Instituto Juan March
de Estudios e Investigaciones

92 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Regulation of Protein Synthesis in Eukaryotes

Organized by

M. W. Hentze, N. Sonenberg and C. de Haro

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Introduction

M. W. Hentze, N. Sonenberg and C. De Haro

An important part of the control of gene expression in eukaryotic cells occurs at the post-transcriptional level. Translation regulation allows the cell to quickly respond to environmental changes. Post-transcriptional regulation of gene expression appears to play a major and often underestimated role in the efficient production of specific proteins by the eukaryotic cells.

The mechanisms of mRNA translation have been a topic of research for many years. The realization that protein synthesis is regulated by biological signals or programs has emerged much more recently. These mechanisms are now beginning to be understood.

This Workshop on "Regulation of Protein Synthesis in Eukaryotes" brought together experts and leaders in this emerging field. The presentations focussed on the responsible translation factors, regulatory RNA sequences, regulatory proteins and a broad scope of biological systems including those from developmental biology, growth control, cell differentiation and viruses. Many new discoveries were presented and discussed for the first time in public. Not only invited papers but also the communications presented as posters in this Workshop were important contributions to grasp the current state and the scenario for future developments on the field.

True to the typical character of the Juan March Workshops, the discussions were intensive, thorough and stimulating. On behalf of ourselves and of all participants we would like to thank the Juan March Foundation for making possible and very pleasant this Workshop.

New ideas and a sense of excitement were what many participants took home in addition to much new information.

Matthias Hentze, Nahum Sonenberg and César de Haro

Session 1: eIF2α kinases and initiation factors

Chair: Nahum Sonenberg

CONTROL OF PROTEIN SYNTHESIS BY PKR AND RIBOREGULATORS

Michael B.Mathews

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The double-stranded (ds) RNA-activated kinase, PKR, is a well-studied regulator of cellular function. In addition to its established roles in translational control and the interferon-induced antiviral response, it has been implicated in diverse processes including apoptosis, differentiation, transformation and tumorigenesis (see refs. 1-3 for reviews). Activation of the kinase is accompanied by its autophosphorylation and leads to the phosphorylation of initiation factor eIF2 on its alpha subunit. This results in translational inhibition mediated via the GTP exchange factor, eIF2B. Although less well documented, other cellular and viral proteins can also serve as substrates for PKR. Conventionally, PKR activation is thought to occur under the influence of dsRNAs, such as viral replicative intermediates, whereas other highly-structured RNAs such as adenovirus VA RNA_I prevent activation. It is increasingly recognized, however, that this picture of PKR regulation is incomplete in that RNAs lacking canonical duplex structure can also activate PKR, and that PKR interacts with cellular proteins that can modulate its function. Data relating to both of these points will be presented.

Work with model RNAs led to the conclusion that PKR activation requires a minimal length of ~30 bp of perfect duplex. We have examined muscle RNAs that do not meet this criterion yet activate PKR. The first of these RNAs consists of repeats of the sequence CUG, found in the 3' untranslated region (UTR) of the myotonin protein kinase gene (DMPK). Myotonic dystrophy (DM) is a neuromuscular disorder caused by the expansion of these repeats. In normal cells, the repeat length is 5-35 copies, whereas in DM patients, the CUG repeat expands to 50 to >3000 copies. The molecular mechanism by which expanded repeats give rise to the disorder is not clear, but it is associated with reduced muscle protein synthesis. We therefore examined the properties of CUG repeat RNAs (from 5-150 repeats), and of repeat RNAs unrelated to DM (CUC and CAG), generated by *in vitro* transcription with T7 RNA polymerase. Gel retardation assays indicate that these RNAs can bind p20, the dsRNA-binding domain of PKR expressed in *E. coli*. The longer the CUG repeat, the higher its affinity for p20. The threshold for binding is 15 repeats. For $(CUG)_{150}$, the binding affinity is higher than that of HIV-1 TAR RNA but lower than that of VA RNA_I. In contrast to the latter, however, long CUG repeat RNAs elicited PKR activation. Thus, $(CUG)_{69}$ and $(CUG)_{15}$ activate PKR, as measured by autophosphorylation and eIF2 phosphorylation, while CUG repeat RNAs with lengths in the normal range (5-35 repeats) do not. This result correlates with the clinical observation that myotonic dystrophy is caused by long CUG repeats but not by short ones. Biophysical and biochemical studies show that long CUG repeat RNAs, but not short ones, adopt extensive hairpin-like structures although they are not formally double-stranded

Activation of PKR and p20 binding is also observed with segments of other muscle RNAs that are not predicted to form extended duplex structures. These sequences include segments of the 3'UTR of alpha-tropomyosin mRNA (4) and of the troponin and alpha-cardiac actin mRNAs which have been linked to the control of muscle cell differentiation (5). The conclusion that PKR can be activated by naturally-occurring RNAs that are incapable of regular base-pairing has profound implications for the regulation of gene expression.

Studies of a second abundant adenovirus RNA species, VA RNA_{II}, which is highly conserved but does not appear to subserve the same function as VA RNA_{I}, led to the identification of two cellular proteins that interact with PKR (6). These are RNA helicase A (RHA) and nuclear factor 90 (NF90). RHA is a DNA and RNA helicase which interacts with RNA polymerase II and the CREB-binding protein CBP, and functions as a transcriptional co-activator. NF90 (also known as M-phase phosphoprotein 4, MPP4) is found as a heterodimer, NF90/NF45, and in complexes with the DNA-dependent protein kinase (DNA-PK) and eIF2. The NF90/NF45 complex is reported to function as a sequence-specific DNA binding protein and transcriptional activator. Like PKR, both RHA and NF90 contain copies of the double-stranded RNA binding motif (dsRBM) and bind to dsRNA as well as VA RNA. RHA and NF90 co-purify from human 293 cell extracts, and they co-immunoprecipitate with PKR from HeLa and 293 cell extracts, implying that these proteins are associated with one another *in vivo*. Interactions between these proteins and with eIF2-alpha also occur in yeast and with proteins synthesized *in vitro* in a wheat-germ translation system. The observations that they bind related RNA ligands and form complexes with one another and with eIF2, suggests that RHA and NF90 may act in concert with PKR to participate in a pivotal mechanism of cellular regulation.

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COUPLING OF *GCN4* TRANSLATION TO AMINO ACID AVAILABILITY BY FACTORS CONTROLLING INITIATOR tRNA^{Met} BINDING TO RIBOSOMES. A. Hinnebusch[1], J. Anderson[1], K. Asano[1], M. Garcia-Barrio[1], H. Qiu[1], G. Pavitt[1], and L. Phan.
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Transcription of amino acid biosynthetic genes in yeast cells is stimulated in response to amino acid starvation by derepression of the transcriptional activator GCN4. Translation of *GCN4* mRNA is induced in starved cells by phosphorylation of translation initiation factor 2 (eIF2) by protein kinase GCN2. GCN2 is activated by uncharged tRNA, presumably through its binding to a histidyl-tRNA synthetase (HisRS)-related domain in GCN2. The regulation of GCN2 activity appears to involve dimerization in the kinase domain, HisRS region and C-terminal ribosome-binding segments, as well as physical interactions between these domains of GCN2. Autophosphorylation in the kinase activation loop is also a prerequisite for GCN2 activation. The GCN1 and GCN20 proteins, which are related in sequence to translation elongation factor EF3, reside in a ribosome-associated complex that mediates the activation of GCN2 by uncharged tRNA in vivo.

Phosphorylation of eIF2 by GCN2 converts eIF2 from a substrate to a competitive inhibitor of its 5-subunit guanine nucleotide exchange factor, eIF2B. The α, β and δ subunits of eIF2B (GCN3, GCD7, and GCD2) comprise a regulatory subcomplex that can bind eIF2-GDP in the absence of the other two subunits, and shows greater affinity for phosphorylated (eIF2[αP]) versus unphosphorylated eIF2. The γ and ε eIF2B subunits (GCD1 and GCD6) form a second subcomplex that is capable of catalyzing nucleotide exchange on phosphorylated or unphosphorylated eIF2, in the absence of the other eIF2B subunits. We propose that interaction of eIF2-GDP with the regulatory subunits in eIF2B leads to a nonproductive mode of binding in which eIF2-GDP is not properly juxtaposed with the catalytic subunits of eIF2B. Phosphorylation of eIF2-GDP would trap eIF2-GDP in this nonproductive state, preventing isomerization of the complex to the conformation required for nucleotide exchange. Point mutations were isolated in each of the eIF2B regulatory subunits (α, β and δ) which reverse the effects of eIF2 phosphorylation on translation in vivo and allow phosphorylated eIF2-GDP to be accepted as a substrate by eIF2B in vitro. According to our model, these mutations allow eIF2B to interact productively with both eIF2-GDP and eIF2[αP]-GDP.

The principal catalytic subunit of eIF2B (GCD6, ε) and the GTPase activating factor for eIF2 known as eIF5, contain homologous bipartite motifs at their C-termini that mediate stable binding, both in vitro and in vivo, to the N-terminal half of the β subunit of their common substrate eIF2. eIF5 and eIF1 (SUI1) are physically associated with the 5-subunit eIF3 complex of yeast through interactions with the *NIP1*-encoded subunit of eIF3. As eIF3 is a component of native 40S subunits, we suggest that eIF5 and eIF1 are recruited to the 40S initiation complex through their interactions with eIF3-NIP1. Both eIF5 and eIF1 are involved in selection of the AUG codon during scanning, and eIF3 also interacts with the mRNA cap-associated factor eIF4G. Thus, eIF3 may play an important role in positioning eIF1, eIF5, and the mRNA on the 40S ribosome in a manner required for recognition of the AUG codon by the eIF2/GTP/Met-tRNAi^{Met} ternary complex.

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TRANSLATIONAL CONTROL BY eIF2α KINASES.

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Phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) is one of the best characterized mechanisms for down-regulating protein synthesis in mammalian cells in response to a variety of different cellular stresses, including nutrient starvation, iron deficiency, heat shock, and viral infection.

Until recently, three distinct eIF2α kinases were known as translational regulators in eukaryotic cells by phosphorylating eIF2α at Serine-51. These kinases are activated by distinct stimuli as follows: the heme-regulated inhibitor (HRI) in rabbit reticulocytes by heme deficiency; the double-stranded RNA-dependent kinase (PKR) in human, mouse and rat cells by the occurrence of double-stranded RNAs after virus infection; and GCN2 in *Saccharomyces cerevisiae* by amino acid deprivation. In contrast to PKR and HRI, which inhibit global protein synthesis in response to stress signals, the activation of GCN2 in yeast leads to increased translation of one mRNA species, *GCN4* mRNA. A fourth eIF2α kinase, termed PEK, has been recently characterized from rat pancreatic islet cells.

We have been studying new members of this family of kinases in order to provide evidence that the eIF2α kinases are present in all eukaryotes from yeast to mammals, trying to determine their role in general translational control as well as in translation of mRNAs that encode key growth regulating proteins in eukaryotic cells.

