Studies of cap-independent mRNA translation in *Drosophila melanogaster*

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Results

Adh	alcohol dehydrogenase
Anti	antisense
AP	alkaline phosphatase
BCIP	5-bromo-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CFP	cyan fluorescent protein
cpm	counts per minute
CrPv	cricket paralysis virus
CSFV	classical swine fever virus
DIG	digoxigenin
Dm	*Drosophila melanogaster*
DNA	desoxyribonucleic acid
DNAse	desoxyribonuclease
dNTP	desoxyribonucleoside triphosphate
dsRNA	double strand RNA
DTT	dithiotreitol
4E-BP	eIF4E binding proteins
eIF	eukaryotic initiation factor
EMCV	encephalomyocarditis virus
EST	expressed sequence tag
EYFP	enhanced yellow fluorescent protein
FLuc	Firefly luciferase
FMDV	foot-and-mouth disease virus
GDP	guanosine 5´-diphosphate
GMP-PNP	guanosine 5´[β,γ-imido] triphosphate trisodium salt
GST	glutathione S-transferase
GTP	guanosine 5´-triphosphate
h	hours
HA	haemaglutinin
HAV	Hepatitis A virus
HCV	Hepatitis C virus
hid	*head involution defective*
HRP	horseradish peroxidase
HS	heat shock
Hsp	heat shock protein
IgG	immunoglobulin G
IRES	internal ribosome entry site
ITAFs	IRES transacting factors
kDa	kilo Dalton
LC/ESI-MS/MS	liquid chromatography electrospray ionization tandem mass spectrometry
LSCM	laser scanning confocal microscope
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Met-tRNAi	methionyl-tRNA-initiator
m^7GTP	7-methylguanosine 5'-triphosphate
µg	microgram
µl	microliter

mg	milligram
ml	milliliter
mM	millimolar
min	minutes
mRNA	messenger RNA
NBT	nitro blue tetrazolium
ng	nanogram
NLS	nuclear localization signal
nm	nanometers
ORF	open reading frame
P	polysomal fraction
PABP	poly A binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomols
PTB	poly pyrimidine tract binding protein
PSIV	*Plautia stali* intestinal virus
Q-TOF	quadrupole time of flight
RLuc	Renilla luciferase
RNA	ribonucleic acid
RNAi	RNA interference
RNAse	ribonuclease
RNP	ribonucleoprotein
rpm	rotations per minute
rpr	*reaper*
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase coupled to PCR
SDS-PAGE	sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
TA	tobramycin aptamer
tRNA	transfer RNA
TUNEL	TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labeling
U	untranslated fraction
UAS	upstream activator sequence
UTR	untranslated region
UV	ultraviolet light
V	volt
wt	wild type

Introduction

PROTEIN SYNTHESIS IN EUKARYOTES

Protein synthesis is the process by which a given nucleotide mRNA sequence is translated into a protein in the ribosomes. The general process of protein synthesis consists of three phases: initiation, elongation and termination. The initiation of protein synthesis is the binding of the ribosomal subunits to a given mRNA and the positioning of the first aminoacyl-tRNA, the methionyl-tRNA initiator (Met-$tRNA_i$), which is placed in the P site of the ribosome at the initiation codon of the mRNA, usually an AUG. The elongation step consists of the sequential addition of amino acids, which are encoded in the mRNA, to the carboxy-terminal end of the nascent polypeptide. This process involves the binding of the aminoacyl-tRNA to the ribosomal A site, the formation of the peptidic bond and the translocation of the mRNA and the peptidyl-tRNA. Termination takes place when a stop codon is positioned at the A site of the ribosome. This process leads to the hydrolysis of the peptidyl-tRNA by the peptidyl transferase and the release of the nascent polypeptide and the uncharged tRNA.

Translational control is one of the most important processes in the regulation of gene expression. While every phase of the translation process is amenable to regulation, the limiting step is most often the initiation of translation.

MECHANISMS OF TRANSLATION INITIATION

Cap-dependent initiation of translation

A general scheme for the initiation of protein synthesis in eukaryotes is shown in Fig. 1. This process requires the ribosomes and a large number of proteins called eukaryotic initiation factors (eIFs). Prior to protein synthesis initiation, the 80S ribosome dissociates into 40S and 60S subunits. This process is catalyzed by the factors eIF3, eIF1A and eIF6, which maintain a pool of dissociated subunits. eIF3 and eIF1A bind the 40S ribosomal subunit, favoring its dissociation from the 60S subunit, while eIF6 binds the 60S subunit. The 40S subunit is then bound to a ternary complex, which consists of eIF2-GTP-Met-$tRNA_i$, to form a 43S preinitiation complex. eIF3 interacts with the ternary complex eIF2-GTP-Met-$tRNA_i$, stabilizes its binding to the 40S subunit and is necessary for the binding of the mRNA to the 40S ribosomes. The binding of the 43S preinitiation complex

to most eukaryotic mRNAs requires the recognition of the cap structure (m^7GpppN, where N is any nucleotide) present at the 5' end of all eukaryotic mRNAs, by the eIF4F complex. This mechanism is called cap-dependent translation. eIF4F is a heterotrimeric complex comprising the cap-binding protein eIF4E, the multiadaptor protein eIF4G and the helicase eIF4A. The binding of eIF4F to mRNA via eIF4E, further recruits the helicase–stimulatory factor eIF4B/eIF4H, and the entire complex is thought to melt secondary structures present in the 5´ untranslated region (5´ UTR) of the mRNA. Once the secondary structure is removed, an interaction between eIF4G and eIF3 (bound to the 40S subunit), bridges the ribosome to the mRNA. The 40S ribosomal subunit, with associated initiation factors, then proceeds to move in the 5' -> 3´direction until an AUG initiation codon is encountered in a favorable context. This process is known as "scanning" and results in the formation of a 48S initiation complex at the initiation codon. Formation of the initiation complex is followed by the release of associated initiation factors. This step is aided by eIF5, which promotes the hydrolysis of GTP carried by eIF2. The released translation factors are then recycled for another round of initiation. Subsequently, the 60S ribosomal subunit joins the preinitiation complex to form an 80S initiation complex. The translation initiation is then followed by a polypeptide elongation step (Gingras *et al.*, 1999; Hershey and Merrick, 2000).

Some mRNAs are thought to be translated by the so called "ribosomal shunting". Ribosomal shunting is a form of discontinuous scanning and was described to mediate translation initiation only on a few mRNAs, including those of *cauliflower mosaic virus 35S* (Futterer *et al.*, 1993), adenovirus late mRNAs (Yueh and Schneider, 1996 ; Yueh and Schneider, 2000) and *human heat shock protein 70* mRNAs (Yueh and Schneider, 2000). This mechanism is not completely understood, but it appears that 40S subunits bind the mRNA in a 5´cap-dependent manner and scan through the 5´proximal part of the mRNA in the usual way until a stable RNA structure is encountered that arrests the scanning.

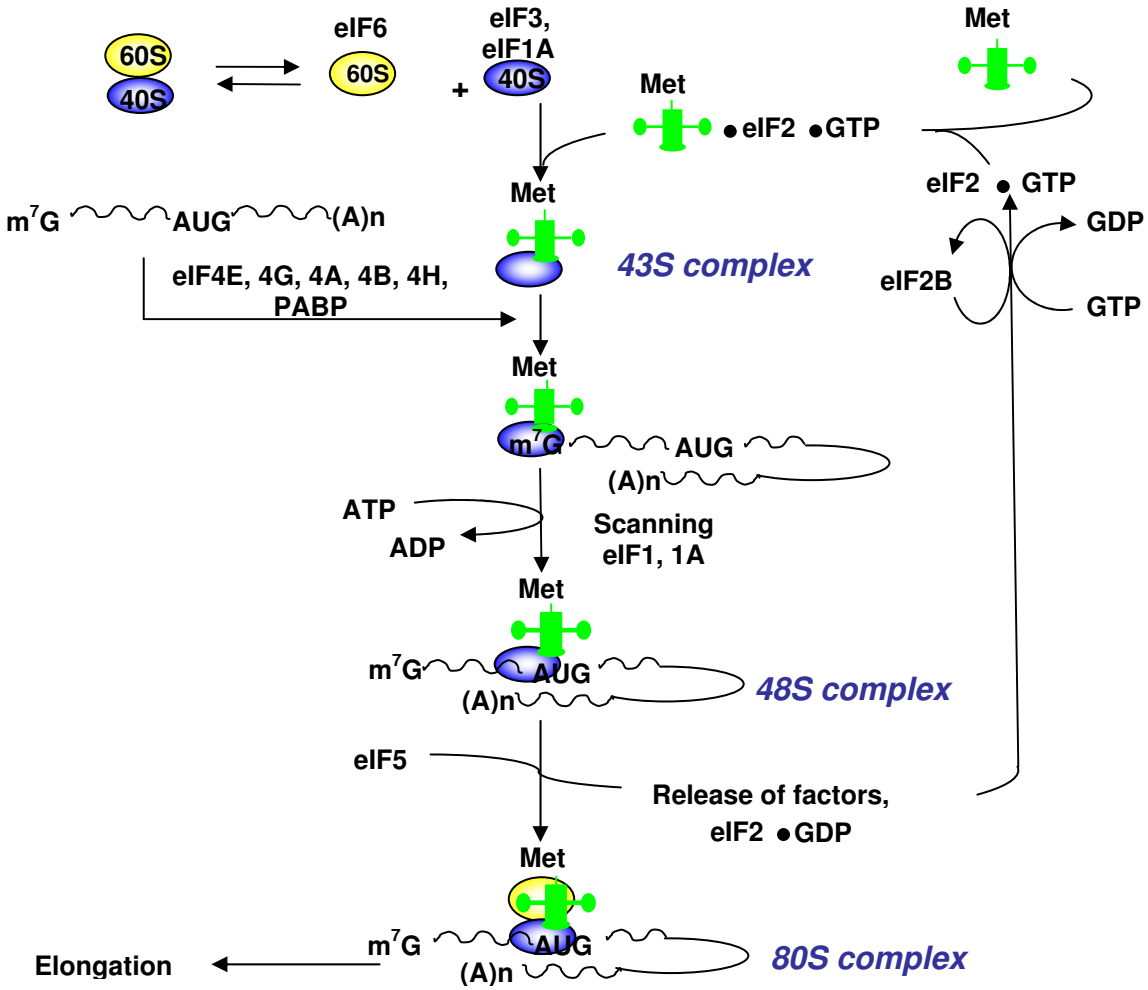


Figure 1. Scheme of the mechanism of eukaryotic translation initiation. Adapted from Hershey and Merrick, 2000.

This arrest is followed by intramolecular shunting of the ribosomal subunit to a downstream landing site, bypassing the RNA hairpin to resume scanning until the next appropriate start codon is encountered. mRNA sequences located between the RNA hairpin and the initiation codon, which are complementary to sequences in the 18S ribosomal RNA, are presumed to participate in the direct association with the 40S subunit to provide a landing site after it has bypassed the hairpins (Yueh and Schneider, 2000).

Internal ribosome entry site-mediated initiation of translation

Some eukaryotic mRNAs recruit the 40S ribosomal subunits in a cap-independent manner. The mechanism they use is called IRES-dependent initiation, and it represents an important way of initiating translation during stress conditions like heat shock, starvation, irradiation or viral infection or during induced or developmentally regulated apoptosis when cap-dependent translation is compromised (Holcik *et al.*, 2000; Hellen and Sarnow, 2001; Stoneley and Willis, 2004). In this mechanism, the ribosome can reach the initiation codon by recognizing sequences present in the 5´UTRs of mRNAs, called internal ribosome entry sites (IRES), without the need of cap recognition (Fig. 2). IRES-mediated translation was first discovered as a strategy used by RNA-viruses to infect cells (Jang *et al.*, 1988; Pelletier and Sonenberg, 1988). The virus shuts off most of the cap-dependent cellular protein synthesis by the cleavage of eIF4G by viral proteases (Fig. 2). The proteolysis of eIF4G separates its N-terminal cap-binding region from the 40S subunit binding region, thus inhibiting cap-dependent translation but not IRES-dependent translation of some viral mRNAs. In addition, the truncated forms of eIF4G, containing the eIF3 and eIF4A binding sites, stimulate translation of some viral IRESs (Ohlmann *et al.*, 1996; Pestova *et al.*, 1996b; Borman *et al.*, 1997).

Different viral IRESs use distinct strategies to interact with the translation machinery and some of the eukaryotic translation factors play an essential role in IRES-dependent translation (Belsham and Jackson, 2000; Jackson, 2000; Hellen and Sarnow, 2001). The encephalomyocaditis virus (EMCV) and foot-and-mouth disease virus (FMDV) IRESs were described to depend on eIF4A, eIF4G, eIF2 and eIF3 for 48S complex formation *in vitro* (Pestova *et al.*, 1996a; Pestova *et al.*, 1996b; Pilipenko *et al.*, 2000). For translation of FMDV eIF4B is essential (Ochs *et al.*, 1999; Rust *et al.*, 1999). The Hepatitis A Virus (HAV) IRES is unique among picornavirus, exhibiting a strong requirement for eIF4E (Ali *et al.*, 2001; Borman *et al.*, 2001). In contrast to picornavirus IRESs, the Hepatitis C Virus (HCV) and the classical swine fever virus (CSFV) IRESs bind the 40S subunit in the absence of eIF4A, eIF4B and eIF4F. These binary ribosomal

complexes require only the addition of eIF2, GTP and Met-tRNA$_i$ to form 48S initiation complexes (Pestova *et al.*, 1998b).

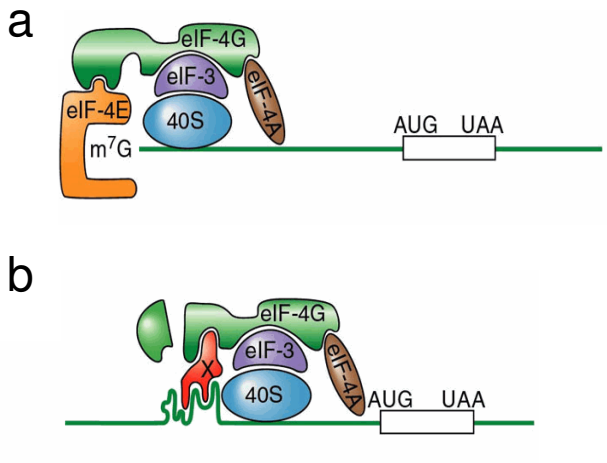


Figure 2. Scheme of the factors involved in the initiation of translation in eukaryotic mRNAs. (a) In most cellular mRNAs the cap structure is recognized by eIF4E. eIF4E binds factor eIF4G that coordinates the activity of eIF3 to recruit the ribosomes. eIF4B and eIF4A unwind the secondary structure of the mRNA. The whole complex then scans the mRNA to reach the AUG. **(b)** Scheme of IRES-dependent translation on picornaviral mRNAs. Viral proteases cleave the eIF4G in a way that can no longer bind eIF4E. Different transacting factors (X) and translation factors recognize the secondary structure of IRES and initiation takes place in the absence of cap binding activity. Taken from Hentze, 1997.

HCV is the only IRES for which a ribosomal protein, rpS5, has been shown to interact directly with IRES sequences (Fukushi *et al.*, 1999; Fukushi *et al.*, 2001). On the other hand, an unexpected IRES-dependent mechanism was discovered in case of the insect cricket paralysis virus (CrPv) and its relative *Plautia stali* intestinal virus (PSIV). These viruses are able to direct protein synthesis initiation at a non-methionine codon in the absence of Met-tRNA$_i$. (Sasaki and Nakashima, 2000; Wilson *et al.*, 2000). Moreover, it was demonstrated that the CrPv IRES can form 80S complexes *in vitro* in the absence of eIF2, Met-tRNA$_i$ or GTP, without a coding triplet in the P site and directing an unusual protein initiation from the A site of the ribosome (Wilson *et al.*, 2000).

Several cellular IRES transacting factors (ITAFs) play important roles in modulating the efficiencies of various picornaviral IRESs. The polypyrimidine tract-binding protein (PTB) binds to all picornavirus IRES and enhances the

translational initiation driven by EMCV IRES (Borovjagin *et al.*, 1994; Kaminski *et al.*, 1995; Pestova *et al.*, 1996a). Initiation of FMDV IRES requires the binding of PTB and a 45-KDa ITAF (ITAF45) to nonoverlapping sites on FMDV IRES and causes conformational changes on the RNA, which enhance its binding to eIF4G/4A (Pilipenko *et al.*, 2000). The La autoantigen binds to the poliovirus IRES, stimulates its activity *in vitro*, and enhances the accuracy of initiation codon selection (Meerovitch *et al.*, 1993). It also binds and stimulates translation driven by HCV, ECMV and coxackiesvirus IRES (Ali and Siddiqui, 1997; Kim and Jang, 1999; Ali *et al.*, 2000; Ray and Das, 2002). PTB as well as a complex between the upstream of N-ras protein (UNR) and an associated WD-repeat protein (UNRIP) synergistically stimulate the rhinovirus IRES (Hunt *et al.*, 1999). Translation of poliovirus but not EMCV or FMDV is also strongly dependent of UNR (Boussadia *et al.*, 2003). Finally, translation driven by poliovirus and HAV IRES requires the poly(rC) binding protein-2 (PCBP-2) (Graff *et al.*, 1998; Walter *et al.*, 1999; Gamarnik and Andino, 2000). In conclusion, different cellular factors and different combinations of eIFs are involved in regulating viral mRNA translation.

Some cellular mRNAs also contain IRES. They include mRNAs encoding for growth factors (FGF-2, PDGF-2/c-Sis, IGF-II, VEGF), proteins involved in cell cycle regulation (PITSLRE, ODC, *Hairless*, CDK inhibitor p27), and development (*Antp, Ubx, Hairless*), apoptotic proteins (XIAP, c-Myc, p97/DAP5/NAT1, Apaf-1), proteins involved in differentiation (PDGF-2/c-Sis), in heat shock response (Bip, human hsp70, Bag-1), oxidative stress (FGF-2), hypoxia (HIF-1α, c-Myc), and irradiation (XIAP) (Hellen and Sarnow, 2001; Stoneley and Willis, 2004).

Some eIFs are involved in IRES-dependent translation of some cellular mRNAs. Overexpression of eIF4G in cultured cells stimulates IRES-dependent translation of ornithine decarboxylase (ODC) mRNA (Hayashi *et al.*, 2000). Caspase-cleaved p97/DAP5/NAT1, a protein with homology to eIF4G but lacking the eIF4E binding site, is unable to support cap-dependent initiation but supports translation through the IRESs of DAP5, c-Myc, XIAP and Apaf-1 (Henis-Korenblit *et al.*, 2002). On the other hand, it is known that several ITAFs participate in the IRES-dependent translation of cellular mRNAs. A phosphorylated form of hnRNP C interacts with the IRES in PDGF2 mRNA

(Sella *et al.*, 1999). PTB inhibits the translation driven by Bip IRES both *in vitro* and in cultured cells (Kim *et al.*, 2000). In contrast, rabbit reticulocytes supplemented with PTB and UNR activate the Apaf-1 IRES (Mitchell *et al.*, 2001). PTB together with poly(rC1) binding protein 1 (PCBP-1) also stimulates Bag-1 IRES-mediated translation initiation *in vitro* and *in vivo* (Pickering *et al.*, 2003). Members of the poly(rC) family, PCBP-1, PCBP-2 and hnRNPK have been found to interact and activate the c-myc IRES (Evans *et al.*, 2003). Finally, the La autoantigen and the hnRNP C1 and C2 stimulate the XIAP mRNA IRES efficiency (Holcik and Korneluk, 2000; Holcik *et al.*, 2003). In most cases, the role of these trans-acting factors in IRES-dependent translation of cellular mRNAs is yet unknown. For Apaf-1 and Bag-1 IRES, a mechanism was reported in which the binding of the corresponding ITAFs induces a specific unwinding of the RNA structure in the region where the ribosome is recruited (Mitchell *et al.*, 2003; Pickering *et al.*, 2004).

In *Drosophila* it has been shown that both cap-dependent and independent initiation can be targets for simultaneous (Wharton *et al.*, 1998) or differential (Niessing *et al.*, 1999) regulation during embryonic development. IRES-dependent initiation has been partially studied in three *Drosophila* genes, *Antennapedia* (*Antp*) (Oh *et al.*, 1992), *Ultrabithorax* (*Ubx*) (Hart and Bienz, 1996; Ye *et al.*, 1997) and *Hairless* (Maier *et al.*, 2002) but the factors regulating these IRESs are still unknown.

The experimental test for IRES activity

The most generalized experiment to demonstrate IRES activity is based on the insertion of the suspected IRES element in the intergenic region of a dicistronic reporter in which the translation of the second reporter cistron must be independent of the translation of the first one. The recent flurry of newly discovered IRES sequences led to a controversy about the experimental criteria that should be applied to an RNA sequence for it to be denoted as an IRES (Kozak, 2001a; Kozak, 2001b; Schneider *et al.*, 2001). The conclusion is that the integrity of the dicistronic mRNA after translation should be assayed to evaluate other possible reasons for translation of downstream sequences, such as RNA cleavage or alternative splicing upon transfection with a DNA construct capable

of generating a functionally monocistronic RNA. It also remains to be determined whether the suspected IRES element can show promoter activity that could also lead to the synthesis of monocistronic mRNAs. By performing these control experiments to search for IRES activity, it was found that eIF4GI 5´UTR, previously shown to have an IRES, indeed posseses intrinsic promoter activity (Han and Zhang, 2002). A similar controversy was put forward for the described IRES of TIF4631 gene that encodes the translation initiation factor eIF4G1 of *Saccharomyces cerevisiae* (Altmann *et al.*, 2004; Mauro *et al.*, 2004; Verge *et al.*, 2004). Van Eden *et al.* found that aberrantly spliced transcripts occurred in cells transfected with a XIAP dicistronic DNA construct. Although this work did not question the presence of an IRES in XIAP 5´UTR, by using dicistronic RNA transfections the authors showed that it is less active than previously described (Van Eden *et al.*, 2004a).

ROLE OF CAP-INDEPENDENT TRANSLATION DURING CELLULAR STRESSES

In response to environmental stress (e.g. heat, oxidative conditions, hypoxia, irradiation), cells shut down protein synthesis to conserve energy for the repair of stress-induced damage. This translation arrest is selective in that translation of constitutively expressed “housekeeping” genes is shut down, whereas translation of stress induced transcripts is maintained or enhanced. It is also known that global cellular protein synthesis is downregulated during cellular processes such as the G2/M phase of the cell cycle and during cell death. Translation of some factors that regulate these processes is refractory to this global translation inhibition.

Control of translation during heat shock in eukaryotic cells

The heat shock response (hs) includes inhibition of protein synthesis above a threshold temperature; only the translation of heat shock protein mRNAs is maintained (Sierra and Zapata, 1994; Schneider, 2000). Heat shock induced mRNAs are neither degraded nor modified during heat shock, and they can be translated *in vitro* (Kruger and Benecke, 1981). Rather the translational apparatus is altered to prevent translation of normally capped mRNAs, acting by

disassembly of polysomes and inhibition of initiation. The inhibition of normal mRNA translation during heat shock is thought to enhance cell survival by limiting the accumulation of misfolded or denatured proteins that result from heat stress. Most members of the heat shock protein (Hsp) family are molecular chaperones, which normally assist protein folding, transport and assembly of multiprotein complexes. During heat shock, they protect other proteins, repair damaged proteins and promote degradation of severely damaged proteins.

Despite years of study, it is still not well understood how heat shock blocks protein synthesis in mammalian cells. Heat shock induces dephosphorylation and possible inactivation of eIF4E in a wide variety of mammalian cells, leading to inhibition of cap-dependent mRNA translation (Duncan and Hershey, 1984; Lamphear and Panniers, 1990). eIF4E-binding proteins (4E-BPs) become hypophosphorylated during heat shock, leading to an increased binding to eIF4E and the inhibition of normal mRNA translation (Feigenblum and Schneider, 1996; Vries *et al.*, 1997). More recently a novel molecular mechanism involving Hsp27 was described for the inhibition of protein synthesis during heat shock. Hsp27 binds specifically to eIF4G, inducing the dissociation of the eIF4F-complex and insolubilization of eIF4G into heat shock granules, thereby resulting in the inhibition of non-heat shock mRNA translation (Cuesta *et al.*, 2000).

The hallmark of heat shock is thus the impairment of eIF4F-dependent mRNA translation. The translation of heat shock mRNAs continues because it requires minimal amounts of eIF4F, by virtue of initiation through internal ribosome entry or by a ribosome shunting mechanism. The Bip mRNA encoding an ER-associated variant of Hsp70 is translated in an eIF4F independent manner by utilizing an IRES element (Macejak and Sarnow, 1991). The mechanism of translation of human hsp70 mRNA during heat shock remains controversial. Yueh and Schneider reported that the 5´UTR of human hsp70 mRNA contains two sequence blocks complementary to the 3´ hairpin of the 18S rRNA (Yueh and Schneider, 2000). This element promotes ribosome shunting on hsp70 during heat shock. These authors also reported that human hsp70 5´UTR did not function as an IRES when inserted internally in a dicistronic mRNA, a fact that had been observed before by Vivinus *et al.* (Vivinus *et al.*, 2001). However, Rubtsova *et al.* have recently proposed that the 5'UTR of the human hsp70

mRNA displays IRES activity which requires integrity of almost the entire sequence of its 5'UTR (Rubtsova *et al.*, 2003).

Although the heat shock response was first discovered in *Drosophila*, the molecular mechanisms that control the preferential translation of heat shock mRNAs are poorly understood. In *Drosophila* the mechanism of cap-dependent inhibition upon heat shock involves the inactivation of eIF4F complex (Zapata *et al.*, 1991). Partial and specific rescue of translation is achieved when heat shock lysates are supplemented with *Drosophila* eIF4F. The mechanism of inactivation of *Drosophila* eIF4F appears to involve the aggregation of translation factors upon heat shock (Sierra and Zapata, 1994). It was also reported that phosphorylation of 4EBP (Duncan and Song, 1999) and dephosphorylation of eIF4E (Duncan *et al.*, 1995) occur upon heat shock in *Drosophila*. In contrast to *hsp70*, for which a shunting and IRES dependent translation have been reported to occur, translation of *Drosophila hsp70* was reported not to involve IRES activity and the complementarities with the 18S rRNA required for a shunting mechanism are lacking (Yueh and Schneider, 2000; Hess and Duncan, 1996)**.** However, the ability to translate during heat shock resides in the *hsp* 5'UTR. The 5' UTR of *Drosophila* heat shock mRNAs (with the exception of hsp83) mRNA are long (150-350 nucleotides) and usually rich in adenosine residues (~50%), suggesting a low potential for secondary structure formation (Ingolia and Craig, 1981; Lindquist and Petersen, 1990). They also contain two conserved sequences near the 5' end and in the middle of the leader, respectively (Holmgren *et al.*, 1981). Although reduced secondary structure is important for translation of *hsp* mRNAs during heat shock, an artificial A-rich *hsp70* 5´UTR does not overcome the translation block, implicating a requirement for specific sequences, structural elements, or both (Lindquist and Petersen, 1990). A detailed mutational analysis of the *Drosophila hsp70* 5'UTR showed that both reduced secondary structure and uncharacterized segments of the 3' region of the leader were required for efficient translation of the mRNA during heat shock (Hess and Duncan, 1996).

Control of translation during apoptosis

Programmed cell death or apoptosis is now recognized as an important physiological process by which cell and tissue growth, differentiation and programs of development are regulated. Induction of apoptosis is associated with a rapid and substantial inhibition of protein synthesis in several cell types due to a decrease in the amount of polysomes (Clemens *et al.*, 1998; Morley *et al.*, 1998; Zhou *et al.*, 1998). Some initiation factors are targets of specific degradation by caspase-mediated mechanisms in a variety of cell types and in response to several inducers of apoptosis (Clemens *et al.*, 2000). Caspase 3 is considered to be an executioner caspase that cleaves a large number of substrates during apoptosis. It was observed that following a proapoptotic stimulus, eIF4GI is cleaved into three fragments by caspase 3, which correlates with the inhibition of protein synthesis (Marissen and Lloyd, 1998; Bushell *et al.*, 1999). This situation is analogous to that produced by cleavage of eIF4G during picornaviral infection. Caspase 3 also mediates the proteolysis of eIF2, eIF4B, eIF4GII, the p35 subunit of eIF3, PABP and the eIF4E-BP, both *in vitro* and in cells induced to enter into apoptosis by a variety of treatments (Satoh *et al.*, 1999; Bushell *et al.*, 2000; Marissen *et al.*, 2000; Tee and Proud, 2000; Marissen *et al.*, 2004). Apoptosis also correlates with alterations in the phosphorylation states of eIF4E, eIF4E-BP1 and eIF2α (Clemens *et al.*, 2000; Tee and Proud, 2000; Saelens *et al.*, 2001; Horton *et al.*, 2002; Li *et al.*, 2002).

As mentioned previously, a number of proteins associated with the regulation of cell death such as the inhibitors of apoptosis XIAP, cIAP-1 and HIAP2, the oncogenes c-myc and Bcl-2 and the proapoptotic proteins p97/DAP5/NAT-1 and Apaf-1 are translated via IRES elements present in their 5´ UTRs (Nanbru *et al.*, 1997; Coldwell *et al.*, 2000; Henis-Korenblit *et al.*, 2000; Holcik and Korneluk, 2000; Sherrill *et al.*, 2004; Van Eden *et al.*, 2004b; Warnakulasuriyarachchi *et al.*, 2004). In this way, IRES-dependent translation can overcome the general inhibition of protein synthesis activity that occurs in apoptotic cells.

Enhanced expression of proteins of the inhibitor of apoptosis (IAP) family would be expected to limit the apoptotic response, whereas synthesis of p97/DAP5 or Apaf-1 would have the opposite effect, providing a positive

feedback and acceleration of apoptosis. The possible outcome for the cell could then depend on the cell type, its state of differentiation, the type and severity of the stress stimulus, intercellular signaling and the abundance, stability and efficiency of translation of a given mRNA.

AN OVERVIEW OF APOPTOSIS IN *Drosophila melanogaster*

A genetic screen for mutants exhibiting a disruption of the pattern of apoptosis was initially performed to identify regulators of cell death in *Drosophila*. A deletion in the region 75C of the *Drosophila* genome was identified as a requirement for the occurrence of embryonic cell death (Abrams *et al.*, 1993; White *et al.*, 1994). This deletion, named ΔH99, showed an absence of virtually all types of programmed cell death that normally occur during *Drosophila* embryogenesis. Mutant embryos contained many extra cells and failed to hatch, but many other aspects of development appeared quite normal. Despite the absence of endogenous programmed cell death, some apoptosis could be induced experimentally by high X-ray doses in ΔH99 embryos. This observation led to the conclusion that the genes contained in this genomic interval encoded activators of apoptosis rather than essential elements of the death machinery itself. Three genes related to apoptosis were identified in the H99 region: *reaper* (*rpr*), *grim* and *head involution defective* (*hid*) (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996). More recently, the gene *sickle* (*skl*) was also identified; it mapps near the ΔH99 deletion (Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002). *rpr, hid, grim* and s*ickle* are transcribed in the same orientation, which may imply a common evolutionary history. All of them share a similar 14-amino-acid sequence at the N-terminus, referred to as the RHG motif. Aside from the RHG domain, the proteins are highly divergent and do not share sequence similarity. The N-terminal region and the RHG domains are both necessary and sufficient for binding the *Drosophila* Inhibitor of apoptosis Proteins (IAPs), DIAP1 and DIAP2. In this way, they all can activate caspase-dependent cell death in part by inhibiting the ability of IAPs to bind caspases and repress their activities. Although RPR, HID and GRIM may interfere with DIAP1 function in distinct ways (Lisi *et al.*, 2000; Ryoo *et al.*, 2002; Yoo *et al.*, 2002), endogenous levels of DIAP1 are significantly reduced (by a posttranslational

mechanism) upon binding of all RHG proteins. DIAP1 downregulation is likely due to its targeting for proteasome-mediated destruction as a consequence of polyubiquitination. Intriguingly, Hay and Kornbluth laboratories reported that GRIM and RPR can exert generalized suppressive effects on protein translation, which may be partly responsible for DIAP1 downregulation in cells expressing these proteins (Holley *et al.*, 2002; Yoo *et al.*, 2002). DIAP1 regulates the *Drosophila* effector caspase DRONC, through ubiquitination and, presumably, degradation (Wilson *et al.*, 2002). The perturbation of this mechanism by reduced IAPs levels results in the stabilization and activation of the caspase cascade and culminates in cell death.

In contrast to the understanding of the programmed cell death core machinery in apoptosis in *Drosophila*, the characterization of apoptosis signaling pathways and the regulation of expression of the different proapoptotic genes is at an early stage. When overexpressed in embryos via heat shock inducible transgenes*, rpr, grim* and *hid* each induce extensive cell death resulting in the demise of the embryo. When expressed ectopically in the *Drosophila* eye, each of these genes is sufficient for inducing massive cell death leading to the ablation of the eye. In contrast, targeted expression of *sickle* does not induce cell death in the *Drosophila* eye. Overexpression of any of these genes in *Drosophila* or mammalian cultured cells results also in the induction of cell death. *rpr* and *grim* are expressed in cells that are fated to die. In contrast, *hid* and *sickle* expression are largely coincident with many cells that undergo cell death, although the correlation is not absolute. For example, the expression of *rpr* mRNA but not *sickle* mRNA is upregulated in *crumbs* mutants (Christich *et al.*, 2002). It was lately discovered that upon an irradiation stimulus, the tumor suppressor protein p53 activates *rpr, hid* and *sickle* transcription (Brodsky *et al.*, 2000; Sogame *et al.*, 2003; Brodsky *et al.*, 2004). It was also found that *rpr*, but not the other proapoptotic genes, is transcribed following the increase in ecdysone that triggers salivary gland cell death (Lee *et al.*, 2003). Taken together, these data suggest that steroid hormone ecdysone, developmental signals and cell cycle perturbation induce differential expression of these genes and thus different proapoptotic pathways are triggered or regulated.

