) that bind to the mRNA during the processing steps (Aguilera, 2005; Moore, 2005). Apart from the aforementioned CBC or PABPs, such RBPs 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 export 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 important for quality control, as faulty or unprocessed mRNAs are not only useless but also potentially harmful if translated in the cytoplasm; this quality control step is coupled to RNA processing and the mRNP composition.

It needs to be emphasized that although we introduced mRNA transcription, capping, splicing, polyadenylation, and nuclear export as sequential events, these events seem to be tightly integrated with each other both spatially and temporally (Aguilera, 2005; Moore, 2005; Proudfoot *et al.*, 2002).

3. Regulation of Translation

Translation can be divided into three major steps: initiation, elongation, and termination. Translation initiation comprises the events that lead up to the positioning of an elongation-competent 80S ribosome at the 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.

Several lines of evidence indicate that initiation is the rate-limiting step for translation. When cells are treated with low doses of elongation inhibitors (e.g., cycloheximide) such that total protein synthesis is only minimally affected, the translational efficiency of most mRNAs is not altered (Lodish and Jacobsen, 1972; Mathews *et al.*, 2007; Walden *et al.*, 1981). Furthermore, the average density of ribosomes along the mRNA is significantly lower than the maximum packing capacity of one ribosome per 30–40 nt (Arava *et al.*, 2003; Lackner *et al.*, 2007; Mathews *et al.*, 2007; Wolin and Walter, 1988). 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 translation initiation (Pestova *et al.*, 2007; Preiss and Hentze, 2003).

Therefore, it is not surprising that most translational regulation is executed at the level of initiation (Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005; Mathews *et al.*, 2007; Preiss and Hentze, 2003).

We will provide an overview of the molecular mechanisms of translation initiation as far as they are directly relevant to the regulation of translation. More detailed reviews of the molecular events of translation initiation in mammalian and yeast cells are available (Hinnebusch *et al.*, 2007; Pestova *et al.*, 2007). Note that much of the molecular data on translation have been acquired using either *in vitro* studies with purified components to reconstitute translation events or genetic studies in the budding yeast *Saccharomyces cerevisiae*. For descriptions of translation elongation and termination, we refer to recent reviews (Ehrenberg *et al.*, 2007; Taylor *et al.*, 2007).

3.1. Mechanisms of translation initiation in eukaryotes

3.1.1. Preinitiation complex formation

Translation initiation starts with the formation of the 43S preinitiation complex (Fig. 5.2). As physiological conditions favor the association of small (40S) and large (60S) ribosomal subunits to form complete 80S ribosomes, but only free ribosomal subunits can initiate translation, it is important that posttermination ribosomes dissociate (Pestova *et al.*, 2001; Preiss and Hentze, 2003). In prokaryotes, this dissociation is achieved through a ribosome-recycling factor, which shows no known eukaryotic equivalent (Kisselev and Buckingham, 2000). The eukaryotic initiation factors (eIFs) eIF3, eIF1, eIF1A, and eIF6 are thought to promote this dissociation in eukaryotes, but its mechanism is unknown. Recent data suggest that the activity of these factors is not sufficient to prevent formation of 80S ribosomes (Pestova *et al.*, 2007; Preiss and Hentze, 2003), and it is thought that dissociation of 80S ribosomes is directly linked to 43S preinitiation complex formation (Pestova *et al.*, 2007).

The first step in 43S preinitiation complex formation is the assembly of the ternary complex (Figs. 5.2 and 5.3). The ternary complex consists of eIF2, a hetero-trimer of α, β, and γ subunits, methionyl-initiator tRNA (Met-tRNA$_i^{Met}$) and GTP, and its assembly is regulated by the guanine nt exchange factor (GEF) eIF2B (Fig. 5.3). GTP is hydrolyzed after recognition of the AUG start codon producing eIF2 bound to GDP, which has a tenfold reduced affinity for Met-tRNA$_i^{Met}$ (Hinnebusch *et al.*, 2007). eIF2B promotes the GDP–GTP exchange to regenerate active eIF2 (Fig. 5.3) (Hinnebusch *et al.*, 2007; Pestova *et al.*, 2007; Preiss and Hentze, 2003). Binding of the active ternary complex to the 40S ribosomal subunit is aided independently by eIF1, eIF1A, and eIF3 in mammalian cells (Pestova *et al.*, 2007; Preiss and Hentze, 2003). In budding yeast, eIF1, eIF3, eIF5, and the ternary complex can be isolated as a multifactor complex

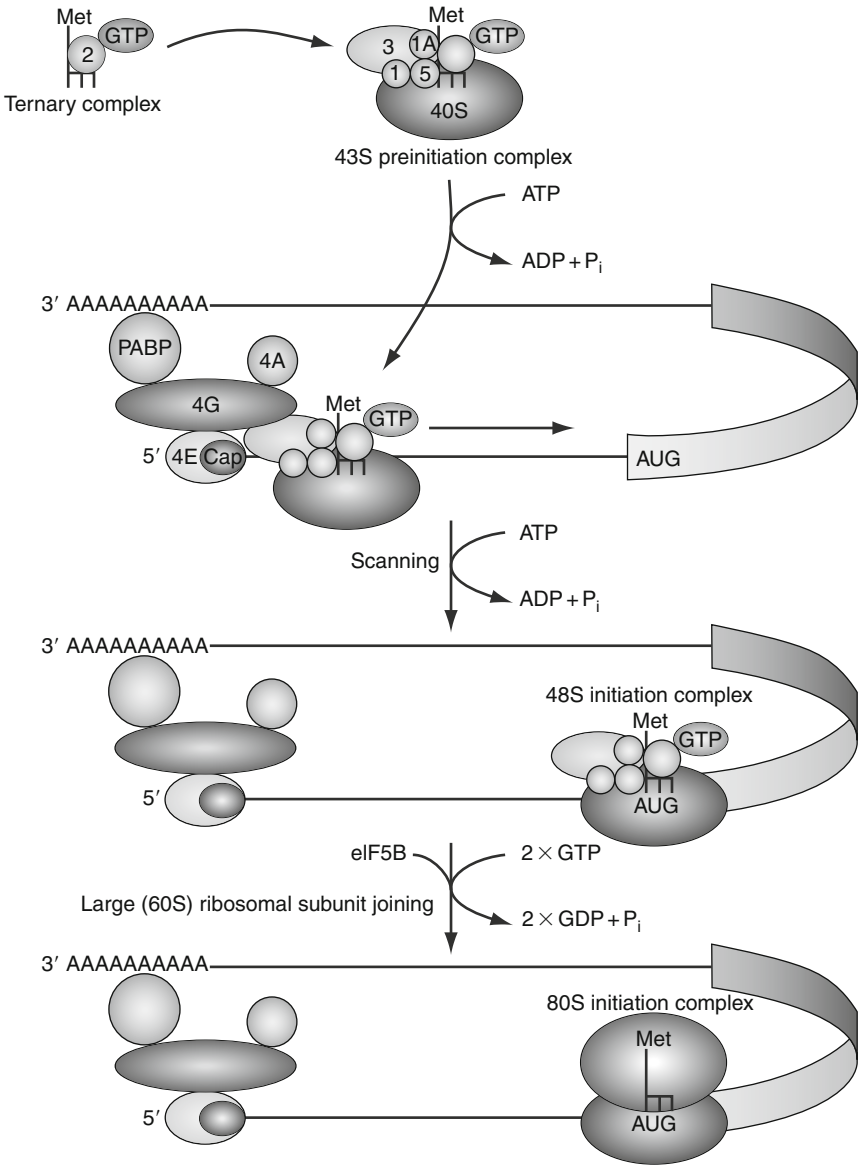

Figure 5.2 Major molecular events that lead to cap-dependent translation initiation. For a detailed description see main text. Reproduced with permission from Gebauer and Hentze (2004).

(MFC), which raises the possibility that this MFC is recruited to the 40S subunit as a preformed unit (Hinnebusch *et al.*, 2007). The 43S preinitiation complex is then ready to bind to the 5′ end of the mRNA.

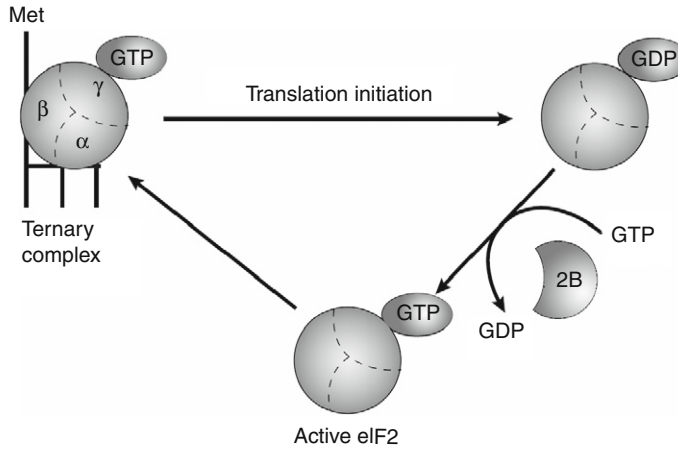

Figure 5.3 Formation of the active ternary complex. The ternary complex consists of eIF2, a hetero-trimer of α, *β*, and *γ* subunits, the initiator tRNA (Met-tRNA$_i^{Met}$) and GTP, and its assembly is regulated by the guanine nucleotide exchange factor (GEF) eIF2B: GTP is hydrolyzed after recognition of the AUG start codon producing eIF2 bound to GDP, which has a tenfold reduced affinity for Met-tRNA$_i^{Met}$. eIF2B promotes the GDP–GTP exchange to regenerate active eIF2. Reproduced with permission from Gebauer and Hentze (2004).

