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Tracking the m^{7}G-cap during translation initiation by crosslinking methods

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ABSTRACT

In eukaryotes, cap-dependent translation initiation is a sophisticated process that requires numerous *trans*-acting factors, the eukaryotic Initiation Factors (eIFs). Their main function is to assist the ribosome for accurate AUG start codon recognition. The whole process requires a 5′-3′ scanning step and is therefore highly dynamic. Therefore translation requires a complex interplay between eIFs through assembly/release cycles. Here, we describe an original approach to assess the dynamic features of translation initiation. The principle is to use the m^{7}Gcap located at the 5′ extremity of mRNAs as a tracker to monitor RNA and protein components that are in its vicinity. Cap-binding molecules are trapped by chemical and UV crosslinking. The combination of cap crosslinking methods in cell-free translation systems with the use of specific translation inhibitors for different steps such as edeine, GMP-PNP or cycloheximide allowed assessing the cap fate during eukaryotic translation. Here, we followed the position of the cap in the histone H4 mRNA and the cap binding proteins during H4 mRNA translation.

1. Introduction

Translation initiation leads to the assembly of an elongation-competent 80S ribosome on the genuine AUG start codon. In eukaryotes, this is achieved through assembly of a 43S pre-initiation complex containing the small ribosomal subunit 40S on the 5′ m^{7}Gcap of mRNA. Next, the so-called 43S slides on the 5′UTR, by a 5′-3′ nucleotide-by-nucleotide inspection that is an ATP-consuming mechanism termed scanning, until the AUG start codon is found [1]. Then, the large ribosomal subunit 60S joins in order to assemble a complete 80S ribosome on the start codon. This sophisticated process is assisted by numerous *trans*-acting factors called eukaryotic Initiation Factors (eIF) [2]. The whole process is highly dynamic with many transient complexes that are difficult to trap. The interplay between the eIF is rather complex and methods to assess the numerous assembly/release cycles of individual players are rare.

Among these, a number of techniques for studying the structure and interaction of proteins with nucleic acids depend on methods of crosslinking. Crosslinking is the process of chemically joining two or more molecules by a covalent bond. It often implies chemical modification of one partner or incorporation of a reactive reagent such as photo reactive nucleotides that alter the reactivity of the original molecule. A great advantage of crosslinking approaches is to enable trapping of transient complexes by linking covalently the molecules that interact temporarily during dynamic processes such as translation initiation. Crosslinking methods provide a rapid mean of obtaining evidence for the proximity of functional groups in structurally complex RNAs and ribonucleoproteins. Accurate identification of the crosslinks is also a way to probe the conformation of the RNA of interest.

The position of the m^{7}G-cap during the whole translation initiation process is of special interest. Indeed, numerous studies have demonstrated that the cap first initiates the scanning process by recruiting the whole machinery at the 5′ extremity of mRNA. However, the positioning of the cap during the scanning process, after 80S formation on the AUG and later on during elongation is so far unexplored. Here, we describe two crosslinking methods using the cap as a bait to trap partner molecules in order to assess the cap positioning and its fate during the whole translation process. In this study, we used the mouse histone H4 mRNA as a model to follow the cap fate during the whole initiation process. This particular mRNA is part of the cell cycle-dependent histone mRNAs which are

Abbreviation: U, units.

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not polyadenylated at their 3′ end [3]. They are massively and exclusively expressed during the S-phase of the cell cycle. They have short UTRs, H4 mRNA being the one with the shorter 5′ UTR of only 9 nucleotides [4]. The translation mechanism of histone H4 mRNA is non canonical. The 5′ m^7Gcap is not accessible but instead is sequestered by a cap binding pocket located in the coding region [5,6]. This enables the internal recruitment of eIF4F on a specific element called 4E-Sensitive Element that is also located in the coding region. Then, the ribosome is dropped on the start codon by a so-called 'ribosome tethering' mechanism. Since there is no scanning, the AUG start codon is positioned accurately in the P site of the ribosome by a direct interaction between an AGG triplet in the coding region of H4 mRNA and the loop of helix h16 of the 18S ribosomal RNA [7]. In order to better characterize the role of the cap in this sophisticated process, we studied its position during the distinct steps of translation initiation and elongation.

First, we monitored the position of the cap in the mRNA alone before translation initiation starts by UV-crosslinking with thio-modified caps. As previously mentioned, we confirmed that the cap is not accessible but rather located on an internal structure formed by H4 mRNA making a cap-binding pocket. Second, we investigated the fate of the cap during H4 mRNA translation initiation and elongation by chemical crosslinking. Thereby, we detected proteins that are positioned near the 5′-end of mRNA in initiation complexes formed during histone H4 mRNA translation.