Objectives

Knowledge about the mechanisms of protein synthesis during stress response and apoptosis derives mainly from studies in mammalian models. However, the role of cap-independent translation during these processes in *Drosophila* has only partially been studied for heat shock mRNAs. In *Drosophila* the *eIF4E1-2* gene encodes two isoforms, namely eIF4E1 and eIF4E2 (Lavoie *et al.*, 1996; Hernández *et al.*, 1997). eIF4E1 is the unique isoform detected in embryos, suggesting that it might play a major role in cap-dependent protein synthesis during embryogenesis (Maroto and Sierra, 1989; Zapata *et al.*, 1994; Hernández and Sierra, 1995). Previous work in our laboratory on the *Drosophila* lethal mutant *l(3)67Af* characterized it as a null mutant for the gene *eIF4E1-2* (Hernández *et al.,* unpublished). The evidence that *l(3)67Af* mutants show widespread induction of apoptosis and upregulation of *rpr* mRNA, raised the hypothesis that *rpr* mRNA and likely other proapoptotic mRNAs must be translated in a cap-independent manner. Because of this genetic evidence and the fact that when we started this work the role of cap-independent translation in apoptosis had not been investigated in *Drosophila*, the aim of this doctoral thesis was:

1) to study the mechanism of translation of *rpr* mRNA (Chapter 1);

2) to extend the study to the *Drosophila* proapoptotic genes *hid, grim* and *sickle* (Chapter 2); and

3) to determine the possible existence of factors involved in cap-independent translation in *Drosophila.* This goal required:

a) setting up a method to isolate mRNA interacting factors involved in translation control during apoptosis by the analysis of factors bound to *rpr* 5´UTR (Chapter 3); and

b) characterizing *Drosophila* eIF4B activity in cap- and IRES-dependent translation (Chapter 4).

Materials and Methods

Fly work

Mutant *l(3)67Af*^{1} *ri*^{1} *e*^{4} / TM3, *Sb*^{1} (Leicht and Bonner, 1988) was obtained from Mid-America *Drosophila* Stock Center (Bloomington, USA). To identify homozygous mutant embryos, the mutation was balanced to *TM3*, *Actin-GFP* chromosome. Wild type, heterozygous and homozygous embryos were hand-sorted *in vivo* by identifying the GFP marker under a fluorescence microscope.

Plasmids

A list and map for the final plasmids used in this thesis are depicted in Table I.

Plasmids used *in vitro* translation assays

Plasmids pA2 (Hart and Bienz, 1996; a gift of M. Bienz), SK+II-Grim-short-cDNA (Chen *et al.*, 1996; a gift of J.M. Abrams), and pBS-*rpr*-cDNA (a gift of G. Vorbrüggen) were used as templates to amplify *Ubx, grim* and *rpr* 5´ UTRs respectively. *hid, sickle* and *Dm-hsp70* 5´UTRs or ORF cDNAs were amplified using a *Drosophila* adult cDNA library as template (a gift from G. Hernández).

Cassettes containing flanking *Sac*I and *Nco*I sites were introduced by PCR onto the 5'UTR cDNA sequences of *rpr* (White *et al.*, 1994), *grim* (Chen *et al.*, 1996), *hid* (Grether *et al.*, 1995), *sickle* (Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002) and *Dm-hsp70* (McGarry and Lindquist, 1985). Amplified products were cloned into the *Eco*RV site of vector pBluescript SK(+) (Stratagene) by T-end ligation to create the plasmids pBS1-*hid* 5´UTR, pBS1-*sickle* 5´UTR, pBS1-*grim* 5´UTR, pBS1-*rpr* 5´UTR and pBS1-*hsp70* 5´UTR. *rpr, hid, grim, sickle* and *hsp70* 5´UTRs were subcloned then into the *Sac*I-*Nco*I site of pLuc-cassette (Gebauer *et al.*, 1999) to create the plasmids p*rpr*-FLuc, p*hid*-FLuc, pg*rim*-FLuc, p*sickle*-FLuc and p*hsp70*-FLuc, respectively.

PCR amplifications were also performed to introduce flanking *Bgl*II sites in the *Ultrabithorax* (*Ubx*) (Hart and Bienz, 1996; Ye *et al.*, 1997) and *rpr* 5´UTR sequences, and *Bgl*II and *Bam*HI sites in the *hid, grim, sickle* and *hsp70* 5´UTR sequences. *Bam*HI and *Bgl*II flanking sites were PCR-introduced in the *maternal caudal (cad)* 5´UTR sequence (Mlodzik and Gehring, 1987) using a *Drosophila* 0-3 hs embryo library as template (a gift of G. Hernández). The PCR products were cloned by T-end ligation into the *Eco*RV site of vector pBluescript SK(+) to create the plasmids pBS2-*rpr* 5´UTR, pBS2-*hid* 5´UTR, pBS2-*grim* 5´UTR, pBS2-*sickle* 5´UTR, pBS2-*hsp70* 5´UTR and pBS2-*cad* 5´UTR. *Ubx* 5´UTR PCR product was

cloned into pGEMT vector to create pGEMT-*Ubx* 5´UTR. *Ubx* 5´UTR was then subcloned into *Sac*I site of pLuc-cassette by T-end ligation to create p*Ubx*-FLuc. Renilla luciferase (RLuc) was amplified by PCR using the plasmid pRLuc-null (Promega) and cloned into the *Hpa*I site of pLuc-cassette to create the dicistronic reporter vector pFLuc/RLuc. pRLuc vector was created by digesting pFLuc/RLuc with *Sma*I/*Bgl*II to remove out the FLuc ORF and subsequent religation. The plasmid pFLuc/*cad*/RLuc was generated by subcloning the *cad* 5'UTR from pBS2-*cad* 5´UTR into the *Bgl*II site of pFLuc/RLuc. A stable synthetic hairpin containing a 62-nucleotide palindromic sequence (Coldwell *et al.*, 2001) and *Bam*HI/*B*glII flanking sites was introduced into the *Bgl*II site of pFLuc/RLuc, between the two reporter cistrons, to create the plasmid pFLuc/hairpin/RLuc. The 5'UTRs to be assayed for IRES activity were subcloned from pBS2-*Ubx* 5´UTR, pBS2-*rpr* 5´UTR, pBS2-*hid* 5´UTR, pBS2-*grim* 5´UTR, pBS2-*sickle* 5´UTR and pBS2-*hsp70* 5´UTR plasmids into *Bgl*II site of pFLuc/RLuc, pFLuc/*cad*/RLuc and pFLuc/hairpin/RLuc. Deletion constructs of *rpr* and *hsp70* 5´UTRs were generated by mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Plasmids used in cell transfection

A cassette containing the entire *rpr* 5' UTR and ORF was generated by PCR amplification using primers that replaced the *rpr* stop codon by a sequence corresponding to the *myc* 9E10 epitope. The cassette [*rpr 5'* UTR-ORF-*myc*] was then cloned into the *Eco*RV site of pBluescript KS (+) and pLuc-cassette was digested with *Sac*I and *Bgl*II to remove the FLuc ORF. The cassette [*rpr 5'* UTR-ORF-*myc*] was further subcloned into the cut pLuc-cassette to create the plasmid p*rpr 5'* UTR-ORF-*myc*-A_{71}.

To create the plasmid pBSAdh-UAS, oligos containing the minimal *Adh* promoter under control of five UAS sites were cloned into the *Hind*III/*Eco*RI site of pBluescript KS (+). Then a cassette containing a reporter *myc*-tagged Cyan Fluorescent Protein (first cistron, *myc*-CFP) was amplified by PCR from pECFP-C1 (Clontech) and cloned into *Eco*RI site of pBSAdh-UAS to create the plasmid pBSAdh-UAS/CFP. Subsequently a cassette containing haemaglutinin-tagged enhanced Yellow Fluorescent Protein (EYFP) bearing a nuclear localization signal (HA-EYFP-NLS, second cistron) was amplified from pEYFP-Nuc (Clontech) and cloned into the *Xba*I site of pBSAdh-UAS/CFP to create the plasmid pBSAdh-UAS-/CFP/EYFP. The cassette Adh-UAS/CFP/EYFP was subcloned into the *Spe*I/*Xba*I

sites of plasmid pCasperBgal to create the plasmid pCFP/EYFP that contains now a polyadenilation SV40. *rpr* 5´ UTR was then cloned into the intercistronic region to create the plasmid pCFP/*rpr* 5´UTR/EYFP. *eIF4E1* 5´UTR (Hernández *et al.*, 1997) was amplified using a *Drosophila* adult cDNA library as template and subcloned in the intercistronic region of pCFP/EYFP to create the plasmid pCFP/*eIF4E1* 5´UTR/EYFP. The promoterless constructs p*rpr*/EYFP and p*eIF4E*/EYFP were made by digestion of pCFP/*rpr* 5´UTR/EYFP and pCFP/e*IF4E1* 5´UTR/EYFP vectors with *Kpn*I, to remove the Adh-UAS promoter and the first cistron, and subsequent religation.

Plasmids used in the tobramycin based affinity purification method.

Primers were designed to amplify by PCR *rpr* 5' UTR mRNA and to introduce either a cassette containing a T7 (italics) promoter immediately upstream of the *rpr* 5' UTR (underlined) (5´-*TAATACGACTCACTATAGGG*TGAATAAGAGAGACACCAGAA CAAA-3´) or the T7 promoter followed by the J6f1 tobramycin aptamer (Hartmuth *et al.*, 2002; bold) (5´-*TAATACGACTCACTATAGGG***GGCTTAGTATAGCGAG GTTTAGCTACACTCGTGCTGAGCC**TGAATAAGAGAGACACCAGAACAA A-3´). In both cases the same primer was used from the 3' including the first 10 amino acids (italics) of the *rpr* ORF (5´-*CTGATCGGGTATGTAGAATGCC ACTGCCAT*TGTTGTTGGTTTATCTTTCTTCG-3´). PCR fragments were cloned into the *Eco*RV site of vector pSL1180 (Amersham Pharmacia Biotech) to create the plasmids pSL-T7-*rpr* and pSL-T7-TA-*rpr*.

Plasmids used in RNAi experiments

Drosophila La autoantigen ORF was amplified by PCR from a *Drosophila* adult cDNA library and cloned into the *Eco*RV site of vector pBluescript SK(+) to create the plasmid pBSK-La.

Plasmids used as templates for synthesis of the probes for *in situ* hybridization and Northern Blot experiments

hid and *sickle* ORF were amplified by PCR from a *Drosophila* cDNA library and cloned into the EcoRV site of vector pBluescript SK(+) to create the plasmid pBS-*hid*-ORF and pBS-*sickle*-ORF.

The Firefly luciferase ORF was PCR amplified from pLuc-cassette and subcloned into pGEM-T vector (Promega) to create pGEM-FLuc in order to use as template for Northern experiments.

***In vitro* translation assays**

Translation extracts were prepared from 0-12 h old *Drosophila* embryos as described (Tuschl *et al.*, 1999). *In vitro* translation was performed as described (Maroto and Sierra, 1988; Gebauer *et al.*, 1999) for the indicated times at 25°C. Translation extracts from heat-shocked embryos were prepared from pools of 0-12 h old embryos which have been treated for 45 min at 37°C and processed without further recovery. Translation extracts from mutant embryos in aliquots of 10 µl were prepared by hand sorting 50-100 homozygous embryos. We observed that different batches of translation extracts or extracts derived from different treatments vary in their absolute translation activity, but the relative activity of each mRNA remains stable. Thus, the comparison of absolute values for different batches of the extracts was not possible. In all cases whenever we indicate the comparison of the absolute values, we have always used the same batch of extract. Either m^7GpppG or ApppG-capped transcripts, and uncapped transcripts were synthesized using T3 RNA polymerase (Ampliscribe mRNA transcription kit, Biozym Diag. GmbH) and using plasmids linearized with Xho I as templates in the presence or absence of m^7GpppG or ApppG (New England Biolabs). The reaction was digested with DNAse I and the transcripts purified using the RNeasy kit (Qiagen). Reporter gene expression (Firelfly and Renilla luciferases) was determined using the Dual-luciferase reporter assay system (Promega) and detected in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). When error bars are shown they represent at least the mean of two experiments. The possible degradation of the transcripts was assessed using [^{32}P]-labeled reporter RNAs for the translation reaction. After completion of the reaction, the RNA was purified and analyzed by denaturing agarose gel electrophoresis and phosphorimaging.

S2 cell transfection

Drosophila Schneider S2 cells were cultured in Schneider's *Drosophila* medium (GIBCO) containing 10% fetal bovine serum (Biochrom), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to 80% confluence and co-transfected in 24 wells-multidish with 150 ng of each dicistronic plasmid to be tested (pCFP/EYFP, pCFP/*rpr* 5´UTR/EYFP, pCFP/eIF4E1 5´UTR/EYFP, p*rpr*/EYFP or p*eIF4E*/EYFP) together with 50 ng of the pActGal4 plasmid by using the Effectene transfection reagent (Qiagen). After 48 h of transfection, the fluorescence signal was acquired

using a Zeiss CLSM 310 Confocal Laser Scanning Microscope and the appropriate filters set. For visualization of CFP an excitation at 458nm and a BP 475-525 filter were used. EYFP was excited at 518nm and visualized with a LP530 filter. RNAs used in cell transfection were prepared from the corresponding linearized plasmids by in vitro transcription in the presence of either m^7GpppG or ApppG, digested with DNAse I and purified using the RNeasy kit (Qiagen). $1x10^6$ *Drosophila* S2 cells were seeded in each well of 6 multi-well plates and transfected with 5 µg of RNA using the Effectene reagent. 6-8 h after transfection cells were harvested by resuspension and split in two tubes in order to assay for reporter gene activity and to determine RNA integrity by Northern Blot. Cells were pelleted by centrifugation and washed once with PBS (130 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO4, 3m M KH_2PO_4, pH 7). For luciferase assay the cell pellet was resuspended in 40 µl of passive lysis buffer of the Dual-Luciferase reporter assay system and 20 µl was used for luciferase measurements. 5 µg of RNA of the construct *rpr 5'* UTR-ORF-*myc*-A_{71} tag were used to transfect $1X10^6$ *Drosophila* S2 cells as described using 18mm round coverslips in the 6 multi-well plates. 6 h after transfection the cells on the coverslips were fixed and used to analyze *rpr-myc* expression and apoptosis induction. Rpr-Myc was detected by immunofluorescence. Apoptosis was assayed by TUNEL staining. The cells remaining in the wells were resuspended and split into two tubes in order to assay *rpr-myc* expression by Western Blot and RNA integrity of the transfected RNA construct by Northern Blot.

Northern Blot

RNA was purified from cell pellets using the RNeasy kit and eluted with 30 µl water. RNA samples (5µl) were separated by electrophoresis in formaldehyde-agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labeled anti-sense RNA probes as described (Hernández and Sierra, 1995). Linearized pBS*rpr 5'* UTR-ORF-myc-A_{71} and pGEM-FLuc plasmids were used as templates to generate the RNA-probes.

Immunofluorescence

S2 cells were grown on coverslips, washed with PBS, fixed in PBS-4% formaldehyde for 10 min at 25°C, permeabilized in PBS-0.1% Triton X-100 for 5 min at 25°C, washed twice with PBS-0.1% Tween 20 (PBT), blocked with PBT-5% BSA for 30

min and incubated with monoclonal antibody 9E10 anti-myc epitope (Santa Cruz) (diluted 1:1 in PBT-5% BSA) overnight at 4°C. After 4 times washing for 10 min with PBT, cells were incubated with Cy3 –conjugated goat anti mouse (Jackson Immunoresearch) (diluted 1:600 in PBT-5% BSA) for 2 h at 25°C, washed 4 times for 10 min with PBT and mounted in Moviol. Images were acquired with a CLSM 310 Confocal Laser Scanning Microscope (Zeiss).

TUNEL assay in S2 cells

Apoptosis was detected by TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labeling (TUNEL) with the *In Situ* Cell Death Reaction Kit, AP (Roche Diagnostics). S2 transfected cells growing on coverslips were washed once with PBS, fixed with PBS-4% paraformaldehyde for 15 min at 25°C, washed twice with PBS, permeabilized with PBS-0.1% Triton X-100 for 5 min at 25°C, rinsed twice for 5 min with PBT and then washed once with buffer 2: label solution from the TUNEL kit (50 µl). Cells were then incubated in a humid atmosphere in the dark for 1 h at 37°C with 50 µl buffer 2 containing 5 µl enzyme solution. The use of fluorescein–dUTP in the label solution allowed the detection of DNA fragmentation by fluorescence microscopy. The cells where rinsed once and washed twice for 10 min with PBT. Cells were blocked with PBT-BSA 5%, for 30 min and then incubated with a monoclonal antibody 9E10 anti-myc epitope (diluted 1:1 in PBT-5% BSA) overnight at 4°C. Cells were washed 4 times for 10 min with PBT and then washed twice for 20 min with PBT-BSA 5%. Cells were then incubated with Cy3 –conjugated goat anti mouse (diluted 1:600 in PBT-5% BSA) for 2 h at 25°C. After being washed 4 times 10 min, cells were mounted in Moviol and images were acquired with a CLS 310 Confocal Scanning Microscope (Zeiss).

Western Blot analysis

Western Blot analysis was performed on a gel loaded with extracts of 10-20 wild type or homozygous embryos (stage 8-12) per lane. 30 µl of sample buffer 1x (NuPAGE) was added to the sorted embryos. After homogenization, sonication and boiling for 2 min. samples were ready to load onto the SDS-PAGE gel (NuPAGE 10% Acrylamide/ Bis-Acrylamide precasted gel using the NuPAGE MES running buffer). For samples obtained from S2 cells, cells were pelleted by centrifugation 4 min at 800 x g and washed once with PBS and stored at -70°C. 50 µl of hypotonic buffer (10 mM

Hepes pH 7.4, 10 mM KAc, 1.5 mM MgAc, EDTA-free protease inhibitors cocktail Complete™ (Roche Diagnostics), 1% NP-40, 2.6 mM DTT) was added. The cells were resuspended by vortexing for 10 seconds, further incubated for 15 min at 4°C, then centrifuged at 10000 x g for 5 min at 4°C and the supernatant collected. Protein concentration of supernatant samples was quantified with the Protein-Assay Kit (Bio-Rad). 5 µg of total protein extracts were mixed with an equal volume of 2x sample buffer, boiled 2 min and resolved by SDS-PAGE. The samples were blotted to nitrocellulose membranes for 30 min at 25V. The membranes were incubated in blocking solution for 1 hour at room temperature. After 3 x 20 min washes with PBT, the membranes were incubated overnight at 4°C with the primary antibody (rabbit anti-Dm-eIF4E-1/2 antibody (1:1500) (Maroto and Sierra, 1989; a gift from J.M. Sierra), rat monoclonal 7FB anti-HSP70 (1:200) (Velazquez and Lindquist, 1984; a gift of S. Lindquist), rabbit anti-Dm-La (1:1000) (Yoo and Wolin, 1994; a gift from S. Wolin), rabbit anti-Dm-eIF4B (1:20.000) or mouse anti-myc monoclonal antibody 9E10 (1:10). After 3 x 20 min washes with PBT, the membranes were incubated for 2 h at room temperature with conjugated-secondary antibody (HRP-conjugated goat anti-rabbit antibody (1:20000, Jackson ImmunoResearch), HRP-conjugated goat anti-mouse antibody (1:5000, Dianova) or preabsorb HRP-conjugated goat anti-rat antibody (1:1500, Jackson ImmunoResearch). The Western Blots were developed using the ECL detection kit (Amersham Pharmacia Biotech).

Polysome analysis

Drosophila melanogaster Oregon R embryos were collected from population cages in apple juice-agar plates and dechorionated. For heat shock treatment, 0-12 h old embryos were heat shocked for 45 min at 37 ˚C and processed without recovery. For apoptosis induction, 0-8 h embryos were exposed to 4,000 radians using a Torrex 150D X-ray irradiator and, after further development for 4 h at 25 ˚C, processed. 150 mg of embryos were homogenized on ice in 300 µl buffer A (30 mM Hepes pH 7.4, 100 mM K acetate, 2 mM Mg acetate, 5 mM DTT, 50 u/ml RNasin, 2 mg/ml heparin, and EDTA-free protease inhibitor cocktail Complete™ (Roche Diagnostics). The homogenate was centrifuged at 14.500 x g for 20 min. 200 µl of the supernatant was layered onto a 10 ml 10%-50% sucrose gradient prepared in 15 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 300 mM NaCl, 1 mg/ml heparin, and centrifuged in a Beckman Ti-SW41 rotor for 2.5 h at 36K at 4 ˚C. UV absorbance was recorded at 254 nm and 0.5

ml fractions were collected. The fractions corresponding to pre-initiation and initiation complexes (40/43/48S and 80S) and the ones corresponding to polysomes were pooled.

Quantitative Real Time RT-PCR

Pooled fractions of pre-initiation/initiation complexes or polysome-bound mRNAs were digested with proteinase K (150 µg/ml) in the presence of 1% SDS for 30 min at 37 ˚C. The digestion was adjusted to sodium acetate 0.3 M and the RNA precipitated with ethanol. The RNA pellet was dissolved in H_2O and digested with RNAse-free DNAse I to prevent any contamination with genomic DNA, further purified using the RNeasy Mini Kit, and quantified by spectrophotometry. 100 ng of RNA was used for quantitative real time RT-PCR using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) in a DNA Engine Opticon System (M. J. Research Inc.). Sequence specific 25-mer oligonucleotides for *Drosophila actin5C*, *hsp70*, *hid, sickle, grim* and *rpr* mRNAs were designed to amplify 100 bp fragments.

RNA preparation and assembly of the RNP complexes

Aptamer-tagged or untagged RNAs were synthesized with the Transcription T7 polymerase kit (Megascript, Ambion) and the plasmids pSL-T7-TA-*rpr* and pSL-T7-*rpr* as templates, respectively. For quantification, [^{32}P]UTP (3000Ci/mmol; 1 Ci =37 GBq) was added to the reaction to 0.23 µM final concentration. The transcription reaction was incubated for 6 hours at 37˚C. The template was digested with DNAse I and the transcripts purified by LiCl precipitation at -20°C following the manufacturer protocol (Megascript, Ambion). Unincorporated nucleotides were removed by spin column chromatography (S-300 HR column, Amersham Pharmacia). The RNAs were finally dissolved in CE buffer (10 mM cacodylic acid-KOH pH 7; 0.2 mM EDTA).

Drosophila embryo extracts, were prepared from 0-12 h old embryos, and cell free translation reactions were carried out as described above. 4x binding buffer (4x BP; 80 mM Tris·HCl, pH 9.1 at 4°C; 4 mM $CaCl_2$; 4 mM $MgCl_2$; 0.8 mM DTT) was freshly prepared and diluted to 1x BP for the preparation of gradient, blocking, washing and elution buffers. RNPs were assembled *in vitro* using either radiolabelled *rpr* 5´UTR or aptamer- *rpr* 5´UTR and embryonic translation extracts. 350 µl cell-free translation reaction were assembled and pre-incubated at 25°C in the presence or absence of 100 mM GMP-PNP and 50 mM cycloheximide. After 4 minutes, 140

pmoles of radiolabelled aptamer-tagged or non-tagged RNAs were added and further incubated for 10 minutes at 25°C. The reaction was then loaded on top of a 10ml 10-30% sucrose gradient prepared in 1x gradient buffer (1x BP, 145 mM KCl and 4 mM $MgCl_2$) and centrifuged in a Beckman Ti-SW41 rotor (15 h, 25K, 4°C). Continuous UV absorbance was recorded at 254nm and 0.4 ml fractions were collected from top to bottom. The fractionation procedure was performed at 4°C. The presence of the target RNA in the fractions was determined by Cherenkov counting. Fractions corresponding to the RNP complex were pooled. Usually 4 fractions of 400 µl were pooled and incubated with 200 µl of tobramycin matrix (see below).

Tobramycin affinity purification of RNP complexes

N-hydroxysuccinimide-activated Sepharose 4 Fast Flow (Amersham, Biosciences) was derivatized with 5 mM tobramycin as described (Hartmuth *et al.*, 2004). All procedures were performed at 4°C. Aliquots of tobramycin matrix (140-200 µl) were blocked overnight with 1,5 ml of blocking buffer (1x BP, 300 mM KCl, 0.1 mg/ml tRNA, 0.5 mg/ml BSA, 0.01% Nonidet P-40) by flipping rotation. The matrix was collected by centrifugation and 2 ml of the pooled fractions containing the *rpr* 5´ RNP-complex were added to 140-200 µl of tobramycin matrix and incubated overnight. The matrix was then washed three times (washing volume, 1.5 ml) with washing buffer (1x BP, 145 mM KCl, 5 mM $MgCl_2$). The bound complexes were eluted with 400 µl of elution buffer (1x BP, 10 mM tobramycin, 145 mM KCl, 4 mM $MgCl_2$) for 10 min at room temperature. Approximately 2-4 pmoles of aptamer-tagged *rpr* 5´UTR RNA was eluted per gradient loaded. Proteins were recovered by ethanol precipitation and analyzed by SDS 10-13% PAGE buffer and silver staining.

Mass Spectrometry

The protein bands visible on silver stained SDS-PAGE were cut out and proteins were in-gel digested with trypsin. Eluted peptides were sequenced by liquid chromatography-coupled electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) on a Q-ToF Ultima instrument (Waters) as described (Hartmuth *et al.*, 2002). The corresponding proteins were identified by searching against all entries in the National Center for Biotechnology Information nonredundant database using MASCOT (Matrix Science, London) as a search engine.

Recombinant protein expression

Drosophila La protein was expressed in *E.coli* using pTrCHisA-La (a gift of S. Wolin) and purified as described (Yoo and Wolin, 1994). The protein was dialyzed against 20 mM Hepes pH 7.8; 0.15 mM EDTA; 10% glycerol; 0.01%NP-40. Recombinant *Drosophila* eIF4B-L and eIF4B-S proteins (Hernandez *et al.*, 2004) were a gift of G. Hernández.

UV-crosslinking

For crosslinking experiments, ^{32}P-labeled RNA probes (*Ubx*, *rpr* and *caudal* 5´UTRs) were generated by transcription of linearized pGEMT-*Ubx* 5´UTR, pBS2-*rpr* 5´UTR and pBS2-*cad* 5´UTR with the T7 RNA polymerase in the presence of α-[^{32}P]ATP and α -[^{32}P]UTP. RNA probes were digested with RNAse-free DNAse I and further purified. RNA integrity was assessed by agarose gel electrophoresis. RNA probes were diluted in 10 mM Hepes-K^+ pH 7.6; 15 mM KCl; 2.5 mM $MgCl_2$. The crosslinking was performed in a final volume of 10 µl in crosslinking buffer (10 mM Hepes K^+ pH 7.6, 1 mM DTT, 5% glycerol, 1 mM ATP; 100 ng/ µl yeast tRNA; 10 µg/ µl heparin), RNA (100,000-600,000 cpm, previously treated for 15 min at 70°C) and either 1.5 µg Dm-eIF4BL, Dm-eIF4BS, Dm-La, GST or BSA (Roche). After incubation for 15 min at room temperature, the samples were transferred to ice and irradiated 35 min at 254nm at 2 cm from the UV light source (UV Stratalinker 2400, Stratagene). Reactions were digested for 45 min at room temperature with 1 µl of a mixture of RNAse A (1 µg/µl) and RNAse T1 (5 µg/µl). The complexes were resolved in 10% SDS-PAGE and analyzed in a Phosphoimager.

RNAi experiments

Sense and antisense RNAs were prepared from linearized pBSK-La using the Ampliscribe mRNA transcription kit in the presence of m^7GpppG, digested with DNAse I and purified using the RNeasy kit. Isoform-specific dsRNAs were produced by hybridization of an equimolar amount of sense and antisense RNAs in 50 mM NaCl and 20 mM Tris-HCl pH 8.0 (3 min at 85˚C, 60 min at 65˚C, chilled on ice and stored at –20 ˚C). The quality of the dsRNA was assessed by agarose gel electrophoresis.

For RNAi experiments to knock down the Dm-La antigen, 1 X 10^6 *Drosophila* Schneider S2 cells were transfected in a 35 mm dish with 10 µg of La antigen dsRNA

using the Effectene reagent. Control cells were mock transfected with the Effectene reagent alone. 24 h after transfection the medium was removed; the cells were resuspended in 3.5 ml of medium and split into three dishes. 72 h after transfection, the cells from one well were transfected again with 10 µg of dsRNA. The cells were again split after 24 h into three wells. 96 h after the second dsRNA transfection, the cells were transfected by triplicate with 5µg of mRNA reporters. Reporter transcripts were synthesized from linearized p*rpr*-FLuc, pRLuc and pFLuc/hairpin/*rpr*/RLuc plasmids using the T3 Ampliscribe mRNA transcription kit in the presence of m^7GpppG or ApppG, digested with DNAse I and purified using the RNeasy kit. 8 h after transfection, cells were harvested and assayed for reporter activity and Western Blot. Reporter gene expression (Firefly and Renilla luciferase) was determined using the Dual-Luciferase reporter assay system and detected in a Monolight 2010 Luminometer. Western Blot analysis was performed as described using 5 µg of protein per lane and revealed with rabbit anti-*Drosophila* La antibody or anti-*Drosophila* eIF4E-1,2 antibody as control.

Embryo double whole-mount *in situ* hybridization

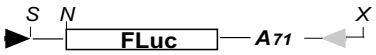
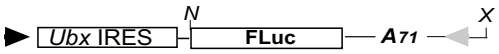
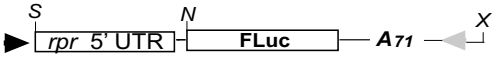
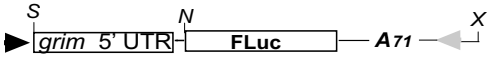
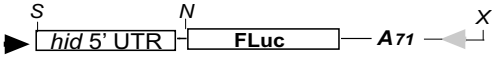
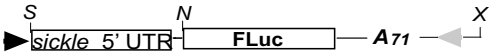
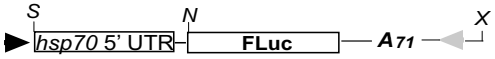
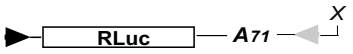
Whole mount *in situ* hybridization of embryos was essentially performed as described by Klinger *et.al.* with the following modifications (Klinger and Gergen, 1993). Linearized pBS-*sickle*-ORF, pBS-*hid*-ORF, SK+II-*Grim*-short-cDNA and pBS-*rpr*-cDNA were used as templates to generate digoxigenin-labeled RNA antisense probes. Linearized pRK27 (a gift of R. Kühnlein) was used as template to generate an antisense fluorescein-labeled GFP probe. The hybridization was performed with 1µl preheated DIG-labeled probes (either *rpr* or *hid* or *grim* or *sickle*) together with 1µl of preheated fluorescein-labeled probe for GFP. The embryos were first incubated with anti-DIG-AP antibody (Boehringer Mannheim) at 1:2000 dilution in PBT. The staining was developed with 1ml SIGMA FAST BCIP/NBT solution (Sigma kit No. B5655) in the dark. The staining reaction was stopped rinsing twice and washing 3 times the embryos for 10 min with PBT. Embryos were then washed with increasing concentration of ethanol (50%, 70% and 100%) for 10 min each. They were incubated two more times with ethanol 100% and left at –20°C overnight. Embryos were washed with ethanol 50%. After two washes for 10 min with PBT, the embryos were washed with Glycine–buffer (100 mM Glycine pH 2.2, 0.1% Tween-20) for 10 min.

They were further washed 1x 10 min and 2x 30 min with PBT. For the detection of fluorescein-labeled GFP probe, AP-coupled anti-fluorescein Fab fragment antibody (Boehringer Mannheim) was used at 1:2000 dilution in PBT and incubated at 4^{o}C overnight. The staining was developed with 1ml Fast-Red-solution (1 pill of Fast-Red TR/Naphtol AS-MX + 1 pill of Tris-HCl Buffer (Sigma kit No. F-4648) diluted in 1 ml H_2O) in the dark. The reaction was stopped by washing 3 times for 15 min with PBT. After removing the PBT, 100% Glycerin was added. The embryos were mounted and images were acquired with an Axioplan Microscope coupled to a Kontron CCD camera.

