TRANSLATIONAL CONTROL OF GENE EXPRESSION IN RAT LIVER IN
RELATION TO MAMMLIAN TARGET OF RAPAMYCIN SIGNALING

A Thesis in
Physiology
By
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Abstract

Previous research demonstrated a correlation between changes in RNA content and protein synthesis in the liver following food intake or endurance exercise. A central protein implicated in the regulation of protein synthesis and ribosome biogenesis is referred to as the mammalian target of rapamycin (mTOR). However, while the protein kinase referred to as the mTOR has been implicated in the regulation of ribosome biogenesis in the liver, inhibition of mTOR does not alter the rate of protein synthesis. The overall objective of the research presented herein was to gain insight into the translational regulation of gene expression in rat liver in response to two physiological perturbations, food intake and endurance exercise. In response to food intake, phosphorylation of two substrates of mTOR, the eukaryotic initiation factor (eIF)4E binding protein-1 4E-BP1 and the ribosomal protein (rp) S6 kinase S6K1 and its substrate rpS6, was increased within 15 min and was sustained for at least 180 min. Activation of S6K1 has been linked to upregulated translation of a subset of mRNAs. To identify translationally regulated mRNAs, polysomal (i.e. actively translated) and nonpolysomal (non-translated) fractions were isolated and subjected to microarray analysis. The mRNAs encoding 78 proteins, including 42 proteins involved in protein synthesis, exhibited increased abundance in polysomes in response to feeding. In contrast, the mRNAs encoding 50 proteins displayed decreased abundance in polysomes in response to feeding.

In another study, the effect of a single bout of treadmill running on signaling through mTOR in liver was examined in 12 h fasted rats. Phosphorylation of a
downstream target of mTOR, 4E-BP1, was decreased after exercise to values even lower than the fasting-induced basal levels. Phosphorylation of another mTOR target, S6K1, was already maximally repressed by fasting and was not further reduced by exercise, and phosphorylation of rpS6 was unaltered by endurance exercise. Neither the global rate of protein synthesis nor the proportion of the mRNA encoding β-actin associated with polysomes was changed after exercise from the basal fasting state. However, the proportion of the mRNAs containing a 5′-TOP sequence associated with polysomes was significantly lower in liver after exercise suggesting that the translation of mRNAs encoding ribosomal proteins was specifically repressed.

Activation of AMP-activated protein kinase (AMPK) occurs in response to extended food deprivation and endurance exercise and could act as a common mediator for the changes in mTOR signaling. For this reason, the role of AMPK activation in the regulation of mTOR was evaluated in the intact liver. Phosphorylation of AMPK either in vivo or in situ was associated with a repression of protein synthesis as well as decreased phosphorylation of a number of targets of mTOR signaling including S6K1, eIF4G, and 4E-BP1. Together, the results demonstrate a coordinated response in the liver to changes in nutrient and hormonal status to alter gene expression through translational control mechanisms in the liver.
## Table of Contents

**Figures**

- vii

**Tables**

- x

**Abbreviations**

- xi

**Acknowledgements**

- xiii

**Chapter I. Background**

1. Overall significance of translational control of protein synthesis in the liver

2. Overview of translational control of protein synthesis

3. Assessment of protein synthesis

4. Assembly of the 43S preinitiation complex

5. Recruitment of 5’-m7GTP capped mRNA to the ribosome

6. Regulation of mTOR through interactions with mTOR-binding proteins and PI 3-kinase/ Akt signaling

7. mTOR-mediated regulation of ribosome biogenesis

8. Physiological conditions under which total protein synthesis in the liver is altered

**Chapter II. Materials and Methods**

27

**Chapter III. Translational Control of Gene Expression in Rat**

- 41

  1. Liver in Response to Meal Feeding

  2. Introduction
Chapter IV. Endurance Exercise Represses Eukaryotic Initiation

Factor 4E-Binding Protein 1 (4E-BP1) Phosphorylation and Translation of Ribosomal Protein mRNA in Rat Liver

Chapter V. Repression of Protein Synthesis and mTOR Signaling in Rat Liver Mediated by the AMP-Activated Protein Kinase (AMPK) Activator 5-Aminoimidazole-4-Carboxamide Riboside (AICAR)
Figures

**Figure 1** Protein synthesis occurs as a three step process beginning with initiation, followed by elongation of the nascent peptide, and ending with termination and release of the protein. 19

**Figure 2** Polysome profile analysis is used to assess the relative translation of mRNAs. 20

**Figure 3** Formation of the 43S preinitiation complex is one rate-controlling process of translation initiation. 21

**Figure 4** Formation of the eIF4F complex recruits 5’m7GTP-capped mRNA to the ribosome. 22

**Figure 5** mTOR phosphorylates three translation initiation factors, 4E-BP1, S6K1, and eIF4G. 23

**Figure 6** Mitogens and amino acids stimulate mTOR kinase activity. 24

**Figure 7** AMPK activation is sensitive to PKA activation and increases in AMP:ATP. 25

**Figure 8** Serum insulin concentration increased within 15 min feeding. 59

**Figure 9** Fractional rate of protein synthesis increases in liver following feeding. 60

**Figure 10** Aggregation of polysomes occurs rapidly following feeding. 61

**Figure 11** eIF2B activity does not change in the liver following feeding. 62
Figure 12 eIF4G (Ser 1108) and eIF4E (Ser 209) phosphorylation are temporally altered in response to feeding.

Figure 13 Hyperphosphorylation of 4E-BP1, S6K1 and S6K1 (Thr 389) as well as rpS6 phosphorylation increases following feeding.

Figure 14 eIF4F complex assembly rapidly increases following commencement of feeding.

Figure 15 Polysome association of five mRNAs increases after feeding in the absence of a change in their relative abundance.

Figure 16 Effect of treadmill running on protein synthesis in rat liver.

Figure 17 Treadmill running specifically repressed the translation of 5'-TOP mRNAs in rat liver.

Figure 18 Phosphorylation of S6K1 and rpS6 did not change in rat liver after treadmill running.

Figure 19 Phosphorylation of 4E-BP1 decreased in rat liver after treadmill running.

Figure 20 Association of 4E-BP1 and 4E-BP2 in rat liver after treadmill running.

Figure 21 Phosphorylation of eIF4G, the insulin receptor β-subunit, and AMPK differed between the two experimental models of treadmill running.
Figure 22 AICAR treatment stimulated AMPK phosphorylation.

Figure 23 AMPK (Thr172) phosphorylation remained stable following in vivo AICAR administration but increased following perfusion of liver with AICAR.

Figure 24 AICAR treatment reduced global rates of liver protein synthesis.

Figure 25 AICAR treatment had no effect on either polysomal aggregation or eEF2 (Thr56) phosphorylation in perfused rat liver.

Figure 26 eIF2α and eIF2Bε phosphorylation and eIF2B activity remained constant following AICAR administration to post-absorptive rats.

Figure 27 AICAR repressed mTOR signaling as assessed by eIF4G(Ser1108) phosphorylation, 4E-BP1 hyperphosphorylation, 4E-BP1(Thr37), and S6K1(Thr389) phosphorylation in liver.

Figure 28 AICAR treatment repressed the assembly of the eIF4F complex.

Figure 29 Serum insulin concentration decreased whereas the phosphorylation of mTOR on Ser2448 increased following in vivo AICAR treatment.
Tables

Table 1 Summary of changes in hormones and nutrients in response to food intake and endurance exercise.

Table 2 Increased polysome association of mRNAs containing a 5’-TOP sequence.

Table 3 Increased polysome association of mRNAs not known to contain a 5’-TOP sequence.

Table 4 Decreased polysome association of mRNAs following food intake.

Table 5 Summary of results presented in Chapters III-V
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4E-BP</td>
<td>eukaryotic initiation factor 4E binding protein</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide 1-β-D-ribonucleoside</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>EAAs</td>
<td>essential amino acids</td>
</tr>
<tr>
<td>eEF</td>
<td>eukaryotic elongation factor</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic initiation factor</td>
</tr>
<tr>
<td>GβL</td>
<td>G protein β-subunit-like protein (also known as mLST8)</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>GCN2</td>
<td>nutrient-regulated protein kinase</td>
</tr>
<tr>
<td>HRI</td>
<td>heme-regulated inhibitor</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>met-tRNA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>initiator methionyl-tRNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin kinase</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly (A)-binding protein</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like ER resident kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>double-stranded RNA-activated protein kinase</td>
</tr>
<tr>
<td>PI 3-K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>raptor</td>
<td>regulatory associated protein of mTOR</td>
</tr>
<tr>
<td>Rheb</td>
<td>ras-homolog enriched in brain</td>
</tr>
</tbody>
</table>
rp: ribosomal protein
S6K1: ribosomal protein S6 kinase 1
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOP mRNA: mRNAs containing a 5’-terminal oligopyrimidine tract
TOS: TOR signaling motif
TSC: tuberous sclerosis complex
TSC1: gene encoding harmatin
TSC2: gene encoding tuberin
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Chapter I: Background

Overall significance of translational control of protein synthesis in the liver

The liver contributes to the regulation of glucose, lipid, and protein metabolism. Following intake of a complete meal, the liver functions largely in the uptake of amino acids (reviewed in Wannemacher, 1972), and growth of the liver is stimulated. The stimulation of liver growth coincides with an increase in protein synthesis and ribosome biogenesis as well as synthesis of specific proteins. A protein kinase, mTOR, regulates transcription of rDNA (Hannan et al., 2003) and translation of ribosomal proteins (reviewed in Meyuhas, 2000), and for this reason, mTOR is a protein that is centrally involved in growth. mTOR is also sensitive to amino acids (reviewed in Kimball & Jefferson, 2004b; Proud, 2004) and mitogens (reviewed in Proud & Denton, 1997; von Manteuffel et al., 1997). Thus, signaling through mTOR likely contributes to the growth of the liver in response to feeding.

One of the most abundantly produced proteins in the liver is albumin, which has multiple functions and is altered in different pathological conditions including kidney dysfunction and sepsis. Several studies suggest that synthesis of albumin is translationally regulated and sensitive to amino acid deprivation (Kuwahata et al., 1998; Ijichi et al., 2003). As with the ribosomal proteins, translation of albumin mRNA is sensitive to inhibition of mTOR (Ijichi et al., 2003), suggesting that signaling through mTOR regulates translation of a larger subset of the proteins synthesized in the liver. Many other blood proteins, possessing various vital
functions critical to the formation of blood clots, stabilization of hormones in the blood, production of hormones that are synthesized in the liver, and transportation of lipids from the liver to peripheral tissues for metabolism. Fibrinogen and other proteins involved blood clotting are synthesized and secreted from the liver. The liver also synthesizes and secretes hormones, including IGF-I and IGF-II, and globulins that bind and stabilize circulating hormones in the blood such as the thyroid hormones. Thus, the growth response of the liver to different physiological circumstances is dependent on translational control of protein synthesis and ribosome biogenesis, and synthesis of specific secreted proteins, e.g. albumin, may be translationally regulated.

**Overview of translational control of total protein synthesis**

Translation of mRNA occurs as a three-step process beginning with translation initiation, followed by elongation of the nascent peptide, and ending with termination (see pg. 19, Fig. 1). Most studies suggest that the rate of protein synthesis is primarily regulated during the process of translation initiation (Kimball & Jefferson, 1991a; Vary & Kimball, 1992), although some studies demonstrate alterations in the rate of elongation (Vary & Kimball, 1992; Horman et al., 2002). Two rate-controlling processes of translation initiation, the formation of the 43S preinitiation complex and the recruitment of $5'\text{-m}^7\text{GTP}$-capped mRNA to the ribosome, have been extensively analyzed under various conditions (reviewed in Proud, 2001, 2002; Kimball & Jefferson, 2004b).
Assessment of protein synthesis

Two widely used techniques for examining protein synthesis are the flooding dose technique (Garlick et al., 1980), which assesses the global rates of protein synthesis, and polysome profile analysis, which assesses the relative aggregation of ribosomes on specific mRNAs. In the flooding dose technique, a sufficient amount of radiolabeled amino acid, e.g. $^3$H-phenylalanine, is injected intravenously to raise the plasma level of the amino acid to a concentration sufficient to equilibrate intracellular pools of amino acid with the plasma pool. After a period of time, such as ten minutes, the tissue is extracted and analyzed for the rate of incorporation of the radiolabel into total protein. Thus, the flooding dose technique assesses the rate of synthesis of all proteins during the duration of the label period.

In contrast to the analysis of global rates of protein synthesis, the relative translation of specific mRNAs is assessed by employing polysome profile analysis. As more ribosomes bind to a single mRNA, it will gain density and progress further into a sucrose density gradient. For polysome profile analysis, samples are prepared and layered over a sucrose density gradient, and the gradients are centrifuged to separate unbound ribosomes, monosomes, and disomes, referred to as the subpolysomal fraction, from bound ribosomes, referred to as the polysomal fraction. The subpolysomal fraction represents mRNAs that are poorly translated while the polysomal fraction contains mRNAs that are efficiently translated. The relative translation of a specific mRNA is assessed as its relative distribution between the
subpolysomal fraction and the polysomal fraction (see pg. 20, Fig. 2). Thus, if the content of a particular mRNA decreases in the subpolysomal fraction and increases in the polysomal fraction, translation of the mRNA is likely elevated. In contrast, if the content of a particular mRNA increases in the subpolysomal fraction and decreases in the polysomal fraction, translation of the mRNA usually decreases. Thus, with the use of a combination of the flooding dose technique and polysome profile analysis, the rate of total protein synthesis as well as translation of specific mRNAs can be examined.

**Assembly of the 43S preinitiation complex**

The formation of the 43S preinitiation complex (see pg. 21, Fig. 3) is reduced following amino acid deprivation (Kimball et al., 1991) and following induction of diabetes (Kimball & Jefferson, 1991b). Formation of the 43S preinitiation complex is mediated through the binding of eukaryotic initiation factor (eIF) 2 to a guanine triphosphate (GTP) molecule, an initiator methionyl (met)-tRNA, and a 40S ribosomal complex (reviewed in Kimball, 1999). The binding of eIF5, results in hydrolysis of the GTP bound to eIF2, and release of the eIF2•GDP complex. In order for subsequent rounds of translation initiation to occur, the GDP bound to eIF2 must be exchanged with GTP by eIF2B, the rate-limiting enzyme for this process.

The activity of eIF2B is inhibited by phosphorylation of Ser51 on the α-subunit of eIF2, phosphorylation of Ser535 on the catalytic subunit of eIF2B, the ε-subunit, and by allosteric effectors. eIF2α (Ser51) is phosphorylated by one of four
kinases: double-stranded RNA-activated protein kinase (PKR), ER-stress regulated kinase (PERK), heme-regulated inhibitor (HRI), and the nutrient-regulated protein kinase (GCN2) (reviewed in Kimball, 1999). PKR becomes activated in response to an accumulation of double-stranded RNA in the cell, which can occur with viral infection (Clemens & Elia, 1997). PERK becomes activated in response to the accumulation of unfolded or misfolded proteins in the ER and comprises part of the unfolded protein response (Shi et al., 1998; Harding et al., 1999). Conditions of heme deficiency are associated with the activation of HRI in reticulocytes (Levin et al., 1976). Finally, GCN2 is activated in response to nutrient deprivation (Hinnebusch, 1986). Under each of these various conditions, activation of the respective kinase results in phosphorylation of eIF2α (Ser51). Phosphorylation of eIF2α (Ser51) promotes its association with eIF2B and competitively inhibits the GTP for GDP exchange activity of eIF2B. Previous work has demonstrated a positive correlation between eIF2B activity and changes in the global rate of protein synthesis. eIF2B activity is inhibited by phosphorylation of its ε-subunit on Ser535 by glycogen synthase kinase 3 (Welsh et al., 1998; Jefferson et al., 1999). Through the inhibition of eIF2B activity, phosphorylation of eIF2α (Ser51) suppresses the rate of global protein synthesis (reviewed in Pain, 1996; Proud, 2001). Finally, a decrease in eIF2B activity has also been associated with an increase in the NADP⁺/NADPH ratio (Dholakia et al., 1986; Kimball & Jefferson, 1991b; Karinch et al., 1993; Crozier et al., 2003) suggesting that eIF2B activity is regulated in a manner other than covalent modification and is sensitive to changes in the redox state of the cell.
Recruitment of 5'-m$^7$GTP-capped mRNA to the ribosome

A second rate-controlling process of translation initiation is the recruitment of 5'-m$^7$-GTP capped mRNA to the ribosome (reviewed in Gingras et al., 1999b) (see pg. 22, Fig. 4). As with the formation of the 43S preinitiation complex, the recruitment of 5'-m$^7$-GTP capped mRNA to the ribosome is sensitive to amino acids (Shah et al., 1999; Anthony et al., 2001b) and mitogens (Pause et al., 1994). This process is regulated through the interaction of a scaffolding protein, eIF4G, with three proteins, eIF3, eIF4E, and eIF4A, that possess individual functions. eIF3 binds to the 43S preinitiation complex, while eIF4E binds to 5'-m$^7$GTP-capped mRNA. Thus, the association of eIF3 and eIF4E with eIF4G helps to guide the 43S preinitiation complex to the 5'-m$^7$GTP-cap. eIF4A, along with eIF4B, acts as an mRNA helicase and removes any secondary structure within 5'-UTRs of mRNAs. Together, eIF4E, eIF4A, and eIF4G form the active eIF4F complex and recruit 5'-m$^7$GTP-capped mRNA to the ribosome.

mTOR signaling regulates the interaction of eIF4E with eIF4G by modulating the association of eIF4E with the eIF4E binding proteins (4E-BPs) (Haghighat et al., 1995; Tsukiyama-Kohara et al., 1996; Poulin et al., 1998), which interact with eIF4E on a region that overlaps with the eIF4G binding site (Mader et al., 1995) (see pg. 23, Fig 5). To date, three different 4E-BPs have been described, and six phosphorylation sites on 4E-BP1 have been identified (reviewed in Gingras et al., 2001). Phosphorylation of these six residues constitutes hyperphosphorylation of 4E-BP1.
Hyperphosphorylation of 4E-BP1, the best characterized of the 4E-BPs, through mTOR signaling (Burnett et al., 1998; Gingras et al., 1999a), promotes its dissociation from eIF4E and permits eIF4F complex formation (Haghighat et al., 1995). Treatment with rapamycin, an inhibitor of mTOR that binds the FKBP12 protein and disrupts the interaction of mTOR and raptor, prevents hyperphosphorylation of 4E-BP1 (Kim et al., 2002) and supports a role for mTOR in the regulation of 4E-BP1 phosphorylation. Phosphorylation of the Thr37 residue on 4E-BP1, an initial phosphorylation event in the sequence of hyperphosphorylation of the protein (Gingras et al., 1999a), is a residue directly phosphorylated by mTOR (Choi et al., 2003). Therefore, formation of the active eIF4F complex is mediated, at least in part, by signaling through mTOR.

