REGULAR ARTICLE

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

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Translational control is a key step in gene expression regulation during apoptosis. To understand the mechanisms of mRNA translation of a pro-apoptotic gene, *reaper* (*rpr*), we adapted the tobramycin-aptamer technique described by Hartmuth *et al.* (*Proc. Natl. Acad. Sci. USA* 2002, *99,* 16719–16724) for the analysis of proteins interacting with *rpr* 5′ untranslated region (UTR). We assembled ribonucleoprotein complexes *in vitro* using translation extracts derived from *Drosophila* embryos and purified the RNA-protein complexes for mas spectrometry analysis. We identified the proteins bound to the 5′ UTR of *rpr*. One of them, the La antigen, was validated by RNA-crosslinking experiments using recombinant protein and by the translation efficiency of reporter mRNAs in *Drosophila* cells after RNAinterference experiments. Our data provide evidence of the involvement of La antigen in the translation of *rpr* and set a protocol for purification of tagged-RNA-protein complexes from cytoplasmic extracts.

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1 Introduction

Apoptosis is a cellular process required for cell selection and for organ and tissue development, but also serves as a mechanism for eliminating cells under stress or malfunctioning ones. Apoptosis has been studied in detail in *Drosophila melanogaster*, and the pattern of apoptotic cells during development has been established [1, 2]. Apoptosis has been related to the control of protein synthesis in various ways [3, 4]. Recent evidence suggests that at least two pro-apoptotic genes from *D. melanogaster*, *reaper* (*rpr*) and *grim*, down-regulate inhibitors of apoptosis (IAPs) by inhibition of general protein synthesis and, in addition, by protein degradation [5, 6]. Apoptosis and other stress-related processes result in a cellular environment characterized by degraded translation factors and impaired protein synthesis [4].

Translation of the majority of eukaryotic mRNAs requires the recognition of the 5′ cap structure (m^7GpppN) by the eukaryotic initiation factor 4E (eIF4E), which interacts with the adaptor protein eIF4G to eventually recruit the mRNA to the small ribosomal subunit. This mode of initiation of protein synthesis is termed cap-dependent. In addition, it has been demonstrated that some cellular mRNAs can be translated in a cap-independent manner. These mRNAs contain 5′ untranslated region (UTR) regulatory

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Abbreviations: IRES, internal ribosome entry site; **RNAi**, RNA interference; **RNP**, ribonucleoprotein; ***rpr***, *reaper*; **TOP**, track of oligopyrimidines; ***Ubx***, *Ultrabithorax*; **UTR**, untranslated region

sequences termed IRES that serve as a landing pad for ribosomes, which can then initiate translation employing a wide variety of translation factors and regulatory proteins [3]. During apoptosis, caspases cleave eIF4G and prevent its association with the cap-binding protein eIF4E. Under these conditions, cap-dependent translation is impaired and cap-independent, IRES-dependent translation plays a major role. The identification of the regulatory proteins that control cap-independent initiation during apoptosis is just emerging and will help to understand the mechanisms of translation.

In *Drosophila*, the pro-apoptotic gene *rpr* is translated in a cap-independent manner that resembles the translation of heat-shock proteins, and its 5' UTR displays IRES activity [7]. One of the main questions regarding the regulation of translation of pro-apoptotic 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. In this work, we adapted the tobramycin affinity-selection method developed by Hartmuth *et al.* [8] for the purification of native ribonucleo protein (RNP) complexes in human pre-spliceosomes to study the formation and components of RNP complexes assembled onto the 5' UTR of *rpr* mRNA. We established a protocol that allows the purification of complexes that can be further analyzed by MS. We identified several RNA binding proteins, some of which have been related to IRES activity. One of them, the La antigen, a ubiquitous RNA-binding protein, was validated as proof-of-concept for our technique. Knock down of La antigen in *Drosophila* cells by RNA interference (RNAi) provided evidence for its role during translation of *rpr* mRNA. 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 shed light on the complexity of the early assembly of the translational machinery on mRNA.