Table I

Plasmids used in this thesis[a]

MONOCISTRONIC REPORTERS FOR *IN VITRO* TRANSLATION ASSAYS

NAME	MAP	SOURCE
pFLuc-cassette		gift of F. Gebauer (Gebauer *et al.* 1999)
p*Ubx* - FLuc		This thesis
p*rpr* - FLuc		This thesis
p*grim* - FLuc		This thesis
p*hid* - FLuc		This thesis
p*sickle* - FLuc		This thesis
p*hsp70* - FLuc		This thesis
pRLuc		This thesis

DICISTRONIC REPORTERS FOR *IN VITRO* TRANSLATION ASSAYS

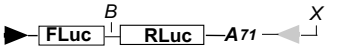
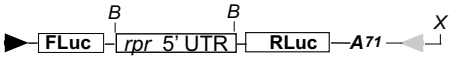
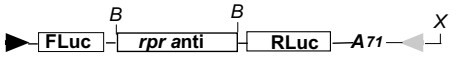
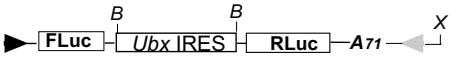
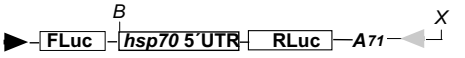
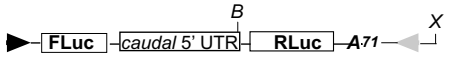
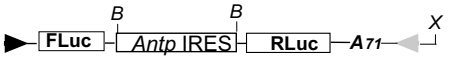
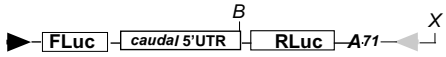
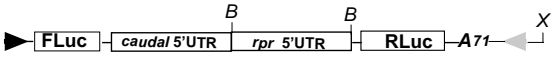
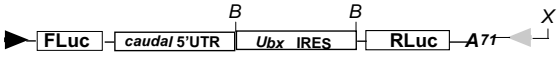
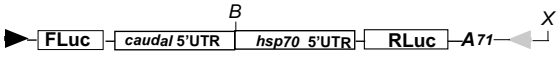
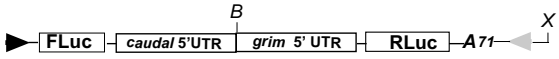
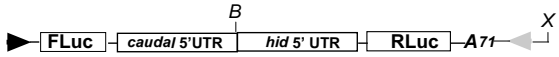
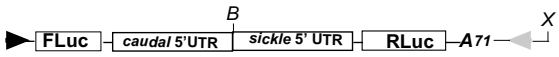
pFLuc / RLuc		This thesis
pFLuc / *rpr* / RLuc		This thesis
pFLuc / *rpr* anti / RLuc		This thesis
pFLuc / *Ubx* / RLuc		This thesis
pFLuc / *hsp70* / RLuc		This thesis
pFLuc / *cad* / RLuc		This thesis
pFLuc / *Antp* / RLuc		This thesis
pFLuc / *cad* / RLuc		This thesis
pFLuc /*cad* / *rpr* / RLuc		This thesis
pFLuc /*cad* / *Ubx* / RLuc		This thesis
pFLuc /*cad* /*hsp70*/ RLuc		This thesis
pFLuc /*cad* /*grim*/RLuc		This thesis
pFLuc /*cad* /*hid* /RLuc		This thesis
pFLuc /*cad* / *sickle* / RLuc		This thesis

Table I
(*continued*)

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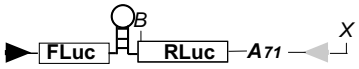
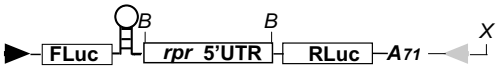
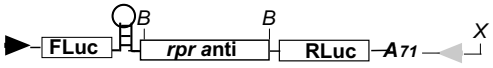
pFLuc / hairpin/ RLuc		This thesis
pFLuc / hairpin / *rpr* / RLuc		This thesis
pFLuc / hairpin / *rpr* anti / RLuc		This thesis
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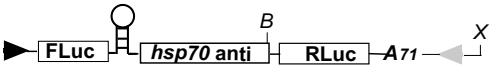
pFLuc / hairpin / *hsp70* anti / RLuc		This thesis
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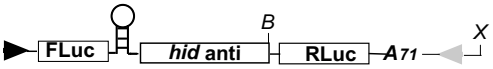
pFLuc / hairpin / *hid* anti / RLuc		This thesis
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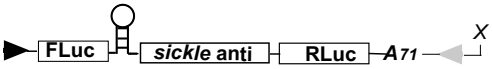
pFLuc / hairpin / *sickle* anti / RLuc		This thesis
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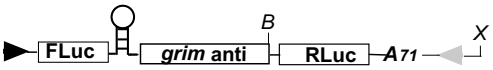
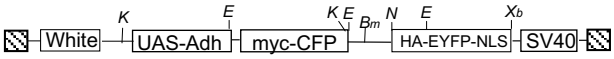
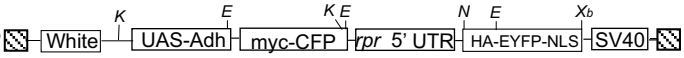
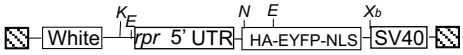
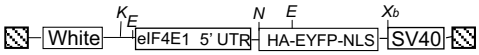
pFLuc / hairpin / *grim* anti / RLuc		This thesis

REPORTERS FOR CELL TRANSFECTION

pCFP/EYFP		This thesis
pCFP/ *rpr* 5'UTR/ EYFP		This thesis
p*rpr*/EYFP		This thesis
peIF4E1/EYFP		This thesis
p*rpr* 5´UTR-ORF -*myc -A71*		This thesis

PLASMIDS USED IN THE TOBRAMYCIN BASED PURIFICATION METHOD

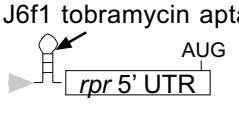
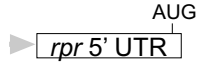
pSL-T7-*rpr*		This Thesis
pSL-T7-TA-*rpr*		This Thesis

Table I
(*continued*)

TEMPLATES USED FOR *IN SITU* HYBRIDIZATION AND NORTHERN BLOTS

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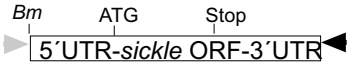
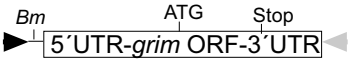
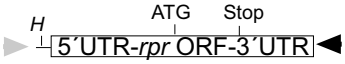
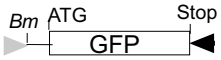
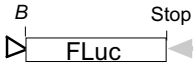
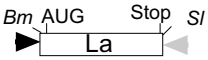
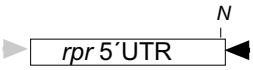
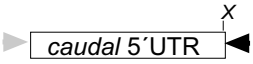
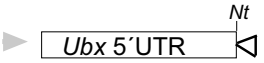
pBS-*sickle*-ORF		gift of G. Hernandez
pBS-*hid*-ORF		This Thesis
pBK+ II-*grim* Short cDNA		gift of J. Abrams (Chen *et al.* 1996)
pBS-*rpr*-cDNA		gift of G. Vorbrüggen
pRK27		gift of R. Künhlein
pGEM-FLuc		This Thesis

TEMPLATES FOR RNAi EXPERIMENTS

pBS-La-ORF		This Thesis

CONSTRUCTS USED FOR CROSSLINKING EXPERIMENTS

pBS2-*rpr* 5´UTR		This Thesis
pBS2-*cad* 5´UTR		This Thesis
pGEMT-*Ubx* 5´UTR		This Thesis

[a] Enzymes used for cloning or for linearizing the templates for *in vitro* transcription are shown: *Bm* (*Bam* HI), *B* (*Bgl* II), *E* (*Eco* RI), *H* (*Hind* III), *N* (*Nco* I), *Nt* (*Not* I), *K* (*Kpn* I), *P* (*Pst* I), S (Sac I), *Sl* (*Sal* I), *Xb* (*Xba* I), *X* (*Xho* I). T3 polymerase promoter (black arrow head), T7 polymerase promoter (gray arrow head) or Sp6 polymerase promoter (white arrow head) are shown. Dashed squares represent P sites. All constructs containing luciferase reporters were derivatives of pFLuc-cassette (Gebauer *et al.*, 1999) which is cloned in pBluescript.

Results

Chapter 1

Internal ribosome entry site drives cap-independent translation of *reaper* in *Drosophila* embryos

reaper mRNA is translated in a cap-independent manner *in vitro* and *in vivo*

RPR is a poor antigenic protein and despite the efforts of many laboratories, good quality antibodies against it are unavailable. Thus, it was not possible to supply direct evidence for ectopic RPR protein expression in *l(3)67Af* embryos. For this reason, *rpr* mRNA translation was analyzed in a cell-free *Drosophila*-embryo translation system (Maroto and Sierra, 1989; Gebauer *et al.*, 1999). For that purpose we prepared *in vitro* transcribed, monocistronic and polyadenylated reporter mRNAs containing the 5'UTRs to be tested either capped with m^7GpppG or uncapped (Fig. 1.1a). The mRNAs were added to the translation reaction at different concentrations and the activity of the encoded reporter gene (FLuc, firefly luciferase) was measured at different time points within a linear response range (Fig. 1.1b). The translation of an uncapped control reporter mRNA (FLuc; Fig. 1.1b) was 1/10th as efficient as the capped control mRNA (cap-FLuc; Fig. 1.1b). In contrast, uncapped reporter mRNA containing the previously defined IRES from the *Ubx* mRNA, *Ubx* –Fluc (Hart and Bienz, 1996; Ye *et al.*, 1997) was translated 5-8 times more efficiently than the uncapped control mRNA (FLuc) (Fig. 1.1b). Similarly, the uncapped reporter mRNA containing the 5'UTR of *rpr* mRNA (*rpr*-FLuc; Fig. 1.1b) was translated at levels equivalent to the capped control transcript cap-FLuc. Hence, *rpr* mRNA 5'UTR enhanced the translation of an uncapped reporter transcript *in vitro*, despite the fact that the stability of uncapped transcripts, measured by the addition of [^{32}P]-labeled transcript to the reaction, was strongly reduced when compared to the capped reporter (Fig. 1.1c) at 90 min. of reaction. To confirm that the difference in translation of the reporters was not due to the different stability of the mRNAs we decided to synthesize mRNAs with an ApppG cap.

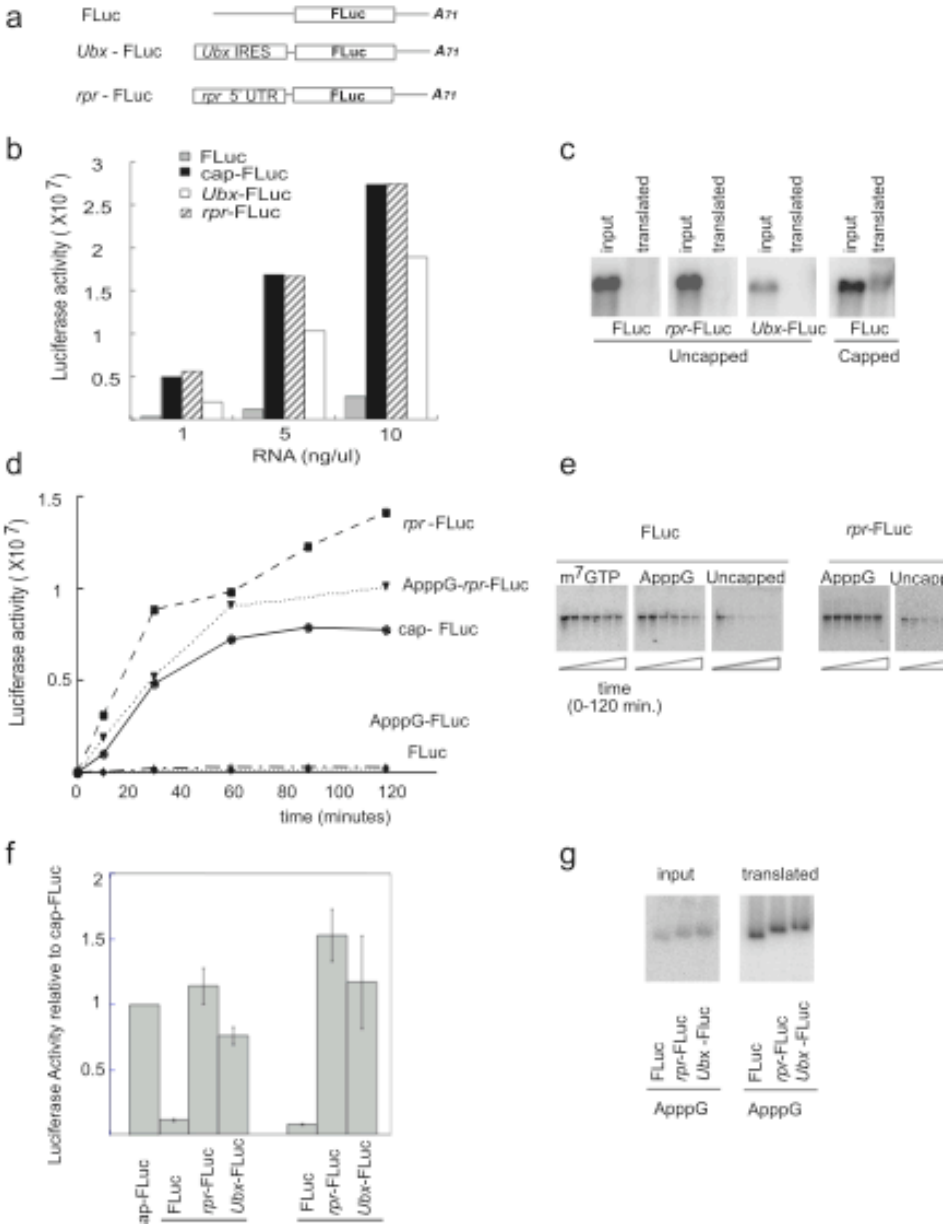


Figure 1.1. *rpr* **5'UTR enhances cap-independent translation *in vitro*. (a)** Reporter monocistronic mRNAs used in *in vitro* translation assays. A_{71} denotes a poly-A tail of 71 nucleotides; FLuc: firefly luciferase. **(b)** Titration of reporter mRNA translation activity in *Drosophila* cell-free extracts. 90 min. of reaction time was used. Luciferase activity is measured in absolute values. **(c)** Stability of the transcripts assayed in (b). Radiolabelled transcripts were added to the translation mixture, purified at the end of the reaction and separated in agarose gels. The same translation reaction was measured in parallel for expression of the reporter gene. **(d)** Translation time-course of trace-labeled reporter mRNAs containing different end modifications. Aliquots were taken at 0, 10, 30, 60, 90 and 120 min, and were used to measure luciferase activity. **(e)** Stability of the mRNAs used in (d). **(f)** Relative translation of uncapped and ApppG-capped transcripts in embryonic translation extracts relative to a m^7GpppG-capped control (Cap-FLuc). **(g)** mRNA stability of the ApppG-capped transcripts assayed in (f).

This cap-analog confers stability to the mRNAs but it is not recognized by eIF4E (Gebauer *et al.*, 1999), and thus, fails to stimulate cap-dependent translation. A time-course experiment was done using the same reporters and luciferase activity (Fig. 1.1d) and RNA stability (Fig. 1.1e) were measured at the indicated time of reaction. The stability of ApppG-capped mRNAs after the translation assay was indeed similar to their m^7GpppG-capped counterparts (Fig. 1.1e). In contrast, their translation profiles mirrored those of their uncapped counterparts (Fig. 1.1d). The use of a polyadenylated reporter mRNA bearing a non-functional cap structure (ApppG instead of m^7GpppG) also resulted in impaired translation (Fig. 1.1d, ApppG-FLuc). In comparison to cap-FLuc, neither uncapped nor ApppG-FLuc transcripts were translated in the extracts (Fig. 1.1d). The translation efficiency of ApppG-*rpr*-FLuc and uncapped-*rpr*-FLuc was equivalent to the capped control (cap-FLuc). Based on the data of the experiments depicted in Fig 1.1b and 1.1d we decided to perform the following experiments by triplicate-duplicate using 5-10 ng of reporter mRNAs and a fixed time between 60-90 min of translation reaction. By using these reaction conditions we were assured that a linear range of RNA concentration would be achieved for all reporters and that Luciferase activity would reach the plateau for these RNA concentrations.

Subsequently, the efficiency of the translation driven by the different 5´ UTRs was compared (Fig. 1.1f). The translation activity of the reporters relative to the cap-dependent translation (Cap-FLuc) was analyzed. In comparison to cap-FLuc, both uncapped and ApppG-FLuc control transcripts were translated 1/10th as efficiently than the capped control. The translation efficiency of ApppG-capped *rpr*-FLuc and ApppG-capped *Ubx*-FLuc was equivalent to the capped control (cap-FLuc), and even more efficiently translated than the capped control upon presence of the cap analog. This was most likely the result of the increased stability of the reporter mRNAs in this experiment (Fig. 1.1g). All ApppG-capped mRNAs were intact after 90 min of reaction indicating that the inefficient translation of the uncapped control was not due to degradation. We concluded that *rpr* 5' UTR (as well as *Ubx* IRES used as a positive control) would confer cap-independent translation to a reporter *in vitro*. The amount of mRNA synthesized per reaction was higher for uncapped mRNAs. Most of the experiments were performed with the uncapped transcripts instead of the ApppG capped ones, because of our demonstration that even if they were less stable they

showed the same profile of translation as their ApppG-capped counterparts (Fig. 1.1d and 1.1f).

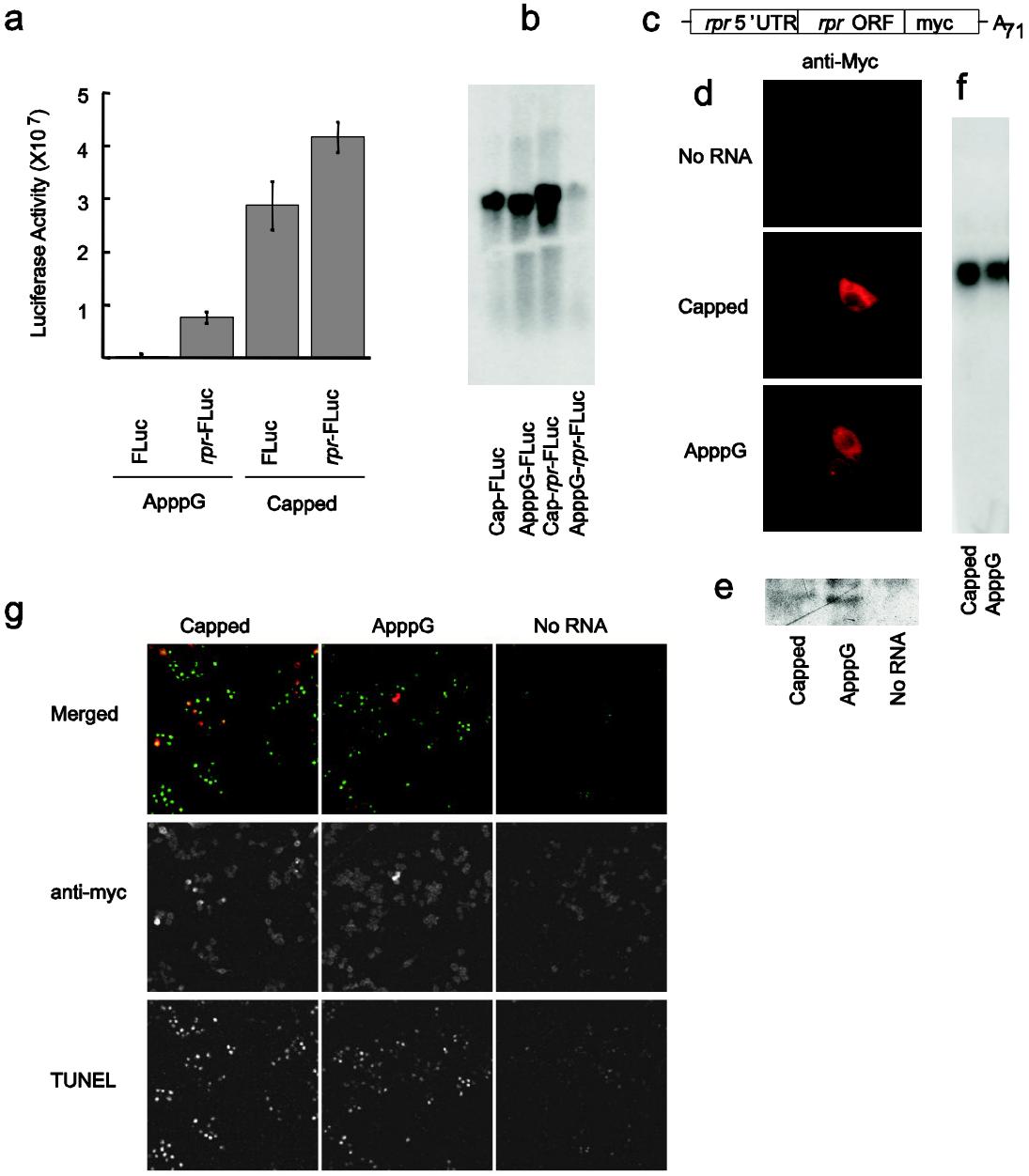


Figure 1.2. *rpr* 5'UTR enhances cap-independent translation *in vivo*. **(a)** Reporter gene activity 6 hours after transfection of S2 cells with ApppG-capped and m^7GpppG-capped transcripts depicted in Figure 1.1a. The bars represent mean and standard deviation of three transfection experiments using 5 µg of reporter mRNA. **(b)** Northern Blot of transfected S2 cells reveals the integrity of the transcripts after 6 hours of incubation. **(c)** *rpr* 5'UTR-ORF-Myc-tagged construct used in an *in vivo* assay. **(d)** Immunofluorescence detection of myc epitope in S2 cells mock-transfected (upper panel, no RNA), transfected with in vitro transcribed, m^7GpppG-capped, polyadenylated *rpr* 5'UTR-ORF-Myc transcript (middle panel) or ApppG-capped, polyadenylated *rpr* 5'UTR-ORF-Myc (lower panel). **(e)** Detection by Western Blot of the translation products from the cells transfected with *rpr*5'UTR-ORF-Myc containing the different end modifications as described in (d) using anti-myc antibody. **(f)** Northern Blot detection of the transcript in the cells corresponding to the transfection experiment shown in (d). **(g)** Transfection of S2 cells with 2 µg of the mRNAs described in (d). After 6 hours of transfection the cells were stained with myc-antibody and assayed for apoptosis by TUNEL technique.

The same effect was observed *in vivo* after transfection of *Drosophila* S2 cells with m^7GpppG- or ApppG- capped reporter mRNAs (Fig. 1.2a). Although there was decreased stability of ApppG-*rpr*-FLuc mRNA by Northern blot analysis (Fig. 1.2b), we observed that the transcript bearing *rpr* 5'UTR was translated at least 10 fold better than the ApppG-capped FLuc control (Fig. 1.2a). To rule out an effect of sequences downstream of the AUG or the *rpr* ORF, a transcript bearing both the *rpr* 5'UTR and the entire *rpr* ORF (*rpr* 5´UTR- ORF-Myc), tagged at the C-terminus with a *myc* epitope, was designed and used to transfect cells (Fig. 1.2c). Transfection of S2 cells with m^7GpppG - or ApppG- capped *rpr* 5´UTR-ORF-Myc mRNAs resulted in the translation of both forms as assessed by immunofluorescence (Fig. 1.2d) and Western blot (Fig. 1.2e) using anti-myc antibody. Northern blot analysis revealed that both transcripts were neither degraded nor cleaved in the experimental conditions used (Fig. 1.2f). It is important to note that the *in vivo* translation of *in vitro* transcribed ApppG-capped RNA indicates cap-independent translation independent of the presence of cryptic promoters in the 5'UTRs (see below). Thus, the results confirmed the existence of a cap-independent mechanism for *rpr* mRNA translation *in vivo*.

We also performed TUNEL staining of these transfected cells to check if the m^7GpppG-capped and ApppG-capped *rpr*-myc mRNAs were able to induce apoptosis. Although the control treatment with Effectene alone (no RNA) resulted in some levels of apoptosis, the cells transfected with m^7GpppG-capped or ApppG-capped *rpr*-myc mRNAs consistently showed more intense TUNEL signal (Fig. 1.2g). However, we failed to observe a correlation between the expression of *rpr* (assayed by detection of the myc epitope) and TUNEL staining in all cells. This result could be rationalized by noting that the expression of *rpr* is one of the earliest steps in the apoptosis cascade and DNA fragmentation one of the last symptoms of the process. Cells expressing *rpr* but showing no TUNEL staining could represent early stages of apoptosis, while those not expressing *rpr* any longer could represent advanced stages of apoptosis. Nonetheless, there was a clear correlation between observed apoptosis and transfection with *rpr* mRNA.

Capping either *rpr*–FLuc or *Ubx* -FLuc reporter mRNAs resulted in even higher levels of absolute translation in wild type extracts with respect to the uncapped transcripts (Fig. 1.3a). This result could either reflect the consequence of increased mRNA stability or the result of the addition of cap-dependent and cap-independent

translation initiation mechanisms. To distinguish between the two possibilities, we performed competition experiments by addition of the cap analog m^7GpppG to the *in vitro* translation reaction in wild type extracts, which mimics the lack of eIF4E in the lysates (Maroto and Sierra, 1988).

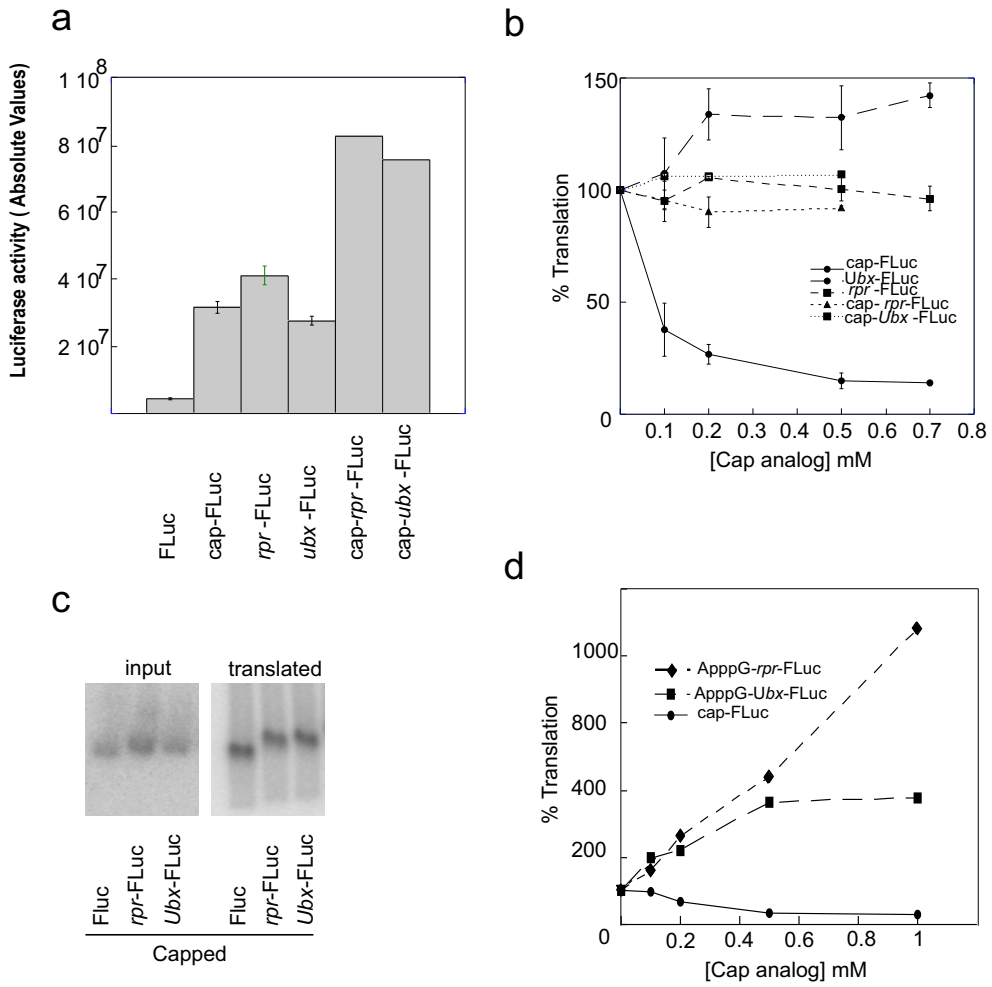


Figure 1.3. Translation of capped *rpr* 5'UTR is not competed by free cap analog. (a) *in vitro* translation of capped and uncapped monocistronic reporter transcripts in translation extracts derived from wild type embryos. **(b)** Competition of cap-dependent translation with increasing concentrations of free cap analog m^7GpppG. The efficiency refers to the translation of the reporter mRNA in the absence of the cap analog. **(c)** Stability analysis of the capped transcripts assayed in (a) and (b). **(d)** Competition of cap-dependent translation with increasing concentration of free m^7GpppG. The translation of mRNAs containing ApppG-end increased while cap-dependent translation decreased.

The addition of 0.1 mM cap analog resulted in a 60% decrease in the translation efficiency of cap-FLuc (Fig. 1.3b). Further addition of the cap analog reached a plateau at 90% inhibition, a residual activity equivalent to the remaining translation level observed when uncapped or ApppG-capped reporter transcripts were

used (Fig. 1.3b, circles and solid line, compare with Fig. 1.1f). On the contrary, the translation of either uncapped or m^7GpppG-capped *Ubx* -FLuc and *rpr*-FLuc was not significantly reduced by the addition of the cap analog (Fig. 1.3b). Transcript degradation was not observed during the translation reaction for the capped mRNAs (Fig. 1.3c). Upon performing the same experiment using ApppG-capped reporters bearing the *rpr* 5´UTR and the *Ubx* IRES their translation increased upon addition of competitor. This result agrees with the increased stability of these mRNAs compared with the uncapped ones, as well as with the availability of free translation factors and regulatory proteins involved in cap-independent translation, since cap-dependent translation of the endogenous mRNAs present in the extracts was blocked (Fig.1.3d). The competition experiments described above suggest the existence of some level of cap-dependent translation on cap-containing reporters. By the addition of cap analog the translation remained constant, possibly due to a decrease in cap-dependent initiation together with an increase on cap-independent initiation. The translation of their ApppG-capped counterparts was greatly increased when cap-dependent translation was blocked because they are translated only by cap-independent initiation mechanism. The stability of the capped and ApppG-capped mRNAs was similar. Their uncapped counterparts were also unaffected by the addition of cap-analog but the reduced stability of these mRNAs likely masked an increase in their translation. We concluded that cap recognition is not required for the translation of the mRNA containing the *rpr* 5' UTR when cap-dependent translation is blocked, although it would contribute to the translation under normal conditions.

Sequence analysis of *rpr* 5´UTR

Analysis of the 5'UTR of *rpr* showed that it does not contain sequence similarities, a complex secondary structure, short ORFs or the polypyrimidin track characteristic of some viral IRESs (Belsham and Jackson, 2000; Jackson, 2000). However, *rpr* 5´UTR is short (170 nucleotides), contains a high content of adenine (45%), and lacks an obvious secondary structure. These features are found to be common in heat shock protein mRNAs 5'UTRs which are translated in a cap-independent manner during heat shocked conditions (Schneider, 2000). Sequence comparison showed that *rpr* mRNA 5'UTR displays blocks of similarity to the 5'UTR of *heat shock protein 70* mRNA from *Drosophila melanogaster* (*Dm-hsp70*; Fig. 1.4a). This led us to consider that *rpr* and *Dm-hsp70* might share similarities in their translation mechanism.

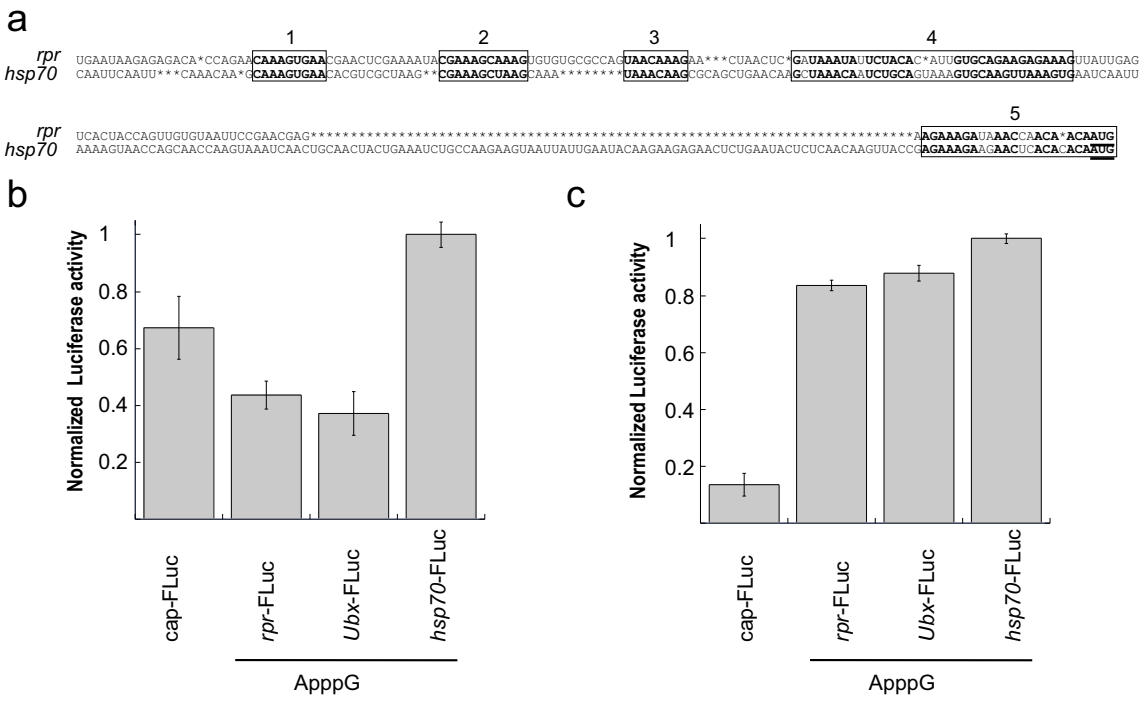


Figure 1.4. *rpr* and *Drosophila hsp70* mRNAs show similarities in their 5'UTRs and both 5'UTRs are able to support cap-independent translation in control and heat shocked extracts. (a) Sequence comparison between the 5'UTR of *Drosophila hsp70* and *rpr* mRNAs. Nucleotides highlighted in bold and in numbered squares indicate motifs of similarity. Monocistronic mRNA reporters expression in translation extracts derived from untreated **(b)** and heat-shocked **(c)** embryos. In all experiments the normalization is referred to the *hsp70*-containing reporter mRNA. Absolute values are not comparable since they were performed with different extracts.

rpr 5' UTR drives translation of the reporter in extracts prepared from heat shocked embryos

To further analyze *rpr* and *hsp70* mRNA translation, we tested the ability of different reporter mRNAs to function *in vitro* in translation extracts derived from untreated and heat shocked embryos, where cap-dependent initiation is severely impaired and translation of heat shock protein mRNAs is favored (Schneider, 2000). In extracts derived from untreated embryos, ApppG-capped *hsp70*-FLuc, *rpr*-FLuc mRNAs and *Ubx*-FLuc were efficiently translated at a level comparable to m^7GpppG-capped FLuc control (Fig. 1.4b, data normalized to *hsp70*-FLuc). In translation extracts derived from heat shocked embryos, translation of the capped FLuc control was dramatically reduced (up to 10 times), as we observed in the competition experiments and those using ApppG-capped transcripts (Fig. 1.1f and 1.3b) when compared to *hsp70*-FLuc

translation (Fig. 1.4c). At the same time, the translation efficiency of ApppG-capped *rpr*-FLuc and ApppG-capped *Ubx*-FLuc reporters increased, likely due to the availability of the translational machinery. These results were in agreement with the data presented above, which show the ability of *rpr*-FLuc to be translated in a cap-independent manner.

rpr 5' UTR drives translation of the reporter in extracts prepared from eIF4E mutant embryos

To prove that *rpr* can be translated in the *eIF4E1/2* mutant, a condition where eIF4E is absent, we performed the same *in vitro* experiments using a translation extract derived from homozygous *l(3)67Af* embryos (Fig. 1.5a). The mutant embryos were hand sorted and were homogenized in 10 µl of translation buffer and used for preparing the translation extract. Because of the low concentration of protein in mutant extracts the translation was not as efficient as in wild type extracts, and thus we compared the relative values between cap-dependent and *rpr*-dependent translation in both extracts. The absolute values were normalized to the cap-FLuc reporter.

