

Dynamics and Transport of Nuclear RNA

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Abstract The messenger RNA (mRNA) molecule passes the genetic information from the genome to the protein synthesis machinery. Decades of study of the spatial characteristics of mRNA distribution in fixed cells and tissues particularly by electron microscopy and *in situ* hybridization approaches, have revealed the sites of synthesis in relation to the nuclear DNA, and the paths taken en route to the nuclear pore. These studies are now complemented by experiments performed in living cells using fluorescent tags that specifically target mRNA transcripts. The use of high-end microscopy equipment improving the detection of mRNA molecules, together with the advent of new fluorescent tags and original means by which to label the mRNAs, allow us to spy on the mRNA within its natural context of the living cell. High-resolution time-lapse imaging has brought to light the dynamics of single molecules of mRNA during RNA polymerase II transcription, nucleoplasmic transport of mRNA-protein complexes (mRNPs), and the final nuclear event of mRNA export through the nuclear pore complex.

1 Introduction

In 1952 James Watson taped a small note on the wall of his room. It was his hypothesis for how protein synthesis takes place in eukaryotic cells. The note said: $\cup \text{DNA} \rightarrow \text{RNA} \rightarrow \text{Protein}$, putting forward the propositions that: (a) RNA is synthesized in the nucleus from the DNA molecule, and (b) that RNA must move into the cytoplasm where it serves as a template for protein synthesis. As Watson describes, his hypothesis was based on two findings (Watson 2011). First, was the experiment that showed that the nucleus (DNA) had no role in protein synthesis. Brachet and Chantrenne used the giant alga *Acetabularia* for these experiments; after cutting the cells in half they found that the half lacking the nucleus could

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sustain protein synthesis for 2 months (Brachet and Chantrenne 1951). The second finding was made by Beadle and Tatum, showing that the genetic information required for protein synthesis is harbored in the cell nucleus, also known as the “one gene–one enzyme hypothesis” (Beadle and Tatum 1941). Only in 1961 did the term messenger RNA (mRNA) emerge following the publication of two back-to-back papers in *Nature*. Meselson, Jacob and Brenner were searching for an RNA associated with the ribosomes that was not ribosomal RNA, and discovered the short-lived messenger RNA (Brenner et al. 1961). Independently around the same time, Watson and colleagues came to the same conclusion (Gros et al. 1961). Although Jacob et al. were ready for publication, Meselson tells that Watson requested that they wait with the submission, and so they did. Thus mRNA was discovered, and the rest is history. In the wide perspective of cell biology, it is safe to say that the mRNA molecule has become on its own right, one of the pillars of modern biology.

The study of mRNA biology has branched out in many directions focusing on the highly regulated processes of nuclear transcription and cytoplasmic translation. This chapter will describe the life cycle of the mRNA molecule in the nucleus, from the time it leaves the transcribing gene and travels toward the nuclear periphery, ending with nucleo-cytoplasmic export through the nuclear pore complex (NPC). We will emphasize the spatial considerations of mRNA dynamics in the nucleus with regard to the temporal information obtained from live-cell studies, and will use the technical developments in microscopy and imaging as stepping stones in describing key discoveries and the progression in our understanding of mRNA dynamics in eukaryotic cells. We will travel from the days where electron microscopy provided the first high resolution glimpses of mRNAs in fixed cells, through the appearance of the fluorescent molecules that lighted up mRNAs in cells, to bring us to the current era of live-cell imaging which provides real-time measurements of mRNA dynamics.

2 The mRNP

Electron microscopy was one of the first commonly used tools to study the appearance, location and even structure of mRNA molecules in the context of the cell. These large complexes, biochemically purified from cells or observed in EM specimens and identified as containing mRNAs, were considered mRNPs, meaning an mRNA molecule coated with a substantial protein component.

It is well recognized by now that mRNA molecules are not simple linear single stranded nucleic acids as depicted in many schemes, and are rather molecules rich in secondary structures that serve as scaffolds for the binding of tens or even hundreds of different proteins (Muller-McNicoll and Neugebauer 2013; Dreyfuss et al. 2002). These proteins have a variety of roles, one being a protective coat against the many mRNA decay factors roaming the cell in search for unprotected mRNA ends to latch onto. Some other functions might be the regulation of mRNA

processing and maturation, mRNP packaging, and determination of export properties. Many of the mRNP proteins assemble co-transcriptionally (Neugebauer and Roth 1997) and accompany the mRNA as it travels to the cytoplasm (Pinol-Roma and Dreyfuss 1992; Visa et al. 1996; Le Hir et al. 2000b). The mRNP is remodeled as some proteins are removed during or following export in a process of mRNP re-modelling, such as the nuclear polyA-binding protein (PABPN1) that is exchanged with the cytoplasmic polyA binding protein (PABPC1), or the nuclear cap binding complex (CBC) that is exchanged for eIF4E (Lejeune et al. 2002; Hosoda et al. 2006). Some of the proteins that assemble on the mRNA in the nucleus can determine the cytoplasmic fate of the mRNA in terms of RNA localization and translation efficiency (Ross et al. 1997) or even function in cytoplasmic mRNA decay (Kataoka et al. 2000; Le Hir et al. 2000a, 2001; Haimovich et al. 2013a, b).

What does an mRNP look like—is it a round granule or perhaps elongated or irregular? How many proteins does it contain? How is mRNA folded within the complex—is it hidden inside or perhaps is it wrapped around? Most of these questions remained unanswered. The extensive biochemical studies on mRNP composition (Sperling and Sperling 1990) have established that many of the RNA-binding proteins coating the mRNA belong to the hnRNP family (Dreyfuss et al. 2002; Dreyfuss 1986), which rapidly and co-transcriptionally bind to the nascent transcripts (Fakan et al. 1986), but their exact role in mRNP biogenesis and transport remains enigmatic for now. The heterogeneous ribonucleoprotein particles (hnRNPs) were characterized using purified hnRNP complexes which contained approximately a 4:1 stoichiometric ratio of protein to RNA. The studied hnRNP particles were 20–25 nm in diameter but may have been part of larger complexes since they were obtained by nuclease digestion. Detection of similar sized particles on actively transcribing genes in *Drosophila* embryo cells was obtained by Beyer and Osheim who implemented the Miller chromatin spreading technique for the detection of RNA polymerase II (Pol II) transcribed mRNAs (Beyer and Osheim 1988; Osheim et al. 1985). They could not identify which active genes were being detected, but could clearly observe multiple nascent mRNAs of increasing length associated with the gene body. Each transcript had 2 RNP particles associated with it, one at the 5' and one usually at a similarly spaced position downstream. However, subsequent studies revealed that these were splicing related particles and were not the mature mRNPs. Performing the Miller spreads at less stringent conditions revealed 50–60 nm granules close to the chromatin, which could be mRNPs (Sperling and Sperling 1990).

