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TOPICAL REVIEW

Mechanisms governing the control of mRNA translation

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Abstract
The translation of cellular mRNA to protein is a tightly controlled process often deregulated in diseases such as cancer. Furthering our understanding of mRNA structural elements and the intracellular proteins and signaling pathways that affect protein expression is crucial in the development of new therapies. In this review, we discuss the current state-of-the-art of detecting and determining the role of mRNA sequence elements in regulating the initiation of mRNA translation and the therapeutic strategies that exploit this knowledge to treat disease.

Introduction

The central dogma of molecular biology of the cell describes the forward flow of information from DNA to RNA to protein, wherein the instructions (nucleic acid sequences) are used by a cell to produce a particular protein with a particular amino acid sequence (Crick 1970). In order for eukaryotic cells and tissues to function properly, each cellular protein must be made available in the correct abundance and location at the proper time. This is a daunting task given the plethora of genes present in the cell, each potentially yielding mRNAs capable of competing for the translational activity of ribosomes to generate a specific protein. The ability of a cell to overcome this hurdle is achieved in large part through the regulation of mRNA translation. While transcription factor-mediated regulation of gene expression controls the production of cellular mRNAs, the regulation of mRNA translation provides a more direct and rapid means of regulating protein expression. Multiple factors govern the translation of mRNA at each step in between when it is initially transcribed and eventually degraded. Translational control is the study of the mechanisms determining which mRNAs are translated into protein under a particular set of conditions, including extracellular stimuli, cell/tissue type and subcellular localization. This capability allows for the precise spatial and temporal fine-tuning of protein levels to permit normal physiological function. At the most basic level mRNA translation can be thought of as the accumulation of cellular biomass that parallels the doubling of DNA content necessary before cytokinesis can ensue. More profoundly, however, one must remember that each protein may fulfill a precise cellular function that may be necessary for a cellular process (e.g. growth, proliferation, survival). As such, deregulated mRNA translation can often be found in disease, including cancer, which exhibits seemingly unregulated growth and proliferation independent of the cues (e.g. contact inhibition) that should suppress the mRNA translation required for such growth. Given the countless, intricate mechanisms controlling global mRNA translation and that of specific mRNAs or functionally related mRNA groups, pharmaceutical targeting of translation holds great therapeutic potential. In this review we describe and discuss the post-transcriptional mechanisms that determine whether a particular mRNA will be efficiently translated into protein with a strong emphasis on the initiation of mRNA translation, its regulation and the development of cancer therapies that exploit our knowledge of this regulation.

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Post-transcriptional regulation of gene expression

In eukaryotes, multiple variables affect the creation of the functional output of a gene: the protein (Lackner *et al* 2007). Clearly, mRNA synthesis, as governed by transcription factor activity and epigenetic factors, is an important determinant, but the regulation of mRNA translation is perhaps equally important, as a single mRNA being translated efficiently by multiple ribosomes can soon out-compete high copy, poorly translating mRNAs (Axelsen and Sneppen 2004). Evidence of this can be seen given the advent of genome-wide microarray technology that has allowed rapid profiling of eukaryotic cells giving an overview of which mRNAs are being highly expressed in, for example, diseased versus healthy tissues. The goal of these studies is often to identify an aberrantly expressed gene product responsible for phenotype of interest. Along these lines, it has been theorized that a molecular diagnosis of the gene product driving a disease will be useful in determining the best course of personalized treatment (Weinstein 2002). Comparative genomic versus proteomic studies have indicated, however, that mRNA expression is only a poor predictor of protein levels in eukaryotes (Gygi *et al* 1999, Ideker *et al* 2001). As a result, post-transcriptional mechanisms controlling gene expression have taken on much importance in understanding the control of the abundance of cellular proteins.

Following the production of pre-mRNAs in the nucleus, multiple events must occur before the translation of the mature mRNA can yield functional proteins (reviewed in Mansfield and Keene (2009)). Initially, most pre-mRNAs must be subjected to splicing to remove introns. Clearly, RNA-binding protein-mediated alternative splicing represents a potential regulatory event. Addition of the poly(A) tail is crucially important for subsequent translation efficiency, as this will allow binding of poly(A) binding protein (PABP). The resulting mRNA must then be exported from the nucleus to the cytoplasm, where translation occurs. In the cytoplasm, mRNA abundance is regulated by stability, a variable that has gained much attention as of late due to the interest in small RNAs (e.g. siRNAs and microRNAs), which target mRNAs for destruction. Finally, the primary focus of this review, the control of mRNA translation itself, is key in determining the cellular abundance of a given protein.

