METHOD

Highly efficient in vitro translation of authentic affinity-purified messenger ribonucleoprotein complexes

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ABSTRACT

Cell-free systems are widely used to study mechanisms and regulation of translation, but the use of in vitro transcribed (IVT) mRNAs as translation substrates limits their efficiency and utility. Here, we present an approach for in vitro translation of messenger ribonucleoprotein (mRNP) complexes affinity purified in association with tagged mRNAs expressed in mammalian cells. We show that in vitro translation of purified mRNPs is much more efficient than that achieved using standard IVT mRNA substrates and is compatible with physiological ionic conditions. The high efficiency of affinity-purified mRNP in vitro translation is attributable to both copurified protein components and proper mRNA processing and modification. Further, we use translation inhibitors to show that translation of purified mRNPs consists of separable phases of run-off elongation by copurified ribosomes and de novo initiation by ribosomes present in the translation extracts. We expect that this in vitro system will enhance mechanistic studies of eukaryotic translation and translation-associated processes by allowing the use of endogenous mRNPs as translation substrates under physiological buffer conditions.

Keywords: in vitro translation; RNA affinity purification; mRNP; *Pseudomonas* phage 7; RNA-binding protein; RNP purification

INTRODUCTION

Eukaryotic protein synthesis is a dynamic molecular process that occurs in three stages: initiation, elongation, and termination/recycling. Cell-free protein expression systems have long been used to study mechanisms and regulation of each step of mRNA translation. Traditional in vitro translation protocols involve incubation of protein-free in vitro transcribed (IVT) mRNAs in translation-competent cytoplasmic extracts (Katzen et al. 2005). The application of RNA interference and CRISPR/Cas technology now allows for relative ease in targeted manipulation of *trans*-acting factors within cell-free translation systems, bolstering their utility for understanding contributions of individual proteins (Rakotondrafara and Hentze 2011). However, existing protocols may fail to recapitulate important mechanistic and regulatory features of eukaryotic translation as they lack mRNA substrates that have undergone proper assembly into ribonucleoprotein complexes (mRNPs).

In all cells, dynamic RNA–protein associations are responsible for carrying out mRNA function and regulation (Moore 2005). RNA-binding proteins have been found to impact each stage of translation, from initiation to recycling (Gehring et al. 2017). Moreover, the ribosome engages in extensive communication with additional mRNP components to determine

rates of mRNA decay (Shoemaker and Green 2012). As mRNP assembly is a context-dependent, highly orchestrated process (Mitchell and Parker 2014), it is important to study mRNPs that have undergone physiological biogenesis in order to accurately study mRNA translation and decay. In addition to the possibility that nonphysiologically assembled mRNPs may fail to recapitulate important processes, previous work has suggested that poor in vitro translation efficiency could in part be due to inappropriate assembly of transcripts into mRNPs upon addition of nonphysiological, IVT mRNA into highly concentrated translation extracts (Panthu et al. 2015). Components of mRNPs could enhance translation through direct mechanisms, such as the role of the exon junction complex protein MLN51 in stimulating translation by engaging eIF3 (Chazal et al. 2013), or by serving more general structural functions that are currently not well understood (Panthu et al. 2015). Thus, a cell-free protein expression system is needed in which endogenously assembled mRNP complexes can be isolated from cells and introduced as substrates for in vitro translation.

RNA-based affinity purification systems have been valuable tools for the isolation and characterization of endogenously assembled RNPs (Slobodin and Gerst 2010; Yoon and Gorospe 2016; Panchapakesan et al. 2017). In particular, a system based on the *Pseudomonas* phage 7 (PP7) coat protein and its cognate hairpin binding site has been used to

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Article is online at <http://www.rnajournal.org/cgi/doi/10.1261/rna.065730.118>.

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investigate proteins associated with noncoding RNAs and the targeted degradation of transcripts by the nonsense-mediated mRNA decay pathway (Hogg and Collins 2007a,b; Hogg and Goff 2010; Ge et al. 2016). The primary use of such techniques has involved identification of mRNP components by mass spectrometry, but the ability to isolate endogenous mRNPs under conditions optimized to maintain RNA–protein interactions raises the possibility that affinity-purified mRNPs may also be used for downstream functional assays.