3.1.2. Recruitment of preinitiation complex to mRNA

Recognition of the m^{7}G cap structure at the 5′ end of the mRNA is mediated by eIF4F, which contains the three subunits eIF4E, eIF4G, and eIF4A (Fig. 5.2): 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 (untranslated region) so that the 43S complex can scan along the mRNA, and eIF4G is thought to act as scaffold protein (Hinnebusch *et al.*, 2007; Pestova *et al.*, 2007; Preiss and Hentze, 2003). In mammalian cells, eIF3 from the preinitiation 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 preinitiation complex to the mRNA involves the cooperative activities of eIF4F, eIF3, eIF4B, and possibly the 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 (Fig. 5.2) (Wells *et al.*, 1998). Furthermore, PABP Pab1p is essential for translation initiation in budding yeast (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).

3.1.3. mRNA scanning and AUG recognition

After proper assembly at the 5′ end of the mRNA, the preinitiation complex needs to scan along the mRNA to find the AUG start codon (Kozak, 1989, 2002). The model of scanning had originally been proposed by Kozak (1999), and despite the fact that most biochemical and genetic data are consistent with the model, direct physical intermediates of the scanning process have not been identified to date. The 43S preinitiation 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 and Kolupaeva, 2002; Pestova *et al.*, 1998). 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 preinitiation complex, which allows scanning in 5′–3′ direction.

3.1.4. Ready to go: Formation of translation-competent 80S subunit

The 43S preinitiation complex recognizes the start codon through formation of base pairs between the anticodon loop of the initiator tRNA and the AUG start codon (Fig. 5.2). This stable complex is known as the 48S initiation complex. Selection of the correct start codon is dependent on eIF1 (Pestova and Kolupaeva, 2002; Pestova *et al.*, 1998). Several events then take place in order for the 60S subunit to join the 48S complex and form the 80S ribosome. eIF5 promotes the hydrolysis of eIF2–GTP, and, as a consequence, most of the initiation factors including eIF2–GDP dissociate 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 ribosome competent for polypeptide synthesis: GTPase activity of eIF5B is stimulated by 60S subunits and even stronger by 80S ribosomes. GTP-bound eIF5B stimulates 60S subunit joining, and GTP hydrolysis occurs after 80S subunit formation and is essential for the release of eIF5B (Lee *et al.*, 2002; Pestova *et al.*, 2000; Shin *et al.*, 2002). Taken together, two steps of GTP-hydrolysis are required for 80S ribosome formation, which also provide a checkpoint for proper start codon recognition.

3.1.5. Cap-independent translation initiation

The cap-dependent events of translation initiation described above are most common for cellular mRNAs. However, a cap-independent way of initiating translation can happen through internal ribosomal entry sites (IRES). IRES are heavily structured sequence elements in 5′ UTRs of some mRNAs with no obvious conserved consensus sequence (Baird *et al.*, 2006). The

structured IRES segment in 5′ UTRs has an active role in the recruitment of the 40S subunit. IRES elements are found in viral mRNAs and also in certain cellular mRNAs that 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 downregulation of cap-dependent translation. In-depth reviews on the topic of IRES are available (Fraser and Doudna, 2007; Hellen and Sarnow, 2001; Jackson, 2005; Spriggs *et al.*, 2005; Stoneley and Willis, 2004).

3.2. Rationale for regulating translation

Why do cells regulate translation and how do they benefit from it? There are several possible answers to this question, which are also addressed by (Mathews *et al.*, 2007). Regulation at the translational level can happen rapidly without the necessity of going through all the upstream processes of gene expression such as transcription, mRNA processing, and 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, such as 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 (Schuman *et al.*, 2006; St Johnston, 2005). The requirement for localized protein production in neurons or during development can only be met by translational regulation, as transcriptional regulation is restricted to the cell nucleus. Translational regulation also provides flexible control of gene expression: given 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 reason for translational regulation lies in the fine tuning of gene expression, and there are numerous examples of genes that are regulated at both the transcriptional and translational levels (e.g., GADD45α or TNF-α; Lal *et al.*, 2006; Saklatvala *et al.*, 2003).

3.3. Targets for translational regulation: Initiation factors, mRNAs, and ribosomes

Translational control can in principle be divided into global regulation of translation and mRNA-specific regulation (Gebauer and Hentze, 2004). Global regulation affects the translational efficiency of most mRNAs through a general tuning of translation, while mRNA-specific regulation only affects the translation of selected mRNAs. In some cases, however, this

simple distinction cannot be made; for example, the general downregulation of cap-dependent translation enhances translation of a subset of IRES-bearing mRNAs (Sections 3.1.5 and 5.1).

What are the targets for translational control at the initiation step and what are the basic principles? A simple answer to this question would be that most translational regulation either inhibits or promotes the association of mRNAs with the translation apparatus. Given the plethora of translation initiation factors, it is not surprising that many of them are targets in translational regulation, and many are controlled posttranslationally (Dever, 2002; Raught and Gingras, 2007). A key target for many regulatory mechanisms is the cap-binding protein eIF4E, which can be bound by inhibitory proteins that subsequently hinder binding of the mRNA (see below for more details). Global regulation of translation is generally mediated through modifications of translation initiation factors.

Another target for translational regulation is the mRNA itself, via *cis*-regulatory elements that are bound by *trans*-acting factors. The *cis*-regulatory elements on the mRNA can be found anywhere along the mRNA, but for most well-characterized examples of translational regulation these elements are present in either the 5′ or 3′ UTRs (Fig. 5.4). mRNA-specific translational regulation happens mostly via RNA-bnding proteins that recognize *cis*-regulatory elements of a given mRNA.

The ribosome itself can also be targeted to exert translational regulation, and several of its protein constituents can undergo posttranslational 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′-

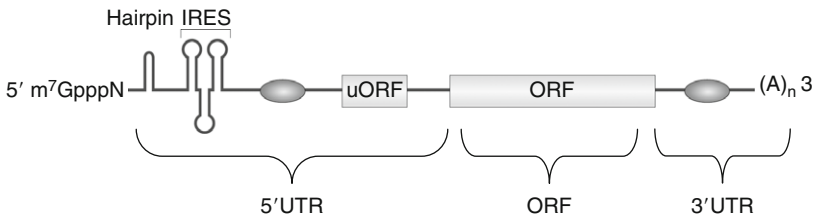

Figure 5.4 *Cis*-acting sequence elements that influence translation initiation of specific mRNAs. The m^7G cap structure at the 5′ end and the poly(A) tail at the 3′ end of mRNAs are both essential elements for cap-dependent translation initiation. Additionally, specific sequence elements in the 5′ or 3′ UTRs (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. Reproduced with permission from Gebauer and Hentze (2004).

terminal oligopyrimidine sequence (TOP mRNAs), prompted the hypothesis that translation of TOP mRNAs is regulated through this phosphorylation (Jefferies *et al.*, 1994). However, recent data contradict this model of a simple causal relationship between rpS6 phosphorylation and translational efficiency: a double knockout of both S6K homologues in mouse cells (Pende *et al.*, 2004) or a knockin of nonphosphorylatable rpS6 (Ruvinsky *et al.*, 2005) do 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 translation initiation (Ruvinsky and Meyuhas, 2006). Ribosomal proteins are also modified through ubiquitination (Spence *et al.*, 2000), methylation (Bachand and Silver, 2004; Swiercz *et al.*, 2005), and a recent report identified ribosomal proteins as targets for NEDDylation (Xirodimas *et al.*, 2008).

In budding yeast and other organisms, many genes encoding ribosomal proteins are duplicated. The open reading frame (ORF) and the protein sequence of the paralogues are similar, while the UTRs and intron sequences can differ. Ribosomal gene pairs were generally considered to be functionally equivalent, and it was thought that the gene pairs were retained to keep up with the cell's strong need to synthesize ribosomal proteins and ribosomes (Warner, 1999). 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), cellular life span (Steffen *et al.*, 2008), or for genes that exhibit deleterious haploinsufficient interactions with actin (Haarer *et al.*, 2007) uncovered specific effects for only one of the paralogues of the ribosomal protein, whereas deletion of the other paralogue would not affect the studied biological process.

Functional specificity among duplicated ribosomal proteins was further corroborated by recent work from Komili *et al.* (2007): localized translation of *ASH1* mRNA in *S. cerevisiae* is dependent on a specific subset of ribosomal proteins. Furthermore, phenotypes and transcriptomes largely differ between mutants in nearly identical paralogues. Taken together, this work is a nice example of a combination of cell biology and systems biology approaches, which reveals that paralogues of ribosomal proteins rarely behave in the same way. The biological reasons for these differences are not clear. One possibility could be that specific ribosomal proteins are involved in cellular processes other than translation. Another intriguing possibility is heterogeneity of ribosomes: the cell could construct various kinds of ribosomes, which differ in terms of paralogue composition and posttranslational modifications, and these specialized ribosomes could play roles in the regulation of translation of specific subsets of mRNAs. Further work will be needed to elucidate the exact mechanism behind this apparent ribosome specialization, especially in light of the similarity between the paralogues, some of which share the exact same protein sequence.