EM studies performed *in situ*, without the chromatin dispersal inherent to Miller spreads, examined the site of formation of newly synthesized mRNAs. The nascent mRNAs were coined 'peri-chromatin fibrils' since they were found in close proximity to chromatin (Fakan 1994; Bachellerie et al. 1975). These fibrils could sometimes be seen forming into single granules of 35–55 nm in diameter (Puvion-Dutilleul and Puvion 1981), and were structurally similar to the best documented example of single mRNP detection by EM, the Balbiani ring (BR) mRNP produced from the salivary glands of the dipteran *Chironomus tentans*.

The BR mRNPs contain extremely long mRNAs (35–40 kb) that are transcribed from the BR puff genes and therefore are easily detected as 50 nm granules that form co-transcriptionally and travel through the nucleoplasm until they reach the NPC (Fig. 1) (Stevens and Swift 1966; Skoglund et al. 1983; Daneholt 1999). The BR granules were spherical and uniformly dense after uranyl staining, and distinguishable from ribosomes or chromatin. These granules provided much insight to the process of mRNA export on account of the large size of the mRNPs, to be discussed later on. Studying the formation of the BR pre-mRNPs has shown that they are structurally diverse particles, mostly due to size and structure of the transcript, and the mRNA processing events that occur on the pre-mRNA (Bjork and Wieslander 2011). Structural analysis of the mature nucleoplasmic BR granules by EM tomography showed that they had similar structure, namely a sphere with a central hole. The particles contained a thick RNP ribbon folded into four domains, with the 5' end at the top of the first domain and the 3' end in the fourth domain, suggesting close proximity between the two ends of the mRNA (Skoglund et al. 1986). A study that purified nuclear mRNPs from yeast found similar configuration, exhibiting a ribbon-like elongated structure with lateral constrictions, and length which was dependent on mRNA size (Batisse et al. 2009). The authors suggest that these 25–30 nm long mRNPs contain mRNA in a condensed manner, since a 1-kb linear mRNA would be 340 nm in length, tenfold longer than

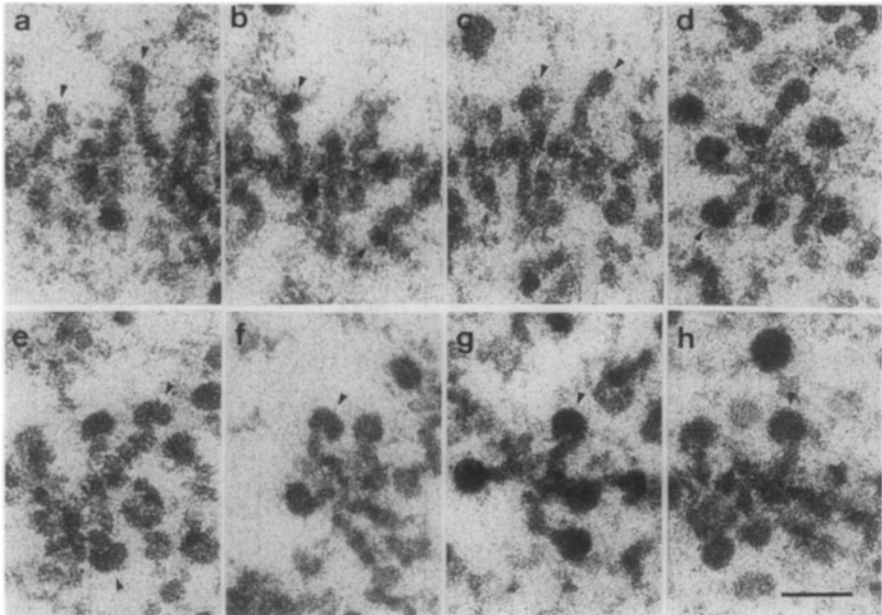


Fig. 1 Electron micrographs of growing Balbiani Ring (BR) RNP particles during various stages of maturation in *C. tentans* cells. Examples are shown from the (a) proximal, (b, c) transitional, and (d–h) distal portions of the gene. Bar = 100 nm. Reprinted by permission from *Cell Press* (Skoglund et al. 1983)

the mRNP particles. Very few studies of this sort document RNP structure, for example, purification of 30–120 nm influenza virus vRNPs (Wu et al. 2007), or *in vitro* generated RNPs with average lengths of ~130 nm (Matsumoto et al. 2003; Skabkin et al. 2004). Altogether, it is difficult to conclude whether there is common architecture for all mRNPs, and more study is required in this field.

3 Detecting Specific mRNAs

It was not always clear that DNA and RNA coexisted in the same cells. In fact, at the beginning of the twentieth century it was “common knowledge” that thymonucleic acid (DNA) was found only in animal cells whereas zymonucleic acid (RNA) was found in plants. At the time, Brachet was working on sea urchin eggs and found that cells producing high levels of protein also contained high concentrations of RNA (Thieffry and Burian 1996). In his studies, DNA and RNA nucleic acids were observed using cytochemical approaches (e.g., Feulgen, Unna and toluidine blue stains) until he finally developed the widely used methyl-green pyronin RNA stain. These staining approaches provided information as to the presence of RNA in all types of cells and within the different compartments of the cells.

A dramatic step forward in RNA observation came with the development of a method that could detect specific RNA or DNA sequences within cells. The *in situ* hybridization (ISH) method developed by Joe Gall used radioactive nucleic acid sequences that were complementary to DNA (or RNA) sequences. Gall and Pardue were the first to detect specific chromosomal regions such as satellite DNA using DNA probes, and the ribosomal DNA genes using radioactive ribosomal RNA as probe (Gall and Pardue 1969; Pardue and Gall 1969, 1970). This method was rapidly applied to many biological systems thus enabling the detection of endogenous genes and RNAs in fixed cells and tissues. The ISH protocol was refined and the hybridizing oligonucleotide sequences were labeled with enzymes that produced a colored stain in place of the radioactive labels. An additional improvement came with the appearance of fluorescence microscopy. Direct labeling of the oligonucleotides with fluorescent fluorophores established the fluorescence *in situ* hybridization (FISH) technique, popularly used in both basic sciences and diagnostics (Levsky and Singer 2003).

