

Metabolic enzymes that bind RNA: yet another level of cellular regulatory network?

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Several enzymes that were originally characterized to have one defined function in intermediary metabolism are now shown to participate in a number of other cellular processes. Multifunctional proteins may be crucial for building of the highly complex networks that maintain the function and structure in the eukaryotic cell possessing a relatively low number of protein-encoding genes. One facet of this phenomenon, on which I will focus in this review, is the interaction of metabolic enzymes with RNA. The list of such enzymes known to be associated with RNA is constantly expanding, but the most intriguing question remains unanswered: are the metabolic enzyme–RNA interactions relevant in the regulation of cell metabolism? It has been proposed that metabolic RNA-binding enzymes participate in general regulatory circuits linking a metabolic function to a regulatory mechanism, similar to the situation of the metabolic enzyme aconitase, which also functions as iron-responsive RNA-binding regulatory element. However, some authors have cautioned that some of such enzymes may merely represent “molecular fossils” of the transition from an RNA to a protein world and that the RNA-binding properties may not have a functional significance. Here I will describe enzymes that have been shown to interact with RNA (in several cases a newly discovered RNA-binding protein has been identified as a well-known metabolic enzyme) and particularly point out those whose ability to interact with RNA seems to have a proven physiological significance. I will also try to depict the molecular switch between an enzyme’s metabolic and regulatory functions in cases where such a mechanism has been elucidated. For most of these enzymes relations between their enzymatic functions and RNA metabolism are unclear or seem not to exist. All these enzymes are ancient, as judged by their wide distribution, and participate in fundamental biochemical pathways.

Keywords: enzymes, mRNA binding, gene expression, translation regulation

One of the methods used most often in detecting direct RNA–protein interaction *in vitro* is electrophoretic mobility shift assay, in which a complex of radioactively labelled RNA with protein is electrophoresed and visualized by autoradiography. The other is filter-binding assay, in which only protein (and protein with bound labelled RNA) binds to the filter that is subsequently measured for ra-

dioactivity. To ensure specific RNA–protein interaction, an excess of unlabelled competitor RNA (for example yeast tRNA) is routinely added to the protein prior to incubation with labelled RNA. In some cases to resolve the RNA–protein complex into specific complexes a combination of heparin and RNase T1 is used. Additionally, to show the specificity of particular RNA–protein interaction, competition ex-

Abbreviations: ARE, AU-rich element; CKBB, creatine kinase brain form B; COLBP, cytochrome *c* oxidase L-form transcript-binding protein; COX, cytochrome *c* oxidase; DHFR, dihydrofolate reductase; eALAS, erythroid isoform of 5-aminolevulinic synthase; FdUMP, fluorodeoxyuridine monophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; GDK, phosphoglycerate kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; gRNA, guide RNA; HADHB, hydroxyacyl coenzyme A dehydrogenase/3-ketoacyl-thiolase/enoyl-coenzyme A hydratase (trifunctional protein), β subunit; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HPIV, human parainfluenza virus; IDH, isocitrate dehydrogenase; IRES, internal ribosomal entry site; IRE, iron-responsive element; IRF, iron-regulatory factor; IRP, iron-regulatory protein; LDH, lactate dehydrogenase; meTHF, N^{5,10}-methylene tetrahydrofolate; MTX, methotrexate; MyHC, myosin heavy chain; RT-PCR, reverse transcription-polymerase chain reaction; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase; uPAR, urokinase-type plasminogen activator receptor; UTR, untranslated region.

Table 1. Metabolic enzymes that interact with RNA

Enzyme	Catalyzed reaction	Binds	References
Aconitases			
Vertebrate cytoplasmic aconitase (IRP1)	citrate \rightarrow isocitrate	ferritin mRNA 5'UTR (IRE) transferrin mRNA 3'UTR (IRE) ferroportin mRNA 5'UTR (IRE) DMT-1 mRNA 3'UTR (IRE)	Hentze & Kuhn, 1996; Hirling <i>et al.</i> , 1994; Klausner <i>et al.</i> , 1993 (reviews); Abboud & Haile, 2000; Gunshin <i>et al.</i> , 2001;
<i>E. coli</i> aconitases		eALAS mRNA 5'UTR (IRE)	Cox <i>et al.</i> , 1991; Dandekar <i>et al.</i> , 1991;
Yeast mitochondrial aconitase (Aco1p)		m-acon mRNA 5' UTR (IRE) acnA and acnB mRNA 3'UTR (IRE)	Kim <i>et al.</i> , 1996; Tang & Guest, 1999;
Enzymes involved in glycolysis and the pentose cycle			
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	3-phosphoglyceraldehyde + NAD ⁺ + P _i \rightarrow 1,3-diphosphoglycerate + NADH + H ⁺	tRNA IFN- α , c-myc, GM-CSF and IL-2 mRNA 3'UTR (ARE) GLUT1 mRNA 3'UTR (ARE) ribosomal RNA TNF- α hammerhead ribozyme HAV RNA 5'UTR (IRES) HAV RNA 3'UTR (ARE) and 3'coding HCV RNA 3'UTR HBV HPIV RNA 3'UTR (U-rich) HDV antigenomic RNA MyHC mRNA 3'UTR	Singh & Green, 1993; Nagy & Rigby, 1995; Nagy <i>et al.</i> , 2000; McGowan & Pekala, 1996; Ryazanov, 1985; Sioud & Jespersen, 1996; Chang <i>et al.</i> , 1993; Schultz <i>et al.</i> , 1996; Yi <i>et al.</i> , 2000; Dollenmaier & Weitz, 2003; Petrik <i>et al.</i> , 1999; Zang <i>et al.</i> , 1998; De <i>et al.</i> , 1996; Lin <i>et al.</i> , 2000a; Kiri & Goldspink, 2002;
Aldolase	fructose-1,6-bisphosphate \leftrightarrow glyceraldehyde-3-phosphate + dihydroxyacetone phosphate	MyHC mRNA 3'UTR	Kiri & Goldspink, 2002;
Lactate dehydrogenase (LDH)	lactate + NAD ⁺ \leftrightarrow pyruvate + NADH + H ⁺	GM-CSF mRNA 3'UTR (ARE)	Pioli <i>et al.</i> , 2002;
Phosphoglycerate kinase (PGK)	1,3-diphosphoglycerate + ADP \rightarrow 3-phosphoglycerate + ATP	uPAR mRNA	Shetty <i>et al.</i> , 2004; Shetty & Idell, 2004;
Glucose 6-phosphate dehydrogenase (G6PDH)	glucose-6-phosphate + NADP ⁺ \rightarrow 6-phosphogluconolacton + NADPH	GLUT1 mRNA 3'UTR (ARE)	McGowan & Pekala, 1996;
Enzymes connected with the TCA cycle			
Glutamate dehydrogenase (GDH)	L-glutamate + NAD(P) ⁺ + H ₂ O \leftrightarrow 2-oxoglutarate + NH ₄ ⁺ + NAD(P)H	cytochrome <i>c</i> oxidase mRNA gRNA 3'oligo(U)	Preiss <i>et al.</i> , 1993; 1995; Bringaud <i>et al.</i> , 1997; Estevez <i>et al.</i> , 1999;
NAD ⁺ -dependent isocitrate dehydrogenase (IDH)	isocitrate + NAD ⁺ \rightarrow α -ketoglutarate + CO ₂ + NADH + H ⁺	yeast mitochondrial mRNA 5'UTR (ARE)	Papadopoulou <i>et al.</i> , 1990; Dekker <i>et al.</i> , 1992; Elzinga <i>et al.</i> , 1993; de Jong <i>et al.</i> , 2000; Anderson <i>et al.</i> , 2002;

Enzymes connected with fatty acid metabolism

Enoyl-CoA hydratase	$\Delta^{2,3}$ - <i>trans</i> -enoyl-CoA + H ₂ O → 3-hydroxyacyl-CoA	mRNA 3'UTR (ARE)	Nakagawa <i>et al.</i> , 1995; Nakagawa & Moroni, 1997; Brennan <i>et al.</i> , 1999;
Flesh fly 3-oxoacyl-CoA thiolase Human HADHB, β subunit	3-oxoacyl-CoA + CoA → acetyl-CoA + fatty acyl-CoA with 2 fewer carbon atoms	sarcotoxin IIA mRNA 3'UTR renin mRNA 3'UTR	Nanbu <i>et al.</i> , 1993; Adams <i>et al.</i> , 2003;

Enzymes involved in thymidylate synthesis cycle

Thymidylate synthase (TS)	Deoxyuridine monophosphate + N ^{5,10} -methylene-tetrahydrofolate → deoxythymidine monophosphate + dihydrofolate	TS mRNA p53 mRNA <i>c-myc</i> mRNA various RNAs	Chu <i>et al.</i> , 1991; Cieřła <i>et al.</i> , 2005; Ju <i>et al.</i> , 1999; Chu <i>et al.</i> , 1999; Chu <i>et al.</i> , 1995; Chu <i>et al.</i> , 1996; Cieřła <i>et al.</i> , 2005;
Dihydrofolate reductase (DHFR)	Dihydrofolate + NADPH + H ⁺ → tetrahydrofolate + NADP ⁺	DHFR mRNA	Chu <i>et al.</i> , 1993; Ercikan-Abali <i>et al.</i> , 1997; Tai <i>et al.</i> , 2002; 2004;
Serine hydroxymethyltransferase (SHMT)	Tetrahydrofolate + Ser ↔ methylenetetrahydrofolate + Gly meTHF → 5-formyl-THF	SHMT mRNA 5'UTR	Liu <i>et al.</i> , 2000;