3.4. 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 misregulation of gene expression during cancer (Schneider and Sonenberg, 2007), in apoptosis (Morley and Coldwell, 2007), in development (Thompson *et al.*, 2007), and in the establishment of synaptic plasticity and, consequently, in learning and memory (Klann and Richter, 2007). Many examples of translational control have been reported both within and outside these areas. Instead of giving a broad overview of these regulatory mechanisms, we 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 posttranscriptional regulation via ARE-elements—are probably conserved for most eukaryotes, although these processes have mostly been studied in budding yeast and mammalian cells. Other regulatory mechanisms—such as the translational regulation of gene expression in *Drosophila* or *Xenopus* development—probably apply specialized mechanisms to meet the specific requirements of gene regulation in different organisms. The underlying principles for these regulatory mechanisms, however, are found in diverse variations in many eukaryotic cells.

3.4.1. 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 downregulation of translation (Holcik and Sonenberg, 2005). One of the best-studied examples for this downregulation is the control of the availability of active ternary complexes (Fig. 5.5). Binding of Met-tRNA$_i^{Met}$ to the 40S subunit through the ternary complex is an essential step in translation initiation as described in Section 3.1.1 (Figs. 5.2 and 5.3). 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 downregulated 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 toward phosphorylated eIF2α–GDP than toward unphosphorylated eIF2α–GDP (Rowlands *et al.*, 1988). There are at least four kinases that have been identified to phosphorylate eIF2α at

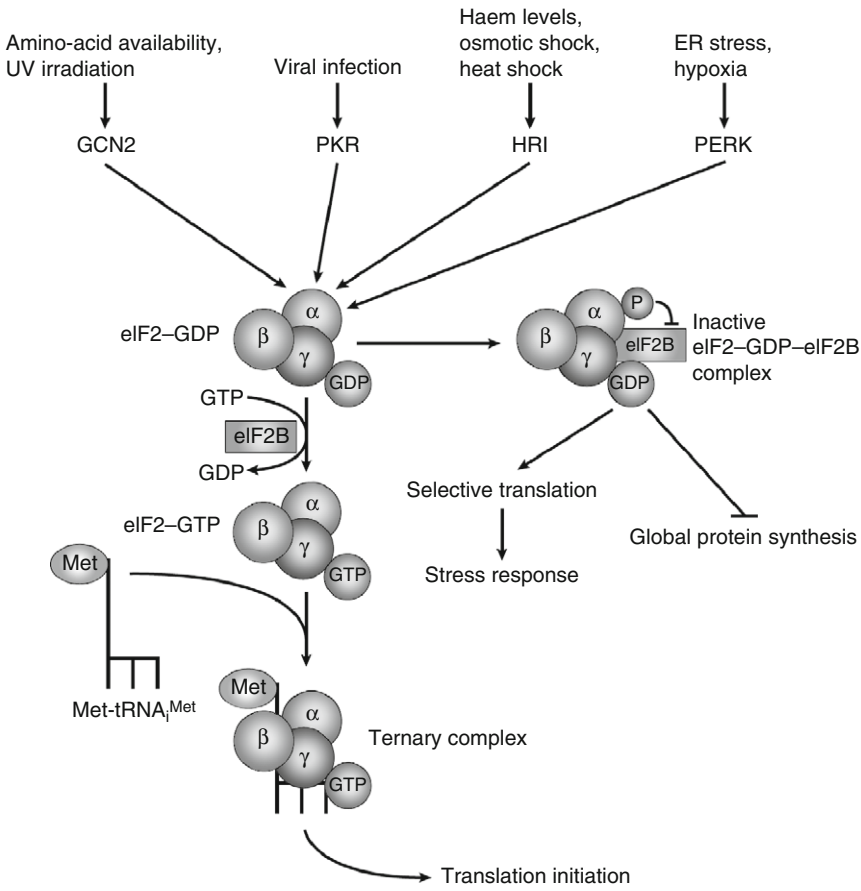

Figure 5.5 Inhibition of global protein synthesis in response to stress through phosphorylation of eukaryotic initiation factor-2α. Several protein kinases (GCN2, PKR, HRI, or PERK) can phosphorylate the α-subunit of eIF2 in response to various stress conditions. This phosphorylation inhibits the GTP–GDP exchange on eIF2 by reducing the dissociation rate of the guanine nucleotide exchange factor eIF2B, thus inhibiting active ternary complex formation. As a consequence, translation initiation is globally downregulated. Reproduced with permission from Holcik and Sonenberg (2005).

Ser51 in the response to various stresses (Fig. 5.5; Dever *et al.*, 2007): the haem-regulated inhibitor (HRI) is induced by haem depletion; general control nondepressible 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).

3.4.2. Regulation through uORFs

Whereas translation of most mRNAs is downregulated by eIF2α phosphorylation, translation of several specific mRNAs can be upregulated in response to reduced availability of ternary complex. Gcn2p kinase is upregulated in response to various starvation conditions in budding yeast, expression of *GCN2* is upregulated through a mechanism that recognizes a lack of amino acids; this is mediated through binding of uncharged tRNAs to the kinase (Dong *et al.*, 2000). Ternary complex formation and global translation are downregulated as a consequence. However, *GCN4*, encoding a master transcriptional regulator that activates transcription of amino acid-biosynthesis genes, is translationally upregulated under these conditions (Hinnebusch and Natarajan, 2002). This upregulation 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, 2005; Hinnebusch and Natarajan, 2002): Under optimal growth conditions and availability of ternary complex, translation usually starts at uORF1 and ribosomes then resume scanning to translate uORF2, uORF3, and uORF4 (Fig. 5.6). However, ribosomes cannot reinitiate translation after

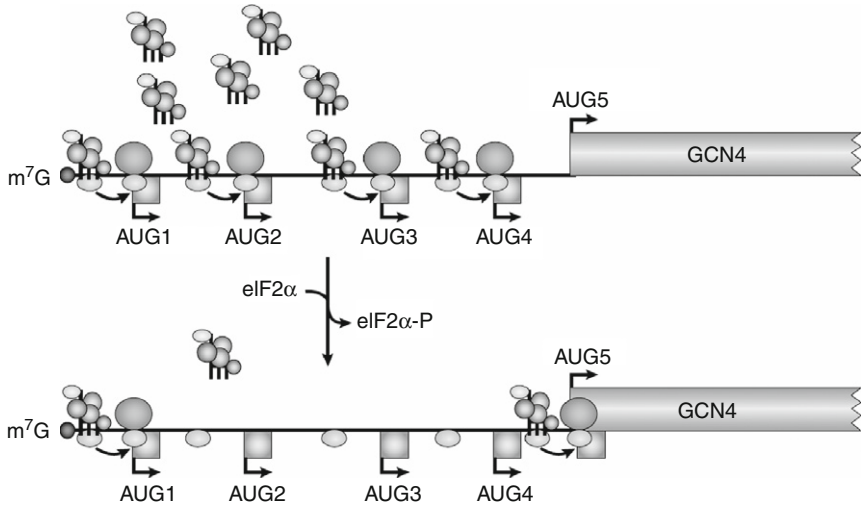

Figure 5.6 Translational regulation of *GCN4* by upstream open reading frames (uORFs). With low levels of eIF2α-phosphorylation and abundant active ternary complex, ribosomes initiate translation at uORF1, resume scanning, and reinitiate translation at uORF2, uORF3, or uORF4. However, they do not resume scanning to reinitiate translation at the start codon of *GCN4*. When cells are starved for amino acids, eIF2α becomes phosphorylated and, as a consequence, the number of active ternary complexes decreases. Under this condition, reinitiation at uORF2–uORF4 happens less frequently and scanning can resume to the actual start codon of *GCN4*, which is then translated. Reproduced with permission from Holcik and Sonenberg (2005).

termination at these latter uORFs and, as a consequence; the main coding region of *GCN4* mRNA is not translated. Upon eIF2α phosphorylation, when ternary complexes become limiting, ribosomes are more likely to resume scanning without reinitiating translation at the downstream uORFs, and translation is initiated at the actual start codon of *GCN4* (Fig. 5.6). The response to amino acid starvation via the Gcn2p kinase seems to be an evolutionarily conserved mechanism, as it was recently shown that Gcn2p activity in the mouse brain is essential for a restricted intake of diets lacking essential amino acids (Hao *et al.*, 2005; Maurin *et al.*, 2005). These studies reveal that the Gcn2 pathway recognizes 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 favors the intake of a balanced diet.

The mammalian transcription factor Atf4 is regulated in a similar way by uORFs in response to ER stress or amino acid starvation (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 (Cvijovic *et al.*, 2007; Iacono *et al.*, 2005).

3.4.3. Regulation by eIF4E inhibitory proteins

An important step during translation initiation is the binding of the m^7G cap by eIF4F (Fig. 5.2). The backbone of this complex is eIF4G, which interacts with eIF4E and the helicase eIF4A. Translation 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 or cell growth, and may also 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, while 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 to inhibit translation initiation. The mRNA specificity for these proteins comes through interactions with sequence-specific elements within the mRNA or through the interaction with RBPs.