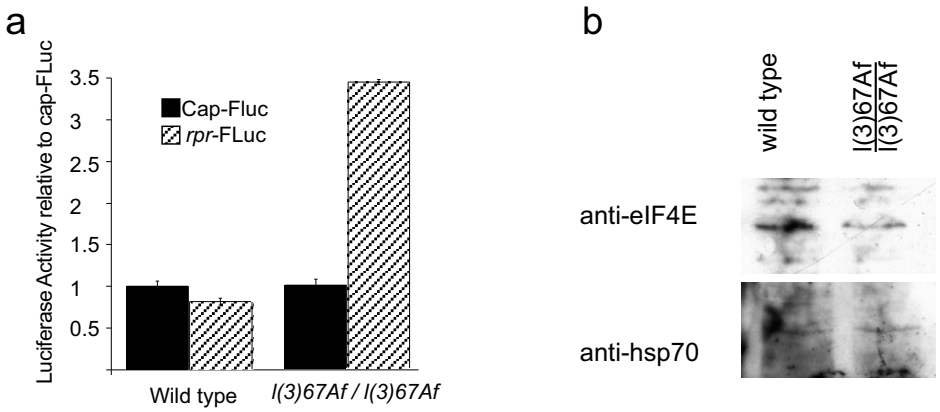


Figure 1.5. *rpr* and *hsp70* are translated in the *Drosophila* eIF4E mutants. **(a)** Translation of cap-FLuc and uncapped *rpr*-FLuc reporter transcripts in embryonic translation extracts prepared from wild-type and *l(3)67Af* homozygous embryos. The translation efficiency is normalized to cap-dependent translation. *rpr*-dependent translation was higher in extracts derived from *l(3)67Af* homozygous embryos. **(b)** Western Blot of protein extracts derived from wild type (+/+) and mutant *(l(3)67Af/l(3)67Af)* embryos. The same membrane was blotted for detection with anti-eIF4E and anti-hsp70 antibodies.

The translation level of both cap-FLuc and uncapped *rpr*-FLuc was similar in wild type extracts. However, in the mutant extracts the relative efficiency of translation of *rpr*-FLuc was 3.5 times higher than translation of the cap-FLuc control mRNA (Fig. 1.5a). This indicates that *rpr*-FLuc was more efficiently translated than a capped transcript in the absence of endogenous eIF4E in the extracts. The expression of Hsp70 protein in mutant embryos was also analyzed by Western Blots (Fig. 1.5b). While eIF4E was reduced in mutant embryos (the remaining eIF4E protein probably represents the maternal protein), Hsp70 level was not affected, as it is expected for a transcript that is able to be translated by a cap-independent mechanism.

rpr and _Dm-hsp70_ mRNA 5'UTRs display IRES activity

To further evaluate the mechanism of cap-independent translation on *rpr* mRNA, we performed *in vitro* translation assays with capped dicistronic reporter transcripts, which either lack intercistronic sequences (FLuc/RLuc, negative control) or bear the IRES of the *Ubx* mRNA (FLuc/*Ubx* /RLuc), the *rpr* 5'UTR (FLuc/*rpr*/RLuc), the *hsp70* 5´UTR (FLuc/*hsp70*/RLuc) or contain the *maternal caudal* 5´UTR (FLuc/*cad*/RLuc), in the intercistronic region (Fig. 1.6a). As expected, the presence of the *Ubx* IRES, increased the efficiency of translation of the second cistron (RLuc) by a factor of 2 (with respect to the first cistron, FLuc) when compared to the control without an IRES (Fig. 1.6b). The presence of *rpr* 5'UTR also resulted in a statistically significant 2-fold increase in the translation of the second cistron relative to the first cistron when compared to the control (Fig. 1.6b). The same effect was observed when a transcript bearing *Dm-hsp70* 5'UTR in the intercistronic region was used. On the contrary, the insertion of *rpr* 5'UTR in anti-sense orientation resulted in the reduction of the second cistron activity. The insertion of a sequence corresponding to the 5´UTR of *maternal caudal* mRNA also resulted in a reduction of the second cistron over a higher translation of the first cistron.

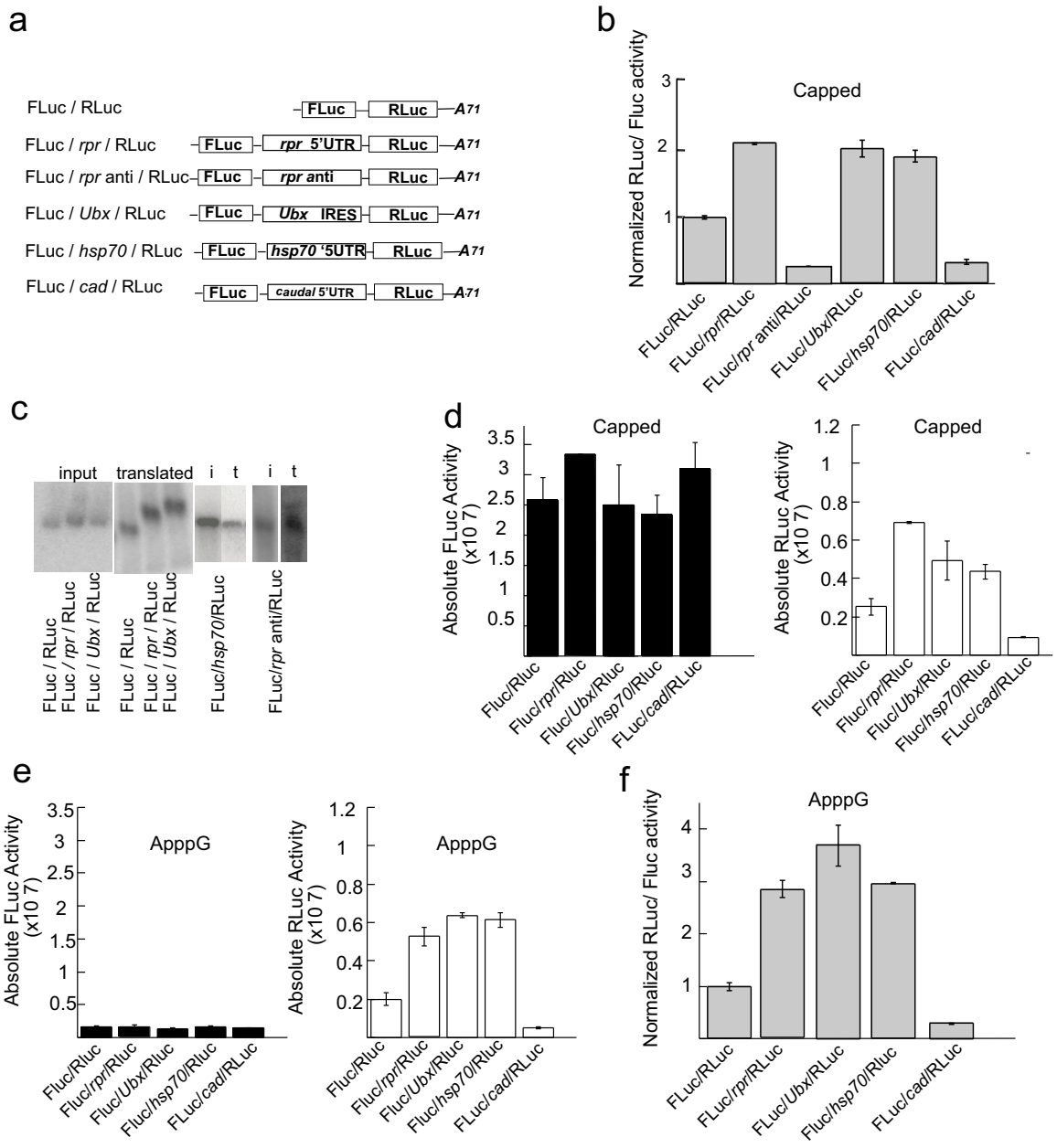


Figure 1.6. *rpr* and *Dm-hsp70* 5'UTR do contain an Internal Ribosome Entry Site that functions *in vitro*. **(a)** Reporter dicistronic mRNAs used in the *in vitro* translation assays. *rpr*-anti indicates a construction in which *rpr* 5'UTR was cloned in inverse orientation with respect to the second cistron AUG. RLuc, Renilla luciferase. **(b)** Normalization of the translation efficiency of dicistronic transcripts. The graphic shows the ratio of RLuc activity (translation of the second cistron) to FLuc activity (first cistron) normalized with respect to the same ratio in the FLuc/RLuc transcript. **(c)** Stability analysis of the dicistronic reporter mRNAs used in (b). **(d)** Absolute values of translation corresponding to the first cistron (FLuc, black bars) and the second cistron (RLuc, white bars) of the constructs indicated in (a) and (b) using m^7GpppG-capped transcripts. **(e)** Absolute values of translation corresponding to the first cistron (FLuc, black bars) and the second cistron (RLuc, white bars) of the constructs indicated in (a) using ApppG-capped transcripts. Note that the scale is the same as in (d). Absolute values are comparable as all the experiments were performed with the same lot of extracts. The activity of the first cistron decreases dramatically, while the activity of the second cistron remains either constant or increases. **(f)** Normalization of the translation efficiency of dicistronic ApppG -capped transcripts. The graphic shows the ratio of RLuc activity (translation of the second cistron) to FLuc activity (first cistron) normalized with respect to the same ratio in the FLuc/RLuc transcript. All experiments represent at least two independent determinations.

The integrity of the transcripts, assessed by the addition of labeled mRNA as tracer and evaluated after the translation reaction, was not affected within the level of detection of the method used, indicating that the translation of the second cistron was not the result of mRNA cleavage (Fig. 1.6c). The absolute values of luciferase activity showed that the relative efficiency of translation of the second cistron over the first one (Normalized to RLuc/FLuc activity) was due to a different activity of the second cistron (Fig 1.6d; RLuc, white bars) since the expression of the first cistron did not change significantly between the different reporters (Fig 1.6d; FLuc, black bars). However, we observed that the second cistron (RLuc, white bars) of the reporter FLuc/RLuc displayed a relatively high level of translation (Fig. 1.6d and 1.6b, normalized data) likely due to ribosomal read-through. Therefore, we could not rule out at this point that the relatively low, albeit significant, IRES activity performed by *rpr* 5'UTR and *hsp70* 5'UTR could have been the consequence of enhanced intercistronic read-through. This argument is relevant if we consider that both *rpr* and *hsp70* 5'UTRs are short and do not have any potential secondary structure. The use of ApppG-capped dicistronic reporter transcripts showed that while the activity of the first cistron was stopped (Fig 1.6e; FLuc, black bars) the second cistron (Fig 1.6e; RLuc, white bars) was still translated (Fig. 1.6e, compare to Fig. 1.6d). Thus, the efficiency of translation of the second cistron over the first cistron compared with the FLuc/RLuc control increased for the dicistronic reporters bearing *rpr*, *Ubx* and *hsp70* 5´ UTR, and not for the reporter containing the *maternal caudal* 5´UTR (Fig. 1.6f).

To address the problem of read-through activity we assayed IRES activity using the dicistronic reporter vector pFLuc/*cad*/RLuc, which contains the 5'UTR of the *Drosophila* maternal *caudal* (*cad*) mRNA downstream of the first cistron (Fig. 1.7a). We had observed that *cad* 5'UTR is not an IRES (Fig 1.6), possesses a significant secondary structure and thus efficiently prevents read-through probably in the same way as observed for hairpins or inactive IRESs. The insertion of the *Ubx* 5'UTR in the reporter FLuc/*cad*/*Ubx* /RLuc resulted in a 12-fold relative increase of the second cistron translation with respect to the control mRNA FLuc/*cad*/RLuc, likely due to the consequence of the decrease in ribosomal read-through in the control RNA (Fig. 1.7b and 1.7c). The analysis of the reporter FLuc/cad/*rpr*/RLuc also showed that the second cistron was efficiently translated with a 14-fold increase relative to the control FLuc/*cad*/RLuc and equivalent to that for FLuc/*cad*/*Ubx* /RLuc (Fig. 1.7b and 1.7c).

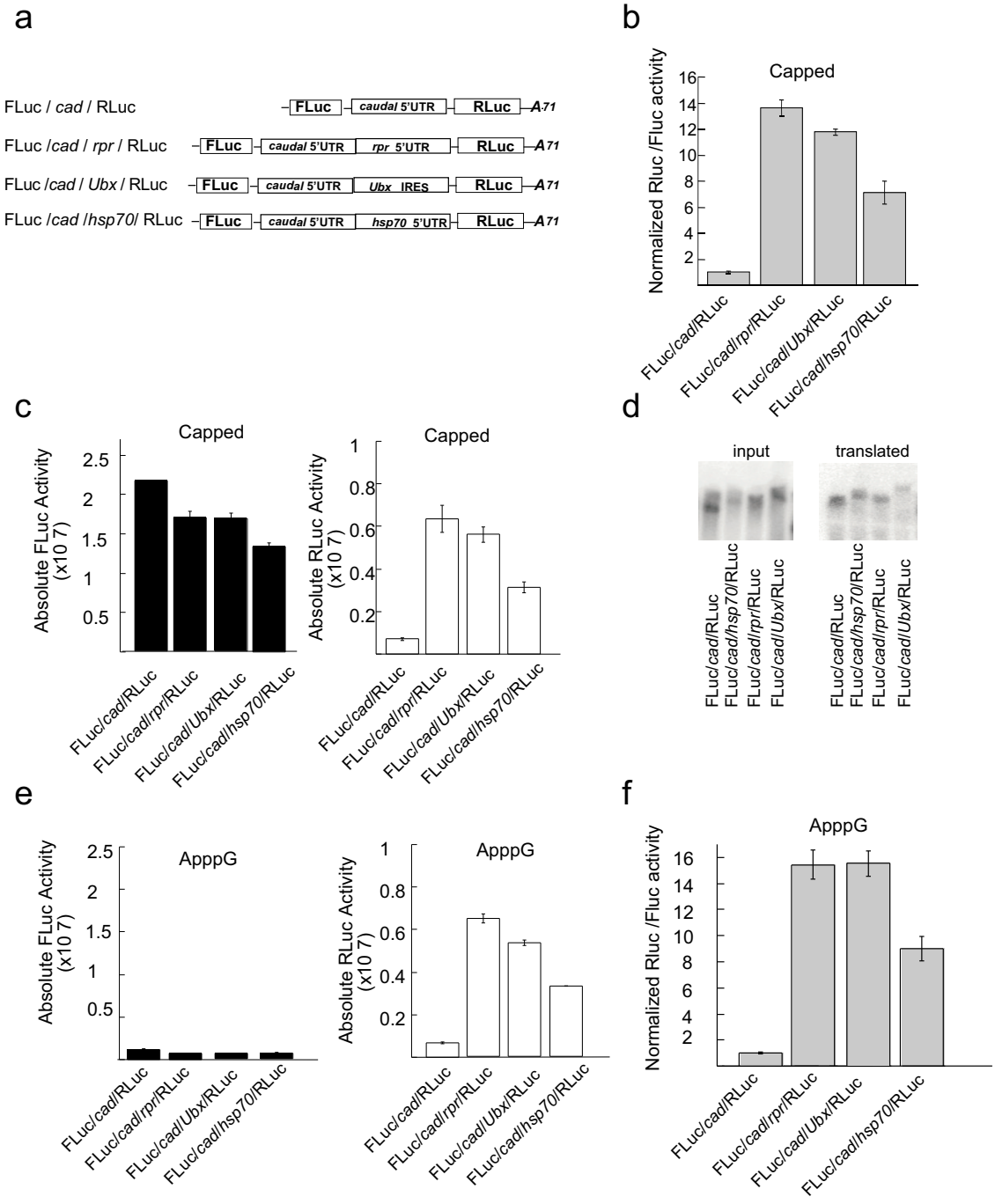


Figure 1.7. *rpr* and *Dm-hsp70* contain a stronger IRES when analyzed in a dicistronic reporter that has less leaky activity of the second cistron. **(a)** Reporter dicistronic mRNAs containing the 5'UTR of *caudal* to prevent read-through of ribosomes. **(b)** Translation efficiency (ratio of RLuc to FLuc activities) of m^7GpppG-capped dicistronic transcripts depicted in (a) is normalized to the ratio derived from the use of FLuc/*cad*/RLuc reporter RNA. **(c)** Absolute values of translation corresponding to the first cistron (FLuc, black bars) and the second cistron (RLuc, white bars) of the constructs indicated in (a) and (b) using m^7GpppG-capped transcript. **(d)** Stability and integrity analysis of the dicistronic reporter mRNAs used in (b) and (c). **(e)** Absolute values of translation corresponding to the first cistron (FLuc, black bars) and the second cistron (RLuc, white bars) of the constructs indicated in (a) but using ApppG-capped transcript. Note that the scale is the same as in (c). Absolute values are comparable as all the experiments were performed with the same lot of extracts. The activity of the first cistron decreased dramatically, while the activity of the second cistron remained either constant or increases. **(f)** Translation efficiency (ratio of RLuc to FLuc activities) of ApppG-capped dicistronic transcripts depicted in (a) and used in (e) normalized to the ratio derived from the use of FLuc/*cad*/RLuc reporter RNA.

The increase of IRES activity was also evident for the insertion of *hsp70* 5'UTR sequence (Fig. 1.7b and 1.7c). In all these cases, the integrity of the reporter mRNAs was unaltered during the translation reaction (Fig. 1.7d). By using ApppG-capped dicistronic transcripts the activity of the first cistron was stopped but the second cistron was still translated (Fig. 1.7e, compare to Fig. 1.7c). The translation efficiency of the second cistron over the first one was the same as with capped-dicistronic transcripts (Fig. 1.7f compared with Fig. 1.7b). Although we established that blocking the read-through improved the detection of the IRES activity of *rpr* and *Dm-hsp70* 5'UTRs, we could not rule out that sequences within *cad* 5'UTR, representing a functional 5' UTR (and translation factors potentially binding therein), could influence the activity of *rpr* and *hsp70* 5'UTRs. To exclude the effect of *cad* 5'UTR on the activity of the second cistron, we inserted a synthetic hairpin of high stability (Coldwell *et al.*, 2001) to prevent read through (Fig. 1.8a).

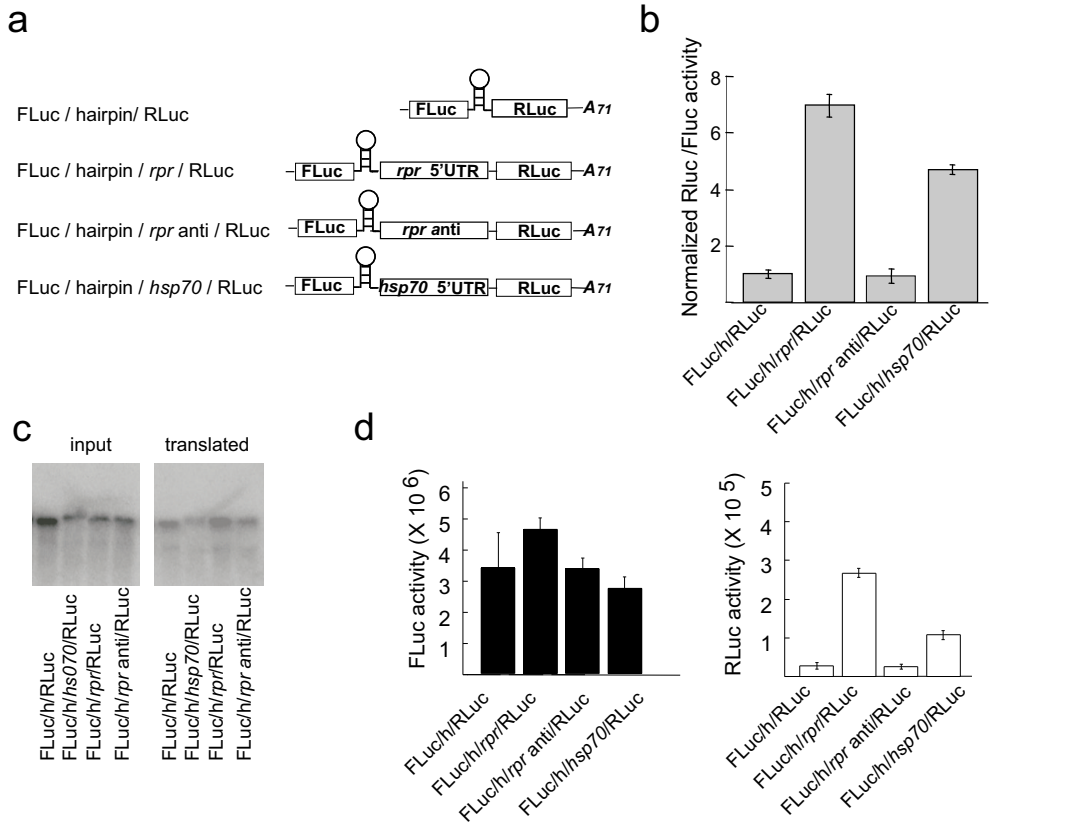


Figure 1.8. *rpr* and *hsp70* 5'UTR showed IRES activity in a vector containing a hairpin that efficiently prevents read-through. (a) Reporter dicistronic mRNAs containing a synthetic, stable hairpin (h) that prevents read-through of ribosomes. **(b)** Translation efficiency of dicistronic transcripts depicted in (a) normalized to the FLuc/hairpin/RLuc. **(c)** Stability analysis of the dicistronic reporter mRNAs used in (b). **(d)** Absolute values of translation corresponding to the first cistron (FLuc, black bars) and the second cistron (RLuc, white bars) of the constructs indicated in (a).

The results of the *in vitro* translation activity and mRNA stability experiment, including a control of the *rpr* 5'UTR inserted in antisense orientation, confirmed the data obtained using the *cad*-based reporter mRNA and strongly indicated that *rpr* and *hsp70* 5'UTRs display IRES activity (Fig. 1.8b, c and d).

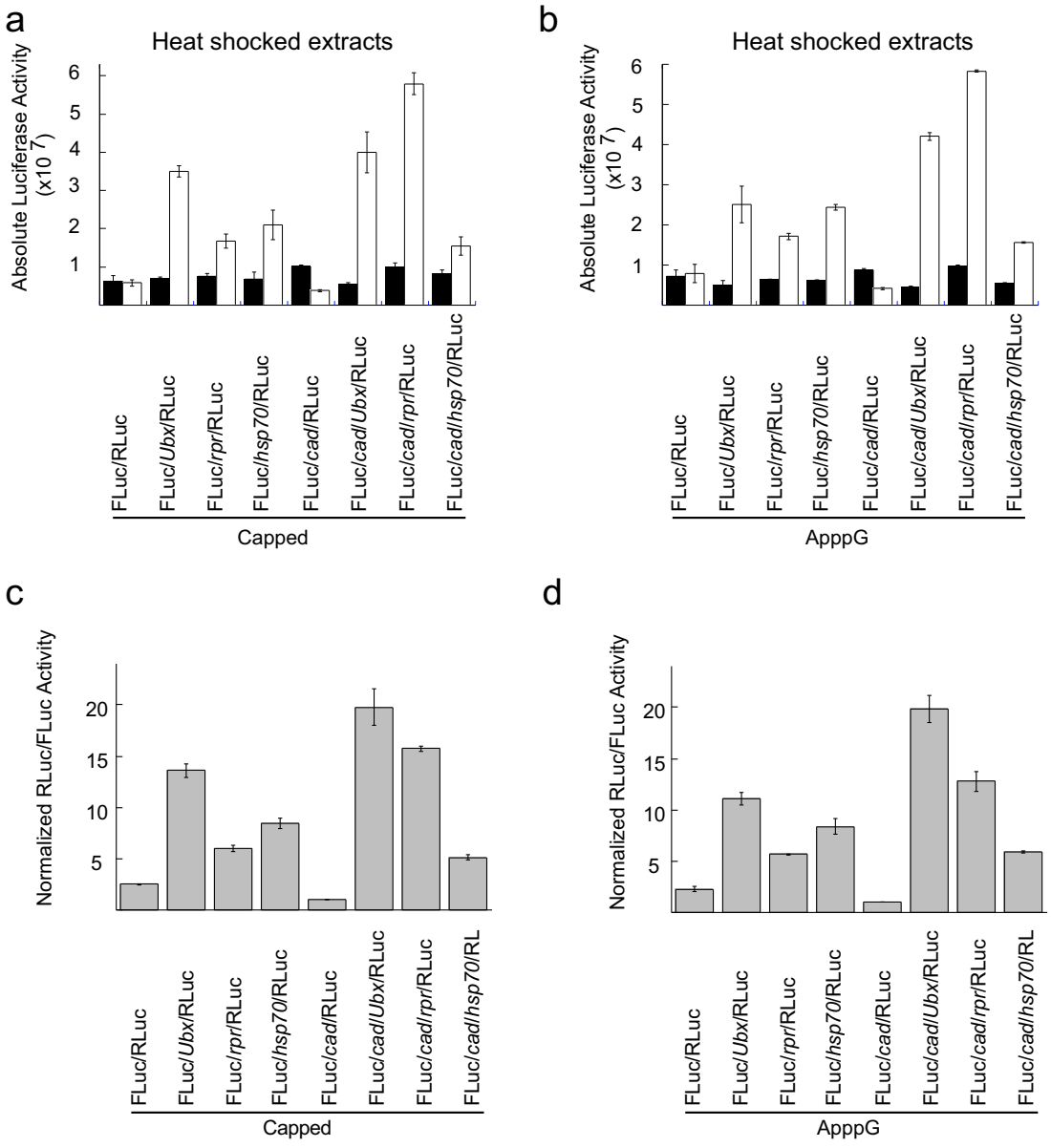


Figure 1.9. *rpr* and *Dm-hsp70* 5'UTR IRES are functional during heat shock. (a) Absolute values of reporter gene expression activity of m^7GpppG-capped transcripts in heat shock extracts. Cap-dependent translation is abolished (first cistron, FLuc, black bars). Translation of the second cistron is higher (RLuc, white bars). **(b)** Absolute values of reporter gene expression activity of ApppG-capped transcripts in heat shocked extracts. (FLuc, black bars; RLuc, white bars). Normalized activity of capped **(c)** and ApppG-capped **(d)** dicistronic mRNA reporters in translation extracts derived from heat-shocked embryos. The graphics shows the ratio of RLuc activity to FLuc activity normalized to the same ratio in the FLuc/*cad*/RLuc transcript.

We next assayed the ability of the different 5'UTRs to support translation of the second cistron in dicistronic reporter mRNAs containing either a functional cap (Fig. 1.9 a,c) or the non-functional cap analog ApppG (Fig. 1.9 b,d) in heat-shocked extracts. A summary of the absolute values for the first and second cistron for all dicistronic mRNAs used is summarized in Fig. 1.9 a,b. Using either set of transcripts the translation of the first cistron was abolished while the second cistron was still translated. The efficiency of translation of the second cistron over the first cistron was significantly higher than the corresponding control vectors used (either FLuc/RLuc, or FLuc/*cad*/RLuc, Fig 1.9 c,d) and similar to the translational efficiency ratios obtained in control conditions. These data demonstrated that the 5'UTR of *rpr* and *Dm-hsp70* mRNAs support IRES-dependent translation also in conditions of heat shock where cap dependent translation is blocked. All the analysis of IRES activity in the different dicistronic constructs used, indicated that *rpr* and *Dm-hsp70* mRNAs are translated in a similar cap-independent fashion, and that both 5'UTRs support IRES activity in control and heat shocked conditions.

To validate our observations *in vivo,* we proceeded to study reporter mRNAs in transfected *Drosophila* S2 cells with the same transcripts used in the *in vitro* experiment shown in Fig 1.6a. We observed that m^7GpppG-capped dicistronic reporter mRNAs bearing either *rpr* or *Dm-hsp70* 5'UTRs display IRES activity as described for the *in vitro* translation assays (Fig. 1.10a, compare with Fig. 1.6b). Northern Blot experiments showed that the transcript were stable and remained intact after the whole experiment (Fig. 1.6b). We finally analyzed the translation of several dicistronic reporter plasmids in DNA transfected S2 cells. The reporter used contains the minimal *Adh* promoter under control of five UAS sites, which is activated by Gal4, and two reporter cistrons: myc-CFP (myc-tagged Cyan Fluorescent Protein, first cistron) and HA-EYFP-NLS (haemaglutinin-tagged enhanced Yellow Fluorescent Protein bearing a nuclear localization signal, second cistron; Fig. 1.10c). Co-transfection of *Drosophila* S2 cells with pActin-Gal4 and either the dicistronic reporter gene without intercistronic sequences or bearing the *rpr* 5'UTR was performed (Fig. 1.10d). The CFP (first cistron) was expressed in both cells transfected with the control plasmid and cells transfected with the *rpr*-containing construct, while EYFP (second cistron) was only detected in the nuclei of cells that contain the *rpr* 5'UTR upstream of the second cistron (Fig. 1.10d). The score of the cells displaying CFP expression showed that none of them expressed EYFP in the absence of *rpr*

5'UTR sequences, but 80% showed nuclear EYFP when *rpr* 5'UTR sequences were inserted. Although we considered that we had demonstrated the presence of an IRES transfecting dicistronic reporter RNA, we had to confirm that the activity determined using the reporter plasmid was not the result of cryptic promoters within *rpr* 5'UTR.

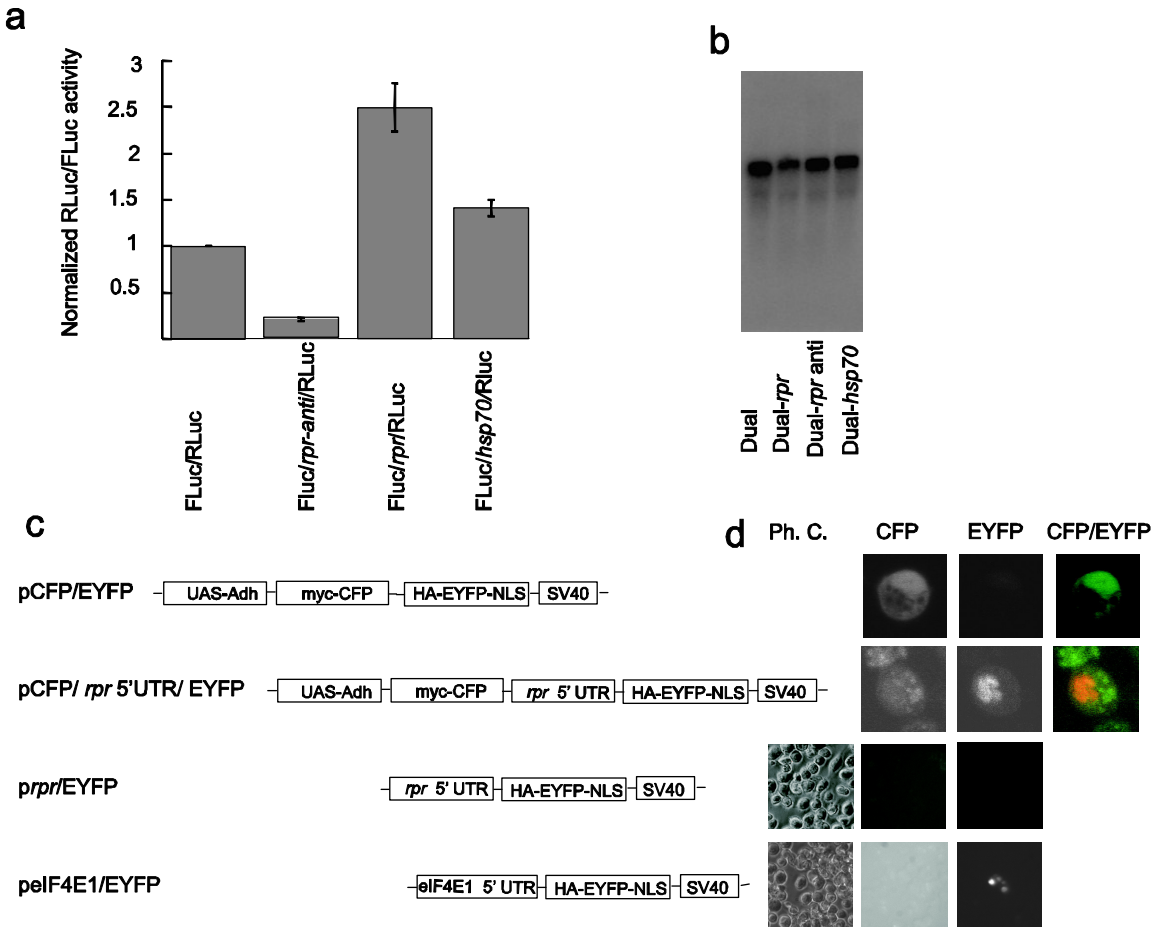


Figure 1.10. ***In vivo*** **analysis of** ***rpr*** **and Dm-*hsp70* IRES. (a)** Relative translation activity of dicistronic reporter gene expression in S2 cells transfected with the m^7GpppG-capped transcripts depicted in Fig.1.6a. **(b)** Northern Blot experiments on transfected S2 cells show that the transcripts are stable after 6 hours incubation. There was no sign of degradation. **(c)** Dicistronic plasmids containing the Cyan Fluorescent Protein ORF (myc-CFP, first cistron, cytoplasmic) and the enhanced Yellow Fluorescent Protein ORF (HA-EYFP-NLS, second cistron, nuclear), under the Gal4-dependent UAS-Adh promoter. SV40, the SV40 t-antigen 3'UTR and polyadenilation signal. **(d)** *Drosophila* S2 cells co-transfected with the plasmids depicted in (c) and *actin*-Gal4, which activates the UAS-Adh promoter. Merged figure displays digitalized colors, CFP (green) and EYFP (red). The panels correspond to the construct used for transfection. For detail see text.

In the absence of the Gal4 we observed some activity of both cistrons indicating a leaky activity of the Adh-UAS promoter in S2 cells. Thus, this strategy was not useful to check for the presence of cryptic promoter in the *rpr* 5' UTR. We

then decided to clone the *rpr* 5'UTR in a promoterless vector and co-transfected the cells with pActinGal4. None of the cells transfected with the p*rpr*/EYFP plasmid expressed EYFP (Fig. 1.10d), while a control bearing the 5'UTR of an alternatively spliced isoform of *Drosophila eIF4E-1* mRNA, which we found to contain a cryptic promoter, indeed expressed EYFP (Fig. 1.10d). We concluded that the entire set of *in vitro* and *in vivo* experiments, designed to assess translation of a variety of reporter mRNAs, its stability and the absence of cryptic promoters, provided clear evidence of IRES activity in the *rpr* and *Dm-hsp70* 5'UTRs.

