RESEARCH ARTICLE

Purification of mRNA-programmed translation initiation complexes suitable for mass spectrometry analysis

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Liquid Chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) is a powerful analytical technique for the identification and mass analysis of complex protein mixtures. Here, we present a combination of methods developed for the extensive/deep proteomic analysis of purified ribosome/mRNA particles assembled in rabbit reticulocyte lysate (RRL). Ribosomes are assembled on chimeric biotinylated mRNA–DNA molecules immobilized on streptavidin-coated beads and incubated with RRL to form initiation complexes. After washing steps, the complexes are trypsin-digested directly on the beads in semi-native condition or after their elution from the beads in denaturing Laemmli buffer. The nanoLC-MS/MS analysis performed on complexes assembled on β-globin, viral HCV, and histone H4 mRNAs revealed significant differences in initiation factors composition in agreement with models of translation initiation used by these different types of mRNAs. Using Laemmli-denaturing condition induces release of deeply buried peptides from the ribosome and eukaryotic initiation factor 3 (eIF3) allowing the identification of the nearly complete set of ribosomal proteins.

Keywords:
Cell biology / mRNA / Ribosome / RNA binding proteins / Translation initiation

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1 Introduction

Half of the energy available in the cell is dedicated to protein biosynthesis. Translation is a highly regulated cellular process and dysfunction of this crucial regulation has severe implications for various physiological processes such as development, stress response, cell proliferation, viral infection, immune response, and cancer [1, 2]. Several molecular mechanisms are involved in the tight regulation of protein biosynthesis in the cell. Most of them target initiation, which is the rate-limiting step of translation and concerns the assembly of ribosomes on AUG start codon. In higher eukaryotes, this process is tightly regulated by numerous eukaryotic initiation factors (eIF) ensuring accurate start codon selection [3]. Nowadays, this step has become a major target for development of therapeutic strategies against cancer [4].

Within the cell distinct subclasses of mRNAs co-exist that are differentially translated in response to physiological states at different stages during cell life. Moreover, stress conditions or viral infections induce an adaptive response of the cell leading to selection by the ribosome of distinct mRNA subclasses to be translated. This propensity of the ribosome to select mRNA for translation adds an additional layer of gene expression regulation that has been termed ribosome code [5]. Although most of the eIF are characterized and a few examples such as hox mRNA expression regulation are elucidated [6,7], the general mechanism of selection of mRNA subclasses is still largely unknown. How does the ribosome select which mRNAs are to be translated at different stages of the cell life, how does the ribosome respond to different stimuli, and what are the *trans*-acting factors?

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Abbreviations: eIF, eukaryotic initiation factors; **RRL**, rabbit reticulocyte lysates

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Therefore, there is an increasing demand for techniques allowing an exhaustive characterization of all the *trans*-acting factors involved in the translation initiation of specific mRNAs. In this study, we developed an approach to identify whole set of proteins present on translation initiation complexes isolated at different stages. We used a combination of methods developed for the purification of ribosome/mRNA particles assembled in RRL and dedicated to extensive/deep proteomic analysis. Ribosomes are assembled on chimeric biotinylated mRNA–DNA molecules immobilized on streptavidin-coated beads and incubated with RRL to form initiation complexes. After washing steps, the complexes are trypsin-digested directly on the beads in semi-native condition or after their elution from the beads in denaturing Laemmli buffer. The nanoLC-MS/MS analysis performed on complexes assembled on β-globin, viral HCV and histone H4 mRNAs revealed significant differences in initiation factors composition in agreement with models of translation initiation used by these different types of mRNAs. Using Laemmli-denaturing conditions induces release of deeply buried peptides from the ribosome and eukaryotic initiation factor 3 (eIF3) allowing the identification of the nearly complete set of ribosomal proteins.

