8
RNA in cells

Valeria de Turris and Robert H. Singer

8.1
Why Study RNA?

In terms of movements and interactions, RNA is one of the most dynamic and flexible molecules in the cell. These characteristics are the keys to understanding its biological roles, in particular in terms of regulation of gene expression. In the cell, the expression of a gene is controlled at different levels. Checkpoints are spread out all along the way, from the triggering of a signal to "open" the chromatin until post-translational modifications and degradation of the protein. In this complex network, RNA occupies a central position since it is the "messenger" (mRNA) sent from the site of information storage (DNA in the nucleus) to everywhere in the cell. Some RNA molecules are coding RNAs, "read" by the ribosome in the cytoplasm to produce functional proteins by ordered loading of the correct amino acids. Other functional RNAs are never translated into proteins (non-coding RNAs, ncRNAs). They can be involved in ribosome assembly and function, such as ribosomal RNA (rRNA), transfer RNA (tRNA) and the small nucleolar RNA (snoRNA); others are implicated in splicing pieces of mRNA together, as are small nuclear RNAs (snRNAs) important components of the spliceosome, the macro-complex that accomplishes this splicing. Moreover, in the last few years new classes of regulatory non-coding RNAs have been discovered: small interfering RNAs (siRNAs) and microRNAs (miRNAs). These ncRNAs are characterized by their tiny dimensions (varying between 21 and 24 nucleotides) and new members are continuously being found. They are involved in multiple functions, from the protection against parasitic nucleic acids, such as viruses and transposons [1], to the control of the expression of specific mRNAs in development and cancer [2]. The discovery of these new RNAs that has strongly improved our understanding of cell defense and regulation also provides tools to manipulate and study gene expression. The relevance of this finding is evident from the 2006 Nobel Prize where Fire and Mello shared the Medicine Prize for the discovery of RNA interference by these small RNAs.

Single Molecule Dynamics in Life Science. Edited by T. Yanagida and Y. Ishii
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ISBN: 978-3-527-31288-7

Another important characteristic of RNA molecules is that they are never "naked" in the cell. They are always associated with proteins, forming ribonucleoprotein particles (RNPs). As a consequence, the RNP is much higher in molecular weight than the RNA alone. Moreover, the composition of the complex is modified over time due to exchange of binding partners, therefore increasing the complexity and the spectrum of possible interactions and functions. These characteristics make the RNA molecule, in all its forms and functions, an exciting and important object of study.

Many questions have been raised about RNP dynamics in the nucleus and in the cytoplasm. Some of them concern how RNAs move, whether they follow rules of free diffusion or energy-dependent movement, and to what extent the environment, such as chromatin in the nucleus or filament networks in the cytoplasm, constrains RNA movements. RNA dynamics range from the sites of nuclear transcription, where maturation occurs, to the specific localization of particular RNAs in the cytoplasm, which creates defined gradients by enrichment in, or exclusion from, particular areas.

8.2 RNA Visualization inside Cells

Because "seeing is believing", during the last decade efforts have been focused on observing the actual dynamics of RNA movement inside a single living cell.

In the next section we outline the most important components to be considered when imaging mRNAs during their movements in living cells: the development of suitable methods to label the RNA, generating a sufficient signal to detect specific individual transcripts, and improvements in imaging technologies. In the subsequent sections we will describe the travel of RNA molecules from transcription sites until their final destination in their respective translational compartments.

8.2.1 Techniques to Label RNA

The intrinsic complexity of the cellular system gives rise to many issues. To address them, different methods have been developed to visualize RNAs. Before choosing a particular approach, pros and cons have to be considered taking into account the cellular system, the target and the aim of the project.

One among the first techniques utilized to study the RNA dynamics in living cells was Fluorescent *In Vivo* Hybridization (FIVH, [3]). This method was developed on the basis of the Fluorescent *In Situ* Hybridization (FISH, [4–6]) and relies on intrinsic abilities of oligonucleotides to recognize and hybridize to a complementary target sequence. The main difference between the two techniques is that FISH applies to fixed cells, while FIVH allows the study of transcripts in living cells. The first methodological improvement of the FIVH technique was the optimization of protocols for oligonucleotide uptake and hybridization *in vivo*. Fluorescent [3] or caged-fluorescent oligo-dT [7] were used to probe the poly(A) tails of all mRNAs and study their movements. The ability to obscure the fluorochrome on the probe by a

protecting, "caging", group allows the movement of mRNA to be studied at a higher resolution. The photolytic unmasking of the fluorochrome, "uncaging", activates the fluorescent probes only at the illuminated region of the nucleus. This distinguishes hybridized probes from the free oligo-dT, because the latter diffuse faster and therefore disappear by dilution in the cellular volume.