Phases of translation

The translation of an mRNA to protein can be divided into three sub-processes (initiation, elongation and termination), each of which requires a particular set of conditions and factors. Figure 1 represents an overview of intracellular signaling events governing mRNA translation initiation and should help readers visualize the concepts being presented in this review. The initiation phase is the rate-limiting step, requires the function of multiple eukaryotic translation initiation factors (eIFs) and starts with the binding of eIF4F to the 5′ cap of the mRNA (Pestova *et al* 1996, Sonenberg 2008). eIF4F is a complex composed of three proteins (eIF4E, eIF4G and eIF4A), each with specific roles crucial for efficient translation of mRNA. eIF4E, the mRNA 5′ cap binding protein, and eIF4A, an ATP-dependent RNA helicase, bind the large scaffolding protein, eIF4G, which contains binding domains for mRNA, PABP and eIF3 (Gingras *et al* 1999, Tahara *et al* 1981). These interactions serve to stabilize and circularize the eIF4F:mRNA complex and to recruit the small ribosomal subunit (Gingras *et al* 1999, Wells *et al* 1998). The closed-loop model proposes that eIF4G's ability to tether the 5′ mRNA cap (via eIF4E) to the poly(A) tail (via PABP) greatly increases translation efficiency (reviewed in Derry *et al* (2006)). Once recruited, the small, 40S ribosomal subunit scans the mRNA until it reaches the start codon AUG or a cognate triplet in a favorable sequence and recruits the large, 60S ribosomal subunit to form the translationally competent 80S ribosome (Pestova *et al* 2007). The terms 40S, 60S and 80S refer to the size-based sedimentation characteristics (Svedberg unit) of these ribonucleoprotein complexes upon ultracentrifugation (figure 2).

Importance of mRNA secondary structure: G-C content

Both ribosome recruitment and scanning are greatly impaired by complex secondary structure in the mRNA 5′-untranslated region (UTR), explaining why some mRNAs are more highly reliant on the helicase activity of eIF4A. As a general rule, the thermal stability (low ΔG) of hairpin-like structures upstream of the start codon inhibits mRNA translation (reviewed in Merrick (1990)). As such, 5′UTRs harboring a high degree of secondary structure (e.g. with high G–C content) often are highly reliant upon eIF4F for translational activity (Svitkin *et al* 2001). Shown in figure 3 are secondary structure/thermal stability predictions (mfold) for the 5′UTRs of three mRNAs known to be translated in a highly eIF4F-dependent manner: VEGF, ODC and cyclin D1 (reviewed in De Benedetti and Graff (2004)). While the 5′UTR of the human GAPDH mRNA, which is efficiently translated even when eIF4F activity is low, shows minimal thermodynamically stable secondary structure, those of cyclin D1, ODC and VEGF each possess long, complex secondary structures. Although these selected examples have been studied extensively as translationally regulated mRNAs, they likely represent the tip of the iceberg. A recent analysis of more than 17 000 human 5′UTRs in the RefSeq database suggests that approximately two thirds may be structured with a predicted $\Delta G < -40$ kcal mol^{-1} (Parsyan *et al* 2009). Interestingly, the 3′UTR, too, regulates the eIF4E dependence, likely through a variety of mechanisms, which may include microRNA binding, PABP-dependent mRNA circularization and/or nucleo-cytoplasmic mRNA export (Fan *et al* 2009, Rousseau *et al* 1996, Santhanam *et al* 2009).

mRNA sequence elements

Independent of thermal stability, multiple sequence elements present within the 5′ and 3′ UTRs of mRNAs have been shown to greatly influence translation efficiency, allowing coordinate expression in response to environmental conditions and/or