We have reported the molecular cloning and characterization of DGCN2, a *Drosophila* eIF2α kinase related to yeast GCN2 protein kinase. Affinity-purified antibodies, raised against a synthetic peptide based on the predicted DGCN2 sequence, specifically immunoprecipitated an eIF2α kinase activity and recognized a 175 kDa phosphoprotein in Western blots of *Drosophila* embryo extracts. Expression of DGCN2 is developmentally regulated and at later stages becomes restricted to a few cells of the central nervous system. The physiological role of DGCN2 in *Drosophila* is currently unclear. Furthermore, it is not certain whether DGCN2 mediates total protein synthesis or controls gene-specific translation. Recently, a GCN2 kinase homologue has been reported from *Neurospora crassa* (CPC3).

We have purified a heme-sensitive eIF2α kinase activity from both mouse liver and NIH 3T3 cell extracts. Furthermore, we have cloned and characterized this mouse liver eIF2α kinase (mHRI), which shows 83 and 94% identities to rabbit and rat HRIs, respectively. Both the purified enzyme and recombinant mHRI exhibited an autokinase and an eIF2α kinase activity, and both activities were inhibited *in vitro* by hemin. In addition, wild-type mHRI, but not the inactive mHRI-K196R mutant was autophosphorylated *in vivo* when they were expressed in 293 cells. The

mHRI mRNA is expressed in all mouse tissues examined being most abundant in the liver, kidney and testis, suggesting that mHRI is a ubiquitous eIF2α kinase of mammalian cells and that it could play a role in translational control of nonerythroid tissues.

Finally, we have also cloned a cDNA from mouse that it is homologous to GCN2 kinase and it represents the first mammalian GCN2 homolog (mGCN2). Northern blot analysis demonstrated that mGCN2 mRNA is expressed in a wide range of different tissues, with the highest levels in liver and brain. Specific mGCN2 antibodies immunoprecipitated and recognized a 190 kDa phosphoprotein in Western blots of mouse liver extracts. Interestingly, serum deprivation but not poliovirus infection, increased eIF2α phosphorylation in mGCN2 transfected human 293T cells.

The molecular mechanisms which regulate the function of the eIF2α kinases in normally dividing cells are largely unknown. In this respect, we have isolated and partially purified, from both mouse and rat liver post-mitochondrial supernatants, an eIF2α kinase inhibitor (mKI) that could be involved in translational control of mammalian cells.

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Induction of apoptosis by the ds-RNA dependent protein kinase (PKR) involves eIF-2α and NF-κB

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The double-stranded (ds) RNA-dependent protein kinase (PKR) is a key mediator of antiviral effects of interferon (IFN) and an active player in apoptosis induced by different stimuli. Two physiological substrates of PKR have been identified, the translation initiation factor eIF-2α and I-κBα the inhibitor of the transcription factor NF-κB. The role of PKR substrates in apoptosis is not well defined. To evaluate the involvement of NF-κB and eIF-2α in the induction of apoptosis by PKR, we have used vaccinia virus (VV) recombinants that inducibly express PKR concomitantly with dominant negative mutants of I-κBα and eIF-2α . We find that while expression of PKR by a VV vector results in extensive inhibition of protein synthesis and induction of apoptosis, co-expression of PKR with a dominant negative mutant of eIF-2α (Ser 51 Ala) reverses both the PKR-mediated translational block and PKR-induced apoptosis. Co-expression of PKR with a dominant negative mutant of I-κBα (Ser 32, 36 Ala) also leads to inhibition of apoptosis while translation remains blocked. Treating cells with two different proteosome inhibitors which block I-kBα degradation, prevents PKR-induced apoptosis supporting results from co-expression studies. In addition, upregulation of Fas mRNA transcription occurred during PKR activation. Our findings provide evidence for the involvement of eIF-2α and NF-κB in the induction of apoptosis by PKR.

UNIVERSAL CONSERVATION IN TRANSLATION INITIATION REVEALED BY ARCHAEAL, YEAST, AND HUMAN HOMOLOGS OF BACTERIAL TRANSLATION FACTOR IF2. T.E. Dever, S.K. Choi, J.H. Lee, W.L. Zoll, W.C. Merrick. Laboratory of Eukaryotic Gene Regulation, NICHD, National Institutes of Health, Bethesda, MD 20892-2716.

A universally conserved step in gene expression is the initiation of protein synthesis at an AUG codon using a specific initiator $tRNA^{Met}$ ($tRNA_i^{Met}$). The delivery of the Met-$tRNA_i^{Met}$ to the ribosome is catalyzed in prokaryotes by the translation factor IF2, a single polypeptide of ~90 kDa. Whereas, in eukaryotes, the heterotrimeric factor eIF2 is thought to catalyze the binding of Met-$tRNA_i^{Met}$ to the ribosome. Recently, bacterial IF2 homologs have been found in archaea and yeast genome sequences, suggesting that the mechanism of translation initiation in archaea and lower eukaryotes may combine features from bacteria and higher eukaryotes. Disruption of the *FUN12* gene encoding yeast IF2, yIF2, resulted in a severe slow-growth phenotype, and analysis of polyribosome profiles by velocity sedimentation of cell extracts in sucrose gradients revealed a translation initiation defect in the *fun12Δ* strain. Translation assays of whole cell extracts from isogenic wild-type and *fun12Δ* strains using a firefly luciferase mRNA showed that the wild-type extract had around 15-fold greater translational activity compared to the *fun12Δ* extract. Translational activity was restored to the *fun12Δ* extract by adding back purified recombinant GST-yIF2 fusion protein, indicating that yIF2 functions directly in translation initiation. Furthermore, both yIF2 and eIF2 were found to stimulate the synthesis of methionyl-puromycin in a reconstituted mammalian translation system, indicating that yIF2 promotes Met-$tRNA_i^{Met}$ binding to ribosomes. The severe slow growth phenotype of a *fun12Δ* strain was partially suppressed by overexpression of $tRNA_i^{Met}$; however, the phenotype was exacerbated by overexpression of eIF2. On the basis of these findings, we propose that yIF2 is a general translation factor that functions with eIF2 in essentially all initiation events to promote binding of Met-$tRNA_i^{Met}$ to the 40S ribosomal subunit.

To further explore the conservation of IF2 proteins in eukaryotes and archaea we isolated a cDNA from human that encodes an IF2 homolog, and we have performed a genetic and biochemical characterization of hIF2, the human IF2 homolog. The hIF2 cDNA predicts a protein of ~139 kDa; however, western analysis suggests a size closer to 175 kDa. The hIF2 mRNA is ubiquitously expressed and shows an expression pattern similar to other translation initiation factors. The expression of hIF2 complemented the slow-growth phenotype of a *fun12Δ* yeast strain. In addition, purified recombinant hIF2 substituted for yIF2 to stimulate translation in extracts from a *fun12Δ* strain. The ability of hIF2 to substitute for yIF2 both in vivo and in vitro demonstrates that hIF2 and yIF2 are functionally equivalent. Expression of an archaeal IF2 protein also partially complemented the slow-growth phenotype of a *fun12Δ* yeast strain. Thus, IF2 is a universally conserved factor in gene expression, and the mechanism of binding Met-$tRNA_i^{Met}$ to the ribosome is more highly conserved among prokaryotes and eukaryotes than previously anticipated.

The prokaryotic, archaeal and eukaryotic IF2 proteins all contain a GTP-binding domain. We have examined the importance of the G-domain in hIF2 by introducing point mutations in conserved motifs of the G-domain. Mutations that are predicted to impair GTP-binding abolished the ability of hIF2 to substitute for yIF2 both in vivo and in vitro. Furthermore, several point mutations in the equivalent positions of yIF2, as well as elsewhere in the G-domain, resulted in loss-of-function or dominant-negative phenotypes. Truncation and deletion mutations in yIF2 have revealed that the N-terminus of yIF2, prior to the G-domain, is dispensable for function in vivo and in vitro. In addition, several portions of the C-terminal region of yIF2 are dispensable for activity in vivo. These yIF2 mutants should be useful in further defining the role of the eukaryotic IF2 proteins in translation initiation.

Session 2: Translational control by viruses and apoptosis

Chair: Michael B. Mathews

TRANSLATION INHIBITION DURING HEAT SHOCK BY CHAPERONE TRAPPING OF eIF4G AND DISPLACEMENT OF eIF4E KINASE, Mnk1

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Mammalian cells respond to heat stress in a highly orchestrated manner. Heat shock activates a specific transcriptional response and largely inhibits protein synthesis, establishing conditions which favor the exclusive translation of heat shock and stress-related mRNAs. The inhibition of non-heat shock mRNA translation during heat stress prevents the continued synthesis of nascent proteins that might misfold due to elevated temperature and impair the cell. mRNAs that are specifically translated in mammalian cells during heat shock generally encode molecular chaperones, such as hsps 100, 90, 70, 60 and 27. A primary function of heat shock chaperones is to trap and bind unfolding protein intermediates, maintaining them in intermediate states of denaturation, thereby facilitating native protein refolding, cell survival and the rate of cell recovery. In mammalian cells, small heat shock proteins, such as hsp27, function as ATP-independent chaperones to physically complex with unfolding protein intermediates, limiting their denaturation and aggregation. During recovery from heat shock, ATP-dependent chaperones such as hsp70 can refold protein intermediates trapped by hsp27 to a native state. There is considerable evidence that only a small subset of proteins enter into chaperone complexes. The accumulation of heat shock proteins hsp70 and hsp27 are causally related in mammalian cells to a more rapid rescue of translation during cell recovery. In fact, increased cell survival correlates strongly with an increased rate of translational recovery after heat stress. Since the extent of translational protection during heat shock, and the rate of translational recovery, are related to the level of accumulation of hsp70 and hsp27, they likely participate in protecting the protein synthetic apparatus.

Heat shock mediates dephosphorylation and inactivation of the 24 kDa cap-binding protein translation initiation factor eIF4E, in a wide variety of cell types. eIF4E comprises the cap binding component of eIF4F, which includes the 50 kDa ATP and RNA-dependent helicase eIF4A, and the 220 kDa scaffolding protein eIF4G. The eIF4F complex binds to the m7GTP (cap) found on most mRNAs, and unwinds 5' secondary structure in the mRNA. The ability of eIF4F to facilitate translation strongly correlates with phosphorylation of eIF4E, which occurs in response to stimulation by serum and mitogenic growth factors, whereas inhibition correlates with decreased phosphorylation of eIF4E, which occurs during heat shock, serum deprivation, mitosis, and infection by certain viruses.

The shutoff of eIF4E phosphorylation and translation during heat shock in mammalian cells has been attributed to the sequestration of eIF4E
by a family of small eIF4E binding proteins known as 4EBP1, 2, 3 that are activated by heat shock and sequester eIF4E in some types of cells. However, several studies indicate that 4EBP sequestration of eIF4E is not sufficient to account for inhibition of eIF4E phosphorylation and

shutoff of protein synthesis during heat shock. First, hypophosphorylated 4EBP can be generated by treatment of cells with rapamycin, resulting in activation of 4EBP but only a very modest inhibition of translation. Second, many cell types appear to express low levels of 4EBPs which are insufficient to sequester eIF4E. Third, heat stress does not mediate hypophosphorylation and activation of 4EBP in some cell types.