Other RNA-binding metabolic enzymes

Uracil phosphoribosyltransferase (PyrR)	uracil + PRPP → UMP + PP _i	<i>pyr</i> mRNA	Ghim & Neuhard, 1994; Turner <i>et al.</i> , 1998; Tomchick <i>et al.</i> , 1998; Chander <i>et al.</i> , 2005;
Creatine kinase brain form B (CKBB)	creatine + ATP → phosphocreatine + ADP	αMyHC mRNA 3'UTR	Vracar-Grabar & Russell, 2004;
Mevalonate kinase (MVK)	mevalonate + ATP → mevalonate-5-phosphate + ADP	luteinizing hormone receptor mRNA (polypyrimidine-rich)	Nair & Menon, 2004;
Catalase	2H ₂ O ₂ → 2H ₂ O + O ₂	catalase mRNA 3'UTR	Clerch <i>et al.</i> , 1996;

Enzymes involved in RNA metabolism displaying RNA-binding activities outside their well-established functions

Aminoacyl-tRNA synthetases	amino acid + tRNA + ATP ↔ aminoacyl-tRNA + AMP + PP _n	various cognate and non-cognate mRNAs	Springer <i>et al.</i> , 1985, 1989; Schray & Knippers, 1991; Miseta <i>et al.</i> , 1991;
Endoribonuclease (CSP41), homologous to nucleotide-sugar epimerases and dehydrogenases	ss and ds RNA cleavage	<i>petD</i> pre-mRNA 3'UTR (stem-loop and ARE)	Yang <i>et al.</i> , 1996; Yang & Stern, 1997; Baker <i>et al.</i> , 1998;

petD, chloroplast gene encoding subunit IV of the cytochrome *b₆f* complex; *pyr*, pyrimidine nucleotide biosynthetic operon.

periments are conducted with the use of unlabelled RNA that binds the protein and competes with labelled RNA or various unlabelled unrelated RNAs that are unable to compete with the labelled RNA. Dissociation constants (K_d) for specific RNA-protein interaction are within low picomolar to high nanomolar values, however, only for few RNA-binding enzymes have they been measured.

IRP1/CYTOSOLIC ACONITASE

The best-studied example of a specific RNA-binding protein – whose interaction with its cognate binding site in mRNA (iron-responsive element, IRE) is regulated in response to metabolic signals – is iron-regulatory protein (IRP), formerly known as iron-regulatory factor (IRF) or IRE-binding protein (IRE-BP) (reviewed by Klausner & Rouault, 1993; Hentze & Kuhn, 1996; Rouault, 2002; Cairo *et al.*, 2002).

The iron-responsive element (IRE), forming a hairpin structure, is highly conserved in evolution for any given gene and is remarkably similar among different genes harboring such elements, suggesting precise structural constraints in the binding to IRPs (Henderson *et al.*, 1994). Studies on plant and yeast cells, which lack endogenous IRP activity, indicate that the binding of IRP to the IRE is sufficient to regulate translation without requirement for additional *cis*-acting sequences or *trans*-acting factors (Kim *et al.*, 1995), although adjacent non-IRE sequences may affect the function of an IRE (Dickey *et al.*, 1988; Dix *et al.*, 1993).

Actually, two structurally and functionally similar cytoplasmic IRPs (IRP1 and IRP2) modulate stability and translation of mRNA encoding proteins involved in regulation of iron homeostasis, i.e. transferrin receptor (TfR, receptor for cellular iron uptake) and ferritin (Ft, iron storage protein). IRPs bind with high affinity ($K_d = 5\text{--}50$ pM; Butt *et al.*, 1996) to IRE in the 3' UTR of TfR mRNA protecting it from degradation and leading to enhanced synthesis of TfR protein. On the other hand, IRPs bind to an IRE in 5' UTR of Ft mRNA causing translational repression and leading to decreased synthesis of Ft protein.

IRP1 was purified from human liver (Rouault *et al.*, 1989) and, after partial peptide sequencing, the cDNA encoding this protein was cloned (Rouault *et al.*, 1990) making it possible to find a significant homology with two eukaryotic mitochondrial aconitases (Hentze & Argos, 1991). Then, bovine cytosolic aconitase was purified, demonstrated to possess an IRE-binding activity and shown by peptide sequencing to be identical with IRP1 (Kennedy *et al.*, 1992). The mitochondrial enzyme lacks the IRE-binding activity, but it also seems to have an additional func-

tion, independent of the enzyme's catalytic activity – it has been found to be associated with mitochondrial DNA and shown to be essential for its maintenance (Chen *et al.*, 2005). IRP1, but not IRP2, can switch between the mRNA-binding and enzymatic activities according to cell iron levels. In iron-replete cells the [4Fe-4S] catalytic cluster is assembled, the IRE-binding site is not accessible, and IRP1 displays aconitase activity; in iron-depleted cells the cluster is lacking and IRP1 apoprotein functions as an RNA-binding protein (Constable *et al.*, 1992; Klausner & Rouault, 1993; Hentze & Kuhn, 1996). This reversible switch allows aconitase/IRP1 to constantly sense iron levels and adapt them to cell requirements without any appreciable change in protein levels. The state of the [4Fe-4S] cluster is also changed by oxygen species and by $\bullet\text{NO}$ generated by endogenous synthases or exogenous donors (reviewed by Drapier, 1997; Hanson & Leibold, 1999; Cairo & Pietrangelo, 2000), providing a direct regulatory linkage between iron metabolism and oxidative stress. The cluster's stability also depends on the phosphorylation state of a serine residue required for RNA binding (Brown *et al.*, 2002). The enzymatic function of cytosolic aconitase, which is the product of a gene distinct from that encoding the mitochondrial TCA cycle aconitase, has been well characterised, but its role in the metabolism of the cell is not well understood and remains to be established. According to studies in yeast (Narahari *et al.*, 2000), an iron-mediated conversion of IRP1 to aconitase would have the advantage of increasing the NADPH level, thus providing reducing equivalents helpful in maintaining the redox balance.

In addition to modulating ferritin and transferrin receptor levels, IRP can regulate other IRE-containing mRNAs, encoding proteins closely related to iron utilization (erythroid 5-aminolevulinic synthase, eALAS), uptake (proton-coupled iron transporter, DMT1) and release (ferroportin) (Cox *et al.*, 1991; Dandekar *et al.*, 1991; Abboud & Haile, 2000; Gunshin *et al.*, 2001). Studies of the aconitase family of proteins in prokaryotes and eukaryotes support the suggestion that IRP may also control the synthesis of proteins that are not direct regulators of iron metabolism, such as mammalian mitochondrial aconitase (Kim *et al.*, 1996; Chen *et al.*, 1997), *Escherichia coli* aconitases A and B (Tang & Guest, 1999), *Drosophila melanogaster* succinate dehydrogenase (Kohler *et al.*, 1995; Gray *et al.*, 1996), and *Bacillus subtilis* cytochrome oxidase (Alen & Sonenshein, 1999), all of which contain an IRE or IRE-like element in the mRNA. The results of these studies point to regulatory link between iron and energy metabolism in vertebrates, invertebrates and bacteria.

Recently, Lin and colleagues (2001) discovered that protein levels of the 75-kDa Fe-S subunit of mi-

tochondrial respiratory chain Complex I were modulated by levels of iron in the cell, whereas mRNA levels were minimally changed. They found that the 5' UTR of the 75-kDa subunit mRNA contained a novel IRE-like stem-loop sequence. RNA-protein gel shift assays demonstrated that a specific cytoplasmic protein, being neither IRP1 nor IRP2 (IRP3?), bound the novel IRE and that the binding of the protein was affected by the iron status. In addition, ferritin IRE was able to compete for binding with this putative IRP. These results suggest that the 75-kDa Fe-S subunit of mitochondrial Complex I may be regulated by a novel IRE-IRP system.

As the numbers of target IRE-containing mRNAs and iron-regulatory proteins seem to be growing, novel IRP-IRE interactions regulating and connecting other essential metabolic pathways are awaiting discovery.