2 Materials and methods

The three distinct mRNAs used in this study were in vitro transcribed by T7 RNA polymerase from PCR templates containing the sequences of mouse H4–12 mRNA (375 nt; accession number X13235), human β globin mRNA (494 nt; accession number BC007075), and HCV IRES (fragment 1–377, accession number KP098533). The same sequence tag 5'-$(CAA)_9$CAC-3' was added at the 3' end of each mRNA during PCR amplification. The PCR products were phenol-extracted and precipitated with ethanol. In vitro transcription and mRNA purification were performed as previously described [8]. Next, a common biotinylated DNA oligonucleotide was ligated by splint ligation to the 3' end of each mRNA by T4 DNA ligase. The resulting chimeric mRNAs β globin-biotin, histone H4-biotin and HCV-biotin were then immobilized on magnetic streptavidin beads (MagSI-STA 600 – MagnaMedics) and used to program rabbit reticulocytes lysates (RRL) ribosomes. The RRL used in this study were purchased from Green Hectares (Oregon, WI). Prior to incubation in RRL, the splint oligonucleotide was removed to prevent cleavage by endogenous RNAse H (which cleaves RNA/DNA duplexes) present in RRL. To do so, the immobilized chimeric mRNAs were incubated 2 min at 95°C to unwind mRNA-splint duplexes and then 10 min at room temperature in the presence of a 10-fold excess of an antisense splint DNA oligonucleotide containing the complementary sequence to trap the splint and avoid re-annealing on the mRNA. The 48S- and 80S-preinitiation complexes were obtained by adding the specific translation inhibitors GMP-PNP (2 mM) and cycloheximide (1 mg/mL), respectively, to the RRL prior to incubation with mRNA. The assembled complexes were then formed by incubating in RRL at 30°C for 5 min. Washing and elution of the complexes were performed as previously described [8]. For elution of the RNP complexes, the DNA moiety of the biotinylated linker was digested with 10 units of RQ1 RNase-free DNase (Promega) over 30 min at room temperature. The eluted RNP were concentrated by ultracentrifugation and resuspended for analysis by MS as previously described [8]. Samples were then trypsin digested and submitted to a mass spectrometric nanoLC-MS/MS analysis. In the case of in solution Laemmli buffer samples, proteins were first precipitated during 2 h with 0.1 M ammonium acetate in 100% methanol. After centrifugation at 12 000× g and 4°C during 15 min, the resulting pellets were washed twice with 0.1 M ammonium acetate in 80% methanol and further dried under vacuum (Speed-Vac concentrator). Pellets were resuspended in 50 μL of 50 mM ammonium bicarbonate and submitted to reduction (5 mM dithiothreitol, 95°C, 10 min) and alkylation (10 mM iodoacetamide, room temperature, 20 min). Samples were then digested during 12 h at room temperature with 150 ng of sequencing-grade trypsin (Promega) and submitted to a mass spectrometric nanoLC-MS/MS analysis. In the case where the three different biotinylated RNAs were immobilized on magnetic streptavidin beads, the samples were first placed on a magnet during 5 min to exchange the surrounding buffer with 50 μL of 50 mM ammonium bicarbonate. The same reduction and alkylating steps were performed (5 mM DTT – 10 mM IAA) before overnight digestion with 150 ng of trypsin. Resulting tryptic digests were dried under vacuum and further re-suspended in 15 μL of water containing 0.1% FA (solvent A) before analysis on a NanoLC-2DPlus system (with nanoFlex ChiP module; Eksigent, Sciex Separations, Concord, Ontario, Canada) coupled to a TripleTOF 5600 mass spectrometer (AB Sciex) operating in positive mode. 5 μL of each sample were loaded on a ChIP C-18 precolumn (300 μm ID × 5 mm ChromXP; Eksigent) at 2 μL/min in solvent A. After 10 min of desalting and concentration in the trap, the system was switched online with the analytical ChIP C-18 analytical column (75 μm ID × 15 cm ChromXP; Eksigent) equilibrated in 95% solvent A and 5% solvent B (0.1% formic acid in ACN). Peptides were eluted by using a 5–40% gradient of solvent B for 60 min at a 300 nL/min flow rate. The TripleTOF 5600 was operated in data-dependent acquisition mode with Analyst software (version 1.5, ABSciex). Survey MS scans were acquired during 250 milliseconds in the 400–1250 *m/z* range. Up to 20 of the most intense multiply charged ions (2^+ to 5^+) were selected for CID fragmentation, if they exceeded the 150 counts per second intensity threshold. Ions were fragmented using a rolling collision energy within a 60 milliseconds accumulation time and an exclusion time of 15 seconds. This so-called "Top20" method, with a constant cycle time of 1.5 seconds, was set in high-sensitivity mode. To obtain optimal mass accuracy, a beta-galactosidase digest (AB Sciex) was injected before each sample using the "Autocal" feature from Analyst software: calibration was performed using the 10 more