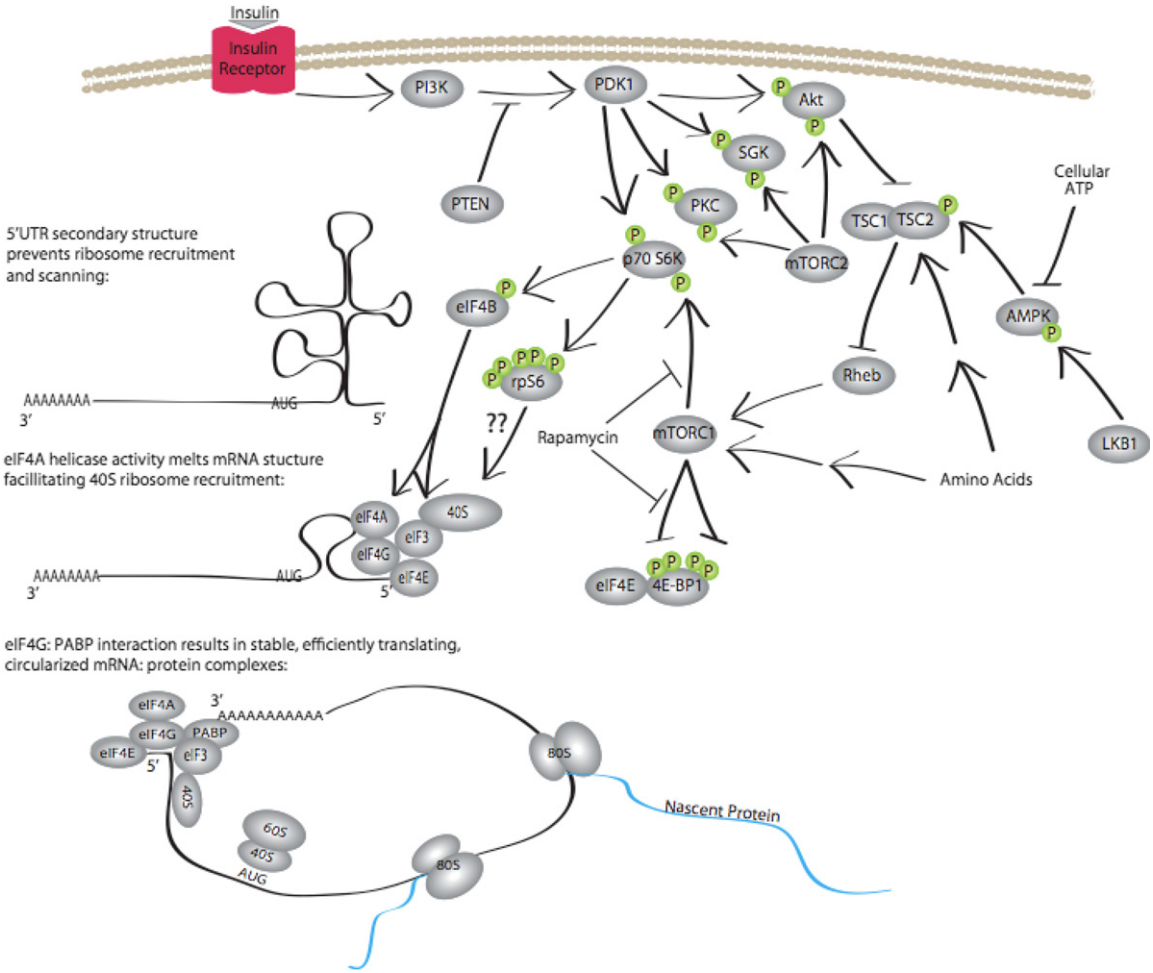

Figure 1. Overview of intracellular regulatory mechanisms controlling mRNA translation. mTOR complex 1 (mTORC1) receives signals reflecting extracellular nutrient (e.g. insulin and growth factors) availability, amino acid abundance and cellular energy levels (ATP). These pro-growth and proliferation cues result in an increase in the translation of translationally repressed mRNAs, via mTOR's ability to phosphorylate 4E-BP1 (freeing eIF4E) and p70 S6 kinase (encouraging 40S small ribosomal subunit recruitment).