2. Material and methods

2.1. In vitro transcription

Synthesis of RNAs containing thio-modified nucleosides can be performed with the T7 RNA polymerase system. The gene encoding mouse histone H4 (HIST1H4C) and the human β-globin mRNA have been previously cloned into pUC19 and YpGlo respectively. Transcription templates are synthesized by PCR amplification from the plasmids. The 5′ primers contain the T7 promoter sequence and the 3′ primers are designed to promote *in vitro* run-off transcription at the desired position. The PCR approach is also used to synthesize transcripts of H4 mRNA with truncated 5′ sequences. For that, new 5′ primers containing the T7 promoter sequence connected with the desired H4 sequences are designed to synthesize the PCR fragments with variable sizes. *In vitro* transcription is performed as follows. PCR DNA template (about 20 μg) is mixed with transcription buffer (40 mM Tris-HCl, pH 8.1, 20 mM $MgCl_2$, 5 mM DTT), 1 mM each ATP, CTP, GTP, UTP, 40 U of RNase inhibitor (RNasin-Promega), 0.2 mg/mL of T7 RNA polymerase, and milliQ water to 100 μL. The reaction mixture is incubated at 37 °C for 1 h. Then, 2 μL of pyrophosphatase (10108987001-Sigma) are added and the mix is further incubated for 30 min. Then, the DNA template is degraded by addition of 2 μL of DNase I RNase-free (04716728001-Sigma) and incubation for 1 h at 37 °C. Usually, to check RNA integrity, an aliquot is mixed with Formamide Dye and loaded on urea-denaturing 4% PAGE and visualized under UV light after ethidium bromide staining. To eliminate unincorporated nucleotides, the remaining RNA sample is loaded on a G25 column. Transcripts are further purified by separation on denaturing 4% PAGE and electro-elution from gel slices using a Biotrap apparatus (Schleicher and Schuell). Purified RNA samples are then phenol extracted to eliminate proteins from the synthesized transcripts. The concentration of purified RNA sample is determined by absorbance measurement at 260 nm. Before use, transcripts are folded in water by incubating at 80 °C for 2 min followed by slow cooling to 35 °C and kept on ice.

2.2. Capping of RNA transcripts

RNA transcripts can be m^7G-capped co-transcriptionally by T7 RNA polymerase in the presence of m^7GpppG cap analogue (New England Biolabs). More efficient capping is obtained with the vaccine-capping enzyme (VCE) using the ScriptCap™ m^7G Capping System (Epicentre Biotechnologies) which caps and methylates the 5′ end of mRNAs to nearly completion (Fig. 1A). The ScriptCap™ m^7G Capping System requires purified mRNA (purified by electro-elution, see 2.1.). When the GTP from the kit is substituted by s^6GTP (Jena Biosciences, 1 mM), a m^7s^6G-capped mRNA suitable for photo-crosslinking studies is synthesized by VCE after a 1-h incubation at 37 °C (Fig. 1B). After capping, enzymes used for the reaction are eliminated by phenol-chloroform extraction and the m^7s^6G-capped mRNAs are precipitated with ethanol in the presence of 250 mM NaCl. The capped mRNAs are recovered by centrifugation; the pellets are dried and then resuspended in autoclaved milliQ water.

2.3. Short-range crosslinking using thio-containing 5′ cap

Two pmoles of *in vitro* synthesized and capped m^7s^6G-histone H4 mRNA are dissolved in water, refolded at 95 °C for 2 min and then incubated at 0 °C, then are irradiated with 312 nm for 30 min at a distance of 8 cm from the bulb using a Bio-Link BLX 312 on ice as previously described [6]. Importantly, previous experiments with histone H4 mRNA demonstrated that it has to be refolded in water to favour the optimal conformation to visualize the UV-crosslink of the cap in its cap binding pocket. However, for other mRNAs, it might be critical to first set-up an optimal refolding protocol that may require Mg^{2+} to reach the optimal conformation. Then, crosslinks are detected by primer extension using fluorescently labelled H4-specific primers by reverse transcriptase. Reverse transcription is performed in final volume of 20 μL. First, 2 pmol of cross-linked mRNA are mixed with 2 pmol of fluorescently (VIC or NED, from Integrated DNA Technologies) labelled primer (H4-rev160: 5′-TGAGGCCGGAGATGCGCTTC-3′) and denatured first at 95 °C for 2 min followed by primer annealing at 65 °C for 5 min and incubation on ice for 2 min. Then, 0.2 U AMV reverse transcriptase (Life Sciences Advanced Technologies Inc.) in its buffer as recommended by the manufacturer is added and supplemented with 1.5 mM of each dNTP. Reverse Transcription extension parameters are: 42 °C for 20 min, 50 °C for 30 min, 60 °C for 10 min. Sequencing reactions are performed in parallel in similar conditions, but containing 0.5 mM of each dNTP and 16.6 μM of ddCTP. Reactions are stopped by phenol-chloroform extraction and ethanol precipitation. Samples are resuspended in 10 μL deionized formamide and loaded on a 96-well plate for sequencing on an Applied Biosystems 3130xl genetic analyzer. The resulting electropherograms are analysed using QuSHAPE [8], which aligns signal within and across capillaries, as well as to the dideoxy references, and corrects for signal decay. Normalized reactivities range from 0 to ~2, with 1.0–1.2 being the average reactivity for highly reactive positions. The average reactivity (*.shape file) is calculated for each H4 mRNA transcript from three independent experiments with Pearson correlation coefficients ≥0.9.

2.4. 5′-end radioactive labelling of RNA transcripts

For chemical crosslinking studies, mRNA transcripts (80 pmol-10 μg) are first 5′-end-labelled. For that, 50 μCi of [α-^{32}P]-GTP (6000 Ci/mmol, 10 mCi/mL, Hartmann Analytic) is used instead of unlabelled GTP during the capping reaction by the ScriptCap™ m^7G Capping System (Fig. 1C). After radioactive capping, capped mRNA transcripts are separated from unincorporated GTP by denaturing PAGE (4%) and eluted overnight from gel slice at 4 °C in