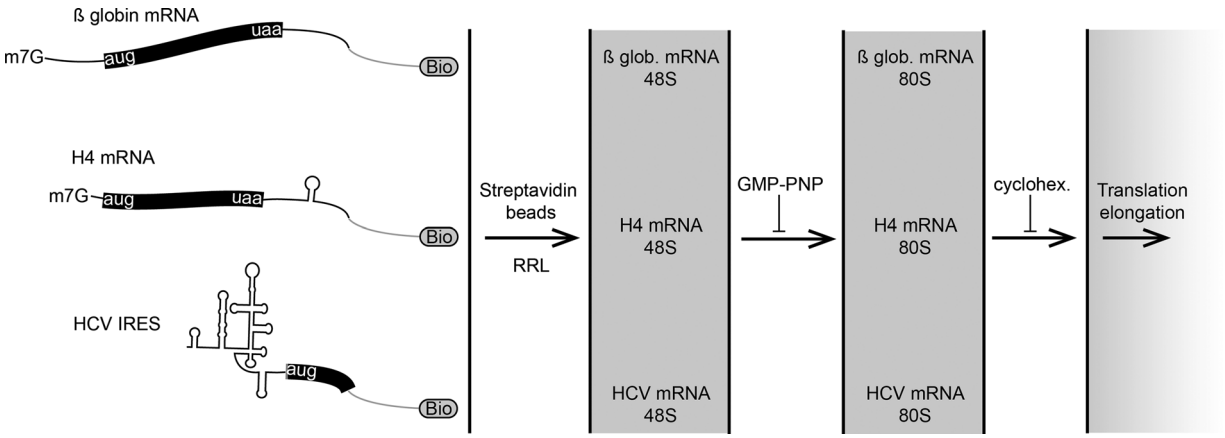

Figure 1. 48S- and 80S-preinitiation complexes purification. Three chimeric mRNAs have been used in this study, namely human β globin mRNA (497 nt + linker biotinylated DNA oligo), mouse histone H4 (375 nt + linker biotinylated DNA oligo) and the IRES (fragment 1–377 + linker biotinylated DNA oligo) from Hepatitis C Virus (HCV). The three mRNAs were immobilized on magnetic streptavidin beads and incubated in RRL in the presence of a non-hydrolysable GTP analog (GMP-PNP) for 48S preinitiation complexes assembly or in the presence of cycloheximide for assembly of 80S particles.

abundant peptides in MS mode and with the 729.3652 *m/z* precursor in MS/MS mode. Moreover, to prevent carry-over due to stationary phase memory, two consecutive washing runs were performed after each sample injection, as well as a blank injection (solvent A) to verify that no peptides were identified due to a carry-over phenomenon.

Data were searched against the complete Human proteome set from the SwissProt database (released 2013/01/09; 43.964 sequences). The fasta file used for the database search was created by adding a decoy database to the original forward database using a Perl script (makeDecoyDB.pl, Bruker). The algorithm used for database search was Mascot (version 2.2, Matrix Science, London, UK) through the ProteinScape package (v3.1, Bruker). Peptide modifications allowed during the search were: N-acetyl (protein), carbamidomethylation (C) and oxidation (M). Mass tolerances in MS and MS/MS were set to 30 ppm and 0.5 Da, respectively, and the instrument setting was specified as ESI-QUAD-TOF. Three trypsin or chymotrypsin missed cleavages were allowed. Protein identifications obtained from Mascot were validated with a FDR < 1% using the ProteinScape Protein Assessment tool. Data were further validated by manually inspecting the quality of the MS/MS fragmentation spectra: a minimum of five consecutive amino acids was requested to validate the spectrum, as well as other fragmentation rules (proline specific fragmentation pattern, major peaks assigned to fragments). Spectral count values were obtained for each protein in each sample and further aligned between the different conditions using the "Compare" tool available in the Proteinscape package. 48S complexes and 80S pre-initiation complexes were assembled on the three distinct mRNAs in two independent experiments and analysed after 'on beads digestion' and in Laemmli buffer. Since the reproducibility was high, the median tendency is reported in this study.

3 Results and discussion

3.1 Complex formation and purification

In order to gain insight into the translation initiation process, we performed an exhaustive analysis of the composition of translation initiation particles. For this purpose, we developed a method to purify translation initiation complexes blocked on in vitro transcribed 3' tagged mRNA. We then determined their composition by liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS).

In order to test our approach, we used three different representative mRNAs, which are translated by distinct previously described translation initiation mechanisms (Fig. 1). Human β globin mRNA is translated by canonical and well-documented cap-dependent mechanism. In this mechanism, the 5' cap of the mRNA is bound by the multi-factor eIF4F. The 43S ribosomal particle is then recruited on eIF4F and undergoes a 5' to 3' scanning process until the AUG start codon is found [9]. In contrast, HCV Internal Ribosome Entry Site (IRES) contains RNA structural elements that are able to promote pre-initiation complex assembly on an AUG located internally in the mRNA without the need of cap and cap-binding factors [10, 11]. The third mRNA, histone H4, is translated by a hybrid mechanism that combines canonical with IRES-mediated features. Indeed, histone H4 translation is strictly cap-dependent, however the ribosome is deposited on the AUG by a tethering mechanism using structural RNA elements without any scanning step [12, 13]. To aid with purification, each of these three mRNA were 3' end tagged with the same 30-nt 5'-(CAA)9CAC-3' sequence. Translation of globin mRNA requires a polyA tail at its 3' end, however since this mRNA is translated in RRL very efficiently in a non-polyadenylated form, the polyA tail was omitted in this study. Alternatively, a genetically encoded polyA track that