2 Materials and methods

2.1 Plasmids

Primers were designed to PCR amplify *rpr* 5' UTR mRNA and to introduce either a cassette containing a T7 promoter immediately up-stream of the *rpr* 5' UTR (5'-TAATACGACTCACTA TAGGGTGAATAAGAGAGACACCAGAACAAA-3') or the T7 promoter followed by the J6f1 tobramycin aptamer ([8]; underlined) (5'-TAATACGACTCACTATAGGGGCTTAGTATAGC GAGGTTTAGCTACACTCGTGCTGAGCCTGAATAAGAGA GACACCAGAACAAA-3'). In both cases the same primer was used from the 3' including the first ten amino acids of the *rpr* ORF (5'-CTGATCGGGTATGTAGAATGCCACTGCCATTGTT GTTGGTTTATCTTTCTTCG-3'). PCR fragments were cloned into the EcoRV site of vector pSL1180 (Amersham Biosciences, Freiburg, Germany) to create the plasmids pSL-T7-*rpr* and pSL-T7-TA-*rpr*. *Drosophila* La auto-antigen ORF was amplified by PCR from a *Drosophila* adult cDNA library and cloned into the EcoRV site of vector pBluescript SK(+) (Stratagene, La Jolla, CA, USA) to create the plasmid pBSK-La. Plasmids *rpr*-firefly luciferase (FLuc) and pFLuc/hairpin/*rpr*/renilla luciferase (RLuc) used in this study are described elsewhere [7].

2.2 RNA preparation and assembly of the RNP complexes

Aptamer-tagged or untagged RNAs were synthesized with the transcription T7 polymerase kit (Megascript; Ambion Austin, TX, USA) and the plasmids pSL-T7-TA-*rpr* and pSL-T7-*rpr* as templates, respectively. For quantification, [α-^{32}P]UTP (3000 Ci/mmol; 1 Ci = 37 GBq) was added to the reaction to 0.23 μM final concentration. After the transcription reaction (6 h, 37°C) the template was digested with DNAse I and the transcripts purified by LiCl precipitation at −20°C. Unincorporated nucleotides were removed by spin column chromatography (S-300 HR column; Amersham Biosciences). 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 as described [10]. Cell-free translation reactions were carried out as previously described [9]. 4× binding buffer (4× 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. RNPs were assembled *in vitro* using either radiolabeled *rpr* 5' UTR or aptamer-*rpr* 5' UTR and embryonic translation extracts. Three hundred fifty microliters of 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 min, 140 pmoles of radiolabeled aptamer-tagged or nontagged RNAs were added and further incubated for 10 min at 25°C. The reaction was then loaded on top of a 10 mL 10–30% sucrose gradient prepared in 1× BP containing 145 mM KCl and 4 mM $MgCl_2$ and centrifuged in a Beckman Ti-SW41 rotor (15 h, 25 K, 4°C). Continuous UV absorbance was recorded at 254 nm 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 was determined by Cerenkov counting of the radioactivity in each fraction. Fractions corresponding to the RNP complex were pooled; usually four fractions of 400 μL were pooled and incubated with 200 μL of tobramycin matrix (see below).

2.3 Tobramycin affinity purification of RNP complexes

N-hydroxysuccinimide-activated Sepharose 4 Fast Flow was derivatized with 5 mM tobramycin as described [11]. All procedures were performed at 4°C. Aliquots of tobramycin matrix (140–200 μL) were blocked overnight with 1.5 mL of blocking buffer (1× BP; 300 mM KCl; 0.1 mg/mL tRNA; 0.5 mg/mL

BSA; 0.01% NP-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 (1× BP; 145 mM KCl; 5 mM $MgCl_2$). The bound complexes were eluted with 400 μL of elution buffer (1× 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 were eluted *per* gradient loaded. Proteins were recovered by ethanol precipitation and analyzed by SDS/10–13% PAGE and silver staining.

2.4 MS

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 LC-coupled ESI tandem MS (LC-MS/MS) on a Q-ToF Ultima instrument (Waters, Milford, MA, USA) as described [8]. The corresponding proteins were identified by searching against all entries in the National Center for Biotechnology Information nonredundant database by using MASCOT (Matrix Science, London, UK) as a search engine and confirmed by cross-search in the *Drosophila* database FlyBase (flybase.org).

2.5 Recombinant protein expression and UV-crosslinking

Dm-La protein was expressed in *Escherichia coli* using pTrCHisA-La and purified as described [12]. The protein was dialyzed against 20 mM HEPES pH 7.8; 0.15 mM EDTA; 10% glycerol; 0.01% NP-40). For cross-linking experiments, ^{32}P-labeled RNA probes (*Ultrabithorax* (*Ubx*) and *rpr*) were generated by transcription of linearized *Ubx*-pBSK and *rpr*-pBSK with the T7 Mega transcription kit (Ambion) and α-[^{32}P]ATP and α-[^{32}P]UTP (Amersham Biosciences). RNA probes were digested with RNAse-free DNAse I (Ambion) and further purified using the RNeasy kit (Qiagen, Hilden, Germany). RNA integrity was assessed by agarose gel electrophoresis. RNA probes were dissolved 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; Mg^{2+} concentration in the reaction varied from 0.25 to 0.375 mM), RNA (600 000 cpm, previously treated for 15 min at 70°C) and either 1.5 μg Dm-eIF4B [13], *Dm*-La, GST, or BSA (Roche, Mannheim, Germany). After incubation for 15 min at room temperature, the samples were transferred to ice and irradiated for 35 min at 254 nm. The reactions were digested for 45 min at room temperature with 1 μL of a mixture of RNAse A (1 μg/μL)/RNAse T1 (5 μg/μL). The complexes were resolved in 10% SDS-PAGE and imaged in a Phosphoimager.