In *Xenopus* oocytes, many mRNAs remain dormant with short poly(A) tails. When the oocytes are stimulated by progesterone for maturation, 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 inhibits the binding between eIF4E and eIF4G (Fig. 5.7),

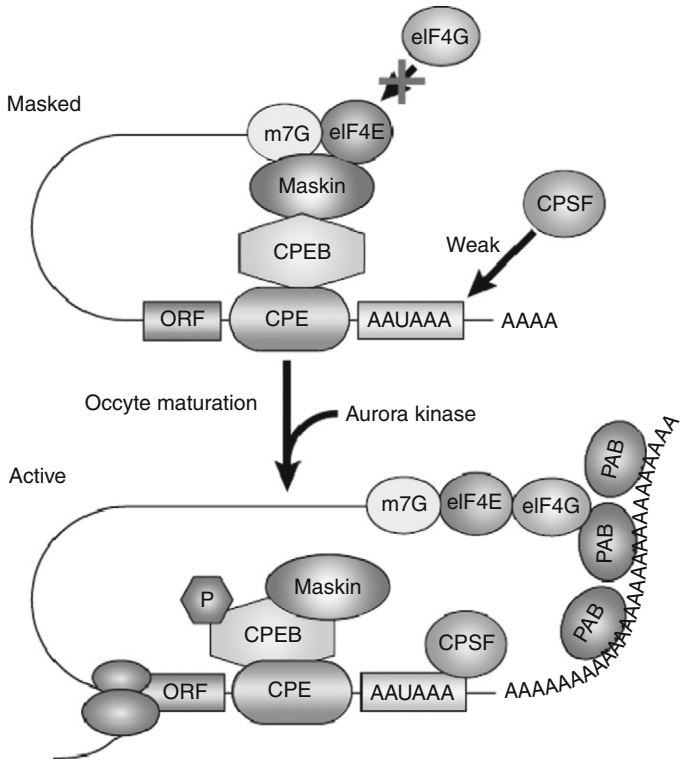

Figure 5.7 Translational regulation by the cytoplasmic polyadenylation element (CPE). mRNAs with a CPE in their 3′ UTRs are translationally repressed in developing oocytes by binding of the cytoplasmic polyadenylation element binding protein (CPEB) and Maskin. Maskin interacts directly with the cap-binding protein eIF4E and prevents its association with eIF4G, which is crucial for translation initiation. CPEB inhibits association of the cleavage and polyadenylation specificity factor (CPSF) with the AAUAAA sequence motif resulting in short poly(A) tails. Oocyte maturation leads to phosphorylation of CPEB. Consequently, Maskin dissociates from eIF4E and CPSF binds to the AAUAAA motif. Binding of CPSF recruits poly(A) polymerase that extends the poly(A) tail. These events lead to translation initiation in the previously translationally repressed mRNAs. Reproduced with permission from Kuersten and Goodwin (2003).

acting as an mRNA-specific 4E-BP (Cao and Richter, 2002). After stimulation of the oocyte to complete meiosis, CPEB stimulates cytoplasmic polyadenylation of the mRNA; the poly(A) tail is bound by PABP, which then can bind eIF4G and displace Maskin (Fig. 5.7; Cao and Richter, 2002). In turn, this cytoplasmic polydenylation also activates the synthesis of C3H-4, which leads to deadenylation of a subset of of mRNAs in a negative feedback loop required to exit meiotic metaphase (Belloc and Méndez, 2008). During translational repression, the CPEB-containing complex also includes PARN, a poly(A)-specific ribonuclease that contributes to the short poly(A) tail of target mRNAs by overriding the polyadenylating activity of the PAP GLD2 (Kim and Richter, 2006). Recently, a combinatorial code of sequence motifs in 3′ UTRs was uncovered that determines not only whether mRNAs will be translationally repressed by CPEB but also the pattern of polyadenylation-dependent translational activation (Piqué *et al.*, 2008).

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 was initially thought to directly bind 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 translation initiation (Nakamura *et al.*, 2004; Nelson *et al.*, 2004; Wilhelm *et al.*, 2003). Nanos and Oskar are examples of mRNAs regulated by Cup.

3.4.4. Other mechanisms of mRNA-specific translational regulation

AU-rich elements (AREs) are present in the 3′ UTR of many mRNAs and are potent sequence elements of posttranscriptional gene regulation. AREs influence the stability or translation of a given mRNA, usually through binding of ARE-specific RBPs (Barreau *et al.*, 2005). AUF1 was the first ARE-binding protein to be identified and was shown to exist in four isoforms (Wilson *et al.*, 1999). Association of ARE-binding proteins of the AUF1 family with AREs promotes degradation of mRNAs encoding cytokines (IL-3, GM-CSF) or cell cycle regulators ($p16^{INK4a}$, $p21^{WAF1/CIP1}$, cyclin D1) (Lal *et al.*, 2004; Raineri *et al.*, 2004; Wang *et al.*, 2005). AUF1 also interacts with the heat-shock proteins hsc70-hsp70, eIF4G, and PABP (Laroia *et al.*, 2002). Despite its role in promoting mRNA decay, recent work shows that AUF1 can induce translation of *MYC* proto-oncogene mRNA (Liao *et al.*, 2007): downregulation of AUF1 abundance by RNA-interference (RNAi) 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 upregulation 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) and the translation initiation factors eIF4A and eIF4E, especially in response to UV radiation (Mazan-Mamczarz *et al.*, 2006) and to TNFα (Gueydan *et al.*, 1999).

Additional 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.

3.4.5. Multistep regulation of translation

As is evident from some of the examples given above, translational regulation can be exerted as a multistep mechanism, which means that more than one mechanism is used to ensure tight translational control for critical proteins whose misexpression 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 RBP that 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; Gebauer *et al.*, 1998; Kelley *et al.*, 1997). SXL binds to sites in the 3′ UTR and the intronic 5′ UTR of *msl*-2 (Fig. 5.8) and represses translation in a dual way: SXL bound to the 3′ UTR inhibits recruitment of the 43S preinitiation complex, and SXL bound to the 5′ UTR can inhibit scanning of the 43S preinitiation complex if it escapes the first inhibitory mechanism (Beckmann *et al.*, 2005). Furthermore, to exert its function via the 3′ UTR, SXL requires the RBP UNR (upstream of N-*ras*) as a corepressor (Abaza *et al.*, 2006; Duncan *et al.*, 2006; Grskovic *et al.*, 2003).

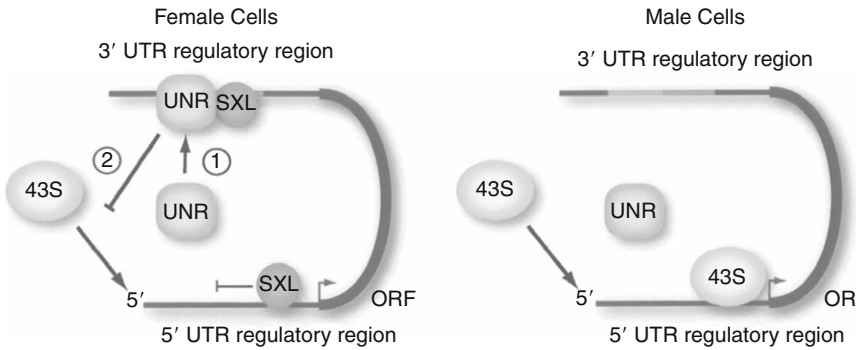

Figure 5.8 Translational regulation of male-specific-lethal (*msl-2*) mRNA in *Drosophila* through a multistep mechanism. *msl-2* translation is inhibited by Sex-lethal (SXL), a female-specific RNA-binding protein (RBP). First, SXL promotes retention of a facultative intron in the 5′ UTR of *msl-2* and 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: binding to the 3′ UTR inhibits recruitment of the 43S preinitiation complex, and binding to the 5′ UTR can inhibit scanning of the 43S preinitiation complex. To exert its function via the 3′ UTR, SXL requires the RNA-binding protein UNR (upstream of N-*ras*) as a corepressor. In male cells, SXL is not expressed and *msl-2*is translated. Reproduced with permission from Duncan *et al.* (2006).

4. Emerging Concepts in Translational Regulation

In the past few years, two new ways to modulate mRNA fate at the posttranscriptional level have attracted much 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; Cougot *et al.*, 2004; Ingelfinger *et al.*, 2002; Lykke-Andersen, 2002; Sheth and Parker, 2003; van Dijk *et al.*, 2002). 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 a connection between P-bodies and miRNA-mediated gene regulation (Liu *et al.*, 2005a,b; Sen and Blau, 2005). These novel concepts will be introduced below, with a focus on their involvement in translational regulation. We will also describe recent examples for how the modulation of alternative transcripts can affect translation.