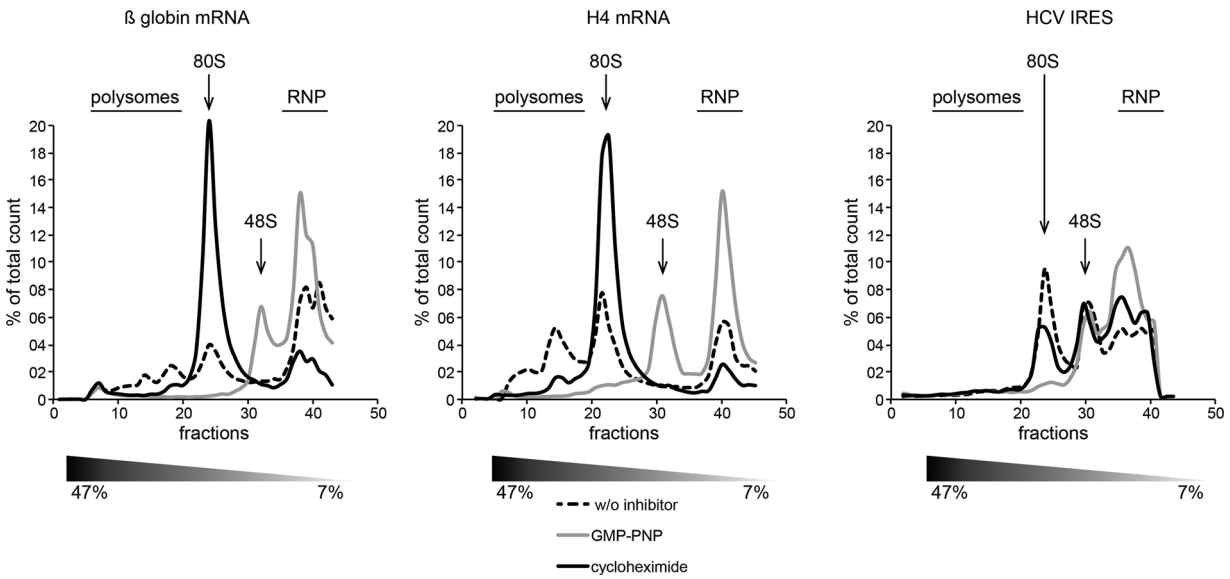

Figure 2. RNA-programmed preinitiation complexes analysed on sucrose gradients. To monitor the assembly efficiency of preinitiation complexes, the 3 mRNAs were first radiolabelled and analysed on sucrose gradients. H4 mRNA and β globin mRNA were 5′ radiolabelled by capping using radioactive ^{32}P α-GTP. The uncapped IRES from HCV was radiolabelled at the 3′ end by ^{32}P pCp ligation. Radioactivity was monitored in the fractions obtained by migration on sucrose gradients (7–47%) after 2h30 centrifugation at 37,000 rpm on SW41 rotor. The curves represent preinitiation complex assembly in presence of cycloheximide (black lines), of GMP-PNP (grey lines) or without any inhibitor (black dashed lines). Positions of 48S-, 80S-particles, polysomes and RNP peaks are indicated above the plots.

mimics a polyA tail could be inserted upstream our tag sequence. We then first tested whether these tagged-mRNAs were efficiently translated. To do this, we incubated them in RRL in the presence or absence of translation inhibitors. GMP-PNP, a non-hydrolysable GTP-analog that inhibits subunit joining was used to accumulate 48S complexes [14] and cycloheximide, a specific translocation inhibitor was used for 80S complexes formation [15]. We monitored the formation of these translation initiation complexes by sucrose gradient centrifugation (Fig. 2). Incubation without any inhibitor led to formation of polysomes with the β globin and histone H4 mRNAs indicating that they were efficiently recognized and translated by the ribosome. Since we used a RNA containing the IRES sequence upstream of a very short ORF, 80S and 48S complexes accumulated but, as expected, no polysomes could be detected with HCV RNA. Inhibition by GMP-PNP led to efficient accumulation of 48S complexes although a significant part of mRNA remains free at the top of the gradient (RNP fraction). Moreover a limited but significant contamination by 80S complexes is observed when GMP-PNP was used. Inhibition and assembly in the presence of cycloheximide was the most efficient for H4 and β globin mRNA, a clear 80S peak was observed with a little contamination of disomes (2 ribosomes). Surprisingly, cycloheximide inhibition with HCV RNA led to accumulation of 80S complexes and a strong 48S peak probably because of limited space on the mRNA used in this study. Since the three tagged-mRNAs were efficiently recognized by the ribosomes, we decided to purify and analyze the composition of these complexes. A tag was used to attach at the 3′ end a DNA linker containing a biotin residue at its 3′ end by splint ligation with T4 DNA ligase (Fig. 3). The resulting chimeric mRNA-DNA molecules were immobilized on magnetic streptavidin beads. The beads were then incubated in RRL to assemble translation initiation complexes on mRNA. Most cellular extracts, including RRL, contain RNAse H activity that cleaves RNA-DNA duplexes. Therefore, to avoid premature cleavage of the mRNA on the beads, an additional step was added in order to eliminate the splint DNA oligonucleotide that remained hybridized to the mRNA after ligation. For this purpose, the mRNAs bound to the beads were heated for 2 min at 95°C in the presence of an excess of another oligonucleotide with complementary sequence to the splint. The mixture was cooled down to room temperature to trap the splint to its antisense oligonucleotide. After this step, the beads were incubated in RRL in presence of GMP-PNP or cycloheximide. After several washing steps, the complex compositions were determined by MS analysis after trypsin digestion on the beads.