An additional tool to label endogenous RNAs based on oligonucleotide hybridization is provided by molecular beacons [8]. These molecules are characterized by a particular stem–loop structure that maintains the fluorophore and its quencher bound close together at each end of the probe. The aim of this system is to overcome the background signal derived from unbound probes, since the fluorescent signal will be visible only when the annealing of the molecular beacon to its target separates the quencher from the fluorophore. However, the stem–loop structure could be destroyed *in vivo* by nuclease activity or protein binding, enabling fluorescence without hybridization. Therefore, an improvement of this tool has been developed exploiting the advantages of Fluorescence Resonance Energy Transfer (FRET, [9–11]). Briefly FRET occurs when two spectrally-matched fluorescent pairs are sufficiently close (<10 nm) and in the correct orientation. The fluorophore excited (donor) by an external source (lamp) does not disperse all the energy in the emission instead it transfers activation energy to the second longer-wavelength fluorophore (acceptor) that in turn will emit. With this technique, opportunely designed pairs of molecular beacons anneal to adjacent sequences on the same RNA target, thus recruiting the donor and the acceptor of the FRET pair close enough to generate the FRET signal [12].

Another method to resolve the background due to unbound probes is the direct *in vitro* labeling of the target RNA before introduction into the cell [13, 14]. In this case, unlike previous techniques, the target RNA is not endogenously produced possibly eliminating some steps in the normal maturation pathway. Since mRNA injected into the cytoplasm will not have contact with the nuclear environment it could assemble a different mRNP complex. For example, mRNAs injected in the cytoplasm may lack all the nuclear factors usually recruited during their travel in this compartment. Furthermore, even if injected into the nucleus, they may be deficient in all the factors deposited during transcription and maturation; processes like splicing and polyadenylation. Nonetheless, these features are not always a con and they have been exploited, for instance, to determine the involvement of nurse cell factors in *bicoid* RNA dynamics in *Drosophila* embryos [15].

A completely different approach relied on the power of fluorescently tagged proteins [16–18]. Green Fluorescent Protein (GFP) and other fluorescent proteins derived from the jellyfish *Aequorea victoria* are extensively used to tag RNA binding proteins. In this case, the binding of the chimeric protein will indirectly label the transcript. GFP-poly(A) binding protein 2 (GFP-PABP2) and the GFP-TBP export factor [19] were used to study the movement of the bulk of endogenous mRNA by Fluorescent Recovery after photobleaching (FRAP) experiments [20]. General RNA binding proteins, like FIVH with oligo-dT, can be used to address endogenous mRNAs but they do not discriminate one transcript from another, showing the dynamics of a population and not of a specific transcript. Furthermore, there is the additional complexity of the off- and on-rates of the protein and its recycling to

another transcript. In any case, the approach was not appropriate for studying the dynamics of a single RNA molecule. The key for this advance came from the bacteriophage MS2 coat protein (MCP) coupled with FP tagging [21]. This phage protein is an RNA-binding protein recognizing specifically a distinctive binding site on a stem–loop folded RNA (Figure 8.1). The high affinity interaction (<1 nM) between the stem–loop and the phage protein make this method highly specific. Since two MCPs bind each stem–loop as dimers, the insertion of several MS2 binding sites (MBS) into the target gene will recruit multiple florescent tagged MCPs on a single molecule (Figure 8.1), providing a powerful system for the detection of single mRNPs distinguishable from the GFP-MCP background.

Developing this system in yeast [21] and in human cells [22, 23] made it possible to probe RNA expression of a specific sequence. A recent study focused on the synthesis of a specific gene array of MBS containing-transcripts by pol II and demonstrated the advantages of the MS2 system in mammalian cells [24]. In this work, integration of results obtained by FRAP, photoactivation, mathematical modeling and computation analysis allowed the quantification of the *in vivo* dynamics and kinetics of pol II transcription.