In addition to regulating 4E-BP1 phosphorylation, mTOR signaling also results in the phosphorylation of three residues in the C-terminus of eIF4G, Ser1108, Ser1148, and Ser1192 (see pg. 23, Fig. 5) (Raught et al., 2000). Rapamycin treatment prevents serum-induced phosphorylation of the serine residues; however, truncation of the N-terminus of eIF4G makes these residues rapamycin insensitive (Raught et al., 2000). Thus, while phosphorylation of Ser1108, Ser1148, and Ser1192 is sensitive to rapamycin, these residues are not directly phosphorylated by mTOR. Although the functional consequence of eIF4G phosphorylation has not been fully elucidated, recent studies demonstrate a positive correlation between phosphorylation of eIF4G (Ser1108) and the rate of total protein synthesis (Lang et al., 2003; Bolster et al., 2004).
**Regulation of mTOR through interactions with mTOR-binding proteins and PI 3-kinase/ Akt signaling**

Mitogens and amino acids stimulate mTOR kinase activity in many different tissues and cell types, by modulating the signaling pathway(s) upstream of mTOR and regulating the interaction of mTOR with various proteins (see pg. 24, Fig. 6). Mitogens and amino acids regulate the activity of mTOR through similar mechanisms, but the signaling pathway(s) stimulated by amino acids is less clearly defined (reviewed in Kimball & Jefferson, 2004a). Activation of the PI 3-kinase/ Akt signaling pathway in response to growth factors, such as insulin and IGF-I, results in activation of mTOR, and prior treatment of cells with a PI 3-kinase inhibitor, LY294002 or wortmanin, prevents growth factor-induced stimulation of mTOR kinase activity. Akt phosphorylates mTOR at Ser2448 (Sekulic et al., 2000), although the consequence of this phosphorylation event on mTOR activity is not clear. Overexpression of Rheb, a GTPase, also promotes phosphorylation of Ser2448, in the absence of any apparent stimulation of Akt (Inoki et al., 2003a) and maintains phosphorylation of S6K1 during amino acid starvation, suggesting it is an important upstream regulator of mTOR. The PI 3-kinase/ Akt signaling pathway also modulates activation of the TSC1/ TSC2 complex, an upstream regulator of Rheb, via phosphorylation of TSC2 on several Ser/Thr residues (Dan et al., 2002; McManus & Alessi, 2002; Potter et al., 2002). Phosphorylation of TSC2 represses the inhibitory effect of the TSC1/ TSC2 complex on Rheb and promotes phosphorylation of S6K1.
Therefore, the PI 3-kinase/ Akt signaling pathway may regulate mTOR through direct phosphorylation of mTOR and/ or phosphorylation of TSC2, an upstream regulator of mTOR.

Several mTOR interacting proteins have been identified including raptor and GβL (reviewed in Kwiatkowski, 2003; Kimball & Jefferson, 2004a). While the mechanism by which raptor and GβL regulate mTOR is unclear, much knowledge has been gained through the use of siRNA constructs. Use of siRNA to decrease expression of either raptor or GβL represses phosphorylation of S6K1 in response to amino acids, suggesting the raptor and GβL function as positive regulators of mTOR signaling (Kim et al., 2003). Raptor is thought to exert its effect on mTOR signaling by acting as a scaffold between mTOR and its substrates 4E-BP1 and S6K1, and this concept is supported by the identification of a binding site for raptor, a TOS motif, on both 4E-BP1 and S6K1 that is necessary for phosphorylation and interaction with raptor (Schalm & Blenis, 2002; Nojima et al., 2003; Schalm et al., 2003). In contrast, the mechanism whereby GβL exerts its effect on mTOR is much less clear although some models propose that raptor and GβL may function together to regulate mTOR signaling (reviewed in Kimball & Jefferson, 2004a).

**mTOR-mediated regulation of ribosome biogenesis**

Ribosome biogenesis requires transcription of ribosomal DNA into rRNA and translation of ribosomal proteins, which together form the ribosome. Signaling
through mTOR also contributes to the regulation of both of these processes. Phosphorylation on Thr389 of S6K1 by mTOR (Pullen & Thomas, 1997; Burnett et al., 1998) leads to maximal kinase activation. Activated S6K1 stimulates phosphorylation of the rDNA transcription factor, UBF, leading to increased transcription of rDNA (Hannan et al., 2003).

Under many experimental conditions, signaling through mTOR is also associated with preferential translation of a class of mRNAs which in turn regulates ribosome biogenesis and cell growth (Jefferies et al., 1994; Jefferies et al., 1997; Schmelzle & Hall, 2000). Subsequent phosphorylation of the S6K1 substrate rpS6 is associated with preferential translation of mRNAs containing a TOP sequence (Meyuhas, 1996; Jefferies et al., 1997). Although the functional consequence of rpS6 phosphorylation is not clear, rpS6 is localized to the mRNA-tRNA binding site of the ribosome, suggesting that rpS6 could function in the selectivity of mRNA translation (Nygard & Nilsson, 1990). mRNAs encoding many components of the translational apparatus contain a 5′-TOP sequence, including those encoding ribosomal proteins, translation elongation factors, and poly (A) binding protein (PABP) (reviewed in Meyuhas, 2000). Several studies report an inhibition of translation of 5′-TOP-containing mRNAs following treatment with rapamycin (Jefferies et al., 1994; Jefferies et al., 1997; Reiter et al., 2004a), suggesting that the mTOR-mediated regulation of S6K1 activity functions in the regulation of ribosome biogenesis and cell growth.
Recent studies in cell culture suggest that mTOR-mediated signaling is not the only controlling factor in the regulation of 5’-TOP translation (Tang et al., 2001; Stolovich et al., 2002). These studies demonstrate a dependence on PI 3-kinase signaling in the stimulation of 5’-TOP translation in response to either amino acids or mitogen stimulation, as treatment of cells in culture with LY294002, a PI 3-kinase inhibitor, severely represses polysomal aggregation on mRNAs containing a 5’-TOP sequence. While treatment of cells in culture with amino acids does not stimulate PI-3 kinase signaling, the stimulation of 5’-TOP translation in response to amino acids is prevented by LY294002, suggesting that basal PI-3 kinase signaling is necessary for the amino acid stimulation of 5’-TOP translation. Together, the results suggest that multiple signaling pathways, including the PI-3 kinase and mTOR signaling pathways, contribute to the regulation of translation of mRNAs containing a 5’-TOP sequence and ribosome biogenesis.

Physiological conditions under which total protein synthesis in the liver is altered

The rate of protein synthesis is tightly regulated under various physiological conditions through the complex interplay among positive and negative signals derived from the nutrients and/or mitogens to which the cell is exposed. In particular the protein content of the diet seems to be a critical component of the response of the liver to feeding. Animals fed a diet devoid of protein do not exhibit an increase in the hepatic rate of protein synthesis one hour later as do animals fed a diet composed of
20% protein (Yoshizawa et al., 1998). In animals fed the protein containing diet, the change in protein synthesis coincides with an increase in 4E-BP1 hyperphosphorylation and decreased phosphorylation of eIF4E (Ser209). Because both meals elicit similar increases in circulating serum insulin concentration, the results suggest that the increase in serum insulin concentrations in response to meal feeding is not sufficient to stimulate protein synthesis. In support of this idea, perfusion of neonatal pigs with varying concentrations of both amino acids and insulin (O'Connor et al., 2004) reveals that insulin does not increase the hepatic rate of protein synthesis whereas amino acids stimulate protein synthesis independent of the insulin concentration. As with the observations of Yoshizawa et al. (Yoshizawa et al., 1998), infusion of amino acids at various concentrations significantly increases hyperphosphorylation of 4E-BP1 and S6K1 and eIF4F complex assembly. Together, these results suggest that translational control of protein synthesis in the liver depends on the amino acid component of the meal and not the increase in serum insulin concentration.

Furthermore, specific amino acids comprising the protein component of the diet are of critical importance to the stimulation of hepatic protein synthesis. In particular, animals fed a diet absent in one or more of the EAAs possess a significantly reduced rate of total protein synthesis compared to rats fed a complete meal containing all amino acids or lacking a non-EAA (Anthony et al., 2001b). The decreased rate of protein synthesis in this model coincides with a decrease in 4E-BP1 and S6K1 hyperphosphorylation and eIF4F complex formation. Unlike other studies
in which the total protein component of the meal is altered, livers from rats fed a diet lacking one or more EAAs also exhibited increased phosphorylation of eIF2α (Ser51) and decreased eIF2B activity. No change in eIF2α (Ser51) phosphorylation or eIF2B activity was observed in the livers of GCN2−/− mice fed a diet lacking leucine (Kimball et al., 2004). Thus, the EAAs in the diet appear to modulate both met-tRNA- and mRNA-binding steps. In contrast, oral administration of leucine, one of the EAAs, has no effect on the rate of total protein synthesis in the liver, despite an increase in 4E-BP1 and S6K1 hyperphosphorylation and eIF4F complex assembly (Anthony et al., 2001a; Reiter et al., 2004a). These results suggest that in liver an increase in protein synthesis is not entirely dependent upon a single EAA or 4E-BP1 and S6K1 phosphorylation. In support of this suggestion, rapamycin treatment of rats results in no change in the rate of protein synthesis in either control liver or liver from rats administered an oral bolus of leucine (Reiter et al., 2004a). Overall, the results support a model in which EAAs are necessary but not sufficient to increase the total rate of protein synthesis in the liver.

In addition to their role in the regulation of the hepatic protein synthesis, EAAs also function in the regulation of translation of specific mRNAs. The best-characterized subset of mRNAs regulated by EAAs are those containing a 5’-TOP sequence. Livers from rats fed a complete meal exhibit increased polysomal aggregation of rpmRNAs, which contain a 5’-TOP sequence, whereas livers from rats fed a diet lacking one or more of the EAAs do not (Anthony et al., 2001b). Following oral administration of leucine, an increase in polysomal aggregation on
rpmRNAs is observed (Anthony et al., 2001a; Reiter et al., 2004a). Rapamycin treatment of the rats prior to oral leucine administration prevents the increase in 4E-BP1 and S6K1 hyperphosphorylation and eIF4F complex assembly, suggesting these events are mediated by mTOR signaling (Reiter et al., 2004a). Additionally, rapamycin treatment inhibits the polysomal aggregation on rpmRNAs in response to leucine administration (Reiter et al., 2004a). The results suggest that the stimulation of 5’-TOP mRNA translation in response to oral administration of leucine is dependent upon signaling through mTOR. Perhaps more importantly, the results also suggest that in response to feeding, specific mRNAs are selected for translational upregulation and contribute to the overall response of the liver to feeding.

As with fasting and feeding, endurance exercise has been implicated in the regulation of protein turnover in the liver. In a chronic model of endurance exercise, rats were subjected to a single bout of endurance exercise for seven consecutive days (Hayase & Yokogoshi, 1992). It was found that protein synthesis in the liver was reduced in exercised compared to the unexercised control animals. In addition to a reduction in protein synthesis, the total weight of the liver along with the protein and RNA content of the liver is reduced, suggesting that endurance exercise also alters the rate of ribosome biogenesis in the liver. Following an acute bout of endurance exercise, protein degradation is stimulated in the liver independent of the nutritional state of the animals, suggesting that protein synthesis could also be repressed in an acute model of endurance exercise (Kasparek et al., 1980). In order to gain insight into the early events leading to decreased protein synthesis and reduced RNA content
in the liver in the chronic model of endurance exercise, the effect of an acute bout of endurance exercise on translational control of protein synthesis must be examined.

AMPK could potentially play a central role in regulating protein synthesis in response to fasting and feeding (Munday et al., 1991) and endurance exercise (Carlson & Winder, 1999; Park et al., 2002). LKB1 phosphorylates AMPK at Thr172 and activates AMPK kinase activity (see pg. 25, Fig. 7). LKB1 forms a complex with various proteins including STRADα/β and MO25α/β, which are required for activation of LKB1 kinase activity (Hawley et al., 2003). LKB1 is also phosphorylated by PKA on Thr431, and mutation of Thr431 to an alanine prevents the inhibition of cell growth by LKB1, suggesting that phosphorylation at Thr431 is critical for the function of LKB1 (Collins et al., 2000). The results also suggest that activation of PKA leads to phosphorylation and activation of LKB1 and, in the presence of the LKB1 interacting proteins, can lead to activation of AMPK (Hawley et al., 2003; Woods et al., 2003). AMPK is also termed the “energy sensor” of the cell, because it becomes activated under conditions that result in an increase in the AMP:ATP ratio, possibly by increased affinity for LKB1 (Woods et al., 2003; Zou et al., 2004). As an “energy sensor”, AMPK promotes conservation of energy within the cell through phosphorylation of various substrates including ACC (reviewed in Hardie & Carling, 1997). Under conditions of prolonged fasting, activation of AMPK and decreased ACC activity has been observed (Munday et al., 1991), and one model of endurance exercise demonstrated an activation of AMPK in the liver that coincided with a reduction in ACC activity (Park et al., 2002).
Because of the association of LKB1 and inhibition of cell growth (reviewed in Boudeau et al., 2003) and because protein synthesis and mTOR signaling are associated with changes in cell growth, the affects of AMPK activation on protein synthesis and mTOR signaling have recently been studied. Activation of AMPK results in a repression of protein synthesis in isolated primary hepatocytes (Dubbelhuis & Meijer, 2002; Horman et al., 2002). In one study, an increase in phosphorylation of eEF2, which decreases the rate of elongation, was observed in response to AMPK activation, suggesting that an attenuation of translation elongation contributes to the repression of total protein synthesis (Horman et al., 2002). In addition, two different groups reported a correlation between AMPK activation and a decrease in phosphorylation of S6K1 and its activity, suggesting that AMPK regulates mTOR signaling and translation initiation (Dubbelhuis & Meijer, 2002; Krause et al., 2002). Although changes in S6K1 often reflect changes in mTOR signaling and eIF4F complex formation, they do not always result in a change in the rate of total protein synthesis in the liver (Anthony et al., 2001a; Reiter et al., 2004a). Indeed, treatment of isolated primary hepatocytes with rapamycin does not alter the rate of protein synthesis despite a similar repression of S6K1 activation, whereas administration of AICAR, an AMP mimic, exhibits a dose-dependent repression of total protein synthesis (Dubbelhuis & Meijer, 2002). These results suggest that the decrease in protein synthesis following AMPK activation is not mediated through the mTOR-dependent regulation of translation initiation (Dubbelhuis & Meijer, 2002). Despite the many studies performed in isolated primary hepatocytes suggesting that
AMPK regulates the rate of global protein synthesis and mTOR-mediated signaling, no such observations have been demonstrated in vivo. Moreover, the results of Horman et al. (Horman et al., 2002) and Dubbelhuis and Meijer (Dubbelhuis & Meijer, 2002) invoke questions as to whether the change in protein synthesis following AMPK activation results from a repression in translation elongation and/or translation initiation.

Following feeding in meal trained rats, the serum concentrations of several hormones and metabolites change (see pg. 26, Table 1) including an increase in the anabolic hormone, insulin, and a decrease in the catabolic hormone, glucagon (Potter et al., 1968). The coordinated change in anabolic and catabolic hormones along with an increase in nutrient availability, i.e. glucose and amino acids, results in subsequent increases in protein and RNA content in the liver (Kosterlitz, 1947). While recent progress suggests that the increase in ribosome biogenesis depends on the amino acid stimulation of mTOR, previous work has focused on examining the translational regulation of mRNAs containing a 5’-TOP. The possibility exists that many other proteins synthesized in the liver are also translationally regulated, and this concept is supported by studies illustrating translational regulation of albumin (Kuwahata et al., 1998; Ijichi et al., 2003). Additionally, depending on the intensity and duration of the endurance exercise, a decrease in serum insulin concentration is observed with a concomitant increase in anabolic hormones, including glucagon and corticosterone (see pg. 26, Table 1) (Naveri et al., 1985; Winder et al., 1988). While a chronic model of endurance exercise displays a reduction in hepatic protein synthesis and
RNA, little information has been gained as to the effects of an acute bout of endurance exercise on translational control of protein synthesis and 5'-TOP mRNA translation. The overall goal of the studies described in Chapters III and IV was to gain a better understanding of the translational regulation of gene expression in the liver using two physiological models: food intake and endurance exercise.

AMPK functions in the regulation of glucose and lipid metabolism under conditions of nutrient deprivation (reviewed in Hardie & Carling, 1997). Studies in isolated primary hepatocytes suggest that AMPK also functions in the regulation of protein synthesis and mTOR signaling. To date, the impact of AMPK activation on protein synthesis and mTOR signaling the intact liver has not been studied. In Chapter V, AICAR treatment was utilized to assess the consequence of energetic stress and AMPK activation on total protein synthesis and mTOR-mediated signaling in the liver.
Figure 1 Protein synthesis occurs as a three step process beginning with initiation, followed by elongation of the nascent peptide, and ending with termination and release of the protein. The blue circles labeled 80S represent a ribosome, while the ovals labeled 60S and 40S represent the 60S and 40S ribosomal subunits, respectively. The small yellow circles represent amino acids, and the solid line represents a strand of mRNA.
Polysome profile analysis is used to assess the relative translation of mRNAs by separating untranslated mRNAs, subpolysomal, from highly translated mRNAs, polysomal. The light green circles represent ribosomes, and the short, curved lines represent a strand of mRNA. The thin pink rectangle represents the sample that is layered over the gradient prior to centrifugation.
Figure 3 Formation of the 43S preinitiation complex is one rate-controlling process of translation initiation. Hydrolysis of the GTP bound to eIF2 produces an inactive complex of eIF2 bound to GDP. The activity of eIF2B is also regulated by phosphorylation of eIF2α on Ser51 and phosphorylation of the catalytic ε-subunit of eIF2B on Ser535.
Figure 4  Formation of the eIF4F complex recruits 5'-m^7GTP-capped mRNA to the ribosome. eIF3 binds to the 43S preinitiation complex, and eIF4E binds to the 5'-m^7GTP-cap. eIF4A, along with eIF4B, removes secondary structure of the mRNA.
Figure 5  mTOR phosphorylates three translation initiation factors 4E-BP1, S6K1, and eIF4G. Phosphorylation of 4E-BP1 promotes its dissociation from eIF4E. mTOR indirectly regulates phosphorylation of eIF4G. mTOR phosphorylation of S6K1 on Thr389 maximally activates the kinase activity and increase phosphorylation of rpS6, which is associated with increased translation of rpRNA. S6K1 also phosphorylates UBF and increases transcription of rDNA. Pink circles with a “P” inside represent a phosphate group.
Figure 6 Mitogens and amino acids stimulate mTOR kinase activity. Stimulation of phosphorylation or activation is indicated by an arrow, e.g. Akt phosphorylates Ser2448 of mTOR and amino acids stimulate Rheb through an unknown mechanism. Perpendicular lines indicate an inhibition of signaling from the regulated protein, e.g. Akt represses the activity of the TSC1/TSC2 complex.
**Figure 7** AMPK activation is sensitive to PKA activation and increases in AMP:ATP. PKA phosphorylates and activates LKB1. Activation of LKB1 promotes its phosphorylation and activation of AMPK. AICAR becomes phosphorylated and mimics an increase in AMP. Pink circles represent phosphorylated residues.
Table 1: Summary of changes in hormones and nutrients in response to food intake and endurance exercise.

<table>
<thead>
<tr>
<th>Hormones/ Metabolites</th>
<th>Food intake</th>
<th>Endurance exercise</th>
<th>Protein synthesis</th>
<th>mTOR signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>↑</td>
<td>↓</td>
<td>Anabolic</td>
<td>Stimulates</td>
</tr>
<tr>
<td>Glucagon</td>
<td>↓</td>
<td>↑</td>
<td>Catabolic</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>↓</td>
<td>↑</td>
<td>Catabolic</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Catecholamines</td>
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<td>↑</td>
<td>Catabolic</td>
<td>N/A</td>
</tr>
<tr>
<td>Glucose</td>
<td>↑</td>
<td>↓</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>↓</td>
<td>↑</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Lactate</td>
<td>↓</td>
<td>↑</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Chapter II: Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratory) were kept on a 12-h light: dark cycle with free access to food (Harlan-Teklad Rodent Chow, Madison, WI) and water prior to experimentation. The experimental protocols used in the studies reported herein were reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine.