RNA binding proteins (reviewed in Keene and Tenenbaum (2002)). Clearly, it would be naïve to attempt to determine the functional importance of an mRNA element based solely on two-dimensional structural predictions, as three-dimensional structures give significantly more insight (for example, Laing and Hall (1996)). Still, empirical data are crucial in establishing the functional importance of mRNA sequence elements, and deletion/mutation analyses are required to determine the impact of a given UTR on translation. A number of such elements have been identified, each with its own putative structure and mode of regulation (reviewed in Keene and Tenenbaum (2002)). mRNAs carrying a 5′ terminal oligopyrimidine tract (5′TOP), for example, are translationally repressed when growth conditions are poor and encode ribosomal proteins, poly(A) binding protein and translation elongation factors (Avni *et al* 1997, Meyuhas 2000). These C–U rich elements at the extreme 5′ end of mRNAs appear to contribute very little to thermal stability/secondary structure, suggesting that other factors make TOP mRNAs repressive to translation.

The precise mechanism by which the translational apparatus selectively up-regulates TOP mRNAs in response to growth signals remains to be determined. It is known, however, that the serine/threonine kinase, named mammalian target of rapamycin (mTOR), promotes TOP mRNA translation (Holland *et al* 2004, Patursky-Polischuk *et al* 2009). The ability of mTOR to integrate signals sensing growth factor, amino acid and ATP availability to regulate catabolic versus anabolic processes has been well studied using the mTOR inhibitor, rapamycin (reviewed in Furic *et al* (2009), Sabatini (2006) and Wullschleger *et al* (2006)). Briefly, when conditions are favorable for growth and proliferation (i.e. sufficient nutrient supply) mTOR modifies its *in vivo* substrates p70 S6 kinase and 4E-BP1, by reversible phosphorylation of specific amino acid residues. p70 S6 kinase activated by mTOR is then able to phosphorylate the small ribosomal subunit protein S6 (rpS6). Similarly, mTOR-dependent phosphorylation of 4E-BP1 at multiple sites (Thr37, Thr46, Ser65 and Thr70) leads to the release of the functional mRNA 5′cap-binding protein eIF4E (Beretta *et al* 1996, Gingras *et al* 2001). As discussed above,

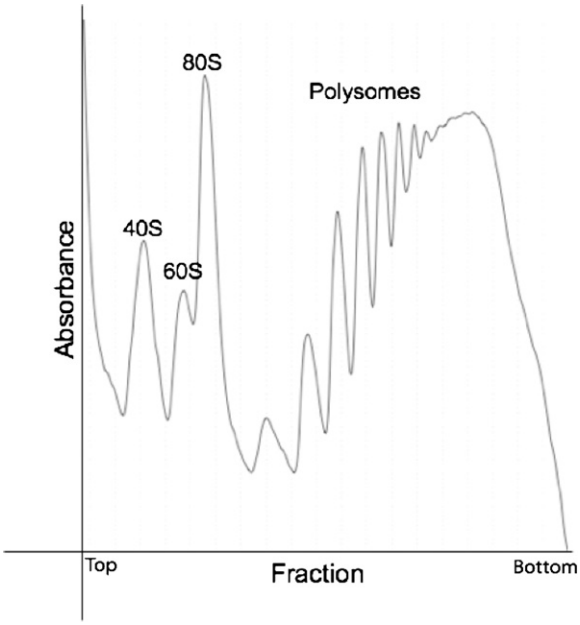

Figure 2. Typical profile drawn during the ultracentrifuge-based sedimentation of ribonucleoprotein complexes. Actively translating mRNAs associated with multiple ribosomes (polysomes) sediment more deeply (right) in a sucrose gradient and can be collected in later fractions.

recognition of the 5′ cap of mRNAs by eIF4E and recruitment of the other eIF4F components, eIF4G and eIF4A, are crucial steps in the initiation of cap-dependent translation. For efficient, stable mRNA cap binding and initiation of mRNA translation, eIF4E must bind eIF4G, an interaction that is subject to competitive inhibition by the eIF4E-binding proteins (4E-BPs) (Pause *et al* 1994, Yanagiya *et al* 2009). In mammals three 4E-BPs exist (4E-BP1, 4E-BP2 and 4E-BP3), each containing the canonical eIF4G binding sequence for eIF4E: Tyr-X-X-X-X-Leu-ϕ, where ϕ represents a hydrophobic amino acid (reviewed in Topisirovic and Borden (2005)), allowing hypophosphorylated 4E-BPs to function as competitive inhibitors of eIF4F formation. This molecular mechanism then links growth signaling through mTOR to increase cap-dependent translation of mRNAs that are dependent on eIF4F.