3.2 MS analysis on mRNA-programmed RRL: H4-HCV-globin mRNA

Using semi-native conditions (‘on bead’ trypsin digest), MS analysis detected a rather limited number of proteins for the 48S complexes (H4:135 proteins, globin: 131 proteins, HCV: 128 proteins) and for the 80S complexes (H4:156 proteins, globin: 153 proteins, HCV: 175 proteins) (Fig. 4A). The majority of these proteins were detected in the

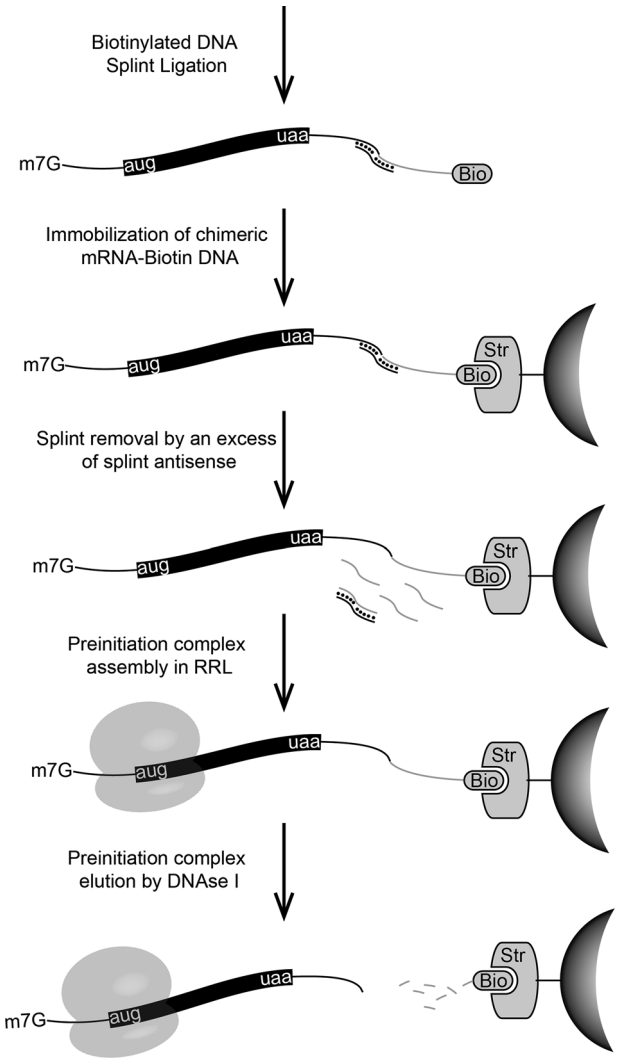

Figure 3. Pre-initiation complex purification. A biotinylated DNA oligonucleotide was ligated at the 3′ end of mRNA by splint DNA ligation using a splint DNA oligonucleotide. The biotinylated chimeric mRNA-DNA molecules were then immobilized on magnetic streptavidin beads. To remove the annealed splint DNA oligonucleotide, the beads were incubated 2 min at 95°C in the presence of a 10-fold excess of an antisense splint DNA oligonucleotide to trap the free splint. After incubation in RRL the preinitiation complexes were analysed by MS directly on beads or after elution by DNAse I digestion of the DNA moiety.

6 mRNA-programmed complexes and we found that ~20 proteins were specific for each mRNA in the 48S complexes and between 18 to 45 proteins were specific for each mRNA in 80S complexes. As expected the small ribosomal proteins were detected in both 48S complexes and 80S complexes. Notably, we identified the full set of small ribosomal proteins except uS19 (see next section). For the large ribosomal subunit, we identified a nearly complete set of large ribosomal subunits for all 3 mRNAs but we never found eL39, eL41, eL42, and eL43. Proteins eL39 and eL41 are relatively small and their protein sequence is not "mass-spec friendly" due to a high arginine and lysine content. Concerning eL42 and eL43, we found in the public repositories of proteomics data PRIDE (http://www.ebi.ac.uk/pride/archive/) and PeptideAtlas (http://www.peptideatlas.org/) the presence of several peptides coming from these two proteins. With this data, the Extracted Ion Chromatogram (EIC) has been reconstituted for each of the observable peptides in our MS dataset. However, none of these peptides were seen, we have no explanation for the absence of eL42 and eL43 in our analysis. Surprisingly, large ribosomal subunits are not only detected in the 80S complexes but also detected in the 48S complexes confirming contamination of 80S complexes in the 48S samples as previously suspected from sucrose gradient analysis. The eIF2 subunits (α, β, and γ) are specific for 48S complexes and can be considered as specific 48S markers. The eIF2α and γ subunits are present in both 48S and 80S complexes although the spectral counts are significantly higher in 48S whereas the β subunit is only detected in 48S. Therefore, cross contamination of 48S by 80S is only limited. Several known RNA binding proteins such as PCBP1 and YBOX1 have been selected for every mRNA used. The putative requirement of these proteins in the translation initiation process has to be confirmed using alternative biochemical and molecular biology approaches. A straightforward assay would be to test the implication of the candidates in a cell-free translation system. For a complete list of detected proteins, see supporting information.