Meal Feeding Protocol

Rats (age 21 days) weighing 45-60 g were maintained on a reverse 12:12-h light: dark cycle with free access to water. The day after arrival, the rats were subjected to meal-training as described previously (Anthony et al., 2001b). Briefly, rats were allowed access to food (Harlan-Teklad Rodent Chow, Madison, WI) for three hours a day, two hours following commencement of the dark cycle for 14 days. On the day of the experiment, rats (100-120g) were randomly assigned to four treatment groups including a fasted control group and groups that were permitted to feed for 15, 60, or 180 min. Ten min prior to sacrifice, a flooding dose (1.0 mL/100 g of body weight) of L-[2,3,4,5,6-3H]phenylalanine (150 mmol/L containing 3.70 GBq/L) was administered via tail vein injection. After injection of [3H]phenylalanine, rats were returned to their cages with free access to food and water. Fasted rats were sacrificed first, followed by the 15 min treatment group, then
60 min treatment group, and finally the 180 min treatment group. All rats were sacrificed 15 min apart by decapitation, trunk blood was collected to measure the amino acid specific radioactivity, and the liver was excised for analysis of protein synthesis and biomarkers of mRNA translation.

**Exercise Protocol**

Five, four, and three days prior to the experiment, rats weighing 200-260g assigned to an exercise group were acclimated to the protocol by allowing them to run on a treadmill (constructed at the Pennsylvania State University machine shop or Exer-6M-Treadmill, Columbus Instrument, Columbus, OH). Food was removed from the cages twelve hours prior to the start of the study for rats in both the control and exercise groups. Using the Pennsylvania State University treadmill (Protocol 1), rats ran at a pace of 26m/min with a 10% incline for 60 min or to the point of exhaustion with a minimum time of 30 min. Rats were motivated to run by a stream of intermittent air. For analysis of the global rate of protein synthesis, rats were immediately injected with a flooding dose (1.0 mL/100 g body wt) of L-[2,3,4,5,6-\(^3\)H] phenylalanine (150 mmol/L) via the tail immediately after the exercise. Ten minutes later, rats were sacrificed via decapitation, blood was collected, and the liver was excised and processed as described below. All serum and tissue samples were stored at -70°C until analysis. Using the Exer-6M-Treadmill (Protocol 2), rats ran at a pace of 26 m/min with a 10% incline for 60 min and were motivated by an intermittent stream of air. Control rats were placed on the grid at the end of the
treadmill and air was blown on them for 2 min. Immediately following the exercise period, the rats were anesthetized with Isofluorane, and one portion of the liver was frozen in place between two aluminum blocks cooled to the temperature of liquid nitrogen for analysis of AMPK. The remaining portion of the liver was excised and processed for analysis of translation initiation and polysomal aggregation as described below. All serum and tissue samples were stored at -70°C until analysis.

**AICAR Administration In Vivo**

On the day of the experiment, rats (225-275g) used for the in vivo studies were injected subcutaneously with AICAR (1 mg/g body weight) (Toronto Chemicals, North York, ON, Canada) in sterile 0.9% NaCl. Control rats were administered an equivalent volume of sterile 0.9% NaCl as previously described (Bolster *et al.*, 2002). A flooding dose (1.0 mL/100 g body wt) of L-[2,3,4,5,6-3H] phenylalanine (150 mmol/L) was injected via the tail vein 50 min following the subcutaneous injection of AICAR for measurement of the rate of synthesis of total mixed liver protein (Garlick *et al.*, 1980). Ten minutes following the injection of radiolabeled phenylalanine, the rats were sacrificed via decapitation, blood was collected, and the liver was excised for analysis of the rate of total protein synthesis. The remainder of the liver was processed for analysis of mTOR mediated signaling and translation control mechanisms. All serum and tissue samples were stored at -70°C until analysis.
Liver Perfusion

The liver was perfused in situ essentially as previously described (Flaim et al., 1982). Briefly, it was perfused through the portal vein for a period of 30 min at a flow rate of 7 mL/min with non-recirculating buffer consisting of Krebs-Henseleit buffer (pH 7.4) containing 11 mmol/L glucose, 3% (w/v) bovine serum albumin (MP Biomedicals Aurora, OH), 30% washed bovine erythrocytes, amino acids at 5 times the concentrations found in the arterial plasma of normal fasted rats, and when present, 2 mmol/L AICAR (Toronto Chemicals). For the final 10 min of the perfusion period, radiolabeled L-[4,5-3H]-leucine (6.7 µCi) was added while the total concentration of leucine was maintained at 5 times the fasted value. Following the 30 min perfusion period, the liver was removed and a portion was immediately clamped between two aluminum blocks pre-cooled to the temperature of liquid nitrogen for analysis of AMPK phosphorylation. The remaining unfrozen portion of liver was used for analysis of protein synthesis and biomarkers of translation initiation or for analysis of polysomal aggregation as described below.

Measurement of Protein Synthesis

The rate of synthesis of total mixed liver protein was measured as previously described (Garlick et al., 1980). Briefly, a portion of the liver (~0.5) was weighed and homogenized by polytron in 7 volumes of buffer consisting of (in mmol/L) 20 HEPES (pH 7.4), 100 KCl, 0.2 EDTA, 2 EGTA, 50 NaF, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamidine, and 0.5 sodium vanadate. An aliquot
of the homogenate (~250 µL) was used to estimate the fractional rate of protein synthesis ($K_s$) as the rate of incorporation of $[^3]$Hphenylalanine into total protein using the serum phenylalanine specific radioactivity as representative of the precursor pool during the incorporation period, i.e. the time elapsed from injection of $[^3]$Hphenylalanine until tissue homogenization (Anthony et al., 2001b). To assess the incorporation of $[^3]$Hphenylalanine in tissue, the 250 µL aliquot was added to 2.5 mL of 1.0 N perchloric acid and boiled for 15 min. The sample was cooled and centrifuged at 2,500 rpm for 10 min at 4°C. The supernatant was aspirated, and the pellet was resuspended in 2.5 mL 0.5 N perchloric acid, vortexed and centrifuged again at 2,500 rpm for 10 min at 4°C. This step was repeated. Then, the pellet was washed twice with 1 chloroform: 2 ethanol: 1 ether as described above. Next, the pellet was washed in ether and dried in a ventilation hood overnight. The following day, the pellet was dissolved and boiled in 3 mL of 0.1 N NaOH for 15 min. An aliquot (500 µL) was added to 10 mL of Formula 989 in duplicate and the DPMs were assessed on a Beckman-Coulter LS6500 Multipurpose Scintillation Counter. A second aliquot (5 µL) was used for analysis of total protein by performing a protein assay (Bio-Rad, Hercules, CA). The specific radioactivity of the tissue was assessed by dividing the radioactivity of the tissue by the protein concentration of the tissue. The remaining homogenate was centrifuged at 10,000 xg for 10 min at 4°C, and the resulting supernatant was used for Western blot analysis.

_Measurement of Protein Phosphorylation Status_
Phosphorylation of eIF4G was assessed by Western blot analysis using an anti-phospho-eIF4G(Ser 1108) antibody (Cell Signaling Technology, Beverly, MA) and normalized to total eIF4G using a polyclonal anti-eIF4G antibody. Phosphorylation of 4E-BP1 was assessed as a change in electrophoretic mobility during SDS-PAGE. eIF4E(Ser209) phosphorylation was evaluated using a polyclonal antibody to eIF4E(Ser209) (Cell Signaling Technology, Beverly, MA) and normalized to total eIF4E using an anti-eIF4E monoclonal antibody (Kimball et al., 1997). Hyperphosphorylation of S6K1 was assessed as decreased migration during SDS-PAGE using a polyclonal anti-S6K1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); S6K1 phosphorylation was also analyzed using a polyclonal antibody that recognizes only the Thr389 phosphorylated form of S6K1 (Cell Signaling Technology, Beverly, MA). rpS6 phosphorylation was evaluated using a 1:1 mixture of polyclonal antibodies that recognize either the Ser 235/236 or Ser 240/244 phosphorylated forms of rpS6 (Cell Signaling Technology, Beverly, MA). Phosphorylation of AMPK was evaluated by Western blot analysis using an anti-phospho-AMPK (Thr172) antibody (Cell Signaling Technology, Beverly, MA) and normalized to total AMPK content using a polyclonal anti-AMPK antibody (Cell Signaling Technology, Beverly, MA). Samples were analyzed by Western blot analysis for relative phosphorylation of the α-subunit of eIF2 employing an anti-phospho-eIF2α (Ser51) antibody (BioSource International, Hopkinton, MA); the blots were normalized for total eIF2α content using a monoclonal antibody (Scorsone et al., 1987) that recognizes the protein irrespective of its phosphorylation
status. eIF2Bε phosphorylation was also analyzed employing an anti-phospho-eIF2Bε (Ser535) (BioSource International, Hopkinton, MA) and normalized to total eIF2Bε content using a monoclonal anti-eIF2Bε antibody (Kimball et al., 1994). Phosphorylation status of eEF2 (Thr56) was analyzed as the signal intensity from a phosphospecific antibody normalized to that of an antibody that recognizes the total protein independent of phosphorylation state. Phosphorylation of mTOR was evaluated by Western blot analysis using an anti-phospho-mTOR (Ser2448) antibody (Cell Signaling Technology, Beverly, MA) and normalized to total mTOR content using a polyclonal anti-mTOR antibody (Cell Signaling Technology, Beverly, MA). All images were captured using ECL or ECL+ (Amersham Biosciences, Buckinghamshire, England) kits according to the manufactures instructions by GeneTools GENOME (K&R Technology, Frederick, MD) software and quantified using GeneTools Syngene software (K&R Technology).

Quantitation of 4E-BP1•eIF4E, 4E-BP2•eIF4E, and eIF4G•eIF4E complexes

eIF4E was immunoprecipitated from the 10,000 xg supernatants using an anti-eIF4E monoclonal antibody (Kimball et al., 1997). Samples were subjected to immunoblot analysis using a polyclonal antibody to eIF4G to assess the association of eIF4G with eIF4E (Kimball et al., 1997). Association of 4E-BP1 or 4E-BP2 with eIF4E was analyzed in a similar manner using a 4E-BP1 polyclonal antibody or a 4E-BP2 (Calbiochem, San Diego, CA) polyclonal antibody. Results were normalized to the amount of eIF4E in the immunoprecipitates.
**Analysis of Polysomal Aggregation**

A portion of the liver (~1g) was weighed and homogenized by dounce in 4 volumes of buffer containing (in mmol/L) 40 HEPES (pH 7.5), 100 KCl, and 5 magnesium chloride. The homogenate was centrifuged at 3,000 xg for 15 minutes. One volume of detergent (10% Triton X-100, 0.24 mol/L deoxycholate sodium salt) was added to nine volumes of the supernatant. An aliquot of the sample was layered over a 10-70% linear sucrose density gradient. The gradients were centrifuged at 90,000 xg for three hours at 4°C in a Beckman SW28 rotor. After centrifugation, the gradients were fractionated on an Isco gradient fractionator while the ultraviolet (UV) absorption at 254 nm was continuously recorded. Five 5.0 mL fractions and a final 2.5 mL fraction were collected for extraction of total RNA as described below.

**RNA Extraction and Purification**

Total RNA was isolated from the sucrose density gradient fractions by performing two phenol:chloroform (5:1) (Ambion, Austin, TX) extractions. The second phenol:chloroform extraction was followed by an RNA precipitation step using 0.1 volume 5 mol/L ammonium acetate (Ambion, Austin, TX) and 2 volumes 100% ethanol. The precipitate was washed with 100% ethanol and dissolved in RNA Storage Solution (Ambion, Austin, TX) containing Anti-RNase (Ambion, Austin, TX) to prevent degradation. RNA from each condition was then pooled into one of two fractions termed subpolysomal (consisting of non-ribosome associated
mRNA, 40S and 60S ribosomal subunits, and mRNA associated with 80S ribosomal subunits alone) or polysomal (consisting of mRNA associated with two or more ribosomes). In the meal-feeding experimental model, subpolysomal RNA from each condition was pooled with subpolysomal RNA from the same treatment group, and polysomal RNA from each condition was pooled with polysomal RNA from the same treatment group for microarray and quantitative real time (QRT) polymerase chain reaction (PCR) analysis. In the endurance exercise experimental model, subpolysomal RNA from each rat liver was pooled, and polysomal RNA from each rat liver was pooled for QRT-PCR analysis. The pooled RNA was further purified using the RNeasy Kit (Qiagen, Valencia, CA) and eluted in RNase free water (Ambion, Austin, TX).

**RNA Extraction from Liver**

Total RNA was extracted from the liver using an RNeasy Kit (Qiagen, Valencia, CA). Samples were eluted in RNase free water (Ambion, Austin, TX). The quality and quantity of the RNA was assessed by measuring the $A_{260}$ and $A_{280}$ with a Beckman Coulter spectrophotomer.

**Microarray Analysis**

DNase I treatment was performed on each pooled RNA sample following purification with the RNeasy Kit (Qiagen, Valencia, CA) using the DNase Free Kit (Ambion, Austin, TX). Next, the quality, purity, and concentration of the RNA were
assessed using a LabChip (Agilent, Palo Alto, CA) analyzed on an Agilent 2100 Bioanalyzer. Microarray analysis was performed in the Juvenile Diabetes Research Foundation Functional Genomics Core Facility at The Pennsylvania State University College of Medicine. Briefly, total RNA (50 µg) from each treatment group was indirectly labeled with CY5 while total RNA (50 µg) from a rat reference library (Stratagene, La Jolla, CA) was indirectly labeled with CY3. Then, the labeled cDNA was hybridized to a Rat Liver Array (MWG Biotech, High Point, NC). The microarrays were analyzed on a Perkin Elmer ScanArray 4000XL, and the data were analyzed using GeneSpring version 6.0.

**QRT-PCR Analysis**

QRT-PCR was performed as previously described (Kubica et al., 2004). Briefly, 1 µg total RNA was incubated with 1 µL Oligo (dT) (Invitrogen, Carlsbad, CA) for 10 min at 70°C, and then the sample was placed on ice. Next, 5 µL 2X reaction buffer, 2 µL 0.1 mmol/L DTT (Invitrogen, Carlsbad, CA), and 1 µL Superscript Platinum Taq Polymerase were added to the reaction mixture. The samples were then incubated at 42°C for 65 min and stored at –20°C until QRT-PCR was performed. Serial dilutions were performed on the samples to a final dilution of 1:16. QRT-PCR was performed using QuantiTect SYBR Green PCR (Qiagen, Valencia, CA) following the manufacturer’s instructions. The primers used for each mRNA analyzed were as follows: eEF1A-upper-5’-CTAATATGCGGTGTCAAG-3’ and lower-5’-CGCAGAGCCTTGTCAGTTG-
3’; GAPDH-upper-5’-GGGCTGCCTTTCTCTTGTA-3’ and lower-5’-TGACTGCTGGCTTCTTTAGA-3’; Hs p 8 a-upper-5’-TGTCTCATCAAGCGCAATA-3’ and lower-5’-GGCCCTTTTACCTTTCTAC-3’; rpS6-upper-5’-ACTGGCTGTCAGAAACTCAT-3’ and lower-5’-CCACATAACCCTCACCCTCT-3’; rpS8-upper-5’-CGTGCCTTGAGATGGATGT-3’ and lower-5’-CGGACAAGCTCGGTTATTGG-3’; rpL26-upper-5’-TCTCACATTGCGAGAGAT-3’ and lower-5’-TGTCCTCGAACACCTGAAC-3’; β-actin-upper-5’-TAGGCACCAGTGTGGATGG-3’ and lower-5’-GCAGCTTGCATTGTAAGAAAGTGT-3’. QRT-PCR reactions and analysis were performed on an MWG Biotech DNA Engine Opticon Continuous Fluorescence Detector using the Opticon Monitor Analysis Software Version 2.01.

**Lambda Protein Phosphatase Treatment**

Frozen liver samples were homogenized in 7 volumes of buffer consisting of (in mmol/L) 20 HEPES, pH 7.4, 2 EGTA, 100 KCl, 0.2 EDTA, 1 DTT, 0.1 PMSF, 1 benzamidine, 1 µg/mL leupeptin, 10 µg/mL aprotinin. Aliquots (65 µL) of homogenates were incubated with 6.5 µL 10X λ protein phosphatase buffer (New England Biolabs, Beverly, MA), 2 µL λ phosphatase (400,000 U/ml; New England Biolabs, Beverly, MA), and 6.5 µL magnesium acetate and incubated at 37°C for 15 min.


Measurement of Serum Insulin Concentration

Serum insulin concentrations were measured using an insulin RIA kit (Linco Research, St. Charles, MO) as previously described (Anthony et al., 2000a).

Assessment of Insulin Receptor Phosphorylation

Frozen liver samples were homogenized by sonication in ice-cold buffer consisting of (in mmol/L) 50 HEPES pH 7.3, 137 NaCl, 1 MgCl₂, 1 CaCl₂, 2 sodium vanadate, 10 sodium pyrophosphate, 10 NaF, 2 EDTA, 2 PMSF, 10 benzamidine, 10% glycerol, 1% NP-40, 1 tablet/ml proteinase inhibitor tablets (Boehringer-Manheim, Manheim, Germany). The samples were then rocked for 15 min at 4°C and centrifuged for 10 min at 18,000 xg. A detergent compatible protein assay (Bio-Rad, Hercules, CA) was then performed, and 500 µg of total protein were added to 30 µL Protein A-Sepharose beads, 5 µL anti-insulin receptor β antibody (sc-711, c-19; Santa Cruz Biotechnology, Santa Cruz, CA), and 900 µL homogenization buffer. Samples were rocked overnight at 4°C and then washed twice with homogenization buffer. Insulin receptor β-subunit was eluted in 30 µL 2X SDS-sample buffer. For analysis of insulin receptor β-subunit tyrosine phosphorylation, Western blot analysis was then performed using anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) and the results were normalized to total insulin receptor β-subunit (Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of eIF2B Activity
A portion of liver (~0.3 g) was homogenized in 5 volumes of buffer consisting of (in mmol/L) 45 HEPES (pH 7.4), 0.375 magnesium acetate, 0.075 EDTA, 95 potassium acetate, 2.03 digitonin, 10% glycerol, and 0.003 microcystin. The homogenate was centrifuged at 10,000 xg for 10 min at 4°C, and the resulting supernatant was analyzed for eIF2B activity as previously described (Kimball & Jefferson, 1991b; Anthony et al., 2001b). An assay mixture consisting of 103 µL of water and 140 µL of assay buffer consisting of (in mmol/L) 52.1 MOPS, pH 7.4, 104.2 KCl, 1.04 dithiothreitol, 2.08 magnesium acetate, 224 GDP and 250 µg/mL bovine serum albumin) was warmed for 30 seconds in a 30°C water bath. Then, 20 µL of liver supernatant and 35 µL of preformed eIF2•[^3]H]GDP complex were added and the mixture was incubated at 30°C. At four time points (0, 1, 2, and 3 minutes), a 60 µL aliquot was removed and added to 2.5 mL of ice-cold wash buffer consisting of (in mmol/L) 50 MOPS, pH 7.4, 100 KCl, and 1 dithiothreitol). The contents were filtered through a nitrocellulose filter disc under vacuum, and the filter was rinsed twice with 2.5 mL of ice-cold wash buffer. The amount of eIF2•[^3]H]GDP complex bound to the filter was measured by scintillation counting. The activity of eIF2B was calculated by plotting the DPM as the dependent variable and time as the independent variable and determining the slope of the nearest fit line through those points. The slope indicated the rate of GDP exchanged as pmols per minute as an indication of eIF2B activity.