ENZYMES INVOLVED IN GLYCOLYSIS AND THE PENTOSE CYCLE

Several NAD⁺-dependent dehydrogenases and kinases, among them the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and phosphoglycerate kinase (PGK), have been shown to bind RNA. Although they were found to bind different RNAs, they share common features associated with this activity. They possess coenzyme binding domains, remarkably similar in three-dimensional structure, consisting of two identical $\beta\alpha\beta$ -folds each associated with a mononucleotide-binding surface (Rossmann fold; Rossmann *et al.*, 1975), whose similarity is not due to a highly conserved primary structure, but rather to homology at crucial positions in order to ensure similar folding patterns (Wierenga *et al.*, 1986). It has been shown that (di)nucleotides (NAD⁺, NADH, ATP) can compete with RNA binding. Results of mutational studies showed that the N-terminal 43 amino-acid residues of GAPDH, which correspond to the first mononucleotide-binding domain of the NAD-binding fold, are sufficient to confer RNA-binding (Nagy *et al.*, 2000). Studies on RNA-binding combined with peptide mapping localized RNA-binding to the predicted N-terminal 6.8-kDa peptide, known to be involved in the formation of the NAD⁺-binding domain of GAPDH (Nagy & Rigby, 1995). These results suggest an involvement of the (di)nucleotide binding fold in RNA binding. Nagy and coworkers (2000) proposed adding the NAD-binding domain to the list of RNA-binding domains/motifs. Most of RNA-binding proteins that contain a Rossmann fold interact with AU-rich elements (ARE) of mRNA. The ARE elements confer message instability as well as translational sup-

pression (Shaw & Kamen, 1986; reviewed by Espel, 2005). The combination of functionally and structurally different sequence motifs, including AU pentamers and nonamers and other U-rich stretches, determines the ultimate destabilizing ability of each particular ARE. Numerous proteins have been reported to specifically recognize these elements and suggested to play a role in the regulation of stability and translatability of various lymphokine, cytokine and proto-oncogene mRNAs.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), aldolase, glucose-6-phosphate dehydrogenase (G6PDH), phosphoglycerate kinase (PGK)

Glyceraldehyde-3-phosphate dehydrogenase has generally been considered to be a housekeeping protein involved mainly in glycolysis, but a number of studies have indicated that it displays diverse activities (reviewed by Sirover, 1999; Ishitani *et al.*, 2003). The protein can act as a kinase, a tubulin- and actin-binding protein, play a role in membrane fusion, and act as a target for nitric oxide (Modun *et al.*, 2000; Mazzola & Sirover, 2002). It has been shown that GAPDH is overexpressed and translocated to the nucleus during apoptosis (Sunaga *et al.*, 1995). In the growing cells GAPDH can act as uracil-DNA-glycosylase and helicase (Meyer-Sigler *et al.*, 1991) and participates in the control of transcription and DNA repair (Ronai, 1993). The enzyme has been shown to cleave RNA (Evguenieva-Hackenberg *et al.*, 2002). GAPDH has been found to bind specifically to sequences critical in the regulation of several mRNAs (see Table 1). These include *cis*-acting elements important in the stability and translation of mRNA, i.e. AU-rich elements in 3'UTR sequences of lymphokine mRNAs, regions involved in viral transcription and replication, i.e. 3'UTR sequences involved in RNA-dependent RNA polymerase binding for mRNA synthesis, and 5' UTR sequences involved in HAV replication and functioning as ribosomal recognition sites (IRES, internal ribosome entry site) enabling cap-independent translation initiation (with $K_d = 0.2$ nM; Yi *et al.*, 2000). The enzyme has also been shown to bind with high affinity ($K_d = 18$ nM) to several tRNAs in a manner that most likely involves both sequence and structure (Singh & Green, 1993). The physiological significance of the *in vitro* studies was confirmed by studies *in vivo*, including identification of GAPDH/lymphokine mRNA association in polysomes (Nagy & Rigby, 1995) or the presence of GAPDH in RNP particles in human parainfluenza virus-infected cells (De *et al.*, 1996). The activity of GAPDH as either a glycolytic enzyme or an ARE-binding protein may be regulated by the local concentration of NAD⁺, NADH, and ATP in dif-

ferent subcellular compartments (polysomes, cytosol, and nucleus). The Rossmann fold of GAPDH appears to be reciprocally regulated between its RNA-binding (inactive in glycolysis) and NAD⁺-binding (active in glycolysis) states *in vivo* (Nagy & Rigby, 1995). Alternatively, post-translational modifications (redox or otherwise) might influence the intracellular location of GAPDH as well as differentially regulate its RNA- or NAD⁺-binding activity. Nagy and Rigby (1995) have found that incubation of GAPDH with the reducing agent 2-mercaptoethanol enhances the binding of both lymphokine 3' UTR and tRNA probes, whereas treatment with an oxidizing agent, diamide, markedly decreases the binding of GAPDH to the IFN- α 3' UTR. On the other hand, Arutyunova and coworkers (2003) have shown that binding of total tRNA to reduced apo-GAPDH is much lower than to the oxidized enzyme form.

Recently, GAPDH and another glycolytic enzyme, aldolase, were identified as proteins complexed with the 3' UTR sequence of myosin heavy chain (MyHC) mRNA (Kiri & Goldspink, 2002). The authors suggest that both enzymes could be involved in transporting the MyHC mRNA to specific regions and/or anchoring of the message in regions of myosin synthesis by association with the cytoskeletal framework. This hypothesis is supported by studies showing that glycolytic enzymes interact with microtubules (Walsh *et al.*, 1989).

Another glycolytic enzyme, lactate dehydrogenase, is a tetramer that may exist in five forms each consisting of combinations of subunits designated H (for heart) and M (for skeletal muscle). LDH-M (the isoenzyme consisting of four identical skeletal muscle type subunits) has been identified as an ARE-binding protein interacting with granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA 3' UTR and mediating ARE-dependent translation (Pioli *et al.*, 2002). The RNA-protein binding *in vitro* and competition with NAD⁺ was demonstrated using UV cross-linking and nitrocellulose filter binding assays. The latter assay indicated that the binding was ARE-specific, as the affinity of purified LDH for mutant AUGUA GM-CSF mRNA was markedly diminished in comparison with wild-type GM-CSF mRNA ($K_d = 1879$ nM and 501.9 nM, respectively). Immunoprecipitation, sucrose density gradient analysis and other data collectively demonstrated that LDH is a polysome-associated protein localized to this compartment by RNA-protein interactions. Moreover, direct association of LDH with AUF1, another ARE-binding protein, that may play multiple roles in the regulation of mRNA turnover, was demonstrated (reviewed by Dean *et al.*, 2004). One potential model for LDH function is that it provides further specificity for the translation and turnover of certain ARE-containing mRNAs through its direct

interaction with both ARE and AUF1 protein. There is also a possibility that LDH may act independently of AUF1 and play a distinct role in mRNA translation and turnover. Although both GAPDH and LDH bind to ARE of GM-CSF mRNA, their roles in post-transcriptional gene regulation seem to be different. LDH, but not GAPDH, interacts with AUF1 and appears to be more specific in its interaction with ARE. In addition, LDH binds to GM-CSF mRNA with greater affinity compared to GAPDH (Pioli *et al.*, 2002).

GAPDH, LDH and an enzyme involved in the pentose cycle, glucose-6-phosphate dehydrogenase (G6PDH), have been shown to specifically bind to various regions of the basal glucose transporter (GLUT1) mRNA 3' UTR (McGowan & Pekala, 1996). The authors hypothesized that binding of one of the dehydrogenases involved in glucose metabolism could control the GLUT1 mRNA half-life and would provide a rapid mechanism for controlling glucose transport, the rate-limiting step of glucose utilization. Comparison of gel mobility shifts obtained with purified enzymes and GLUT1 mRNA 3' UTR to those obtained using authentic cell extracts indicates that GAPDH and G6PDH may play a role in intact cells.

Phosphoglycerate kinase, like several other glycolytic enzymes, has been shown to possess a wide range of biological activities in addition to its well-characterized role in glycolysis, among them possible regulation of virus transcription through interaction of PGK (and another glycolytic enzyme, enolase) with tubulin (Ogino *et al.*, 2001). Recently, the protein purified from cultured human bronchial epithelial cells that specifically reacted with urokinase-type plasminogen activator receptor (uPAR) mRNA has been identified as PGK (Shetty *et al.*, 2004). Expression of uPAR controls several cellular functions, including epithelial cell adhesion, signaling and mitogenesis, because most of the biological activities of urokinase plasminogen activator (uPA) are dependent on its association with uPAR (Vassali *et al.*, 1991; Chapman, 1997). The uPA action yields plasmin, a serine protease, which has been implicated in the pathogenesis of several lung diseases. PGK expressed in both eukaryotic and prokaryotic cells bound *in vitro* to the coding region of uPAR mRNA (but not to the ARE of uPAR 3' UTR) as shown by gel mobility and Northwestern assays, and a direct *in vivo* interaction was confirmed by combined immunoprecipitation-RT-PCR (Shetty *et al.*, 2004). PGK overexpression in cultured cells inhibited cell surface uPAR expression, decreased rate of proliferation and migration of cells most likely through destabilization of uPAR mRNA. Evidence indicate that phosphorylation of PGK may play a major role in regulating its ability to bind uPAR mRNA (Shetty & Idell, 2004). The physiologic relevance of the con-

nection between uPAR mRNA decay and glycolysis remains unknown.