The ability to detect bulk RNA within cells as well as specific RNA targets led to important observations regarding the location of mRNA within the cell. In electron microscopy studies the nascent mRNA transcripts were observed as fibers protruding from the DNA (peri-chromatin fibrils), and transcription was found to take place in the peripheral areas of chromosomal regions that were in contact with the nucleoplasmic surroundings, whereas more internal regions of a chromosome were seen to be less transcriptionally active (Zirbel et al. 1993; Verschure et al. 1999). mRNA molecules that had left the site of transcription seemed to be randomly dispersed within the nucleoplasm, usually in between chromatin dense

regions (Singh et al. 1999; Pante et al. 1997). With the detection of nuclear bodies containing pre-mRNA splicing factors [termed inter-chromatin granules or nuclear speckles (Spector 1993)] it was thought that these might serve as the sites of splicing activity during the pathway an mRNA takes from the gene to the nuclear pore (Huang and Spector 1992). However, it turned out that bulk mRNA or specific transcripts were distributed throughout the nucleoplasm (Zachar et al. 1993; Dirks et al. 1995; Snaar et al. 2002) and no particular accumulations of mRNA could be detected. Even though poly(A) FISH detected considerable RNA signal in nuclear speckles, the actual accumulation was probably not more than twofold higher than the nucleoplasm (Fay et al. 1997). In the light of our current knowledge that many long non-coding RNAs (lncRNA) are transcribed by RNA polymerase II, contain poly(A) tails, and are nuclear retained [it was known early on that much of the nuclear RNA never left the nucleus (Perry et al. 1974; Herman et al. 1976)], it is possible that ncRNA is a substantial component of nuclear speckles as indicated by the detection of the MALAT/NEAT2 ncRNA in speckles (Hutchinson et al. 2007). In any case, the exact function of these nuclear speckle bodies remains controversial and they do not specifically accumulate mRNAs within (Lamond and Spector 2003; Hall et al. 2006). Intriguingly, some mRNA labeling studies demonstrated fiber-like tracks in the nucleus (Lawrence et al. 1989) suggesting that mRNAs could transport on a filamentous nuclear network, reminiscent of the actin or tubulin cytoskeletal highways observed in the cytoplasm. To date, such a nuclear transport system has not been detected.

The popularity of RNA FISH combined with conventional fluorescence microscopy provided much qualitative information on mRNA distribution in cells and tissues, but lacked a quantitative angle. Moreover, many of the protocols suffered from high background issues that did not enable the detection of single molecules. This required modification of the technique such as signal enhancement that will easily differentiate between the real molecules and the background. To overcome these issues, Singer and colleagues developed a single molecule RNA FISH approach in which each mRNA transcript of interest was targeted by a series of five short complementary DNA oligonucleotides (approx. 50 nucleotides long), and each oligo (or probe) was labeled with several (3–5) fluorophores. Thereby, each transcript was labeled with many fluorophores, providing a strong point of fluorescence detectable by fluorescence imaging as well as high signal versus the background fluorescence of the specimen and non-specifically bound fluorescent probes. This approach enabled the detection of actively transcribing genes in mammalian cells and the counting of nascent and cellular mRNAs (Femino et al. 2003; Shav-Tal et al. 2004b). For instance, quantification of single molecules of β -actin mRNA showed that a quiescent population of cells contained 500 ± 200 β -actin mRNAs per cell, whereas a proliferating population had up to ~ 1500 copies per cell (Femino et al. 1998). At high activation levels the transcribing β -actin alleles harbored ~ 30 nascent transcripts suggesting the presence of numerous RNA polymerase II enzymes associated with the DNA along the β -actin gene body. Using differently labeled probe sets to the 5'-untranslated region (UTR) and the 3'UTR they could determine a rate of 1.1–1.4 kb/min for RNA Pol II transcription.

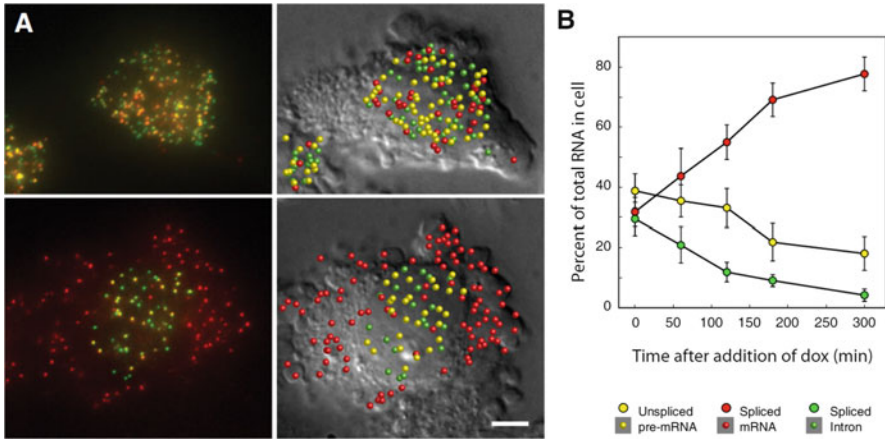


Fig. 2 Demonstration that pre-mRNA molecules dispersed in the nucleus are capable of being spliced. (a) *Upper panels* show composite RNA FISH images of cells in which a gene containing array with an intron was transcriptionally induced for a brief period (2 h). Many pre-mRNA molecules are seen scattered within the nucleoplasm with little accumulation of spliced mRNA molecules in the cytoplasm. *Lower panels* show images from the same batch of cells as above, but in which induction was followed by a period of suppression (2 h). There was a decrease in the proportion of pre-mRNA molecules in cells fixed after the chase period, with a concomitant increase in spliced mRNA molecules in the cytoplasm. Raw images are shown on the *left* and overlays with colored balls identifying the RNA species are presented on the *right*. (b) Percentage of the three different RNA species in individual cells as a function of time after the addition of doxycycline (dox). Doxycycline turns off new RNA synthesis within several minutes. Even though the proportion of spliced mRNAs continues to increase after 3 h, the overall number of RNAs declines due to degradation. *Error bars* represent 95% CI. Reprinted by permission from *Cell Press* (Vargas et al. 2011)