Coupling this method with other emerging tools like Fluorescence Correlation Spectroscopy (FCS), a means of resolving molecular events within rapid time frames, for example the dynamics of the specific steps of transcription or for splicing, will be likely to yield valuable kinetic data.

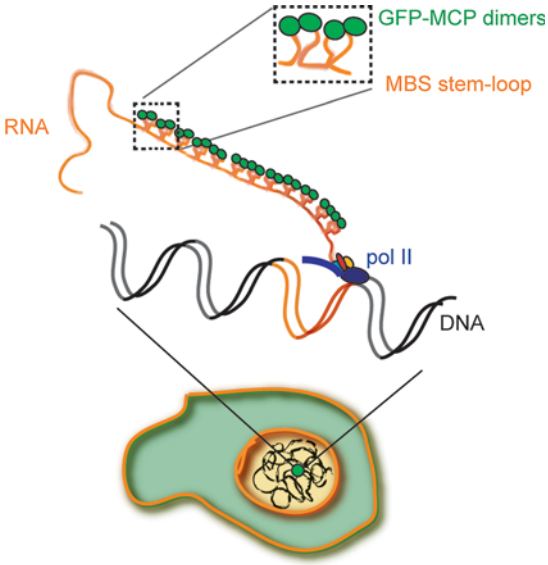


Figure 8.1 Schematic representation of a specific RNA labeled with the MS2 system. Upon stable insertion of the modified gene in the cell, its active transcription site will be visualized by fluorescence due to the binding of the GFP-MCP dimers at the MCB stem–loop regions. For simplicity only a single transcribing polymerase is depicted. Multiple active polymerases at the site will increase the RNA production therefore the intensity, allowing the site to be detected over the signal background.

The concept behind the MCP system led to the creation of an alternative system based on the U1A splicing factor and its recognition sequences [25–27]. This approach works well in yeast but is not exploitable in mammalian cells, which contain the endogenous U1A protein.

8.2.2
Advancements in Imaging Technologies

A critical aspect in single-molecule imaging is the development of the best optical system to match the requirements for imaging single moving particles. This includes acquiring images at rates as fast as, or faster than, the particle movements projected optically onto the capture chip; applying a minimal amount of light to avoid phototoxicity and bleaching of the sample, and all of this while maximizing the signal-to-noise ratio and tracking the particles in three dimensions (3D) and in time ("4D" imaging). In the past, the classical confocal microscope was preferred for imaging fluorescently-labeled cells, but now alternative options are available. The confocal platform itself has been recently modified to increase speed and enable fast imaging in the z-plane, in time and with different wavelengths simultaneously. More advances have also been made in the wide-field epifluorescent microscope. The improvements in the Charge-Coupled Device (CCD) camera, that converts incident photons at the detector into electronic information, and the technology of electron-multiplying CCDs (EMCCDs) which detect very weak signals, are providing higher levels of sensitivity than the photomultiplying tubes (PMT) used in the confocal microscope. In this latter instrument, photons are first converted into electrons which then converge in an electron multiplier where a system of electrodes amplifies the signal by a secondary emission process. However, the major difference between confocal and wide-field imaging is that the confocal microscope discards photons that are not within the image plane and this loss of information reduces the sensitivity required to detect single molecules. Instead, with the wide-field microscope all the information from "out of focus" photons collected during the acquisition process becomes important and useful. In fact applying the images deconvolution algorithms, a "point spread function" will allow "reconstructing" them in three dimensions. In this way the light is reassigned to its point of origin to recreate an image with a signal-to-noise ratio much higher than that in a confocal microscope [28]. In conclusion, the developments in biophotonics, imaging technologies, bioinformatics and computational analysis are continuously increasing their relevance and indispensable roles in the discovery of new principles of cellular and molecular biology in living cells.

8.3
RNA Dynamics in the Nucleus

The birth site of RNAs, namely the transcription site or "RNA factory", is located in the nucleus. Transcription by RNA polymerase I (pol I) occurs inside the nucleoli,

while pol II and pol III are active in the nucleoplasm. During transcription by those enzymes, RNAs are matured and released from the sites. They then move towards the nuclear envelope to translocate in the cytoplasm through the nuclear pore structure. Beyond this elementary information, what are the actual RNA dynamics in all these processes?