2.6 RNAi and cell transfection

Sense and antisense RNAs were prepared from linearized pBSK-La using the Ampliscribe mRNA transcription kit (Biozym Diagnostics, Olendorf, Germany) in the presence of m^7GpppG (New England BioLabs, Beverly, MA, USA), digested with DNAse I and purified using the RNeasy kit (Qiagen). 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. *Drosophila* Schneider S2 cells (1×10^6) were transfected in a 35 mm dish with 10 μg of dsRNA using the Effectene reagent (Qiagen). Control cells were mock transfected with the Effectene reagent alone. Twenty-four hours after transfection, the medium was removed, the cells were resuspended in 3.5 mL of medium, and split into three dishes. Seventy-two hours 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. Ninety-six hours after the second dsRNA transfection, the cells were transfected by triplicate with 5 μg of mRNA reporters. Reporter transcripts were synthesized from linearized *rpr*-FLuc, pRLuc, and pFLuc/hairpin/*rpr*/RLuc plasmids using the T3 Ampliscribe mRNA transcription kit (Biozym Diagnostics) in the presence of m^7GpppG or ApppG (New England BioLabs), digested with DNAse I and purified using the RNeasy kit (Qiagen). Eight hours after transfection, cells were harvested and assayed for reporter activity and Western blot. Reporter gene expression (firefly and sea pansy luciferase) was determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) and detected in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA, USA). Western blot analysis was performed loading 5 μg of protein *per* lane and revealed with rabbit anti-*Drosophila* La antibody [12] and using anti-*Drosophila* eIF4E-1 [14] as control.

3 Results and discussion

3.1 Isolation of RNP complexes assembled onto the 5' UTR of *rpr* from embryonic cytoplasmic extracts

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 [8]. We introduced a 40 nt RNA aptamer that binds with high affinity to the aminoglycoside antibiotic tobramycin (hereafter referred as TA) at the 5' end of a sequence bearing the *rpr* 5' UTR and the ten first amino acids of the ORF (aptamer-tagged *rpr* 5' UTR, TA-*rpr*). The choice of the 5' or 3' end of the target RNA for the aptamer fusion needs to be evaluated either by experimental trial of both fusions or by

previous functional analysis of the target RNA. We decided to fuse the aptamer at the 5' end for two reasons: (i) the fusion of a hairpin to the 5' end of *rpr* 5' UTR does not affect the capacity of *rpr* 5' UTR to drive translation of the second cistron of a dicistronic reporter [7], (ii) the analysis of the predicted secondary structure after the fusion does not show changes in the individual structure of both aptamer and *rpr* 5' UTR (data not shown). It only remained to be determined whether the aptamer binding capacity was affected by the fusion of *rpr* 5' UTR. Thus, we assayed first the binding of radiolabeled TA-*rpr* and nontagged *rpr* to tobramycin-derivatized Sepharose. Sixty percent 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 (data not shown). More than 80% of the bound TA-*rpr* could be eluted with 5 mM 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 because it released more than 50% of pre-bound TA-*rpr* from the tobramycin matrix, which bound nonspecific ribosomal RNAs and proteins. This is most 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 RNAs in the translation extracts competes with TA-*rpr* for the binding to the matrix and results in the release of TA-*rpr* from the matrix. This represents a drawback of the technique when 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 (Fig. 1). To isolate the earliest steps of the assembly, 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 pre-initiation 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 min of incubation of the RNA with cytoplasmic extract in the absence of inhibitors the RNA was present in RNP particles that sedimented as an ~20S complex (Fig. 2a). The incorporation of RNA into RNPs was increased in the presence of inhibitors, which block the initiation steps of translation (Fig. 2b). This indicates that the RNP represents a step prior to the assembly of the ribosome. 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. 2d) was much more efficient than the binding of untagged *rpr* (0.27%, Fig. 2c), validating the appropriateness of the approach. Almost no TA-*rpr* was washed away and the elution rate represented 65% of the bound RNA (Fig. 2d). 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). 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. 3a): 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 types of protein purification [15].