***rpr* and *hsp70* mRNAs are associated to polysomes after heat-shock and apoptosis induction in embryos.**

To obtain definitive evidence of the ability of the endogenous *rpr* and *hsp70* mRNAs to be translated *in vivo*, during a situation where cap-dependent translation is impaired, we separated ribosomal fractions from translation extracts derived from 0–12 h old *Drosophila* embryos under normal conditions of development or after either heat shock or irradiation with X-rays, which induce the transcription of *hsp70* mRNA and *rpr* mRNA, respectively. The heat-shocked embryos mimicked the absence of eIF4E in the *l(3)67Af* homozygous mutant and we used them for polysome analysis because the acquisition of enough staged homozygous mutant embryos by hand sorting was unworkable. By ultracentrifugation in sucrose gradients, we isolated mRNAs associated with two well-defined ribonucleoprotein fractions: 1- initiation complexes (43S, 48S, 80S), which correspond to untranslated or just initiated mRNAs (U in Fig. 1.11a), and 2- the polysomal fraction that contained mRNAs being efficiently translated into proteins (P in Fig. 1.11a). When compared to untreated embryos (Fig. 1.11a, left panel), the sucrose profile of heat-shocked embryos (Fig. 1.11a, middle panel) showed a sharp decrease of polysomes, reflecting the inhibition of translation that occurs during heat shock. A reduction in the amount of polysomes was also observed in sucrose profiles derived from X-ray irradiated embryos (Fig. 1.11a, right panel). The effect of irradiation was less dramatic than the effect of heat shock and might have reflected a smaller fraction of cells damaged after irradiation compared to the number of cells responding to heat shock.

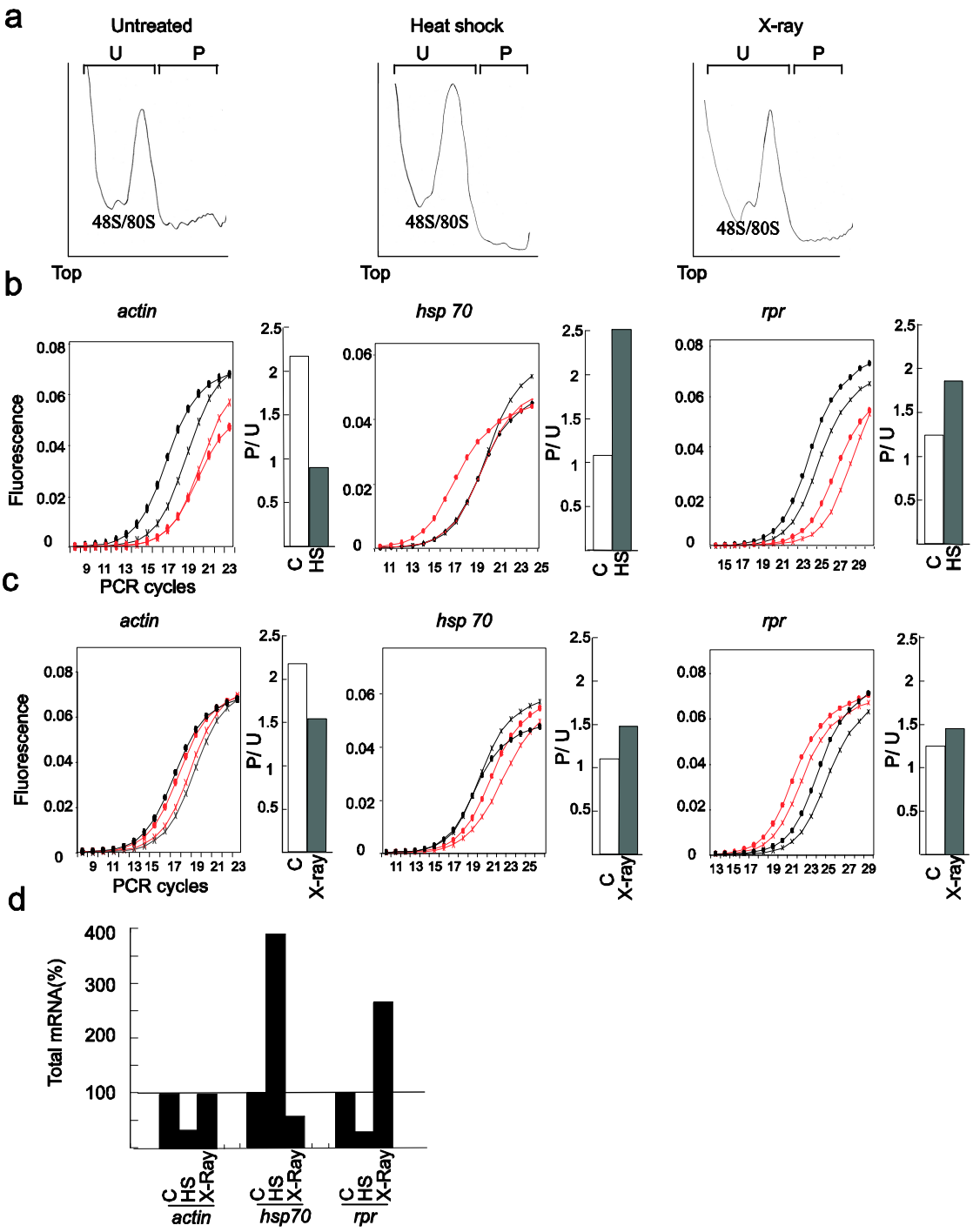


Figure 1.11. Recruitment of *actin5C*, *hsp70,* and *rpr* mRNAs to polysomes during heat shock and apoptosis in *Drosophila* embryos. (a) Sedimentation profiles in sucrose gradients from untreated (left), heat-shocked (middle) or X-ray-irradiated (right) *Drosophila* embryos. The position of ribosomal complexes 48S and 80S is indicated. (U), fraction containing untranslated/initiated mRNAs. (P), polysomal fraction. **(b)** Amplification of *actin5C*, *hsp70,* and *reaper* mRNAs in U and P fractions by real-time quantitative-RT-PCR experiments from heat-shocked embryos. **(c)** Same experiment as in (b) conducted X-ray-irradiated embryos. The amplification course shows the mRNA present in the control (black lines) or treated embryos (red lines) either in the fraction P (closed circles) or U (crosses). The insets represent the P/U ratio in the control (white bars) or treated (grey bars) embryos for each mRNA tested. **(d)** Comparison of the total amounts of mRNAs from the experiments performed in (b) and (c). The amount of mRNA was calculated as P + U and normalized to untreated embryos.

We then studied the presence of the transcripts corresponding to *Actin5C* (a cap-dependent transcript; Fig. 1.11b, left panel), *hsp70* (Fig. 1.11b, middle panel), and *rpr* (Fig. 1.11b, right panel) mRNAs in both untranslated and polysome-associated fractions derived from untreated and heat-shocked embryos. The fraction of polysome-associated mRNAs with respect to the untranslated ones provides an indication of their level of translation (P/U in Fig. 1.11c,d insets). *Actin5C* mRNA (Fig. 1.11b, left panel) is actively translated under normal conditions as indicated by a P/U ratio of 2, while a ratio of 1 was observed for *hsp70* mRNA (Fig. 1.11b, middle panel) and *rpr* mRNA (Fig. 1.11b, right panel). This implies that all three mRNAs were translated, although *hsp70* and *rpr* mRNAs were present in lower amount than *Actin5C*. After heat shock treatment (Fig. 1.11b, red lines), we observed an increase of the total amount of *hsp70* mRNA and a decrease in the levels of *Actin5C* and *rpr* mRNAs relative to untreated embryos (Fig. 1.11d). However, a major change was evident: the total amount of *Actin5C* mRNA associated with polysomes decreased (P/U < 1), while the ratio of polysome-associated *rpr* mRNA remained stable and that of *hsp70* mRNA increased (Fig. 1.11b). Our data demonstrate that during heat shock the translation of a cap-dependent mRNA decreases, while both *rpr* and *hsp70* mRNAs are still actively translated, irrespective of their relative level of transcription.

It has been shown that irradiation with X-rays induces the transcription of *rpr* mRNA in embryos and, in consequence, apoptosis, and that the induction of apoptosis results in impaired translation (Clemens *et al.*, 2000). Thus, we conducted an equivalent experiment using translation extracts derived from X-ray-irradiated *Drosophila* embryos to provide evidence for the ability of *rpr* to be translated during apoptosis induction (Fig. 1.11c). As expected, the overall transcription of *rpr* mRNA increased (Fig. 1.11d), no major changes were observed in the levels of *Actin5C* mRNA, while transcription of *hsp70* was reduced. However, the association of *Actin5C* mRNA to polysomes decreased (P/U=1.5; Fig. 1.11c, left panel) and the level of *hsp70* and *rpr* mRNAs associated with polysomes increased slightly (P/U=1.5; Fig. 1.11c, middle and right panels). Although the results were less dramatic than the ones observed in heat-shocked embryos, perhaps due to the number of cells effectively undergoing apoptosis, cap-dependent translation decreased whereas cap-independent initiation was maintained or was even slightly elevated. Our data indicate that during apoptosis mRNAs are poorly translated reflecting a lower initiation rate, and suggest that cap-dependent but not cap-independent translation is preferentially impaired. The

increase of *rpr* mRNA associated with polysomes indicates that *rpr* mRNA is translated *in vivo* under conditions of diminished cap-dependent initiation both through heat shock or irradiation. The higher proportion of *hsp70* and *rpr* mRNAs in polysomes can be attributed to the increased availability of the basic translation machinery upon inhibition of cap-dependent translation, eventually resulting in an important increase of the absolute mass of *HSP70* and *RPR* protein synthesized during heat shock and apoptosis, respectively.

Role of the sequence regions shared by *rpr* and *hsp70* 5'UTR for cap-independent translation

Nothing is known about the proteins involved in the translation control of the first IRES described in *Drosophila,* the *Ubx* and the *Antp* IRES. The same is true for *rpr* and *hsp70* IRES. To study the possible existence of specific factors required for *hsp70*, *rpr* and *Ubx* IRES to drive translation, we performed competition experiments by using free 5´UTRs and analyzing the translation of several 5´UTR-FLuc reporters (Fig. 1.12). Cap-dependent translation (Cap-FLuc) was reduced by the addition of a 10-fold excess of either *rpr*, or *hsp70* or *Ubx* 5´UTRs (Fig. 1.12a, left panel). However, the addition of *rpr* 5´UTR but neither *hsp70* 5' UTR nor *Ubx* 5´UTRs reduced the translation of *rpr*-FLuc. The same result was observed for the translation of *hsp70*-FLuc and *Ubx*-FLuc transcripts. Their translation was only reduced in the presence of their corresponding free 5´ UTRs. This observation raises the hypothesis that translation driven by *rpr*, *Ubx* and *hsp70* 5´UTR use factors needed by cap-dependent translation but these factors are not shared between them. The same competition experiment was carried out using heat shocked extracts (Fig.1.12a, right panel). In this case the addition of every free 5´ UTR blocked either cap-dependent (the residual one) as well as cap-independent translation driven by the mentioned IRES. This result is in agreement with a limited amount of functional translation machinery during heat shock conditions and with an involvement of part of the canonical translation factors on IRES dependent translation of *Ubx, rpr* and *hsp70* mRNAs.

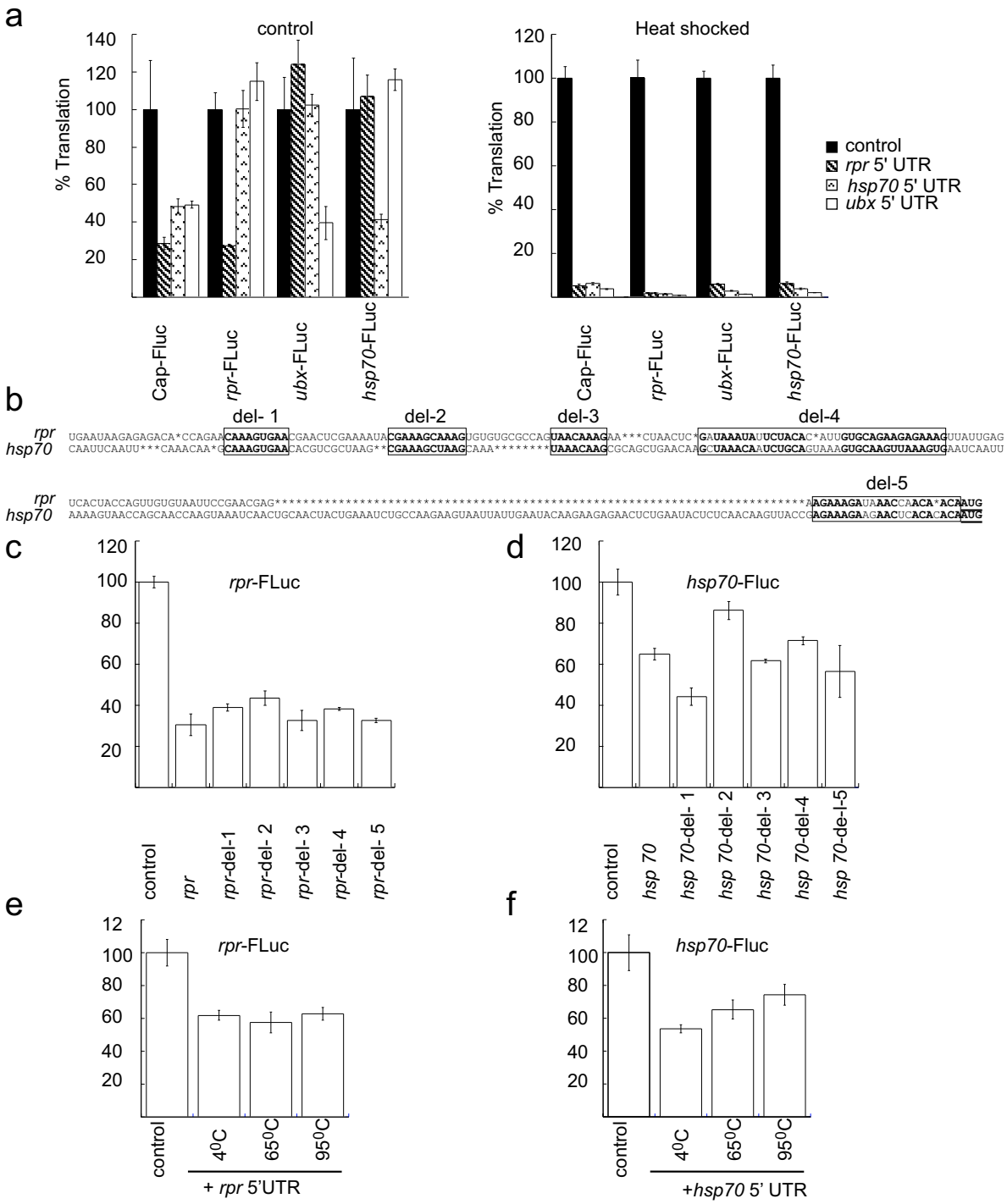


Figure 1.12. Specific competition experiments with free 5' UTR. (a) Translation efficiency of Cap-FLuc, *rpr*-FLuc, *ubx*-FLuc and *hsp70*-FLuc transcripts upon addition of 10 molar excess of free *rpr*, *hsp70* or *ubx* 5' UTRs. 100% corresponds to the value of translation of each reporter in the absence of competitor 5' UTR. The experiment was performed using untreated *Drosophila* translation extract (left panel) or heat shock treated extracts (right panel). **(b)** *rpr* and *hsp70* 5' UTRs sequence alignment showing the deleted sequence blocks (del). These deletion were used in competition experiment shown in (c) and (d). **(c)** Translation efficiency of *rpr*-FLuc transcript competed with the addition of 10 molar excess of free wild type and deleted *rpr* 5' UTR. **(d)** Translation efficiency of *hsp70*-FLuc transcript competed with the addition of 10 molar excess of free wild type and deletion mutants of *hsp70* 5' UTR. **(e)** Translation efficiency of *rpr*-FLuc transcript competed with the addition of free wild type *rpr* 5' UTR preincubated at the indicated temperatures. **(f)** Translation efficiency of *hsp70*-FLuc transcript competed with the addition of free *hsp70* 5' UTR preincubated at the indicated temperatures.

We have shown before that *rpr* mRNA 5'UTR shares blocks of similarity with the 5'UTR of *Drosophila hsp70* mRNA (Fig. 1.4a). In order to investigate the importance of these sequence blocks on *hsp70* and *rpr* translation we performed competition experiments.

Translation of *rpr*-FLuc and *hsp70*-FLuc reporters was competed by the addition of their corresponding 5´UTRs bearing deletions on the homology sequence blocks (Fig 1.12c,d). The deletion constructs are depicted in Fig. 1.12b. All the deleted constructs competed efficiently the translation driven by their 5´UTR-FLuc counterparts. The del-2 and del-4 mutants consistently showed a significantly lower efficiency in blocking the translation of *rpr*-FLuc and *hsp70*-FLuc reporters (Fig 1.12c,d). To check if secondary structure might be necessary for the translation of *rpr* and *hsp70* mRNAs, the free 5´UTRs were denatured by heating to 95^{o}C prior to the addition to the *in vitro* translation reaction (Fig. 1.12e,f). Under these conditions there was no difference on the repression of translation of *rpr*-FLuc and *hsp70*-FLuc by the free 5´UTRs. Taken together these observations lead to the conclusion that the homology blocks are likely dispensable or redundant and that secondary structure does not play any role for translation of *rpr* and *hsp70* mRNAs.

Discussion

The mutant *l(3)67Af* (Leicht and Bonner, 1988) is a null allele of the *Drosophila eIF4E1-2* gene (Hernandez *et al*., unpublished). *l(3)67Af* is recessive embryonic lethal which shows up-regulation of the proapoptotic gene *rpr* and widespread induction of apoptosis. *rpr* was also found to be a direct transcriptional target of *Drosophila*-p53 following irradiation DNA damage (Brodsky *et al.*, 2000). Mammalian p53 is a tumor suppressor gene that functions as a transcription factor by controlling expression of genes that affect the cell cycle, induce DNA repair or regulate apoptosis. However, it was recently reported that overexpression of p53 causes a decrease in translation initiation that was associated with a dephosphorylation of 4E-BP1, an increase in the association of eIF4E with 4E-BP1 and a decreased association of eIF4E with eIF4G (Horton *et al.*, 2002), a condition that is known to impair cap-dependent translation. In both situations, after induced transcription, *rpr* mRNA must escape translation inhibition in order to exert its apoptotic function. Here, we demonstrated that *rpr* can be translated in the absence of cap-binding activity *in vitro*

by using translation extracts derived from *eIF4E* mutant or heat shocked embryos or by competition with a cap-analog. We revealed that *rpr* and *hsp70* mRNAs, two antagonizing genes induced in response to stress, are translated in an IRES-dependent manner *in vitro* and that their endogenous mRNAs are associated with polysomes *in vivo* in conditions of diminished cap-dependent translation mediated by stress. Our experiments are consistent with the observation of Joshi-Barve *et al*. (Joshi-Barve *et al.*, 1992) that *hsp70* but not *actin* mRNA is associated to polysomes when cap-binding activity is reduced in HeLa cells by antisense eIF4E RNA, which we now extend to the translation of those genes during stress and apoptosis.

IRESs have been identified in some cellular mRNAs that encode apoptotic regulators such as XIAP, Bag-1, Bip, and Apaf-1 (Holcik *et al.*, 2000). Thus, IRES-dependent translation is a common theme during apoptosis. The resemblance and similar behavior of *rpr* and *Dm-hsp70* mRNA 5'UTRs correlates with the ability of both *rpr* and *Dm-hsp70* mRNAs to be translated under heat shock and X-ray irradiation conditions, as our polysome analysis showed. A very recent finding suggests that human-*hsp70* mRNA might also support IRES activity (Rubtsova *et al.*, 2003), in contradiction to previously published evidence (Vivinus *et al.*, 2001). IRES capability of *human-hsp70* 5'UTR remains a matter of controversy and evidence has been provided suggesting that *human-hsp70* mRNA also uses a shunting mechanism (Yueh and Schneider, 2000). The mechanism of shunting demonstrated by Yueh and Schneider (Yueh and Schneider, 2000) for the translation of human-*hsp70* mRNA involves the pairing of sequences within the 5'UTR and the 18S rRNA. In agreement with the observation of Yueh and Schneider (Yueh and Schneider, 2000) for *Dm-hsp70* 5'UTR, we could not find any pairing possibility between *rpr,* or *Dm-hsp70* 5'UTR mRNAs to *Drosophila* 18S rRNA, nor did the similarity we observed between *rpr* and *Dm-hsp70* does extend to human-*hsp 70* mRNA, indicating that the shunting mechanism established for human-*hsp70* mRNA might not necessarily apply to the *Drosophila* homolog. The evidence presented here shows that both *Dm-hsp70* and *rpr* mRNAs are indeed translated in an IRES dependent manner and that their 5'UTRs show sequence similarity, although most of the homology blocks between *rpr* and *hsp70* 5'UTR seem to be dispensable or redundant for reporter mRNA translation in a cap-independent context.

Discrepancy with respect to the existence and nature of cellular IRESs has recently arisen (Kozak, 2001a; Kozak, 2001b; Schneider *et al.*, 2001). A positive

consequence of this debate has been that, while IRESs do indeed exist, the experimental standards required to prove their activity have increased. We consider that we have fulfilled them in this study. Canonical IRES, namely the 5'UTR of certain viruses, implies a complex RNA secondary structure, some times the presence of several short ORFs and a polypyrimidine track (Belsham and Jackson, 2000). Cellular IRESs are less characterized and show enormous heterogeneity (Jackson, 2000). In this context, the IRESs of *rpr* and *Dm-hsp70* appear to be unusual, suggesting that rather than being defined structures, IRESs may constitute the intrinsic activity of certain mRNAs. Accordingly, the definition of IRES can be very broad and implies a variety of mechanisms, including shunting. IRESs such as the ones described in this study may reflect the selective advantage of an unstructured 5'UTR with the ability to direct the passive recruitment of ribosomes without the requirement of other factors such as the helicase eIF4A and, possibly, without scanning. Our experiments using translation extracts from heat-shocked embryos indicate that the IRESs of *rpr* and *Dm-hsp70* are indeed translated in an eIF4F-independent manner. This mechanism might be advantageous under stress conditions where the amount of eIF4F complex decreases and the availability of idle ribosomes increase.

HSP70 and *RPR* act via different pathways in response to stress (Abrams, 1999; Schneider, 2000). Heat shock treatment does not result in increased apoptosis, likely due to the counter-acting role of HSP70, which is produced at higher rates. HSP70 blocks apoptosis by binding to Apaf-1 (Saleh *et al.*, 2000; Beere and Green, 2001) and to the apoptosis-inducing factor AIF (Ravagnan *et al.*, 2001). Thus, a simple mechanism of translation would allow *Dm-hsp70* mRNA to escape the repression of translation by caspase-mediated cleavage of translation factors, at least during the initial steps of apoptosis. It is a challenging idea that at least in *Drosophila*, in which *rpr* and other proapoptotic genes have been identified, early apoptosis genes may be translated in a similar manner as *heat shock protein* mRNAs. The hypothesis that heat shock response and apoptosis evolved in a similar context using common molecular mechanisms during cellular stress has recently been put forward (Beere and Green, 2001). Here, we provide evidence supporting the idea of a shared mechanism of protein synthesis in genes involved in different developmental processes, one protecting and the other one eliminating cells at risk.

Chapter 2

Mechanism of translation of *Drosophila hid*, *grim* and *sickle* proapoptotic genes

As we have previously mentioned, *eIF4E* mutant embryos die in early stages of embryogenesis due likely to widespread apoptosis. In this mutant, upregulated and widespread transcription of *rpr* mRNA was observed. Based on these data we have investigated and proved that *rpr* mRNA is translated in an IRES-dependent mechanism. This can account for its translation in the absence of the cap binding activity in the *eIF4E* mutant (Chapter 1). We thus asked here whether the early proapototic genes, *grim, hid* and *sickle* play a role on triggering cell death in *eIF4E* mutant embryos, and whether they share a common mechanism of translation initiation.

hid and *sickle,* but not *grim,* are upregulated in the *eIF4E* mutant

We analyzed the mRNA expression of *hid, grim* and *sickle* genes by *in situ* hybridization in *eIF4E* mutant embryos. To identify homozygous embryos, the mutant chromosome was balanced with *TM3*, *Actin-GFP* chromosome. Double *in situ* hybridization using the GFP probe and specific probes were used to detect the expression of the proapoptotic genes in mutant embryos (Fig. 2.1b). Wild type and heterozygous embryos were distinguished from mutant homozygous embryos by *in situ* hybridization using a GFP probe (embryos without GFP signal are homozygous for the *eIF4E* mutation) (Fig. 2.1a). As it has been observed for *rpr* (Chapter 1), *hid* and, in lesser extent, *sickle* mRNA were up-regulated in the homozygous eIF4E mutant embryos. The expression of *grim* mRNA did not seem to be affected in mutant embryos. As was mentioned in the introduction, activation of the transcription of *Drosophila* proapoptotic genes varies between different development stages and/ or in different cell types and in response to different apoptotic stimuli. It is not surprising that the absence of eIF4E differentially triggers the transcription of the proapoptotic genes.

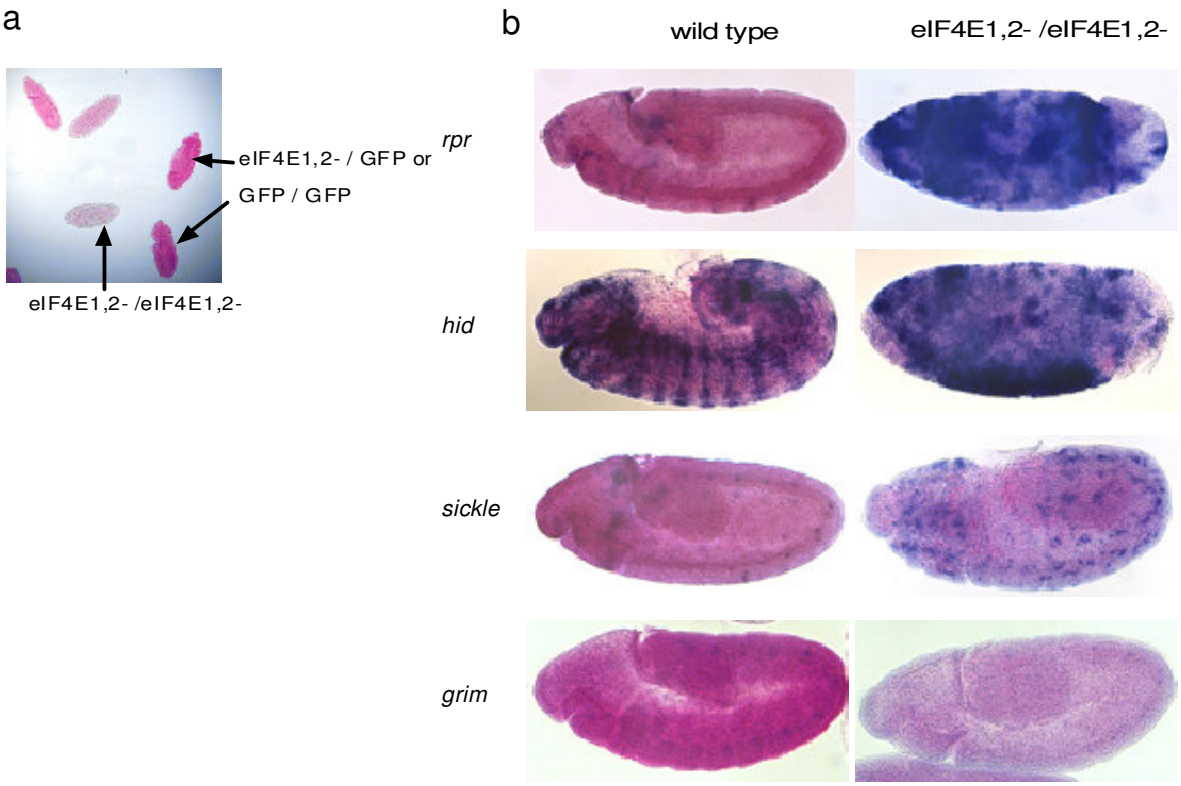


Figure 2.1. Expression of proapoptotic mRNAs in *eIF4E* mutant embryos. (a) The chromosome bearing the mutation on *eIF4E* was balanced with a chromosome marked by a transgene *Actin-GFP*. Homozygous and heterozygous embryos for GFP are distinguished from the homozygous mutant embryos by *in situ* hybridization using an antisense GFP probe (red staining). **(b)** Double *in situ* hybridization in *eIF4E* mutant embryos with a GFP probe (red) and probes for the different proapoptotic *Drosophila* genes (blue). *rpr*, *hid* and in a lesser extent *sickle*, are upregulated in mutants embryos. *grim* expression is not affected.

hid and *grim,* but not *sickle,* are able to be translated under reduced concentration of functional eIF4F complex

Although, the mechanism of transcriptional activation of all these genes in the *eIF4E* mutant remains to be elucidated, the presence of those mRNAs in embryos that lack the cap-binding protein eIF4E prompted us to study how they can be translated. We introduced *sickle*, *hid* and *grim* 5´ UTRs in the monocistronic vector containing Firefly luciferase as a reporter (Fig. 2.2a) and used them to perform *in vitro* translation assays. As expected, the uncapped FLuc control mRNA was not translated efficiently in the extracts, whereas the uncapped reporters containing the 5´UTR of *hid*, *grim* and *sickle* conferred translation to the reporter at levels equivalent to the capped-FLuc control vector (Fig 2.2b). The same was observed for *rpr* and *hsp70*

mRNAs which were used as controls in this experiment. Capped reporters containing the 5´ UTR for proapoptotic genes were translated 1-3 times more efficiently than their uncapped counterparts (Fig. 2.2b) as was observed previously for *rpr* and *Ubx* mRNAs (Fig 1.3a). All capped mRNAs showed the same stability after 90 min of reaction (Fig 2.2c).

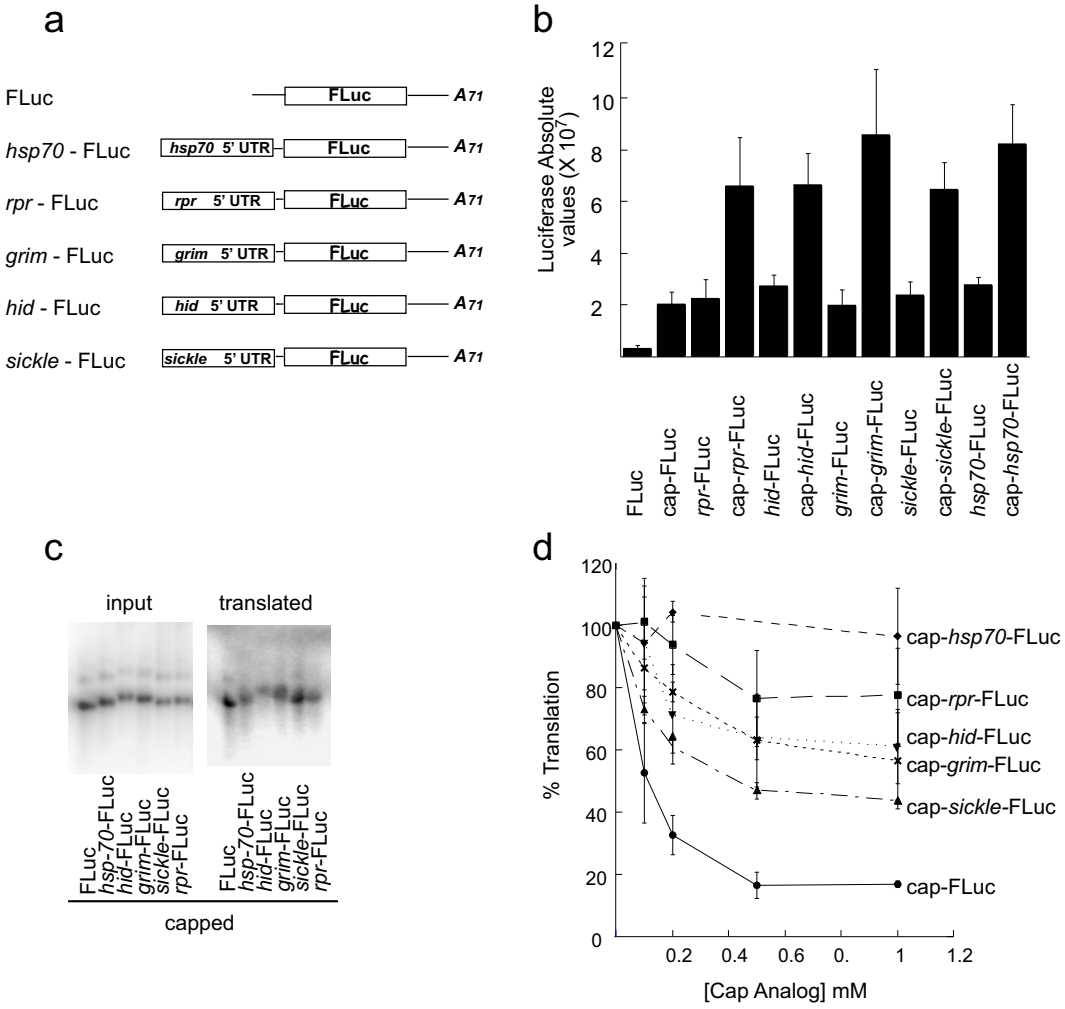


Figure 2.2. Translation driven by different *Drosophila* proapoptotic mRNAs 5'UTRs is competed at different degrees by free cap analog. (a) Reporter monocistronic mRNAs used in *in vitro* translation assays. **(b)** *in vitro* translation of capped and uncapped monocistronic reporter transcripts in translation extracts derived from wild type embryos. **(c)** Stability analysis of the capped transcripts assayed in (b). **(d)** Competition of cap-dependent translation with increasing concentrations of free cap analog m^7GpppG. The efficiency refers to the translation of the reporter mRNA in the absence of the cap analog.