Here we demonstrate that affinity-purified mRNP complexes can be substrates for efficient cell-free protein synthesis. By monitoring production of NanoLuciferase (NLuc) protein from PP7-purified mRNPs (Hall et al. 2012), we show that affinity-purified mRNPs are rapidly translated in cytoplasmic extracts prepared from human cell lines (Rakotondrafara and Hentze 2011). A comparison to standard IVT mRNA substrates reveals a greater than 100-fold translation preference for correctly packaged mRNPs. Notably, maximal mRNP translation activity occurs in physiological K^+ and Mg^{2+} ionic concentrations, in contrast to the nonphysiological salt requirements of IVT mRNAs. Further, use of standard small molecule inhibitors of translation, harringtonine and puromycin, indicates that the translation activity of purified mRNPs arises from both elongation by copurified ribosomes and de novo initiation in extracts. We expect our in vitro system will provide a versatile tool to study contributions of mRNA-binding proteins and RNA modifications to protein synthesis and translation-dependent processes under cellular ionic conditions.

RESULTS AND DISCUSSION

Isolation and characterization of endogenously assembled mRNP complexes

To develop a system allowing translation from highly purified genuine mRNP substrates, we began by assessing the composition of affinity-purified mRNPs assembled on tagged mRNAs expressed in human cells (Hogg and Collins 2007b; Hogg and Goff 2010). We used the PP7 coat protein and its cognate RNA hairpin binding site to isolate complexes associated with two different mRNAs containing GFP open reading frames: one with an unstructured 5'UTR that supports efficient translation (GFP-PP7) and one containing a strong cap-proximal RNA structure (HP-GFP-PP7; $\Delta G = -35$ kcal/mol) that prevents translation initiation (Fig. 1A, B; Supplemental Fig. 1A; Babendure et al. 2006). As a control, purifications were performed using extracts lacking tagged mRNAs (mock). Importantly, only minor background purification of proteins was observed in mock samples, indicating that the mRNPs were purified to near-homogeneity (Fig. 1B). Both SDS-PAGE and mass spectrometry revealed significant differences in composition between the two tagged mRNAs, consistent with their distinct translational states (Fig. 1B; Supplemental Table 1). Specifically, the translationally inac-

FIGURE 1. Isolation and characterization of endogenously assembled mRNPs. (A) Schematic of PP7-tagged mRNAs used as mRNP purification substrates. mRNAs with an unstructured 5'UTR are efficiently translated (*top*), while mRNAs with a stable 5' hairpin are translationally repressed (*bottom*). (B) SDS-PAGE gel stain of proteins copurified with PP7-tagged mRNAs with (+) and without (–) a cap-proximal structure that prevents translation initiation. Mock purifications from extracts lacking tagged mRNAs were performed as controls.

tive mRNAs were associated with fewer low-molecular-weight ribosomal proteins, while recruiting several additional RNA-binding proteins. Mass spectrometry of the purified complexes identified more than 160 proteins represented by at least five peptide spectral counts in each sample. While the makeup of individual mRNPs within the purified population will be heterogeneous, this level of protein identification makes it possible to perform downstream functional assays on complexes whose typical composition has been determined with high confidence.

Affinity-purified mRNPs are functional substrates for in vitro translation

To allow for easy manipulation of translation reaction components, we elected to use established methods for in-house generation of translation-competent extracts from human tissue culture cells (Pisarev et al. 2010; Rakotondrafara and Hentze 2011). For the experiments described herein, actively dividing HEK-293 cells were harvested at subconfluency, and cytoplasmic extracts were prepared as previously described (Rakotondrafara and Hentze 2011). As substrates for in vitro