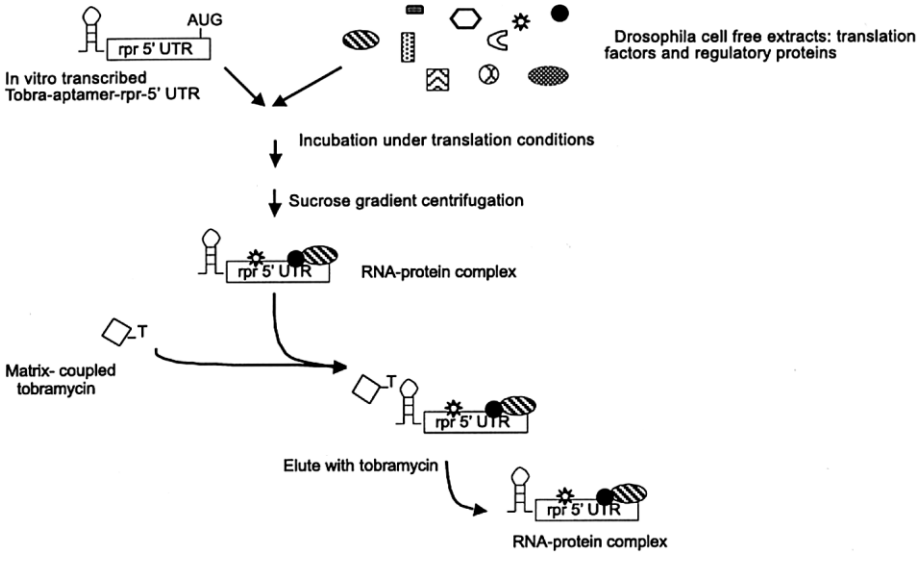

Figure 1. Scheme of the RNP purification strategy.

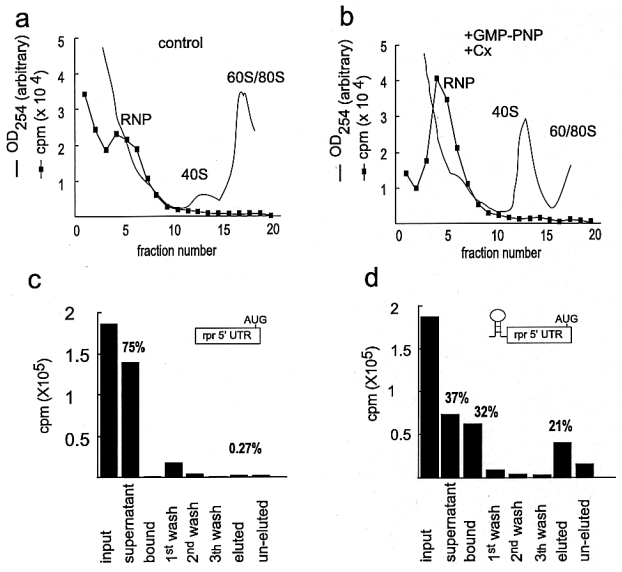

Figure 2. Purification of RNPs assembled on *rpr* 5′ UTR. Sucrose gradient separation of RNP containing radiolabeled tobramycin-aptamer-tagged RNA in the absence (a) or presence (b) of GMP-PNP and cycloheximide. Absorbance was recorded at 254 nm (black lines) and the amount of RNA was measured by Cherenkov 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 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.

3.2 *rpr* 5′ UTR-interacting factors isolated by tobramycin-affinity-selection

To identify the proteins forming part of the RNP complex, the procedure was scaled up to 60 pmoles of bound tagged-*rpr* 5′ UTR (Fig. 3b). The eluted proteins were purified, fractionated by SDS-PAGE, and analyzed by LC-MS/MS (Fig. 3b). Seventeen proteins were identified as part of the *rpr* 5′ UTR RNP complex (Tables 1, 2), among them several RNA-binding proteins (Table 1).

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 [16]. La antigen binds the 5′ UTR of several viral mRNAs and cellular mRNAs, such as hepatitis C virus (HCV) [17, 18], poliovirus [19], encephalomyocarditis virus (ECMV) [20], Coxsackie virus [21], X-linked inhibitor of apoptosis (XIAP) [3, 22], and human immunoglobulin heavy chain-binding protein [23] to stimulate translation. It was also reported that La is present on polysomes where it is associated with TOP mRNAs [24, 25]. GAPDH is a housekeeping gene involved in glycolysis, but several studies indicate that it also participates in various cellular processes including mRNA transport and translation [26]. GAPDH binds hepatitis A virus (HAV) 5′ UTR, which exhibits IRES activity, and the overexpression of GAPDH suppresses HAV IRES activity in transfected cells [27, 28]. GAPDH also binds AU-rich RNAs in the 3′ end of parainfluenza virus, HCV, and the pregenome of hepatitis B virus (HBV) [29–31]. The interaction with RNA is mediated by the NAD(+)-binding region of GAPDH. Therefore, it was not surprising that thioredoxin reductase 1-spliced variant, other protein containing NAD/FAD binding domains, was also found in the *rpr*-RNP. Interestingly, thioredoxin reductase is a mediator of retinoic acid and INF induced cell death in mammalian cells [32], suggesting a role for the regulation of other pro-apoptotic genes.