4.1. P-bodies and translation

P-bodies were first visualized by various groups using microscopy to localize factors involved in mRNA decay such as DCP1, DCP2, XRN1, and LSM (Bashkirov *et al.*, 1997; Cougot *et al.*, 2004; Ingelfinger *et al.*, 2002; Lykke-Andersen, 2002; Sheth and Parker, 2003; van Dijk *et al.*, 2002). In mammalian cells, GW182 protein is another marker of P-bodies, which are therefore sometimes also referred to as GW bodies (Eystathioy *et al.*, 2002, 2003).

mRNA decay in eukaryotes can be controlled in different ways via endonucleolytic or exonucleolytic pathways (Parker and Song, 2004; Wilusz *et al.*, 2001). Exonucleolytic degradation is usually initiated by deadenylation of poly(A) tails. Transcripts are then degraded from their 5′ ends by the exonuclease XRN1, following removal of the 5′ cap (decapping), which is the most common route for decay. 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 are 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 leads 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), are also 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 are also 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 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 (Andrei *et al.*, 2005; Sheth and Parker, 2003), which supports the 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 two states: actively translated and associated with polysomes or translationally repressed and associated with P-bodies. When yeast cells are exposed to stress translation is inhibited at the level of initiation, which is reflected by a strong decrease in polysomes (Coller and Parker, 2005). While translation gets downregulated, P-bodies increase in size (Coller and Parker, 2005). After removal of the stress, P-bodies decrease in size and polysomes re-form, even in the absence of new transcription (Fig. 5.9; Brengues *et al.*, 2005). Therefore, P-bodies in yeast seem to serve as sites of mRNA storage, which can be released back

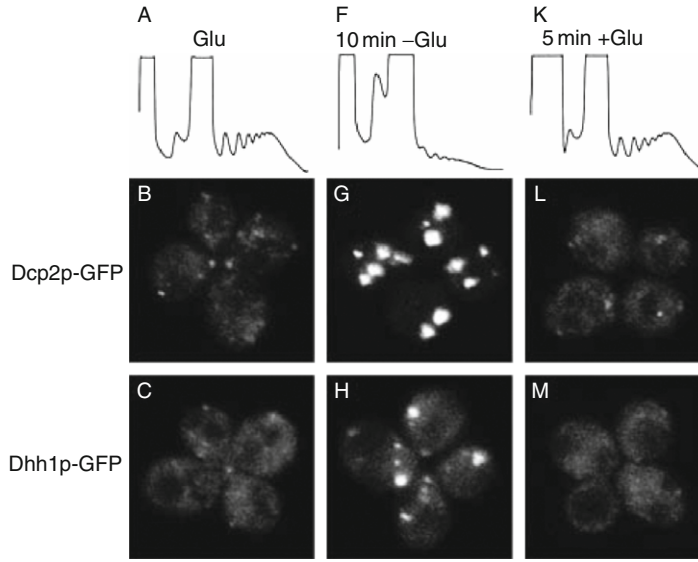

Figure 5.9 Movement of mRNAs between polysomes and P-bodies. Glucose starvation leads to inhibition of translation, which is evident from diminished polysomes (A, F). Translational inhibition also results in increased numbers and sizes of P-bodies, which are visualized using the green fluorescent protein (GFP)-tagged reporters Dcp2p (G) and Dhh1p (H), whose presence in P-bodies is dependent on mRNAs. After the readdition of glucose, polysomes reappear (K) and P-bodies largely disappear (L, M). These findings are consistent with a move of mRNAs from polysomes to P-bodies after the inhibition of translation, and reentering of mRNAs into the polysome pool after translation is restored. Reproduced with permission from Brengues *et al.* (2005).

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 the size and number of P-bodies (Andrei *et al.*, 2005; Brengues *et al.*, 2005; Cougot *et al.*, 2004; Sheth and Parker, 2003; 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; Chu and Rana, 2006; Ferraiuolo *et al.*, 2005; Kedersha *et al.*, 2005; Wilczynska *et al.*, 2005). However, the exact mechanism of how mRNAs shuttle into P-bodies and become translationally repressed is not known.

Another kind of cytoplasmic foci linked to translational repression can be observed in mammalian cells after exposure to stress: stress granules (SGs) contain translationally silent mRNAs, which are associated with preinitiation complexes lacking the ternary complex and which can shuttle 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, translation initiation factors, and ARE-binding proteins (Kedersha *et al.*, 2005). Despite these differences, fusion events and close associations between SG and P-bodies are evident (Kedersha *et al.*, 2005; Wilczynska *et al.*, 2005).

4.2. Regulation by small RNAs

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

miRNAs and siRNAs are short RNAs of 21–26 nt and are distinguished based on their biogenesis (Jackson and Standart, 2007; Kim, 2005): miRNAs are derived from longer precursors that include a ~70 nt imperfectly base-paired hairpin segment; siRNAs are of similar length but are derived from perfectly complementary RNA precursors. Despite the different modes of biogenesis, processing of 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 RNAi, exogenously introduced siRNAs target mRNAs for endonucleolytic cleavage (Tomari and Zamore, 2005), which has now also been described for miRNAs in plants (Allen *et al.*, 2005; Llave *et al.*, 2002) and mammals (Yekta *et al.*, 2004). Initially, it was thought that perfect base pairing between the miRNA/siRNA and the target mRNA favors endonucleolytic cleavage, whereas imperfect base pairing results in target repression by alternative mechanisms. However, endonucleolytic cleavage can still occur even with 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 downregulation of protein levels is usually greater than the downregulation of mRNA abundance, suggesting regulation at the level of translation (Jackson and Standart, 2007). The classic example is *lin-4* miRNA regulating *lin-14* mRNA in *Caenorhabditis elegans* through

interaction with its 3′ UTR (Arasu *et al.*, 1991; Wightman *et al.*, 1991). This regulation 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 *lin-14* repression (Petersen *et al.*, 2006): luciferase expression is strongly downregulated without large changes in mRNA abundance, and repressed mRNAs are 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 ribosome drop-off at various points along the ORF resulting from miRNA repression (Petersen *et al.*, 2006). It is hard to understand, however, how the polysomal distribution under repressed conditions with continuous ribosome drop-off would be similar to the distribution in an activated state (Jackson and Standart, 2007).

In contrast to the idea that miRNAs regulate mRNAs after translation initiation, two reports point toward initiation as the regulated step (Humphreys *et al.*, 2005; Pillai *et al.*, 2005). Using the same *CXCR4* system, Humphreys *et al.* (2005) show a similar strong downregulation at the protein level of a luciferase reporter mRNA bearing four partially complementary binding sites for the *CXCR4* siRNA. However, this downregulation is not seen with IRES-containing mRNAs. Furthermore, the downregulation is dependent on the 5′ cap and 3′ poly(A) sequences. Pillai *et al.* (2005) have also used luciferase reporters, with either one perfectly complementary or three imperfectly complementary target sites for *let-7* miRNA. Expression of the reporter is downregulated, and reporter mRNA containing imperfect *let-7* target sites is found in lighter polysomal fractions upon expression of *let-7* miRNA. Furthermore, using *in vitro* synthesized mRNAs, it has been 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 repression at the level of translation initiation.

What could be the reason for the discrepancies in miRNA-mediated translational repression reported by these various groups? First, in their study, Petersen *et 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 cotransfected with the miRNA. Second, the number, origin, specificity, and location of target sites on the reporter might influence the observed effect.

Furthermore, in a recent paper, Thermann and Hentze (2007) describe the formation of heavy miRNPs after repression by the *miR2* miRNA in *Drosophila*. These miRNA–mRNA assemblies, which the authors call "pseudo-polysomes" show the same sedimentation characteristics as polysomes, but form even under conditions of effectively blocked 60S subunit joining (Thermann and Hentze, 2007). It is not clear what these pseudo-polysomes are, but it is tempting to speculate that they represent smaller RNA–protein assemblies that combine to form particles similar to P-bodies. However, no such formation of pseudo-polysomes has been observed using a mouse cell-free translation system to study miRNA-mediated translational repression *in vitro* (Mathonnet *et al.*, 2007), but this system further supports the case of translational repression by miRNAs at the level of initiation: repression of a luciferase reporter is not due to mRNA degradation but due to inhibition of translation. Furthermore, two other groups who use *in vitro* systems for the study of miRNA-mediated translational repression come to a similar conclusion: Wakiyama *et al.* (2007) apply a cell-free system with extracts from HEK297F cells, in which miRNA pathway components are overexpressed to recapitulate the *let*-7 miRNA-mediated translational repression. In their systems, both the cap and the poly(A) tail are required for translational repression, which again points toward initiation as the regulated step (Wakiyama *et al.*, 2007). Additionally, *let*-7 miRNA mediates the deadenylation of the target mRNA, and the authors conclude that this deadanylation step is not a mere consequences of translational repression as it still happens when translation is repressed by cycloheximide. Wang *et al.* (2006) use a rabbit reticulocyte lysate *in vitro* translation system in conjunction with luciferase mRNA reporters that contain imperfect complementary binding sites to the *CXCR4* siRNA to study miRNA-mediated translational repression. Apart from showing again that a cap and the poly(A) tail are required for translational repression via miRNAs, they also show that increasing poly(A) tail length alone on the reporters can increase miRNA silencing (Wang *et al.*, 2006).

All these studies build a strong case in favor of a scenario, in which miRNAs repress translation at the initiation step. A recent study also shows that human Ago2, one of the effector proteins of miRNA-mediated repression, possesses a cap-binding motif, which is involved in translational repression (Kiriakidou *et al.*, 2007). However, it is also possible that miRNAs exert their repression on translation through different mechanisms, and that repression of translation initiation is only one aspect or an early effect by miRNA-mediated repression of gene expression. As a consequence, it will be important and necessary to validate the regulatory mechanism for each miRNA-target pair individually. Furthermore, translation could also be indirectly influenced by miRNAs, for example, by acting on the adenylation status of the 3′ end of mRNAs (Giraldez *et al.*, 2006; Wakiyama *et al.*, 2007; Wang *et al.*, 2006; Wu *et al.*, 2006).