ENZYMES CONNECTED WITH THE TCA CYCLE

Glutamate dehydrogenase (GDH)

Several subunits of mammalian cytochrome *c* oxidase (COX) have two tissue-specific isoforms referred to as the L-form (for liver) and H-form (for heart). The H-isotypes are only found in the heart and skeletal muscle and are regulated at the level of transcription, whereas the L-isotypes are present in all tissues, and several observations have suggested that their regulation is likely to occur at the level of mRNA expression. A protein termed COLBP (cytochrome *c* oxidase L-form transcript-binding protein) has been shown to recognize sequences or structures within the 3' UTRs of transcripts encoding the L-, but not H-forms of COX (subunits VIII and VIIa). Moreover, tissue-specific distribution of COLBP activity paralleled the presence of the liver isopeptides in the mature oxidase complex (Preiss *et al.*, 1993), which indicated that COLBP may mediate the tissue-specific regulation of COX liver isoform mRNA expression, possibly by increasing mRNA stability. COLBP has been identified as mitochondrial matrix glutamate dehydrogenase (GDH) (Preiss & Lightowlers, 1993; Preiss *et al.*, 1995). Binding of mRNAs for COX subunits by GDH *in vitro* was inhibited by GTP and NADH in combination, but not by either alone, and partial inhibition of complex formation was noted in the presence of ADP. Mammalian L-glutamate dehydrogenase, catalyzing the reversible oxidative deamination of glutamate to α -ketoglutarate and ammonia, utilizing either NAD⁺ or NADP⁺, has potential importance in linking several metabolic pathways. It possesses many complex properties, being allosterically regulated by numerous effectors and exhibiting concentration-dependent polymerization from a basic hexameric unit to massive polymers (Fisher, 1985). Immunohistochemical data showed two distinct mitochondrial matrix locations for GDH, one soluble and one membrane-associated (de Duve *et al.*, 1962; Knecht *et al.*, 1986). Scatchard analysis and surface plasmon resonance techniques have shown that only a subset of GDH is able to bind RNA, and this subset was shown to be phosphorylated (Preiss *et al.*, 1995). The mutually exclusive subcellular locations for the nuclear-encoded COX subunit mRNA and GDH seem incompatible with an *in vivo* interaction and consequently with any role in transcript protection or expression. However, it has been hypothesized that the RNA-binding domain of inner-mitochondrial-membrane-associated GDH may span both membranes at contact sites

and function to localize several species of cytosolic mRNA at the mitochondrial periphery, anchoring the translation complex to the mitochondrial surface (Preiss *et al.*, 1995; Lightowlers *et al.*, 1996). In accord, a radiolabelled mRNA transcript was found bound to intact mitochondria (Preiss *et al.*, 1995). However, this interesting hypothesis has not been, to my knowledge, verified by further studies.

Studies in trypanosomes provided two facts confirming the affinity between GDH protein and certain sequences or structures in COX mRNA 3' UTR: i) the 3' UTR of cytochrome oxidase subunit II (COX II) mRNA serves exclusively as a *cis* guide RNA for the coding region of the COX II mRNA (Golden & Hajduk, 2005), and ii) mitochondrial GDH from *Leishmania tarentolae* has been identified as a protein that binds to 3' oligo(U) of guide RNA (gRNA) (Bringaud *et al.*, 1997). Guide RNAs are small RNAs (40–70 nt), typically transcribed from maxicircle DNA independent of their own cognate mRNA. They are complementary to fragments of mature RNA (Blum *et al.*, 1990; Pollard *et al.*, 1990), whose editing involves the insertion and deletion of uridine residues at multiple sites mainly within their coding regions (reviewed by Benne, 1994; Estevez & Simpson, 1999; Stuart *et al.*, 2005). Taking into account that GDH may bind UTP in competition with NADP(H) and that *L. tarentolae* *in vitro* editing activity was also inhibited by high concentrations of NADP(H), it was proposed that GDH might be involved in editing reaction and could represent a link between mitochondrial metabolism and RNA editing (Bringaud *et al.*, 1997). However, the results of GDH-knockout experiments in the homologous bloodstream *T. brucei* suggest that the gRNA-binding properties of GDH may not play a role in RNA editing (Estevez *et al.*, 1999).

Despite the very interesting hypotheses regarding the RNA-binding activities of GDH in mammals and trypanosomes, there is no conclusive proof for a physiological role of these interactions.

NAD⁺-specific isocitrate dehydrogenase (IDH)

Another mitochondrial dehydrogenase, NAD⁺-specific isocitrate dehydrogenase, that is proposed to regulate flux through the TCA cycle primarily *via* a positive allosteric response to AMP (Kornberg & Pricer, 1951; Hathaway & Atkinson, 1963), has been demonstrated to bind specifically to 5' UTRs of the eight major yeast mitochondrial mRNAs, encoding inner membrane-localized components of electron transport chain, including three subunits of cytochrome *c* oxidase, three subunits of ATP synthase and apocytochrome *b* (Dekker *et al.*, 1992). Most leader sequences of these transcripts are very long and AU-rich, and are not capped at their 5' ends.

Specific protein binding sites are localized in different positions with respect to the start codon, but they share secondary structural features that may direct binding of IDH. Unlike for the dehydrogenases described above, IDH-mRNA complex formation in gel shift assays appeared not to be affected by addition of either substrate, cofactor or allosteric effectors of the enzyme (Elzinga *et al.*, 1993; Anderson *et al.*, 2002); sedimentation experiments, however, have shown that the binding of RNA by IDH is affected by AMP in the presence of isocitrate. The enzymatic activity of IDH has been found to be allosterically inhibited by mRNA (Anderson, *et al.*, 2000) and this inhibition is not prevented by the presence of NAD⁺ (Elzinga *et al.*, 1993; Anderson *et al.*, 2002). In addition, the amino-acid residues identified by site-directed mutagenesis as necessary for RNA binding by IDH were not important for enzyme activity (Elzinga, 2001). Thus the catalytic and RNA-binding sites seem to be distinct. It is not surprising, since crystallographic analyses of related enzymes (Hurley *et al.*, 1991; Imada *et al.*, 1991) have revealed that the NAD⁺-binding sites of decarboxylating dehydrogenases lack the classical Rossmann fold.

The relevance of the IDH-RNA interaction *in vivo* is suggested by studies of de Jong *et al.* (2000), who showed the rates of mitochondrial translation to be increased 1.5–3-fold in yeast strains lacking IDH as compared to normal yeast strain, in accord with a similar increase of the translation product's turnover. They suggested that IDH may suppress translation of mRNAs in the mitochondrial matrix until the transcripts are in the vicinity of mRNA-specific membrane-bound translational activators (Grivell, 1995) and thus ensure correct localization of electron transport chain components. Since AMP modulates IDH enzymatic activity and apparently also IDH association with RNA, mitochondrial levels of AMP may be a primary determinant of both the catalytic function in the tricarboxylic acid cycle and the rates of translation of mitochondrial mRNAs. It seems possible that the interaction of IDH with mitochondrial mRNAs could provide a mechanism for coordinate control of metabolite flux through the TCA cycle and of mitochondrial gene expression at the level of translation.

ENZYMES CONNECTED WITH METABOLISM OF FATTY ACIDS

Enoyl-CoA hydratase

The protein termed AUH was originally purified from human brain using affinity chromatography on the AU-rich element (ARE) of interleukin 3, and was found to exhibit enoyl-CoA hydratase activity

(Nakagawa *et al.*, 1995). AUH amino-acid sequence exhibited significant homology to both long- and short-chain rat mitochondrial enoyl-CoA hydratases (Kamijo *et al.*, 1993). The protein was able to bind ARE-containing 3' UTR of GM-CSF mRNA, as shown by Northwestern blot analysis. Recombinant AUH has been shown to bind specifically to ARE-containing mRNA 3' UTRs in GM-CSF, IL-3, c-fos, c-myc and adenoviral AdIVwt transcripts. However, the primary structure of AUH does not contain either a Rossmann fold-forming sequence or any previously described RNA-binding motifs. RNA-binding analysis of deletion mutants revealed a 20 amino-acid sequence that was necessary and sufficient for RNA binding. A distinctive feature of this segment is that it contains multiple lysine residues which provide a positive charge (Nakagawa & Moroni, 1997). The crystal structure of human AUH protein revealed the distances between these Lys residues to be similar to those between RNA phosphate groups, suggesting that the so called "lysine comb" may bind to ssRNA (Kurimoto *et al.*, 2001). AUH protein was found to bind to a solid ARE-displaying matrix with retained enzymatic activity, suggesting that the RNA binding domain is different from the catalytic domain (Nakagawa *et al.*, 1995). The 20-amino-acid RNA-binding domain of human AUH, as well as sequences thought to constitute the enzyme's active site, are 100% conserved in murine AUH. Murine AUH has also been shown to be a bifunctional protein exhibiting enzymatic and RNA-binding activities (Brennan *et al.*, 1999). It has been shown, using Western blot analysis and immunoelectron microscopy, that AUH is located in the mitochondria of mouse cells and therefore allows the possibility that ARE-mediated RNA decay and/or processing mechanisms may also function in this organelle. As an ARE-binding protein that contains intrinsic enoyl-CoA hydratase activity, AUH may provide a link between mitochondrial metabolic pathways and RNA stability.