3.3 'On-bead' digestion versus Laemmli buffer

After analyzing carefully the protein composition obtained by translation complexes assembled on mRNAs used in this work and assembled on other mRNAs (data not shown), we noticed that some proteins were more difficult to find than others and did not always appear in the analysis. We hypothesized that this was due to incomplete 'on-bead' trypsin digestion. Therefore, we developed another strategy to denature the proteins more drastically by incubating beads in Laemmli buffer, which contains SDS, prior to trypsin digestion [16]. The denatured proteins were then separated from the beads using the magnetic stand and the proteins contained in the flow through were precipitated by 0.1 M ammonium acetate in 100% methanol. Protein pellets were resuspended and proteins were reduced, alkylated, and digested by trypsin. In order to compare this strategy with trypsin digestion directly on the beads, we focused on 40S ribosomal subunits in the 48S preinitiation complexes assembled on each of the three mRNAs. Using 'on bead' digestion, we found all ribosomal subunits with the exception of ribosomal uS19. However, after Laemmli buffer treatment, uS19 was detected in the 48S complexes assembled on the three mRNAs, indicating that this ribosomal protein might need drastic denaturation for digestion with trypsin (Fig. 4B). We also performed this analysis with 80S complexes and in this case, uS19 was efficiently

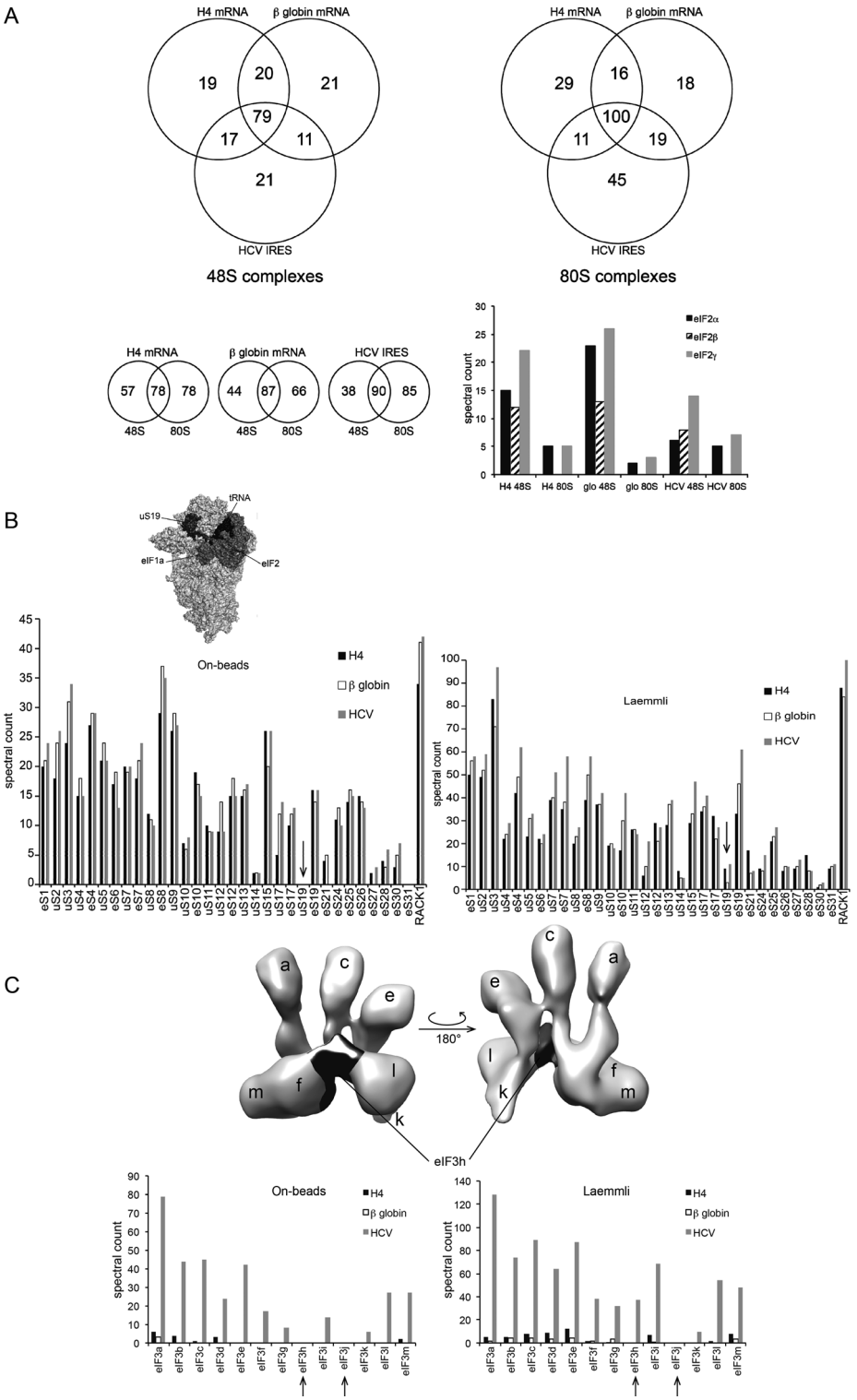

Figure 4. MS analysis of 48S- and 80S-preinitiation complexes. (A) Venn diagrams showing the total number of proteins identified in 48S and 80S complexes with the 3 mRNAs; histogram showing the LC/MS-MS spectral counts of eIF2 subunits in H4-, β globin- and HCV-programmed 48S and 80S complexes (B) Cryo-EM structure of the yeast *Kluyveromyces lactis* partial 48S intermediate containing eIF1 (hidden by eIF2 on this view), eIF1a, eIF2 bound to $tRNA^{Met}_i$ on the small ribosomal 40S and histograms showing the LC/MS-MS spectral counts after on-beads trypsin digestion or on eluted complexes of small ribosomal subunits in H4-, β globin-, and HCV-programmed 48S and 80S complexes. Ribosomal subunits are named according to the recently established nomenclature [23]. The position of ribosomal protein uS19 is also indicated (figure adapted from [17]). (C) Cryo-EM structure of human eIF3 and histograms showing the LC/MS-MS spectral counts after on-beads trypsin digestion or on eluted complexes of small ribosomal subunits in H4-, β globin-, and HCV-programmed 48S complexes. The core subunits a, c, e, f, k, l and m are shown in grey and eIF3h is shown in dark (figure adapted from [24]).