Other proteins identified are not yet directly related to IRES-dependent translation, such as the products encoded by the genes 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 [33]. PEP is associated with active ecdysone and heat shock-inducible puffs on polytene chromosomes [34] and also binds to *hsp70* transcripts with high affinity [35]. The latter observation suggested a role of PEP in mRNA transport or stability [35]. 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 [7]. MXC, finally, contains a La lupus-like domain and RNA binding domains of the class RNP-1 and RBD. Although not yet directly related to translation, loss of MXC promotes uncontrolled malignant growth [36], 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 ([37], http://biodata.mshri.on.ca/fly_grid/) revealed that MXC interacts with Nanos (NOS), a protein involved in translational regulation in the early embryo [38]. The ribosomal associated-protein CG14792 (Stubarista, STA) was also identified to be a component of the *rpr* 5′ UTR–RNP complex. STA 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.* [39] and Török *et al.* [40]. Dm-p40 and its yeast homolog (*YST1*) are components of the small ribosomal subunit and Dm-p40 might play a role during the assembly of the translational machinery on *rpr* 5′ UTR–RNP complex [40, 41]. These proteins might represent enhancers or repressors of *rpr* mRNA translation during early phases of the translational machinery assembly, possibly regulating the assembly of the 40S ribosomal subunit onto the mRNA.