Thiolase

In a search for factors that bind to the ARE of flesh fly (*Sarcophaga peregrina*) defense protein, sarcotoxin IIA mRNA, Nanbu *et al.* (1993) purified a protein that also had a strong affinity for poly(A), poly(U), and poly(C) and some affinity for poly(G) and poly(I). These results suggested that the protein may interact with poly-pyrimidine rich regions or the poly(A) tract of RNAs. From the partial amino-acid sequence it was shown that this protein has a striking similarity to rat mitochondrial and peroxisomal thiolases and yeast cytoplasmic thiolase. The protein has been demonstrated to have 3-oxoacyl-thiolase activity (see Table 1) similar to rat mitochondrial thiolase. Moreover, purified rat mitochondria thio-

lase also had an RNA-binding activity, which suggests that this activity is an intrinsic feature of the thiolase (Nanbu *et al.*, 1993). The effect of the binding of sarcotoxin IIA mRNA by flesh fly thiolase on mRNA stability has not been tested and the possible physiologic role of this interaction has not been, to my knowledge, elucidated.

Hydroxyacyl coenzyme A dehydrogenase/3-ketoacyl-thiolase/enoyl-coenzyme A hydratase (trifunctional protein), β subunit (HADHB)

Renin, secreted by renal juxtaglomerular cells, is rate-limiting in angiotensin II generation and tightly regulated in order to ensure expression appropriate to need. One of the levels of this control is renin mRNA half-life. Although renin mRNA does not contain a classical ARE, an AU-rich region at the very end of the 3' UTR has high homology to a region of *Sarcophaga peregrina* sarcotoxin IIA mRNA. This 34-nucleotide sequence had a destabilizing effect on a linked reporter gene. Yeast three-hybrid screening of a fetal kidney cDNA library, with the renin 3' UTR as a bait, isolated hydroxyacyl coenzyme A dehydrogenase/3-ketoacyl-thiolase/enoyl-coenzyme A hydratase (trifunctional protein), β subunit (HADHB) as a novel mRNA binding protein. Various gel separation and antibody techniques, as well as use of recombinant HADHB, confirmed this result (Adams *et al.*, 2003). Immunoprecipitation and RT-PCR confirmed association of HADHB with renin mRNA *in vivo*. Upon decreasing HADHB mRNA with RNAi in renin-expressing human pulmonary adenocarcinoma cells, renin protein and mRNA stability increased, pointing to destabilization of renin mRNA by HADHB. Also the thiolase activity may be important in renin mRNA turnover, since 4-bromocrotonic acid (4-BCA), a specific thiolase inhibitor, reduced binding of cell extract proteins, among them HADHB, to renin 3' UTR *in vitro*, and cultured cells treated with 4-BCA had elevated renin protein levels over the course of several hours (Adams *et al.*, 2003). The cleavage of disulphide bonds, that are now regarded rather as switches for protein function than mere structural motifs (Hogg, 2003), might affect the function of certain renin mRNA-binding proteins. Intracellular imaging revealed distinct localization of HADHB to the inner mitochondrial membrane, yet similarly to GDH that binds nuclear-encoded COX subunit mRNA, the data support an ability of HADHB to bind renin mRNA.

Since several other mitochondrial proteins, among them AUH and 3-oxoacyl-CoA thiolase from *S. peregrina*, can bind mRNA, a novel potential link between mitochondrial processes (such as β -oxidation) and mRNA metabolism is emerging.

ENZYMES INVOLVED IN THYMIDYLATE SYNTHESIS CYCLE

All three enzymes involved in thymidylate synthesis cycle, thymidylate synthase (TS), dihydrofolate reductase (DHFR) and serine hydroxymethyltransferase (SHMT) have been shown to bind their own mRNA within the 5' UTR or/and the coding region of the message and to inhibit the cognate mRNA translation.

TS catalyzes the formation of thymidine monophosphate using the $N^{5,10}$ -methylene tetrahydrofolate cofactor (meTHF) as the reductant and donor of one-carbon group. The second product of TS reaction, dihydrofolate, is reduced by an NADPH-utilizing enzyme, DHFR, and the resulting tetrahydrofolate is then a substrate for the third member of the cycle, pyridoxal-5'-phosphate-dependent enzyme SHMT which converts it back to meTHF using the serine as the donor of one-carbon group.

The reaction catalyzed by TS is the only *de novo* source of TMP, a substrate for DNA synthesis. DHFR activity provides the key intermediate in one-carbon transfer reactions and is required for the *de novo* synthesis of dTMP, purines and certain amino acids, whereas SHMT plays a pivotal role in channeling metabolites between amino-acid and nucleotide metabolism. The levels of all three enzymes increase in proliferating cells, including a variety of tumours, and this increase appears to be regulated at both transcriptional and posttranscriptional levels. ST and DHFR are targets in anticancer therapies, in which analogues of folate or pyrimidine are used to inhibit enzyme activity. Development of resistance to drugs has necessitated the identification of alternate targets and SHMT is strongly considered for the design of specific drugs (Rao *et al.*, 2000).

Thymidylate synthase (TS)

The RNA-binding activity of thymidylate synthase has been discovered by Chu and coworkers, who showed, using gel mobility shift assay, that human TS formed a complex with its own mRNA (Chu *et al.*, 1991). Using cell-free rabbit reticulocyte model system they also demonstrated the inhibition of TS mRNA translation by exogenously added TS protein. Addition of a TS substrate (deoxyuridine monophosphate, dUMP) or inhibitor (5-fluorodeoxyuridine monophosphate, FdUMP) prevented both the complex formation and the inhibition of translation by human TS (Chu *et al.*, 1994b). These results and several reports describing elevation in TS enzyme level (not connected with mRNA level increase) after short-term exposure to fluoropyrimidines on *in vitro* and *in vivo* models as well as in clinical tumour specimens (Spears *et al.*, 1982; Wash-

tien, 1984; Swain *et al.*, 1989) prompted the authors to formulate the hypothesis that TS mRNA translation is autoregulated by its own protein end product and that accumulation of FdUMP and dUMP after fluoropyrimidine treatment results in a decrease of the intracellular level of unbound TS, which then results in enhanced TS mRNA translation. This hypothesis has been elaborated in following papers from the same laboratory and summarized in several review articles (Chu & Allegra, 1996; Schmitz *et al.*, 2001; Liu *et al.*, 2002; Tai *et al.*, 2004b).

Using a series of truncated TS RNAs in gel mobility shift and UV cross-linking assays the sites in human and *E. coli* TS mRNA molecules responsible for TS protein binding were identified. In human TS mRNA one binding site contained a putative stem-loop structure that incorporated the translational start sequence and another was contained within a 200-nt sequence of the protein-coding region (Chu *et al.*, 1993b; Lin *et al.*, 2000b). In bacterial TS mRNA three sequences (each 200–300 nucleotides long) were indicated as responsible for binding (Voeller *et al.*, 1995).

As far as the RNA-binding site on the enzyme molecule is concerned, the responsible domain(s) has not been fully recognized yet. Site-directed mutagenesis of cysteins on human TS protein together with RNA gel shift and *in vitro* translation assays have determined that Cys-180 plays a critical role in mediating RNA recognition (but not in the TS enzymatic activity, since the mutated TS expressed over 80% of the wild-type enzyme catalytic activity), whereas TS protein mutated in Cys-195 (active site cysteine, crucial for TS catalytic activity) retained the RNA-binding activity (Lin *et al.*, 2003). On the other hand, mutation of homologous active site Cys-146 in bacterial thymidylate synthase made the enzyme unable to bind its own mRNA (Voeller *et al.*, 1995). Recently Voeller *et al.* (2002) studied the interaction of mRNA with synthetic 17-mer peptides, corresponding to successive sequences of TS, and showed that, for the human enzyme, short peptides at the dimer interface are responsible for mRNA binding. The latter would indicate that mRNA could be bound only by the monomer, and not by the dimer. To prove it, the authors demonstrated that a 50-h dialysis, with the use of a membrane letting only monomers through, allowed about 50% of the enzyme activity to appear on the other side of the membrane. It should be mentioned that mammalian thymidylate synthase is active as a dimer, as a result of a non-symmetric arrangement of its identical subunits, both participating (although unequally) in the structure of each of the two active centers (Carreras & Santi, 1995). That is why the above result should probably be interpreted in terms of a monomer-dimer equilibrium in solution, with great excess of the dimer (we

have tested that electrophoretic separation of a large amount of recombinant rat thymidylate synthase under non-denaturing conditions in a polyacrylamide gradient gel did not allow identification of a free monomer; Cieřła and Rode, unpublished).

The results of other studies by the Chu's group showed that repression of thymidylate synthase mRNA translation by exogenously added TS protein in rabbit reticulocyte lysate may be mimicked by an antisense oligonucleotide (Schmitz *et al.*, 1998). However, in parallel, results of other authors were published, concerning the *in vivo* influence of antisense oligoribonucleotides complementary to the part of human thymidylate synthase mRNA that contains the translation initiation site and appears to be one of the critical elements for the binding by the human enzyme of its own mRNA. Transfection of cells of two human tumour lines (MCF-7 and HeLa) with vectors expressing those antisense oligoribonucleotides did not cause lowering of TS cellular level (DeMoor *et al.*, 1998). Other data, indicating also that results of *in vitro* studies do not necessarily correspond to *in vivo* conditions, were published (Kitchens *et al.*, 1999). The results of that work indicated that prolongation of the enzyme protein half-life, resulting from enzyme stabilization, rather than autoregulation by the thymidylate synthase protein of its own translation, is the primary mechanism of TS induction by fluoropyrimidines.