The single molecule RNA FISH approach underwent another level of simplification by Raj, van Oudenaarden and Tyagi making it easily applicable in many laboratories (Raj et al. 2008). In place of the unique fluorophore conjugation procedures required for labeling the probes in 3–5 different positions within one probe, the DNA probes were typically labeled only at one end, and signal amplification was obtained by the use of between 40 and 100 short probes to the known mRNA sequence (compared to five probes in the previous approach). This approach and others are now commercially available [reviewed in (Pitchiaya et al. 2014)]. Subsequent studies have used these single molecule techniques to quantify mRNA expression levels in different types of cells and tissues generating a broad picture of stochastic behavior in gene expression patterns (Fig. 2), for instance see (Yunger et al. 2010; Raj et al. 2006; Vargas et al. 2011; Levsky et al. 2002; Zenklusen et al. 2008; Itzkovitz et al. 2012; Hansen and van Oudenaarden 2013; Waks et al. 2011; Battich et al. 2013; Chou et al. 2013; Hoyle and Ish-Horowicz 2013; Lee et al. 2014).

4 Bringing mRNAs to Life

Do drive RNA detection from fixed cell imaging to real-time imaging, Pederson and Politz applied the FISH method to living cells. Much of the initial detections of RNA by FISH in fixed cells were performed using an oligo(dT) fluorescent probe that hybridized with the poly(A) tails of all mRNAs, thus detecting the bulk poly(A)-containing populations of nuclear RNAs. This approach in living cells and the detection of several sub-populations based on their nuclear mobility [one of the first applications of fluorescence correlation spectroscopy (FCS) in the study of molecule mobility in living cells], suggested the existence of different mRNA populations that may vary in size (Poltiz et al. 1995, 1998). This study brought upon a whole new set of scientific questions and motivated the generation of new approaches for RNA labeling in living cells. It is important to note two of the major obstacles that had to be addressed in future development of studies in living cells. First, since excess oligo(dT) probe roamed the nucleus and could not hybridize with mRNA, it was difficult to distinguish between the mRNA-probe fraction and the unbound probe, hence the required use of FCS that could help differentiate between the populations. But the latter did not provide a solution for examining where in the nucleus do the mRNAs actually travel. Second, as with FISH in fixed cells, it became important to be able to examine specific mRNA transcripts rather than only the bulk poly(A) population.

An elegant approach helped solve the first issue of detection. Instead of labeling bulk mRNA with a fluorescent oligo(dT) probe, a caged fluorophore was attached to the probe, and only by specific activation of the caged fluorophore could the probe become detectable (Poltiz et al. 1999). In this manner, Politz and colleagues activated only a small portion of the probe in one area of the nucleus, and subsequently could follow the fluorescently tagged mRNA molecules over time. If mRNA were a non-mobile molecule one would expect the uncaged fluorescent signal bound to the mRNA to remain in one spot. This was in fact not the case at all, and the movement of the hybridized uncaged signal could be tracked over time. Importantly, this study included labeling of the DNA using the Hoescht 33342 dye that can be applied to living cells, and unequivocally demonstrated that mRNA traveled throughout all the nucleoplasmic space that was not occupied by chromatin (Fig. 3). In light of the abovementioned accumulation of poly(A) signal in nuclear speckles, it was later on shown that mRNA passed through nuclear speckles with the same mobility as within the rest of the nucleoplasm, not showing any “rest-stops” at which it might pause for further processing (Poltiz et al. 2006; Molenaar et al. 2004).

The appearance of green fluorescent protein (GFP) at the doorstep of cell biology expanded the toolbox for mRNA tagging in living cells. For instance, instead of using oligo(dT) probes, the group of Carmo-Fonseca used the natural nuclear poly(A)-binding protein (PABPN1) fused to GFP, to bind to the poly(A) region of mRNAs (Calapez et al. 2002). This study used fluorescence recovery after photobleaching (FRAP) to measure the nuclear mobility of the different