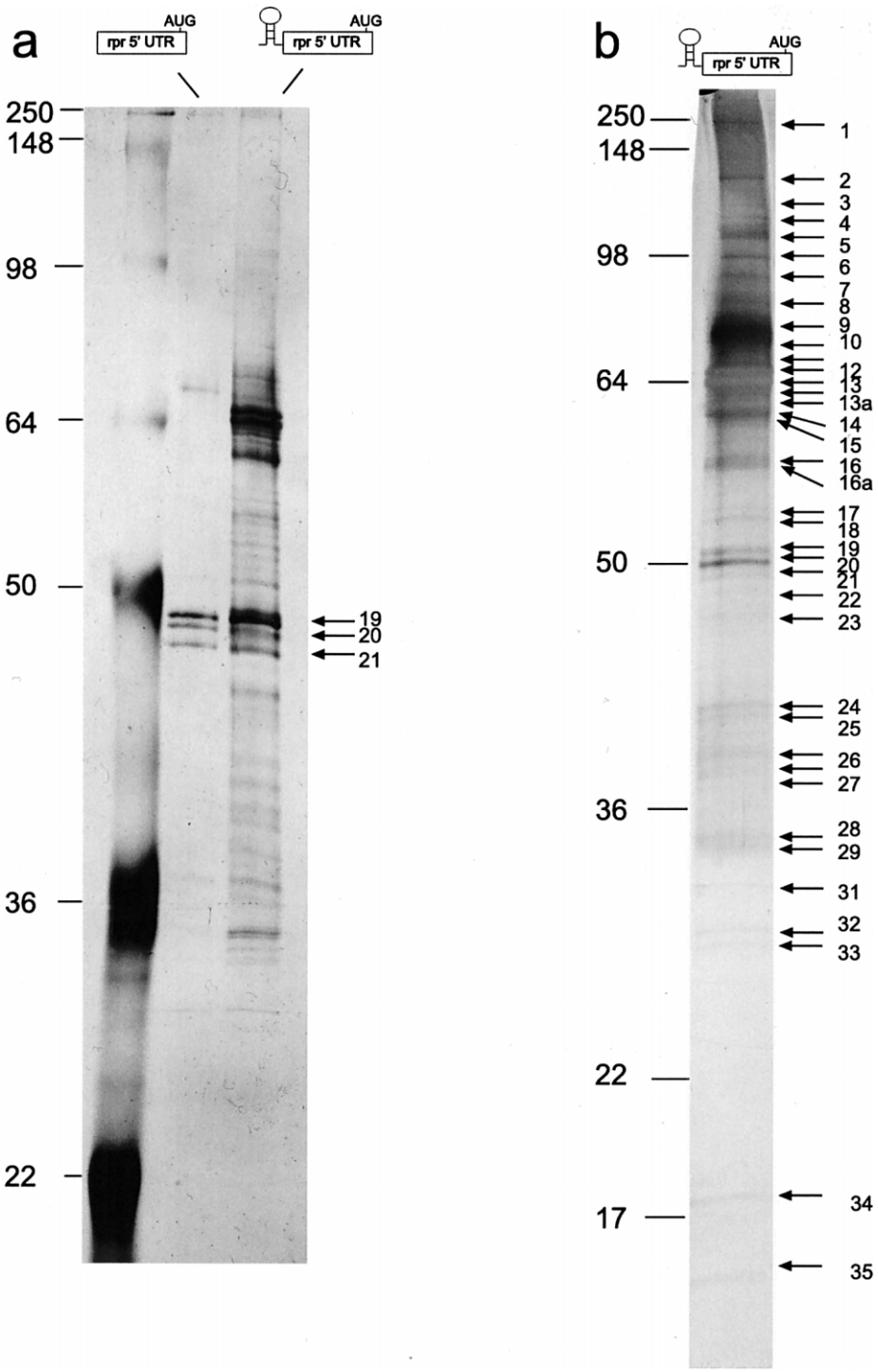

Figure 3. Protein content of RNP complexes eluted with tobramycin. (a) RNPs assembled on the untagged (left lane) or tagged (right lane) RNAs were incubated with the tobramycin matrix. After binding and washing the complexes were subsequently eluted with tobramycin. Binding of 20 pmol of RNA was achieved for the tagged RNA. Proteins were analyzed by PAGE and visualized by silver staining. (b) Scaled up purification of the reaction to reach 60 pmol of RNA bound to the matrix in order to perform MS analysis. Arrowheads and numbers represent the bands further analyzed by MS. 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.

Proteins involved in splicing were also purified. The products of the genes CG5352 and CG1249 belong to the Sm protein class, which bind as heterometric complexes to various RNAs recognizing short U-rich stretches. *rpr* 5' UTR also contains U-rich sequences that could serve as a target to Sm protein interactions. In *Drosophila*, CG5352 (the small ribonucleoprotein B) forms part of the U1 snRNP [42]. Recently, it was reported that Hfq, a protein required to mediate translational repression driven by OxyS RNA on their target genes in bacteria, shows the hallmarks of the Sm proteins [43]. 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. U1 snRNP displays an associate serine kinase ac-

Table 1. RNA-binding proteins identified by MS in *rpr* 5′ UTR-ribonucleoprotein complexes

Protein	BN	SC	PN	AC	Features	Biological processes
RNA binding proteins						
YPS	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
PEP	5, 6	208	5	CG6143	RNA-binding, C_2H_2 Zn-finger domain	Binding to hsp70 mRNA and DNA
La-autoantigen like	16, 16′	52, 58	1[c], 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
MXCs	6	174	4	CG12058	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[b], 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[a]	CG14792	Ribosomal S2 domain	Structural constituent of the 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 ribonucleoprotein particle protein B	32	52	1	CG5352	Sm motif, pre-mRNA splicing factor activity	Nuclear mRNA splicing, via spliceosome
Putative small ribonucleoprotein 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[a]	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. 3b; SC, score; PN, number of peptides identified by LC-MS/MS; AC, gene denomination according to the *Drosophila* annotated database (flybase.org).

a) MS/MS on the Q-TOF revealed a Y-type series of at least four amino acids. Peptide tolerance was 50 ppm and MS/MS tolerance was 50 mm.

b) Observed with low score and one peptide matched in two bands.

c) La-autoantigen protein was confirmed by MALDI-MS in two independent affinity purification experiments.

Table 2. Other proteins identified by MS 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
Thioredoxin reductase 1-spliced variant	16, 16′	101	4	CG2151	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	Anti-oxidant activity; NOT glutathione-disulfide reductase activity; thioredoxin-disulfide reductase activity; sulfur metabolism; thioredoxin pathway; INF an retinoic acid-induced cell death
Transacetolase	12	29	1[a)]	CG8036	Transketolase, thiamin diphosphate-binding fold (THDP-binding)	Transketolase activity, pentose-phosphate shunt
Enolase	18	95	4	CG17654	Enolase *C*-terminal and *N*-terminal domain-like	Phosphopyruvate hydratase activity, glycolysis

BN, band numbering in Fig. 3b; SC, score; PN, number of peptides identified by LC-MS/MS; AC, gene denomination according to the *Drosophila* annotated database (flybase.org).