Zhang and Rathod (2002) have recently shown that bifunctional DHFR-TS protein of the protozoan parasite that causes malaria, *Plasmodium falciparum*, also binds to its own mRNA and inhibits DHFR-TS translation. However, in contrast to the corresponding individual human enzymes, DHFR and TS (Ericikan *et al.*, 1993; Chu *et al.*, 1993a; 1994), this interaction is not impaired by substrates or antimetabolites of either enzyme. Considering a direct correlation between the latter difference in translational regulation of the target enzymes in human and parasitic cells and the selectivity of the antimalarial, antifolate drug WR99210, the authors suggested the antifolate's inability to relieve translational inhibition in parasites as co-responsible for the selectivity. However, this correlation has been questioned recently, since on challenging the parasite's cultures with fluoropyrimidines targeted to thymidylate synthase, as well as with antifolates targeted to DHFR, among them WR99210, at levels close to their respective IC₅₀ values, DHFR-TS was found to be upregulated (Nirmalan *et al.*, 2004). Thus, the demonstrated *in vitro* insensitivity of the DHFR-TS-invoked translational repression to substrates/inhibitors does not seem to affect the expected result in intact cells. Interestingly, also our studies on recombinant thymidylate synthase of another parasite, the nematode *Trichinella spiralis*, showed the enzyme to bind its own mRNA

and repress *in vitro* translation of the mRNA, with neither activity being impaired by dUMP, meTHF or FdUMP (Cieřła *et al.*, 2005). These results prompted our further comparative studies that showed that thymidylate synthases from *T. spiralis*, rat and human bind and inhibit translation of their cognate mRNAs, with both phenomena being not abrogated by the presence of ligands (for comparison of the primary structures of the TSs see: Takeishi *et al.*, 1985; Cieřła *et al.*, 1995; Dąbrowska *et al.*, 2004). However, while the *T. spiralis* thymidylate synthase is capable of both binding and inhibiting translation of its own mRNA, the same enzyme preparation used in the two assays revealed that translation was affected at a considerably lower protein/mRNA molar ratio (in the range 5–20) than was binding (≥ 1000). This finding suggested either the presence in the reticulocyte preparation of a factor strengthening the mRNA–protein interaction or a mechanism whereby thymidylate synthase impaired translation differently than by mRNA binding (e.g. interaction of the enzyme with the ribosome). The fact that TS mRNA binding by TS protein exhibits saturation at a very high protein/mRNA molar ratio (evident also in the Chu's papers, although the authors did not comment on that) indicated that only a small fraction of the enzyme is capable of RNA binding. This points to a possibility of the binding being dependent on some posttranslational modification (Fu *et al.*, 1985; Knirsch & Clerch, 2001; Haegebarth, 2004) and a lack of a mechanistic relation between the two (mRNA binding and translational repression) phenomena. It should be noted that in view of our results being in agreement with those of Zhang and Rathod (2002), but in striking contrast to those of Chu and coworkers, the model of translational autoregulation of TS expression (Chu *et al.*, 1991) may require a correction.

Further studies on the thymidylate synthase potential to bind RNA, employing immunoprecipitation-RT-PCR technique on an extract from colon cancer cells overexpressing TS protein demonstrated that the protein may *in vivo* form complexes (with similar affinity) not only with its own mRNA, but also with several other RNAs such as those for c-myc, p53, IFN-induced 15 kDa protein, zinc finger protein 8, kinesin heavy chain and mitochondrial complete RNA (Chu *et al.*, 1994a; 1996; 1999). TS protein binding to 28S and 18S ribosomal RNA, albeit with a much lower affinity, may be a result of a physical interaction at the level of polysome (Chu *et al.*, 1996). Much effort has been devoted to investigate two of these cross-interactions, namely the binding of TS protein to c-myc and p53 mRNAs. Using immunoprecipitation-RT-PCR and gel mobility shift assay a 165-nt sequence on c-myc RNA that bound the TS protein was located in the 3' part of the pro-

tein-coding region (Chu *et al.*, 1995). This sequence, similarly to full-length c-myc mRNA, repressed the inhibition of TS mRNA translation by TS protein in rabbit reticulocyte lysate. Similar experiments involving TS protein and p53 mRNA have pointed to a sequence contained within 498 nucleotides in the protein-coding region of the p53 message as the TS protein-binding region (Chu *et al.*, 1999). In *in vitro* translation system TS protein inhibited p53 mRNA translation. To address the role of this interaction *in vivo* Chu and coworkers examined the level of p53 in cells overexpressing TS. They found that the p53 protein was not detectable in human colon cancer and rat hepatoma cell lines overexpressing TS protein. The expression of p53 mRNA was the same in parental and resistant cell lines suggesting that the suppression of p53 expression in TS overexpressing cells was a direct result of a decreased translational efficiency of p53 mRNA (Chu *et al.*, 1999; Ju *et al.*, 1999). The authors suggest that a direct interaction between TS protein and p53 mRNA controls p53 expression *in vivo* (Ju *et al.*, 1999). On the other hand, Longley *et al.* (2003) have not observed decreased expression of the p53 protein in any of the studied human cell lines overexpressing TS, which indicates lack of direct regulation of p53 synthesis by TS.

Dihydrofolate reductase (DHFR)

DHFR, the second enzyme involved in the thymidylate synthesis cycle is critical in maintaining the cellular pool of reduced folates. The intracellular level of DHFR is regulated by concentrations of serum factors, changes in cAMP levels, viral infections, exposure to antimetabolites such as MTX and fluoropyrimidines (Bertino *et al.*, 1963; Kellems *et al.*, 1979; Gudewicz *et al.*, 1981; Schuetz *et al.*, 1988). Alterations in DHFR expression result from gene amplification (Hanggi & Littlefield, 1976; Kaufman *et al.*, 1979) as well as from both transcriptional (Santiago *et al.*, 1984) and posttranscriptional (Bertino *et al.*, 1965; Cowan *et al.*, 1986) regulatory mechanisms. DHFR levels acutely increase after exposure of malignant cells to the antifolate methotrexate, and are independent of DHFR mRNA level and gene copy number (Domin *et al.*, 1982; Bastow *et al.*, 1984). After transcriptional as well as mRNA stability changes and inhibition of enzyme degradation by MTX were ruled out (Domin *et al.*, 1982), it has been proposed that this phenomenon may result from a translational control mechanism (Bastow *et al.*, 1984). It has been shown using gel mobility shift assay, UV cross-linking and nitrocellulose filter-binding methods that DHFR can bind specifically to its own mRNA (Chu *et al.*, 1993a; Ercikan *et al.*, 1993). Studies with the use of rabbit reticulocyte *in vitro* translation system have shown that DHFR protein inhibits the trans-

lation of its own mRNA (Ercikan *et al.*, 1993). Substrates (dihydrofolate and NADPH) or MTX markedly diminished the binding of DHFR protein to its mRNA and released the translation arrest (Chu *et al.*, 1993a; Ercikan-Abali *et al.*, 1997). A model of the autoregulation was proposed: DHFR binds to its cognate mRNA when the metabolic needs of the cell are satisfied or the cell is in a quiescent state, whereas when substrate levels are high and/or an inhibitor is present, DHFR is no longer able to bind to its own mRNA, allowing for translation of new DHFR protein.

To characterize the *cis*-acting element(s) within DHFR mRNA, a series of truncated transcripts were used in binding experiments. A 100-nt sequence within the coding region, containing two putative stem-loop structures, was identified as a binding site (Ercikan-Abali *et al.*, 1997). *In vitro* and *in vivo* studies further localized this *cis*-acting element to an 82-nucleotide sequence (Tai *et al.*, 2004a). In parallel, studies aiming at identification of amino-acid residues involved in the interaction with mRNA were carried out. A site-directed mutagenesis study has pointed to Cys-6 as being essential, and the Ile-7, Arg-28 and Phe-34 residues as being important for RNA binding. The fact that catalytically inactive mutant proteins retain their RNA-binding activity has indicated that the catalytic domain is distinct from the RNA-binding one (Tai *et al.*, 2002). It remains unclear as to whether these amino acids interact directly with the RNA or whether they maintain the protein in a conformational state that allows the proper binding domain of the protein to interact with the target mRNA. More recently Skacel *et al.* (2005) used Chinese hamster ovary cells lacking DHFR, in which wild-type and mutant human DHFR-EGFP fusion proteins were expressed, to functionally identify amino acids essential for the upregulation of DHFR caused by antifolates. The results indicated that the upregulation appeared to be independent of the catalytic activity and of the enzyme's affinity for MTX. Since two of the critical residues (Leu-22 and Ser-118) reside in the NADPH binding pocket and the third (Glu-30) is involved in conformational changes upon the binding of NADPH to DHFR, the NADPH-binding site of the enzyme may be involved in mRNA binding. Skacel *et al.* (2005) have proposed a new model for translational regulation of DHFR by MTX in which the enzyme exists as at least two conformers: one with bound NADPH, and the other bound to DHFR mRNA. The conformers are in equilibrium and can interconvert. Binding of MTX or dihydrofolate to the DHFR-NADPH complex shifts the equilibrium towards the catalytic function of DHFR, whereas MTX or dihydrofolate binding to the DHFR-mRNA complex leads to a

conformational change releasing the mRNA, resulting in resumption of DHFR synthesis.