We therefore asked whether the shutoff of cap-dependent protein synthesis during heat shock is mechanistically linked to the induced expression of heat shock chaperones. Studies examined the control of the eIF4E kinase, a MAP kinase known as Mnk1, in cells at normal and heat shock temperature. At normal temperature, Mnk1 complexes with eIF4G, which is shown to be essential for phosphorylation of eIF4E. However, formation of the Mnk1-eIF4G complex and the phosphorylation of eIF4E are shown to be inhibited by heat shock, thereby blocking cap dependent protein synthesis. The mechanism for inhibition of translation during heat shock is shown to involve heat shock induced hsp70 protein, which specifically binds eIF4G, displaces the eIF4E kinase Mnk1, and selectively sequesters unfolding eIF4G in perinuclear complexes with hsp27, thereby blocking eIF4E phosphorylation. Experimental induction of hsp70 in the absence of thermal denaturation (using the proteasome inhibitor MG132) does not induce hsp27, and it does not mediate sequestration of eIF4G, displacement of Mnk1, dephosphorylation of eIF4E or shutoff of translation. Thus, chaperone trapping of eIF4G actually constitutes a fundamental mechanism by which cap-dependent protein synthesis is inhibited during cell heat shock. Since eIF4E-Mnk1 kinase activity was found to remain intact during heat shock, these results also implicate the release of Mnk1 in the propagation of downstream signalling events involved in the heat stress response.

A human sequence homologue of Staufen is an RNA-binding protein that is associated to polysomes and localizes to the rough endoplasmic reticulum

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In the course of a two-hybrid screen with the NS1 protein of influenza virus, a human clone was identified capable of coding for a protein with high homology to the Staufen protein from *D. melanogaster* (dmStaufen). Using these sequences as a probe, cDNAs were isolated from a l cDNA library. The encoded protein (hStaufen-like) contained 4 dsRNA binding domains with 55% similarity and 38% identity to those of the dmStaufen, including identity at all residues involved in RNA binding. A recombinant protein containing all dsRNA binding domains was expressed in *E. coli* as a His-tagged polypeptide. It showed dsRNA binding activity *in vitro*, with an apparent Kd of 10^{-9} M. Using a specific antibody, a main form of the hStaufen-like protein could be detected in human cells, with apparent molecular mass of 60-65 kDa. The intracellular localization of hStaufen-like protein was investigated by immunofluorescence, using a series of markers for the cell compartments. Co-localization was observed with rough endoplasmic reticulum, but not with endosomes, cytoskeleton or Golgi apparatus. Furthermore, sedimentation analyses indicated that hStaufen-like protein associates with polysomes.

The interaction of hStaufen-like protein with influenza NS1 protein has been studied both in vivo and in vitro. In addition to the interaction detected in the two-hybrid system in yeast, both proteins can co-immunoprecipitate from influenza virus-infected cells, when co-expressed in cultured cells or when mixed in vitro. Furthermore, both hStaufen-like and NS1 co-fractionate in the polysomes of influenza virus-infected cells.

These results will be discussed in relation to the possible functions of the hStaufen-like protein.

INFLUENZA VIRUS NS1 PROTEIN BINDS TO eIF-4GI.

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The influenza virus NS1 protein is a multistep post-transcriptional modulator, which regulates polyadenylation, splicing and nucleocytoplasmic transport of cellular mRNAs. In addition, NS1 protein is able to enhance the translational rate of viral, but not cellular mRNAs. Previous experiments showed that this specific enhancement takes place at the initiation step. In order to understand the mechanism by which NS1 stimulates viral translation, we started several strategies to find cellular targets for the influenza virus protein. Here we show that NS1 protein interacts *in vivo* with eIF4GI, the 220 kDa subunit of the cap binding complex eIF4F.This *in vivo* interaction occurs both in influenza virus infected cells and in transfected cells individually expressing NS1 from cloned cDNA. *In vitro* binding assays indicate that the NS1-binding domain of eIF4GI is located in the amino terminal half of the protein, between residues 1 and 393, in a region where no other component of the translational machinery is known to interact. Finally, we demonstrate through an overlay assay that NS1 and eIF4GI bind directly, in an RNA-independent manner.

It has been described that 1) the NS1-dependent enhancement of viral translation requires the 5'-UTR of viral mRNAs and 2) influenza virus infection is not compatible with poliovirus infection where the eIF4GI is cleaved. Collectively with the results reported here, the data suggest a model where NS1 recruits eIF4GI to the 5'-UTR of the viral mRNA allowing specific translational enhancement of the influenza virus messengers.

THE ART OF SELFISH PERSUASION: HOW VIRUSES HIJACK THE CELLULAR PROTEIN SYNTHESIZING MACHINERY AND EVADE THE HOST DEFENSE.

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This seminar will focus on virus-host interactions, evasion of the host defense, and the control of protein synthesis. Two very different RNA viruses, influenza virus and hepatitis C virus (HCV), will be discussed. In the first part of the presentation we will describe mechanisms by which these viruses evade the host cell interferon defense system. Viruses downregulate the interferon induced protein kinase, PKR, to avoid its antiviral properties and resultant negative effects on overall protein synthetic rates. Influenza virus activates a complex cellular pathway, which includes the co-chaperone P58IPK as well as the established molecular chaperones hsp40 and hsp70, to downregulate PKR activity. Influenza may also utilize a secondary strartegy to downregulate PKR via the action of the NS1 nonstructural protein which we have found binds to and inactivates PKR.

HCV downregulates PKR through the action of the NS5A nonstructural protein. For HCV, these anti-PKR strategies may likely have therapeutic consequences. The second portion of the presentation will concentrate on the devious mechanisms devised by influenza virus to regulate selective mRNA translation. We have found that the influenza virus mRNA 5'UTR (untranslated region) is critical in directing selective viral protein synthesis. Indeed this portion of the viral mRNA is both necessary and sufficient to trick the host cell protein synthesizing machinery. Using the yeast three-hybrid system we have now identified a cellular RRM containing RNA binding protein, GRSF-1, that likely plays a key role in directing selective viral mRNA translation. This is the first example of a cellular protein binding to an influenza virus RNA of any kind. The GRSF-1 protein binds to specific regions of the influenza virus mRNA 5'UTR and most importantly upregulates translation of only mRNAs containing the influenza virus 5' leader. A model will be presented to describe possible mechanisms of GRSF-1 action.

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Selective regulation of polypeptide chain initiation factor phosphorylation and degradation during apoptosis

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Polypeptide chain initiation factors are subject to a range of regulatory processes in mammalian cells. These mechanisms result in changes in the activities of several initiation factors in response to extracellular and intracellular signals. Examples are the phosphorylation of the α subunit of eIF2 by the protein kinases PKR, HCR and GCN2 [1], the phosphorylation of eIF4E by MAP kinase-regulated pathways [2,3] and the phosphorylation of proteins such as 4E-BP1, which binds and sequesters eIF4E, by a PI 3-kinase and mTOR-regulated pathway [4]. In addition, alterations in the concentrations of key (potentially rate-limiting) factors may play an important role in the control of translational activity.

Recently we have been investigating changes in protein synthesis and in the levels and activity of initiation factors that occur during the early stages of apoptosis in human lymphoid and other tumour cell lines. When the B cell and T cell tumour-derived cell lines BJAB and Jurkat, respectively, are subjected to conditions that induce apoptosis, protein synthesis is rapidly inhibited. This is accompanied by early degradation of initiation factor eIF4G and a slower rate of loss of eIF2. There are no major losses of certain other key initiation factors such as eIF4E and eIF4A under the same conditions. The disappearance of full length eIF4G is accompanied by the appearance of smaller forms of the protein, including a major product of approximately 76 kDa [5]. Such effects are observed when apoptosis is induced by serum deprivation, stimulation of the CD95 (APO-1/Fas) receptor, or treatment of cells with the protein synthesis inhibitor cycloheximide or the DNA damaging agent etoposide. Both total cytoplasmic eIF4G and eIF4G associated with eIF4E are degraded with a half-life of about 2-4 hours. Exposure of cells to the compounds Z-VAD.FMK or Z-DEVD.FMK, which inhibit a number of specific proteases (caspases) required for apoptosis, protects eIF4G from degradation and blocks the appearance of the ca. 76 kDa product [5]. Such treatment also completely protects protein synthesis from inhibition in cells stimulated through the CD95 receptor [6].

BJAB cells are very sensitive to the effects of the macrolide immunosuppressant rapamycin, which inhibits the mTOR/$p70^{s6}$ kinase signalling pathway, blocks the phosphorylation of the eIF4E binding proteins (4E-BPs) and inhibits the association of eIF4G with eIF4E. However, in spite of the fact that exposure of BJAB cells to rapamycin rapidly inhibits overall protein synthesis, this does not lead to the acute degradation of eIF4G [5]. Moreover, rapamycin prevents the cleavage of eIF4G in cells deprived of serum or stimulated through the CD95 receptor, although the drug has no effect in cells treated

with cycloheximide or etoposide.

In another cell line (MCF-7 breast carcinoma), which is deficient in caspase-3 activity, eIF4G is not cleaved upon induction of apoptosis with tumour necrosis factor and cycloheximide or with staurosporine. *In vivo* expression of caspase-3 in these cells restores eIF4G cleavage following either treatment. *In vitro*, recombinant caspase-3 can cleave the eIF4G contained in cell extracts or presented as purified eIF4F to yield 76 kDa and other fragments which are apparently identical to those seen *in vivo* in apoptotic cells. Cleavage *in vitro* is not affected by the addition of the eIF4E binding protein 4E-BP1. These results indicate that caspase-3 activity is both necessary and sufficient for eIF4G degradation and suggest that cleavage is independent of the association of eIF4G with eIF4E.

In parallel with the changes described above, a number of other events occur that may contribute to the inhibition of protein synthesis during the early stages of apoptosis. These include increased phosphorylation of the α subunit of eIF2, dephosphorylation of eIF4E and increased association of 4E-binding proteins with eIF4E. Thus the progress of apoptosis is characterized by a complex programme of changes in several initiation factors, including the specific fragmentation or complete degradation of some and alterations in the state of phosphorylation of others.

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Session 3: Translational control by the 5' end: the cap and the 5'UTR

Chair: Michael J. Clemens

THE mRNA 5'-CAP BINDING PROTEIN COMPLEX, eIF4F: SIGNALING PATHWAYS THAT CONTROL ITS ACTIVITY. Nahum Sonenberg, Department of Biochemistry, McGill University, Montreal, Quebec, CANADA H3G 1Y6

Cellular mRNAs contain a cap structure (m^7GpppN) at their 5' terminus, which facilitates 40S ribosome binding to the mRNA. Cap function is mediated by the initiation factor eIF4F, which consists of three subunits: a. eIF4E, the cap-binding polypeptide. b. eIF4A, an RNA helicase. c. eIF4G, which forms a platform for the binding of eIF4A, eIF4E, and other initiation factors. eIF4E plays a key role in regulating translation and in control of cell growth, and proliferation. In addition, eIF4E can transform NIH 3T3 cells in culture when overexpressed. eIF4E is phosphorylated in response to cell treatment with a vast array of extracellular stimuli including hormones, growth factors, and G-coupled receptor agonists.