We then performed competition experiments using free cap-analog to mimic the absence of eIF4E in the extracts. The different reporters showed different levels of

inhibition (Fig. 2.2d). The translation of a m^7GpppG-capped reporter containing the 5´UTR of *hsp70* (Cap-*hsp70*-FLuc) was unaffected by the addition of the cap-analog as expected for its well known cap-independent translation mechanism of protein synthesis. On the other hand, the translation of the m^7GpppG-capped FLuc (Cap-FLuc) was reduced more than 80 % compared to its translation in control conditions. The translation of m^7GpppG-capped *rpr*- FLuc (Cap-*rpr*-FLuc) was the less affected one among the proapoptotic genes. The translation of m^7GpppG-capped-*hid*-FLuc (Cap-*hid*-FLuc) and m^7GpppG-capped-*grim*-FLuc (Cap-*grim*-FLuc) reached a plateau at 40% of inhibition. The translation of m^7GpppG-capped *sickle*-FLuc was the most affected (50% of inhibition) in the absence of eIF4E. This experiment showed that different 5´UTRs can confer different levels of cap-independent translation to the reporter as measured by the sensitivity to the addition of cap analog.

We then tested the ability of the same reporters to drive translation in heat shocked extracts where cap-independent translation is severely impaired. In extracts derived from untreated embryos the uncapped- FLuc reporters bearing *hid*, *grim* and *sickle* 5´UTRs were translated *in vitro* at a level comparable to cap-FLuc and uncapped *rpr*-FLuc and uncapped *hsp70*-FLuc as we had observed before (Fig. 2.3a).

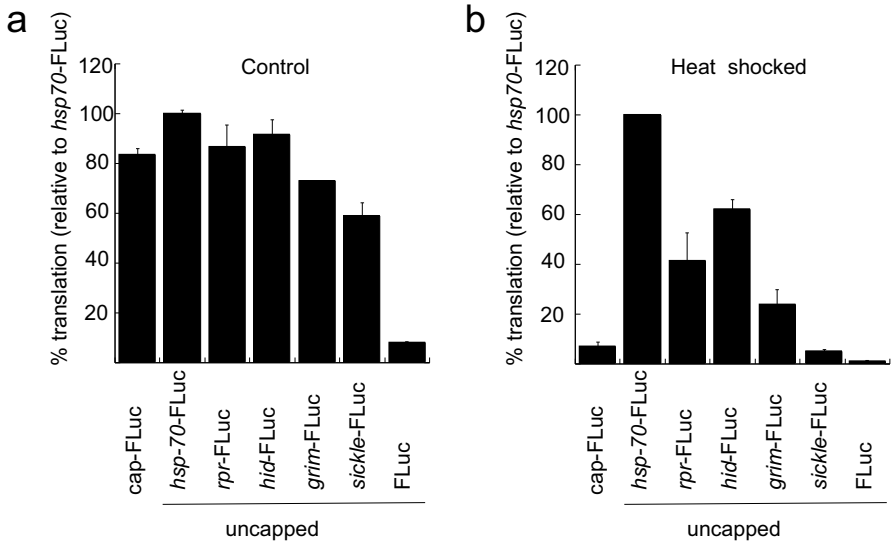


Figure 2.3. Translation of apoptotic genes in heat shocked extracts. Monocistronic mRNA reporters translated in extracts derived from untreated **(a)** and heat shocked **(b)** embryos. In all experiments the normalization is referred to the h*sp70*-FLuc reporter mRNA. Absolute values are not comparable as they were performed with different extracts.

In translation extracts derived from heat shocked embryos the cap-dependent translation was reduced (Cap-FLuc). Translation of uncapped *sickle*-FLuc was also dramatically reduced at a level comparable to the capped-FLuc control (Fig. 2.3b). Uncapped *rpr*-FLuc, *hid*-FLuc and *grim*-FLuc were able to drive translation in heat shocked extracts but with less efficiency than uncapped *hsp70*-FLuc reporter (Fig. 2.3b).

In the experiment of Fig. 1.4c we observed that the translation efficiency of ApppG-*rpr*-FLuc was even higher than that of ApppG-capped *hsp70*-FLuc during heat shock. This discrepancy could be due to a different stability of the reporters used or to a different degree of the heat shocked treatment in order that *hsp70* translation is more favorable. However, this result in combination with the competition experiment using free cap analog (Fig. 2.2d and Fig. 2.3b) showed that translation driven by the 5´UTR of *sickle* is more sensitive to the absence of a functional eIF4E or eIF4F complex than the translation driven by *rpr*, *hid* and *grim* 5´UTRs. Since the translation of uncapped *sickle*-FLuc was as efficient as translation driven by the Cap-FLuc control and much more efficient than the translation of the uncapped FLuc control (Fig. 2.2b and 2.3a), we inferred that its translation was independent of the presence of a m^7GpppG end. We can not distinguish whether the translation of *sickle* is exclusively eIF4E- or, most probably, eIF4F-dependent. We conclude that *rpr*, *hid* and *grim* are able to be translated in a cap-independent and eIF4F-independent manner while *sickle* translation might be eIF4F-dependent.

***hid* and *grim,* but not *sickle,* display IRES activity**

We observed previously that *rpr* and *hsp70* showed IRES activity *in vitro* and *in vivo*. To evaluate whether this mechanism also controls cap-independent translation of the other proapototic genes we performed *in vitro* translation assays with the dicistronic transcripts that had shown reduced read-through in previous experiments (FLuc/ hairpin/RLuc and FLuc/cad/RLuc, Fig. 2.4, Fig. 2.5 and Fig.2.6). The insertion of the different 5´UTRs downstream of the hairpin in the FLuc/hairpin/RLuc did not affect the expression of the first cistron significantly (Fig. 2.4b, FLuc, black bars). However, while the presence of *rpr*, *grim*, and *hid* 5´ UTRs increased the efficiency of the translation of the second cistron (Fig. 2.4b, RLuc, white bars), the presence of *sickle* 5´UTR in the dicistronic vector did not show an effect on the translation of the second

cistron when compared with the control FLuc/hairpin/RLuc vector, in agreement with previous data.

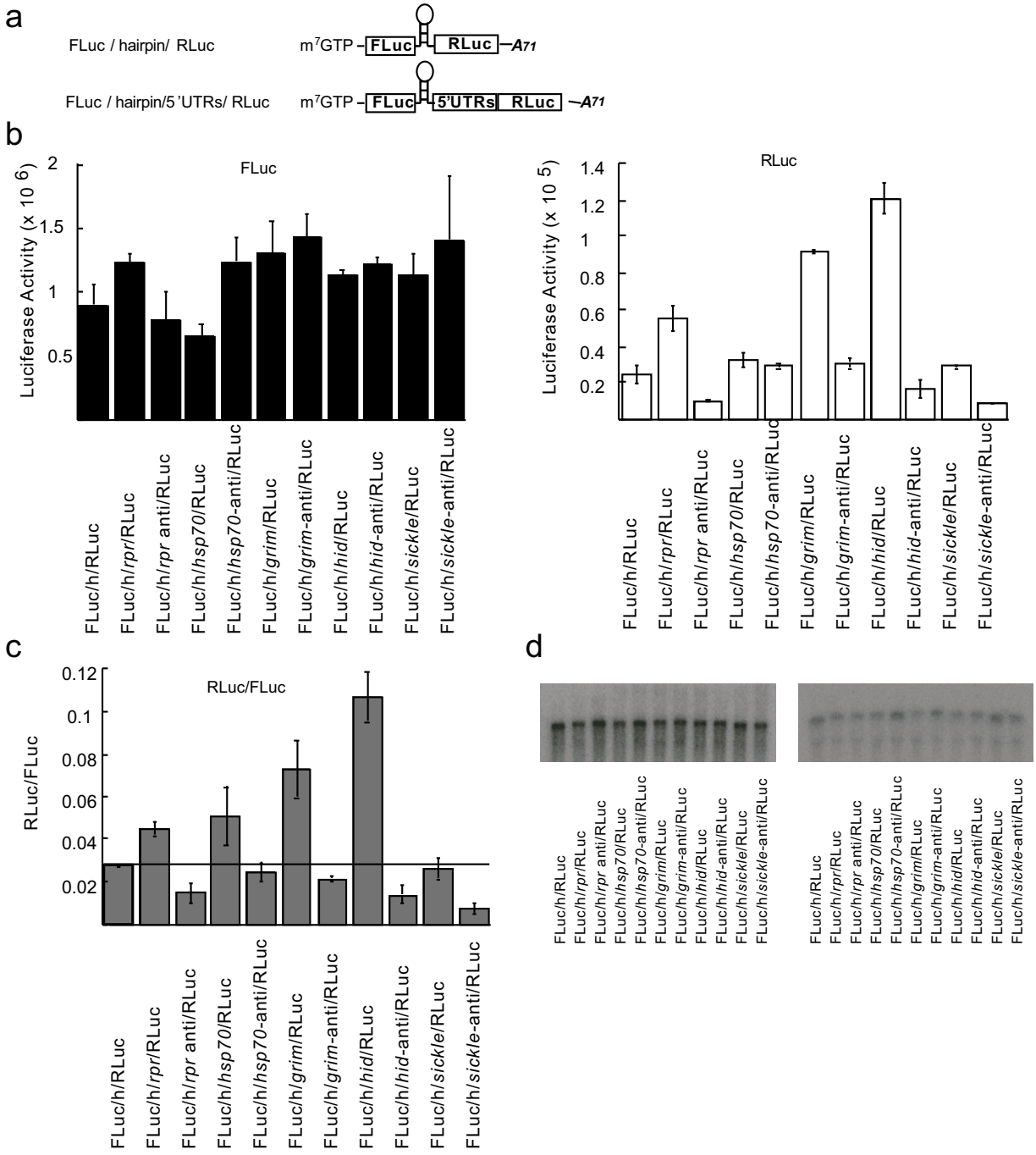


Figure 2.4. *rpr*, *hid* and *grim* 5'UTR, but not *sickle* 5'UTR, show IRES activity in a vector containing a stable hairpin. **(a)** Reporter dicistronic mRNAs containing a synthetic, stable hairpin that prevent read-through of ribosomes. The different 5' UTR where cloned downstream of the hairpin. **(b)** *In vitro* translation of the capped dicistronic transcripts containing the different 5'UTR in sense and antisense orientation. Absolute values of Firefly luciferase activity (FLuc, first cistron, black bars) and values of the Renilla luciferase activity (RLuc, second cistron, white bars) are shown. **(c)** Efficiency of translation of the second cistron over the first cistron (RLuc/FLuc values) of the experiment depicted in (b). **(d)** Stability analysis of the dicistronic reporter mRNAs used in (b) and (c). The minor band observed in the transcripts before and after translation probably is the result of an incomplete transcription due to the presence of the stable hairpin that blocks the polymerase activity. Since this band is observed in same amounts in all the transcripts could not account for the efficiency of translation of the different reporters tested.

The insertion of all the 5´UTR sequences in antisense orientation resulted in a reduction or no increase of the second cistron expression. The efficiency of translation of the second cistron with respect to the first cistron increased for *rpr*, *hid* and *grim* 5´UTRs bearing dicistronic reporters but not for the one with *sickle* 5´ UTR inserted in the intercistronic region (Fig. 2.4c, see the bars above of the cut-off line). The stability and integrity of the transcripts after the translation reaction were not affected (Fig. 2.4d).

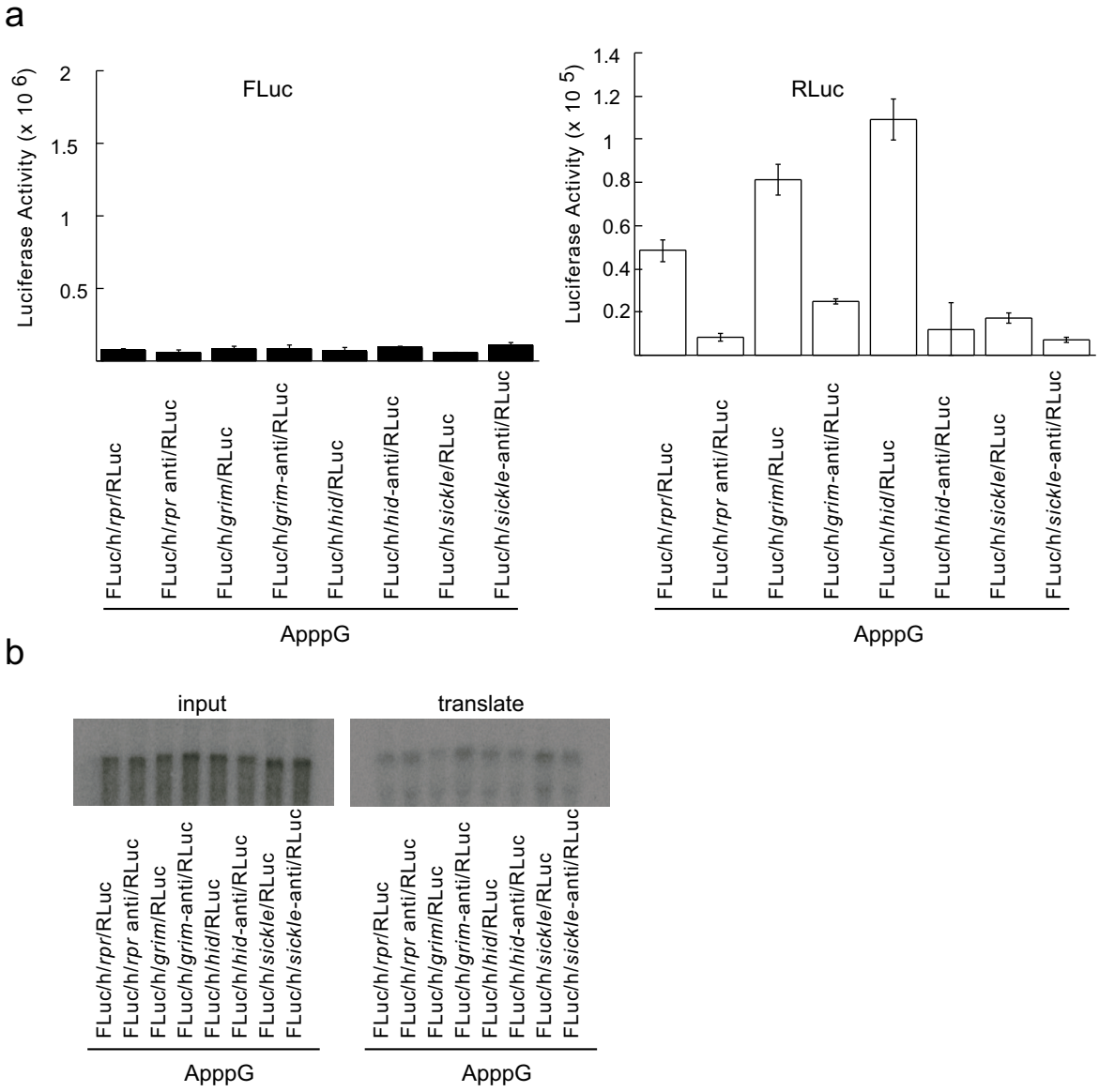


Figure 2.5. *rpr*, *hid* and *grim* 5'UTR but not *sickle* 5'UTR ApppG-capped transcript showed IRES activity. (a) *In vitro* translation of the ApppG-capped dicistronic transcripts containing the different 5'UTRs in sense and antisense orientation. Absolute values of Firefly luciferase activity (FLuc, first cistron, black bars) and values of the Renilla luciferase activity (RLuc, second cistron, white bars) are shown. The translation of the first cistron is reduced and the second cistron is expressed at the same rate as was observed by the m^7GpppG-capped counterparts in Fig. 2.4 (b). The same scale as in figure 2.4 (b) was used. **(b)** Stability analysis of the dicistronic reporter mRNAs used in (a).

The same reporters used in Fig. 2.4a were capped with the ApppG analog and used for *in vitro* translation. The graphs showing the absolute values of the first cistron (Fig. 2.5a, FLuc, black bars) and second cistron (Fig 2.5a, RLuc, white bars) are shown with the same scale as in the corresponding graphs in Fig. 2.4. The translation of the first cistron was abolished by the presence of the ApppG while the second cistron was still been translated with the same efficiency. Again the stability and integrity of the transcripts after the translation reaction was not affected (Fig. 2.5 b).

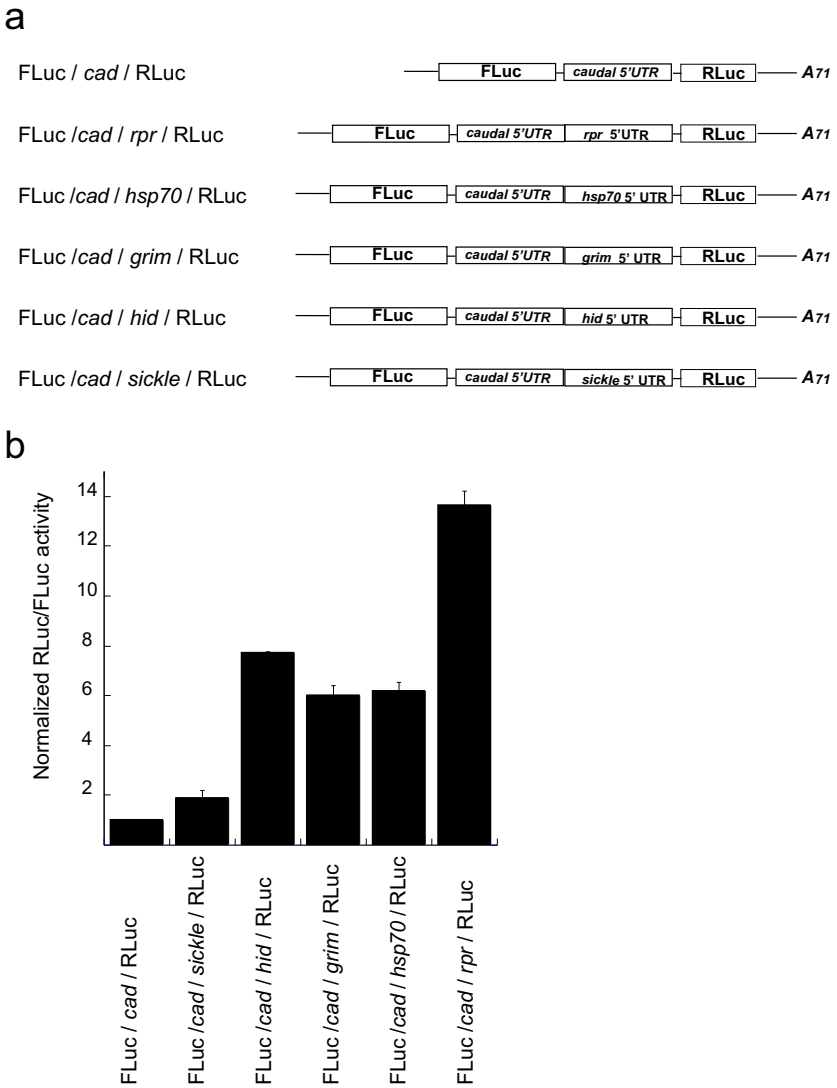


Figure 2.6. *rpr*, *hid* and *grim* 5'UTR but not *sickle* 5'UTR show IRES activity in a vector containing the 5'UTR of *maternal caudal* mRNA (*cad*) to prevent read-through. (a) Reporter dicistronic mRNAs used for *in vitro* translation. **(b)** Translation efficiency (RLuc/FLuc activity) of dicistronic transcripts depicted in (a) normalized to the efficiency in the FLuc/ *cad* /RLuc vector.

We corroborated the IRES activity of *hid* and *grim* by inserting their 5´UTR sequences into the dicistronic reporter FLuc/*cad*/RLuc (Fig. 2.6a). We used this m^7GpppG dicistronic vectors for *in vitro* translation (Fig. 2.6b). The presence of *hid*, *grim* and *rpr* 5´UTR increased the efficiency of translation of the second cistron (RLuc) with respect to the first cistron (FLuc), when compared to the control FLuc/*cad*/RLuc vector. On the contrary the efficiency of translation of the reporter bearing the *sickle* 5´UTR was almost the same as in the control vector. We conclude that the proapoptotic genes *rpr*, *hid* and *grim* 5´UTR displays IRES activity while *sickle* 5´UTR appears not to share this mechanism of translation.

Recruitment of *hid* and *grim*, but not *sickle*, to polysomes during heat shocked conditions

To obtain evidence if the endogenous proapoptotic mRNAs are being translated *in vivo* under conditions that block cap-dependent initiation, we separated ribosomal fractions from extracts derived from 0–12 h old *Drosophila* embryos grown under normal conditions or after either heat shock or irradiation with X-rays as done for the analysis of *hsp70* mRNA and *rpr* mRNA (Fig. 1.11). We then studied the presence of the transcripts corresponding to *hid*, *grim* and *sickle* (Fig. 2.7) and *Actin5C, hsp70* and *rpr* mRNAs (shown before in Fig. 1.11) in both untranslated (U) and polysome-associated (P) fractions derived from untreated and heat shocked embryos (Fig 2.7a). The fraction of polysome-associated mRNAs compared to the untranslated ones provided an indication of their level of translation (P/U in Fig. 2.7 insets). *hid, grim* and *sickle* were translated in normal conditions as indicated by a P/U ratio of almost 1.5 for both mRNAs (compare with the *Actin5C* mRNA with P/U ratio of 2, and *hsp70* and *rpr* mRNAs with a P/U ratio of 1 in Fig. 1.11b). After heat shock treatment we observed an increase of the total amount of *hsp70* mRNA and a decrease in the levels of *Actin5C*, *rpr, hid, grim*, and *sickle* mRNAs relative to untreated embryos (Fig. 2.7c). The amount of *hid* and *grim* mRNA associated with polysomes remained stable as observed by the measurement of *rpr* mRNA (Fig. 2.7a compared with Fig. 1.11b), while the ratio of polysome-associated *sickle* mRNA decreased (P/U < 1), as observed for *Actin5C* mRNA (Fig. 2.7a compared with Fig. 1.11b). These data demonstrate that translation rates of a cap-dependent mRNA (*Actin5C*) and a eIF4F-dependent transcript (*sickle*) decrease upon heat shock, while *rpr, hid, grim* and *hsp70* mRNAs are still actively translated, irrespective of their relative level of transcription.

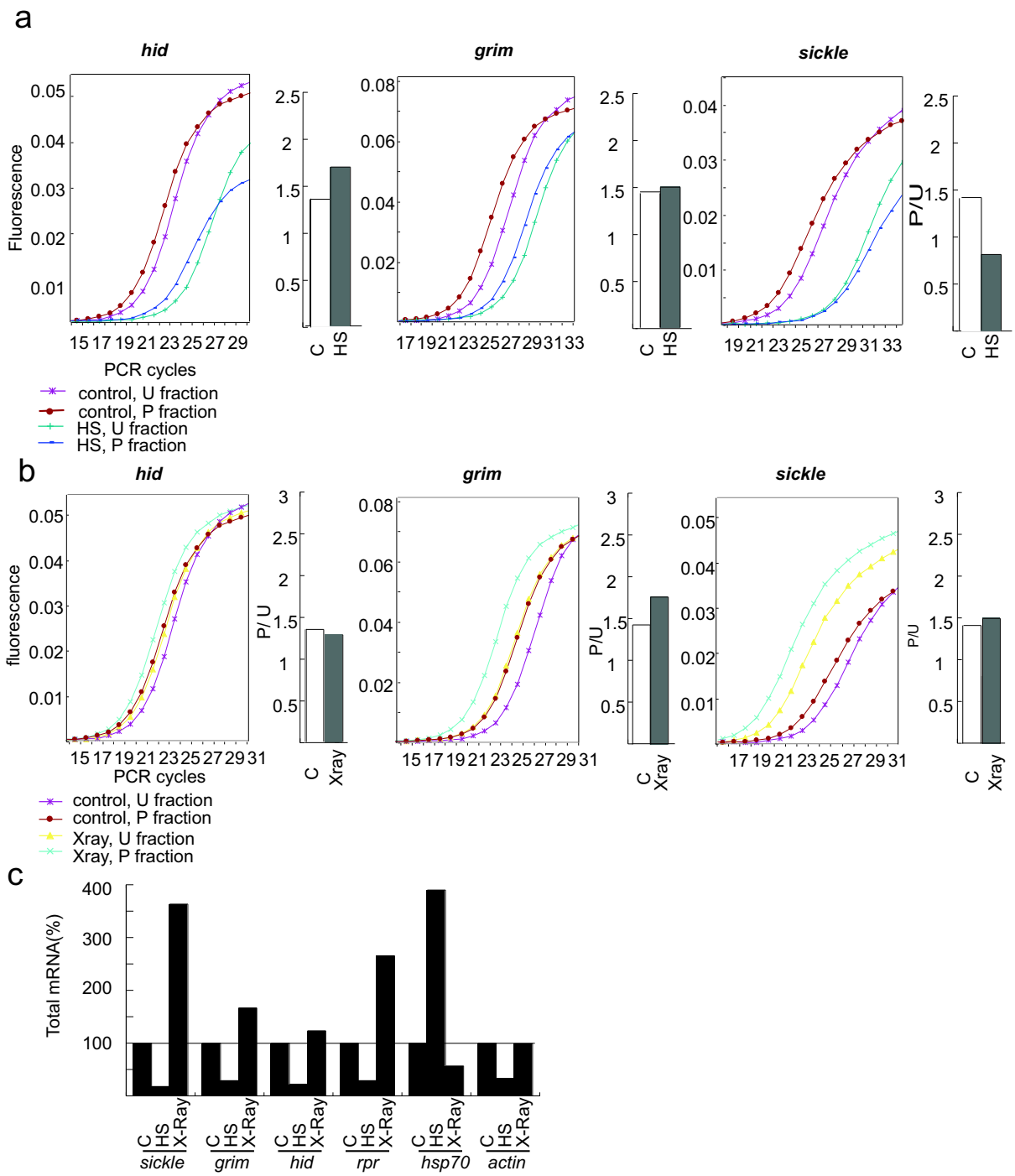


Figure 2.7. Recruitment of *grim*, *hid* and *sickle* mRNAs to polysomes upon heat shocked and X-ray treatment. (a) Amplification of *hid*, *grim* and *sickle* mRNAs in U and P fractions by real time quantitative RT-PCR experiments from heat shocked embryos. The amplification course shows the mRNA present in the control in the P fraction (red line), control in the U fraction (violet line) and during heat shock treatment in the P fraction (blue) and in U fraction (green). The insets represent the P/U ratio in the control (white bars) or treated (grey bars) embryos for each mRNA tested. **(b)** Same experiment as in (a) but using X-Ray treated embryos. The amplification course shows the mRNAs present in P (turquoise) and in U fraction (yellow) during X-ray treatment. The amplification course for the mRNAs present in control are the same depicted in (a). **(c)** Comparison of the total amounts of mRNAs from the experiments performed in (a) and (b). The amount of mRNA is calculated as P+U and normalized to untreated embryos. The values for *rpr, actin* and *hsp70* mRNAs were taken form Figure 1.11d.

We then performed the equivalent experiment using translation extracts derived from X-ray-irradiated *Drosophila* embryos (Fig. 2.7b, see also Fig 1.11c). As expected, while the overall transcription of *rpr, hid, grim* and *sickle* mRNA increased (Fig. 2.7c), no major changes were observed in the levels of *Actin5C* mRNA, while transcription of *hsp70* was reduced. While the association of *Actin5C* mRNA to polysomes decreased (P/U=1.5; Fig. 1.11c, left panel) the level of *hid, grim* and *sickle* associated with polysomes remained constant (Fig. 2.7b), and that of *hsp70* and *rpr* mRNAs slightly increased (P/U=1.5; Fig. 1.11c, middle and right panels).

Free *rpr, hsp70* and *Ubx* 5' UTRs do not compete the translation driven by *hid, sickle* and *grim* 5' UTRs

The addition of free *rpr*, *hsp70* and *Ubx* 5' UTRs competed for translation of a cap-dependent reporter. A specific inhibition of the translation driven by *rpr*, *hsp70* and *Ubx* IRES was only observed upon addition of the corresponding 5' UTR counterparts (Fig. 1.12), suggesting that specific factors may control the translation of these IRES in agreement with their different physiological roles. *rpr, hid* and *grim* genes are also involved in triggering early steps of apoptosis in *Drosophila* and display IRES activity. Although they are expressed in different cell types in *Drosophila* development and may respond to different signals, a common IRES dependent mechanism, involving similar translation factors or regulatory proteins, cannot be excluded. Thus, we performed competition experiments by using free *rpr*, *hsp70* and *Ubx* 5´UTR and analyzed the translation of *hid*-FLuc, *grim*-FLuc and *sickle*-FLuc reporters (Fig. 2.8). Translation driven by *sickle*-FLuc reporter was unaffected by the presence of either free *rpr, hsp70* or *Ubx* 5' UTRs. This result was surprising since translation of *sickle* was shown to require an intact eIF4F complex, the main complex involved in the cap-dependent translation of the Cap-FLuc reporter whose translation was reduced by the presence of these free 5' UTRs. Translation driven by *grim* and *hid* IRES was also not affected by competition with *rpr, hsp70* and *Ubx* 5´ UTRs. These preliminary results indicated that although *sickle, rpr, hid* and *grim* would be able to be translated during apoptosis, a condition under which cap-dependent translation of most mRNAs could be compromised, different regulatory factors would be involved in their translation regulation. Cross competition experiments using free *hid, sickle* and *grim* 5' UTRs and RNA stability experiments will be necessary to reach a definitive conclusion.

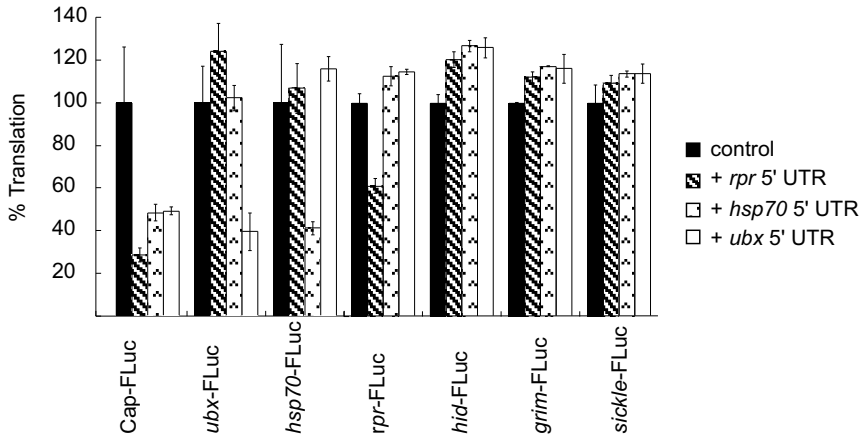


Figure 2.8. Specific competition experiments with free 5' UTR. Translation efficiency of the indicated reporters upon addition of 10 molar excess of free *rpr*, *hsp70* and *ubx* 5' UTRs. 100% corresponds to the value of translation of each reporter in the absence of competitor 5' UTR.

Discussion

We showed that *hid, grim* and *rpr* 5´UTRs display IRES activity and they confer translation of an uncapped reporter mRNA under heat shocked conditions and in the presence of free cap-analog. We also observed that the endogenous *hid, grim* and *rpr* mRNAs can be translated during heat shock and X-ray conditions leading to the impairment of cap-dependent translation of *Actin5C*. We conclude that cap-independent translation might play a generalized role during apoptosis in *Drosophila* like it happens in mammals.

On the other hand, endogenous *sickle* mRNA showed different translation behavior during heat shock and X-ray treatment. eIF4F complex aggregates in a way that is unable to support cap-dependent translation during heat shock (Sierra and Zapata, 1994; Schneider, 2000). The reduction of polysome-associated *sickle* mRNA during this stress condition is in agreement with a reduced translation of *sickle*-FLuc reporter in heat shocked extracts, the high sensitivity to the competition with free cap-analog and the fact that *sickle* does not display IRES activity. These observations indicate that *sickle* mRNA translation does not necessary need intact eIF4E since uncapped *sickle*-FLuc reporter was efficiently translated in *in vitro* translation extracts. We suggest that other components of the eIF4F complex or other unidentified eIF4E interacting proteins may be involved in *sickle* mRNA translation.

The rate of protein synthesis is down-regulated in mammalian cells following induction of apoptosis. The inhibition occurs at the level of the initiation of translation and is accompanied by phosphorylation of the α subunit of initiation factor eIF2 and caspase dependent cleavage of initiation factors eIF4G, eIF4B, 4EBP1, eIF2α, the p35 subunit of eIF3 and PABP (Clemens *et al.*, 2000). However the mechanism of inhibition of translation during apoptosis is not well known in *Drosophila*. Degradation or cleavage of some of the translation factors which could explain for this phenomenon in *Drosophila* was not observed by experiments done in the laboratory (data not shown). In this context the inhibition of translation during apoptosis induced by X-Ray in *Drosophila* would involve the modification or degradation of a factor(s) dispensable for *sickle, hid, grim* and *rpr* dependent translation.

hsp 5´UTRs have a high content of adenines (46-50%) (Ingolia and Craig, 1981). This feature is necessary for efficient translation during heat shock, since it decreases the likelihood of secondary structure formation (Hess and Duncan, 1996). A 38% adenine fraction in the 5´UTR Hsp83 suffices to confer efficient translation upon heat shock. The 5´UTR of, *hid*, *rpr*, *grim* and *sickle* have 50%, 45%, 37% and 34% adenine content, respectively and they are translated in this order of efficiency in heat shocked extracts. Thus, a correlation seems to exist between the low secondary structure in a 5´UTR bearing a high adenine content and the ability to maintain translation activity under heat shock conditions, for which a reduction of functional eIF4F complex likely derives from a loss of unwinding activity of eIF4A. Indeed, mRNAs containing stable secondary structure in the 5´UTR are more susceptible to translation inhibition by a dominant mutant form of mammalian eIF4A (Svitkin *et al.*, 2001).

We did not detect sequence homology between either *hid* or *grim* 5´UTRs with *hsp70* 5´UTRs as was the case for *rpr* 5´UTR. However, competition experiments with free 5' UTRs suggest that specific sequences within the 5´UTR of the proapoptotic genes and likely different factors that recognize these sequences may be involved in the control of their translation. These factors could act differentially upon different stimuli and in different cell types to trigger the apoptosis pathways.