A further addition to the ever-growing number of mechanisms by which miRNAs can affect gene expression comes from the surprising finding that members of the miRNA pathway and miRNAs themselves can also function in the upregulation of translation. Under serum starvation and thus cell cycle arrest, TNFα becomes translationally upregulated, which is dependent on AREs in the mRNA (Section 3.4.4). In order to identify the ARE-binding proteins, Vasudevan *et al.* (2007) used a biochemical approach and found that miRNA-related proteins, fragile-X-mental-retardation-related protein 1 (FXR1) and Ago2, were both associated with the AREs under serum starvation. The authors could further demonstrate that FXR1 and Ago2 are both directly involved in the translational upregulation of TNFα mRNA (Vasudevan and Steitz, 2007). Furthermore, the same authors studied if actual miRNAs are involved in this process and they could show that miRNA *369-3* directs the association of FXR1 and Ago2 with the AREs of TNFα (Vasudevan *et al.*, 2007). Furthermore, they also show that other miRNAs (*let*-7 and *CXCR4*) have the same stimulating effect on the translation of target transcripts upon cell cycle arrest. The authors suggest that miRNA function oscillates during the cell cycle: they repress translation of targets in proliferating cells, whereas they can mediate translation activation in a state of cell cycle arrest (Vasudevan *et al.*, 2007). They further speculate that such a switch between repressive and activating function could be the cause for the sometimes contradictory results documented for miRNA function in different experimental systems.

To date, no definitive mechanism for miRNA-dependent gene regulation has been established, which is not surprising given the recent emergence of this field. Furthermore, it seems unlikely that there is one unifying mechanisms that will explain miRNA function. Instead, it seems more likely that miRNA-mediated regulation is involved in several aspects of gene expression through a variety of diverse mechanisms, many of which remain to be identified. However, as publications in this area keep pouring in, we should soon obtain a better picture of the full extent of gene regulation by miRNAs.

4.3. Interplay between miRNAs and P-bodies

Several recent reports have found connections between gene regulation via miRNAs/siRNAs and P-bodies. Pillai *et al.* (2005) show that mRNAs that are translationally repressed by *let*-7 miRNA localize to P-bodies or to cytoplasmic foci adjacent to P-bodies. Argonaute proteins, the effector of miRNA-mediated regulation, also localize to P-bodies (Liu *et al.*, 2005b; Sen and Blau, 2005); these proteins 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.*, 2005a).

A recent report 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 the *CAT-1* 3′ UTR, which is negatively regulated by the *miR-122* miRNA. In Huh7 cells, *miR-122* is endogenously expressed, CAT-1 protein levels are significantly downregulated, and both *CAT-1* and *miR-122* are present in P-bodies. However, after exposure to stress, *CAT-1* mRNA can escape the translational repression, and this derepression and exit from P-bodies is dependent on ARE elements in its 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.

The above examples strongly suggest that P-body components are important for gene regulation via miRNA/siRNA-mediated repression. However, the P-body environment or P-body components important for this interaction remain to be determined. Recent work suggests that disruption of P-bodies does not necessarily affect siRNA-mediated regulation (Chu and Rana, 2006). Therefore, concentration of miRNAs and their targets in P-bodies could be a consequence rather than a prerequisite of miRNA/siRNA-mediated gene regulation. Taken together, regulation of gene expression via small RNAs and sequestration to P-bodies, and the interplay between mRNA translation and decay adds further complexity to posttranscriptional control. As 30% of human genes are potential miRNA targets (Lewis *et al.*, 2005), it is entirely possible that miRNAs exert their functions in a combinatorial way: a given mRNA could be regulated by several miRNAs, and a given miRNA could target several mRNAs. Clearly, further research will be needed to elucidate the molecular events behind these regulatory mechanisms.

4.4. Translational regulation through alternative transcripts

As pointed out above, translational control is by no means independent of other layers of gene regulation, and virtually every step upstream of translation can influence the translational efficiency of a given mRNA (Fig. 5.1). Here, we will provide examples of recent work that describes how changes in transcript structure can ultimately effect translation.

In the fission yeast *Schizosaccharomyces pombe*, the trancription factor Sre1p, an ortholog of the mammalian sterol regulatory element binding protein (SREBP), is essential for anaerobic growth and activates transcription under low-oxygen conditions. However, the general transcriptional activation via Sre1p does not necessarily include an upregulation of protein levels: *tco1*, a gene potentially involved in oxygen-regulated lipid transport and a target of Sre1p, is downregulated at the level of translation under low-oxygen conditions (Sehgal *et al.*, 2008). This downregulation is paradoxically mediated by an upregulation of transcription: under low-oxygen

conditions, Sre1p directs transcription of *tco1* from an alternative promoter, resulting in a transcript with an extended 5′ UTR compared to the transcript under normal oxygen levels. This longer transcript forms a stable structure in its 5′ UTR, explaining the downregulation at the level of translation (Sehgal *et al.*, 2008).

Another study shows that changes in the 5′ transcript structures can also induce translation: Law *et al.* (2005) examined a population of mRNAs that are only weakly translated in rapidly growing budding yeast cells. These weakly (or ''undertranslated'') mRNAs were identified based on data from genomic studies, which combine sucrose-gradient centrifugation with global measurements of transcripts using microarray technology (Section 5.1). Gene Ontology categories such as responses to stress and external stimuli were enriched within the undertranslated transcripts, and 17 transcripts chosen for detailed study showed indeed an increase in translation in response to the corresponding stimulus such as nitrogen starvation, pheromone response, or osmotic stress (Law *et al.*, 2005). Interestingly, the majority of these transcripts also showed an altered 5′ structure in response to the stimulus, which again illustrates the interconnectivity between regulation at the level of transcription and translation. The authors speculate that the altered transcript structure arises through the use of alternative promotors and that this mechanism of translational control allows low-level transcription and maintenance of open chromatin structures while avoiding protein production of the corresponding gene (Law *et al.*, 2005).

5. Global Approaches to Identify Targets of Posttranscriptional Gene Regulation

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

As gene expression is often regulated at posttranscriptional levels, it is important to also gain an understanding of these regulatory processes and their targets on a genome-wide scale. In the same way, that DNA and its interactions with transcription factors and chromatin modifiers is integral to transcriptional regulation, mRNA and its association with RBPs is crucial for posttranscriptional gene regulation. Consequently, recent work of many groups has focussed on large-scale analyses of mRNA–protein interactions

and mRNA dynamics. Many of these studies employ microarray-based approaches to unravel a variety of processes such as (1) the global association of mRNAs with specific RBPs, (2) mRNA stability, and (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 posttranscriptional regulatory mechanisms, and building on this knowledge can in turn be useful to examine the underlying molecular mechanisms of the regulatory processes. Here, some of these techniques and resulting findings will be introduced.

5.1. Translational profiling

Translational efficiency can be measured on a genome-wide scale by assessing the number of ribosomes that are bound to a given mRNA. This is achieved by combining the traditional method of polysome profiling with microarray technology, referred to as translational profiling (Fig. 5.10):

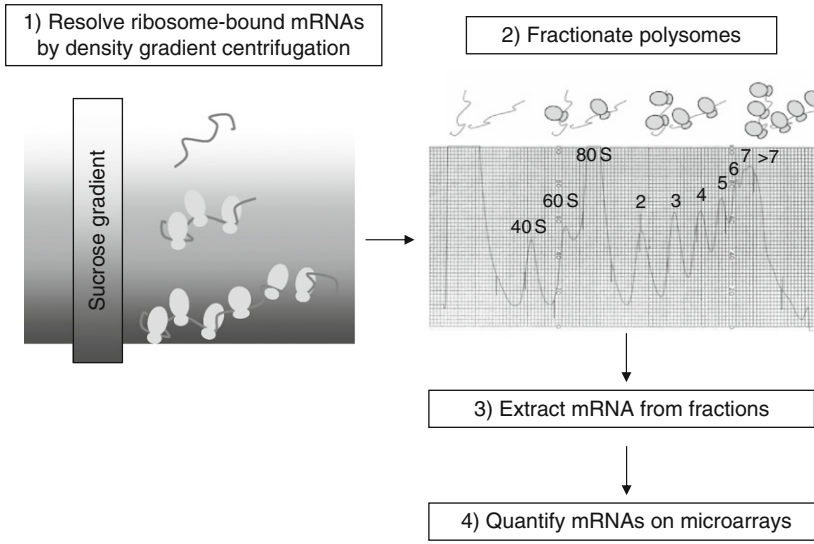

Figure 5.10 Genome-wide measurement of translation is achieved by combining polysome profiling with microarray technology, referred to as translational profiling. mRNAs are resolved on a sucrose gradient by ultracentrifugation according to their density, which reflects the number of associated ribosomes. The gradient is fractionated and a polysome profile is obtained by measuring RNA abundance. From the light to the heavy fractions: free mRNAs, 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 the different fractions are then extracted and quantified using microarrays.