One of the signaling pathways, which is responsible for eIF4E phosphorylation is the Ras-Raf-Erk pathway. The direct kinase, which phosphorylates eIF4E *in vivo* and *in vitro*, is Mnk1, which is a substrate for the MAP kinases, Erk and p38. Mnk1 does not bind directly to eIF4E, but utilizes the eIF4G subunit as a docking site to phosphorylate eIF4E. The binding site of Mnk1 on eIF4G was mapped to a 150 amino acids fragment at the carboxy-terminus. A mutant eIF4E, which is defective in eIF4G binding, could not be phosphorylated *in vivo*.

We are studying a family of proteins, termed 4E-BPs (eIF4E binding proteins), which bind to eIF4E, and inhibit specifically cap-dependent translation initiation. 4E-BPs compete with eIF4G for binding to eIF4E through a shared binding motif. Thus, 4E-BPs prevent the formation of a functional eIF4F complex. The interaction between 4E-BPs and eIF4E is dramatically diminished upon their phosphorylation in response to extracellular stimuli, concomitant with the relief of translational repression of capped mRNAs. A rapamycin-sensitive signal transduction pathway effects phosphorylation of 4E-BPs. PI3-kinase and Akt/PKB are upstream components in this signal transduction pathway. In addition, the PI3-kinase-related, rapamycin-sensitive protein FRAP/mTOR serves as a checkpoint of this pathway. FRAP/mTOR phosphorylates two sites on 4E-BP1 in an *in vitro* immune-kinase assay, suggesting that these are priming sites for the subsequent phosphorylation of the remaining sites. The study of signaling to the translation machinery is important for the understanding cell growth and proliferation control.

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CELLULAR RNA-BINDING PROTEINS REQUIRED FOR INTERNAL INITIATION OF TRANSLATION

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Internal initiation of translation was first discovered in the animal picornavirus RNAs and was subsequently extended to RNAs of the hepatitis C virus (HCV)/pestivirus family. It requires a substantial cis-acting RNA element, ~450 nt. long in the case of the picornaviruses and ~330 nt. for HCV, which is generally known as the IRES (for "internal ribosome entry segment").

The function of picornavirus IRESes requires virtually all the canonical translation initiation factors needed for the scanning mechanism except for eIF4E, the N-terminal part of eIF4G, and possibly eIF1A. Small (40S) ribosomal subunits do not bind to picornavirus IRESes in the absence of canonical initiation factors; it seems likely that the critical factor is the central fragment of eIF4G which appears to bind to the IRES at a specific site and delivers the 40S subunit to the correct internal initiation site, probably via the intermediary action of eIF3 (which can interact with both eIF4G and the 40S subunit). In addition, at least some picornavirus IRESes have a specific binding site for eIF4B.

IRESes of the HCV/pestivirus group are very different, and internal initiation in this case is a much closer analogue of the prokaryotic mechanism of initiation. Small (40S) ribosomal subunits bind to the IRES close to the correct initiation site, even in the absence of canonical initiation factors or other non-ribosomal proteins. Internal initiation promoted by these IRESes does not require eIF4A, 4B, 4E or 4G, and is probably also independent of eIF1 and eIF1A. All that appears to be required is the eIF2/Met-tRNAi/GTP ternary complex, eIF3, which has a specific binding site on the IRES that appears to be close to, but probably not overlapping with the 40S ribosome binding site, and presumably also eIF5 for subunit joining.

Apart from canonical translation initiation factors, internal initiation dependent on picornavirus IRESes, but probably not HCV/pestivirus IRESes, may require other cellular proteins, generally RNA-binding proteins. In this respect there are major differences between the two main groups of picornavirus IRES (neither of which includes hepatitis A virus): (i) the cardio-/aphthovirus and (ii) the entero-/rhinovirus IRESes. Enterovirus (e.g. poliovirus) and especially rhinovirus IRESes function very inefficiently in rabbit reticulocyte lysates unless the system is supplemented with HeLa cell extract. Using this as a functional assay to purify the HeLa cell activities required for rhinovirus IRES activity, we find that there are two such factors each individually capable of stimulating IRES activity, but acting synergistically when tested together. One of these was identified as polypyrimidine tract binding protein (PTB), an RNA-binding protein with 4 degenerate RRMs which has been previously implicated as a regulator of alternative splicing of pre-mRNA; and the other as a complex of a novel GH-WD repeat protein (p38) with unr, a cytoplasmic protein with five copies of the "cold shock" RNA-binding domain. Site-directed mutagenesis has shown that all five copies of the cold shock domain are necessary

for stimulation of rhinovirus IRES activity. In addition rhinovirus IRES activity also requires poly(C) binding protein 2 (PCBP-2), although this protein, which has 3 KH domains, is normally present in reticulocyte lysates in sufficient abundance to be not limiting. Surprisingly, the activity of the poliovirus IRES, which is much higher in unsupplemented reticulocyte lysate than that of the rhinovirus IRES, is not stimulated by unr but does show dependency on both PTB and PCBP-2.

In contrast, cardio- and aphthovirus IRESes function very efficiently in reticulocyte lysates without the need for addition of HeLa cell extract. However, these IRESes bind PTB with high affinity, and it was previously suggested that this binding is necessary for the function of the IRES. (Reticulocyte lysates do contain some PTB, though not enough for efficient entero-/rhinovirus IRES activity as these IRESes bind PTB with much lower affinity than do the cardio-/aphthovirus IRESes.) Subsequently it was shown that this PTB-dependence of the cardiovirus IRESes was an artefact caused by (i) the fact that the IRES studied in this work had acquired an additional A-residue in an A-rich bulge, and (ii) the use of a heterologous reporter cistron. When the true wild-type cardiovirus IRES is linked to viral polyprotein coding sequences, internal initiation does not require PTB, even though the high affinity of the IRES for this protein is unchanged. On the other hand, the foot-and-mouth disease virus IRES does show a partial dependence on PTB even when viral coding sequences serve as reporter.

It is striking that all these non-canonical trans-acting factors required for internal initiation (PTB, PCBP-2 and unr) are RNA-binding proteins with multiple RNA-binding domains, all of which, so far, appear to be necessary for their function in internal initiation. This suggests that these proteins may make multi-point contact with the IRES, which could help to attain or maintain the appropriate tertiary structure necessary for internal initiation. Thus, rather than acting as direct catalysts of internal initiation, we suggest that their function is related to maintenance of the required higher order RNA structure of the IRES.

eIF4E, eIF4G, PABP and their Roles in Cap-Dependent and Cap-Independent Translation

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Initiation factors of the eIF4 class as well as poly(A)-binding protein (PABP) play key roles in the recruitment of mRNA to the small ribosomal subunit and in the discrimination between different types of mRNAs (1). The choice of mRNAs translated can be influenced by modifying the intracellular levels or activities of these initiation factors. The modifications that have been documented to date include proteolysis, phosphorylation, sequestration in complexes with binding proteins, and binding to other initiation factors. Furthermore, isoforms of initiation factors have been described, as has the existence of regulated transcription of their genes. Our laboratory has recently been studying the modifications and isoforms of initiation factors involved in the recruitment of mRNA.

In the nematode *C. elegans*, polysomal mRNAs from 70% of the genes contain an atypical cap structure, $m_3^{2,2,7}$GTP. This cap structure is poorly recognized by mammalian eIF4E, suggesting that *C. elegans* may possess a specialized form of eIF4E that can recognize $m_3^{2,2,7}$GTP. An earlier analysis of the *C. elegans* genomic sequence database revealed the presence of three eIF4E-like genes, which were then named *ife-1*, *ife-2*, and *ife-3* (2). Further investigation has revealed two additional eIF4E genes, *ife-4*, and *ife-5*. All five recombinant eIF4E isoforms were retained on m^7GTP-Sepharose, but IFE-1 and -5 were also retained on $m_3^{2,2,7}$GTP-Sepharose. Furthermore, binding of IFE-1 and IFE-2 to m^7GTP-Sepharose was inhibited by $m_3^{2,2,7}$GTP. These results suggest that IFE-1, -2 and -5 bind both m^7GTP- and $m_3^{2,2,7}$GTP-containing mRNA cap structures. RNA interference assays indicated that not all five isoforms were needed for viability; various combinations of eIF4E isoforms permitted nematodes to survive.

The eIF4 initiation factors act by forming aggregates of increasing complexity during assembly of the 48S initiation complex. Our previous studies showed that the N-terminal one-third of eIF4G-1 contains the eIF4E-binding site while the C-terminal two-thirds contains the eIF3 and eIF4A binding sites (3). A more detailed analysis of recombinant eIF4G fragments has now indicated that an RNA-binding region is located in aa 487-721, whereas the smallest region of eIF4G that can bind eIF3 is aa 819-922. Both titration and competition experiments are consistent with a single site for eIF3 binding on eIF4G and a 1:1 stoichiometry. One of the two eIF4A-binding sites on eIF4G-1 is located in aa 486-720 and is distinct from the eIF3-binding site. The other eIF4A-binding site is located in aa 922-1160. Binding occurs with a 1:1 stoichiometry at each individual site, but an eIF4G fragment containing aa 394-1404 binds eIF4A with a 2:1 stoichiometry, contrary to previous models. The addition of both eIF4A and eIF3 simultaneously to the central domain of eIF4G produced enhanced binding of each ligand rather than competition, suggesting cooperative binding. Rate and equilibrium constants for the various interactions were determined by surface plasmon resonance.

One modification of initiation factors that has been extensively studied is the cleavage of eIF4G by the 2A or L proteases of some picornaviruses. Although considerable evidence

suggests that the shut-off of host mRNA translation is mediated by the cleavage of eIF4G-1, several observations are at variance with this view. Recent evidence indicates that PABP participates directly in translation initiation (4, 5). We have now found that PABP is also proteolytically cleaved during coxsackieviral infection of HeLa cells. The cleavage of PABP was better correlated temporally with the host shut-off and onset of viral protein synthesis than the cleavage of eIF4G-1. Amino acid sequencing indicated that cleavage occurs uniquely in human PABP between aa 490 and 491, separating the four N-terminal RNA-recognition motifs (80%) from the C-terminal homodimerization domain (20%). The N-terminal cleavage product of PABP was less efficient than full length PABP in restoring translation to a PABP-dependent rabbit reticulocyte lysate translation system. These results suggest that the cleavage of PABP may be another mechanism by which picornaviruses alter the rate and spectrum of protein synthesis.

To study the effect of 2A protease action *in vivo*, we turned to the *Xenopus* oocyte system. *Xenopus* oocytes accumulate maternal mRNAs which are then recruited to ribosomes during meiotic cell cycle progression in response to progesterone and coincident with poly(A) elongation of the affected mRNAs. Prior to stimulation, most protein synthesis (~70%) does not require intact translation factor eIF4G (6). We have now addressed the requirement of eIF4G in the recruitment of mRNAs during meiosis. Cleavage of eIF4G by coxsackievirus protease 2A (under conditions that did not cleave PABP) inhibited progesterone-induced meiotic progression in 88% of the oocytes, prevented the recruitment of maternal mRNAs encoding cyclin B1, c-Mos, D7 and B9, and disrupted the association of eIF4G with poly(A)-binding protein. Poly(A) elongation, however, was not inhibited by eIF4G cleavage. B9 and cyclin B1 mRNAs, once recruited, were removed from polyribosomes by subsequent cleavage of eIF4G, indicating that eIF4G is required for maintenance of maternal mRNA translation. The expression of a cleavage-resistant variant of human eIF4G-1 (G486E) significantly restored the ability to translate exogenous β-globin mRNA and to recruit c-*mos* mRNA in response to progesterone. These results indicate that intact eIF4G plays an essential role in the poly(A)-dependent recruitment of maternal mRNAs during meiotic cell cycle progression but does not participate in the synthesis of most proteins. (Supported by Grant GM20818 from the NIGMS.)