a) MS/MS on the Q-TOF revealed a Y-type series of at least four amino acids. Peptide tolerance was 50 ppm and MS/MS tolerance was 50 mm.

tivity that is specific to the SR domain of SF2/ASF proteins. Therefore, it was not surprising to find SR proteins as part of the purified RNP complexes such as the ones encoded by CG6987 (the *Drosophila* SF2 homolog), CG10203 (Xl6, a homolog to the human serine/arginine-rich 7 protein), and CG10851 (52 K active chromatin boundary protein, also called B52/SRp55). The role of SR protein in translation is just emerging. While this manuscript was in preparation, it was reported that SR protein SF2/ASF is associated to polyribosomes and participates in translational control [44]. 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 [45] allowed us to detect several potential binding sites within the *rpr* 5′ UTR (data not shown).

Other proteins found forming part of the 5′ UTR *rpr* RNP complex, such as a family of aminopeptidases and metabolic enzymes, have not yet been reported to display either RNA binding activity or protein-protein contacts with the other putative *rpr* 5′ UTR interacting proteins (the absence of protein-protein interactions was deducted from the *Drosophila* protein-protein interaction database [37], see http://biodata.mshri.on.ca/fly_grid/). Therefore, with the exception of the previously mentioned thioredoxin reductase 1-spliced variant, they cannot yet be fully interpreted and their possible role on mRNA metabolism will require further investigation.

3.3 La protein interacts with *rpr* 5′ UTR *in vitro* and affects *rpr* translation *in vivo*

A critical aspect in proteome research is the validation of the identified proteins. To validate the efficiency of the tobramycin-aptamer-tag purification method 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 is also ubiquitous and has a wide variety of targets, which makes it a typical case for validation.

The RNA binding activity of *Dm*-La antigen was confirmed by crosslinking assays using radiolabeled 5′ UTRs derived from *rpr* and *Ubx* mRNAs. *Ubx* translation is mediated by an IRES [46], and genetic evidence suggests that La may control *Ubx* mRNA translation [47]. Therefore, it was used as control. We determined that recombinant *Dm*-La antigen, but neither BSA nor GST crosslinked to *Ubx* (Fig. 4b) and *rpr* (Fig. 4c) 5′ UTRs. We also used the translation initiation factor 4B (eIF4B), a factor that was not puri-

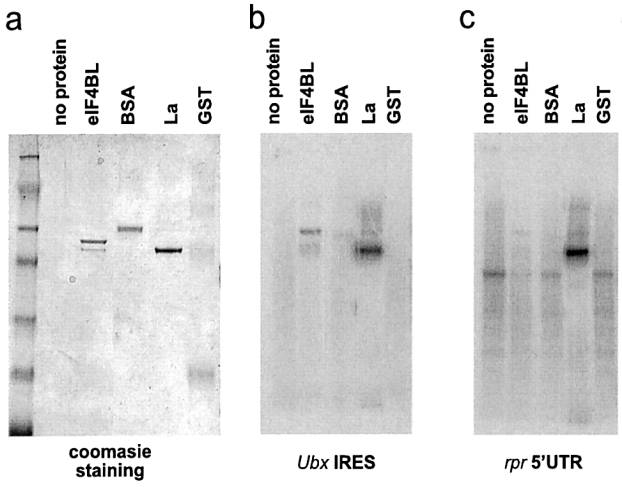

Figure 4. *Dm*-La protein binds to *rpr* 5′ UTR. Crosslinking experiments were carried out in the absence of protein or the presence of GST, BSA, recombinant *Dm*-eIF4B-L or *Dm*-La proteins, with radiolabeled *Ubx* (b) or *rpr* (c) 5′ UTRs. (a) Coomassie staining of the gel shown in (c). Molecular mass markers are shown on the left.

fied using TA-*rpr,* but that binds *Ubx* 5′ UTR [13]. Supporting our data, eIF4B recognizes *Ubx* 5′ UTR (Fig. 4b), while it does not bind *rpr* 5′ UTR (Fig. 4c).