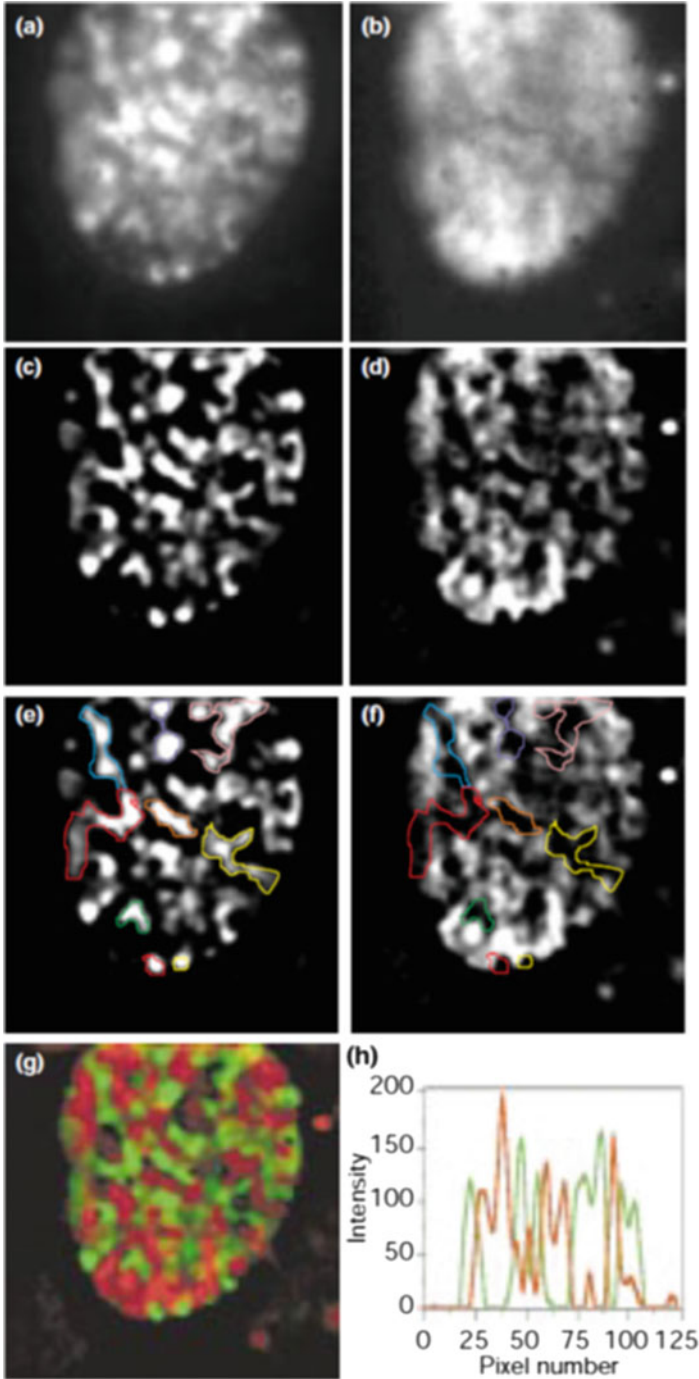


Fig. 3 Intranuclear localization of uncaged fluorescein labeled (FL) FL-oligo(dT) compared to chromatin distribution. Cells were incubated sequentially with caged FL-oligo(dT) and Hoechst 33342 and three-dimensional stacks in both (a, c, e) *blue* (Hoechst-labeled chromatin), and (b, d, f)

populations of moving molecules and could distinguish between free versus mRNA-bound GFP-PABPN1. Still, it was not possible to detect specific mRNAs. To this end, the laboratory of Singer generated a unique tagging sequence that could be inserted into a gene of interest, and subsequently the mRNA molecule would contain the tagging sequence within, that would be bound by a specific RNA-binding protein (RBP). In order for this tag not to interact with eukaryotic RBPs, the chosen sequence was taken from the MS2 bacteriophage, which contains an MS2-coat protein (MCP) that binds to a unique stem-loop structure in the phage MS2 RNA (Bertrand et al. 1998). The MS2 sequence was introduced as a series of 24 sequence repeats into the 3'UTR of a mammalian gene, thus forming 24 stem-loops in the mRNA transcribed from the gene, to be bound by GFP-MS2-CPs (Fig. 4).

The binding of the many GFP-CP RBPs to this specific mRNA immediately as this region was transcribed allowed the detection of the mRNA during transcription (Janicki et al. 2004; Darzacq et al. 2007; Boireau et al. 2007; Brody et al. 2011), co-transcriptional mRNA splicing (Martin et al. 2013; Coulon et al. 2014), release from the gene and nucleoplasmic travels (Shav-Tal et al. 2004a), and the final nuclear point of mRNA export (Mor et al. 2010b; Grunwald and Singer 2010). It is notable that this technique has been successfully implemented in prokaryotes as well as in almost every eukaryotic model organism used in experimental biology (Fig. 5) (Lionnet et al. 2011; Bertrand et al. 1998; Chubb et al. 2006; Muramoto et al. 2012; Golding and Cox 2004; Golding et al. 2005; Bothma et al. 2014). Additional RNA tagging platforms based on similar repeated sequences, known as PP7 and λ_N (Coulon et al. 2014; Martin et al. 2013; Schonberger et al. 2012; Daigle and Ellenberg 2007), have since emerged thus expanding the possibilities for simultaneous mRNA tagging in living cells (Hocine et al. 2013). The dynamics of single mRNPs could then be followed in living human cells showing that mRNPs travel by diffusion at rates that are between 10 and 100 fold slower than single proteins or small complexes (Shav-Tal et al. 2004a). Movement and diffusion rates of mRNPs have since been measured by a variety of additional mRNA tagging techniques (Vargas et al. 2005; Shav-Tal and Gruenbaum 2009; Siebrasse et al. 2008; Ishihama and Funatsu 2009; Thompson et al. 2010; Tyagi 2009; Bratu et al. 2003; Kubota et al. 2010; Santangelo et al. 2009; Gohring et al. 2014), altogether highlighting the bulkiness of the large mRNP particle as it

Fig. 3 (continued) green (uncaged FL-oligo(dT)), channels were captured and restored. (a, b) Raw and (c, d) restored midsections show the distribution of Hoechst signal and uncaged FL-oligo (dT) signal in the same nucleus. (e, f) The same images as in (c, d) but high intensity regions of Hoechst signal were (e) outlined and (f) the outlines superimposed on the oligo(dT) image. (g) A color encoded overlay in which the Hoechst signal is *green* and the oligo(dT) signal is *red*. (h) A plot (linescan) of the intensity (arbitrary units) versus pixel number for the Hoechst (*green*) and oligo(dT) (*red*) signals as they vary along a line across the middle of (g). For (a–g), each image is $\sim 19 \times 19$ mm. Reprinted by permission from *Cell Press* (Politz et al. 1999)