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"The Role of the p70s6k Signal Transduction Pathway in Cell Growth".

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Mitogens induce the coordinated activation of a number of anabolic events which culminate in cell growth and division. Recent studies have defined the distinction between growth and proliferation, demonstrating the dominance of growth in this process. An important component of the growth response is the generation of new translational machinery, required to accommodate the increased demand for additional proteins. The enhanced expression of protein synthetic components, most notably ribosomal proteins and elongation factors, is largely controlled at the translational level. In parallel, it has been shown that the immunosupressant rapamycin, a bacterial macrolide, specifically blocks the translational upregulation of these mRNA transcripts as well as the increased phosphorylation of the activation of S6K1 and the phosphorylation of 40S ribosomal protein S6. More striking, the effects of rapamycin on the translational upregulation of these mRNA transcripts and S6 phosphorylation can be reversed by a rapamycin resistant mutant of S6K1. Thus studies to date strongly imply that S6K1, through S6 phosphorylation is intimately involved in the regulation of translation and that the effects of rapamycin on cell growth are elicited through this pathway. However, corroborative physiological studies in animals are lacking. Due to its powerful genetics, low genetic complexity and high conservation of signaling pathways, *Drosophila melanogaster* has emerged as a model metazoan for gaining insight into the physiological role of specific signaling components and uncovering their genetic interactions with potential downstream targets. We have recently shown that P-element induced mutation of *Drosophila* homologue of S6K1, S6K1 causes a *Minute-like* phenotype in *Drosophila*, which can be rescued by expression of the *Drosophila* or the mammalian S6K1. Only 25% of the

expected number of homozygous flies emerge as adults, with a three delay in development and a reduced body size. Northern blot analysis and subcloning of a RT-PCR product from homozygous flies revealed the presence of cryptic transcripts, suggesting that that the expression of *dS6K* was suppressed but not abolished. More sever alleles were obtained by generating imprecise P-element excisions that removed all or part of the *dS6K* gene. Seven lines were recovered, five of which were rescued by expression of the *dS6K* or mammalian S6K1 transgene and all of which failed to complement one another. Molecular characterization of one of the rescued alleles, $dS6K^{1}$, revealed that the imprecise excision had removed part of the first exon and a large portion of the contiguous intervening sequence. The few flies which survived emerged after a five day developmental delay, were significantly smaller in size than the $dp70s6k^{07084}$ homozygous flies, and lived no longer than two weeks. Dominant genetic interactions between *dS6K* mutants and ribosomal protein S6 mutants, support the hypothesis that the effects of the kinase on cell growth are mediated through S6 phosphorylation. To test this possibility we plan to generate gain of function mutations in *DS6*. In summary, loss of *D*S6K function severely affects growth, development and viability.

MOLECULAR INTERACTIONS BETWEEN CAP BINDING PROTEINS AND ASSOCIATED FACTORS FROM SACCHAROMYCES CEREVISIAE

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To understand the structure and function of the cap-binding complex in S. cerevisiae, we are overexpressing eIF4G, eIF4E, eIF4A and p20 - or parts of these proteins - to study their molecular interactions and biochemical properties in vitro and in vivo.

p20 is a small protein (161 amino acids) which acts as an inhibitor of protein synthesis by binding to eIF4E and thereby impeding its interaction with eIF4G. The amino-terminal sequence of p20 (aa 1 to 10) shows homology to a motif present in the eIF4E-interaction domain of eIF4G1 (aa 449 to 458) and is supposed to be required for proper eIF4E-binding (Altmann et al., 1997). We have further analysed this domain and show now that the amino-terminal amino acid motif (10 aa) on p20 is not sufficient, at least the 13 amino-terminal amino acids are required for efficient eIF4E-binding.

Furthermore, the amino-terminal eIF4E-interaction domain on p20 is located close to an hydrophilic amino acid stretch (between aa 28 to 90) with RNA-binding activity. A similar modular arrangement, eIF4E - RNA binding sites in close vicinity, is also found in eIF4G1 suggesting that binding of p20 and eIF4G to RNA is required to stabilize the interaction with eIF4E at the cap-structure of mRNA.

eIF4A is assumed to be recruited to the mRNA through its interaction with eIF4G. We demonstrate that yeast eIF4G1, like its mammalian and plant counterparts, interacts directly with eIF4A. The interaction is probably transient, since only a small portion of these factors were found in a complex in growing cells. The eIF4A-binding site has been mapped to amino acids 542 to 883 of yeast eIF4G1. This region is homologous to the central domain of the mammalian factor which has been shown to bind eIF4A.

Expression in yeast of the eIF4G1 domain that binds eIF4A results in cell growth inhibition. In an eIF4A-dependent cell free system the eIF4G domain inhibits translation in a dose-dependent manner. Both, inhibition of translation and cell growth can be specifically overcome by increasing the eIF4A concentration. These findings suggest that the interaction of eIF4G with eIF4A is required for translation and cell growth.

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Session 4: Translational control by the 3' end: the poly A and the 3'UTR

Chair: Alan Hinnebusch

RIBOSOME RECRUITMENT TO mRNAs AND ITS REGULATION

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The translation of cellular mRNAs is determined by the 5' cap structure, the 3' poly-A tail and specific regulatory sequences usually contained within the 5' or 3' untranslated regions of the mRNAs. Specific regulatory elements have been identified in both the 5' untranslated regions (Iron-responsive elements, sex-lethal binding sites) and in the 3' untranslated regions (Differentiation Control element, DICE) of specific translationally regulated mRNAs. Their specific binding proteins (iron regulatory proteins, IRPs; sex lethal, SXL, hnRNP K, hnRNP E1) have been identified and cloned, and their mechanism of regulation studied in vivo and in vitro. Results will be discussed with regard to the regulation of translation initiation during cell differentiation, in metabolic control and in early development.

Furthermore, the role of general translation initiation factors involved in the recruitment of the 43S translation initiation complex will be discussed, also in the light of the mechanism by which translational regulatory proteins can act.

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CPE-Mediated Masking and Unmasking of mRNA

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The translational regulation of many maternal mRNAs in Xenopus laevis is controlled by cytoplasmic poly(A) elongation. Generally, mRNAs in oocytes that are translationally dormant have relatively short poly(A) tails, and it is only when these tails are elongated during oocyte maturation, or after fertilization, does translation ensue. Two 3' UTR elements are necessary for polyadenylation during maturation, the CPE (consensus, UUUUUAU) and the polyadenylation hexanucleotide AAUAAA. The CPE is bound by CPEB, a protein that is necessary for polyadenylation.

We suspected that cyclin B1 mRNA, which undergoes polyadenylation and translational activation during oocyte maturation, might be repressed initially in oocytes by a trans acting factor. To test this possibility, we injected large amounts of cyclin B1 3' UTR into oocytes. This procedure resulted in endogenous cyclin B1 synthesis in the absence of poly(A) elongation. To determine the B1 3' UTR sequence that was responsible for mRNA unmasking, we performed a mutational analysis. Surprisingly, the CPE was the most efficient unmasking sequence. Furthermore, when placed in the context of a nonspecific polylinker, the CPE efficiently unmasked cyclin B1 mRNA. However, other U-rich sequences that resemble the CPE in structure, but which do not function as CPEs in injected oocytes, did not lead to mRNA unmasking.

We appended the CAT coding sequence with various 3' UTRs containing or lacking a CPE and AAUAAA. The cyclin B1 3' UTR repressed translation of the chimeric mRNA following oocyte injection. Moreover, when attached to the 3' end of CAT mRNA, a polylinker sequence containing multiple CPEs repressed translation in a CPE dose-dependent manner. When such injected oocytes were induced to mature with progesterone, only the CAT chimeric mRNA that contained a CPE and AAUAAA hexanucleotide was unmasked, as evidenced by high CAT activity. In other words, unmasking of mRNA required cytoplasmic polyadenylation.

Because the CPE acts both negatively and positively vis-à-vis translation, we thought that a repressor protein, in addition to the activator CPEB, might bind this sequence. However, an extensive analysis revealed that only CPEB bound the CPE. Thus, to begin to build a testable model, we asked whether an mRNA cap was necessary for the masking process. Indeed, efficient masking required cap-dependent translation. Based on these data, we will propose a hypothesis to explain CPE-mediated masking and unmasking of mRNA.

The 3' Untranslated Region of AMV Coat Protein mRNA Regulates Competitive Translational Efficiency

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Our research interests include characterizating translational control mechanisms that enhance virus replication potential. The focus of our work is the identification of nucleotide sequences in non-polyadenylated viral mRNAs that promote efficient and competitive mRNA translation, thereby favoring viral gene expression over the host.

Alfalfa mosaic virus is a single stranded RNA virus of positive polarity. Like cellular mRNAs, AMV RNAs are capped; however, they lack a poly(A) tail. AMV RNAs are further distinguished by the lack of both 3' pseudoknots and the 3'-terminal transfer RNA-like structure that is found on a number of plant viral RNAs (1). AMV RNA 4 encodes the viral coat protein, and this mRNA was recognized nearly two decades ago as an efficient and competitive mRNA (2,3). Although substantial evidence suggests that the poly(A) tail, poly(A) binding protein (PABP), and 5'-3' interactions facilitate cellular mRNA translation (4-6), the relationship of these findings to translating non-adenylated mRNAs is not clear.

Our results suggest the 3' untranslated region (3' UTR) of AMV coat protein mRNA is required for competitive translation (7). We have used *Xenopus laevis* oocytes to study AMV coat protein mRNA because they are translationally saturated, and therefore represent a naturally competitive translational environment (8). When AMV coat protein mRNAs lacking the 3' UTR are microinjected into *Xenopus* oocytes, the transcripts are stable, but translate poorly. Similar results are obtained when the mRNAs are translated under competitive conditions in a wheat germ translation extract. The results suggest that 3' untranslated sequences are important for determining competitive translational activity of the viral mRNA, allowing the viral sequences to out-compete cellular mRNAs for limiting translational components.

The 170-nucleotide 3' UTR of the AMV coat protein mRNA has nine AUG triplet nucleotides and, correspondingly, nine potential open reading frames. One hypothesis to explain the competitive role of the 3' UTR is that the downstream open reading frames permit ribosome reinitiation and facilitate 3'-5' end ribosome recycling (7). If the hypothesis were correct, a prediction would be that impeding ribosome movement into the 3' UTR would diminish translation. We therefore inserted a stable stem loop between the termination codon of the AMV coat protein mRNA and the 3' UTR. The results of the experiment showed that the mRNA containing this stable hairpin structure translated as efficiently as the wild type AMV coat protein mRNA, suggesting that ribosome reinitiation and 3'-5' ribosome recycling do not determine competitive potential.