Usually, cells are treated with the elongation-inhibitor cycloheximide, which "traps" ribosomes on the mRNAs they are translating. Cellular lysates are then resolved according to their density on a sucrose gradient by ultracentrifugation. As the ribosome is a large macromolecular complex with a molecular mass above three megadalton (Taylor *et al.*, 2007), the density of the mRNA–ribosome particles is determined by the number of ribosomes bound to mRNAs. The sucrose gradient is then fractionated and a polysome profile is obtained by measuring RNA abundance (Fig. 5.10; right panel). mRNAs from the different fractions can then be extracted and globally quantified using microarrays. In most studies that applied this approach to study translational regulation, the pool of mRNAs associated with polysomes was compared to the pool of untranslated (or poorly translated) mRNAs or total mRNA preparations to define translationally regulated transcripts (Bachand *et al.*, 2006; Bushell *et al.*, 2006; Dinkova *et al.*, 2005; Iguchi *et al.*, 2006; Johannes *et al.*, 1999; Kash *et al.*, 2002; Kuhn *et al.*, 2001; Qin and Sarnow, 2004; Rajasekhar *et al.*, 2003; Spence *et al.*, 2006; Thomas and Johannes, 2007). Other studies used more than 10 fractions spaced along the polysome profile, which were all probed to microarrays to obtain higher-resolution data of ribosome association for mRNAs (Arava *et al.*, 2003; Lackner *et al.*, 2007; MacKay *et al.*, 2004; Preiss *et al.*, 2003; Qin *et al.*, 2007).

Using translational profiling, the effect on global and mRNA-specific translational regulation was examined in a variety of conditions. Examples are the exposure of cells to environmental stress such as hypoxia, treatment with rapamycin, heat shock, or change in carbon source (Grolleau *et al.*, 2002; Kuhn *et al.*, 2001; Preiss *et al.*, 2003; Thomas and Johannes, 2007), the translational regulation during the mitotic cell cycle, meiosis, or during recovery from cell cycle arrest (Iguchi *et al.*, 2006; Qin and Sarnow, 2004; Serikawa *et al.*, 2003), the dependence of mRNAs on specific translation initiation factors (Dinkova *et al.*, 2005; Johannes *et al.*, 1999), and 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): they examined the requirement for cap-dependent translation initiation by analyzing the association of mRNAs with polysomes in poliovirus-infected cells with reduced eIF4G concentrations. Most of the examined mRNAs showed the expected downregulation in translation, whereas a small percentage remained associated with polysomes or even showed increased polysome association. These mRNAs are probably translated via IRES-mediated translation initiation, and include 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 knockout 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 egg laying defects. Using translational profiling, several mRNAs could be identified that showed changes in their polysomal association without corresponding alteration in total mRNA levels. Interestingly, these mRNAs were 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) measured the translational response in budding yeast cells to the transfer from a fermentable (glucose) to a nonfermentable (glycerol) carbon source. This shift resulted in a global downregulation of translation. mRNAs encoding ribosomal proteins were strongly downregulated 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 showed increased association with polysomes, and most of these mRNAs also showed increased abundances in their total mRNA levels. A similar connection between changes in total mRNA levels and polysome association was described in a study that examined translational regulation in response to treatment with rapamycin and heat shock (Preiss *et al.*, 2003). mRNAs that showed increased abundance in response to the treatment often showed increased translational efficiency, and a similar correlation was evident for mRNAs with decreased abundance. A similar relationship between changes in total mRNA levels and translational efficiency was observed in response to treatment with mating pheromone in budding yeast (MacKay *et al.*, 2004). This coordination between changes in transcript levels and translation was termed "potentiation" (Preiss *et al.*, 2003). Further studies will be required to determine whether potentiation happens through coordinated yet independent regulation of transcription and translation or whether it is a consequence of favored translation of mRNAs from *de novo* transcription over aged transcripts. For example, *de novo* transcription could influence mRNP composition or could simply provide "intact" messages with long poly(A) tails, which are then more efficiently translated (Lackner *et al.*, 2007; Beilharz and Preiss, 2007).

Translational profiling was recently applied to study translational changes in response to hypoxia (Thomas and Johannes, 2007). When PC-3 cells were grown under hypoxic conditions, translation was globally downregulated, concomitant with mammalian target of rapamycin (mTOR) inactivation and phosphorylation of eIF2α (Section 3.4.1), and mRNAs encoding ribosomal proteins were found to be most sensitive to the global translational downregulation. Again, several mRNAs were identified that escaped the translational downregulation and still were associated with polysomal fractions under hypoxic conditions (Thomas and Johannes, 2007). The authors suggested 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 downregulated in this condition. These sets of mRNAs could only be identified using genome-wide, unbiased approaches, as their involvement in certain biological processes is unexpected and could not have been anticipated by more hypothesis-driven approaches.

Arava *et al.* (2003) and Lackner *et al.* (2007) provide comprehensive views of translational efficiency in rapidly growing budding and fission yeast cells, respectively. mRNA extracted from 12–14 fractions across the polysomal profile were analyzed on microarrays, and the translation profiles were used to determine the average number of ribosomes associated with a given mRNA on a genome-wide scale. This approach revealed several interesting findings. For most mRNAs, 70–80% of the transcripts were associated with polysomal fractions. Among the few mRNAs not associated with polysomal fractions, several were known to be translationally regulated. Furthermore, ribosomes were 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. Moreover, integration of high-resolution translational profiling data with other global data sets revealed that translational efficiency is aligned with mRNA half-lives, transcriptional efficiency, mRNA stability, and poly(A) tail lengths in both budding and fission yeasts (Beilharz and Preiss, 2007; Lackner *et al.*, 2007), highlighting a substantial coordination between different layers of gene regulation. Qin *et al.* (2007) used a high-resolution translational profiling approach to study the extent of translational control during early *Drosophila* embryogenesis. Accordingly, mRNAs that were known to be spatially repressed by translational mechanisms in the early fly embryo had only a small portion of their transcripts associated with polysomal fractions.

5.2. Proteomic approaches to study translational regulation

Currently, translational profiling is the method of choice to examine translational regulation on a genome-wide scale. 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. Two studies combined the measurement of absolute protein levels using proteomics and total mRNA levels using microarrays (Lu *et al.*, 2007; Newman *et al.*, 2006). Newman *et al.* (2006) exploited a collection of yeast strains, where 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 and mRNA levels were measured using microarrays. Lu *et al.* (2007) used a mass spectrometry approach together with a novel algorithm to make absolute measurements of protein levels. Both studies concluded that changes in protein levels were largely due to changes in the abundance of the corresponding mRNAs, but certain mRNAs were identified for which changes 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 are disadvantages to these proteomic approaches: in the case of the GFP-tagged strain collection, the tag could interfere with translational regulation via sequence elements in the UTR or with protein targeting and turnover, and mass spectrometry approaches do not yet manage to identify every expressed protein in the cell and are biased toward abundant proteins. However, as these techniques improve, they will become increasingly important for the genome-wide study of translational control.

5.3. mRNA turnover

mRNA turnover is regulated by multiple mechanisms (Parker and Song, 2004; Wilusz *et al.*, 2001). Deadenylation of transcripts 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 are degraded via endonucleolytic mechanisms, for example, via the RNAi machinery (Tomari and Zamore, 2005). Furthermore, NMD serves as a 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 nonstop 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 different times after the transcription block, mRNAs are isolated and probed on microarrays (Fig. 5.11; Mata *et al.*, 2005). Using this approach, genome-wide mRNA stability has been determined in various organisms such as yeast (Grigull *et al.*, 2004; Wang *et al.*, 2002), 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 (Grigull *et al.*, 2004; Wang *et al.*, 2002). mRNAs encoding transcription factors,

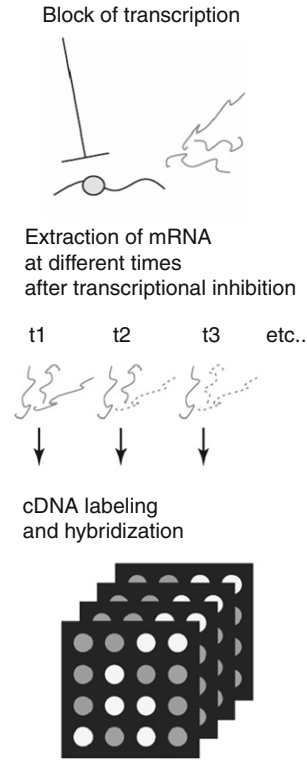

Figure 5.11 Genome-wide measurements of mRNA half-lives. Transcription is blocked using drugs or mutants of RNA polymerase II. At different times after the transcriptional block, mRNAs are isolated and quantified using DNA microarrays to deduce mRNA half-lives. Reproduced with permission from Mata *et al.* (2005).

parts of the transcriptional machinery, proteins involved in ribosome biogenesis and the translation machinery generally show short half-lives, whereas mRNAs encoding central metabolism proteins show longer half-lives (Grigull *et al.*, 2004; McCarroll *et al.*, 2004; Wang *et al.*, 2002; Yang *et al.*, 2003). Short half-lives for mRNAs involved in transcription or translation could be advantageous for rapid regulation of these central processes in response to changing environmental conditions. Note, however, that the transcriptional shutdown itself, and the use of drugs or RNA polymerase II mutants in these experiments could trigger cellular stress responses (Grigull *et al.*, 2004). Thus, the short half-lives of mRNAs involved in transcription and translation could reflect a response to stress, and half-lives for the same mRNAs may actually be longer in unstressed cells at steady state.