detected in the three mRNAs even with 'on bead' trypsin digestion. This suggests that in the 80S complex, uS19 might be more accessible than in the 48S. The structure of yeast *Kluyveromyces lactis* 48S complex has been recently elucidated by cryoEM (Fig. 4B) [17]. The uS19 is buried in the central part of the 40S subunit and somehow hidden by eIF1a and ternary complex eIF2/tRNA/GTP. In addition, eIF3, which is present in the 48S complex but not visible in this structure might add additional steric hindrance to uS19 digestion [18]. The fact that uS19 was not detected without SDS treatment

might be explained by limited access of trypsin to this protein in the 48S complex.

The multi-subunit eIF3 was also used to compare on-beads trypsin digestion with Laemmli buffer treatment. For complexes of 48S-globin mRNA, 'on bead' digestion allowed the detection of a single subunit (subunit a). Five subunits were detected on 48S-H4 mRNA (a, b, c, d, m), whereas 11 subunits were detected in 48S-HCV (a, b, c, d, e, f, g, i, k, l, m). This discrepancy might be explained by different affinities to the mRNA. Indeed, it is well known that HCV IRES binds tightly and directly to eIF3 [19, 20]. In the case of globin mRNA, eIF3 does not interact tightly with the mRNA and therefore the affinity of eIF3 in 48S complex is much weaker and might be partially released during the washing steps after complex assembly. With the globin mRNA only subunit a was detected suggesting that this is the only subunit that interacts directly with this mRNA. Histone H4 is also believed to interact more tightly to eIF3 therefore explaining the intermediate number of subunits between globin and HCV complexes. When the beads were treated with Laemmli buffer, eight additional subunits were detected in the 48S-globin mRNA and five additional subunits were detected in the 48S-histone H4 mRNA. In the case of HCV, Laemmli treatment allowed the detection of eIF3h. The cryoEM structure of eIF3 shows that eIF3h is located in the centre of the core subunits, which probably explains why it was not detected without Laemmli treatment (Fig. 4C). Finally, we searched for the presence of eIF3j. eIF3j is a loosely attached subunit of eIF3 that has been shown to interact with the ribosomal 40S subunit in the path of the mRNA, and to compete with mRNA binding [21]. eIF3j was never detected in our 48S-complexes with the three mRNAs even when treated with Laemmli buffer indicating that eIF3j was most probably absent from the assembled complexes. Since mRNA and eIF3j are competing for the same binding site on the ribosome, it seems reasonable that eIF3j was absent in the assembled 48S complexes. In conclusion, the differences observed between 'on bead' digestion and after Laemmli buffer treatment might bring some structural information of the analyzed complexes and/or their binding affinity in addition to the composition of these complexes.