FIGURE 2. In vitro translation of endogenously assembled mRNPs under physiological buffer conditions. (A) Scheme for in vitro translation of endogenously assembled mRNPs. Distinct cell populations were used to express and assemble PP7-tagged mRNAs into mRNPs (*top*) and produce translation-competent extracts (*bottom*). Following affinity purification, mRNPs immobilized on magnetic beads were introduced to extracts for cell-free translation. Translation can result both from copurified ribosomes (blue) and de novo initiation of ribosomes present in translation extracts (orange). (B) Affinity-purified NLuc mRNPs were incubated in translation extracts in the presence of 1.5 mM Mg²⁺ and 135 mM K⁺, and NLuc luminescence was monitored at the indicated time points. Results from seven independent experiments are shown; error bars indicate SD. (C) Affinity-purified NLuc mRNPs were translated under the indicated K⁺ concentrations, with constant 1.5 mM Mg²⁺. Results from two independent experiments are shown; error bars indicate SD. (D) mRNP translation reactions as above performed with the indicated Mg²⁺ concentrations and 135 mM K⁺. Results from two independent experiments are shown; error bars indicate SD.

translation of endogenously assembled mRNPs, we used PP7-tagged mRNAs encoding the small, bright, rapidly folding NLuc protein (Hall et al. 2012). We transfected HEK-293 cells with constructs expressing tagged NLuc mRNAs from the strong CMV promoter, prepared whole-cell extracts, and isolated the tagged mRNPs using tandem protein A-tagged PP7 coat protein under similar conditions to those used for mass spectrometry. The mRNPs bound to magnetic beads were then transferred to translation extracts and incubated at 37°C for translation (Fig. 2A). Following the shift to 37°C, we observed rapid increases in the NLuc signal, illustrating that this system can be used for quantitative assessment of translation occurring on endogenously assembled mRNPs with high temporal resolution (Fig. 2B).

Maximal in vitro mRNP translation activity depends upon physiological K⁺ and Mg²⁺ ionic concentrations

The report describing the in vitro translation extracts used here recommended use of 50 mM potassium acetate (KOAc)

and 2.5 mM magnesium acetate (MgOAc) as a starting point for optimization (Rakotondrafara and Hentze 2011). As these values deviate from commonly cited intracellular concentrations of ~140 mM K⁺ and ~1 mM free Mg²⁺, we next tested the mono- and divalent ion requirements for efficient translation of mRNP substrates (Hille 2001; Romani 2011). In these and subsequent experiments, we determined relative translation activity by calculating the fold increase in luminescence at the indicated time points. This approach corrects for differences in raw luminescence values caused by minor variability of mRNA expression levels among biological replicate transfections. In line with physiological conditions, increasing potassium concentration led to increased mRNP translation activity, up to the highest concentration tested of 170 mM (Fig. 2C). Lowering Mg²⁺ from 2.5 mM to 1.5 mM had a minimal effect on translation activity, but concentrations below 1.5 mM impaired NLuc production (Fig. 2D). Because physiologically relevant levels of potassium and magnesium ions (135 mM K⁺ and 1.5 mM Mg²⁺) supported high levels of translation, we used these conditions for further experiments.

In vitro translation of purified mRNPs is much more efficient than standard IVT mRNA substrates

To allow comparison to standard in vitro translation methods, we used T7 polymerase to transcribe the same NLuc mRNA sequence in vitro. The in vitro transcribed mRNAs (IVT mRNAs) were subsequently capped and polyadenylated to promote translation. In contrast to the behavior of the mRNP described above, translation of IVT mRNAs was strongly inhibited by physiological buffer conditions (Fig. 3A). For appropriate comparison of translation efficiencies

from IVT and mRNP substrates, we therefore used 50 mM KOAc and 2.5 mM MgOAc for translation of IVT mRNAs. To determine the efficiency of translation from mRNP and IVT mRNA substrates, we quantified the amounts of mRNA present in mRNP purifications by northern blotting, using the IVT mRNA as a standard (Supplemental Fig. 1B). Division of NLuc values by the quantities of NLuc mRNA present in each reaction revealed that translation from the purified mRNPs was dramatically more efficient than from the IVT mRNAs, with a greater than 100-fold preference for translation from the mRNPs (Fig. 3B).