8.3.1
Dynamics in Transcription

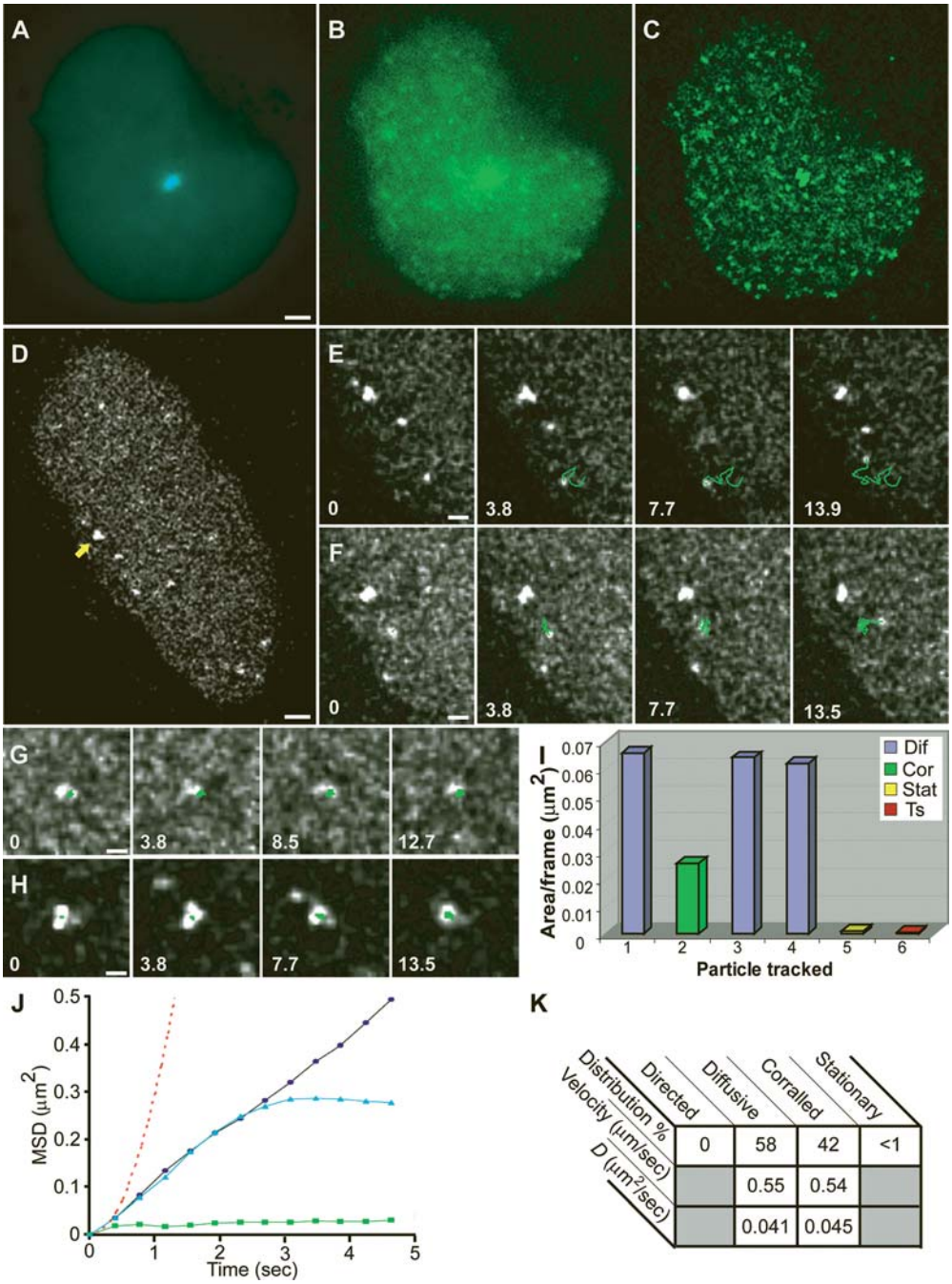
Most of our knowledge about transcription comes from ensemble measurements using methods such as the Northern blot, RT-PCR and microarray. These analyses only provide results that are averages for specific molecules in a population and obscure the differences among all cells. Therefore, only a single cell approach can provide insight into the dynamic behavior and responses to specific stimuli of an individual cell. Exploiting the MS2 system in bacteria, *E. coli* transcripts were tracked and details of prokaryotic transcription revealed [29, 30]. It was demonstrated that transcription in prokaryotes occurred in "bursts" with an average of 6 min of activation for approximately 37 min of inactivity and that RNA partitioning during cell division was random, decreasing the correlation between RNA and protein at the beginning of the cell cycle. Recently, the same approach was used in the eukaryote *Dictyostelium discoideum* for the characterization of the transcription of an endogenous developmental gene [31]. Discrete "pulses" of gene activity were found with an estimated mean time of 5–6 min on or off. The length and intensity of the pulse were consistent during development. This was surprising, considering the strong changes in transcriptional stimuli occurring throughout differentiation of this organism. The important conclusion for this developmental system was that the number of pulses during development did not increase, but rather there was an increase in the number of cells that became committed to transcribing the gene. Initiation of synchronous transcription in neighboring cells was observed to be more frequent than predicted by random events. Furthermore, a "transcriptional memory" existed in cells that had already transcribed that gene; they were more prone to restart transcription than cells that had never expressed it.