Originally, it was thought that mTOR-dependent phosphorylation of p70 S6 kinase and rpS6 regulated TOP mRNA translation; however, more recent studies, including those using a genetically modified 'knock-in' mouse with rpS6 phosphorylation sites mutated, have ruled out a role of p70 S6 kinase-mediated phosphorylation of rpS6 in regulating TOP mRNA translation (Ruvinsky *et al* 2005, Stolovich *et al* 2002, Tang *et al* 2001). While all nuclear transcribed mRNAs are capped, certain mRNAs are more dependent on eIF4E than others for translation. Recent near genome-wide studies have attempted to elucidate which mRNA targets are translated in an eIF4E-dependent manner (Larsson *et al* 2006, Mamane *et al* 2007). These studies combine microarray technology with ultracentrifuge-based fractionation and collection of polysome-associated mRNA to estimate mRNA translation efficiency. In addition to finding

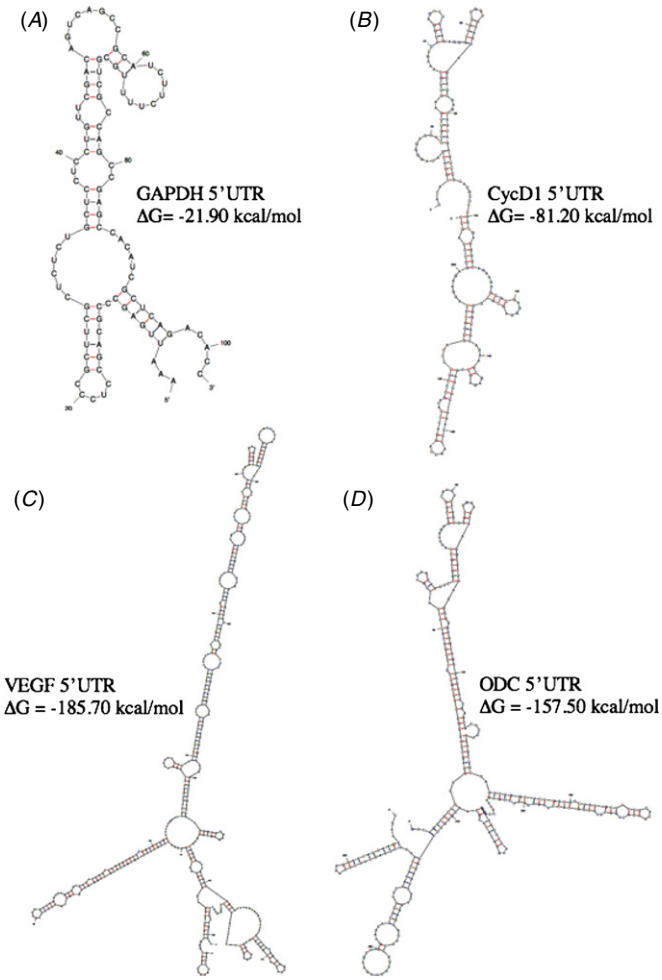

Figure 3. Secondary structure/thermal stability predictions (mfold) of the 5′UTRs of commonly studied targets of eIF4F-mediated translation, cyclin D1 (*B*), VEGF (*C*) and ODC (*D*), suggest a high degree of G–C base pairing relative to the relatively unstable 5′UTR of GAPDH (*A*).

specific mRNAs translationally up-regulated upon eIF4E over-expression, such studies have the potential to identify novel sequence elements conferring eIF4E translational dependence. Interestingly, a number of ribosomal proteins are translated in an eIF4E-dependent manner using this approach, raising the question of whether mTOR signaling to 4E-BPs is at least partially responsible for the regulation of TOP mRNA translation in response to growth signals.