To test whether enhanced translation of the mRNP substrates could in part be due to proper capping, polyadenylation, or other covalent mRNA modifications, we phenol-extracted the mRNA from the affinity-purified mRNPs and subsequently introduced the protein-stripped mRNA to translation extracts. Similar to the IVT mRNA, affinity purified but protein-free mRNA was translated five- to 10-fold more poorly under physiological ionic strength conditions than in buffer supplemented with 50 mM KOAc and 2.5 mM MgOAc (Supplemental Fig. 1C). Comparing translation of protein-stripped and IVT mRNAs translated under low-ionic strength conditions to translation of purified mRNPs under physiological ionic conditions, the endogenously expressed mRNA was translated with greater efficiency than the capped and polyadenylated IVT mRNA, but was still much less efficiently translated than the intact mRNP substrates, particularly at early time points (Fig. 3B,C; Supplemental Fig. 1D). These data indicate that endogenous mRNA capping, polyadenylation, or other covalent modifications contribute to enhanced translation efficiency of purified mRNPs, but that optimal translation of mRNPs under physiological conditions requires copurified protein components.

FIGURE 3. Translation of mRNPs is much more efficient than that of standard IVT mRNA substrates. (A) Quantification of IVT NLuc mRNA translation, with recommended 2.5 mM Mg²⁺ and 50 mM K⁺ versus physiological 1.5 mM Mg²⁺ and 135 mM K⁺. Results from two independent experiments are shown; error bars indicate SD. (B) NLuc activity produced by translation of mRNPs or in vitro transcribed mRNAs was normalized to mRNA content quantified by northern blotting to determine translation efficiency. Results from seven (mRNP) and four (IVT) independent experiments are shown; error bars indicate SD. (C) NLuc activity produced by translation of mRNPs or protein-stripped mRNAs was normalized to mRNA content quantified by northern blotting to determine translation efficiency. Results from four independent experiments are shown; error bars indicate SD.

Translation activity of purified mRNPs is attributable to both elongation from copurified ribosomes and de novo initiation

Our mass spectrometry experiments indicated that 80S ribosomes could be copurified with translation-competent tagged mRNPs, suggesting that the mRNPs were bound to ribosomes in the act of translation (Figs. 1B, 4A; Supplemental Table 1). Further, we noticed that the initial rates of NLuc production from the mRNP and IVT mRNA substrates were different, with the mRNP immediately producing active NLuc protein and the IVT mRNA expressing active NLuc after a lag period of 2.5 min (Fig. 4B). These findings suggested that translation of the mRNPs could be divided into two phases: elongation from copurified ribosomes and de novo initiation of ribosomes present in the translation extracts.

We next devised strategies to disrupt different steps in mRNP translation to test this hypothesis and develop approaches capable of studying translation performed by these distinct pools of ribosomes (Fig. 4A). To abolish the contribution of run-off translation from mRNP-bound ribosomes,

FIGURE 4. mRNP substrates for in vitro translation allow independent analysis of run-off translation and de novo initiation. (A) Scheme for mRNP purification and in vitro translation to separate run-off translation from de novo initiation. mRNP complexes were affinity purified in the absence or presence of EDTA and then introduced to cell-free extracts with no translation inhibitor added, puromycin, or harringtonine. The effects of these treatment conditions on run-off translation versus de novo initiation are shown on the right. Ribosomes copurified with mRNPs are shown in blue, while ribosomes provided by cytoplasmic translation extracts are orange. (B) Representative data from Figure 4E and Supplemental Figure 2C, expanded to show initial rates of mRNP translation during the first 5 min. (C) Quantification of NLuc mRNP translation, with mRNPs purified in the absence of EDTA treatment and translation extracts supplemented with puromycin (+) or ddH₂O (–). Results from two independent experiments are shown; error bars indicate SD. (D) Quantification of NLuc mRNP translation, with mRNPs purified in the presence of EDTA and translation extracts supplemented with puromycin (+) or ddH₂O (–). Results from two independent experiments are shown; error bars indicate SD. (E) Quantification of NLuc mRNP translation, with mRNPs purified in the absence of EDTA treatment and translation extracts supplemented with harringtonine (+) or DMSO (–). Results from two independent experiments are shown; error bars indicate SD. (F) Quantification of NLuc mRNP translation, with mRNPs purified in the presence of EDTA and translation extracts supplemented with harringtonine (+) or DMSO (–). Results from two independent experiments are shown; error bars indicate SD.

we treated the purified mRNPs with EDTA prior to their incubation with translation extracts. Either alone or with EDTA pretreatment of purified mRNPs, we also treated translation extracts with puromycin, an aminoacyl tRNA analog that binds the ribosome A-site and causes premature chain termination and ribosome release to inhibit all translation elongation (Cannon 1968; Blobel and Sabatini 1971). Similarly, we inhibited initiation in translation extracts with harringtonine, which traps initiated 80S complexes at the start codon by blocking recruitment of subsequent aminoacyl tRNAs and interfering with peptidyl-transferase activity (Fresno et al. 1977).