To further explore the role of 3' untranslated nucleotides in AMV coat protein mRNA translation, we used electrophoretic mobility bandshift experiments (EMSA) to test protein interactions. Biochemical data now suggest that poly(A) binding protein (PABP) binds specifically to the 3' UTR of the non-adenylated AMV coat protein mRNA. PABP interactions with the AMV CP mRNA 3' UTR were not expected; however, Dreyfuss and colleagues proposed some years ago that important PABP function may be related to interactions with non-

poly(A) nucleotide sequences (9). The RNAs of several clinically important human viruses, including the flaviruses, *i.e.* dengue fever virus and yellow fever virus, are non-adenylated. Because of the resourceful adaptations shown by viruses, the absence of a poly(A) tail may be related to replicative advantage over the host (10).

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Exploring the yeast eIF4G protein complex through biochemical and genetic means

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The yeast *Saccharomyces cerevisiae* contains two functionally related eIF4G proteins, eIF4G1 and eIF4G2. In order to understand in greater detail how these adapter proteins interact with other components of the translation initiation machinery, we have undertaken an extensive biochemical and genetic analysis of them. First, we have created a series of point mutations in the RRM2 region of yeast Pab1p, and have analyzed them for their ability to prevent association of Pab1p with eIF4G, to prevent Pab1p from mediating poly(A) tail-dependent translation, and to prevent Pab1p from stimulating cap-dependent translation. Results from these experiments indicate that binding to eIF4G and stimulation of poly(A)-dependent translation by Pab1p requires different amino acids then does stimulation of cap-dependent translation by Pab1p. Second, we have continued to define the residues within yeast eIF4G that are required for eIF4E binding. Using a combination of different approaches, we have discovered that a 70 amino acid fragment of eIF4G provides an optimal binding surface for eIF4E, and that this fragment appears to be unfolded in the absence of eIF4E. These studies suggest that the association of eIF4E with eIF4G could be regulated via modifications that prevent eIF4G folding. Finally, we have discovered through a dosage suppression screen of the temperature-sensitive eIF4G2 mutation *tif4632-V610D*, followed by several different biochemical analyses, that the yeast eIF4A protein TIF1 functionally and physically associates with yeast eIF4G. These data are in accord with recent reports documenting the association of eIF4A with an internal region of mammalian eIF4G, and they provide the first genetic evidence for the importance of this interaction.

TRANSLATIONAL REGULATION OF TUMOR NECROSIS FACTOR BIOSYNTHESIS

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Post-transcriptional mechanisms are playing a very important role in the expression of numerous genes. Messenger RNAs encoding growth factors like cytokines, and oncoproteins such as c-fos, c-myc, have very short half-lifes and can be translationally regulated (1,2). Abnormal expression of such genes can lead to severe disorders including tumor formation (3). The precise identification of the sequences which determine the half-life of a messenger RNA or the level of its translation should lead in the near future to the discovery of new therapeutic tools.

In our laboratory, we focus on the study of the translational regulation of the Tumor Necrosis factor-α (TNF-α) mRNA. TNF-α is mainly produced by activated macrophages. Upon exposure to various stimuli (lipopolysaccharides (LPS), viruses, IL-12, ...), macrophages produce important amounts of TNF. In addition to the 20-fold increase in transcriptional activity, TNF gene expression is upregulated (200-fold) at the translational level in LPS-stimulated macrophages (4,5). The translational regulation of TNF mRNA is mediated by a conserved AU-rich element (ARE) located in the 3' untranslated region (UTR). Similar AREs are also found in other cytokine mRNA 3'UTRs (6). In quiescent cells, the translation of TNF mRNA is blocked. However, translational derepression occurs when the macrophage cells are stimulated with LPS. This translational regulation is a crucial mechanism that ensures a strong but highly inducible control of TNF biosynthesis.

Gel retardation experiments performed in our laboratory show that TNF mRNA ARE can form two complexes of different electrophoretic mobilities with proteins present in macrophage cytoplasmic extracts (7). Whereas the complex of low electrophoretic mobility is formed independently from the activation state of the macrophages, the second complex is formed upon activation of the cells with LPS or viruses. Furthermore, herbimycin A which is a potent inhibitor of TNF mRNA translation, abrogates the formation of this inducible complex, indicating a tight correlation between the formation of this inducible complex and TNF mRNA translation. Further studies revealed that the two complexes bind different sequence elements within the ARE. By UV-crosslinking experiments, we identified a 55-kDa polypeptide as a component of the inducible complex (8).

In order to identify the proteins involved in both complexes, we designed a cloning strategy based on the differential screening of a RAW 264.7 macrophage cDNA expression library with TNF mRNA 3'UTR riboprobes containing or not the ARE. By this method, we isolated the cDNA encoding the short 40-kDa isoform of the RNA-binding protein TIAR. We have shown that TIAR specifically binds TNF ARE and corresponds to the protein involved in the constitutive complex. Moreover, analysis of TIAR subcellular localization by immunostaining reveals that TIAR is mainly found in the cytoplasm of macrophages correlating with its ability to form complex I from S100 cytosolic extract. TIAR belongs to the TIA RNA-binding protein family with four members identified so far (9). Work is in progress to assess the functional role of TIAR in the translational regulation of TNF mRNA.

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Session 5: Developmental regulation

Chair: Matthias W. Hentze

Localization-dependent translation of *oskar* mRNA

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The establishment of antero-posterior and dorso-ventral polarity of the *Drosophila* embryo relies on the correct localization of maternally provided determinants in the oocyte, during oogenesis. One such determinant is *oskar*, which induces formation of the posterior pole plasm, the germ plasm of *Drosophila*, at the posterior pole of the egg[1]. The pole plasm contains the determinants of both the germline and of the abdomen, and is therefore crucial to proper development of the embryo. During oogenesis, *oskar* is localized as an RNA to the posterior pole of the oocyte. *oskar* mutants and mutants in which *oskar* RNA remains unlocalized in the oocyte develop into embryos lacking an abdomen and a germline. Conversely, deliberate mislocalization of *oskar* to the anterior pole of the oocyte causes pole plasm assembly at the anterior and the resulting embryos develop an ectopic abdomen and functional germ cells in the place of the head. Hence, *oskar* is both necessary and sufficient to induce pole plasm assemby. Furthermore, correct localization of *oskar* activity to the posterior pole is imperative, to ensure proper establishment of antero-posterior polarity.

Prior to its localization to the posterior pole via its 3' untranslated region (3'UTR), *oskar* RNA is translationally repressed by Bruno protein which binds to several regions within the 3' UTR[2] We have found that, when the mRNA reaches the posterior pole, its translation is derepressed by an active process that requires a specific element in the 5' region of *oskar* mRNA[3]. This novel type of element is a translational derepressor element, whose functional interaction with the previously identified repressor region in the *oskar* 3'UTR is required for activation of *oskar* mRNA translation at the posterior pole. The derepressor element only functions at the posterior pole, suggesting that a locally restricted interaction between trans-acting factors and the derepressor element may be the link between mRNA localization and translational activation. We also observe the specific interaction of two proteins with the *oskar* mRNA 5' region; one of these also recognizes the 3' repressor element. Ongoing experiments are aimed at understanding the

mechanisms by which translational repression and derepression of *oskar* are effected, and at obtaining mutants in the genes encoding the RNA-binding proteins that we have identified and testing their involvement in localization-dependent translation.

To begin to understand the molecular mechanisms by which *oskar* translation is regulated - how repression of *oskar* by Bruno is effected, and ultimately, how this repression is alleviated - we are reconstituting *oskar* translational regulation in vitro. *Drosophila* embryo extracts sustain efficient cap and poly(A)-dependent translation of a variety of mRNAs (Gebauer, Corona, Becker and Hentze, personnal communication). We are making use of these and ovarian extracts, of recombinant Bruno and other RNA-binding proteins suspected to play a role in *oskar* translation, to reproduce and characterize regulation of *oskar* translation in vitro.

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TRANSLATIONAL REGULATION IN DROSOPHILA GERMCELLS

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nanos (*nos*) mRNA is localized to the posterior pole of the Drosophila early embryos where Nanos protein is synthesized. Nos encodes an evolutionary conserved protein with a novel $(CCHC)_2$ Zn-binding domain. Nos acts together with *pumilio* (*pum*) to negatively control translation of the maternally provided product of the transcription factor *hunchback* (hb^M). Consequently, Hb^M protein is distributed in a gradient complementary to that of Nanos. Absence of Hb^M protein from the posterior region of the embryos is necessary for normal abdomen formation. Pum contains a novel RNA-binding motif composed of eight sequence repeats that bind specifically to sequences in the 3' UTR of *hb* RNA. A similar RNA-binding motif with similar but not identical sequence specificity is present in a "Pum-like" protein from humans. We are investigating the mechanisms by which Nos and Pum control translation.

nos and *pum* affect targets in addition to Hb. Maternally provided Nos protein is present in the primoridal germ cells (PGC) when they form and persists throughout embryogenesis. To study the role of *nanos* in germ cell development, we generated eggs that lack Nos and Hb^M. These eggs, when fertilized by wild-type sperm, give rise to normally segmented embryos that form PGCs which lack maternal Nos protein. We find that germ cell migration is dramatically affected resulting in male and female flies that lack germ line. Nos and Pum also play a role in early oogenesis where both gene are required for the normal development of the germ line stem cells.

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Homeodomain-dependent translational control

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The *Drosophila* body organizer Bicoid (Bcd) is a maternal homeodomain protein that forms a concentration gradient along the longitudinal axis of the embryo. It acts as an anterior determinant during *Drosophila* embryogenesis. Bcd plays two roles in embryogenesis by activating transcription of zygotic genes and by repressing the translation of maternal *caudal* (*cad*) mRNA. The repression of cad mRNA translation generates a complementary gradient of Cad protein in the embryo. The biological relevance of the repression of anterior Cad translation is to prevent disruption of morphogenetic events during head formation. Both regulatory functions of Bcd involve the homeodomain (HD). The HD is a well-established DNA-binding motif consisting of three α-helices. It interacts with the 3´ UTR of *cad* mRNA to represses translation. The target of Bcd is called Bcd-Binding Region (BBR), a RNA sequence spanning 110 nucleotides. The helix III of the homeodomain is similar to the RNA-binding domain of HIV Rev, an arginine-rich motif. Most of amino acid residues within helix III are involved in both DNA- and RNA-binding activity indicating a shared recognition domain. However, one amino acid residue, when mutated, impairs RNA but not DNA-binding. This mutation abolishes Bcd-mediated translational control but not transcription activation in the embryo. Biochemical and biophysical experiments revealed that DNA and RNA binding by a HD might occur in a similar way. Co-transfection experiments in cultured Drosophila cells indicated that Bcd represses translation in a cap-dependent manner. The cell culture system was used to determine protein domains other than the HD involved in translational control. It showed that Bcd-dependent translational repression requires, in addition to the HD, a putative PEST domain located nearby the HD The results indicate that distinct portions of Bcd are necessary to mediate the different regulatory functions. Rescue experiments with Bcd-deficient embryos expressing transgene-derived Bcd variants indicated that amino acid replacements within the PEST domain prevented translational repression of *cad* mRNA. PEST domains are know to be involved in protein degradation. Blocking the 26S proteasome pathway in cell culture did not prevent translational control by Bcd. This result suggest that translational control by Bcd is independent of protein degradation processes. However, ubiquitination and phosphorylation might be required to repress *cad* mRNA translation.