The study of transcriptional dynamics is at its beginning and yet very promising. Developing of sensitive systems to observe specific transcription in real-time in mammalian cells, will open new frontiers and most likely reveal new insights into gene expression. A first step in this direction has been made in the study of pol II transcription *in vivo* [24]. Transcription of a specific locus was monitored by FRAP of YFP-pol II recruited to the active site as well as both FRAP and photoactivation of GFP-MCP labeling the transcribed RNA. A systems-modeling approach combined with quantification and testing of the model using transcription inhibitors provided sufficient resolution to demonstrate a faster transcription rate coupled with pausing steps during elongation. Variations in the period length and percentage of pol II pausing could possibly correlate with the appearance of transcriptional "bursting" after relief of the block caused by the upstream pausing polymerase.

8.3.2
A Journey from the Transcription Site to the Nuclear Envelope

The mechanism of RNA movement in the nucleus has been addressed by various approaches [32–35]. One of the hypotheses was that the mRNPs moved from transcription sites to the nuclear envelope guided by some internal structures similar to a railroad, and driven by receptors or a transporting complex. Since the elements of the cytoskeleton, such as actin, nuclear myosin and other related proteins are found in the nucleus and have even been shown to be involved in the transcriptional process [36–38], it has been proposed that nuclear transport machinery that relied on these skeletal structures, including nuclear motor proteins, might exist. Although FRAP experiments on GFP-actin protein show that actin polymerization occurs in the nucleus and that those structures are highly dynamic [39], their involvement in nuclear transport has never been observed to date. Instead many studies inferred that the movement of mRNPs is a combination of Brownian motion and ATP-dependent movements [19, 35]. Most of the questions focused on the relevance and the actual meaning of the ATP requirement in RNA movements inside the nuclear environment. Since directed movements are never observed in the nucleus, the energetic demand may not supply molecular motors but more likely could be used to release RNPs from stalling during random interactions with nuclear structures on their way, such as dense chromatin domains, chromatin scaffolds or the cytoskeleton. Rather than imagine RNP moving on tracks [40], we can envisage the particles moving by diffusion inside a system of interconnected sinusoidal "channels" of fluid phase bounded by dense chromatin domains [41]. The RNPs will travel in this network of interchromatin space and occasionally interact with other complexes and/or domains becoming trapped within areas of high-density chromatin. Reversion from stationary to mobile depends on the consumption of ATP [19, 35]. Single particle tracking shows that RNP motion is energy-independent and not directed [23]. The observation of corralled, and in rare cases, constrained movements highlights the existence of dense and inaccessible structures hindering the free diffusion of large molecular complexes such as mRNPs (Figure 8.2). ATP has an essential role in chromatin remodeling; decondensation of chromatin after energy depletion could be responsible for affecting motility by trapping mRNPs within high-viscosity regions of DNA strands. The caveat in the ATP-depletion experiments is that drug treatments have many pleiotropic effects that impair a clear discrimination between direct or indirect causes.

The RNP could dynamically interact with the environment and change its protein partners during the journey from transcription site to the nuclear envelope, eventually arriving at the proper composition to interact with the export machinery [42–44]. The correct processing of the mRNA will deposit specific proteins on the transcript, like flags indicating that the particle is ready to be exported, or whether it still needs processing or has to be retained and degraded. Some proteins involved in mRNA transport are (respectively yeast/mammalian homolog) Yra1p/Aly of the REF (RNA and Export Factor binding) family of hnRNP-like proteins and Mex64p/TAP [43–45]. The first pair is an RNA-binding protein and the second the



Distribution %	Directed	Diffusive	Corralled	Stationary
Velocity (μm/sec)	0	58	42	<1
D (μm²/sec)		0.55	0.54	
		0.041	0.045	

adaptor bridging the RNA/REF complex with the NPC component, namely nucleoporins with FG repeats (see below).