As expected, puromycin treatment completely prevented translation from the mRNPs, whereas EDTA pretreatment disrupted the initial phase of mRNP translation but allowed robust activity over time (Fig. 4C,D; Supplemental Fig. 2A,B). Translation of IVT mRNAs was completely inhibited by both puromycin and harringtonine, as protein synthesis is limited to de novo initiation on these mRNAs (Supplemental Fig. 2C, D). Importantly, harringtonine treatment of extracts used to translate mRNPs purified with intact ribosomes (i.e., –EDTA) allowed an initial burst of translation activity followed by a plateau, consistent with the hypothesis that the initial phase of mRNP translation is due to elongation by copurified ribosomes. In contrast, harringtonine prevented any translation of mRNPs that had been pretreated with EDTA (Fig. 4E,F). Together, these results suggest that the initial phase of translation from mRNPs is driven by run-off translation from copurified ribosomes, after which initiation by extract-provided ribosomes predominates. Moreover, these data indicate that, while maximal translation of purified mRNPs involves both run-off and de novo translation, high

translation efficiency of mRNP substrates is also achieved when all translation is initiated by extract-derived ribosomes (Supplemental Fig. 2A,B).

The NLuc mRNAs used in the preceding experiments are minimal transcripts containing a short, vector-derived 3'UTR. To test whether the use of mRNPs as substrates also promotes translation of mRNAs with more complex features, we constructed translation reporters in which the NLuc ORF was introduced to a widely used β globin minigene containing two efficiently spliced introns and either the 111 nt β -globin 3'UTR or the ~1.3 kb 3'UTR of the human nonsense-mediated decay factor SMG5 (Lykke-Andersen et al. 2000; Singh et al. 2008; Hogg and Goff 2010; Ge et al. 2016). As with the minimal NLuc mRNAs, the spliced β globin mRNAs were much more efficiently translated from either untreated or EDTA-pretreated mRNPs than from IVT mRNAs (Fig. 5A,B; Supplemental Fig. 2E,F). Interestingly, the magnitude of translation enhancement observed with mRNP translation was similar across all of the mRNAs tested, even though the β globin-derived mRNAs were less efficiently translated than the minimal NLuc mRNAs in each condition (cf. Fig. 5A,B to Fig. 3B and Supplemental Fig. 2C). Together, these findings show that mRNAs with distinct sequences and features are preferentially translated as affinity-purified mRNPs and suggest that this method will serve as a flexible tool for studies of translation.

Conclusions

Together, our data show that affinity-purified mRNP complexes assembled in vivo can be used as highly efficient substrates for in vitro translation under physiological conditions.

FIGURE 5. mRNP substrates with more complex features also promote translation in vitro. (A) Schematic of the PP7-tagged NLuc β wt β TP mRNA used as a mRNP purification substrate (top). NLuc activity produced by translation of the NLuc β wt β TP mRNPs in the presence (+) or absence (–) of EDTA or the corresponding in vitro transcribed mRNAs was normalized to mRNA content quantified by northern blotting to determine translation efficiency (bottom). Results from two (–EDTA) and five (+EDTA and IVT) independent experiments are shown; error bars indicate SD. (B) Schematic of the PP7-tagged NLuc β wtSMG5TP mRNA used as a mRNP purification substrate (top). NLuc activity produced by translation of the NLuc β wtSMG5TP mRNPs in the presence (+) or absence (–) of EDTA or the corresponding in vitro transcribed mRNAs was normalized to mRNA content quantified by northern blotting to determine translation efficiency (bottom). Results from two (–EDTA) and four (+EDTA and IVT) independent experiments are shown; error bars indicate SD.