Statistical Analysis
All data were analyzed by InStat 3.0 (GraphPad Software, San Diego, CA). When more than two treatment groups were compared, data were analyzed using an ANOVA multiple comparisons test and Tukey post-hoc analysis unless otherwise stated. For comparisons between two treatment conditions, a pooled two-tailed t-test was utilized unless otherwise stated. The level of significance was set at p<0.05 for all statistical tests.
Chapter III. Translational Control of Gene Expression in Rat Liver in Response to Meal Feeding

Introduction

The protein and RNA content of the liver falls during food deprivation, and both macromolecules are rapidly replenished upon commencement of feeding (Kosterlitz, 1947; Munro, 1968). In rat liver, the fasting to feeding transition results in a rapid aggregation of ribosomes on mRNA, an effect that is sustained for up to five hours (Kosterlitz, 1947; Yokogoshi & Yoshida, 1986). The observed accumulation of polysomes suggests a stimulation of the process involving the initiation of mRNA translation, which would explain in part the accompanying increase in protein and RNA content. As discussed in Chapter I, further evidence for the involvement of translation initiation in the feeding-induced increase in protein and RNA content of the liver is provided by the reported increase in phosphorylation of 4E-BP1 and S6K1 (Yoshizawa et al., 1997; Anthony et al., 2001b), two biomarkers of signaling through mTOR, one hour following food intake (Yoshizawa et al., 1997; Yoshizawa et al., 1998). However, rapamycin does not alter the global rate of protein synthesis in the liver. Thus, mTOR signaling is activated in response to feeding or leucine administration; however, it is unlikely that activation of the pathway accounts for the stimulation of global rates of protein synthesis (Anthony et al., 2001a; Reiter et al., 2004a). On the other hand, the stimulation of mTOR
signaling may play an important role in the feeding-induced increase in RNA content of the liver through activation of ribosome biosynthesis (Reiter et al., 2004a).

The overall objective of the studies described in this Chapter was to gain insight into the mechanism(s) involved in the regulation of protein synthesis and the translation of selected mRNAs in the liver in response to feeding. Given previous observations of increased signaling through mTOR following food intake, the hypothesis tested herein was that in response to feeding, the polysome association of mRNAs containing a 5’-TOP sequence would be rapidly stimulated and sustained throughout the remainder of the 180 min time course. To maximize the chance of seeing novel changes in translational regulation of gene expression, a time course analysis was performed to investigate both rapid and delayed changes in protein synthesis and mRNA translation. An increase in mTOR signaling, as indicated by increased 4E-BP1 and S6K1 phosphorylation paralleled with an increase in polysome association of 5’-TOP mRNAs. In contrast, the increase in protein synthesis did not coincide with 4E-BP1 or S6K1 phosphorylation, but instead coincided with an increase in eIF4G(Ser1108) phosphorylation, a decrease in eIF4E(Ser209) phosphorylation, and a decrease in the association of 4E-BP2 with eIF4E. The results support previous work demonstrating that activation of mTOR-mediated signaling in the liver does not lead to an acute increase in the global rate of synthesis of total mixed liver protein synthesis but instead is involved in the regulation of translation of specific mRNAs (Reiter et al., 2004a). Furthermore, the
results demonstrate a rapid and sustained increase in protein synthesis and ribosomal protein mRNA translation in response to feeding.
Results

To control for a consistent consumption of food within the different treatment groups, the animals were subjected to meal-training as described in Chapter II. As shown in Fig. 8A on pg. 59, the amount of food consumed in each treatment group was elevated by 15 min and maximal by 60 min. The change in food intake paralleled the increase in serum insulin concentration, which was also elevated by 15 min and sustained for the remainder of the time course (see pg. 59, Fig. 8B). The rate of synthesis of total mixed liver protein, as analyzed using the flooding dose technique (Garlick et al., 1980), was not significantly different from the fasted control value 15 min following commencement of feeding (see pg. 60, Fig. 9). However, by 60 and 180 min the synthetic rate increased to 118% and 128% of the fasted control value, respectively (see pg. 60, Fig. 9).

To further analyze the effect of feeding on protein synthesis, the aggregation of ribosomes on mRNA was assessed by fractionation of liver samples on sucrose density gradients, separating free ribosomes, referred to as subpolysomal, from mRNA-bound ribosomes, i.e. polysomal. Within 15 min of the commencement of feeding, the relative proportion of ribosomes in polysomes increased whereas the proportion of free 40S and 60S ribosomal subunits, i.e. nonpolysomal, decreased, providing evidence of a stimulation of translation initiation (see pg. 61, Fig. 10). The stimulation of ribosomal aggregation was maximal by 60 min with no additional change from 60 min to 180 min (see pg. 61, Fig. 10). Therefore, changes in
polysomal aggregation began to occur prior to the increase in global rates of protein synthesis (see pg. 60, Fig. 9).

Increased protein synthesis mediated through a stimulation of translation initiation is often associated with an increase in the activity of eIF2B (Shah et al., 1999; Anthony et al., 2001b). For this reason, eIF2B activity was analyzed at various times after the commencement of feeding. As seen in Fig.11 on pg. 62, no change in eIF2B activity was observed at any of the time points, suggesting that a change in eIF2B activity is not necessary for the increase in protein synthesis observed in the present study.

To gain better insight into the molecular mechanisms contributing to the increased rate of protein synthesis, phosphorylation of eIF4E (Ser209) and eIF4G (Ser1108) was evaluated. The functional consequence(s) of eIF4E (Ser209) phosphorylation is not well understood, but one study reports that phosphorylation of the cap-binding protein contributes to preferential translation of selected mRNAs including some cell cycle proteins (reviewed in Scheper & Proud, 2002). In the present study, a significant decrease in eIF4E (Ser209) phosphorylation was observed within 60 min, which was sustained at 180 min when compared to the fasted control values (see pg. 63, Fig. 12A). Thus, the decrease in eIF4E (Ser209) phosphorylation that occurred in response to food intake temporally paralleled the increased rate of protein synthesis.

The functional consequence of eIF4G (Ser1108) phosphorylation remains to be elucidated; however, eIF4G (Ser1108) phosphorylation was previously shown to
correspond with an increased rate of protein synthesis in skeletal muscle (Lang et al., 2003; Bolster et al., 2004). In response to food intake, phosphorylation of eIF4G (Ser1108) tended to increase within 15 min, was maximal by 60 min, and was sustained at 180 min (see pg. 63, Fig. 12B). Thus, eIF4G (Ser1108) phosphorylation followed a similar time course to that of polysome aggregation.

Signaling through mTOR is typically assessed by examining the phosphorylation status of various mTOR substrates including S6K1 and 4E-BP1 (reviewed in Gingras et al., 2001; Wang et al., 2003). S6K1 is phosphorylated on multiple residues and resolves into multiple electrophoretic bands when subjected to SDS-PAGE with the most highly phosphorylated form of the protein, the δ-band, migrating the slowest. S6K1 hyperphosphorylation, analyzed in this manner, was significantly elevated within 15 min of feeding and remained elevated through the remainder of the 180 min time course (see pg. 64, Fig. 13A). Phosphorylation of S6K1 (Thr389) results in maximal kinase activity and is directly catalyzed by mTOR (Pullen & Thomas, 1997). Examination of this site at 15 min demonstrated increased phosphorylation that was sustained for 180 min (see pg. 64, Fig. 13B). Together, the data suggest that the activity of S6K1 was rapidly increased and sustained during the 180 min period. As further evidence of the activation of S6K1, phosphorylation of its substrate, rpS6, was examined. A significant increase in rpS6 phosphorylation was evident by 15 min and was also sustained until 180 min (see pg. 64, Fig. 13C), further suggesting that S6K1 activity was rapidly stimulated following commencement of feeding and remained active through 180 min.
Similar to S6K1, the migration of 4E-BP1 decreases during SDS-PAGE as its phosphorylation increases and results in resolution of the protein into multiple electrophoretic forms. The slowest migrating band, the γ-band, corresponds to the most highly phosphorylated form of the protein and is unable to associate with eIF4E. Examination of 4E-BP1 revealed a significant increase in phosphorylation within 15 min following commencement of feeding, an effect that was sustained throughout the remainder of the 180 min time course (see pg. 64, Fig. 13D). Thus, unlike the changes in eIF4E (Ser209) and eIF4G (Ser1108) phosphorylation, the feeding induced stimulation of protein synthesis was delayed relative to the stimulation of mTOR signaling.

Because phosphorylation of 4E-BP1 typically results in alterations in the association of eIF4E with its various binding partners, the association of eIF4E with either 4E-BP1, 4E-BP2, or eIF4G was assessed. Hyperphosphorylation of 4E-BP1 typically results in its dissociation from eIF4E, which is one mechanism of augmenting eIF4F complex assembly and stimulating translation initiation. As seen in Fig. 14A on pg. 65, the amount of 4E-BP1 associated with eIF4E was significantly reduced within 15 min of the commencement of feeding compared to fasted controls and remained lower throughout the remainder of the time course. 4E-BP2, a second eIF4E binding protein, is also expressed in the liver (Tsukiyama-Kohara et al., 1996), and its dissociation from eIF4E exposes the eIF4G binding site and permits eIF4G binding to eIF4E (Mader et al., 1995). Unlike the result obtained for 4E-BP1, a shift in mobility of 4E-BP2 on SDS-PAGE was not observed (data not shown).
Moreover, the time course for dissociation of the 4E-BP2•eIF4E complex was delayed compared to 4E-BP1•eIF4E, and following feeding a significant reduction in 4E-BP2 association with eIF4E did not occur until 60 min and was sustained for the remainder of the 180 min time course (see pg. 65, Fig. 14B). The decreased association of 4E-BP2 with eIF4E coincided temporally with the increased rate of protein synthesis. However, while dissociation of 4E-BP2 from eIF4E was delayed compared to 4E-BP1, both eIF4E binding proteins remained dissociated from eIF4E for an extended period of time. Dissociation of 4E-BPs allows eIF4G to bind to eIF4E (Mader et al., 1995) and, therefore, the amount of eIF4G bound to eIF4E was assessed. A significant increase in eIF4E•eIF4G association was observed within 15 min (see pg. 65, Fig. 14C). The increased association of eIF4E and eIF4G was maintained for 60 min but then returned to the fasted value by 180 min. Thus, eIF4E•eIF4G association was rapidly stimulated in parallel with increased phosphorylation and dissociation of 4E-BP1; however, unlike the changes in the association of 4E-BP1 or 4E-BP2 with eIF4E, the response was not sustained for 180 min.

Highly translated, i.e. polysome associated, mRNAs can be separated from non-translated, i.e. subpolysomal, mRNAs by fractionation on sucrose density gradients (see pg. 20, Fig. 2). Favorably translated mRNAs are bound to a greater number of ribosomes and migrate further through the sucrose gradient whereas free ribosomes and mRNAs associated with only one ribosome, monosomes are less dense and remain in the upper portion of the gradient. As aggregation of
polyribosomes increases, the amount of mRNA in the polysomal fraction increases whereas mRNA in the subpolysomal fraction decreases. In order to assess translational control of a large set of mRNAs, the polysome associated mRNAs from each time point was compared to a rat RNA reference library (Stratagene) on a rat liver specific gene microarray (MWG Biotech). Increased association of a specific mRNA with the polysome fraction would suggest increased translation whereas decreased association would indicate decreased translation. The acceptable level of change for an increase was set at a 1.5-fold increase between two time points. The results of the microarray analysis revealed that 78 mRNAs displayed increased polysome association at one or more time points after feeding (see pg. 66 and 67, Tables 2 and 3). Of these mRNAs, 42 function in the regulation of protein synthesis and are thought to contain a 5’-TOP sequence, with 40 of the mRNAs encoding ribosomal proteins (see pg. 66, Table 2). Another 36 mRNAs not known to contain a 5’-TOP sequence also displayed increased polysome association following the microarray analysis (see pg. 67, Table 3). Decreased polysome association of 50 mRNAs (see pg. 68, Table 4) was also observed but none of these mRNAs were confirmed by QRT-PCR.

Whereas an increase in the abundance of an mRNA in the polysomal fraction suggests that translation of that mRNA is enhanced, a similar finding could also occur if the total amount of that particular mRNA increased in response to feeding. To confirm that the changes reported in Tables 2 and 3 on pg. 66 and 67, respectively, represented a redistribution of mRNA from the nonpolysomal to the
polysomal fraction, rather than a change in expression, the distribution of GAPDH, an mRNA that did not change in its polysome association in the microarray analysis, and of rpL26, rpS6, rpS8, eEF1α, and Hsp8a, mRNAs whose polysomal contents were elevated, was assessed in both the subpolysomal fraction and the polysomal fraction by QRT-PCR. The ratio of mRNAs encoding rpL26, rpS6, rpS8, eEF1α, and Hsp8a in the polysome compared to the subpolysome fraction increased after feeding (see pg. 69, Fig. 15A) whereas a change in GAPDH distribution was not observed (see pg. 69, Fig. 15A). As further evidence that the increased abundance of particular mRNAs in the polysomal fraction was not due to increased expression, QRT-PCR analysis of mRNAs was performed on total RNA. No significant change in mRNA expression of GAPDH was observed (see pg. 69, Fig. 15B). Further, when normalized to the GAPDH expression, no significant change in rpL26, rpS6, rpS8, eEF1α, and Hsp8a mRNA content was observed (see pg. 69, Fig. 15C), confirming the translational regulation of these mRNAs. Thus, following commencement of feeding, a rapid stimulation of 5′-TOP mRNA translation, as indicated by increased polysome association of rpS6, rpS8, rpL26, and eEF1α, and non-5′-TOP mRNA translation, as noted by increased polysome association of Hsp8a mRNA, was observed. While the significance of increased translation of non-5′-TOP mRNAs remains unclear, the microarray data suggests that a novel mechanism(s) for translational regulation of gene expression may be altered in response to food intake in the liver.
Discussion

The increased nutrient availability and serum insulin concentration that accompany ingestion of a complete meal initiate a cascade of events in the liver that ultimately lead to an increase in the protein and RNA content of the tissue. The previously observed rapid and sustained aggregation of ribosomes in response to feeding suggested the involvement of translation initiation in the feeding response. To gain insight into the temporal response of the liver to feeding, translational control of protein synthesis as well as translation of selected mRNAs were evaluated at different time points following commencement of feeding. Overall, the results presented in this chapter demonstrate that protein synthesis as well as ribosomal protein mRNA translation undergo rapid and sustained stimulation in the liver following meal feeding contributing to the previously observed increases in protein and RNA content.

One of the earliest events in the response to feeding is a stimulation of intracellular signal transduction pathways that subsequently result in phosphorylation of a number of proteins that either mediate or control mRNA translation. Phosphorylation of these biomarkers of mRNA translation leads to an increase in the translation of most mRNAs, although some mRNAs are affected to a greater or lesser extent than others. One rate-controlling process in translation initiation is assembly of the eIF4F complex (see pg. 22 and 23, Fig. 4 and 5), which is regulated in part through an mTOR-mediated signaling pathway. Previous studies demonstrated an increase in phosphorylation of 4E-BP1 at one time point, i.e. 60 min
following feeding (Yoshizawa et al., 1997; Yoshizawa et al., 1998; Kimball et al., 2000). In the present chapter, phosphorylation of two proteins, 4E-BP1 and S6K1, lying downstream of mTOR in the PI-3 kinase signal transduction pathway was maximally increased within 15 min after the start of feeding and remained elevated through the 180 min time point suggesting that mTOR-mediated signaling was rapidly stimulated and sustained. Enhanced polysome formation was also observed within 15 min of feeding, but was not maximal until the 60 min time point, suggesting that the stimulation of polysome aggregation was delayed relative to the changes in 4E-BP1 and S6K1 phosphorylation. Increased incorporation of \(^{3}\)H\)phenylalanine into total mixed liver proteins was further delayed and was not statistically significant until 60 min after the commencement of feeding. Overall, the results support a scenario wherein increased nutrient and hormone signaling in response to feeding induces a sequence of events beginning with activation of the mTOR-mediated signaling pathway and progressing through phosphorylation of biomarkers of mRNA translation (e.g. 4E-BP1, S6K1, and rpS6), increased association of ribosomes with mRNA, and upregulated translation of selected mRNAs. Feeding also induces a global stimulation of protein synthesis but this response is delayed compared to mTOR-mediated signaling and may be due to activation of other signaling pathways, a suggestion that is consistent with the observation that administration of rapamycin does not inhibit global rates of protein synthesis in the liver (Anthony et al., 2001a; Reiter et al., 2004a).
The changes in mTOR-mediated signaling were accompanied by enhanced incorporation of mRNAs encoding a number of selected proteins into polysomes. In particular, a number of mRNAs that contain 5’-TOP sequences exhibited a preferential shift into polysomes after feeding, suggesting that phosphorylation of S6K1 and rpS6 might be involved in the process. Results of previous studies provide support for such a suggestion. For example, inhibition of mTOR signaling in cells in culture using rapamycin results in a decrease in both S6K1 activity and rpS6 phosphorylation as well as reduced association of 5’-TOP mRNAs with polysomes (Jefferies et al., 1994; Jefferies et al., 1997). Similarly, a recent study reports that rapamycin prevents the leucine-induced redistribution of ribosomal protein mRNAs into polysomes in rat liver (Reiter et al., 2004a), although it does not prevent its shift into monosomes (i.e. mRNA associated with a single ribosome). The latter result agrees with reports suggesting that S6K1 activation and rpS6 phosphorylation may not be sufficient for enhanced translation of 5’-TOP mRNAs (Tang et al., 2001; Stolovich et al., 2002). Despite the uncertainty of the dependence of 5’-TOP mRNA translation on S6K1 activation or rpS6 phosphorylation, it is clear that feeding rapidly promotes an increase in the polysome association of ribosomal protein mRNAs in the liver. Moreover, the enhanced association of ribosomal protein mRNAs with polysomes observed in the present study, in combination with previous studies reporting an mTOR-mediated increase in rDNA transcription (Hannan et al., 2003), provides a likely explanation for the observed increase in ribosome content in response to feeding (Kosterlitz, 1947; Munro, 1968).
Additionally, the results of the microarray analysis provided insight into the translational upregulation of 78 different mRNAs and downregulation of 50 mRNAs (Table 4). While 42 of the 78 mRNAs have been demonstrated or are believed to contain a 5′-TOP sequence, 36 mRNAs were identified that are not known to contain such a sequence. Of the non-5′-TOP mRNAs, Hsp8a was confirmed to shift from the subpolysomal fraction to the polysomal fraction by QRT-PCR. Thus, it is possible that some of the 36 non-5′-TOP mRNAs may be regulated by a novel mechanism(s) that could involve S6K1. Interestingly, albumin mRNA incorporation into polysomes was observed to increase by microarray analysis and was previously demonstrated to be translationally regulated in response to amino acids (Kuwahata et al., 1998; Ijichi et al., 2003). The translational response to branched-chain amino acids was repressed by rapamycin suggesting mTOR-dependent signaling regulates translation of albumin mRNA (Ijichi et al., 2003). However, because the rat albumin mRNA lacks a 5′-TOP sequence (accession #M16825), the results suggest that its translation as well as other mRNAs may be regulated through unique mechanisms that have not yet been identified.

As with S6K1 phosphorylation, 4E-BP1 hyperphosphorylation was rapidly stimulated and sustained for an extended period of time. The increase in phosphorylation of 4E-BP1 paralleled the decrease in 4E-BP1•eIF4E association. Thus, like S6K1 and rpS6 phosphorylation, the changes in 4E-BP1 correspond with the observed stimulation of 5′-TOP translation. In contrast, the association of 4E-BP2•eIF4E followed a more delayed time course, with a decrease in association not
observed until 60 min that continued through 180 min. Thus, the change in 4E-BP2•eIF4E association coincided more closely with the observed increase in global rates of protein synthesis. 4E-BP2 has not been studied extensively although it shares 60% sequence identity with 4E-BP1 and the residues phosphorylated on 4E-BP1 are fairly well conserved (Tsukiyama-Kohara et al., 1996). The conclusion from most previous studies is that 4E-BP1 and 4E-BP2 are regulated in the same manner and serve the same function, to sequester eIF4E. However, the data presented in the present work and elsewhere (von Manteuffel et al., 1997) suggest that 4E-BP1 and 4E-BP2 are differentially regulated raising the possibility that they may serve different functions in the regulation of the translational control of protein synthesis. It is interesting to speculate that 4E-BP1 may primarily be involved in the regulation 5’-TOP translation whereas 4E-BP2 may function more in the regulation of global rates of protein synthesis. Future studies using mice lacking either 4E-BP1 or 4E-BP2 could address this issue.