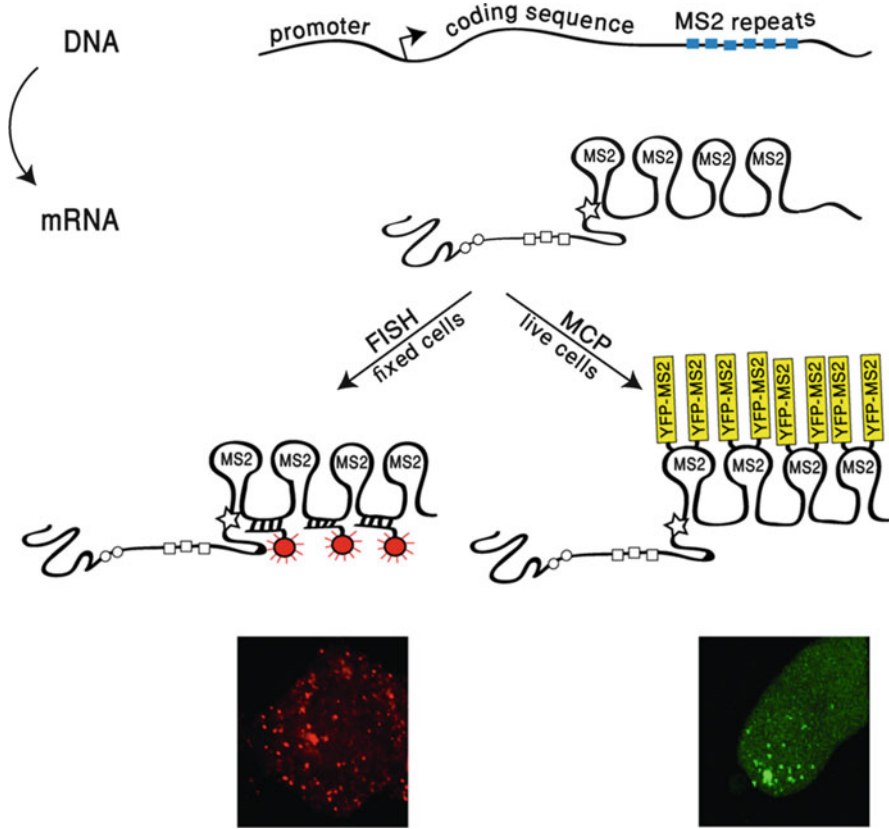


Fig. 4 The MS2 mRNA tagging system. The MS2 sequence, when transcribed, forms repeated stem-loop secondary structures in the mRNA molecule. To observe transcription in living cells, the YFP-MCP protein (can also be GFP/CFP/mCherry etc.) is transfected, and then binds the MS2 loops. For use by RNA FISH in fixed cells, fluorescently tagged DNA oligonucleotides complementary to MS2 repeated sequences, hybridize to the target mRNA. *Empty circle, square and star* indicate RBPs. At the *bottom*, images corresponding to each of the methods: single mRNPs are detected in the nucleus of 293 T cells using GFP-MCP (*right*), or by RNA FISH (*left*)

travels through the nucleoplasm, randomly moving to end up at the exit point at the nuclear pore.

Finally, these studies following bulk RNA and specific mRNA movement in cells showed that the nuclear mRNA movement was diffusion-based and that there was no energy-requiring process utilized by the cell to drive mRNA nucleocytoplasmic transport (Politz et al. 1998; Shav-Tal et al. 2004a; Politz et al. 2006). Although some studies could find an effect of ATP depletion on the mobility of mRNA in living cells (Molenaar et al. 2004; Calapez et al. 2002), it seems that was an indirect effect, while the primary site of energy depletion was on global chromatin structure thereby affecting the structure of the inner nuclear space and confining the movement of mRNPs within the nucleoplasm (Shav-Tal

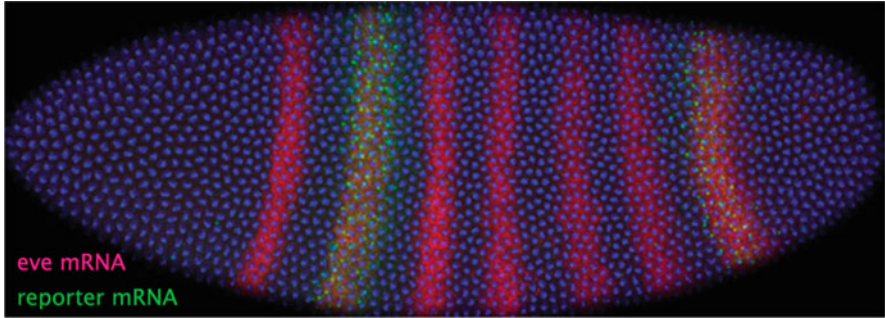


Fig. 5 Confocal image of a transgenic *Drosophila* embryo carrying an *eve* > MS2 transgene. The mRNA is labeled via *in situ* hybridization with probes for the reporter gene (*green*) and endogenous *eve* mRNAs (*red*) in the same embryo during nuclear cycle 14. *Eve* > MS2 transcripts identify authentic stripe 2 and stripe 7 expression patterns. Reprinted by permission from *PNAS* (Bothma et al. 2014)

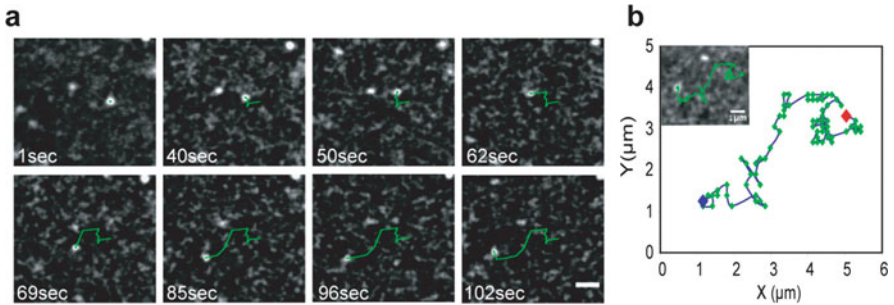


Fig. 6 Analysis of mRNP kinetics in the cell nucleoplasm. **(a)** Frames of a diffusive nucleoplasmic mRNP labeled with the YFP-MS2-CP tracked for 102 s (*green* track). **(b)** The full tracked movement from **(a)**. *Red*, start of track; *blue*, end of track. *Bar* = 1 μm . Reprinted by permission from *Nature Publishing Group* (Mor et al. 2010b)

et al. 2004a; Mor et al. 2010b). To this end we can conclude that the bulk of genetic information moving from the nucleus into the cytoplasm in the form of messenger RNA molecules reaches its cytoplasmic destination by diffusion, and that the cell does not require energy investment for this step of message transport, in contrast to the cytoplasm where some of the mRNAs must be transported by molecular motors (Shav-Tal and Singer 2005). We could track the movement of single mRNPs in the nucleoplasm of mammalian cells (Fig. 6) and showed that the timescale of nucleocytoplasmic transport ranged from 5 to 40 min on average, with longer mRNAs prone to longer transport times (Ben-Ari et al. 2010; Mor et al. 2010b). Therefore, the timeframe of this path is determined by the particle size of the mRNP that would be influenced by mRNA length and the number of proteins coating the mRNA, as well as the biophysical properties of the nucleoplasmic space in which the mRNPs

travel and the hindrance of the chromatin structure (Mor and Shav-Tal 2010; Mor et al. 2010a; Roussel and Tang 2012).