This model assumes that only fraction of the enzyme, the one being in proper conformation, is able to interact with mRNA. Furthermore, other data indicate that mRNA binding by DHFR is not a sole determinant of the translational upregulation in response to antifolate treatment. Raltitrexed, a relatively weak DHFR inhibitor, is significantly less effective than MTX in abolishing the RNA binding activity (Tai *et al.*, 2002), but it is almost equally effective in causing translational upregulation in cells expressing DHFR-EGFP fusion protein from a transgene (Skacel *et al.*, 2005).

To document *in vivo* activity, various DHFR sequences contained within the coding region were cloned in the 5' end of luciferase reporter gene, and transient transfection experiments were performed using human colon cancer RKO cells. In cells transfected with a plasmid containing DHFR mRNA *cis*-acting element, the luciferase activity was decreased by 50% in comparison with cells transfected with unmodified luciferase reporter plasmid, but could be restored by treatment of these cells with MTX or siRNA targeting DHFR mRNA (Tai *et al.*, 2004).

It should be noted that DHFR protein may also interact with the translation machinery, as we have found recently that chicken liver DHFR represses the translation of thymidylate synthase mRNA *in vitro*, although it does not bind to TS mRNA in gel shift assay (Cieřla *et al.*, 2005).

Serine hydroxymethyltransferase (SHMT)

SHMT is an enzyme that in human exists both in the cytosol (cSHMT) and in mitochondria (mSHMT). Sequence analysis of the respective genes (Stover *et al.*, 1997; Girgis *et al.*, 1998) revealed considerable similarity between the two isoenzymes, although the cytosolic gene was longer than its mitochondrial counterpart. Complete knockout of mSHMT in *Saccharomyces cerevisiae* leads to glycine auxotrophy, but knocking out of cSHMT does not render the organism auxotrophic for glycine (Kastanos *et al.*, 1997), suggesting different metabolic roles for the two isoenzymes. cSHMT is positioned to play key roles in regulating folate-activated one-carbon metabolism (Girgis *et al.*, 1997). The 5' UTR of cSHMT message is alternatively spliced, creating a full-length 5' UTR composed of exons 1 and 2 and a shorter UTR that results from excision of exon 2. Both spliced forms have been predicted to form stable stem-loop structures (Girgis *et al.*, 1998), which may indicate tightly regulated protein expression (Byrne *et al.*, 1995). Human cSHMT protein caused inhibition of *in vitro* translation of luciferase gene with the long or short form of cSHMT 5' UTR

fused to the 5' end. The inhibition was independent of amino acid and folate substrate binding to the cSHMT enzyme. In gel shift experiments the cSHMT protein bound to the 5' UTR of cSHMT mRNA and this interaction was not disturbed by enzyme substrates, either. These results, together with the results of ternary complex absorbance studies and Scatchard analysis, suggest that the catalytic domain of cSHMT and the binding site for mRNA are independent. Additionally, the short form of cSHMT mRNA 5' UTR could effectively inhibit cleavage of allothreonine catalyzed by cSHMT by decreasing K_m , which showed that induction of conformational changes in the enzyme by mRNA binding influenced its catalytic properties (Liu *et al.*, 2000). 5-Formyltetrahydrofolate (5-formyl-THF), a product of the second reaction catalyzed by cSHMT (see Table 1) is a slow, tight-binding inhibitor of the enzyme (Stover & Schirch, 1991). 5-Formyl-THF binds to and dissociates quickly from the cSHMT-glycine binary complex when pyridoxal-5'-phosphate is in its external aldimine form. The conversion of the external aldimine to the glycine quinonoid is slow and this step is associated with a conformational change in the enzyme. The off-rate of generation of 5-formyl-THF from the cSHMT-glycine-5-formyl-THF ternary complex is rate-limited by the slow conversion of the quinonoid ternary complex to the external aldimine ternary complex. The binding of cSHMT mRNA 5' UTR shifts the equilibrium from the quinonoid ternary complex to the external aldimine ternary complex and thereby permits rapid release of the inhibitor from the enzyme. Therefore, mRNA binding may also play a role in limiting the inhibitory effects of 5-formyl-THF (Liu *et al.*, 2000). However, additional studies are required to determine if cSHMT can regulate its translation *in vivo*.

Recently Yang and Meier (2003) suggested a novel noncatalytic function for yeast cytosolic hydroxymethyltransferase in cell size control, possibly through the regulation of ribosome biogenesis *via* a chaperone of small nucleolar ribonucleoprotein particles, Srp40p.

Of interest is the possible connection between folate-dependent one-carbon metabolism and iron metabolism that has been elucidated recently by Oppenheim *et al.* (2001). The authors have found that human MCF-7 cells expressing rat heavy (but not light) chain ferritin (which is an endogenous chelator influencing free iron regulatory pool) exhibits markedly increased expression of cSHMT. Increased heavy-chain ferritin expression did not alter the cSHMT mRNA level, but it did increase its translation rate and this was mediated, at least in part, through the cSHMT transcript 5' UTR. It is not clear if heavy-chain ferritin interacts directly with the cSHMT 5' UTR, or if it influences the activity of

other mRNA-binding proteins that may be sensitive to decreased intracellular iron concentrations. MCF-7 cells with increased expression of cSHMT displayed increased efficiency of *de novo* thymidylate biosynthesis, pointing to cSHMT activity as the rate-limiting step of the thymidylate cycle in these cells.

OTHER RNA-BINDING METABOLIC ENZYMES

PyrR/uracil phosphoribosyltransferase

The bacterial pyrimidine biosynthetic operon (*pyr*) encodes all of the enzymes for the *de novo* biosynthesis of UMP, and two additional cistrons encoding uracil permease and the regulatory protein PyrR (Lerner *et al.*, 1987; Kahler & Switzer, 1996). In many bacteria PyrR acts by binding to specific sequences on *pyr* mRNA and causing transcriptional attenuation (Switzer *et al.*, 1999) when intracellular levels of uridine nucleotides are elevated ($K_d = 10.2$ nM at saturating UMP and 216 nM when dUMP was omitted from the binding mixture (Turner *et al.*, 1998).

The uracil phosphoribosyltransferase (UPRTase) activity of PyrR was first discovered by Ghim and Nuehardt (1994), but the protein has little amino-acid sequence resemblance to other bacterial uracil phosphoribosyltransferases and its catalytic activity (see Table 1) is likely to be extremely low under physiological conditions (Turner *et al.*, 1998). The role of the enzymatic function of PyrR is not known; *Bacillus subtilis* possesses an additional UPRTase that has been shown to be quantitatively more important than PyrR (Martinussen *et al.*, 1995). The crystal structures of *B. caldolyticus* PyrR revealed binding of Mg^{2+} , UMP and GMP and these ligands affected the binding of PyrR to *pyr* RNA. The binding was enhanced by UMP or UTP, and Mg^{2+} acted synergistically with either nucleotide to increase the affinity of PyrR for RNA. GMP bound in the UPRTase active site reduced the affinity of the protein for RNA (Chander *et al.*, 2005).

Creatine kinase (CKBB)

Altered cardiac workload regulates the translation and localization of the α myosin heavy chain (α MyHC) messenger RNA through the 3' UTR by protein-RNA interactions. Gel shift analysis and microsequencing techniques were used to identify the proteins binding to α MyHC mRNA 3' UTR from neonatal rat heart (Vracar-Grabar & Russell, 2004). The proteins were identified as creatine kinase brain B isoform (CKBB), phosphoglycerate kinase (PGK), lactate dehydrogenase (LDH) and fatty acid binding protein (FABP), but Western blotting of the purified proteins and the cytosolic fraction from whole heart

of neonatal rat preincubated with MyHC mRNA 3' UTR confirmed only CKBB. LDH, PGK and FABP might be involved in an interaction with α MyHC mRNA 3' UTR but not directly as binding proteins. They might have a more supportive role than a loosely associated complex with CKBB, further providing a link between different pathways of energetic metabolism and contractile protein synthesis. The authors (Vracar-Grabar & Russell, 2004) have not found any known consensus RNA binding domains within the CKBB protein sequence, nonetheless the secondary structure of α MyHC mRNA 3' UTR revealed a hairpin loop with several bulges that might be potential sites for protein binding. Increasing concentrations of ATP enhanced the interaction of CKBB with α MyHC mRNA 3' UTR, suggesting an involvement of CKBB autophosphorylation. The autophosphorylation may depend on pH and the available levels of ATP, ADP and creatine phosphate important for the primary function of "energy shuffling" in the cell. The finding that CKBB, an energy transduction enzyme, binds to the mRNA 3' UTR of the faster ATP-consuming α MyHC suggests a possible regulatory linkage between the metabolic state of the cell and myosin isoform expression. Removal of calcium and magnesium ions had a potentiating effect on the formation of CKBB- α MyHC mRNA 3' UTR complex *in vitro*, but since CKBB is not itself a calcium binding protein, a simple calcium regulatory mechanism is unlikely.