8.3.3
Transport through the Nuclear Pore Complex

The dynamics of particle translocation through the nuclear pore complex (NPC) are still unclear and the mechanism for transport is under investigation [46]. Three types of transport are associated with the pore in the nuclear envelope (NE): restricted diffusion, facilitated diffusion and unidirectional receptor-dependent transport. If the molecule does not interact with the nucleoporins, protein constituents of the NPC, it is defined as "inert" and permeates through the internal channel by restricted diffusion with a rate inversely proportional to its molecular mass, with a limiting size of 50 kDa. Particles interacting directly with the nucleoporin FG repeats, usually transport receptors like NTF2 and transportin 1, are subjected to facilitated translocation. Both these mechanisms are passive bidirectional processes while transport mediated by the receptor is an active unidirectional transport that proceeds against the concentration gradient of the cargo proteins. A cargo is an inert molecule that cannot diffuse freely through the pore. Instead they harbor specific signals (Nuclear Localization Signal, NLS and Nuclear Export Signal, NES) bound by the adaptor to be translocated to the right compartment [47]. Also in this case the translocation process *per se* is not an energy-consuming task, since is not directly coupled with ATP hydrolysis. The real energy driving the transport mediated by importin and exportin proteins is the chemical potential of the RanGTP gradient maintained by NTF2 and RanGEF. This latter protein recharges the RanGDP imported into the nucleus by NTF2 with GTP. The RanGTP gradient, higher in the nucleus than in the cytoplasm, is important for the correct directionality of the cargo transport, since association of the receptor with the cargo is influenced by its level. Importins load the cargo at low levels of RanGTP in the cytoplasm while in the nucleus high RanGTP levels trigger the replacement of the cargo with RanGTP. The exportins work in the opposite direction and with an opposite mechanism: they load the cargo only in combination

Figure 8.2 Live-cell imaging and single-particle tracking of individual mRNPs in the nucleus of a mammalian cell. Images from time-lapse films acquired from a cell co-transfected with (A) CFP-Lac repressor that marks the insertion locus and (B) YFP-MCP. (C) Reduction of noise for tracking of mRNPs was obtained by deconvolution. Bar, 2 μm. (D) Tracking of mRNP (arrow, transcription site) (bar, 2 μm) showed (E) diffusing particles, (F) corralled particles, (G) stationary particles, and (H) the transcription site. Tracks are marked in green, and time in seconds from the beginning of tracking for each particle that appears in each frame. Bars, 1 μm. (I) Plot of the area per frame traveled throughout the tracking period. Diffusive particles are shown in blue, corralled in green, stationary in yellow, and transcription site in red. (J) Mean-square displacement (MSD) of tracked nucleoplasmic particles versus time indicated the presence of three types of characterized movements: diffusive (black circles), corralled (blue triangles), and stationary (green squares). Directed movement was never detected (red dotted line). (K) Table summarizing the mean velocities and diffusion coefficients of tracked particles at 37 °C. (Adapted from [23]).

with RanGTP in the nucleus and release both when they reach the cytoplasm. In both cases, once in the cytoplasm, RanGTP is hydrolyzed to RanGDP to disassemble the complex.

The messenger RNAs rely on the adaptor protein TAP for their transport in the cytoplasm. The TAP-mediated export of the mRNAs appears to be unlinked from the concomitant binding of RanGTP [48], and its marginal role in the process is due to its involvement in nuclear import of TAP and other proteins rather than in the mRNA transport itself. In that case the unidirectional movement seems to be maintained by a highly conserved DEAD-box ATPase/RNA helicase essential for mRNA export, Dbp5p [49]. This is a shuttling protein that associates with the RNA early in transcription and translocates into the cytoplasm with the complex. On the cytoplasmic side of the NPC multiple binding sites for Dbp5p anchor the helicase in this region where Dbp5p is activated by the concomitant presence of Gle1 and Inositol-P_6 [50, 51]. Remodeling of the mRNP causes the release from the NPC and the recycling in the nucleus of the proteins involved in the transport, thus avoiding a possible backward movement of the complex. Although we have considerable information about different kinds of interactions and the mechanism of receptor-mediated cargo transport, how molecules actually translocate through the pore is still unclear.