A CELL-FREE SYSTEM TO STUDY TRANSLATION IN DROSOPHILA: INSIGHTS INTO THE TRANSLATIONAL CONTROL OF MALE-SPECIFIC-LETHAL-2 mRNA BY SEX-LETHAL

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Regulation of translation plays a major role in Drosophila development. The molecular mechanisms underlying translational control in flies, however, are poorly understood, at least in part because of the lack of in vitro systems that accurately recapitulate events of regulated translation. We have developed a cell-free translation system from Drosophila embryos that yields amounts of protein similar to the rabbit reticulocyte lysate. Importantly, the system reproduces the strong synergism observed in vivo between the cap structure and the poly(A) tail to activate mRNA translation, making it the first in vitro system from higher eukaryotes able to recapitulate this regulatory feature.

This advance has provided us with a tool to study a key regulatory step in dosage compensation. Dosage compensation in Drosophila is controlled by inhibition of the expression of the protein male-specific-lethal-2 (MSL-2) in females. This is achieved through translational repression of msl-2 mRNA by the female-specific RNA binding protein Sex-lethal (SXL) (1-3). SXL binding sites in the 5' UTR are necessary for this control mechanism, while the role of SXL binding to additional sites in the 3' UTR is less clear. We are investigating this subject using the homologous in vitro translation system. Insights into the mechanism of the inhibition of msl-2 mRNA translation by SXL will be presented.

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POSTERS

TRANS-ACTING ELEMENTS IN THE TRANSLATIONAL CONTROL OF 5' TERMINAL OLIGOPYRIMIDINE mRNAs.

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The translation of ribosomal protein mRNA, and of other 5' Terminal OligoPyrimidine (TOP) mRNAs, is regulated according to the growth status of the cell by rapidly changing the fraction of mRNA associated with polysomes. Localization changes of these mRNA have been shown in many eukaryotic cultured cell systems following growth rate variations, as well as in amphibians and mammals during development. Sequence elements involved in translational control have been identified in the 5' UTR of TOP-mRNAs. In particular the polypyrimidine tract at the 5' end of the messengers is necessary although probably not sufficient. *In vitro* experiments have shown that La protein can specifically bind the terminal oligopyrimidine tract whereas Cellular Nucleic Acid Binding Protein (CNBP) can bind a downstream region in the 5' UTR of TOP-mRNA. Their binding features suggest a model in which the 5'UTR structure and the interaction with the proteins can modulate the translation of the rp-mRNA. To evidence the possible role of La in the translational regulation we have used gene constructs of wt and mutant forms of La under control of an inducible promoter in stable transfection experiments in the *Xenopus* cell line B 3.2. We have examined in detail three cell clones expressing i) wt La, ii) a C-terminus truncated form (La_{1-194}) and iii) an N-terminus truncated form (La_{93-427}). The effect of the expression of the various forms of La on TOP-mRNA translation has been analyzed both in growing and serum-deprived cells. The results indicate that La can act as a positive effector in TOP-mRNA translation possibly by counteracting translational repression by a negative factor. In fact the percentage of TOP-mRNA on polysomes is higher in cells overexpressing wt La compared to controls. Moreover the effect is evident expecially in resting cells suggesting that La could attenuate the efficacy of a translational repressor, possibly CNBP.

CLONING AND CHARACTERIZATION OF HRI AND GCN2 eIF2α KINASES FROM MOUSE CELLS

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Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) is one of the best characterized mechanisms for inhibition of protein synthesis in response to various stress conditions. Four distinct protein kinases are responsible of this inhibition phosphorylating eIF2α at Serine-51. They are three mammalian eIF2α kinases, the double-stranded RNA-dependent kinase (PKR), the heme-regulated inhibitor (HRI) and the pancreatic eIF2α kinase (PEK); and the GCN2 eIF2α kinase from *S. cerevisiae*, *D. melanogaster* and *N. crassa*.

Recently, we have purified a heme-sensitive eIF2α kinase activity from mouse liver and NIH 3T3 cells. We have also cloned from mouse liver the cDNA corresponding to this kinase (mHRI), homologue of the rat and rabbit HRIs (Berlanga *et al.*, 1998). In addition, we have cloned and characterized a novel eIF2α kinase from mouse liver which represents the first GCN2 homologue in mammals (mGCN2). The mGCN2 cDNA is 5212 bp in length, containing an open reading frame encoding a polypeptide of 1648 amino acids with a predicted molecular mass of 186 kDa. The *in vitro* translation product encoded by mGCN2 cDNA had an apparent molecular mass of 190 kDa. Affinity-purified antibodies, raised against a synthetic peptide based on the predicted mGCN2 sequence, specifically immunoprecipitated and recognized in Western-blot both the product of *in vitro* translation of mGCN2 cDNA and an 190 kDa phosphoprotein from mouse liver extracts. Northern-blot analysis showed that mGCN2 mRNA is expressed in a wide range of tissues. In order to investigate the function of mGCN2 in mammals, we have expressed the protein in human 293T cells. In these mGCN2 transfected cells, serum deprivation increased eIF2α phosphorylation, whereas poliovirus infection had no effect. Interestingly, poliovirus infection resulted in partial proteolysis of mGCN2. These findings suggest that GCN2 could play an important role in translational control and in cellular response to viral infection in mammals.

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Characterization of a ribosomal shunt in the sendai virus P/C mRNA.

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The Sendai virus (SeV) P/C mRNA expresses 8 primary translation products using a combination of ribosomal choice and co-transcriptional mRNA editing. The longest ORF of the mRNA starts at AUG^{104} (the 2nd initiation site) and encodes the 568 aa P protein, an essential subunit of the viral polymerase. The 1st (ACG^{81}), 3rd(ATG^{114}), 4th(ATG^{183}) and 5th (ATG^{201}) initiation sites are used to express a C-terminal nested set of polypeptides in the +1 ORF relative to P, namely C', C, Y1 and Y2, respectively (collectively named the C proteins). Leaky scanning accounts for translational initiation at the first three start sites (a non-ATG followed by ATGs in progressively stronger contexts). Consistent with this, changing $ACG^{81/C'}$ to ATG (GCCATG^{81}G; $ATG^{81/C'}$) ablates expression from the downstream $ATG^{104/C}$ and $ATG^{114/C}$ initiation codons. However, expression of the Y1/Y2 proteins remains normal in this background. We now have evidence that initiation from $ATG^{183/Y1}$ and $ATG^{201/Y2}$ takes place via a ribosomal shunt or discontinuous scanning. Scanning complexes appear to assemble at the 5' cap, and then scan ca. 50 nts of the 5'UTR before being translocated to an acceptor site at or close to the Y initiation codons. No specific donor site sequences are required, and translation of the Y proteins continues even when their start codons are changed to ACG, GCG. CAT and AAA. Curiously, ATG codons (in good contexts) in the P ORF, placed either 16 nts upstream of Y1, 28 nts downstream of Y2, or between the Y1/Y2 codons, are not expressed even in the ACG^{Y1}/ACG^{Y2} backgrcund. This indicates that $ATG^{183/Y1}$ and $ATG^{201/Y2}$ are privileged start sites within the acceptor site probably because the shunt delivers the scanning complex directly to these codons. The shunt appears to be promoted by barriers which would otherwise prevent a scanning complex from arriving at the Y start codons (either stable stem-loop structures or ATG initiation codons) probably because these barriers pause the scanning complex and promote its translocation to the acceptor site.

Hsp90 binds and regulates the ligand-inducible eIF-2α kinase Gcn2

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The protein kinase Gcn2 stimulates translation of the yeast transcription factor Gcn4 upon amino acid starvation. Using genetic and biochemical approaches, we show that Gcn2 is regulated by the molecular chaperone Hsp90 in budding yeast: (i) Several Hsp90 mutant strains exhibit constitutive expression of a *GCN4-lacZ* reporter plasmid; (ii) Gcn2 and Hsp90 form a complex *in vivo* as well *as in vitro*; (iii) the specific inhibitors of Hsp90, geldanamycin and macbecin I, enhance the association of Gcn2 with Hsp90 and inhibit its kinase activity *in vitro*; (iv) *in vivo*, macbecin I strongly reduces the levels of Gcn2; (v) the Hsp90 co-chaperones Cdc37, Sti1, and Sba1 are required for the response to amino acid starvation. Thus, Hsp90 plays a crucial role for the maturation and regulation of Gcn2. By analogy to the steroid receptors, we propose that the inhibitory Hsp90 complex is released from Gcn2 upon binding the activating ligand, uncharged tRNA.

Involvement of phosphatidylinositol 3-kinase signaling pathway in control of TNFα translation in T cells.

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The superantigen Toxic Shock Syndrome Toxin -1 can induce Tumor Necrosis Factor (TNF)α expression in T cells. The expression of TNF α by CD4+ T cells can be inhibited by either, wortmannin or LY 294002, two phosphatidylinositol 3-kinase inhibitors. The inhibitory effect is not transcriptional as wortmannin does not change the mRNA steady state of TNF α at any of the concentrations tested, and LY 294002 when preincubated with PBMC at its median inhibitory concentration (IC_{50} 1.4 μM), inhibited significantly the expression of TNF α but not its mRNA. Immunoprecipitation of pulse-labeled intracellular TNF α or Interleukin 2, showed a specific decrease in the synthesis of both cytokines on cells treated with phosphatidylinositol 3-kinase inhibitors. Growth factor stimulation of T cells enhanced specifically the translation of TNF α, this effect being wortmannin-dependent.

In Jurkat T cells the expression of TNF α can be induced by the superantigen staphylococcal enterotoxin D presented by B cells. Jurkat T cells were transfected with a constitutively active form of phosphatidylinositol 3-kinase and activated with superantigen. The expression of TNF α in supernatant was enhanced in transfected cells whereas the mRNA levels for TNF α did not change. Expression of a dominant negative mutant of phosphatidylinositol 3-kinase inhibited significantly the secretion of TNF α but not its mRNA. These results implicate the phosphatidylinositol 3-kinase signaling pathway in the control of translation of TNF α in T cells.

RNA-Protein Interactions at the 5′ End of the Poliovirus Genome Control Viral Translation *Andrea V. Gamarnik and Raul Andino. Department of Microbiology and Immunology, University of California, San Francisco. USA.*

Initiation of translation of picornavirus RNAs occurs by a mechanism of internal entry of ribosomes. This process requires a long and highly structured segment of the 5′UTR generally known as the internal ribosomal entry site (IRES). The mechanism of ribosome-IRES recognition is still unknown, but it has been shown that several canonical initiation factors, as well as other cellular proteins, participate in this process.

We have found that an RNA element of 90 nucleotides located upstream of the poliovirus IRES controls cap-independent translation and promotes viral RNA synthesis. This RNA element, which folds into a cloverleaf-like structure, specifically interacts with two proteins: the cellular poly(C) binding protein PCBP and the viral precursor of the protease-polymerase 3CD. We found that the binding of PCBP to this RNA element enhances viral translation, while interaction with the viral protein 3CD represses translation and promotes negative strand RNA synthesis. Footprinting analysis and mobility shift assays revealed that the binding of 3CD induces structural changes within the poliovirus 5′UTR and increases the binding affinity of PCBP for the cloverleaf RNA. These findings indicate that the formation of RNP complexes upstream of the ribosome entry segment regulate viral cap-independent translation. To understand the mechanism of viral translational control, we are currently investigating interactions between the RNP complexes formed at the 5′ end of the viral genome and the ones formed within the IRES and the viral 3′UTR.