Unlike the increased phosphorylation of mTOR substrates in response to feeding, a decrease in eIF4E (Ser209) phosphorylation was observed within 60 min and maintained through the remainder of the time course. The result was not entirely unexpected as oral administration of leucine results in a significant reduction in eIF4E (Ser209) phosphorylation in skeletal muscle, despite a stimulation of protein synthesis and mTOR-mediated signaling (Anthony et al., 2000b). However, the results of the present work demonstrate once again a correlation between a decrease in eIF4E (Ser209) phosphorylation and an increase in the global rate of total protein
synthesis in rat liver, although the functional consequences of decreased eIF4E (Ser209) phosphorylation are unclear.

eIF4G (Ser1108) phosphorylation increases in cells in culture following serum stimulation (Raught et al., 2000) and parallels the increased rate of protein synthesis (Lang et al., 2003; Bolster et al., 2004), but the functional consequence of the increase in phosphorylation is unclear. Phosphorylation of eIF4G (Ser1108) corresponded with the aggregation of ribosomes as indicated by the polysome profile analysis and the observed maximal elevation in phosphorylation at 60 min paralleled the increase in the global rates of protein synthesis. Thus, while the functional consequence of eIF4G (Ser1108) phosphorylation is not certain, the data supports a model in which eIF4G (Ser1108) phosphorylation functions in the regulation of the global rates of total protein synthesis. One possible function of eIF4G (Ser1108) phosphorylation that has not been explored is that it may increase the affinity of eIF4G for other components of the eIF4F complex such as eIF4A. Another possibility might be to increase the association of eIF4E with eIF4G. However, in the present study eIF4E•eIF4G association decreased by 180 min, a time when eIF4G (Ser1108) phosphorylation was still elevated. The basis for the maintenance of protein synthesis at a time when eIF4G•eIF4E association had returned to the fasted control value is unclear; however, it is possible that once mRNA is associated with polysomes the requirement for the eIF4G•eIF4E complex in maintaining mRNA in polysomes is reduced, resulting in a sustained elevation of protein synthesis. Thus, further study of the functional consequence of eIF4G
phosphorylation is needed to better understand its importance in the regulation of global protein synthesis.

Another rate-controlling process in translation initiation is the assembly of the 43S preinitiation complex consisting of the eIF2•GDP•initiator methionyl-tRNAi complex bound to the 40S ribosomal complex (see pg. 20, Fig. 2). The rate-limiting enzyme for this process is eIF2B, which catalyzes the exchange of GDP bound to eIF2 for GTP. The activity of eIF2B is regulated by phosphorylation of eIF2α (Ser51), which competitively inhibits the guanine nucleotide exchange activity of eIF2B. Previous work has demonstrated a correlation between eIF2B activity and changes in the global rate of protein synthesis. Feeding animals a diet devoid of one or more essential amino acids stimulated an increase in eIF2α (Ser51) phosphorylation, a decrease in eIF2B activity, and a decrease in the rate of global protein synthesis (Anthony et al., 2001b). However, one hour following feeding of a complete meal, Yoshizawa et al. (Yoshizawa et al., 1998) did not observe any significant change in eIF2B activity. The possibility remained that an increase in eIF2B activity occurred during the first 60 min of feeding. For this reason, eIF2B activity was assessed in the present work, and no change in its activity, as measured by an in vitro activity assay, or in eIF2α (Ser51) phosphorylation (data not shown) was observed at any time. The results suggest that the changes in protein synthesis and polysome aggregation were not regulated through modulation of eIF2B activity. Overall, a role for changes in phosphorylation of eIF4E and eIF4G as well as reduced association of eIF4E with 4E-BP2 must be considered because the time
course for changes in those biomarkers is the same as that observed for alterations in global rates of protein synthesis. Additionally, the results illustrate a rapid and sustained stimulation of mTOR-mediated signaling that coincides with an increase in polysomal association of many ribosomal protein mRNAs as well as other mRNAs that are not known to possess a 5′-TOP sequence and emphasize the coordinated response to increase the rate of protein synthesis and ribosome biogenesis. Finally, following feeding, hepatic protein synthesis as well as ribosomal protein mRNA translation undergo rapid and sustained stimulation, which is likely mediated by both mTOR-dependent and independent signaling events.
Figure 8  Serum insulin concentration increased within 15 min of feeding. A) Food intake was assessed by weighing food before and after feeding. Food intake is expressed as the mean ± SEM, n=9 per group. *p<0.05. Serum insulin concentrations were assessed as discussed in Chapter II and normalized to control values. Values are expressed as means ± SEM, *p<0.05 vs. control.
**Figure 9** Fractional rate of protein synthesis is increased in liver following feeding. The fractional rate of synthesis of total liver mixed protein using the flooding dose technique as described in Chapter II. Values represent means ± SEM (n=7-9) * p<0.05 vs. fasted controls (Ks=1.4098 nmols phenylalanine/ (mg protein • hr)), using a Duncan’s post-hoc analysis.
Figure 10  Aggregation of polysomes occurred rapidly following feeding. Polysome profiles were analyzed by sucrose density gradient centrifugation as described in Chapter II. Fractions were collected as denoted by the vertical lines in each panel. Fractions 1-3 were pooled and are referred in the text as subpolysomal (white) and fractions 4-6 were pooled and are referred to as polysomal (gray). The percentage of subpolysomal and polysomal area under the curve are listed above their representative fractions. Values are expressed as means ± SEM. The results are representative of 9-10 gradients that were analyzed at each time point, *p<0.05 vs. fasted control.
Figure 11 eIF2B activity did not change in the liver following feeding. eIF2B activity was assessed as described in Chapter II. Values are expressed as means ± SEM, n=9-10.
Figure 12  eIF4E (Ser209) and eIF4G (Ser1108) phosphorylation were temporally altered in response to feeding. A) eIF4E (Ser209) phosphorylation was assessed by Western blot analysis using an anti-eIF4E (Ser209) polyclonal antibody and normalized to total eIF4E using an anti-eIF4E monoclonal antibody. A representative blot for eIF4E (Ser209) is shown in the inset. Values are expressed as means ± SEM (n=6). *p<0.01 vs. fasted controls and 15 min fed value. B) eIF4G phosphorylation was determined by Western blot analysis using a polyclonal antibody that recognizes eIF4G phosphorylated on Ser1108 and were normalized to total eIF4G that was detected with an anti-eIF4G polyclonal antibody. A representative blot for phosphorylated eIF4G is shown in the inset. Values are expressed as means ± SEM (n=12). *p<0.01 vs. fasted controls and 15 min fed value.
Figure 13 Hyperphosphorylation of 4E-BP1, S6K1, and S6K1 (Thr389) as well as rpS6 phosphorylation increased following feeding. A) Hyperphosphorylation of S6K1 was evaluated by Western blot analysis. The results are expressed as the intensity of the β, γ, and δ bands compared to the intensity of all bands and normalized to the fasted control values. Values are expressed as means ± SEM (n=15). *p<0.001 vs. fasted control values. B) S6K1 (Thr389) phosphorylation was assessed by Western blot analysis using a polyclonal antibody that recognizes S6K1 when phosphorylated on Thr389. A representative blot for S6K1 (Thr389) is shown in the inset. Values are expressed as means ± SEM (n=6). *p<0.01 vs. fasted control values. C) rpS6 phosphorylation was determined by Western blot analysis using a mixture of two polyclonal antibodies that recognize the Ser235/236 and Ser240/244 phosphorylation sites of rpS6. A representative blot for rpS6 phosphorylation is shown in the inset. Values are expressed as means ± SEM (n=6). *p<0.01 vs. fasted control values. D) Hyperphosphorylation of 4E-BP1 was determined by Western blot analysis. Hyperphosphorylation of 4E-BP1 was determined as the intensity γ band compared to the intensity of all bands and normalized to the fasted control values. A representative blot is shown in the inset. The results are expressed as means ± SEM (n=9-10). *p<0.001 vs. fasted control values.
Figure 14  eIF4F complex assembly rapidly increased following commencement of feeding.  A) Association of 4E-BP1 with eIF4E was examined by performing Western blot analysis on eIF4E immunoprecipitates using a polyclonal antibody that recognizes all forms of 4E-BP1 and normalizing to the total amount of eIF4E immunoprecipitated as detected with an anti-eIF4E monoclonal antibody. A representative blot for 4E-BP1 is shown in the inset. Values are expressed as means ± SEM (n=6). *p<0.01 vs. fasted controls. B) The association of 4E-BP2 with eIF4E was assessed by performing Western blot analysis on eIF4E immunoprecipitates using a polyclonal 4E-BP2 antibody and normalizing to the relative amount of eIF4E immunoprecipitated as detected with an anti-eIF4E monoclonal antibody. A representative blot for 4E-BP2 is shown in the inset. Values are expressed as means ± SEM (n=13-15). *p<0.01 vs. fasted controls. C) Association of eIF4G with eIF4E was evaluated by immunoprecipitating eIF4E with a monoclonal anti-eIF4E antibody followed by Western blot analysis using an anti-eIF4G polyclonal antibody and normalized to the relative amount of eIF4E. A representative blot for eIF4G is shown in the inset. Values are expressed as means ± SEM (n=6). *p<0.01 vs. fasted controls and 180 min.
Table 2: Increased Polysome Association of 5’-TOP mRNAs

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Figure 15  Polysome association of five mRNAs increased after feeding in the absence of a change in their relative abundance.  A) QRT-PCR was performed on pooled samples from both the subpolysomal and polysomal fractions for the indicated mRNA. ■-eEF1α, ●-rpS8, ■-rpS6, ●-rpL26, ▲-Hsp8a, △-GAPDH. Values are expressed as the mean of the ratio of polysomal:subpolysomal (n=2).  B) Total mRNA extracted from frozen liver was analyzed for the relative content of GAPDH using QRT-PCR. Values are expressed as means ± SEM (n=4).  C) Total mRNA extracted from frozen liver was analyzed for the indicated mRNA by QRT-PCR analysis. Values are expressed as the mean (n=4).
Chapter IV. Endurance Exercise Represses Eukaryotic Initiation Factor 4E-Binding Protein 1 (4E-BP1) Phosphorylation and Translation of Ribosomal Protein mRNA in Rat Liver

Introduction

Many metabolic processes in the liver are altered in response to endurance exercise. Fatty acid (Park et al., 2002) and glucose (Dohm & Newsholme, 1983; Winder et al., 1991) metabolism change quickly from energy consuming to energy conserving or producing processes. Moreover, following chronic exposure to endurance exercise, protein synthesis in the liver is reduced and protein degradation is elevated to provide amino acids that can be used for gluconeogenesis (Kasparek et al., 1980; Hayase & Yokogoshi, 1992). The reduction in protein synthesis is in part a result of a fall in the RNA content of the liver (Hayase & Yokogoshi, 1992), suggesting that ribosome biogenesis is repressed in response to endurance exercise.

Ribosome biogenesis is a complex process requiring the coordinated synthesis of both ribosomal RNA and ribosomal proteins. Acutely, both processes are controlled by a signal transduction pathway involving a protein kinase referred to as mTOR (reviewed in Schmelzle & Hall, 2000). Activation of mTOR mediated signaling results in a specific increase in the translation of mRNAs encoding ribosomal proteins as well as activation of RNA polymerase I. Together, the increased synthesis of ribosomal proteins and elevated transcription of ribosomal DNA lead to enhanced ribosome biogenesis.
The goal of the present study was to establish whether or not global rates of protein synthesis and/or translation of ribosomal proteins was depressed in liver immediately following a single bout of endurance exercise, and if so, if signaling through mTOR was also repressed. The hypothesis tested herein was that, in response to a single bout of endurance exercise, the translation of mRNAs containing a 5’-TOP sequence is reduced. To test this hypothesis, a model of endurance exercise was utilized wherein rats ran on a treadmill for one hour or to exhaustion. In the present work, two different exercise regimens on different treadmills referred to as Protocol 1 and Protocol 2, were utilized (see pg. 28, Chapter II). Most of the observations were conserved in both treadmill models. Translation of mRNAs containing a 5’-TOP sequence was assessed by measuring the proportion of the mRNAs encoding rpS6, rpS8 and eEF1α associated with ribosomes, i.e. being actively translated. Phosphorylation of S6K1, rpS6, 4E-BP1, eIF4G, the β-subunit of the insulin receptor, and AMPK was assessed and rates of global protein synthesis were examined. It was found in both treadmill models of endurance exercise that immediately after treadmill running, the proportion of the mRNAs containing a 5’-TOP sequence that was associated with ribosomes was reduced, suggesting that the rate of translation of TOP mRNAs was repressed. Moreover, phosphorylation of 4E-BP1 was decreased whereas phosphorylation of S6K1 and rpS6 was unchanged. Repressed 4E-BP1 phosphorylation and 5’-TOP mRNA polysome association was observed in the absence of a change in global rates of protein synthesis. In one treadmill model, a decrease in phosphorylation of eIF4G and the β-subunit of the
insulin receptor and increased phosphorylation of AMPK was observed whereas no change in any of these parameters was observed in the other treadmill model. Overall, the results are consistent with a specific downregulation of TOP mRNA translation in liver immediately after endurance exercise.
Results

As illustrated in Fig. 16A on pg. 86, in the ten minutes immediately following treadmill running, the incorporation of radiolabeled phenylalanine into total mixed liver protein in the exercised rat livers was not significantly different from that of the control livers, suggesting that the global rate of hepatic protein synthesis was unaffected in liver immediately after endurance exercise. To further analyze the effect of endurance exercise on protein synthesis, the aggregation of ribosomes on mRNA was assessed by fractionation of tissue homogenates on sucrose density gradients, separating free ribosomes, referred to as subpolysomal, from mRNA-bound ribosomes, i.e. polysomal. As shown in Fig. 16B on pg. 86, no significant change in the aggregation of ribosomes was observed in response to endurance exercise.

To determine whether or not the translation of mRNAs containing a 5’-TOP motif was affected immediately after a single episode of treadmill running, the distribution of the mRNAs encoding eEF1α, rpS8, and rpS6 between the polysomal (i.e. actively translated) and nonpolysomal (i.e. non-translated) fractions was compared to the distribution of two non-TOP mRNAs, β-actin and GAPDH. In control rats, the proportion of the mRNAs encoding β-actin and GAPDH present in the polysomal fraction compared to the nonpolysomal fraction was approximately greater then 2:1, and exercise had little or no effect on their distribution (see pg. 87, Fig. 17A). In contrast, the polysome:nonpolysome ratios of eEF1α, rpS8, and rpS6 mRNAs were approximately 0.65, 0.55, and 0.55, respectively, in livers from control rats (see pg. 87, Fig. 17B), and the values decreased significantly to 0.45, 0.43, and...
0.39, respectively, in livers from exercised animals (see pg. 87, Fig. 17B). Thus, the shift in distribution of eEF1α, rpS8, and rpS6 mRNAs from polysomal to nonpolysomal fractions after exercise suggests that the synthesis of the three proteins was repressed in liver in response to exercise. The relatively high proportion of TOP mRNAs present in the nonpolysome fraction in control liver was most likely due to the animals being fasted for 12 h prior to the study, since food deprivation is known to cause the translation of these mRNAs to be depressed (Anthony et al., 2001a; Anthony et al., 2001b; Reiter et al., 2004a).

The phosphorylation status of S6K1 and in particular its substrate rpS6 has been implicated in the regulation of 5'-TOP mRNA translation (reviewed in Meyuhas, 2000). Our previous studies (Anthony et al., 2001a; Reiter et al., 2004a) have shown that the gel electrophoresis protocol used herein resolves S6K1 into as many as four electrophoretic forms under activating conditions. As shown in Fig. 18A on pg. 88, only two forms of S6K1 were detected by protein immunoblot of liver extracts from control rats, and the fastest migrating α-form was the predominant band. Such a result suggests that S6K1 was predominantly in the α-form, which would be expected in a fasted animal. Any further exercise-induced reduction in S6K1 phosphorylation was not detected due to the relative insensitivity of the gel-shift analysis used for assessing its phosphorylation state. Consistent with these results, the phosphorylation state of rpS6 was also unaltered in response to an acute bout of endurance exercise (see pg. 88, Fig. 18B). Thus, the repressed translation of
mRNAs containing a 5’-TOP sequence occurred in the absence of a detectable change in S6K1 and/or rpS6 phosphorylation.

Inhibition of signaling through mTOR, for example by treatment with rapamycin, is one mechanism for preferentially repressing the translation of TOP mRNAs (Jefferies et al., 1994; Jefferies et al., 1997). Therefore, the effect of exercise on the phosphorylation state of an alternative target of mTOR signaling pathway, i.e. 4E-BP1 (Burnett et al., 1998), was assessed. When 4E-BP1 is subjected to electrophoresis on SDS-polyacrylamide gels, it resolves into multiple isoelectric forms based on its phosphorylation state whereby hypophosphorylated form, the α-form, exhibits the greatest and hyperphosphorylated form, the γ-form, the least mobility. As seen in Fig. 19A on pg. 89, the proportion of 4E-BP1 present in the α-form was significantly reduced in liver from exercised compared to non-exercised rats. Moreover, phosphorylation of 4E-BP1 on Thr37, a residue reportedly directly phosphorylated by mTOR (Choi et al., 2003), was reduced to 46% of the control value following exercise (see pg. 89, Fig. 19B).

Phosphorylation of 4E-BP1 is one mechanism through which assembly of the eIF4F complex is promoted. Less highly phosphorylated forms of 4E-BP1 (the α- and β-forms that are resolved during SDS-polyacrylamide gel electrophoresis) bind to eIF4E and prevent it from binding to a second translation initiation factor, eIF4G, to form the active eIF4F complex (reviewed in Gingras et al., 1999b). Thus, when eIF4E is bound to 4E-BP1 it cannot bind to eIF4G and form the eIF4F complex and as a result binding of many mRNAs to ribosomes is repressed. As shown in Fig. 20A
on pg. 90, analysis of the eIF4E immunoprecipitates revealed that 4E-BP1 binding to eIF4E was elevated in response to exercise. However, unlike 4E-BP1, the binding of 4E-BP2, a second protein that binds to the same domain on eIF4E as 4E-BP1 and eIF4G, to eIF4E is unchanged after exercise (see pg. 90, Fig. 20B). Thus, exercise specifically affected 4E-BP1 and not 4E-BP2 binding to eIF4E in liver at the time point assessed.