For many mRNAs, short half-lives correlate with the presence of ARE elements in their 3′ UTRs, but not all rapidly decaying mRNAs have ARE elements (Raghavan *et al.*, 2002; Yang *et al.*, 2003). No obvious correlation between mRNA stability and mRNA features such as ORF length, transcriptional efficiency, or mRNA abundance seems to exist (Wang *et al.*, 2002), but mRNA half-lives are globally aligned with poly(A) tail lengths and translational efficiency in fission yeasts (Lackner *et al.*, 2007).

In a recent study, Shock *et al.* (2007) determined the global decay rates of mRNAs at various stages during the intraerythrocytic development cycle of *Plasmodium falciparum*, the pathogen causing human malaria. Interestingly, as the parasite passes through the examined developmental stages, decay rates decrease globally for essentially all examined mRNAs, which suggests that posttranscriptional control is the main mechanism of gene regulation in this pathogen. Such genome-wide regulation of mRNA turnover, however, has not 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). In addition to its known involvement in mRNA quality control, a new aspect of this pathway was detected in these global studies: hundreds of mRNAs accumulated as a consequence of NMD inactivation, 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, which is inhibited by amino acid depletion, 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 could increase the abundance of these transcripts to turn on amino acid biosynthesis (Mendell *et al.*, 2004). Thus, these genome-wide studies reveal 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 and Weissman (2006) showed that the inositol-requiring enzyme 1 (IRE-1), which functions in activating the UPR due to accumulation of misfolded proteins in the ER, is involved in the specific and immediate degradation of a subset of mRNAs during the UPR. IRE-1 plays a role 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 was identified, whose repression was dependent on IRE-1 but not on XBP-1, and IRE-1 mediates the degradation of these mRNAs (Hollien and Weissman, 2006).

5.4. RNA-binding proteins and their target RNAs

Central to virtually all aspects of posttranscriptional gene regulation, from mRNA processing and export to mRNA decay and translation, is the interplay between mRNAs and RBPs. Some RBPs bind most of the transcripts in the cell, whereas others bind only a small set of specific mRNAs, exerting a specialized control to those mRNAs (Hieronymus and Silver, 2004; Keene, 2007; Mata *et al.*, 2005; Moore, 2005). Furthermore, RBPs may 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 regulation via RBPs has come from the genome-wide identification of their targets via ''RBP Immunoprecipitation followed by chip analysis'' (RIP-chip, Fig. 5.12): 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 precipitate, purified, labeled, and hybridized onto microarrays. In one of the first studies to employ this technology, Tenenbaum *et al.* (2000) used cDNA-filter arrays containing ~600 murine genes to identify mRNAs associated with the RBPs HuB, PABP, and eIF4E, which are all 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 associated with only few mRNAs. Furthermore, the pattern of association of mRNAs with HuB was 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 for five members of the Pumilio family of RBPs in budding yeast (Puf1p-Puf5p). Dozens to hundreds of mRNAs were associated with each of the five Puf proteins, and the subsets of mRNAs bound to each of RBP were enriched for common functional groups or subcellular localizations. Puf1p and Puf2p associated with mRNAs encoding membrane-associated proteins; Puf3p nearly exclusively bound mRNAs that encode mitochondrial proteins; Puf4p associated with nucleolar ribosomal RNA-processing factors; and Puf5p associated with mRNAs encoding chromatin modifiers and components of the spindle pole body. Furthermore, distinct sequence motifs were enriched in the 3′ UTR of mRNAs bound by Puf3p, Puf4p, and Puf5p (Gerber *et al.*, 2004). A related motif was identified in mRNAs that coimmunoprecipitate with the *Drosophila* Pumilio protein (Gerber *et al.*, 2006). Many of the mRNAs associated with Pumilio in *Drosophila* also encode functionally related proteins, but these mRNAs are not related to the mRNAs associated with Puf3p in budding yeast (Gerber *et al.*, 2006).

RIP-chip approaches were also used to identify global targets of RBPs involved at other levels of posttranscriptional gene regulation such as

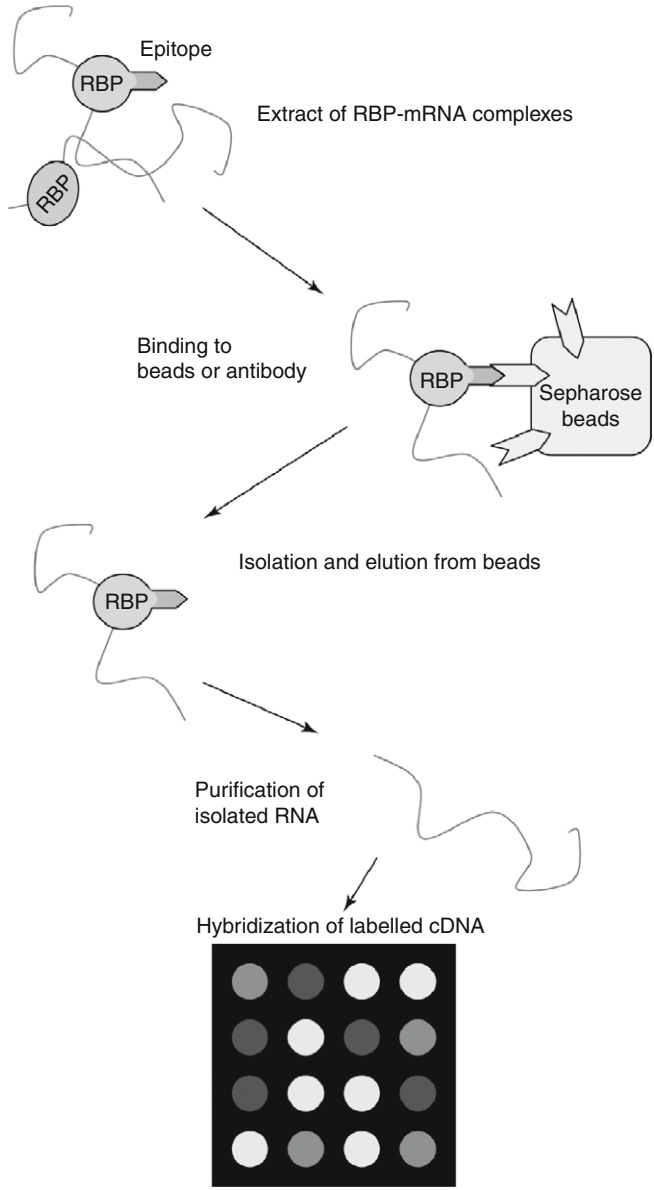

Figure 5.12 Genome-wide determination of mRNA targets of RNA-binding proteins (RBPs) by "RBP Immunoprecipitation followed by chip analysis" (RIP-chip). RBPs are immunopurified together with their associated mRNAs, followed by mRNA isolation, labeling, and hybridization onto microarrays. Reproduced with permission from Mata *et al.* (2005).

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, whereas mRNAs targeted by a certain group of RBPs often share functional specificity. Furthermore, RIP-chip studies also provided clues to unexpected functions of RBPs. An example is the identification of previously unknown mRNAs associated with the yeast La protein (Lhp1p). Lhp1p is involved in the biogenesis of noncoding RNAs transcribed by RNA polymerase III, and thus many noncoding 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*, which encodes a transcription factor required for the UPR. Follow-up experiments indicate that Lhp1p plays a role in the translational regulation of *HAC1* mRNA (Inada and Guthrie, 2004, 387).

Recently, RIP-chip approaches were also employed to measure translation on a global scale. In this case, the RBP is an epitope-tagged ribosomal subunit, and polyribosomal complexes corresponding to ribosome-bound mRNAs are immunopurified. 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 to isolate polyribosomes in *Arabidopsis*. The authors also probed the mRNA from these immunopurified complexes with microarrays 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 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 complementary tool to study translational regulation in varying conditions or different cellular subtypes, and also to identify substrates of potential ribosomal subtypes containing different protein isoforms (Section 5.1).

6. Concluding Remarks

Translation is a complex process mediated by large ribonucleoprotein machines, the ribosomes. Maintaining maximal translational output is a major effort and energetically very costly. Cells therefore globally tune the

translation of transcripts to the physiological requirements dictated by environmental or intrinsic conditions. Besides this global translational tuning, cells also use a great variety of transcript-specific mechanisms to adjust the production of selected proteins to current needs. The elegant mechanistic studies reviewed here provide deep insights into several sophisticated processes of translational regulation, while the powerful genome-wide analyses provide overviews of the targets and global strategies for translational control, thus complementing more traditional studies.

Although much has been learnt about translational control, this level of gene regulation is still relatively poorly understood compared to transcriptional regulation. More work is required to obtain a full picture on the extent and role of translational regulation in different organisms and in different conditions. Recent data on translational and other posttranscriptional regulation via small RNAs further add to the complex picture of gene expression control. The great abundance and diversity of noncoding RNAs emerging from current studies raises the possibility that more of these RNAs play important roles in translational regulation. Proteins are the readout of translational control, and future progress in proteomic approaches should further advance our understanding of translational and posttranslational regulation. Cells integrate multiple regulatory levels to fine-tune gene expression, and it is not well understood how the different processes of translational control are coordinated with each other and with additional levels of gene regulation. An ultimate goal is to obtain a systems-level understanding of multilevel gene expression programs to help predict the contribution of translational regulation for different genes and for different biological processes. It seems certain that scientists working in this fascinating field will not become bored any time soon.

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