3.4 Washing steps with various spermidine concentrations

After complex formation, the beads were washed by several buffers to eliminate unspecific interacting compounds bound to the beads, biotinylated mRNA and/or streptavidin. The washing steps can be done in more or less stringent conditions. This is crucial and depends on the nature of the complex studied and the stringency of these washing steps has to be adapted for each experiment. In order to assess this issue, we performed washing steps using two different spermidine concentrations (0.25 mM and 250 mM). 'On bead' trypsin digests of HCV-programmed 48S and 80S were analysed by LC/MS-MS (Fig. 5). We focused this analysis on

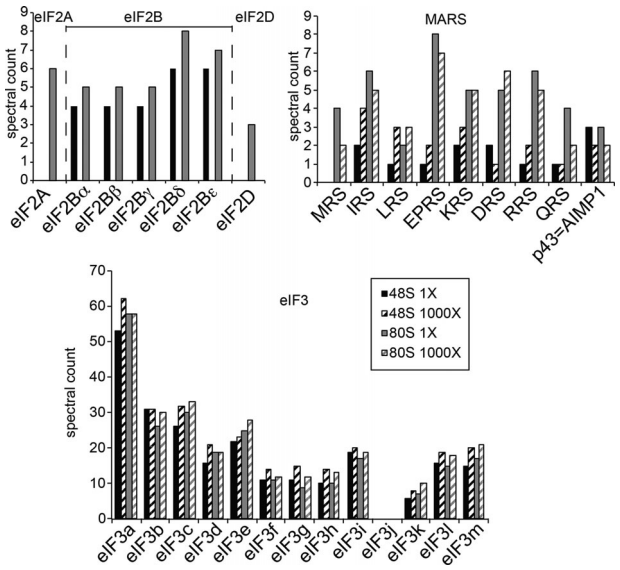

Figure 5. Effect of spermidine on MS analysis. Histograms showing the LC/MS-MS spectral counts of eIF2A, eIF2D and eIF2B and eIF3 subunits and MARS complex components after washing with 0.25 mM spermidine (1X) or 250 mM spermidine (1000X) and on-beads trypsin digestion of small ribosomal subunits in HCV-programmed 48S and 80S complexes.

the translation initiation factors eIF2A, eIF2B, eIF2D, eIF3, and the Multi Aminoacyl-tRNA Synthetase (MARS) complex. As already mentioned, 12 of the 13 eIF3 subunits were detected in both 48S and 80S complexes, whereas the loosely attached eIF3j, which competes with the mRNA, was absent. Washing with 0.25 mM and 250 mM spermidine did not change the composition of the complexes indicating that eIF3 subunits are rather resistant to spermidine treatment. Concerning the MARS complex, increased spermidine concentration during the washing steps seemed to stabilize the complex in the 48S particles since several components such as IRS, LRS, EPRS, RRS had increased spectral counts at the higher spermidine concentration. In contrast, when the 80S complex was formed, we saw that spermidine concentration did not have any effect on the MARS complex. The only exception was for MRS, which was only detected in the 80S complex suggesting a different spermidine sensitivity. Detection of auxiliary factor AIMP1 was not influenced by the spermidine concentration as previously mention for eIF3 subunits. The two other auxiliary factors of the MARS complex, AIMP2 and AIMP3 were not detected in our experiments. Actually, AIMP2 is the core of the MARS complex and its depletion induces its complete dissociation. AIMP2 interacts with AIMP1 and with most of the aminoacyl-tRNA synthetase components and therefore might be poorly accessible to trypsin digestion. On the contrary, AIMP3 is located in the periphery of the MARS complex and only interacts with MRS, which was only detected in 80S particles [22]. In the case of the MARS complex, spermidine has opposite effects on the detection of

its components. Increasing the concentration of spermidine concentration enables the detection of eIF2A and eIF2D in 80S, whereas the five subunits of eIF2B are detected in both 48S and 80S. In these cases, high spermidine concentrations seem to stabilize the complexes. In conclusion, the washing steps can be modulated by varying the spermidine concentrations, however, increasing its concentration can be without any effect on some complexes or improve the detection of several components of other complexes. Testing various spermidine concentrations during washing steps might help to determine the exhaustive composition of translation initiation complexes.

4 Concluding remarks

Starting from crude rabbit reticulocyte lysates and using a novel pull-down method developed in our laboratory [8], we purified pre-initiation ribosomal complexes assembled on three different mRNAs. The complexes were trypsin-digested directly on the beads in semi-native conditions or after their elution from the beads in denaturing Laemmli buffer. The nanoLC-MS/MS analysis revealed significant differences in initiation factors composition in agreement with models of translation initiation used by these different types of mRNAs. More remarkably, the Laemmli-denaturing protocol appeared to be more sensitive in terms of protein resolution and induced release of deeply buried peptides from the ribosome and large eukaryotic initiation factor such as eIF3. This strategy allows extensive listing of *trans*-acting factors required for translation initiation. Since the MS senstivity is rather high, limited contamination of 48S complexes by 80S complexes and vice versa might be detrimental for accurate specific listing of each complex. A further development of this method will be to combine our mRNA-programmed pull-down method with sucrose gradient separation in order to avoid cross-contamination of 48S and 80S pre-initiation complexes.

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The authors declare no conflict of interest.

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