While most of the alterations in translation control were observed in both treadmill models, three endpoints did not display the same response. In cells in culture, Ser1108 on eIF4G is not directly phosphorylated by mTOR, but instead mTOR phosphorylates residues near the N-terminus of the protein that permit subsequent phosphorylation of Ser1108 by another kinase(s) (Raught et al., 2000). Thus, phosphorylation of eIF4G on Ser1108 was assessed as an indirect measure of signaling through mTOR. Using Protocol 1, as shown in Fig. 21A on pg. 91, treadmill running resulted in a reduction in eIF4G (Ser1108) phosphorylation to 41% of the control value. In contrast, using Protocol 2, no change in eIF4G (Ser1108) phosphorylation was observed (see pg. 91, Fig. 21B). Activation of the insulin receptor and AMPK phosphorylation were assessed as two possible signaling pathways that contribute to the regulation of 4E-BP1 phosphorylation and 5’-TOP mRNA polysome association. To determine if insulin receptor signaling was further repressed in the liver following a single bout of endurance exercise in both treadmill models, phosphorylation of the β-subunit of the insulin receptor on tyrosine residues was measured by protein immunoblot analysis following immunoprecipitation of the
insulin receptor β-subunit. Upon binding of insulin to the α-subunit of the insulin receptor, the intracellular domain of the β-subunit trans-phosphorylates on tyrosine residues (Lee et al., 1993). Subsequently, the β-subunit undergoes autophosphorylation, resulting in activation of the protein kinase activity of the receptor, the first step in signal transduction through the insulin receptor (White et al., 1988). As shown in Fig. 21C on pg. 91, phosphorylation of the insulin receptor β-subunit in liver of rats subjected to Protocol 1 was reduced to approximately 25% of the control value, suggesting that insulin signaling is reduced in liver in response to endurance exercise whereas no change was observed in livers from rats subjected to Protocol 2 (see pg. 91, Fig. 21D). AMPK activation has been observed in several studies examining the effect of endurance exercise on liver (Carlson & Winder, 1999; Park et al., 2002). Therefore, phosphorylation of AMPK on Thr172, an event associated with activation of the kinase (Hawley et al., 1996), was examined. As shown in Fig. 21E and 21F on pg. 91, no change in phosphorylation of AMPK on Thr172 was observed in liver from exercised compared to control rats in either treadmill model. However, the mobility of AMPK during electrophoresis was reduced in rat livers in response to the exercise following Protocol 1, suggesting that residues other than Thr172 might be phosphorylated. To test this idea, liver extracts were treated with λ phosphatase prior to electrophoresis and protein immunoblot analysis. It was found that after treatment with λ phosphatase, the mobility of AMPK was increased, and the protein focused as a single distinct band rather than a diffuse smear, suggesting that AMPK was partially phosphorylated on residues other than
Thr172 (see pg. 91, Fig. 19G). Whether or not phosphorylation of AMPK on residues other than Thr172 might modulate the activity of the kinase is unknown. However, no change in activity of AMPK was detected in AMPK-immunoprecipitates (data not shown).
Discussion

In the present study, reduced phosphorylation of 4E-BP1 paralleled the repression of 5'-TOP mRNA translation following a single bout of endurance exercise, whereas phosphorylation of another substrate of mTOR, S6K1 was not affected. A likely explanation for no detectable change in S6K1 phosphorylation is that, because the rats were fasted for 12 h prior to the start of the study, S6K1 was predominantly present in the α and β forms, even in the control rats. A similar finding has been reported previously (Gautsch et al., 1998) in skeletal muscle of fasted rats subjected to a single episode of treadmill running. In that study, S6K1 was in the α and β forms in both fasted control and exercised rats. The finding of a relatively small proportion of 4E-BP1 in the hyperphosphorylated γ-form in control rats is also consistent with this idea. Attempts to measure changes in S6K1 phosphorylation using site-specific antibodies (e.g. anti-phospho-Thr389 antibodies) were unsuccessful due to lack of signal, a result that is consistent with the finding that S6K1 was predominantly in the α and β forms. Overall, the results agree with previous studies (Kimball et al., 2000; Anthony et al., 2001a) that signaling through mTOR was partially repressed by fasting and extend earlier studies to show that immediately after a single bout of endurance exercise, signaling through mTOR was decreased further. The finding that 4E-BP1, but not S6K1, phosphorylation was decreased in the liver after exercise suggests that S6K1 may be more sensitive to fasting-induced changes in phosphorylation compared to 4E-BP1, or is controlled through both mTOR-dependent and independent signaling pathways.
The decrease in 4E-BP1 phosphorylation that occurred after exercise was associated with enhanced association of eIF4E with 4E-BP1; however, no change in binding of eIF4E to 4E-BP2 was observed. This result is surprising because the five best characterized phosphorylation sites are conserved between the two eIF4E binding proteins (Mader et al., 1995), suggesting that phosphorylation of 4E-BP1 and 4E-BP2, and therefore their binding to eIF4E, might be regulated through similar mechanisms. Moreover, the binding domain on 4E-BP1 for eIF4E (amino acids 51-61) exhibits extensive homology (9/11 identical, 11/11 similar) to the corresponding domain on 4E-BP2 (Mader et al., 1995), further suggesting that the interaction of eIF4E with 4E-BP1 and 4E-BP2 is similar. However, in HEK293 cells transfected with a constitutively active form of Akt, a kinase thought to be downstream of PI 3-kinase and upstream of mTOR in the insulin signaling pathway (reviewed in Gingras et al., 2001), 4E-BP2 is phosphorylated on fewer residues than 4E-BP1 (Gingras et al., 1998). Furthermore, although both 4E-BP1 and 4E-BP2 are phosphorylated in adenovirus-infected cells, the binding of 4E-BP1 to eIF4E is reduced to a significantly greater extent compared to the decrease in 4E-BP2 binding (Gingras & Sonenberg, 1997). Thus, although both proteins compete with eIF4G for binding to eIF4E, and therefore block assembly of the active eIF4F complex, it is likely that distinct, as well as overlapping, signaling pathways exist to regulate phosphorylation of the two proteins. This concept is supported by the previous chapter in which the dissociation of 4E-BP1 and 4E-BP2 followed different time courses. In the present chapter, reduced phosphorylation of 4E-BP1 immediately after exercise enhanced
formation of the 4E-BP1•eIF4E complex. The observation that the binding of eIF4E to 4E-BP2 was unaffected suggests that the signaling pathway(s) that modulate 4E-BP1 phosphorylation after exercise do not signal to 4E-BP2, or that such signaling is insufficient to induce dissociation of the 4E-BP2•eIF4E complex. Further delineation of the signaling pathways that regulate 4E-BP2 phosphorylation will be needed to understand the basis for the differential regulation of eIF4E binding to 4E-BP1 and 4E-BP2 in liver after exercise.

In contrast to the results of previous studies showing that global rates of protein synthesis are reduced in liver in response to chronic endurance exercise (Kasparek et al., 1980; Hayase & Yokogoshi, 1992), no change was observed following a single bout of treadmill running in the present study. This result suggests that the effect of exercise on global rates of protein synthesis in liver may be chronic rather than acute and is consistent with the observation that RNA content is reduced in livers of chronically exercised rats (Hayase & Yokogoshi, 1992). The fall in RNA content in response to chronic exercise is presumably due, at least in part, to a fall in ribosome content. The finding in the present study that the translation of mRNAs encoding specific ribosomal proteins is decreased is consistent with an effect of exercise to repress ribosome biogenesis in liver. However, the mechanism through which exercise acts to repress translation of mRNAs encoding ribosomal proteins is unclear. Under a variety of conditions, translation of TOP mRNAs is associated with increased phosphorylation and activation of S6K1 (reviewed in Meyuhas & Hornstein, 2000). Despite these observations, activation of S6K1 is not the only
mechanism through which TOP mRNA translation is regulated. In two recent studies (Barth-Baus \textit{et al.}, 2002; Stolovich \textit{et al.}, 2002), translation of TOP mRNAs was reported to be increased with no detectable change in phosphorylation of either S6K1 or rpS6. Instead, TOP mRNA translation was enhanced in cells expressing constitutively active forms of either PI 3-kinase or Akt (Stolovich \textit{et al.}, 2002), two kinases thought to be upstream of mTOR in the insulin signal transduction pathway. In the present study, the finding that translation of mRNAs encoding ribosomal proteins was repressed immediately after treadmill running with no detectable change in phosphorylation of S6K1 or rpS6 suggests that the incompletely defined signaling pathway involving PI 3-kinase and Akt reported by Stolovich \textit{et al.} (Stolovich \textit{et al.}, 2002) may be downregulated in liver in response to endurance exercise.

Two possible mechanisms through which endurance exercise might repress signaling through mTOR in liver are activation of AMPK and/or repressed signaling through the PI 3-kinase pathway. Several investigations of the effect of endurance exercise on various metabolic processes have reported that AMPK is activated in liver in response to exercise (Carlson \& Winder, 1999; Park \textit{et al.}, 2002). In part, the stimulation of AMPK is a consequence of the increased AMP and decreased ATP contents that are observed in liver in response to exercise (Dohm \& Newsholme, 1983). However, phosphorylation of AMPK on Thr172 is also an important determinant of AMPK activation (Hawley \textit{et al.}, 1996). In both models of treadmill running, phosphorylation of AMPK on Thr172 was unaffected. In part, the lack of effect on Thr172 phosphorylation might be due to the fasted state of the animals,
which could lead to constitutive activation of AMPK in liver. The strong signal observed for Thr172 is consistent with this idea. In the present study, although AMPK phosphorylation was enhanced in livers from rats following Protocol 1, as assessed by changes in migration during SDS-polyacrylamide gel electrophoresis, phosphorylation on Thr172 was unaffected. The identity of the other site(s) phosphorylated in response to exercise are unknown, however, it is tempting to speculate that phosphorylation of the putative residues after treadmill running might alter the ability of AMPK to regulate the mTOR signaling pathway. Several studies have implicated activation of AMPK in the regulation of protein synthesis and mTOR-mediated signaling (Bolster et al., 2002; Dubbelhuis & Meijer, 2002; Krause et al., 2002). The effect of AMPK activation on translational control of protein synthesis will be further discussed in Chapter V.

A second possible basis for the reduction in polysomal aggregation on mRNAs containing a 5’-TOP sequence in exercised rats is the fall in plasma insulin concentrations that has been previously observed in fasted rats that ran at a similar intensity (Winder et al., 1988). In a variety of cell types, insulin promotes mTOR activation through a signal transduction cascade involving PI 3-kinase (reviewed in Gingras et al., 2001). In the present study, phosphorylation of the β-subunit of the insulin receptor in liver was decreased in one model of treadmill running, Protocol 1, compared to control rats but did not change in the liver of rats following to Protocol 2. The results of both protocols suggest that the repression of insulin receptor activation is not necessary for the repression of 5’-TOP mRNA translation in the liver
following an acute bout of endurance exercise. Attempts to analyze PI 3-kinase signaling downstream of the receptor were unsuccessful as phosphorylation of Akt on its activation site, Ser 473, was undetectable in the fasted control livers. The possibility remains that a repression of signaling through PI 3-kinase may be altered through a different receptor signaling pathway and at least partially responsible for the decreased signaling downstream of mTOR; however, the results suggest that a detectable decrease in signaling through the PI 3-kinase signaling pathway is not required for a decrease in 5’-TOP translation.

While many endpoints were conserved between the two endurance exercise models, three phosphorylation changes were not conserved, the repression of eIF4G and insulin receptor β-subunit tyrosine phosphorylation and the increased phosphorylation of AMPK at sites other than Thr172. The reason for the discrepancies between the two models of treadmill running is not clear; however, several differences in the experimental protocol may have contributed to one or more of the discrepancies. First, greater than 90% of the animals following the Protocol 1 were unable to continue to run longer than 30 min while those animals that followed Protocol 2 continued to run with little motivation throughout the 60 min exercise period. Thus, rats following Protocol 1 stopped running earlier than rats following Protocol 2, suggesting that they were exposed to a greater amount of stress than rats following Protocol 2. Another indication of a greater degree of stress in rats following Protocol 1 was the observed increase in phosphorylation of AMPK at sites other than Thr172. As discussed in Chapter V, phosphorylation of AMPK at sites
other than Thr172 could be indicative of an activation of AMPK towards specific substrates including mTOR, and this hypothesis is supported in this chapter by the decreased phosphorylation of eIF4G (Ser1108), another rapamycin-sensitive phosphorylation event (Raught et al., 2000). Another confounding variable between the two treadmill models was the way in which the animals were sacrificed. Rats used in Protocol 1 were sacrificed immediately following the exercise bout via decapitation. Blood loss resulting from decapitation leads to an ischemic effect on the liver and has been shown to increase the phosphorylation of AMPK (Thr172) compared to livers that were frozen in place as in Protocol 2 (unpublished results). For this reason, rats subjected to Protocol 2 were anesthetized with Isoflurane, and the liver was subsequently frozen in place. Anesthesia as well as the manner of extraction could have contributed to one or more of the discrepancies between the two models. However, the conserved changes observed in the liver including the repression of 5’-TOP mRNA polysome association and 4E-BP1 are likely due to the effects of an acute bout of endurance exercise and could account for the reduction in RNA content and total protein synthesis in the chronic model of endurance exercise.
Figure 16  Treadmill running had no effect on protein synthesis in rat liver. Rats were randomly divided into control (light gray bar) or exercise (dark gray bar) groups, and the rats in the exercise group were run on a treadmill as described in Chapter II. A) The fractional rate of protein synthesis was evaluated using the flooding dose technique as described in Chapter II. Livers from five rats per group were analyzed. Control rat liver Ks=1.56 nmols phenylalanine/ (mg protein•hr). B) Aggregation of ribosomes was assessed as described in Chapter II. A representative profile is shown for control livers (left) and liver from exercised rats (right), n=6 per group.
Figure 17 Treadmill running specifically repressed the translation of TOP mRNAs in rat liver. The distribution of mRNA encoding eEF1α, rpS8, rpS6, β-actin, and GAPDH between polysomal and sub polysomal fractions was assessed as described in Chapter II. Results were normalized to the total amount of RNA recovered in the individual fractions and are expressed as a total of the combined sub polysomal and polysomal values. A) Distribution of mRNAs encoding β-actin and GAPDH as a ratio of polysomal:sub polysomal (light gray bars) control (n=5-6) and (dark gray bars) exercised rats (n=5-6). Values are expressed as means ± SEM. B) Distribution of mRNAs encoding eEF1α, rpS8, and rpS6. Values are expressed as means ± SEM. * denotes significant difference from controls (p<0.05) as assessed by a one-tailed t-test.
Figure 18 Phosphorylation of S6K1 and rpS6 did not change in rat liver after treadmill running. A) Hyperphosphorylation of S6K1 was assessed by Western blot analysis as described in Chapter II. A representative blot is shown in the inset. C, control rats; E, exercised rats. B) rpS6 phosphorylation was determined by Western blot analysis using a mixture of two polyclonal antibodies that recognize the Ser235/236 and Ser240/244 phosphorylation sites of rpS6. A representative blot for rpS6 phosphorylation is shown in the inset. Values are expressed as means ± SEM (n=6).
**Figure 19** Phosphorylation of 4E-BP1 decreased in rat liver after treadmill running. A) Hypophosphorylation of 4E-BP1 was measured by Western blot analysis and quantitated as the fraction of the protein present in the hypophosphorylated α-form. Results are presented as a fraction of the control value and expressed as the mean ± SEM. A representative blot is shown in the inset; the α, β, and γ forms of the protein are denoted to the left of the inset. C, control rats; E, exercised rats. *p=0.0032 compared to control (n=12). B) 4E-BP1 phosphorylation on Thr37 was measured by Western blot analysis using a polyclonal antibody that specifically recognizes 4E-BP1 phosphorylated on Thr37 as described in Chapter II. A representative blot is shown in the inset. Results are presented as a fraction of the control value and expressed as mean ± SEM. C, control rats; E, exercised rats. * p=0.0013 compared to control (n=6).
Figure 20  Association of 4E-BP1 or 4E-BP2 with eIF4E in rat liver after treadmill running.  A) The association of 4E-BP1 with eIF4E was measured by Western blot analysis of eIF4E immunoprecipitates and normalized to the relative amount of eIF4E in the immunoprecipitates as described in Chapter II. Results are presented as a fraction of the control value and expressed as the mean ± SEM. A representative blot of 4E-BP1 is shown in the inset. C, control; E, exercised rats. Control rats (light gray bar; n=6); exercised rats (dark gray bar; n=6). * p=0.0011 compared to control. B) The association of 4E-BP2 with eIF4E was measured by Western blot analysis using the same eIF4E immunoprecipitates as in panel and normalized to the amount of eIF4E in the immunoprecipitates. Results are presented as a fraction of the control value and expressed as the mean ± SEM. Control rats (light gray bar; n=6); exercised rats (dark gray bar; n=6). A representative blot of 4E-BP2 is shown in the inset. C, control; E, exercised rats.
**Protocol 1**

A. eIF4G (Ser10) Phosphorylation (Relative to Fasted Control)

B. eIF4G (Ser10) Phosphorylation (Relative to Fasted Control)

C. Insulin Receptor γ Subunit Phosphorylation (Relative to Fasted Control)

D. Insulin Receptor γ Subunit Phosphorylation (Relative to Fasted Control)

E. AMPK (Thr72) Phosphorylation (Relative to Control)

F. AMPK (Thr72) Phosphorylation (Relative to Control)

**Protocol 2**

G. AMPK (Thr72) Phosphorylation (Relative to Control)

H. AMPK (Thr72) Phosphorylation (Relative to Control)

**λ phosphatase**
Figure 21  Phosphorylation of eIF4G, the insulin receptor β-subunit, and AMPK differed between Protocol 1 and Protocol 2. Panels A, C, and E represent results using Protocol 1, and Panels B, D, F, and H represent results from Protocol 2. A) eIF4G (Ser1108) phosphorylation was assessed by Western blot analysis as described in Chapter II and normalized to total eIF4G. Control rats (light gray bar, n=12) or exercised rats (dark gray bar, n=12). Values are expressed as means ± SEM, *p<0.05. A representative blot of eIF4G (Ser1108) is shown in the inset: C, control and E, exercised. B) eIF4G (Ser1108) phosphorylation was assessed in the liver of control rats (light gray bar, n=6) or exercised rats (dark gray bar, n=6). Values are expressed as means ± SEM. A representative blot of eIF4G (Ser1108) is shown in the inset: C, control and E, exercised. C) Phosphorylation of the β-subunit of the insulin receptor on tyrosine residues was measured as described in Chapter II and normalized to the total insulin receptor β-subunit detected. Control rats (light gray bar, n=6) or exercised rats (dark gray bar, n=6). Results are presented as a fraction of the fasted control value and are expressed as means ± SEM, *p=0.0014 vs. control. A representative blot of the phosphorylated insulin receptor β-subunit is shown in the inset, C, control and E, exercised. D) Phosphorylation of the β-subunit of the insulin receptor on tyrosine residues was measured by Western blot analysis. Control rats (light gray bar, n=6) or exercised rats (dark gray bar, n=6). Results are presented as a fraction of the fasted control value and are expressed as means ± SEM. A representative blot of the phosphorylated insulin receptor β-subunit is shown in the inset, C, control and E, exercised. E) Phosphorylation of AMPK on Thr172 was measured in the liver of a control rat (light gray bar, n=6) or exercised rat (dark gray bar, n=6) by Western blot analysis using an antibody that recognizes AMPK phosphorylated on Thr172 as described in Chapter II and normalizing to total AMPK α. Results are presented as a fraction of the control value and as means ±SEM. F) Phosphorylation of AMPK on Thr172 was measured in the liver of a control rat (light gray bar, n=6) or exercised rat (dark gray bar, n=6) by Western blot analysis. Results are presented as a fraction of the control value and as means ±SEM. G) Liver homogenates were incubated with (+) or without (-) λ phosphatase as described in Chapter II prior to Western blot analysis. A representative blot is shown. C, control; E, exercised rats.
Chapter V. Repression of Protein Synthesis and mTOR Signaling in Rat Liver Mediated by the AMP-Activated Protein Kinase (AMPK) Activator 5-Aminoimidazole-4-Carboxamide Riboside (AICAR)

Introduction

AMPK acts as an energy sensor that responds to changes in the intracellular ratio of AMP:ATP (reviewed in Hardie & Carling, 1997). Activation of AMPK results in a stimulation of a variety of cellular processes involved in ATP production, e.g. glucose uptake (Vincent et al., 1996; Mu et al., 2001; Rutter et al., 2003) and fatty acid oxidation (Carling et al., 1989; Munday et al., 1991; Henin et al., 1995; Park et al., 2002), and a repression of energy consuming processes, e.g. fatty acid (Henin et al., 1995) and protein (Bolster et al., 2002; Dubbelhuis & Meijer, 2002; Horman et al., 2002) synthesis. The repression of protein synthesis by AMPK is associated with decreased signaling through the protein kinase referred to as mTOR (Dubbelhuis & Meijer, 2002; Krause et al., 2002; Inoki et al., 2003b). mTOR phosphorylates several proteins that play important roles in regulating the initiation phase of mRNA translation, including 4E-BP1 and S6K1 (Burnett et al., 1998) (see pg. 23, Fig. 5), and also regulates mRNA elongation through modulation of eEF2 kinase activity (Wang et al., 2001; Browne & Proud, 2004). 4E-BP1 binds to the mRNA cap binding protein eIF4E and prevents it from associating with a second initiation factor, eIF4G (Mader et al., 1995), and inhibits the mRNA binding step in translation initiation (Haghighat et al., 1995). Activation of S6K1 promotes
phosphorylation of its substrate, rpS6, and is associated with preferential translation of mRNAs containing a 5’TOP adjacent to the m7GTP cap (Jefferies et al., 1997). Such mRNAs include those encoding eEF1α and eEF2 as well as those encoding many of the ribosomal proteins (Levy et al., 1991; Jefferies & Thomas, 1994; Meyuhas, 2000).