To further determine the role of *Dm*-La in *rpr* mRNA translation we used the RNAi 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 reporter mRNAs (Fig. 5a and b, upper panels). *Dm*-La antigen is a highly abundant protein and requires a double RNAi transfection to significantly reduce the levels. After 72 h post-transfection with dsRNA against *Dm*-La mRNA, the cells were transfected again with the same dsRNA and finally, after another 96 h of incubation, they were transfected with the reporter mRNAs. In this condition, we reduced the amount of *Dm*-La antigen to less than 10% of the total protein, as assessed by Western blot (Fig. 5a and b, lower left panels). Cap-dependent translation was measured by the activity of RLuc, which derived from a capped transcript (Cap-RLuc). At the same time cap-independent activity of *rpr* 5′ UTR was measured by the activity of FLuc derived from a reporter mRNA bearing the 5′ UTR of *rpr* (ApppG-*rpr*-FLuc) and a nonfunctional cap structure (ApppG) that prevents the assembly of the eIF4F complex and, at the same time, prevents RNA destabilization. Simultaneous transfection with the two reporter mRNAs is required to normalize the effect on the *rpr* reporter (Fig. 5a, upper panel). The ratio of firefly luciferase (ApppG-*rpr*-FLuc) to renilla luciferase (Cap-RLuc) will indicate the relative effect of reduced *Dm*-La. A statistically significant, although not dramatic, reduction on the translation mediated by *rpr* 5′ UTR is measured at low levels of La protein (Fig. 5a, lower right panel). This observation indicates that the efficiency of *rpr*-dependent translation against cap-dependent translation is reduced in cells devoid of *Dm*-La protein. The remaining level of FLuc can be attributed to the fact that 10% of endogenous *Dm*-La, a very abundant protein, is still enough to promote some level of translation. We must note that the absolute values for each experiment are not comparable between transfections. La

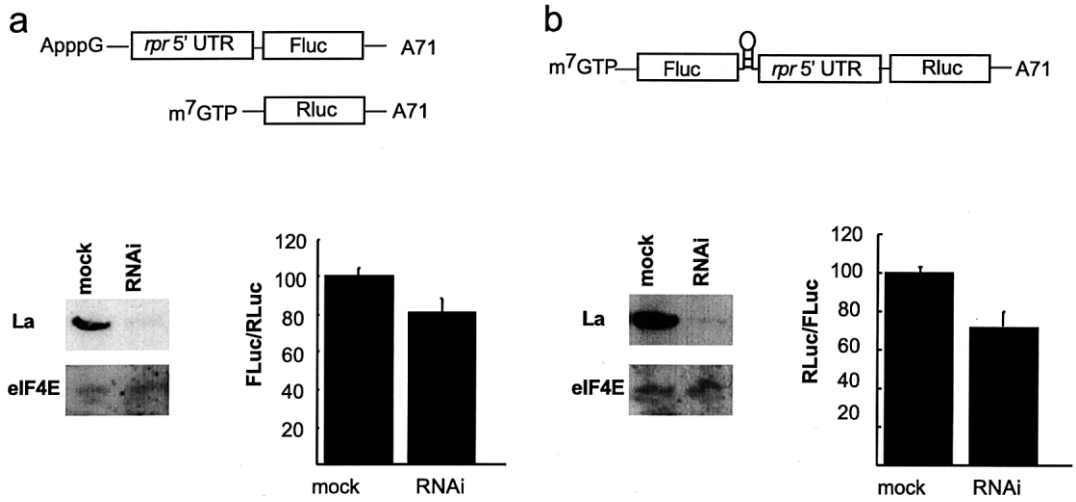

Figure 5. RNAi La knock down reveals a role for La protein on translation. *Drosophila* S2 cells were mock transfected or transfected twice with a mixture of the dsRNAs *Dm*-La as described (see Section 2). After 96 h of the second round of transfection the cells were cotransfected with Cap-RLuc and 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.

antigen is involved in other processes apart from translational control, such as processing, transport, or nuclear retention of some transcripts. This could imply 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 (FLuc) and *rpr*-dependent in the second one (RLuc) in a single transcript (Fig. 5b, upper panel). In agreement with our previous observation, the efficiency of *rpr*-dependent translation compared to cap-dependent translation is reduced in S2 cells with reduced levels of La antigen (Fig. 5b, low right panel). We, therefore, concluded that La antigen is required for the efficient translation of *rpr* mRNA, which validates our proteomic approach for the identification of proteins relevant to translational control. We are currently performing a complete screening using RNAi to determine the function of the other RNA-binding proteins identified in our assay.

4 Concluding remarks

The use of tobramycin-RNA-aptamers, fused to an RNA under investigation has been successfully used for the purification of nuclear RNP complexes [8] and a similar approach has also been described for the purification of yeast splicing factors using a streptomycin aptamer [48]. However, one problem that arises when using this technique to isolate cytoplasmic RNA-protein complexes is the likely binding of tobramycin (and, expectedly, streptomycin) 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 to be useful for the isolation of RNPs and the identification of the protein components by MALDI-TOF MS or LC-MS/MS. 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 the target of regulatory factors that control translation. A systematic screen for RNA binding proteins will provide information about the proteins assembled in particular UTRs and, in consequence, contribute to decipher the mechanisms of control.

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