Chapter 3

Proteomic analysis of *reaper* 5´ UTR-interacting factors isolated by tobramycin affinity-selection reveals a role for La antigen in *reaper* mRNA translation

We described that the *Drosophila* proapoptotic gene *rpr* is translated in a cap-independent manner that resembles the translation of heat shock proteins, and its 5' UTR displays IRES activity (Chapter 1). One of the main questions regarding the regulation of translation of proapoptotic genes is the nature of factors bound to the 5' UTR of the mRNA and new protein-RNA complex purification techniques are required for the analysis of the interactions. We adapted the tobramycin affinity-selection method developed by Hartmuth *et al.* (Hartmuth *et al.*, 2002) for the purification of native RNP complexes in human pre-spliceosomes to study the formation and components of RNP complexes assembled onto the 5' UTR of *rpr* mRNA. We wanted to establish a protocol that allows the purification of complexes that can be further analyzed by mass spectrometry.

Analysis of *rpr* 5´UTR-interacting factors isolated by tobramycin affinity-selection.

To isolate RNP complexes assembled with the 5' UTR of *rpr* mRNA, we modified a tobramycin affinity-selection method recently developed to purify spliceosomes under native conditions (Hartmuth *et al.*, 2002). We introduced a 40 nt RNA aptamer at the 5' end of a sequence bearing the *rpr* 5' UTR and also encoding the 10 first aminoacids of the ORF (aptamer-tagged *rpr* 5´UTR, TA-*rpr*). The aptamer binds with high affinity to the aminoglycoside antibiotic tobramycin. To prove that the aptamer binding capacity was not affected by the fusion of *rpr* 5' UTR we assayed first the binding of radiolabelled TA-*rpr* (Fig. 3.1a, left panel) and non-tagged *rpr* (Fig. 3.1a, right panel) to tobramycin-derivatized Sepharose. 60% of aptamer-tagged *rpr* 5' UTR was bound to the beads while only 2% of the untagged *rpr* 5´UTR bound to the tobramycin matrix.

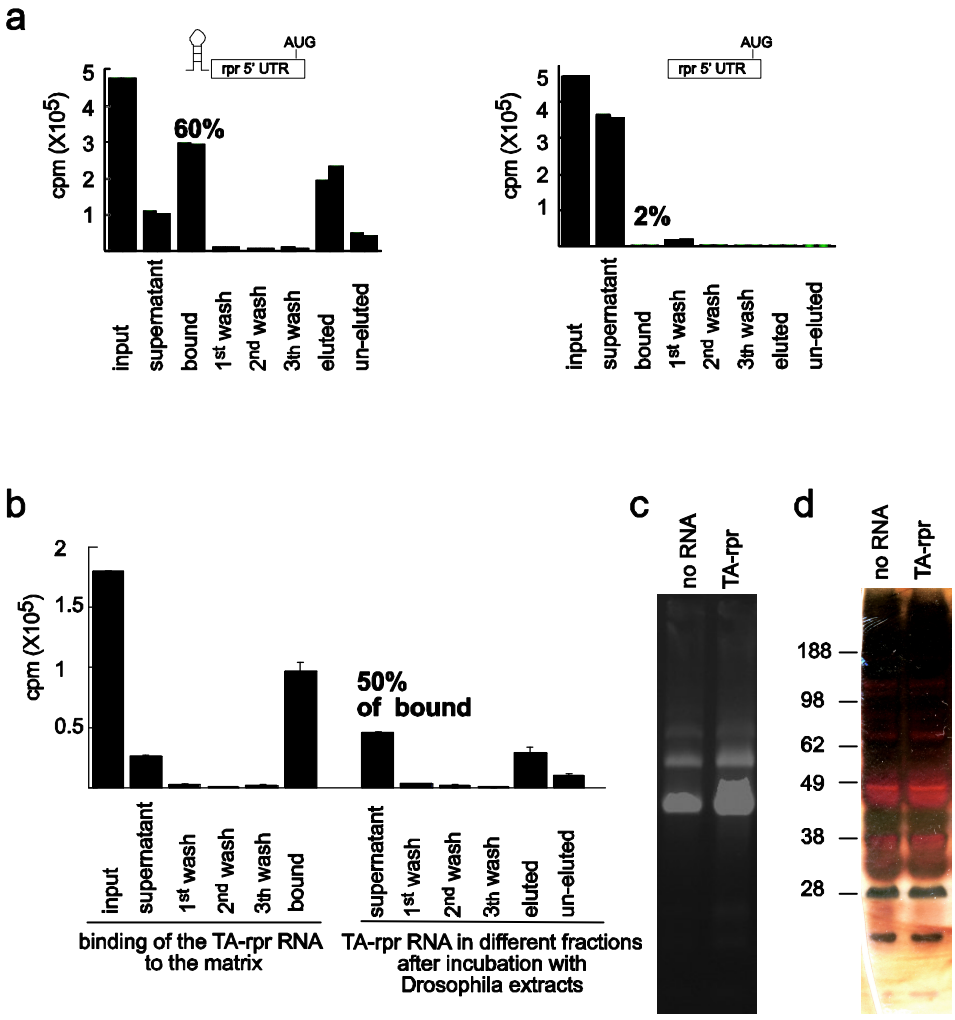


Figure 3.1.(a) Specificity of the interaction of the aptamer-tagged *rpr* 5' UTR (TA-*rpr*) with the tobramycin matrix. The radioactivity of the fractions for the TA-*rpr* (left panel) and untagged-*rpr* 5' UTR (right panel) is shown. Fractions referred to as "bound" and "un-eluted" correspond to the amounts of different RNAs that remained bound to the matrix after washing and elution, respectively. **(b)** TA-*rpr* RNA was first bound to the column and then incubated with *Drosophila* extracts under translation conditions. The radioactivity of the fractions is shown. Note that more than 50% of pre-bound RNA appears in the supernatant after incubation with the extract. As control, tobramycin matrix was incubated with *Drosophila* extracts (no RNA). **(c)** RNA extracted from the eluted fractions after incubation of matrix containing TA-*rpr* RNA or matrix alone (no RNA) with translation extracts. The RNA was analyzed by agarose gel electrophoresis. Unspecific binding of ribosomal RNAs was observed. **(d)** Proteins were precipitated from the same eluted fractions described in (c) by TCA precipitation, analyzed on a 10% SDS-PAGE and detected by silver staining. Unspecific binding of proteins to the tobramycin matrix was observed.

More than 80% of the bound TA-*rpr* could be eluted with 5mM tobramycin. TA-*rpr* was thus immobilized onto the tobramycin matrix and subsequently incubated with cytoplasmic extracts under translation conditions.

This approach, successful in the assembly of spliceosomes, failed to work with translation extracts and released more than 50% of TA-*rpr* from the matrix (Fig. 3.1b). We also observed unspecific binding of ribosomal RNA (Fig. 3.1c) and

proteins (Fig. 3.1d) to the tobramycin matrix upon addition of embryonic extracts in the absence of pre-bound RNA. This is likely due to the fact that tobramycin is an aminoglycoside antibiotic targeting the decoding aminoacyl site (A) on the 16S bacterial ribosomal RNA, but also binds the A-site on the 18S human counterpart.

We concluded that the presence of free *Drosophila* 18S ribosomal RNA in the translation extracts likely competes with TA-*rpr* for the binding to the matrix and results in the release of TA-*rpr* from the matrix. *Drosophila* 18S is likely binding to the tobramycin matrix as part of 43S, 80S complexes and this could account for the unspecific binding of the translation apparatus to the matrix. This represents a drawback in the technique if it is to be used in the assembly of RNP starting from cytoplasmic extracts. To overcome this problem we decided to first separate different RNPs by sucrose gradient ultracentrifugation following the purification strategy shown in Fig. 3.2.

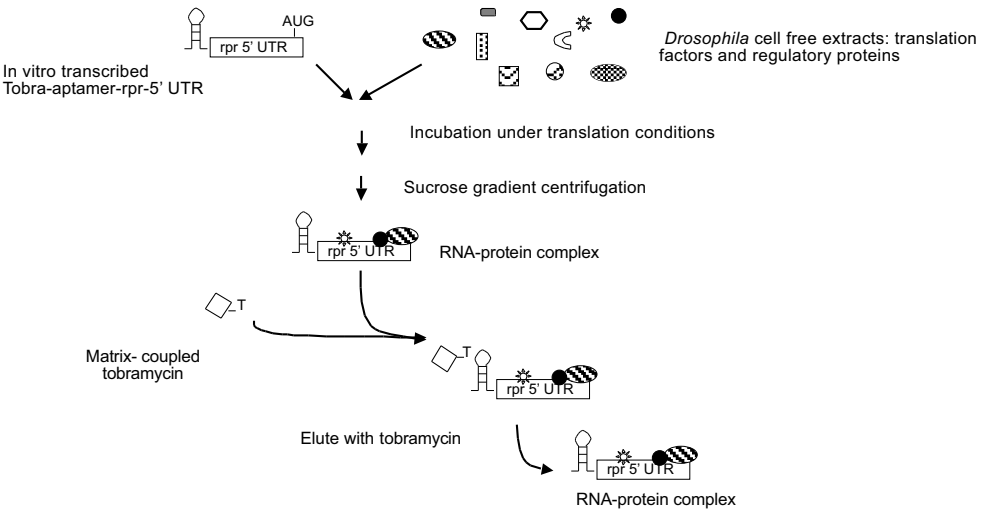


Figure 3.2. Scheme of the RNP purification strategy.

To isolate the earliest steps in the assembly of the RNP complex, the translation reaction was carried out in the presence or absence of cycloheximide, a compound that inhibits translation elongation, and GMP-PNP, a GTP analog that inhibits the joining between the 43S preinitiation complex positioned at the initiation codon and the 60S ribosomal subunit. The reaction was incubated at different times and then loaded on a 10-30% sucrose gradient. After 10 minutes of incubation of the

RNA with cytoplasmic extract in the absence of inhibitors the RNA was present in RNP particles that sedimented as a ~20S complex (Fig. 3.3a). The incorporation of RNA into RNPs was increased in the presence of inhibitors, which blocks the initiation steps of translation (Fig. 3.3b). This result indicates that the RNP represents a step prior to the assembly of the ribosome.

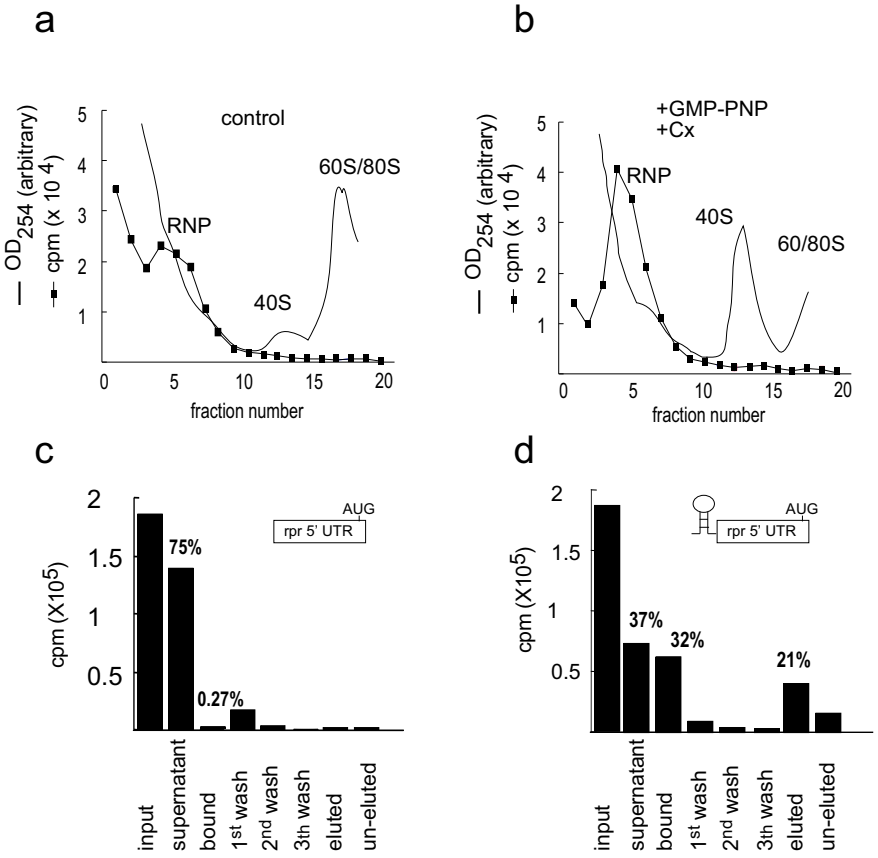


Figure 3.3. Purification of RNPs assembled on *rpr* 5' UTR. Sucrose gradient separation of RNP containing radiolabelled tobramycin-aptamer-tagged *rpr* RNA in the presence **(a)** or absence **(b)** of GMP-PNP and cycloheximide. Absorbance was recorded at 254 nm (black lines) and the amount of RNA was measured by Cherenkov counting (squares). RNP complex formation increased in the presence of inhibitors (b). Specificity of the interaction of the tobramycin-aptamer-tagged *rpr* 5´ UTR - RNP with the tobramycin matrix, **(c)** and **(d)**. Pooled fractions of the sucrose gradient containing either the tobramycin-aptamer-tagged **(d)** or untagged *rpr* RNA **(c)** were incubated with the tobramycin matrix, washed and eluted as described in the text and the radioactivity in the indicated fractions was measured by Cherenkov counting.

The fractions corresponding to the RNP complex containing TA-*rpr* and *rpr* as reference were incubated with the tobramycin matrix. The matrix was then washed and the bound RNA-protein complexes were eluted with tobramycin. The binding of TA- *rpr* RNP (32%, Fig. 3.3d) was much more efficient than the binding of untagged *rpr* (0.27%, Fig. 3.3c), validating the appropriateness of the approach. Almost no TA-

rpr was washed away and the elution rate represented 65% of the bound RNA (Fig. 3.3d). Thus, using this protocol we obtained a significant differential binding between tagged and untagged RNA, and a reasonable elution rate.

Proteins were then isolated from the eluate and analyzed by denaturing SDS-PAGE (Fig. 3.4). We determined the optimum amount of bound TA-*rpr* to be at least 20 pmoles to obtain enough protein to be detected by silver staining. The eluate from the affinity selection exhibited a distinct protein pattern, with only a small number of proteins present in the untagged-*rpr* 5´UTR (Fig. 3.4a): Yolk protein 1, Yolk protein 3 and Vitellogenin (on bands 19, 20 and 21). These proteins are components of the embryonic yolk and they are present in high concentration in cytoplasmic and nuclear preparations from embryos. They usually attach to solid supports such as sepharose and are present in all kind of protein purification (Rivera-Pomar *et al.*, 1996).

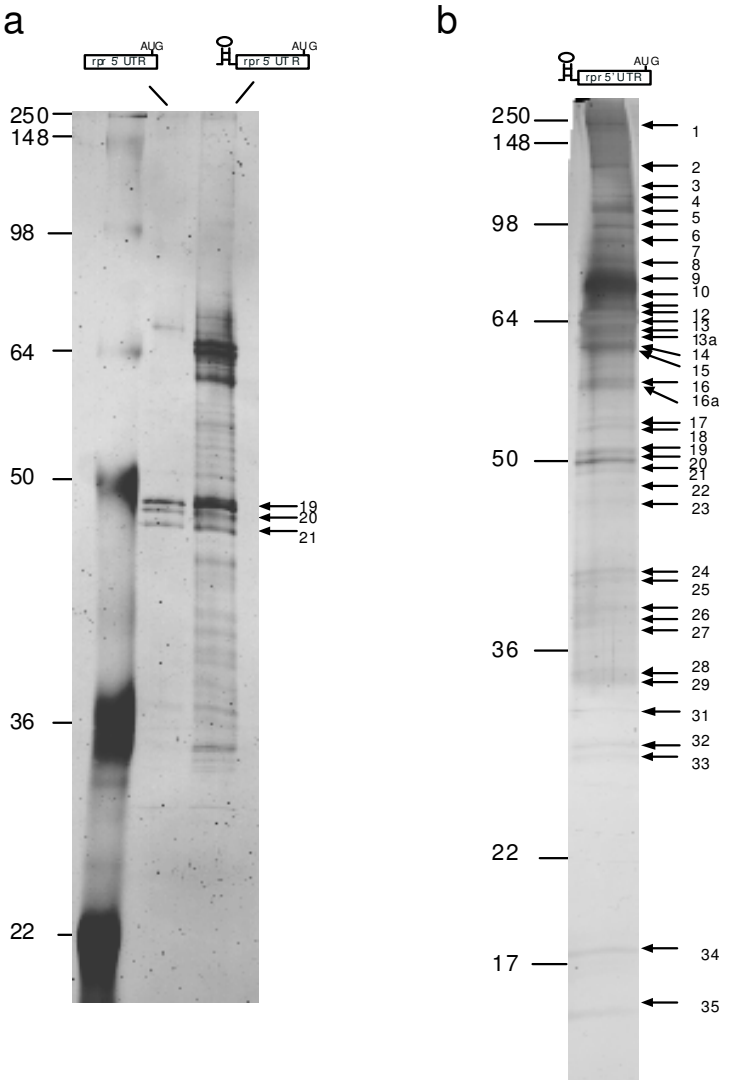


Figure 3.4. Protein content of RNP complexes eluted with tobramycin. (a) RNPs assembled on the untagged (left lane) or tagged (right lane) RNAs were incubated with the tobramycin matrix. After binding and washing the complexes were subsequently eluted with tobramycin. Binding of 20 pmols of RNA was achieved for the tagged RNA. Proteins were analyzed by PAGE and visualized by silver staining. **(b)** Scaled up purification of the reaction to reach 60 pmols of RNA bound to the matrix in order to performe mass spectrometry analysis. Arrowheads and numbers represent the bands further analyzed by mass spectrometry. Bands 19, 20, and 21 represent unspecific binding to the matrix and were shown to correspond to Yolk protein 1, Yolk protein 3 and Vitellogenin.

Table II

RNA-binding proteins identified by mass spectrometry in *rpr* 5' UTR-ribonucleoprotein complexes

Protein	BN	SC	PN	AC	Features	Biological processes
RNA binding proteins						
Ypsilon schaetal	16´ 22	373	12	CG5654	Cold-shock DNA binding domain RNA binding	oogenesis, regulation of transcription from Pol II promoter, oskar mRNA localization and translation
Protein on ecdysone puff	5, 6	208	5	CG6143	RNA-binding, C2H2 Zn-finger domain	binding to hsp70 mRNA and DNA
La-autoantigen like	16, 16´	52 58	1(***) 1	CG10922	RNP-1, RBD, Lupus La protein RNA-binding domain	RNA binding; Pol III transcription termination factor activity; 5S rRNA primary transcript binding; tRNA metabolism HCV, Cosakiesvirus and Xiap IRES and Top mRNAs translation.
multi sex combs	6	174	4	G10922	RNP-1, Lupus La, RBD binding domains	RNA binding, hemocyte differentiation Loss of normal mxc can promote uncontrolled malignant growth
Glyceraldehyde 3-P dehydrogenase	26, 27	22 26	1(**) 1	CG12055	NAD(P)-binding Rossmann-fold domains, Glyceraldehyde-3-phosphate dehydrogenase-like, C-terminal domain	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity, glycolysis HAV IRES translation
Stubarista p40 ribosomal protein	25	32	1(*)	CG14792	ribosomal S2 domain	structural constituent of ribosome, nucleic acid binding, protein biosynthesis
Splicing						
SF2	31	179	6	CG6987	RNA binding, pre-mRNA splicing factor, RNP-1, RBD domains, arginine/serine-rich motif	mRNA splice site selection, nuclear mRNA splicing, via spliceosome
xl6	31	45	1	CG10203	RNA binding, pre-mRNA splicing factor RNP-1, RBD, Zn-finger CCHC type, retrovirus zinc finger-like domains, arginine/serine-rich motif	mRNA splice site selection, nuclear mRNA splicing, via spliceosome
Small ribonucleo protein B	32	52	1	CG5352	Sm motif , pre-mRNA splicing factor activity	nuclear mRNA splicing, via spliceosome
putative small ribonucleo protein D2	35	51	2	CG1249	Sm motif of small nuclear ribonucleoproteins, SNRNP	pre-mRNA splicing factor activity; nuclear mRNA splicing, via spliceosome
52 K active chromatin boundary protein (B52/SRp55)	1, 4	29	1(*)	CG10851	RNA binding, pre-mRNA splicing factor, RNP-1, RBD domains, arginine/serine-rich motif	pre-mRNA splicing factor activity; nuclear mRNA splicing, via spliceosome

BN, band numbering in Fig. 3.4b; SC, score; PN, number of peptides identified by LC/ESI-MS/MS; AC, gene denomination according to the *Drosophila* Annotated Database (flybase.org). (***) La- autoantigen protein was confirmed by MALDI-MS in two independent affinity purification experiments. (*) MS/MS on the Q-TOF revealed a Y-Type series of at least 4 amino acids. Peptide tolerance was 50 ppm and MS/MS tolerance was 50 mm.(**) Observed with low score and one peptide matched in two bands.

To identify the proteins forming part of the RNP complex, the procedure was scaled up to have 60 pmoles of bound tagged- *rpr* 5´UTR (Fig. 3.4b). The eluted proteins were purified, fractionated by SDS–PAGE and analyzed by LC/ESI-MS/MS (Fig. 3.4b). 17 proteins were identified as part of the *rpr* 5' UTR RNP complex, and they are depicted in Table II and Table III. Among them several RNA-binding proteins were found and are depicted in Table II.

Table III

Other proteins identified by mass spectrometry in *rpr* 5' UTR- ribonucleoprotein complexes

Protein	BN	SC	PN	AC	Features	Biological processes
putative cytoplasmic aminopeptidase	16, 16´	189	4	CG7340	leucyl aminopeptidase (EC3.4.11.1) aminopeptidase activity (EC.4.11.-)	proteolysis and peptidolysis.
Dipeptidase C	16	50	1	CG5663	metallopeptidase family M24, Proline dipeptidase, Creatinase/prolidase N-terminal domain, creatinase/aminopeptidase	dipeptidyl-peptidase activity; X-Pro dipeptidase activity, proteolysis and peptidolysis gene transcribed in dying salivary glands
Lipophorin	8	56	2		lipid transporter activity	lipid transport
Tioredoxine reductase 1-spliced variant	16, 16´	101	4	G2151	mercuric reductase, Pyridine nucleotide-disulfide oxidoreductase, class I, FAD-dependent pyridine nucleotide-disulfide oxidoreductase, FAD/NAD(P)-binding domain, FAD/NAD-linked reductases, dimerization (C-terminal) domain	antioxidant activity; NOT glutathione-disulfide reductase activity; thioredoxin-disulfide reductase activity; sulfur metabolism; thioredoxin pathway INF an retinoic acid-induced cell death
Transketolase	12	29	1(*)	CG8036	transketolase, Thiamin diphosphate-binding fold (THDP-binding)	transketolase activity, pentosa-phosphate shunt
Enolase	18	95	4	CG17654	enolase C-terminal and N-terminal domain-like.	phosphopyruvate hydratase activity, glycolysis

BN, band numbering in Fig. 3.4b; SC, score; PN, number of peptides identified by LC/ESI-MS/MS; AC, gene denomination according to the *Drosophila* Annotated Database (flybase.org). (*) MS/MS on the Q-TOF revealed a Y-Type series of at least 4 amino acids. Peptide tolerance was 50 ppm and MS/MS tolerance was 50 mm.

La protein interacts with *rpr* 5´ UTR *in vitro* and affects *rpr* translation *in vivo*.

An important aspect in proteome research is the validation of the identified proteins. To validate the efficiency of the tobramycin-aptamer-tag purification method as a tool to identify proteins involved in translation regulation, we used the La antigen as proof-of-concept for our assay. La has been described to bind IRESs, but it is also ubiquitous and has a wide variety of targets. The RNA binding activity of *Dm*-La antigen was confirmed by cross-linking assays using radiolabelled 5´UTR derived from the IRES-containing mRNA *rpr*, and *Ubx*. *Ubx* translation is mediated by an IRES element (Hart and Bienz, 1996) and genetic evidence suggests that La may control *Ubx* mRNA translation (Bai and Tolias, 2000). Therefore, it was used as control. We determined that recombinant *Dm*-La antigen, but not BSA and GST crosslinked to *Ubx* (Fig. 3.5b) and *rpr* (Fig. 3.5c) 5´ UTRs. We also used the translation initiation factor 4B (eIF4B), an abundant factor that was not purified in our assay using *rpr* 5' UTR, but that binds *Ubx* 5' UTR (see Chapter 4). Supporting our purification data, we observed that eIF4B recognizes *Ubx* 5' UTR (Fig. 3.5b), while it does not bind *rpr* 5' UTR (Fig. 3.5c).

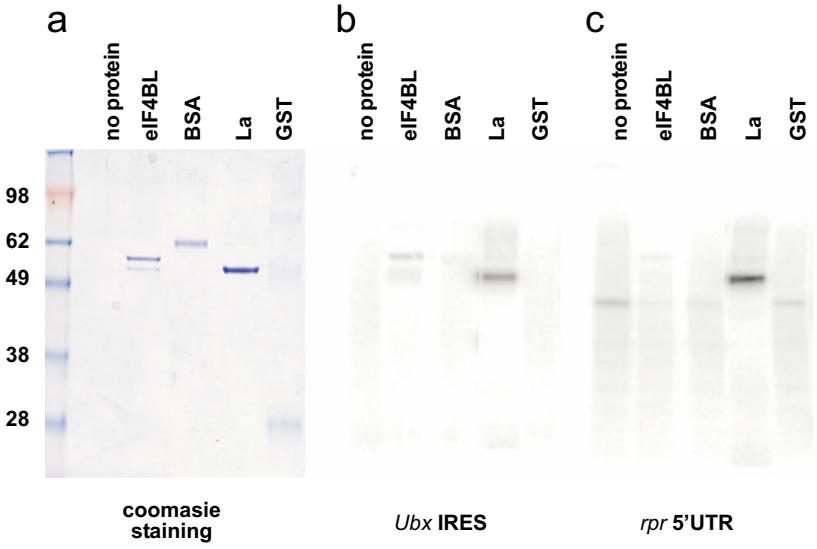


Figure 3.5. *Dm*-La protein binds to *rpr* 5´ UTR. Crosslinking experiments were carried out in the absence of protein or the presence of GST, BSA, recombinant *Dm*-eIF4B-L or *Dm*-La proteins, with radiolabelled *Ubx* **(b)** or *rpr* **(c)** 5´ UTRs. **(a)** Coomassie staining of the gel shown in (c). Molecular mass markers are shown on the left.

To further determine the role of *Dm*-La in *rpr* mRNA translation we used the RNAi (RNA interference) technique to knock down *Dm*-La from *Drosophila* S2 cells. The analysis of the effect on *rpr* translation was performed by double transfection of the *Dm*-La-defective cells with cap-dependent and *rpr*-dependent reporters mRNAs (Fig. 3.6a,b, upper panels). *Dm*-La antigen is a very abundant protein and requires a double RNAi transfection to significantly reduce the levels. After 72 hours post-transfection with dsRNA against *Dm*-La mRNA, the cells were transfected again with the same dsRNA and finally, after another 96 hours of incubation, they were transfected with the reporter mRNAs. Under this condition we reduced the amount of *Dm*-La antigen to less than 10% of the total protein, as assessed by Western Blot (Fig. 3.6a,b lower left panels).

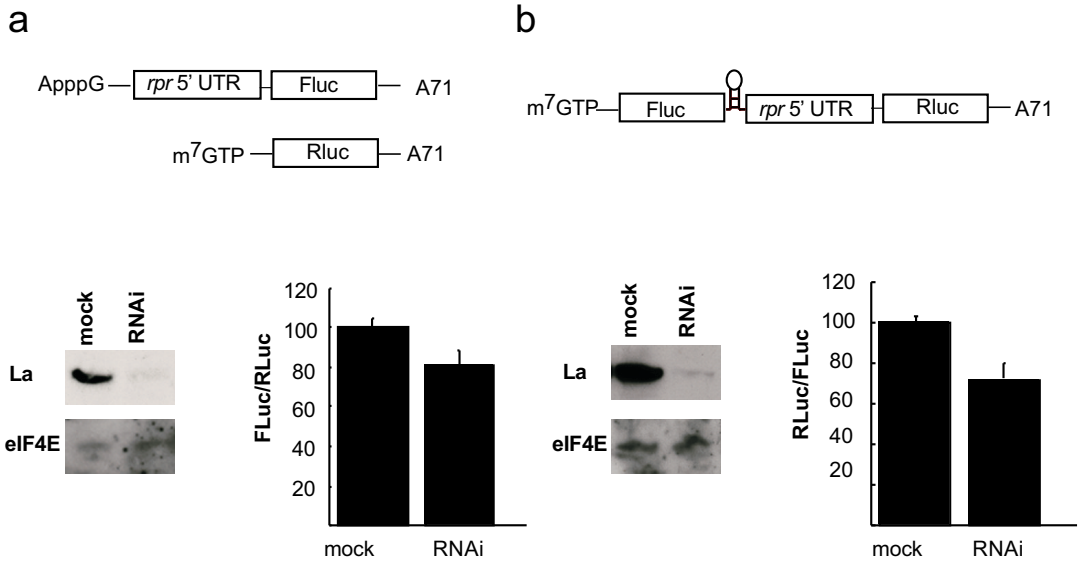


Figure 3.6. RNAi La knock down revealed a role for La protein on translation. *Drosophila* S2 cells were mock transfected or transfected twice with a mixture of the dsRNAs *Dm*-La as described (see Materials and Methods). After 96 h of the second round of transfections the cells were cotransfected with Cap-RLuc and ApppG-*rpr*-FLuc transcripts **(a)** or with FLuc/hairpin/*rpr*/RLuc alone **(b)**. Cells were harvested after 8 h of incubation and Luciferase activity and Western Blot was performed. Ratio of *rpr*-dependent and cap-dependent translation was determined as the ratio of FLuc/RLuc activities in (a) and RLuc/FLuc values in (b). The level of *Dm*-La knock down in (a) and (b), was assayed by Western blot using anti-*Dm*-La and anti-eIF4E antibodies as control.

Cap-dependent translation was measured by the activity of renilla luciferase (RLuc), which derived from a capped transcript without an active 5' UTR (Cap-RLuc). At the same time, the activity of *rpr* was measured by the activity of firefly luciferase (FLuc) derived from a reporter mRNA that contains a non-functional cap

structure (to prevent RNA degradation after transfection) and the 5' UTR of *rpr* (ApppG-*rpr*-FLuc). Transfection with two reporter RNAs is necessary to normalize the effect on the *rpr* reporter (Fig 3.6a, upper panel), since we noted that the transfection efficiencies between experiments are not comparable. The ratio of firefly luciferase (ApppG-*rpr*-FLuc) to renilla luciferase (Cap-RLuc) indicates the relative effect of reduced *Dm*-La. We observed a low, although significant reduction on the translation mediated by *rpr* 5' UTR (Fig. 3.6a, low right pane l). This observation showed that the efficiency of *rpr*-dependent translation against cap-dependent translation was reduced in cells devoid of *Dm*-La protein. The level of reduction can be attributed to the remaining *Dm*-La protein. La antigen is involved in other processes apart from translational control, such as processing, transport or nuclear retention of some transcripts, implying that the result described above might not necessarily represent translational control. Therefore, we used a dicistronic reporter Cap-FLuc/hairpin/*rpr*-RLuc, which displays the following two activities: cap-dependent in the first cistron (firefly luciferase, FLuc), and *rpr*-dependent in the second one (renilla luciferase, RLuc) in a single transcript (Fig. 3.6b, upper panel). In agreement with our previous observation, the efficiency of *rpr*-dependent translation compared to cap-dependent translation was reduced in S2 cells with reduced levels of La antigen (Fig. 3.6b, low right panel). We conclude that La antigen is required for the efficient translation of *rpr* mRNA, thus validating our proteomic approach for the identification of proteins relevant to translational control.

Discussion

The use of tobramycin-RNA-aptamers fused to an RNA under investigation has been successfully used for the purification of nuclear RNP complexes. However, one problem that arises using this technique to isolate cytoplasmic RNA-protein complexes is the likely binding of tobramycin to *Drosophila* 18S rRNA. Although tobramycin specifically affects prokaryote translation, the structure of the aptamer resembles a hairpin loop in 18S rRNA and the efforts to assemble and purify complexes in a solid support failed, likely due to the competition by 40S ribosomes. We used a method that assembles the translation reaction *in vitro* and separates RNP complexes by ultracentrifugation before affinity purification. This procedure proved useful for the isolation of RNPs and the identification of the protein components by MALDI-TOF-MS or LC/ESI-MS/MS.

Two RNA-binding proteins identified in our experiments have previously been reported to be involved in IRES-mediated translation in mammalian cells: CG10922 (*Drosophila* homolog of La-autoantigen) and CG12055 (glyceraldehyde 3P-dehydrogenase, GAPDH). La autoantigen is a conserved and abundant RNA-binding protein with high affinity for poly (U)-rich sequences. It is mostly nuclear, and associates with newly synthesized RNA polymerase III transcripts. It is involved in transcription termination, tRNA processing and transcript transport (Wolin and Cedervall, 2002). La antigen binds the 5´ UTR of several viral mRNAs and cellular mRNAs, such as Hepatitis C Virus (HCV) (Ali and Siddiqui, 1997; Ali *et al.*, 2000), poliovirus (Meerovitch *et al.*, 1993), encephalomyocarditis virus (ECMV) (Kim and Jang, 1999), coxsackievirus (Ray and Das, 2002), X-linked inhibitor of apoptosis (XIAP) (Holcik and Korneluk, 2000), and human immunoglobulin heavy chain-binding protein (Kim *et al.*, 2001) to stimulate translation. La protein is also involved in the regulation of translation of Terminal oligopyrimidine (TOP) mRNAs. TOP mRNAs encode for ribosomal proteins and other components of the translational apparatus, and bear a TOP stretch at the 5´end, which is necessary for the regulation of their translation. La protein binds TOP mRNAs *in vitro* (Pellizzoni *et al.*, 1996), activates their translation *in vivo* (Crosio *et al.*, 2000) and it is present on polysomes where it is associated with TOP mRNAs (Cardinali *et al.*, 2003). GAPDH is a housekeeping gene involved in glycolysis, but several studies indicate that it also participates in various cellular processes such as endocytosis, DNA replication and repair, and RNA transport, and translation (Sirover, 1999). GAPDH binds Hepatitis A Virus (HAV) 5´ UTR, which exhibits IRES activity, and the overexpression of GAPDH suppresses HAV IRES activity in transfected cells (Schultz *et al.*, 1996; Yi *et al.*, 2000). GAPDH also binds AU-rich RNAs in the 3´end of parainfluenza virus and Hepatitis C Virus (HCV), and the pre-genome of Hepatitis B virus (HBV) (De *et al.*, 1996; Zang *et al.*, 1998; Petrik *et al.*, 1999). The interaction with RNA is mediated by the NAD(+)-binding region of GAPDH. Therefore, it was not surprising other protein containing NAD/FAD binding domains CG2151 (thioredoxin reductase 1-spliced variant) was also found in the *rpr*-RNP. Interestingly, thioredoxin reductase is a mediator of retinoic acid and INF induced cell death in mammalian cells (Beere and Green, 2001), suggesting a role for the regulation of proapoptotic genes.