In skeletal muscle of animals subjected to endurance exercise, the ratio of AMP:ATP increases in skeletal muscle resulting in AMPK activation (Sahlin & Broberg, 1990; Tullson & Terjung, 1991; Stephens et al., 2002). Endurance exercise is also associated with a reduction in protein synthesis (Hayase & Yokogoshi, 1992) and attenuation of signaling through mTOR (Reiter et al., 2004b) in the liver. However, although hyperphosphorylation of AMPK corresponds with repressed protein synthesis and decreased signaling through mTOR in the liver under such conditions, it is unclear whether activation of AMPK is sufficient to explain the observed changes. Thus, an alternative explanation for the repressed protein synthesis and decreased signaling through mTOR in the liver during endurance exercise might be a change in hormone or nutrient availability. In this regard, a decrease in either plasma insulin or amino acid concentrations or an increase in glucocorticoid levels might repress protein synthesis and signaling through mTOR in an AMPK-independent manner (Shah et al., 2000).

To test the hypothesis that activation of AMPK represses protein synthesis and signaling through mTOR in the intact liver, in the studies described in this Chapter, AICAR was administered to rats in vivo to directly activate AMPK and, in a
separate study, was infused into isolated perfused rat liver preparations *in situ*. In both the *in vivo* and *in situ* systems, activation of AMPK by AICAR was associated with decreased protein synthesis and phosphorylation of proteins downstream of mTOR, suggesting that activation of AMPK is sufficient to repress signaling through mTOR in rat liver.
Results

The relative phosphorylation of AMPK was assessed by gel-shift analysis as well as through the use of a phosphospecific antibody for Thr172, phosphorylation of which allows for maximal kinase activation (Hawley et al., 1996). As shown in Fig. 22 on pg. 107, AICAR-treatment in either the in vivo experimental model or the in situ liver perfusion experimental model resulted in reduced mobility of AMPK on SDS-PAGE compared to untreated controls. The reduced mobility of AMPK was most likely due to increased phosphorylation of AMPK because treatment of samples with lambda phosphatase prior to Western blot analysis resulted in a collapse of the signal into the fastest migrating band (see pg. 107, Fig. 22A). Assessment of the phosphorylation status of AMPK, quantitated as the proportion of the protein present in the slower migrating forms divided by the total intensity of the signal, demonstrated AICAR-induced hyperphosphorylation of the kinase (see pg. 107, Fig. 22B and 22C). While no significant change in AMPK phosphorylation at Thr172 was observed in the in vivo experimental model (see pg. 108, Fig. 23A), a significant increase was observed in the in situ liver perfusion experimental model comparing AICAR-treated livers to the controls (see pg. 108, Fig. 23B). Thus, both experimental models exhibited an increase in hyperphosphorylation of AMPK in response to AICAR, as assessed by a gel-shift analysis, whereas an increase in phosphorylation at the Thr172 site was only observed in the perfused liver.

Because AMPK phosphorylation increased, the flooding dose technique (Garlick et al., 1980) was utilized to assess the impact of AICAR treatment on the
rate of protein synthesis in rat liver. Following AICAR treatment, the rate of synthesis of total mixed liver protein was repressed to 18% and 45% of the control values in the \textit{in vivo} model (see pg. 109, Fig. 24A) and \textit{in situ} liver perfusion model (see pg. 109, Fig. 24B), respectively.

To assess whether translation initiation and/or elongation was repressed in response to AMPK activation, polysomal aggregation in the \textit{in situ} model was examined by sucrose density gradient centrifugation. In the present study, no change in polysomal aggregation was observed following perfusion of liver with AICAR suggesting that inhibition of both translation initiation and elongation contributed proportionately to the repression of protein synthesis (see pg. 110, Fig. 25A). To evaluate more directly the effect of AICAR treatment on elongation, the relative phosphorylation of eEF2 was evaluated. As shown in Fig. 25B on pg. 110, eEF2 phosphorylation at Thr56 was unaltered in livers perfused with AICAR suggesting that phosphorylation of this translation factor did not contribute to the reduction in the rate of elongation in this experimental model.

One mechanism involved in the regulation of global rates of protein synthesis involves the binding of met-tRNA\textsubscript{i} to the 40S ribosomal subunit (see pg. 20, Fig. 2), a process mediated by the initiation factors eIF2 and eIF2B (reviewed in Webb & Proud, 1997; Kimball, 1999). As shown in Fig. 26A and 26B on pg. 111, eIF2\textalpha phosphorylation at Ser51 and eIF2B\varepsilon phosphorylation at Ser535 were unchanged in the liver following \textit{in vivo} AICAR administration. Furthermore, eIF2B activity, as measured by an \textit{in vitro} activity assay, did not change in response to AICAR
administration (see pg. 111, Fig. 26C). Hence, the data suggest that the modulation of eIF2B activity is not involved in the marked repression of protein synthesis in the liver following administration of AICAR.

The effects of AICAR on mTOR-mediated signaling were assessed in both experimental models by quantitation of eIF4G phosphorylation at Ser1108, 4E-BP1 phosphorylation at Thr37, and S6K1 phosphorylation at Thr389 and hyperphosphorylation of 4E-BP1. A significant reduction in eIF4G phosphorylation at Ser1108 to 25% of the control value in the in vivo model and 56% in the in situ liver perfusion model was observed in response to AICAR administration (see pg. 112, Fig. 27A and 27B). A reduction in 4E-BP1 hyperphosphorylation, as assessed as the intensity of the γ-band divided by the intensity of all three bands, was observed in both experimental models following AICAR treatment (see pg. 112, Fig. 27C and 27D) as well as a reduction in 4E-BP1 phosphorylation at Thr37 (see pg. 112, Fig. 27E and 27F). Finally, phosphorylation of S6K1 on residue Thr389 was evaluated by Western blot analysis. No detectable S6K1 phosphorylation at the Thr389 site was observed in either the control or AICAR-treated liver in the in vivo model (see pg. 112, Fig. 27G); however, following perfusion of liver with AICAR, a decrease in S6K1 phosphorylation at Thr389 to 15% of the control value was observed in response to AICAR treatment (see pg. 112, Fig. 27H). Overall, the results suggest that phosphorylation of several mTOR substrates is repressed in the liver in response to AICAR administration.
Hyperphosphorylation of 4E-BP1 promotes its dissociation from eIF4E allowing eIF4E to bind to eIF4G (Mader et al., 1995). Because hyperphosphorylation of 4E-BP1 decreased in response to AICAR administration, the association of eIF4E with either 4E-BP1 or eIF4G was evaluated in eIF4E immunoprecipitates. As illustrated in Fig. 28A and 28B on pg. 114, the association of 4E-BP1 with eIF4E increased to 140% of the control value in the in vivo model and 280% of the control value in the in situ liver perfusion model in response to AICAR treatment. Further, following in vivo AICAR administration, the increase in eIF4E•4E-BP1 association paralleled the marked reduction in the relative amount of eIF4E•eIF4G association (see pg. 114, Fig. 28C); however, the reduction in eIF4E•eIF4G association was not significant in the liver perfusion model (p=0.1623) (see pg. 114, Fig. 28D). Thus, the results of both experimental models suggest that AICAR treatment leads to an increase in 4E-BP1•eIF4E association.

Finally, because AICAR administration is associated with decreased phosphorylation of mTOR on Ser2448 in skeletal muscle (Bolster et al., 2002), the serum insulin concentrations and mTOR phosphorylation on Ser2448 were assessed in the present models. As shown in Fig. 29A on pg. 115, following in vivo AICAR treatment, the serum insulin concentration decreased to 42% of the control value. Decreased signaling through the PI-3 kinase/ Akt signaling pathway is typically associated with a decrease in phosphorylation of mTOR on Ser2448 (Sekulic et al., 2000; Bolster et al., 2002). However, in the present study, an increase in phosphorylation of mTOR on Ser2448 was observed in the in vivo model (see pg.
115, Fig. 29B), but this observation was not observed in the \textit{in situ} model (see pg. 115, Fig. 29C). The reason for the increase in phosphorylation of mTOR on Ser2448 in the \textit{in vivo} model is not clear, but it suggests that this phosphorylation event does not always reflect changes in PI-3 kinase/ Akt signaling.
Discussion

In the studies described in this chapter, administration of AICAR to rats in vivo or addition of the AMP mimic to isolated perfused rat liver preparations resulted in hyperphosphorylation of AMPK, decreased global rates of protein synthesis, and reduced phosphorylation of several biomarkers of the mRNA binding step with no change in phosphorylation of eEF2 or two biomarkers of the met-tRNAi binding step in translation initiation. Three of the biomarkers of the mRNA binding step examined are downstream targets of mTOR signaling, 4E-BP1, S6K1, and eIF4G (see pg. 23, Fig. 5) (Pullen & Thomas, 1997; Burnett et al., 1998; Raught et al., 2000). The finding that phosphorylation of all three proteins is reduced by AICAR treatment in vivo and in situ strongly suggests that activation of AMPK represses signaling through mTOR. Moreover, because this effect was seen in the perfused liver, this indicates a direct effect of AICAR on the liver per se as opposed to AICAR eliciting some secondary effect (e.g. a decrease in serum insulin concentration or an increase in serum glucocorticoid concentration) that in turn affected the liver. This suggestion is also supported by previous studies in isolated hepatocytes (Dubbelhuis & Meijer, 2002; Krause et al., 2002). In those studies, activation of AMPK with AICAR was shown to prevent the amino acid-induced activation of S6K1. Because amino acids promote S6K1 activation through an mTOR-dependent signaling pathway (Anthony et al., 2001a; Reiter et al., 2004a), the previous studies concluded that activation of AMPK in hepatocytes represses signaling through mTOR. Moreover, activation of AMPK by fructose or glycerol (Dubbelhuis & Meijer, 2002)
or anoxia or oligomycin (Krause et al., 2002) similarly attenuates activation of S6K1 by amino acids, and expression of a constitutive active AMPK variant in human corneal epithelial (HCE)-T cells represses S6K1 activity in the absence of activators of AMPK (Kimura et al., 2003). Combined, the results demonstrate that activation of AMPK per se and not an indirect effect of AICAR on mTOR is responsible for the effect of the AMP mimetic in repressing signaling through mTOR. Although AICAR represses signaling through mTOR in liver (present study) and isolated hepatocytes (Dubbelhuis & Meijer, 2002; Krause et al., 2002), the effect may not be universal. Thus, although AICAR inhibits S6K1 activity in HCE-T cells and H4IIE hepatocytes, it has no effect on S6K1 activity in CHO-IR or HEK293 cells (Kimura et al., 2003). However, in HEK293 cells, 2-deoxyglucose both activates AMPK and inhibits S6K1 activity, suggesting that the lack of S6K1 inhibition by AICAR in those cells is not due to a lack of AMPK, but instead may be a result of insufficient phosphorylation of AICAR by adenosine kinase to generate enough 5-aminomimidazole-4-carboxamide 1-β-D-ribofuranosyl-5’-monophosphate (ZMP), the actual activator of AMPK (Sabina et al., 1985).

The question of how activation of AMPK results in decreased signaling through mTOR is unresolved. One study reports that activation of AMPK in C2C12 myotubes using AICAR results in enhanced phosphorylation of IRS-1 on Ser789 and that AMPK directly phosphorylates IRS-1 on Ser789 in vitro (Jakobsen et al., 2001). Moreover, phosphorylation of this residue reportedly is associated with a reduction in IRS-1-associated phosphatidylinositol (PI)3-kinase activity (Jakobsen et al., 2001).
Because PI3-kinase is upstream of mTOR (Brunn et al., 1996), AMPK-mediated phosphorylation of IRS-1 may explain in part the reduction in mTOR signaling. However, in HCE-T cells activation of AMPK by AICAR has no effect on Akt phosphorylation, an event downstream of PI3-kinase, even though AICAR clearly represses S6K1 phosphorylation (Kimura et al., 2003). Thus, phosphorylation of IRS-1 on Ser789 may not explain completely the repression of mTOR signaling by AMPK. An alternative explanation is provided by results from a recent study showing that AMPK directly phosphorylates TSC2 and that such phosphorylation enhances tuberin function (Inoki et al., 2003b). TSC2 is a GTPase-activator protein that negatively regulates a protein referred to as Rheb (see pg. 24, Fig. 6) (Inoki et al., 2003a; Zhang et al., 2003). Because Rheb is a positive regulator of mTOR signaling (Saucedo et al., 2003), activation of TSC2 by AMPK and subsequent inhibition of Rheb would be expected to repress mTOR-dependent signaling. Whether or not AICAR treatment results in TSC2 phosphorylation in liver is unknown.

In the chapter, the inhibition of signaling through mTOR by AICAR was associated with a decrease in the global rate of protein synthesis. However, in liver mTOR signaling does not appear to control global rates of protein synthesis. In this regard, administration of the specific mTOR inhibitor rapamycin to rats in vivo strongly attenuates phosphorylation of both 4E-BP1 and S6K1 (Reiter et al., 2004a). In addition, rapamycin prevents completely the activation of S6K1 by amino acids in isolated rat hepatocytes (Dubbelhuis & Meijer, 2002; Krause et al., 2002). However, rapamycin does not inhibit global rates of protein synthesis in liver in vivo (Reiter et
al., 2004a) and does not prevent the stimulation of protein synthesis by amino acids in hepatocytes (Dubbelhuis & Meijer, 2002). Instead, inhibition of signaling through mTOR is associated with decreased translation of a subset of mRNAs such as those containing a 5'-TOP sequence (Reiter et al., 2004a). Thus, AMPK-mediated repression of mTOR-dependent signaling may have a preferential, rather than global, effect on mRNA translation.

Other steps in mRNA translation that could contribute to global changes in mRNA translation include those involved in the binding of met-tRNA_i to the 40S ribosomal subunit and those that mediate translation elongation. The binding of met-tRNA_i to the 40S ribosomal subunit is mediated by the eIF2•GTP complex (see pg. 20, Fig. 2) (reviewed in Webb & Proud, 1997; Kimball, 1999). During a late step in the initiation process the GTP bound to eIF2 is hydrolyzed to GDP and eIF2 is released from the 40S ribosomal subunit as an eIF2•GDP binary complex. In order for eIF2 to bind to met-tRNA_i, the GDP bound to eIF2 must be exchanged for GTP, a process catalyzed by the guanine nucleotide exchange protein, eIF2B. Mechanisms for regulating eIF2B activity include phosphorylation of eIF2 on Ser51 of its α-subunit and phosphorylation of eIF2B on Ser535 of its ε-subunit, both of which repress the activity of the protein (reviewed in Webb & Proud, 1997; Kimball, 1999). However, in the present study, no change in eIF2α(Ser51) or eIF2Bε(Ser535) phosphorylation or eIF2B activity was observed in liver of AICAR-treated rats, suggesting that inhibition of the met-tRNA_i binding step is not responsible for the global inhibition of protein synthesis caused by AICAR.
The best characterized mechanism for regulating translation elongation is phosphorylation of eEF2 on Thr56 (Ovchinnikov et al., 1990; Price et al., 1991). Phosphorylation of eEF2 on Thr56 is associated with decreased rates of elongation. However, no changes in eEF2 phosphorylation at Thr56 were observed in the present study, suggesting that AICAR does not promote phosphorylation of eEF2 on Thr56 in the liver. This result is in contrast to a report that activation of AMPK by AICAR, oligomycin, or carbonyl cyanide m-chlorophenylhydrazone (CCCP) increased eEF2 phosphorylation at Thr56 in rat ventricular myocytes (McLeod & Proud, 2002). In that study, eEF2 phosphorylation at Thr56 was maximal within 5 min of the start of treatment, and returned to control values within 20 min. In the present study, livers were analyzed 60 min after the administration of AICAR to rats in vivo, or after 30 min of the start of perfusion. Thus, eEF2 phosphorylation at Thr56 may have increased soon after administration of AICAR, but returned to control values before the livers were harvested for analysis.

An apparent discrepancy between the in vivo and in situ models used herein is the finding that AICAR promoted dephosphorylation of S6K1 on Thr389 in isolated perfused rat livers, but not in livers of animals administered AICAR in vivo. The different results obtained using the two models is likely a result of animals used for the in vivo study being fasted overnight prior to administration of AICAR, whereas for the in situ study, livers were perfused with medium containing amino acids at 5 times the level measured in plasma of fasted rats and 11 mM glucose. Thus, in the in vivo model, S6K1 phosphorylation at Thr389 was almost completely
dephosphorylated in control animals, whereas S6K1 phosphorylation at Thr389 in control perfused livers was enhanced as a result of provision of amino acids. The finding that 4E-BP1 hyperphosphorylation was greater in livers perfused in the absence of AICAR compared to livers from control rats in vivo likewise supports this suggestion. These results also support the idea that activation of AMPK by AICAR suppresses the enhanced phosphorylation of S6K1 and 4E-BP1 caused by amino acids, and are in agreement with reports by others in isolated rat hepatocytes (Dubbelhuis & Meijer, 2002; Krause et al., 2002).