STRUCTURAL CHARACTERIZATION AND EXPRESSION OF *DROSOPHILA* GENES ENCODING TRANSLATION INITIATION FACTORS INVOLVED IN mRNA BINDING TO THE RIBOSOME

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A rate-limiting step of protein synthesis initiation in eukaryotes is the binding of the mRNA to the 43S preinitiation complex. It is well established that the m^7G cap structure of eukaryotic mRNAs plays a crucial role in this binding. The function of this structure is mediated by a protein complex termed eIF4F, composed of at least two polypeptides: eIF4E, which contains the cap-binding site, and eIF4G, which coordinates the action of other initiation factors to facilitate the binding of the mRNA and the small ribosomal subunit. An additional polypeptide, eIF4A, is frequently found in the eIF4F complex isolated from mammals. eIF4A, in conjunction with eIF4B, exhibits a bidirectional ATP-dependent RNA helicase, that is enhanced when eIF4A is associated with eIF4F.

In order to understand the binding of mRNA to the ribosome during protein synthesis initiation in *Drosophila*, we have started a systematic study of the structure and expression of the genes encoding *Drosophila* eIF4E, eIF4G, eIF4A and eIF4B. Single copy genes containing several introns were found for eIF4E, eIF4G and eIF4A in *Drosophila*. While eIF4G is encoded by a single mRNA, the two eIF4E isoforms are synthesized from three mRNAs generated by an alternative splicing of a precursor transcript. Interestingly, the *eIF4A* gene also gives rise two mRNAs by alternative splicing, but both encoding the same eIF4A protein. The interactions between these proteins are being analyzed by the two-hybrid system (in collaboration with V. Lalioti). The expression of *eIF4E*, *eIF4A* and *eIF4G* is spatially and temporally controlled during embryonic development. A preferential accumulation in the pole cells was noticed for the eIF4E and eIF4A transcripts. The possible role of these genes during embryonic development of *Drosophila* is being studied by phenotype analysis of null alleles and of transgenic flies overexpressing these genes (in collaboration with S. Campuzano and R. Rivera-Pomar).

Functional analysis of the gene-regulatory role of translation initiation factor 5A

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Translation initiation factor 5A (eIF5A) is a small abundant protein of 16-18 kDa originally isolated from the post ribosomal wash of rabbit reticulocyte lysate. The amino acid sequence is well conserved in eukaryotes and archaea. eIF5A is the only protein that contains the amino acid hypusine derived from the posttranslational modification of lysine 50. The added butylamine group is transferred from the polyamine spermidine, and is subsequently hydroxylated.

The role of eIF5A in the cell is poorly understood, but some evidence would suggest its involvement in the formation of the first peptide bond based on its ability to stimulate catalysis of methionyl-puromycin *in vitro*. This reaction only occurs when eIF5A is in its hypusinated form suggesting that hypusine is required for functional activity. Further, treatment of chinese hamster ovary cells with inhibitors of deoxyhypusine synthase, the first enzyme in the biosynthesis of hypusine, causes arrest of cell proliferation. Studies in *Saccharomyces cerevisiae* have shown that although cell growth was arrested when eIF5A was depleted, protein synthesis still occurred at about 70% of the wild type rate. This suggests that eIF5A is not involved in general protein translation but rather may be specific for a small subset of mRNAs.

Polyamines such as spermidine as well as being the substrate for hypusination, function as alifatic cations in many aspects of nucleic acid metabolism. They are also required for cellular proliferation and thus there is a need for an inducible and balanced production of polyamines. Three polyamine metabolic proteins S-adenosylmethionine decarboxylase (SAM-DC), ornithine decarboxylase (ODC) and ODC-antizyme are regulated in response to intracellular concentrations of polyamines. Translation of the mRNAs encoding SAM-DC and ODC is controlled at the level of initiation and ODC-antizyme mRNA translation is controlled at elongation. SAM-DC and ODC contain uORFs in their 5' untranslated regions that confer regulation. Although polyamines affect translation of SAM-DC, ODC and ODC-antizyme mRNAs *in vitro* the physiological regulator has yet to be determined.

The goal of our studies are to clarify the role of eIF5A in cellular proliferation and its connection to translational regulation of polyamine metabolic enzymes. We utilise *in vitro* translation assays with luciferase reporter mRNA harbouring the regulatory sequences from SAM-DC, ODC and ODC-antizyme, to test several aspects of the trans-acting regulation, be it small or large molecules. To confirm the physiological relevance of the *in vitro* findings we will continue with transient expression of the luciferase reporter mRNAs under pharmacological alterations of the polyamine levels and metabolism.

The hepatitis C virus internal ribosomal entry site adopts a specific metal ion dependent fold that contains two independent regions and a long-range tertiary interaction.

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Internal ribosome entry site (IRES) action is a poorly understood mechanism of translation initiation, present in both cellular and viral mRNA. Hepatitis C virus (HCV) initiates translation through a cap-independent mechanism which is driven by an IRES located in the 5' untranslated region of the genomic mRNA. The IRES RNA provides the recognition site for ribosomal binding and subsequent preinitiation complex formation at or near the start codon. The secondary structure and minimum sequence requirements of the HCV IRES have been determined, but the presence and characteristics of any higher order folding have not been investigated. We have performed chemical and enzymatic probing experiments on the HCV IRES which clearly show that the IRES adopts a specific higher order fold in the presence of metal ions. Fe(II)-EDTA probing experiments show that upon folding, the RNA backbone in certain helix junctions is protected from solvent, and RNase T1 probing experiments show that certain conserved loop regions become hypersensitive to enzyme cleavage upon folding. We have used these probing techniques to demonstrate that folding of the RNA is a cooperative event that involves several metal ions and is driven primarily by charge neutralization. To examine the effect of mutations that effect the IRES efficiency (defined as the ability to initiate translation in a dicistronic assay) on IRES folding, we have probed a number of junction and loop mutations using Fe(II)-EDTA and RNase T1. These experiments clearly show that these mutations affect the ability of the IRES to fold, reveal that the IRES contains at least two independently folded regions, and identify a previously unsuspected long range tertiary interaction. Based on these data, we propose a model for the global architecture of the HCV IRES as an extended, but specific shaped scaffold, the structure of which is a critical determinant of translation control. We are currently testing this model using small angle X-ray scattering (SAXS) experiments to determine the size and shape of the IRES.

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INVOLVEMENT OF THE APHTHOVIRUS RNA REGION LOCATED BETWEEN THE TWO FUNCTIONAL AUGS IN START CODON SELECTION.

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Initiation of translation in picornavirus RNAs occurs internally, mediated by an element termed IRES. In the aphthovirus RNA, the IRES element directs translation initiation at two in-frame AUGs separated by 84 nucleotides. We have found that bicistronic constructs which contained the IRES element followed by the fragment including the aphthovirus start codons in front of the second gene mimicked the translation initiation pattern of viral RNA observed in infected cells. In those constructs, the frequency of initiation at the first AUG was increased by a sequence context which resembled the favorable consensus for cap-dependent translation, although initiation at the second site was always preferred. In addition, we have found that initiation at the second start codon was not diminished under conditions in which the first initiation codon was blocked by antisense oligonucleotide interference. Interestingly, mutations that positioned the second AUG out-of-frame with the first AUG, did not interfere with the frequency of initiation at the second one. On the contrary, IRES-dependent translation initiation in bicistronic constructs lacking the sequences present between functional AUGs in the viral RNA, was sensitive to the presence of out-of-frame initiator codons and hairpins in the spacer region. This remarkable difference in start codon recognition was due to the nucleotide composition of the RNA that separated the IRES from the initiator codon. Thus, our results indicate that the region located in the aphthovirus RNA between functional AUGs is involved in start codon recognition, strongly favoring selection of the second start AUG as the main initiator codon.

Phosphorylation and Meiosis-Promoting Activity of CPEB

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Translational recruitment of dormant maternal mRNAs is in part controlled by cytoplasmic polyadenylation. During oocyte maturation, polyadenylation is mediated by the U-rich CPE, which resides in the 3' UTR of responding mRNAs, and its binding protein CPEB. Although the precise function of CPEB is unclear, it may play two roles in translational control; that of an initial mRNA masking factor, as well as a polyadenylation-inducing factor. Close to the onset of polyadenylation, CPEB becomes phosphorylated and is partially degraded. Because CPEB phosphorylation does not appear to affects its RNA binding affinity, it may regulate CPEB activity by inducing its destruction, or by affecting its ability to interact with other proteins.

The carboxy-terminal portion of CPEB contains two RRMs and a zinc finger that are conserved among different species. The amino-terminal region, however, is much less conserved with the exception of two short motifs: one with loose homology to the cyclin destruction box, and one that resembles PEST sequences. Moreover, the amino-terminal region contains six of seven possible sites of phosphorylation by cdc2, which we suspect is one of the kinases that phosphorylates CPEB.

To address the importance of CPEB phosphorylation and the role of its amino-terminus in the activity and stability of the protein, we are subjecting recombinant protein to in vitro phosphorylation with ^{32}P-ATP using extracts prepared from immature and mature oocytes as the source of kinase(s). Following re-isolation of the protein from extracts, it was digested with trypsin and subjected to 2-dimensional phospho-peptide mapping. These data show that there are at least six maturation-specific phosphorylation sites target by two kinases, one of which is probably cdc2.

We have substituted alanine residues for several of the phospho-amino acids and well as constructed a number of deletion mutants that affect the cyclin and PEST destruction boxes as well as putative protein-protein interaction domains. These mutants were then tested for (I) their effect on CPEB stability in mRNA-injected oocytes, (ii) their effect on meiotic maturation, and (iii) their on the interaction with other proteins. The results of these tests will be presented. We will also present a model for the regulation of CPEB activity during oocyte maturation.

Translational control by the MAPK-activated protein kinase Mnk1.

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The protein kinase Mnk1 phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) both *in vitro* and *in vivo*. eIF4E binds to the cap structure and interacts with eIF4G that serves as a scaffold protein for eIF4F complex assembly. Phosphorylated eIF4E shares enhanced cap- as well as eIF4G-binding affinity. It is considered as an important modulator of cell growth and proliferation. We have shown recently that the carboxy-terminal region of eIF4G provides a docking site for Mnk1 to phosphorylate eIF4E (Pyronnet *et al.*1999, EMBO J, in press). Here we have mapped the eIF4G-binding site in the first 48 N-terminal amino-acids (48NT) of Mnk1. We also report that, *in vivo*, activated Mnk1 is linked to such opposing responses as the induction and the inhibition of translation. In response to a strong signal, Mnk1 causes a decrease in the biosynthesis of a reporter protein. In contrast, moderate Mnk1 activity induces translation of the reporter. The induction of translation appears specific to Mnk1 activity and requires the first 48 amino-acids as both kinase-dead Mnk1 and activated, 48NT-deleted Mnk1 still cause a decrease in translation when highly expressed, but have no effect when expressed to moderate levels. This suggests that, depending on its level of expression, Mnk1 can phosphorylate disparate substrates that differentially affect translation.

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