5 Exit to the Cytoplasm

Export of mRNAs through the NPC and into the cytoplasm, where they are to reach the translation machinery, is an irreversible step, thus making this a key point of regulation in gene expression. One of the main systems used to visualize mRNA export are the abovementioned exceptionally large BR mRNPs easily observed by EM in the salivary glands cells of *C. tentans*, and their detection on the nucleoplasmic and cytoplasmic sides of the NPC allowed the examination of mRNPs during export. In an early study, Stevens and Swift showed images of the BR granule re-structuring to form a rod shape during translocation through the pore (Stevens and Swift 1966). Immunoelectron microscopy images demonstrating co-localization of the RNA helicase Dbp5 with the BR mRNP as it changes shape, implied the involvement of a helicase in this transformation (Zhao et al. 2002). Indeed, the remodeling of the mRNP on the cytoplasmic side is thought to prevent its return back into the nucleus, suggestive of a molecular ratchet mechanism imposing directionality on nucleo-cytoplasmic mRNA export (Stewart 2007). Using TEM and SEM (transmission and scanning EM, respectively), Kiseleva et al. were able to provide remarkable pictures of the BR mRNPs during the act of transport through the pore. Based on the analysis of these images, a model was proposed for mRNA export, in which the nuclear basket ring exhibits dynamic restructuring in order to allow the passage of the mRNP through it (Kiseleva et al. 1996, 1998; Daneholt 1997). The passage of the mRNP seems to be directional since Mehlín, Daneholt and Skoglund showed by applying 3D technology on high resolution images of BR mRNPs, that the 5' region of the mRNA is first to

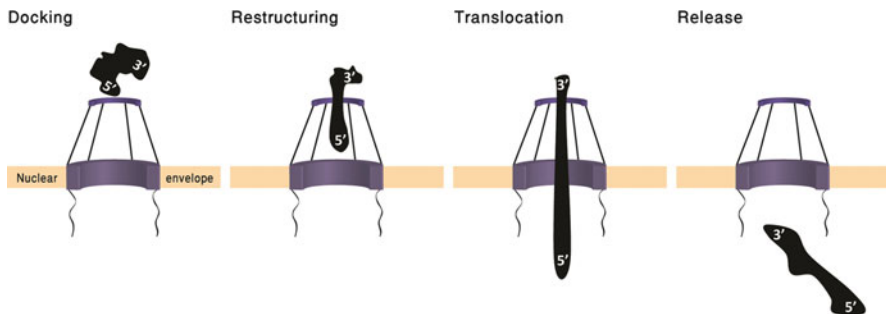


Fig. 7 A schematic overview of BR mRNP export, based on (Daneholt 1997). *Left to right*: the mRNP granule docks at the ring of the nuclear basket. Then, the mRNP changes shape to a rod-like structure and begins to enter the central channel of the pore, with the 5' region inserted first. Next, the mRNP translocates through the pore, crossing from the nucleus to the cytoplasm, and finally released from the NPC

enter the pore (Fig. 7), suggestive of functional interactions between mRNP proteins situated at the 5'-end of the mRNA and the pore proteins, prior to translocation (Mehlin et al. 1992). Supporting information that this was a general phenomenon in mammalian cells as well, was provided by the group of Robin Reed who used RNA immunoprecipitation experiments in human cells to show that the transcription-export protein complex (TREX) is situated on the first exon near the 5'-end of the mRNA (Cheng et al. 2006).

Other groups were motivated to search for proteins that may act as porters in nucleocytoplasmic transport. For instance, in a study conducted by the Dreyfuss laboratory during their studies of hnRNP proteins, the hnRNP A1 mRNA-binding protein was shown to shuttle between the nucleus and the cytoplasm (Michael et al. 1995). This seemed a suitable quality for a carrier of mRNAs, and indeed specific amino acid sequences were identified as crucial for export activity, and termed nuclear export signals (NES). In accordance, the titration of NES-receptors with NES-conjugated peptides in *Xenopus* oocytes cells resulted in mRNA export inhibition (Pasquinelli et al. 1997), suggesting that the NES-mediated mRNA export pathway is limited by NES-receptor availability. Meanwhile, the RBP Crm1 was discovered. This protein also possessed nucleocytoplasmic shuttling properties and was suspected as a carrier of mRNAs. Use of leptomycin B which specifically inhibits CRM1, caused poly(A) RNAs to accumulate in the nucleoplasm thus strengthening the notion of RBP-facilitated transport in mammalian cells (Watanabe et al. 1999). Using inhibition of LMB in heterokaryons of HeLa cells and *Xenopus* A6 cells demonstrated that hnRNP A1 can still translocate from the HeLa cells into the *Xenopus* cells, thus implying that the Crm1 export pathway and hnRNP A1 export are separable (Lichtenstein et al. 2001). The Crm1 pathway is currently considered important mostly in protein transport.

Accumulating data indicated separate export pathways for ncRNAs and mRNAs, the latter involving the mammalian protein TAP/NXF1 (or yeast Mex67). When constitutive transport element (CTE) containing mRNAs taken from viral RNAs, were microinjected by the Izaurralde group concomitantly with recombinant TAP into nuclei of *Xenopus* oocytes, an increase in mRNA export was registered. Indeed, the C-terminal domain of TAP interacts directly with the FG-repeat domains of different nucleoporins (Nups) both on the nuclear and cytoplasmic sides of the NPC (Bachi et al. 2000). The export machinery also interacts with upstream events of gene expression (Luna et al. 2008). For instance, coupling between pre-mRNA splicing and export was shown after the microinjection of ³²P-labeled pre-mRNA and mRNA into nuclei of *Xenopus* oocytes, and the observed increased export of spliced mRNAs whereas only 5% of the unspliced mRNA underwent export (Luo and Reed 1999). This issue was re-examined by looking at β -globin mRNA distribution by RNA FISH. Quantifying single mRNA cellular localization demonstrated higher cytoplasm to nucleus ratio of the spliced mRNAs compared to unspliced transcripts (Valencia et al. 2008), demonstrating the enhancing effect of splicing on mRNA export.

Quite surprisingly, one RNA FISH study has shown regarding the signal sequence coding region (SSCR) amino acid sequence used to localize secreted

proteins to the ER, that this same sequence but on the mRNA nucleotide level will allow the mRNAs of these proteins to export independently of the TREX proteins but in a TAP mediated process (Palazzo et al. 2007). RNA FISH localization assays also helped in sorting out export pathways. For instance, influenza A vRNAs in MDCK cells showed co-localization with GFP-TAP thus implying that the TAP host cellular export mechanism is exploited for the packaging of influenza A virus (Wang et al. 2008). Currently, it is realized that many more RBPs and post-translational modifications are involved in defining the mRNA export process (Tutucci and Stutz 2011).