Deregulated mRNA translation in cancer

Cancer is often explained by the uncoupling of cell growth and proliferation from the intra- and extra-cellular cues that should keep it check. Upstream signaling proteins (PTEN, LKB1 and TSC2) that normally suppress mTOR signaling under nutrient poor conditions (figure 1) are often lost in cancer yielding unrestrained mTOR-dependent growth and mRNA translation (reviewed in Faivre *et al* (2006)). As such, it has been suggested that cancers driven by hyperactive mTOR signaling should be hypersensitive to treatment with mTOR inhibitors, and to this end two rapamycin analogs

have acquired FDA approval as anti-cancer agents (Abraham and Eng 2008, Choo and Blenis 2006). While perhaps all eukaryotic cells and tissues require mTOR activity for growth and proliferation, recent work has shown that non-proliferating cells can tolerate mTOR loss, suggesting that novel mTORC1/mTORC2 (see below) inhibitors might be used to safely treat humans (Nardella *et al* 2009).

Whether the efficacy of mTOR inhibitors in blocking cancer growth is mediated by 4E-BPs or other downstream targets remains unclear; however, much data supports the therapeutic potential of blocking eIF4E activity. eIF4E over-expression has long been known to promote tumorigenic transformation (Lazaris-Karatzas *et al* 1990), and expression of 4E-BP1 mutants lacking the inhibitory mTOR phosphorylation sites blocks tumorigenesis (Avdulov *et al* 2004). RNA interference-based knockdown of eIF4E shows promise in the clinical treatment of tumors (Graff *et al* 2008). Similarly, cell permeable peptides or small molecules that mimic the inhibitory binding of 4E-BP1 to eIF4E induce rapid apoptosis (Herbert *et al* 2000, Moerke *et al* 2007). Finally, recent work has demonstrated the importance of mTOR-dependent 4E-BP1 phosphorylation in tumorigenesis using a lymphoma model (Hsieh *et al* 2010). Taken together, these studies demonstrate that targeting eIF4E-dependent translation and subsequent growth of tumors with hyperactive mTOR signaling is a feasible therapeutic strategy. Similarly, each molecular mechanism regulating mRNA translation may provide a novel therapeutic approach. The flow of information from the outside of the cell to each specific mRNA is subject to multiple points of regulation, allowing the potential for therapies targeting a single mRNA, functionally related groups of mRNAs, or large groups of mRNAs.

mTOR signaling: new roles elucidated with new inhibitors

The mTOR inhibitor rapamycin has proved to be a very specific and potent molecular tool in the elucidation of mTOR-dependent molecular events. mTOR exists in at least two distinct protein complexes (mTORC1 and mTORC2), and rapamycin primarily inhibits the mTORC1 complex (Sarbassov *et al* 2004). In order to function, rapamycin must bind both FK506-binding protein/peptidyl-prolyl *cis–trans* isomerase FKBP12 and the FKBP-rapamycin binding (FRB) domain of mTOR to function as an mTOR inhibitor (Brown *et al* 1994); however, just how rapamycin specifically inhibits mTORC1 and not mTORC2 is currently unknown. Proposed theories include (a) the mTORC2 component Rictor forming a physical barrier precluding FKBP12 rapamycin access to mTOR (Jacinto *et al* 2004, Sarbassov *et al* 2004) and (b) rapamycin preventing mTORC1 complex formation or promoting dissociation of existing complexes (Hara *et al* 2002, Kim *et al* 2002, Oshiro *et al* 2004). Future work elucidating the mechanism will likely reveal novel drug-targetable molecular events regulating mTORC1 versus mTORC2 activity.