Overall, the results of the present study extend earlier observations in isolated rat hepatocytes to show that activation of AMPK in liver represses signaling to multiple downstream targets of mTOR. AICAR administration also inhibits global rates of protein synthesis in liver, and results from sucrose density gradient analysis suggest that the reduction is a result of an inhibition of both translation initiation and elongation. Therefore, future studies will be required to define the mechanism(s) through which activation of AMPK causes a repression of global rates of protein synthesis as well as the functional consequences of the downregulated mTOR-mediated signaling.
AICAR treatment stimulated AMPK phosphorylation. A) Liver samples from the in vivo AICAR study were prepared in the presence of phosphatase inhibitors or in the absence of phosphatase inhibitors followed by lambda phosphatase treatment as described in Chapter II. The results are representative of 6 samples per group that were analyzed: C, control; A, AICAR treated. B and C) Hyperphosphorylation of AMPK was evaluated by Western blot analysis using an anti-AMPK α antibody (in vivo model B and in situ model C). Results are expressed as the proportion of AMPK in the hyperphosphorylated form. B) Light gray bar, control rats (n=11); dark gray bar, AICAR-treated rats (n=5). *p<0.0001 vs. control. C) Light gray bar, control livers perfused without AICAR (n=11); dark gray bars, livers perfused with AICAR (n=11). *p=0.0192 vs. control.
AMPK phosphorylation at Thr172 was unaffected following \textit{in vivo} AICAR administration but was increased following \textit{in situ} perfusion with AICAR. AMPK phosphorylation at Thr172 was assessed by Western blot analysis using an anti-phospho-AMPK (Thr172) antibody and normalized to the total amount of AMPK detected with an anti-AMPK α antibody. A) Light gray bar, control rats (n=4); dark gray bar, AICAR treated rats (n=5). B) Light gray bar, livers perfused without AICAR (n=10); dark gray bar, livers perfused with AICAR (n=9), * p=0.0192 vs. control. Representative blots are shown in the insets: C, control; A, AICAR treated.
Figure 24  AICAR treatment reduced global rates of liver protein synthesis. Rates of protein synthesis were assessed using the flooding dose technique as described in Chapter II and are expressed as a percentage of their respective controls. Values represent the mean ± SEM for each group. A) Light gray bar, control rats (n=6); dark gray bar, AICAR treated rats (n=8). *p<0.001 vs. control. Control Ks=3.11 nmols phenylalanine/ (mg protein•hr). B) Light gray bar, livers perfused without AICAR (n=8); dark gray bar, livers perfused with AICAR (n=7). *p<0.001 vs. control. Control Ks=22.9 mg protein/(g of liver•hr).
Figure 25 AICAR treatment had no effect on either polysomal aggregation or eEF2 phosphorylation at Thr56 in perfused rat liver. A) Polysomal aggregation was analyzed as described in Chapter II. Control, left panel and AICAR treated, right panel. The results are representative of 8 livers that were analyzed in each treatment group. B) eEF2 phosphorylation at Thr56 was assessed as describe in Chapter II and was normalized to the total amount of eEF2. The relative phosphorylation is expressed as a fraction of the control value (n=8, control and n=7, AICAR). A representative blot of eEF2 (Thr56) phosphorylation is shown in the inset: C, control; A, AICAR perfused rat liver.
Figure 26  eIF2α and eIF2Bε phosphorylation and eIF2B activity were unaffected by *in vivo* administration of AICAR.  A) eIF2α phosphorylation at Ser51 was assessed as described in Chapter II and normalized to the total amount of eIF2α.  A representative blot of eIF2α phosphorylation is shown in the inset: C, control; A, AICAR treated.  Values are expressed as means ± SEM for each group, n=10 per condition.  B) eIF2Bε phosphorylation at Ser535 was evaluated as described in Chapter II and normalized to the total amount of eIF2Bε.  A representative blot of eIF2Bε phosphorylation is shown in the inset: C, control; A, AICAR treated.  Values are expressed as means ± SEM for each group, n=10 per condition.  C) eIF2B activity was assessed by an *in vitro* activity assay as described in Chapter II.  Values are expressed as means ± SEM for each group, n=6 control and n=4 AICAR treated.
**Figure 27** AICAR treatment repressed mTOR-mediated signaling as assessed by eIF4G phosphorylation at Ser1108, 4E-BP1 hyperphosphorylation, 4E-BP1 phosphorylation at Thr37, and S6K1 phosphorylation at Thr 389. Panels A, C, E, and G represent results using the in vivo model, and panels B, D, F, and H represent results from isolated liver perfusion preparations. A) eIF4G phosphorylation at Ser1108 was assessed in liver of control rats (light gray bar, n=6) or AICAR-treated rats (dark gray bar, n=6) by performing Western blot analysis using an anti-phospho-eIF4G (Ser1108) antibody and then normalized to the total amount of eIF4G. A representative blot of eIF4G phosphorylation is shown in the inset: C, control; A, AICAR treated rat. Values are expressed as means ±SEM for each group, *p=0.0024.

B) eIF4G phosphorylation at Ser1108 in livers perfused in the absence (light gray bar, n=7) or presence (dark gray bar, n=7) of AICAR. A representative blot is shown in the inset: C, control, A, AICAR-treated. Values are expressed as means ±SEM for each group, *p=0.0002.  C) Hyperphosphorylation of 4E-BP1 was measured by Western blot analysis in liver of control rats (light gray bars, n=10) and AICAR-treated rats (dark gray bar, n=10) as described in Chapter II. Results are expressed as the percent of the protein present in the hyperphosphorylated γ-form relative to total. A representative blot is shown in the inset: C, control, A, AICAR-treated. *p<0.0001 vs control.  D) Hyperphosphorylation of 4E-BP1 in livers perfused in the absence (light gray bar, n=8) or presence (dark gray bar, n=8) of AICAR. A representative blot is shown in the inset: C, control, A, AICAR-treated. *p<0.0001 vs control.  

E) 4E-BP1 phosphorylation at Thr37 was measured by Western blot analysis in liver of control rats (light gray bars, n=10) and AICAR-treated rats (dark gray bar, n=10) using an anti-phospho-4E-BP1(Thr37) antibody (note that this antibody cross reacts with 4E-BP1 phosphorylated on Thr45). A representative blot is shown in the inset: C, control, A, AICAR-treated. *p<0.001 vs control.  F) 4E-BP1 phosphorylation at Thr37 in livers perfused in the absence (light gray bar, n=8) or presence (dark gray bar, n=7) of AICAR. A representative blot is shown in the inset: C, control, A, AICAR-treated. *p<0.0028 vs control.  

G) S6K1 phosphorylation at Thr389 was measured by Western blot analysis in liver of control rats (n=10) and AICAR-treated rats (n=10) using an anti-phospho S6K1(Thr389) antibody. n.d., none detected.  H) S6K1 phosphorylation at Thr389 in livers perfused in the absence (light gray bar, n=8) or presence (dark gray bar, n=7) of AICAR. A representative blot is shown in the inset: C, control, A, AICAR-treated. *p<0.0001 vs control.
**Figure 28** AICAR treatment represses the assembly of the eIF4F complex. A) The amount of 4E-BP1 associated with eIF4E was examined by Western blot analysis of eIF4E immunoprecipitates as described in Chapter II using a polyclonal antibody that recognizes all forms of 4E-BP1 and normalizing to the amount of eIF4E in the immunoprecipitates as detected with a monoclonal eIF4E antibody. Light gray bar, control rats (n=13); drak gray bar, AICAR treated rats (n=13). Values are expressed as means ± SEM for each group. A representative blot of 4E-BP1 is shown in the inset: C, control; A, AICAR treated rat. *p=0.0068 vs. control. B) 4E-BP1 associated with eIF4E in livers perfused in the absence (light gray bar, n=7) and presence (dark gray bar, n=6) of AICAR. A representative blot of 4E-BP1 is shown in the inset: C, control; A, AICAR treated. *p=0.002 vs. control. C) eIF4G associated with eIF4E was measured by Western blot analysis of eIF4E immunoprecipitates as described in Chapter II and normalized to the amount of eIF4E detected in the immunoprecipitate. Light gray bar, control (n=13); dark gray bar, AICAR treated (n=13). A representative blot of eIF4G is shown in the inset: C, control and A, AICAR. *p=0.0229 vs. control. D) eIF4G associated with eIF4E in livers perfused in the absence (light gray bar, n=4) or presence (dark gray bar, n=3) of AICAR. A representative blot of eIF4G is shown in the inset: C, control and A, AICAR treated.
Figure 29  The serum insulin concentration decreased whereas phosphorylation of mTOR on Ser2448 increased following in vivo AICAR treatment. A) Serum insulin concentrations were assessed by RIA as discussed in Chapter II and normalized to control values. Values are expressed as means ± SEM, *p=0.001 vs. control. B) Phosphorylation of mTOR on Ser2448 was assessed by Western blot analysis as described in Chapter II and normalized to total mTOR. Light gray bar, control rats (n=6); drak gray bar, AICAR treated rats (n=6). Values are expressed as means ± SEM for each group. A representative blot of phosphorylated mTOR is shown in the inset: C, control; A, AICAR treated rat, *p=0.0224 vs. control. C) Phosphorylation of mTOR on Ser2448 was assessed by Western blot analysis as described in Chapter II and normalized to total mTOR. Light gray bar, livers perfused in the absence of AICAR (n=8); drak gray bar, livers perfused in the presence of AICAR (n=7). Values are expressed as means ± SEM for each group. A representative blot of phosphorylated mTOR is shown in the inset: C, control; A, AICAR treated rat.
Chapter VI. Conclusions and Future Directions

The results of Chapters III-V are summarized in Table 5 on pg. 123. Food intake, endurance exercise, and AICAR administration each have different effects on the global rate of protein synthesis in the liver. Food intake stimulates the global rate of protein synthesis in the liver, and this stimulation is sustained for an extended period of time. In contrast, a single bout of endurance exercise did not alter the rate of protein synthesis, and AICAR administration resulted in a significant repression in the global rate of protein synthesis. The causative factor(s) for the change in protein synthesis in response to food intake or AICAR administration is difficult to explain. Although changes in signaling through mTOR were observed in both models, several studies indicate that rapamycin-treatment has no effect on protein synthesis in the liver (Reiter et al., 2004a) or isolated primary hepatocytes (Dubbelhuis & Meijer, 2002). Thus, a rapamycin-insensitive mechanism must contribute to the change in hepatic protein synthesis following food intake or AICAR administration. In both models, the change in the rate of global protein synthesis displayed a positive correlation with the change in eIF4G(Ser1108) phosphorylation. These results emphasize the need for a greater understanding of the function of eIF4G phosphorylation. Furthermore, while most of the work presented above focused on the regulation of translation initiation, the results of the polysome profile analysis suggest that both translation initiation and elongation are decreased following AICAR treatment. Therefore, the possibility exists that a coordinated change in translation initiation and elongation is responsible for the observed changes in total protein
synthesis following food intake and AICAR administration. Further study is needed to define additional targets of AMPK and the mTOR-independent regulation of total protein synthesis.

Translational control of gene expression in the liver is altered to respond to physiological perturbations. In response to food intake, mTOR signaling was rapidly activated as indicated by the increased phosphorylation of two of its substrates, 4E-BP1 and S6K1. The increased phosphorylation of 4E-BP1 and S6K1 coincided with an elevation in the polysome content of mRNAs containing a 5’-TOP sequence, suggesting that one or both of these phosphorylation events contributed to the previously observed stimulation of total RNA content following feeding. In contrast, following a single bout of treadmill running, a decrease in phosphorylation of one substrate of mTOR, 4E-BP1, was observed, whereas S6K1 phosphorylation or phosphorylation of its substrate, rpS6, did not change. The decrease in 4E-BP1 phosphorylation paralleled an increase in the association of 4E-BP1 with eIF4E and a decrease in the polysome association of 5’-TOP mRNAs. The initial changes in translation of ribosomal proteins could contribute to the subsequent reduction in total RNA content observed in response to chronic exposure to endurance exercise (Hayase & Yokogoshi, 1992).

While studies suggest that translation of 5’-TOP mRNAs is regulated by the activity of S6K1 and phosphorylation of its substrate rpS6 (Meyuhas, 1996; Jefferies et al., 1997), two recent studies suggest otherwise. In these studies, the stimulation of 5’-TOP translation in response to amino acids or mitogens occurred in the absence of
a change in rpS6 phosphorylation (Tang et al., 2001; Stolovich et al., 2002). In these studies, the authors suggested that translation of mRNAs containing a 5’-TOP sequence relied more heavily on signaling through the PI-3 kinase signaling. However, they did acknowledge that, because rapamycin-treatment did repress translation of mRNAs containing a 5’-TOP sequence to a lesser extent, the possibility existed that another substrate of mTOR could function in this regulation. In the present study, an increase in S6K1 phosphorylation and rpS6 phosphorylation coincided with increased polysome association of 5’-TOP mRNAs following feeding. However, immediately following an acute bout of endurance exercise, a decrease in polysome association of mRNAs containing a 5’-TOP sequence was observed in the absence of a change in rpS6 phosphorylation, providing the first in vivo evidence that a change in rpS6 phosphorylation is not required for a change in translation of mRNAs containing a 5’-TOP sequence. Thus, the possibility exists that another mTOR-substrate is involved in the regulation of 5’-TOP mRNA translation. One common element of both the feeding and exercise induced changes in polysome association of 5’-TOP mRNAs is 4E-BP1. The phosphorylation state of 4E-BP1 consistently paralleled with the changes observed in 5’-TOP translation. Combined the results suggest that 4E-BP1 could also function in the regulation of translation of mRNAs containing a 5’-TOP sequence.

Further, as discussed in Chapter I, regulation of 4E-BP1 under various conditions has been extensively studied while little is known about the other 4E-BPs. The results of the feeding and endurance exercise model demonstrate for the first time
in vivo distinct regulation of 4E-BP1 and 4E-BP2, suggesting that, while both proteins bind to and sequester eIF4E, they may each possess unique functions. In support of this idea, as discussed in Chapter IV, different signaling pathways could regulate phosphorylation of 4E-BP1 compared to 4E-BP2 (Grolleau et al., 1999). Previous studies using of differentiating myeloid cells suggest that 4E-BP1 and 4E-BP2 could also function in the regulation the expression of different subsets of genes. In these studies, two different cell lines, a HL-60 promyelocytic leukemia cell line and an U-937 monoblastic cell line, were stimulated with retinoic acid or DMSO to differentiate. HL-60 cells differentiate into granulocytes whereas U-937 cells differentiate into monocytes. Of interest were the changes in 4E-BP1 and 4E-BP2. In the case of the HL-60 promyelocytic cells, following treatment with DMSO or retinoic acid the protein content of 4E-BP1 decreased and the protein content of 4E-BP2 increased. In contrast, following treatment of the U-937 cells with DMSO or retinoic acid, the phosphorylation of 4E-BP1 decreased with no change in content of either 4E-BP1 or 4E-BP2. From these results, the authors concluded that 4E-BP1 and 4E-BP2 could function in the regulation of gene expression and through this mechanism regulate the differentiation of the two cell lines.

Based on the results of the polysome profile analysis and the eIF4E association data, future studies are needed to gain better insight into the regulation and function of both 4E-BP1 and 4E-BP2. One useful model for these analysis is the development of 4E-BP1, 4E-BP2, and 4E-BP1/2 knockout mice. Little published data is available in terms of any phenotypic perturbations resulting from the deletion
of 4E-BP2 or both 4E-BP1 and 4E-BP2. In the case of the 4E-BP1 knockout mouse, one report identified a loss of white adipose tissue and an increase in the abundance of brown adipose tissue (Tsukiyama-Kohara, K. et al., 2001), further supporting the hypothesis that expression and/or regulation of the different 4E-BPs regulates the expression of different subsets of genes. A second advantage of the use of the mice is that physiological experiments, similar to those described in Chapters III and IV, could be performed to assess the necessity of 4E-BP1 and/or 4E-BP2 on the translational regulation of gene expression as well as total protein synthesis in liver and other metabolically active tissues. However, the possibility exists that in the knockout models the remaining 4E-BPs could compensate for the loss of one or more of the other 4E-BPs. For this reason, the use of gene silencing technology in cell culture could provide a better model. In this case transient transfection of siRNA for either one or more of the three known 4E-BPs could be performed prior to stimulation with mitogens or amino acids and followed by polysome profile analysis to assess translation of 5′-TOP and non-TOP mRNAs. Furthermore, treatment with rapamycin or PI-3 kinase inhibitors could also be performed to assess the relative necessity of the binding proteins in the repression of 5′-TOP and non-TOP translation following mitogen or amino acid stimulation.

Additionally, the results of the microarray analysis of polysome associated mRNAs following feeding identified 36 mRNAs that did not contain a 5′-TOP sequence suggesting additional mechanisms are present and regulate expression of specific genes. The translational regulation of one of these mRNAs, Hsp8a, was
confirmed by QRT-PCR. While the polysomal distribution of all of the non-TOP mRNAs was not examined, the results suggest that a large number of mRNAs may be regulated through a unique manner(s) and respond to similar signaling mechanisms as the 5’-TOP mRNAs. Several possible models could be involved in the translational regulation of these mRNAs such as a high degree of secondary structure in the 5’-UTR and/ or the association of RNA binding proteins. Phosphorylation of 4E-BP1 promotes its dissociation from eIF4E and formation of the eIF4F complex. As the eIF4F complex also includes an RNA helicase, dissociation of 4E-BP1 and eIF4E is associated with increased translation of mRNAs with a high degree of secondary structure (reviewed in Schepers & Proud, 2002). Thus, some of the mRNAs lacking a 5’-TOP sequence could contain a high degree of secondary structure and allow for greater regulation of their expression. In contrast, mRNA interacting proteins have been implicated in regulating translation of specific proteins (reviewed in Dreyfuss et al., 2002). Thus, the structure of the 5’-UTR of mRNAs lacking a 5’-TOP sequence may alter the ability of the ribosome to recognize the start site or the ability of regulatory proteins to interact with and modulate translation of a particular RNA.

The present work examined translational regulation of gene expression in the liver using two physiological models: food intake and endurance exercise, and the regulation of mTOR signaling in response to AMPK activation. Changes in translation of mRNAs containing a 5’-TOP sequence displayed a positive correlation with 4E-BP1 phosphorylation, suggesting that 4E-BP1 may contribute to the translational regulation of mRNAs containing a 5’-TOP sequence. Additionally, the
results of the feeding study suggest that a large number of mRNAs, that appear to lack a 5’-TOP sequence, are translationally regulated. The results also suggest that AMPK is an important mediator of translational control through its regulation of signaling through mTOR. Together, the results emphasize the need for further study into the mechanism through which AMPK exerts its effects on mTOR and total protein synthesis, the role of 4E-BP1 in regulating 5’-TOP translation, and alternative mechanisms for regulating translation of specific mRNAs in the liver.
Table 5: Summary of Data Presented in Chapters III-V

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Meal Feeding</th>
<th>Exercise Protocol 1</th>
<th>Exercise Protocol 2</th>
<th>AICAR In vivo</th>
<th>AICAR In situ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Synthesis</td>
<td>↑</td>
<td>N/C</td>
<td>N/A</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Serum Insulin</td>
<td>↑</td>
<td>↓</td>
<td>N/A</td>
<td>↓</td>
<td>N/A</td>
</tr>
<tr>
<td>Polysome Aggregation on 5’-TOP mRNA</td>
<td>↑</td>
<td>↓ (n.s)</td>
<td>↓</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>S6K1 (Thr389)</td>
<td>↑</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>↓</td>
</tr>
<tr>
<td>rpS6</td>
<td>↑</td>
<td>N/C</td>
<td>N/C</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4E-BP1 Hyperphosphorylation</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>eIF4G (Ser1108)</td>
<td>↑</td>
<td>↓</td>
<td>N/C</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>eIF4E•eIF4G</td>
<td>↑</td>
<td>↓</td>
<td>N/D</td>
<td>↓</td>
<td>↓ (n.s)</td>
</tr>
<tr>
<td>eIF4E•4E-BP1</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>eIF4E•4E-BP2</td>
<td>↓</td>
<td>N/C</td>
<td>N/C</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>AMPK Hyperphosphorylation</td>
<td>N/C</td>
<td>↑</td>
<td>N/C</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>AMPK (Thr172)</td>
<td>N/C</td>
<td>N/C</td>
<td>N/C</td>
<td>N/C</td>
<td>↑</td>
</tr>
</tbody>
</table>

Key

↑ - increased
↓ - decreased
N/A - not available
N/C - no change
N/D - not detectable
n.s. - not significant
Literature Cited


DHALAKIA, J. N., MUESER, T. C., WOODLEY, C. L., PARKHURST, L. J. & WAHBA, A. J. (1986). The association of NADPH with the guanine nucleotide exchange


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AK Reiter, DR Bolster, SJ Crozier, SR Kimball, and LS Jefferson. Repression of Protein Synthesis andmTOR Signaling in Rat Liver Mediated by the AMP-Activated Protein Kinase Activator 5-Aminoimidazole-4-Carboxamide Riboside. AJP: Endocrinology and Metabolism. Submitted