A further visual demonstration of the importance of Nups in mRNA export was obtained by the injection of antibodies to Nup153 into *C. tentans* salivary gland cells, and as a result, the export of BR mRNPs and rRNA was blocked (Soop et al. 2005). This study suggested that mRNP entry into the nuclear basket is a two-step process; first the mRNP binds to the tip of the basket fibrils and only then is it transferred through the basket by a Nup153-dependent process. Later on, live-cell studies following the behavior of single mRNPs in the human nucleus during blockage of mRNA export (using a dominant negative form of Dbp5) showed that indeed mRNP binding to the NPC occurred independently of export itself (Hodge et al. 2011). To provide compelling evidence as to the role of the already suspected DEAD-box ATPase Dbp5 in mRNA export, Lund and Guthrie employed oligo (dT) cellulose chromatography to extract mRNPs from *Saccharomyces cerevisiae* and quantified the bound fraction of Mex67-GFP on Dbp5 mutants compared to wild-type Dbp5. This resulted in an increase of the bound protein on the Dbp5 mutant, implying that Dbp5 is an active participant in the removal of Mex67 and as the terminator of the mRNA export process (Lund and Guthrie 2005).

It is almost dogmatically accepted that all import and export to the nucleus can only follow through the NPCs. Therefore, the field was overwhelmed by the demonstration of an alternative export pathway independent of NPCs. This process resembles herpes virus budding. The studied large mRNP granules in *Drosophila* synapses were found to exit the nucleus via budding through the inner and the outer nuclear membranes (Speese et al. 2012). However, to date, this is the exception rather than the rule, and in fact even the exact path taken by the mRNP inside the pore is not clearly defined. EM examination of various cargoes moving through the pore has distinguished between two pathways, central and peripheral. Use of nanometer sized RNA-gold conjugates offered the opportunity to examine different sub-classes of regular sized RNAs (mRNA, rRNA, tRNA) by EM to test questions regarding the exact pathway of passage within the pore and the competitive nature of RNA export, rather than using radiolabeled RNAs (Jarmolowski et al. 1994). The Mattaj group conjugated DHFR mRNA, tRNA and U1 snRNA to gold particles and microinjected them into *Xenopus* oocyte nuclei and found that only RNA species of the same type could inhibit export by competition (Pante et al. 1997). Analyzing these gold-mRNA conjugates at NPCs showed that mRNA passes through the center of the NPC, in accordance with an earlier study (Dworetzky and Feldherr 1988). In contrast, when Cook and colleagues examined mRNA localization at the pore using indirect immunogold labeling in HL-60 cells, the labeled transcripts

localized at the side of the pore channels and not in the center (Iborra et al. 2000). Huang and Spector presented similar findings using electron microscope pre-embedding *in situ* hybridization with eosin photo-oxidation to monitor poly (A) RNA in HeLa cells, to reveal a stronger staining in the periphery of the pores indicative for transport through the side of the NPC channel (Huang et al. 1994).

The well-known hypothesis termed “gene gating” proposed by Gunter Blobel (Blobel 1985), argued that NPCs play an active part in nuclear organization through interactions between NPC constituents and DNA sequences. It was proposed that the proximity of a gene to the nuclear envelope would facilitate export. Although some studies in yeast strengthen the latter (Casolari et al. 2004; Cabal et al. 2006), most studies in living mammalian cells demonstrate mRNAs slowly diffusing through the whole nucleoplasm on their way to the nuclear pores, as discussed above (Sheinberger and Shav-Tal 2013). mRNA export however, was always considered a rapid event, since not much mRNA was detected within the pores by the different staining approaches used. With the improvement of imaging techniques and rapid live-cell imaging, these type of studies could focus on the detection of mRNP dynamics at the pore. Large MS2-tagged mRNPs were visualized exiting the nucleus at an estimated time frame of 500 ms or less, and were seen to approach the NPC in a compact form to then emerge in the cytoplasm as a disorganized open structure, implying remodeling of the large mRNP during passage through the pore (Mor et al. 2010a, b). In a study performed on endogenous MS2-tagged β -actin mRNAs together with labeled NPCs, export kinetics were measured using super-registration which employs high-sensitive cameras and provide a time resolution of 20 ms. This revealed that a total of 180 ms was required for passage through the NPC, and that the longer dwelling times occurred at the nuclear and cytoplasmic sides (~80 ms) while the movement through the central channel was significantly faster (5–20 ms) (Grunwald and Singer 2010). Another rapid imaging approach could show that export times were in the range of ~12 ms (Ma et al. 2013). This study could also reconstruct a 3D pathway of mRNPs traveling through the NPC in live cells, and found that mRNPs moved along the periphery of NPC and not through the central axial channel that was used by small passively diffusing molecules. Even BR mRNPs were followed in live salivary gland cells. They were indirectly tagged by a fluorescently labeled hrp36 protein (hnRNP A1 homologue) and export times ranging from 65 ms to 6 s were measured (Siebrasse et al. 2012). They could also detect significant binding times at the pore before export, pointing to a rate-limiting step occurring at the nuclear basket.

Future studies will enable direct examination of both mRNPs and NPCs in living cells to better understand the structural changes both undergo as the large mRNP complex travels through the channel in the NPC. It seems probable that both structures must transform to some extent but exactly what happens is not well understood. For instance, using wavelength anomalous dispersion on NPC crystals derived from *Rattus norvegicus*, Melcak et al. proposed that circumferential sliding of Nup58/45 affects the pore diameter and allows transport of macromolecules, potentially explaining how mRNPs translocate through the pores (Melcak et al. 2007). Another study proposed that the nucleoplasmic basket filaments are

connected at their distal ends, and only when an mRNP engages with this structure, does the nuclear basket ring form and continue to dilate as the mRNP passes through (Kiseleva et al. 1998). As a consequence, the filaments shorten, potentially assisting the mRNP approach to the central channel. It will be important to better understand the exact areas of interactions and molecular events of binding and remodeling of mRNA-associated RBPs moving through the NPC. These studies will take us further to examining the connections of nuclear trafficking in human disease and as potential targets for pharmaceutical intervention (Mor et al. 2014).

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