Other identified RNA binding proteins have not yet been implicated in IRES-dependent translation, such as CG5654 (*Drosophila* Y box protein, Ypsilon Schaetal,

Yps), CG6143 (*Drosophila* protein on ecdysone puffs, PEP), and CG12058 (multi sex combs, mxc). Yps is involved in the localization and translation of *oskar* mRNA (Mansfield *et al.*, 2002). PEP has not yet been implicated in translational control. It is associated with active ecdysone and heat shock-inducible puffs on polytene chromosomes (Amero *et al.*, 1993). It also binds to *hsp70* transcripts with high affinity (Hamann and Stratling, 1998). The latter observation suggested a role of PEP in mRNA transport or stability (Hamann and Stratling, 1998). In this regard, it is remarkable that *rpr* 5' UTR and *Drosophila hsp70* 5' UTR both exhibit a high degree of homology and a similar cap-independent translation mechanism (Chapter 1). Multi sex combs, finally, contains an La lupus like domain and RNA binding domain of the class RNP-1 and RBD. Although not yet directly related to translation, loss of Multi sex combs promotes uncontrolled malignant growth (Santamaria and Randsholt, 1995), an antagonist effect to the function of *rpr*. Moreover, the analysis of the *Drosophila* protein-protein interaction deducted from the *Drosophila* protein-protein interaction database ((Giot *et al.*, 2003), http://biodata.mshri.on.ca/fly_grid/) revealed that mxc interacts with nanos (nos), a protein involved in translational regulation in the early embryo (Johnstone and Lasko, 2001).

The ribosomal associated-protein Stubarista (CG14792) was also identified to be a component of the *rpr* 5´ UTR-RNP complex. Stubarista is the *Drosophila* homolog of the human ribosomal-associated protein p40, which may have a role in translation initiation, as suggested by Melnick *et al.* (1993) and Török *et al.* (1999) (Melnick *et al.*, 1993; Török *et al.*, 1999). Dm-p40 and its yeast homolog (*YST1*) are components of the small ribosomal subunit (Demianova *et al.*, 1996; Török *et al.*, 1999) and Dm-p40 might play a role during the assembly of the translational machinery on *rpr* 5' UTR-RNP complex.

Proteins involved in splicing were also purified. CG5352 and CG1249 belong to the Sm protein class. Sm proteins bind as heterometric complexes to various RNAs on recognizing short U-rich stretches. They assemble on the spliceosomal small nuclear (sn) RNAs forming the Sm core RNP complexes, which are essential for the stability and biogenesis of the snRNAs. In *Drosophila*, CG5352 (the small ribonucleoprotein B) and CG1249 (the small ribonucleoprotein D2) forms part of the U1 snRNP (Labourier and Rio, 2001). Recently it was reported that Hfq, a protein required for mediating translational repression driven by OxyS RNA on their target mRNAs in bacteria, shows the hallmarks of the Sm proteins (Zhang *et al.*, 2002). Hfq

is tightly associated with the ribosome and could increase the interaction between an sRNA and its target mRNA by bringing the sRNA into the proximity of the ribosome. *rpr* 5´ UTR also contains U rich sequences that could serve as a target to Sm protein interactions. As U1 snRNP displays an associate serine kinase activity that is specific for the SR (serine-arginine) domain of SF2/ASF proteins, it was not surprising to find SR proteins as part of the purified RNP complexes such as CG6987 (the *Drosophila* SF2 homolog), CG10203 (Xl6, a homolog to the human serine/arginine-rich 7 protein) and CG10851 (52K chromatin boundary protein, also called B52/SRp55 SR). The role of SR protein in translation is just emerging. It was reported that SR protein SF2/ASF is associated to polyribosomes and participates in translational control (Sanford *et al.*, 2004). The presence of an exonic enhancer (ESE) known to bind SF2/ASF in an mRNA can stimulate its translation *in vivo*. Software designed to predict the presence of SF2/ASF-binding sites (Cartegni *et al.*, 2003) allowed us to detect several potential binding sites within the *rpr* 5´ UTR.

Other proteins forming part of the *rpr* 5´ UTR RNP complex like a family of aminopeptidases and metabolic enzymes have not yet been reported to display either RNA binding activity or protein-protein contacts with the others putative *rpr* 5´ UTR interacting proteins (the absence of protein-protein interactions was deduced from the *Drosophila* protein-protein interaction database (Giot *et al.*, 2003), http://biodata.mshri.on.ca/fly_grid/). Therefore, with the exception of the above mentioned thioredoxin reductase-1, they cannot yet be fully interpreted and their possible role on mRNA metabolism will require further investigation. These proteins might play a role during translation, during the assembly of the translational machinery or in the priming of the mRNA before ribosome recognition. Our data shed light on the complexity of the early assembly of the translational machinery on mRNA.

The validation *in vitro* and *in vivo* of the La antigen, which was identified in complex with *rpr* 5' UTR, indicates that the approach is useful for the determination of protein assembled onto an mRNA. 5' and 3' UTRs are targets of regulatory factors that control translation. A systematic screen for RNA binding proteins will provide information about the proteins assembled in particular UTRs and consequently contribute to the decipherment of the translational control mechanisms.

Chapter 4

Two functionally redundant *Drosophila* eIF4B isoforms are involved in cap-dependent but not IRES dependent translation

To further understand translation initiation mechanisms in *Drosophila*, we analyzed one translation factor whose mammalian homolog has been described to be involved in the IRES driven translation of picornaviral RNAs, the translation initiation factor eIF4B.

eIF4B is part of the protein complex involved in the recognition and binding of the mRNA to the ribosome. Its main function is assumed to be an involvement in the scanning process since eIF4B stimulates ATP-dependent unwinding of the mRNA 5'UTR by eIF4F/eIF4A. eIF4B transiently associates with eIF4F (Grifo *et al.*, 1983; Jaramillo *et al.*, 1991; Methot *et al.*, 1994) and stimulates the ATP-dependent RNA-helicase activity of eIF4A and eIF4F *in vitro* (Abramson *et al.*, 1987; Lawson *et al.*, 1989; Rozen *et al.*, 1990; Jaramillo *et al.*, 1991; Altmann *et al.*, 1993; Pause *et al.*, 1994). There is also genetic evidence and *in vitro* experiments suggesting that eIF4B bridges eIF3 to the 40S subunit, thereby facilitating the binding of 40S ribosomes to the mRNA (Benne and Hershey, 1976; Coppolecchia *et al.*, 1993; Methot *et al.*, 1996; De la Cruz *et al.*, 1997). Although it is established that mammalian eIF4B is involved in IRES-dependent translation (Meyer *et al.*, 1995; Pestova *et al.*, 1996a; Ochs *et al.*, 1999; López de Quinto and Martínez-Salas, 2000; López de Quinto *et al.*, 2001; Ochs *et al.*, 2002), the possible participation on IRES-dependent translation of eIF4B from any other systems has not been characterized. For that reason, we investigated the role of *Drosophila* eIF4B on cap-dependent and IRES-dependent translation. *Drosophila eIF4B* gene is a single-copy gene that encodes two isoforms, namely eIF4B-L and eIF4B-S, generated by an alternative recognition of two polyadenylation signals during transcription termination and a subsequent alternative splicing of the two pre-mRNAs (Hernandez *et al.*, 2004).

Binding of Dm-eIF4B-L and Dm-eIF4B-S to RNA

Dm-eIF4B isoforms contain one putative RNA recognition motif (RRM) that is

conserved in other eIF4Bs (Hernandez *et al.*, 2004). To test whether Dm-eIF4B isoforms bind to RNA, RNA binding activity was assayed by cross-linking to the radiolabelled 5´UTR of *Drosophila Ultrabithorax* and *caudal* mRNA. As shown in Figs. 4.1a and 4.1b, both RNAs are crosslinked to Dm-eIF4B recombinant proteins, but not to GST.

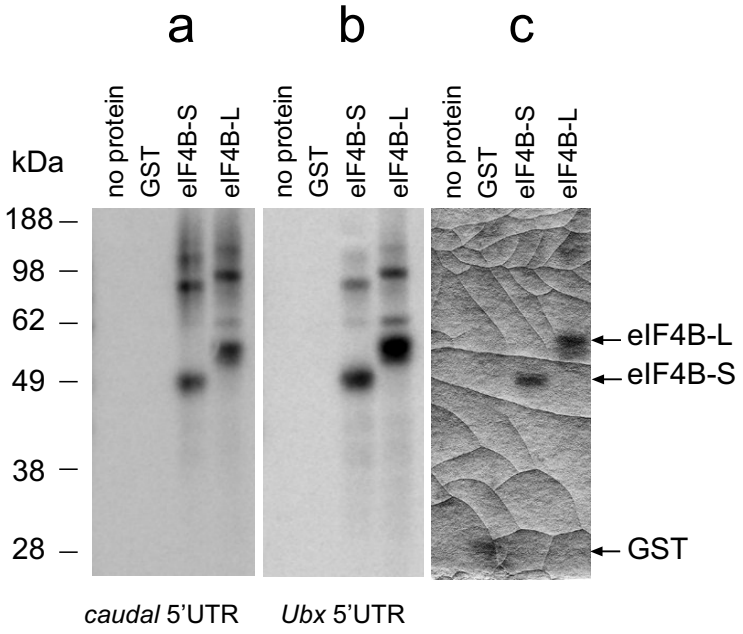


Fig. 4.1. Dm-eIF4B-L and Dm-eIF4B-S are RNA-binding proteins. (a-b) Crosslinking experiments were carried out in the absence of protein or the presence of GST, recombinant Dm-eIF4B-L or Dm-eIF4B-S proteins, with *caudal* **(a)** or *Ultrabithorax* **(b)** radiolabelled 5´UTRs. **(c)** Coomassie staining of the gel showed in (b). Molecular mass markers are shown on the left.

Redundant function of Dm-eIF4B-L and Dm-eIF4B-S in translation

In order to study the effect of Dm-eIF4B isoforms in cap- and IRES-dependent translation, we analyzed the effect of both proteins on the translation of different mRNA reporters in the *in vitro Drosophila* translation system. The cap-FLuc reporter was used to monitor the cap-dependent translation as shown in Fig. 4.2a. While the addition of BSA to the translation extracts did not have any effect on the translation of the reporter mRNA, translation of firefly luciferase (FLuc) mRNA increased up to two fold upon the addition of either recombinant Dm-eIF4B-L, Dm-eIF4B-S or an equimolecular mix of both to the translation system (Fig. 4.2a), indicating a positive, specific and redundant effect on cap-dependent translation.

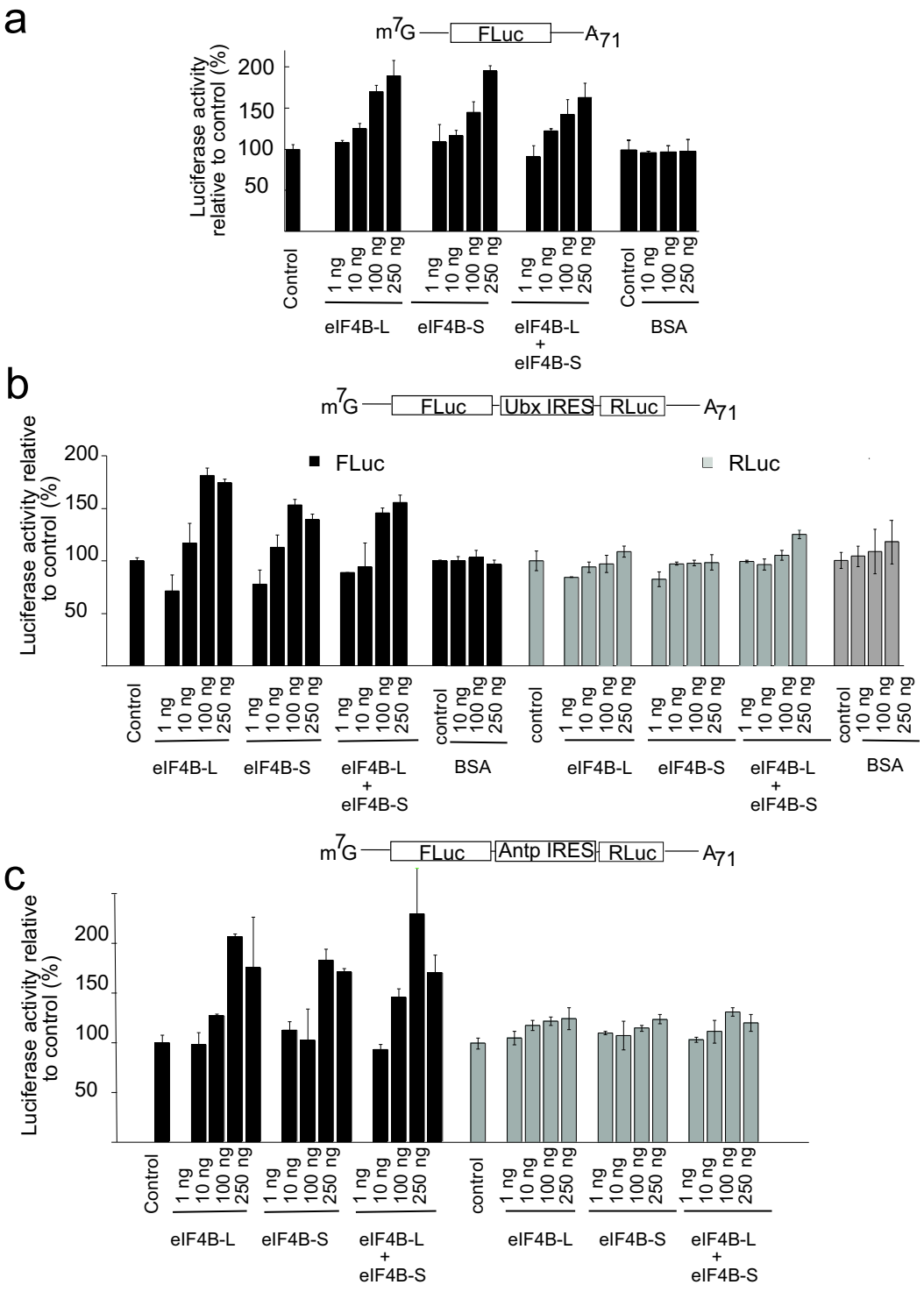


Fig. 4.2. Recombinant Dm-eIF4B-L and Dm-eIF4B-S enhance cap-dependent, but not IRES-dependent translation in *Drosophila* cell-free extracts. (a) Cap-dependent translation using cap-FLuc reporter mRNA in the absence or presence of increasing amounts of the indicated proteins. **(b-c)** Effect of the addition of the indicated proteins on cap-dependent (black bars, FLuc) and IRES-dependent (grey bars, RLuc) translation using the dicistronic mRNA (drawn on top of each figure) is shown. The data of two independent experiments are presented as percentage of control samples (no protein added).

To assess the IRES-dependent translation, we performed similar experiments but using a capped dicistronic reporter mRNA bearing firefly luciferase (FLuc) as the first cistron, and *Renilla* luciferase (RLuc) as the second cistron. The IRES of *Ultrabithorax (Ubx)* served as an intercistronic element. We then analyzed the effect of addition of recombinant eIF4B proteins or BSA as a control on FLuc/*Ubx*/RLuc mRNA in our translational assay. Simultaneous determination of both cap-dependent and IRES-dependent initiation indicated preferential translation of the first cistron when eIF4B-L or eIF4B-S, but not BSA was added (Fig. 4.2b, black bars). However, no significant effect on IRES-dependent initiation was detected upon addition of the Dm-eIF4B proteins or BSA (Fig. 4.2b, grey bars). Similar results were obtained using the IRES of *Antennapedia* (*Antp*) (Oh *et al.*, 1992) (Fig. 4.2c). We conclude that cap-dependent initiation is enhanced by Dm-eIF4B-L and Dm-eIF4B-S and that their effect on cap-dependent translation is equivalent. Our results also indicate that IRES-dependent translation is not affected by any of both forms of Dm-eIFB.

We then translated cap-FLuc and *Ubx*-FLuc mRNA in the presence of purified IgG against Dm-eIF4B-L (Fig. 4.3a-c). Addition of anti-Dm-eIF4B-L IgG (but not IgG purified from pre-immune serum) resulted in a two-fold decrease of cap-FLuc translation (Fig. 4.3a). Conversely, anti-Dm-eIF4BL IgG had only a minor effect on translation of *Ubx*-FLuc mRNA (Fig. 4.3b). Translation of the first cistron of dicistronic transcript FLuc/*Ubx*/RLuc (Fig. 4.3c, black bars) was also affected by anti-Dm-eIF4B-L, but had no significant effect on IRES-dependent translation of the second cistron (Fig. 4.3c, gray bars). Inhibition of cap-dependent translation caused by the anti-Dm-eIF4B-L IgG could be reversed to some extent by addition of recombinant Dm-eIF4B-L or Dm-eIF4B-S to the cell-free extract (Fig. 4.3d). The relative stimulation of luciferase synthesis obtained in the Dm-eIF4B-inhibited lysate was similar to that obtained in the non-inhibited lysate. Taken together, these results suggest that both Dm-eIF4B isoforms have a redundant positive effect on cap-dependent mRNA translation but do not intervene in IRES-dependent translation.

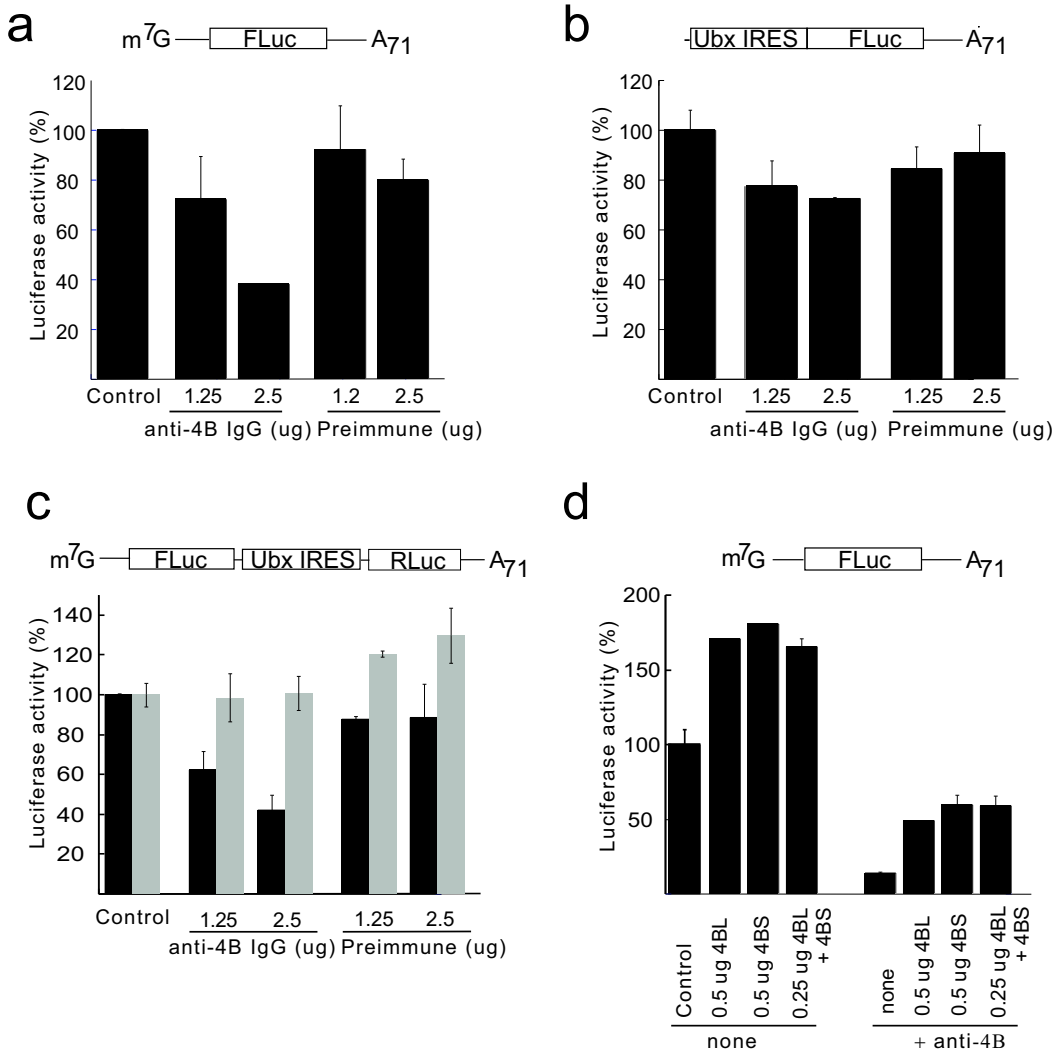


Fig. 4.3. Inhibition of cap- but not IRES-dependent translation by anti-eIF4B antibodies and rescue of the translational activity by recombinant Dm-eIF4B proteins. (a-c) Differential inhibition of cap-dependent *versus* IRES-dependent translation after incubating extracts with antibodies against *Drosophila* eIF4B. Translation of the monocistronic reporter mRNAs cap-FLuc **(a)**, *Ubx*-FLuc **(b)** and the dicistronic capped FLuc/*Ubx*/RLuc **(c)** in the absence or the presence of purified IgG fraction from pre-immune serum or from serum containing anti-eIF4B antibodies. In **(c)** cap- and IRES-dependent translation values are indicated as black and grey bars, respectively. **(d)** Rescue of the translational activity of an eIF4B-immunodepleted extract by addition of recombinant proteins Dm-eIF4B-L and Dm-eIF4B-S. The data of two independent experiments are represented as percentage of the control samples without adding protein.

Discussion

We have investigated the translation properties of two isoforms of eIF4B from *Drosophila melanogaster,* Dm-eIF4B-L and Dm-eIF4B-S. We observed that both Dm-eIF4B isoforms preferentially promote cap-dependent translation, which is sensitive to immunodepletion and the addition of recombinant protein Dm-eIF4B.

Since the excess of eIF4B or the presence of anti-Dm-eIF4B antibodies did not show a significant effect on the *in vitro* activity of *Ultrabithorax* and *Antennapedia*- IRES, we conclude that, eIF4B might not be involved in the translation of these mRNAs under normal conditions. *In vitro* binding of Dm-eIF4B to these two IRESs is likely due to unspecific RNA binding activity. However, this explanation can be controversial since Dm-eIF4BL didn't bind to rpr 5´ UTR (Chapter 2) but it does to *Ubx* IRES. Whether this implies a more important role of eIF4B for the translation of viral or some specific cellular IRESs in *Drosophila* has not been established and requires further investigation. On the other hand, a role of eIF4B in IRES dependent translation during conditions that compromised cap-dependent translation and where IRES-dependent translation is favored such as heat shock or apoptosis can not also be discarded.

In spite of these uncertainties, we can conclude that eIF4B has a role in cap-dependent translation in *Drosophila* embryonic extracts. It has been proposed that eIF4B, eIF4A and eIF4F are involved in the scanning process complementing the role of eIF1 and eIF1A, and that in the presence of eIF4F, eIF4B and eIF4A, scanning is less dependent on eIF1 and eIF1A (Pestova *et al.*, 1998a; Pestova and Kolupaeva, 2002). We observed that reduction of eIF4B decreases the level of cap-dependent translation while addition of recombinant eIF4B increases translation activity *in vitro*, arguing for a central although not exclusive role of eIF4B in the initiation process. Indeed RNAi-based knock-down of both isoforms of *Drosophila* eIF4B in cultured cells show reduced rates of protein synthesis and, accordingly of cell proliferation. Consistently, over-expression of Dm-eIF4B-L in *Drosophila* cell cultures and in developing eye imaginal discs promotes cell proliferation (Hernandez *et al.*, 2004). Although it was not proven that this effect is a direct effect due to enhanced translation rates, it correlates with our *in vitro* experiments demonstrating enhanced cap-dependent translation by the addition of recombinant Dm-eIF4B-L.

Conclusions

- The 5'UTR of *rpr* shares homology to the 5' UTR of *Drosophila hsp70* mRNA and both *rpr* and *hsp* 70 5' UTRs show IRES activity.

- The 5´UTR of the proapoptotic genes *hid* and *grim,* but not *sickle* 5' UTR, display IRES activity.

- We suggest that two antagonizing stress responses, one protecting (heat shock) and one eliminating the cells under risk (apoptosis), share a common mechanism of initiation of translation.

- We set up a protocol for purification of tagged-RNA-protein complexes from cytoplasmic extracts.

- Using this protocol we identified 17 proteins as part of the rpr 5´UTR RNP-complex and showed that La antigen is involved in the translation of *rpr.*

- Drosophila eIF4B promotes cap- but not IRES-dependent translation.

Summary

Control of protein synthesis is a key step in the regulation of gene expression during apoptosis and the heat shock response. Under such conditions, cap-dependent translation is impaired and Internal Ribosome Entry Site (IRES)-dependent translation plays a major role in mammalian cells. Although the role of IRES-dependent translation during apoptosis has been mainly studied in mammals, its role in the translation of *Drosophila* apoptotic genes has not been yet studied. The observation that the *Drosophila* mutant embryos for the cap-binding protein, the eukaryotic initiation factor eIF4E, exhibits increased apoptosis in correlation with up-regulated proapoptotic gene *reaper* (*rpr*) transcription constitutes the first evidence for the existence of a cap-independent mechanism for the translation of *Drosophila* proapoptotic genes. The mechanism of translation of *rpr* and other proapoptotic genes was investigated in this work.

We found that the 5'UTR of *rpr* mRNA drives translation in an IRES-dependent manner. It promotes the translation of reporter RNAs *in vitro* either in the absence of cap, in the presence of cap competitors, or in extracts derived from heat shocked and eIF4E mutant embryos and *in vivo* in cells transfected with reporters bearing a non functional cap structure, indicating that cap recognition is not required in *rpr* mRNA for translation. We also show that *rpr* mRNA 5'UTR exhibits a high degree of similarity with that of *Drosophila heat shock protein 70* mRNA (*hsp70*), an antagonist of apoptosis, and that both are able to conduct IRES-mediated translation. The proapoptotic genes *head involution defective* (*hid*) and *grim*, but not *sickle*, also display IRES activity. Studies of mRNA association to polysomes in embryos indicate that both *rpr*, *hsp70, hid* and *grim* endogenous mRNAs are recruited to polysomes in embryos in which apoptosis or thermal stress was induced. We conclude that *hsp70* and, on the other hand, *rpr*, *hid* and *grim* which are antagonizing factors during apoptosis, use a similar mechanism for protein synthesis. The outcome for the cell would thus depend on which protein is translated under a given stress condition. Factors involved in the differential translation driven by these IRES could play an important role. For this purpose, we undertook the identification of the ribonucleoprotein (RNP) complexes assembled onto the 5'UTR of *rpr* mRNA. We established a tobramycin-affinity-selection protocol that allows the purification of specific RNP that can be further analyzed by mass spectrometry. Several RNA binding proteins were identified as part of the rpr 5´UTR RNP complex, some of which have been related to IRES activity. The involvement of one of them, the La

antigen, in the translation of *rpr* mRNA, was established by RNA-crosslinking experiments using recombinant protein and *rpr* 5´UTR and by the analysis of the translation efficiency of reporter mRNAs in *Drosophila* cells after knock down of the endogenous La by RNAi experiments. Several uncharacterized proteins were also identified, suggesting that they might play a role during translation, during the assembly of the translational machinery or in the priming of the mRNA before ribosome recognition. Our data provide evidence for the involvement of La antigen in the translation of *rpr* mRNA and set a protocol for purification of tagged-RNA-protein complexes from cytoplasmic extracts. To further understand the mechanisms of translation initiation in *Drosophila*, we analyzed the role of eIF4B on cap-dependent and cap-independent translation. We showed that eIF4B is mostly involved in cap-, but not IRES-dependent translation as it happens in mammals.

Zusammenfassung

Die Kontrolle der Proteinsynthese ist ein entscheidender Schritt bei der Regulation der Genexpression während der Apoptose und bei der Hitzeschockantwort (*engl*. heat shock response). Unter diesen Bedingungen ist die „Cap"-abhängige Translation beeinträchtigt und die interne Ribosomen Eintrittsstellen-abhängige (*engl. I*nternal *R*ibosome *E*ntry *S*ite, *IRES*) Translation spielt eine wichtige Rolle. Während die Funktion der IRES-abhängigen Translation während der Apoptose in Säugern untersucht wurde, bleibt ihre Funktion bei der Translation von Apoptosegenen in *Drosophila* weitestgehend unerforscht. Die Beobachtung, dass die *Drosophila*-Mutante für das „Cap"-bindende Protein, den eukaryotischen Initiationsfaktor 4E (*engl*. *e*ukaryotic *I*nitiation *F*actor 4E, *eIF* 4E), während der Embryogenese verstärkt apoptotisch wirksam ist und in Korelation mit der hochregulierten Transkription des proapoptotischen Genes *reaper* (*rpr*) verläuft, ist der erste Hinweis für die Existenz eines „Cap"-unabhängigen Mechanismus bei der Translation proapoptotischer Gene in *Drosophila*. Daher war die Untersuchung des Translationsmechanismus von *rpr* und anderen proapoptotischen Genen Gegenstand dieser Arbeit.

Wir konnten feststellen, dass die 5'UTR der *rpr* mRNA die Translation auf eine IRES-abhängige Art und Weise steuert. Es treibt die Translation der Reporter-RNAs *in vitro* entweder in Abwesenheit des „Caps", in der Anwesenheit von „Cap"-Kompetitoren, oder in Extrakten, welche von hitzeschockbehandelten und eIF4E mutierten Embryonen stammen und *in vivo* in Zellen, welche mit einem Reporter transfiziert wurden, welcher eine nichtfunktionelle „Cap"-Struktur enthält, voran. Dies ist ein Hinweis darauf, dass die Erkennung des „Caps" in der *rpr* mRNA für die Translation nicht benötigt wird. Wir konnten zudem feststellen, dass die 5'UTR der *rpr* mRNA einen hohen Grad an Sequenzübereinstimmung mit dem Hitzeschockprotein 70 (*engl*. *h*eat *s*hock *p*rotein 70, *hsp*70), einem Antagonisten der Apoptose, aufweist, und dass beide Faktoren die Fähigkeit besitzen IRES-vermittelte Translation durchzuführen. Die proapoptotischen Gene „*h*ead *i*nvolution *d*efective" (*hid*) und *grim*, jedoch nicht *sickle*, zeigen auch IRES-Aktivität. Untersuchungen zur mRNA-Assoziation an Polysomen in Embryonen zeigten, dass die endogenen mRNAs von *rpr*, *hsp70*, *hid* und *grim*, in denen Apoptose oder thermaler Stress induziert wurde, an die Polysomen rekrutiert wurden. Wir kommen zu dem Schluß, dass zum einen *hsp70* und außerdem *rpr*, *hid* und *grim*, welche antagonisierende Faktoren während der Apoptose sind, von einem ähnlichen Mechanismus bei der Proteinsynthese Gebrauch machen. Die Folge für die Zelle hängt einerseits davon ab,

welches Protein unter den gegebenen Streßbedingungen translatiert wird, wobei Faktoren, welche an der durch IRES-gesteuerten differentiellen Translation beteiligt sind, dabei eine wichtige Rolle spielen könnten. Aus diesem Grund beschlossen wir die *R*ibo*n*ukleo*p*roteinkomplexe (*RNP*-Komplexe) zu identifizieren, welche auf der 5'UTR der *rpr* mRNA assembliert sind. Wir etablierten ein Protokoll zur Tobramycinaffinitätsselektion, das die Aufreinigung spezifischer RNPs ermöglicht, welche dann weiter mittels Massenspektrometrie analysiert werden können. Verschiedene RNA-bindende Proteine, von denen einige in Beziehung mit der IRES-Aktivität stehen, konnten als ein Teil des *rpr* 5'UTR RNP-Komplexes identifiziert werden. Die Beteiligung eines Proteins, des La Antigens, an der Translation der *rpr* mRNA, konnte mittels RNA-Quervernetzung unter Verwendung rekombinanter Proteine und der *rpr* 5'UTR und außerdem durch die Analyse der Translationseffizienz von Reporter mRNAs in *Drosophila* Zellen nach dem „Knockdown" des endogenen La Proteins mittels RNA*i* (*engl*. RNA *i*nterference), bestätigt werden. Es konnten zudem auch mehrere uncharakterisierte Proteine identifiziert werden, die möglicherweise eine Rolle während der Translation, während der Assemblierung der Translationsmachinerie oder bei der Markierung der mRNA vor der Erkennung durch das Ribosom spielen könnten. Unsere Daten geben einen Hinweis darauf, dass das La Antigen an der Translation der *rpr* mRNA unmittelbar beteiligt ist und stellen ein Protokoll für die Aufreinigung von RNA-Proteinkomplexen aus Zellextrakten bereit. Um ferner den Mechanismus der Translationsinitiation in *Drosophila* zu verstehen, analysierten wir den Einfluss des eIF4B (*engl*. eukaryotic Initiation Factor 4B) auf „Cap"-abhängige und „Cap"-unabhängige Translation. Wir konnten zeigen, dass eIF4B größtenteils an der „Cap"-, jedoch nicht der IRES-abhängigen Translation beteiligt ist.

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