One strategy proposed to inhibit both mTORC1 and mTORC2 activities is the use of small molecule inhibitors that compete with ATP for its binding pocket within the kinase domain of mTOR (Edinger *et al* 2003, Sabatini 2006). Multiple nonspecific phosphatidylinositol-3 kinase (PI3K) and PI3K-like kinase (PIKK) inhibitors including LY294002, wortmannin, PI-103, compound 401 and NVP-BEZ235 can efficiently block mTOR kinase domain activity due to the structural similarity between these structurally related enzymes (Ballou *et al* 2007, Fan *et al* 2006, Maira *et al* 2008). While some of these compounds were successful in demonstrating the efficacy of inhibiting mTOR in combination with specific PI3K isoforms, it remains to be determined whether their lack of specificity will prevent success in clinical trials. Recently, however, specific mTOR kinase domain inhibitors capable of blocking mTORC1 and mTORC2 activities have been described and have allowed the elucidation of rapamycin-insensitive mTORC1 activity toward 4E-BP1 phosphorylation sites Thr37 and Thr46 (Feldman *et al* 2009, Thoreen *et al* 2009). While rapamycin is efficient at blocking mTORC1-dependent phosphorylation of Ser65 and Thr70, these new inhibitors fully block 4E-BP1 phosphorylation at all four sites and should be useful in the elucidation of new 4E-BP-dependent phenomena, as full 4E-BP1 dephosphorylation, including at Thr37 and Thr46, correlates best with eIF4E binding (Livingstone *et al* 2009).

As shown in figure 1, mTORC1 phosphorylates 4E-BPs and the hydrophobic motif site (Thr389) on p70 S6 Kinase, whereas mTORC2 is the *in vivo* kinase for the hydrophobic motif site of other key AGC kinase family members: Akt, SGK and PKC (Garcia-Martinez and Alessi 2008, Hresko and Mueckler 2005, Ikenoue *et al* 2008, Sarbassov *et al* 2005). Given the importance of these rapamycin-insensitive mTOR substrates, it has been proposed that non-rapamycin analog mTOR inhibitors will more potently target mTOR-dependent tumors (Edinger *et al* 2003, Sabatini 2006). The therapeutic use of mTOR kinase inhibitors is likely to have multiple effects apart from reducing eIF4E-dependent mRNA translation. Blockage of mTORC2-dependent Akt activation, for example, should negatively impact a multitude of survival signals repressing apoptosis of cancer cells.

Summary and outlook

While mRNA transcription is, in part, responsible for a protein's cellular abundance, mRNAs are subject to multiple steps regulating their translation to a specific protein. Translational control allows spatial and temporal regulation of protein production, along with operon-like coordinate expression of groups of functionally related proteins depending on the 5′ and 3′ UTRs of the encoding mRNAs (Keene and Tenenbaum 2002). The bioinformatic, technical and genetic tools reviewed herein represent the current state-of-the-art in our ability to study mRNA translational regulation and are the basis for the development of disease therapies aimed at controlling it. The success of anti-cancer agents known to directly suppress eIF4E-dependent mRNA translation suggests that one can identify, characterize and control mRNA translation for therapeutic benefit. Future advances in the identification and grouping of mRNA elements will be a key in elucidating novel regulatory processes

controlling their translation. Clearly, nucleotide sequences and crude structural predictions will give only limited information regarding the homology of two mRNA regions, so more complex, three-dimensional, structural predictions will have to be utilized to achieve this goal of elucidating previously unidentified motifs.

Each newly identified mRNA regulatory event may be subject to its own set of regulatory mechanisms governed by, among other factors, RNA binding proteins and signaling pathways. While TOP mRNAs, for example, have been subject of significant study, the molecular mechanisms governing their translation remain unclear. Most evidence suggests a role for mTOR-dependent phosphorylation of targets, including 4E-BPs, in this process, but even the study of this well-defined group of mRNAs has demonstrated a need for improved methods in the study of translational control.

Once it is determined, however, how a regulatory event modulates translation of a group of mRNAs responsible for a particular phenomenon (e.g. growth, apoptosis, proliferation), one can use this knowledge to develop new therapies that target entire groups of functionally related proteins. The development of mTOR inhibitors as anti-cancer agents strongly supports this notion, since mTOR-dependent translation of eIF4F translational targets is necessary for the deregulated growth of cells seen in cancer. Targeting downstream processes should lead to more specific therapies, however. If the goal is to diverge from traditional chemotherapies, one would like to target a process that is specific to the diseased state. Understanding the signaling flow allows one to understand how one might devise a general therapy (by targeting all of mTOR or Akt signaling) or a very specific therapy (by targeting one downstream RNA or RNA binding protein).

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