Mevalonate kinase (MK)

Luteinizing hormone receptor (LHR) mRNA-binding protein, termed LRBP, has been identified in rat and human ovary (Kash & Menon, 1999). It bound with high affinity to a polypyrimidine-rich bipartite sequence in the coding region of LHR mRNA. Further studies have demonstrated that in an *in vitro* reconstituted mRNA decay system, the addition of partially purified LRBP causes accelerated degradation of LHR mRNA (Nair *et al.*, 2002). N-terminal microsequencing, followed by homology search revealed the LRBP identity as mevalonate kinase (MVK). Mevalonate kinase is an enzyme involved in *de novo* synthesis of cholesterol. In the ovary, the interaction of LH with LH receptor is a key event in the regulation of synthesis of steroid hormones from cholesterol (Dufau, 1998). Increased cellular cholesterol inhibits sterol-responsive genes, including key enzymes in cholesterol synthesis (Brown & Goldstein, 1990). Hence, it is conceivable that under this condition MVK functions as an mRNA-binding protein for the regulation of LHR mRNA levels, leading to the down-regulation of LHR expression. This type of regulation appears to be cell-specific (Kash & Menon, 1998). The me-

valonate kinase LHR mRNA-binding activity was inhibited in the presence of ATP and mevalonate, which indicated that the RNA recognition site of mevalonate kinase might involve the ATP/mevalonate binding region of the protein, or that binding of substrates, ATP and mevalonate, might induce a conformational change in MVK, making it fully functional as a kinase enzyme and rendering it unable to bind to LHR mRNA. Treatment of human embryonic kidney cells with mevastatin (an inhibitor of hydroxymethylglutaryl-CoA reductase that converts hydroxymethylglutaryl-CoA to mevalonate) to deplete cellular mevalonate resulted in an increase in LHR mRNA binding activity of mevalonate kinase. The data support a novel function of rat mevalonate kinase as an LHR mRNA-binding protein in the posttranscriptional regulation of LH-receptor expression in the ovary.

Catalase

Clerch *et al.* (1996) have found that NADPH-depleted bovine liver catalase (from Sigma, but not from Calbiochem or Boehringer) binds rat catalase mRNA 3' UTR.

The catalase mRNA 3'UTR *cis* element involved in the binding is composed of a stem-loop structure and a series of linear CA dinucleotide repeats (Clerch & Massaro, 1992). The catalase protein/catalase RNA complex formation was redox-sensitive, inhibited by NADPH and competitively eliminated by a CA dinucleotide repeat (Clerch, 1995). These and other results have indicated that binding of catalase protein to its mRNA involves the dinucleotide binding domain of catalase. Since NADPH is not required for catalase enzymatic activity but serves to protect catalase from inactivation, the authors speculated that NADPH binding to catalase performed an additional function unrelated to its catalytic activity: that is, to regulate the binding of catalase to RNA. Studies aimed at elucidating if these observations have any physiological importance or whether catalase is only a molecular fossil of the RNA world (Kyrpides & Ouzounis, 1995), have not been, to my knowledge, undertaken.

ENZYMES INVOLVED IN RNA METABOLISM DISPLAYING RNA-BINDING ACTIVITIES OUTSIDE THEIR WELL-ESTABLISHED FUNCTIONS

Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases are another example of multifunctional enzymes which, beside

their primary function of activation of amino acids and transfer to the cognate tRNAs, are involved in many processes such as apoptosis, embryogenesis, synthesis of chlorophyll, alarmons AP₄A, histidine, and also in regulation of gene expression on the level of transcription and translation (reviewed by Ivanov *et al.*, 2000, and by Kamińska *et al.*, 2002).

E. coli threonyl-tRNA synthetase (ThrRS) regulates the translation of its own mRNA by binding to it in a region called the operator, located in front of the ribosomal binding site within the 5' UTR. The primary and secondary structures of the operator resemble those of the anticodon arm of several tRNA^{Thr} isoacceptor species and are recognized by ThrRS in an analogous way (Springer *et al.*, 1989). The enzyme competes with the 30S ribosomal subunit which binds to adjacent translation initiation site. Thus, when the cellular concentration of free synthetase is high, the enzyme binds to threonine synthetase mRNA and competes with and excludes ribosome binding. On the contrary, if the synthetase concentration is low, the ribosome competes effectively with the enzyme and threonine synthetase mRNA is efficiently translated (Springer *et al.*, 1985). Two regions within the synthetase have been identified genetically that are essential to the autoregulation and also are involved in aminoacylation (Springer *et al.*, 1989).

Human glutamyl-tRNA synthetase (GlnRS) is also able to bind to its own mRNA with affinity comparable to the quantitative estimates for the interaction of several synthetases with their cognate tRNAs ($K_d = 200$ nM; Schray & Knippers, 1991). The enzyme contains two binding regions, one unspecific and the other, responsible for specific binding to a secondary structure RNA element in the 3' UTR, located in the core region defined as the part of the human enzyme showing high degree of amino-acid sequence similarity with bacterial and yeast synthetases, and therefore considered to be important for the basic enzymatic function. An obvious possibility is that GlnRS recognizes structural features in the mRNA binding site that also occur in tRNA^{Gln}. The physiological role of this interaction is unclear. One possibility would be that bound GlnRS induces degradation of mRNA (Schumperli, 1988; Marzluff & Pandey, 1988).

A protein isolated from polysomal messenger ribonucleoprotein (mRNP) particles from cultured murine erythroleukemia cells was identified as seryl-tRNA synthetase (SerRS). It contains a sequence motif that is shared by both EF-1 α and GlnRS (Miseta *et al.*, 1991). This region in EF-1 α is located between two sequences that are believed to be involved in GTP binding (Brands *et al.*, 1986). Sequences corresponding to the well described RNA-binding protein consensus (Adam *et al.*, 1986) were not found in

SerRS. The results of UV-crosslinking after treatment of cell lysates with edeine, which inhibits the association of 50S ribosomal subunits with 48S initiation complex, suggest that SerRS may participate directly in the initiation phase of protein synthesis. Given the dimeric structure of SerRS, it could be involved in the association of mRNA with 18S rRNA (Miseta *et al.*, 1991).

It is very likely that more aminoacyl-tRNA synthetases are involved in regulation of gene expression. A role in the initiation phase of protein synthesis has also been suggested for leucyl-tRNA synthetase (Clemens, 1990) and some evidence indicates that *Neurospora* mitochondrial tyrosyl-tRNA synthetase functions in splicing group I introns (Cherniak *et al.*, 1990).

CSP41/endoribonuclease

Most chloroplast pre-mRNAs and mature mRNAs contain an inverted repeat sequence in their 3' UTR that can fold into a stable stem-loop structure, which are required for correct 3'-processing of the pre-mRNAs and for stabilization of mature mRNAs, by impeding processive 3' to 5' exonucleolytic degradation. Spinach CSP41 protein is part of a protein complex (containing also stem-loop-binding proteins CSP55 and CSP29) that binds to the 3' UTR of *petD* precursor mRNA, a chloroplast gene encoding subunit IV of the cytochrome *b₆/f* complex (Chen *et al.*, 1995). The RNA-protein complex formation requires not only the stem-loop structure, but also an AU-rich element (box II) immediately upstream of the stem-loop. The CSP41 protein has been purified and nuclear *csp41* gene cloned and expressed in bacteria (Yang *et al.*, 1996). Although the amino-acid sequence of CSP41 protein did not show a significant overall similarity to any nuclease in the data bases, it had a substantial nonspecific endoribonuclease activity, degrading both single-stranded and double-stranded RNAs (but not DNAs), with preference for RNAs containing a stable stem-loop structure (Yang & Stern, 1997). Experimental data suggest that the function of CSP41 could be altered upon interactions with CSP51 and CSP29 (Chen *et al.*, 1995; Yang *et al.*, 1996) by suppression of its RNase activity. CSP41 could have two functions in chloroplast mRNA metabolism. First, free CSP41 could directly cleave chloroplast mRNA and pre-mRNA in the 3' stem-loop structure to initiate bulk mRNA degradation, and second, as CSP41, along with CSP51 and CSP29, forms a stable mRNA-protein complex with the 3' stem-loop structure of pre-mRNA, its RNase activity could be suppressed and the entire complex could serve as a steric hindrance to 3' to 5' processive exoribonucleases, and thus ensure efficient 3' end maturation. Although some RNases have RNA-

binding activities to target their substrates (Court, 1993), the RNA-binding activity of CSP41 does not enhance the specificity or efficiency of its RNase activity *in vitro* (Yang & Stern, 1997), indicating that this RNA-binding activity could be involved in a second function.

CONCLUDING REMARKS

It is obvious that the list of RNA-binding metabolic enzymes or RNA-binding proteins with intrinsic enzymatic activity is far from being complete. Higher and higher degree of complexity of the metabolic regulatory network is being revealed and well-known proteins with metabolic function established long ago, are now being found to be multifunctional. The relationship between enzymatic functions of proteins described in this paper and their role in gene expression is in most cases unclear and it is likely that it may not exist at all. The switch between those two functions may be triggered by many phenomena, among them the metabolic state of the cell, posttranslational modifications, localization of proteins to different cellular compartments, or intermolecular interactions. All those novel metabolic connections, regulations and mechanisms are awaiting discovery and elucidation.

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