A combination of this approach with mass spectrometry allows sensitive determination of the protein composition of the purified mRNPs, which can in turn be readily controlled by depletion or overexpression of factors in the cells used to express the tagged mRNAs. We expect that this flexibility will enable mechanistic studies of the impact of specific mRNA-binding proteins and RNA modifications in the regulation of translation and mRNA stability. Further, this system can be manipulated with standard small molecule inhibitors of translation to isolate distinct phases of translation and to study contributions of copurified and extract-derived ribosomes. These findings suggest that this approach may also be useful for studies of specialized ribosomes and tissue-specific ribosome-associated factors (Shi and Barna 2015).

MATERIALS AND METHODS

Plasmids

Plasmids for expression of PP7-tagged GFP mRNAs and the PP7 coat protein were previously described (Hogg and Goff 2010). The NLuc ORF (Promega) was introduced into the HindIII-XbaI sites of pcDNAGFP, replacing the GFP ORF. For β globin minigene-derived plasmids, the NLuc ORF was introduced into the HindIII site of pc β wt β (Lykke-Andersen et al. 2000) or pc β wtSMG5TP (Ge et al. 2016) by the sequence and ligation-independent cloning method (Li and Elledge 2007), such that the β globin 5'UTR was deleted and the NLuc ORF was fused in frame to the β globin ORF. To allow mRNP purification, the PP7 hairpin was inserted into the XbaI site of the resulting pcNLuc β wt β plasmid, downstream from the wild-type β globin 3'UTR.

Cell culture and transfections

For in vitro translation reactions, HEK-293 Tet-off cells (632107; Clontech) were cultured in DMEM (11965-092; Gibco) supplemented with 10% FBS (A31606-02; Gibco) and 1% penicillin/streptomycin with L-Glutamine (10378-016; Gibco). Transfection of cells with pcNLucTP, pcNLuc β wt β TP, or pcNLuc β wtSMG5TP was performed by calcium phosphate as previously described (Hogg and Collins 2007a,b). Expression of the PP7-tagged GFP mRNAs was validated by cotransfecting HEK-293 Tet-off cells with pLVX-mCherry plasmid (Clontech) using Lipofectamine 3000 Reagent (Invitrogen). Cells were harvested 24-h post-transfection in 1 $\times$ Passive Lysis Buffer (Promega) and the levels of GFP and mCherry fluorescence detected using an Infinite F200 Pro microplate reader and associated i-control 1.9 software (Tecan).

mRNP purification and preparation of IVT RNA

PP7-based mRNP purification was performed as previously described (Hogg and Goff 2010). For SDS-PAGE, complexes were eluted with RNase A/T1 Cocktail (AM2286; Ambion), diluted 1:50 in HLB150, as previously described (Hogg and Goff 2010). For mass spectrometry, samples were eluted in HLB supplemented with 1 M NaCl. For isolation of protein-stripped mRNAs, PP7-based mRNP purifications were performed and mRNA phenol-

extracted using TRIzol (15596026; Invitrogen). Capped and polyadenylated in vitro transcribed NLuc RNA was prepared using ApaI-linearized NLuc template DNA and the mMessage mMachine T7 Transcription (AM1344; Ambion) and Poly(A) Tailing (AM1350; Ambion) kits according to manufacturer instructions. NLuc β wt β TP and NLuc β wtSMG5TP IVT mRNAs were prepared using PCR-amplified cDNA as a template for the in vitro transcription reactions (PCR primers: 5' Forward: TAA TAC GAC TCA CTA TAG GGA GAC; 3' Reverse: AGG AAA GGA CAG TGG GAG TG).

Generation of translation-competent cytoplasmic extract

HEK-293 Tet-off translation-competent cytoplasmic extract was generated as previously described (Rakotondrafara and Hentze 2011) with the following modification: A 27G 1/4-in needle (305136; BD PrecisionGlide) was used to homogenize the cells.

In vitro translation

In vitro translation assays were conducted by adapting the protocol described by Rakotondrafara and Hentze (see [Supplemental Protocol](#) for details; Rakotondrafara and Hentze 2011). A total reaction volume of 50 μ L per sample was prepared on ice as follows: 16 mM HEPES-KOH, pH 7.6, 6.55 mg/mL creatine phosphate (10621722001; Roche), 0.1 mg/mL creatine kinase (10127566001; Roche), 0.8 mM ATP (P0756S; New England BioLabs), 0.1 mM spermidine, 0.1 mM complete amino acid mix (L4461; Promega), 135 mM potassium acetate (for purified mRNPs) or 50 mM potassium acetate (for IVT mRNA and protein-stripped PP7-purified mRNA), 1.5 mM magnesium acetate (for purified mRNP) or 2.5 mM magnesium acetate (for IVT mRNA and protein-stripped PP7-purified mRNA), and 20 μ L of equilibrated (10 mg/mL) translation-competent cytoplasmic extract.