A key role is suggested for the FG repeats in the nucleoporins [47, 52, 53]. Since these phenylalanine-rich domains are able to interact with each other and with the transport receptors, several models have been developed to describe the possible movements inside the NPC. A Brownian affinity-gating model proposes the formation of an internal channel with binding sites at the tunnel entrance that facilitate the access of the bound molecules but completely exclude those that are unbound [52]. Inside the channel, the particles move by Brownian motion. Macara [47] proposed instead that the channel walls are actually covered with the FG repeats allowing the molecules to jump from one repeat to another while inert molecules can diffuse in the channel. Another possibility is the formation of a meshwork by interaction among the FG domains creating a permeability barrier that restricts the passage for inert molecules [53]. This selective phase model proposes that the nucleoporins form this sieve-like structure within the pores, and transient interactions with the FG repeats would allow the bound particle to "dissolve" into this structure. The carrier would help the cargo to translocate by masking domains that enable them to interact positively with the meshwork.

Fundamental insights into the translocation process require further investigation and higher resolution structures of the intact NPC, a goal that can be achieved only by single molecule approaches. These methods can provide unique information on topographic properties and kinetic processes with excellent spatial and time resolution. To this end, a single-molecule far-field fluorescence microscopy approach was applied to the NPC of permeabilized human cells [54], allowing the measurement of dwell times of NTF2 and transportin with and without their specific cargo molecules bound. These data highlight that binding at the NPC is not the rate-limiting step and that particles can translocate simultaneously via multiple parallel pathways.

8.4
RNA Dynamics in the Cytoplasm

Once the transcripts reach the cytoplasm, they move from the pore to disperse in the environment. We can divide them into two different classes of RNAs based on their final distribution: non-localizing and localizing RNAs. The first will uniformly distribute in the cytoplasm while the latter will be confined or enriched in specific areas. Nevertheless both have the ability to move. Studies with inert tracers suggest that the cytosol is heterogeneous with viscoelastic behavior, allowing limited diffusion for particles of sizes similar to RNPs [55–58]. Therefore, unlike the situation in the nucleus, the particles may require active transport if diffusion is impaired or inefficient. However their dynamics should differ to allow the observed specific compartmentalization. In particular since distances are much larger in the cytoplasm, for instance the distal region of a neuron, a mechanism is required to facilitate transport.

8.4.1
Non-localizing RNA

Most mRNAs, such as housekeeping mRNAs, appear to belong to the non-localizing class although their distribution may in fact be non-homogeneous (for instance mRNAs for mitochondrial proteins appear to be near mitochondria [59]). Their role is to spread out in the cytosol to ensure that their protein products will be generally and uniformly available. The dynamics of single and specific RNAs in living cells has been observed and measured by exploiting the MCP system in COS cells [60].

Three reporter genes with the MBS inserted and different 3′UTR (3′ UnTranslated Region) sequences either from human growth hormone (hGH mRNA) gene, SV40 (SV mRNA) or β-actin (as a control for known localizing RNA) were used. The first two reporters exhibited four possible movements (Figure 8.3): static (33–40%), corralled (∼ 40%), diffusional (15–25%) and directed (2–5%). Interestingly, individual particles were able to switch between these movements and no correlation was observed between a specific behavior and a particular area in the cells. Since active transport is usually associated with cytoskeleton components, this hypothesis was investigated by treating cells with specific drugs against microtubules and microfilaments. The results confirmed the crucial role played by the cytoskeleton in anchoring static particles, supplying tracks for directed motion and creating restricted areas not accessible to the particles, possibly transforming their diffusion into corralled motion. The new finding that "non-localizing" RNPs are also subjected to directed movements suggests the involvement of active transport by molecular motors on microtubules similar to localized RNPs. Actually, both RNP classes moved with the same average speed (1–1.5 μm/s) but the localized RNP classes used active motion more frequently and for longer distances. Therefore, molecules switch stochastically between various movements, but each RNA will have a specific probability of displaying each of the four movements dependent on its sequence. If a sequence enables the recruitment of factors interacting directly with the motors