Following the final wash in HLB150 + 0.1% NP-40, purified mRNPs were washed once in translation wash buffer (10 mM HEPES-KOH, pH 7.6, 135 mM potassium acetate, 1.5 mM magnesium acetate) then immediately resuspended in the 50 μ L of prepared translation reaction. A 6 μ L aliquot was reserved in a prechilled microcentrifuge tube on ice as time point 0 and the remaining reaction directly transferred to a 37°C ThermoMixer (5382000015; Eppendorf) with ThermoTop lid (5308000003; Eppendorf) set to shake at 1500 rpm for 15 sec every 2 min. At indicated time points, 6 μ L aliquots were taken and placed into prechilled microcentrifuge tubes on ice as conducted at time point 0. For IVT mRNA reactions, 0.5 picomoles of the corresponding IVT mRNA was added to the 50 μ L prepared translation reaction, and in vitro translation was conducted as with the purified mRNPs. For experiments with translation inhibitors, 2 μ L of 1 mg/mL harringtonine (H0169; LKT Laboratories) or 1 μ L of 1 mg/mL puromycin (P9620; Sigma-Aldrich) were added to each 50 μ L translation reaction. To examine the effect of run-off translation, 25 mM (final) EDTA was included during the PP7-based mRNP purification.

To determine the amount of NLuc produced at each time point, 2 μ L of the reserved reaction was diluted in 50 μ L of ddH₂O and then combined with prepared NanoGlo Luciferase Assay Reagent (N1130; Promega) in a 96-well white-bottom plate (655904; Greiner Bio-one) according to manufacturer instructions. NLuc luminescence was detected using an Infinite F200 Pro microplate

reader and associated i-control 1.9 software (Tecan). NLuc luminescence was determined by subtracting the background at time 0 from each individual time point within a sample (Figs. 2B, 4B). Translation efficiency was calculated by dividing the background-subtracted NLuc luminescence by the amount of recovered RNA within an experiment (Figs. 3B,C, 5A,B; Supplemental Fig. 2A). Relative NLuc activity values indicate fold change in luminescence and were determined by dividing the NLuc luminescence at each time point by the average at time 0 across samples within a trial to adjust for minor fluctuations in mRNA expression and recovery (Figs. 2C,D, 3A, 4C–F; Supplemental Fig. 2C,D).

Northern blotting

One-tenth of the washed mRNP bead complexes was reserved prior to addition to the in vitro translation reaction and RNA isolated with TRIzol. Samples were resolved on formaldehyde/agarose gels and a ³²P-labeled in vitro transcribed probe against NLuc was used for detection of mRNAs. Northern blots were imaged on a Typhoon scanner (GE Healthcare) and quantification was performed using ImageStudio software (LI-COR).

Mass spectrometry

Purified mRNPs were precipitated in cold acetone (Thermo Scientific), subjected to trypsin digestion, and analyzed on a LTQ Orbitrap Velos mass spectrometer (Thermo Electron), as previously described (Ge et al. 2016). MASCOT software (Matrix Science) was used to determine peptide and protein identities, and Scaffold (Proteome Software) was used for further analysis. Proteins identified by five or more spectral counts were considered putative mRNP components.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank Stacey Baker for assistance in establishing the mRNP translation method and members of the Hogg and Ferré-D'Amaré laboratories and Nicholas Guydosh for helpful discussions and critical reading of the manuscript. Mass spectrometry was performed by Stephen Swatkoski and Marjan Gucsek in the NHLBI Proteomics Core. This work was supported by the Intramural Research Program, National Institutes of Health, National Heart, Lung, and Blood Institute.

Received January 17, 2018; accepted April 25, 2018.

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RNA 2018 24: 982-989 originally published online May 3, 2018

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