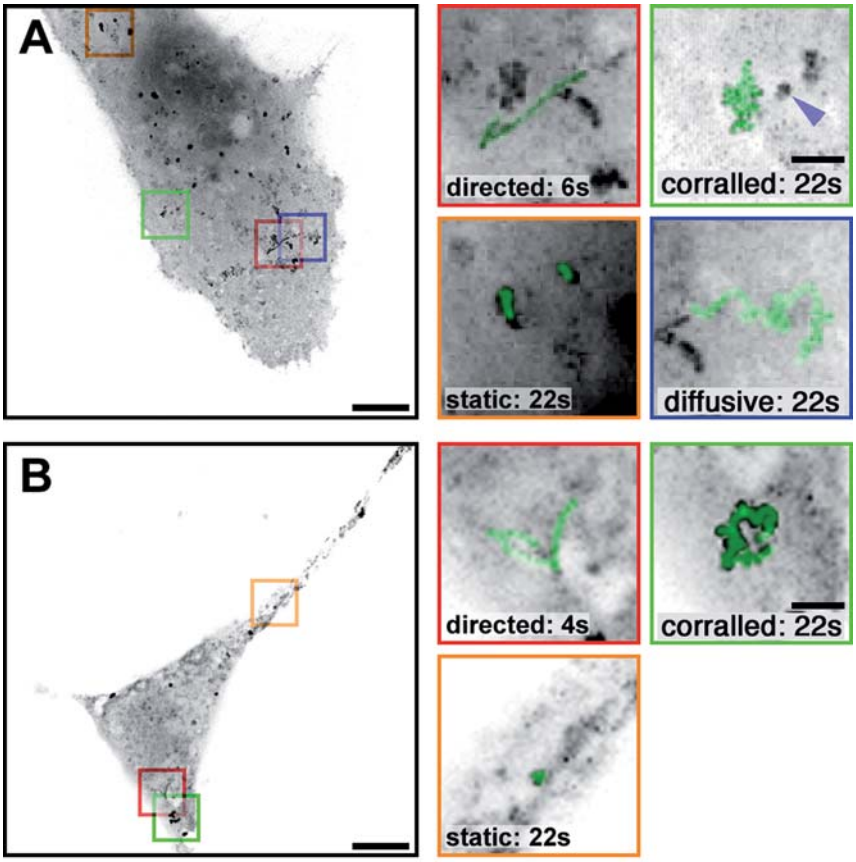


Figure 8.3 Dynamics of single mRNA molecules in the cytoplasm of mammalian cells. Direct movements are also observed in the cytoplasm. (A) Cos cells transiently expressing the reporter hGH mRNAs and the MCP-GFP were imaged live. Left: a maximum intensity image projection of 200 frames on one image. The scale bar represents 10 µm. Right: panel magnifications: the scale bar represents 2 µm. mRNA track superimposed (green) from each of the indicated boxed regions. The blue arrow points to a static particle in the vicinity of a corralled mRNA. (B) COS cells transiently expressing the reporter SV mRNA and MCP-GFP were imaged as in A. The scale bar represents 10 µm. Right: panel magnifications, track of mRNA movement superimposed (green) on an enlargement from each of the indicated boxed regions. The scale bar represents 2 µm. (Adapted from [57]).

or with adaptors, a specific outcome will be determined as the RNA distribution will be a result of the motor direction and its persistence.

8.4.2
RNA Localization

RNA localization is involved in the regulation of many processes inside the cell and often acts in concert with translation and RNA degradation. Most of its effects give

rise to an asymmetric distribution of factors, creating in turn polarized cells [61]. This polarity has important consequences in many processes, such as development, differentiation, cell motility and neuron functionality. Different means are used to reach this goal but the most effective seems to be RNA localization associated with local translation to generate proteins only in the targeted area. The elements required for the localization are sequences *in cis* on the RNA, called "zipcodes" or Localization Elements (LEs), and the *trans*-acting factors recognizing and binding them [62–64]. Examples are: the β-actin localization zipcode [65–67] and its trans-acting factor, ZBP1 [68, 69] in fibroblasts; Vg1 LE with hnRNP I, Vera and 40 LoVe in *Xenopus* oocytes [70–73]; the A2RE signal in the Myelin Basic Protein (MBP) with hnRNP A2 in neurons [13, 74] and the *ASH1* zipcode with She2p in yeast *S. cerevisiae* [21, 75, 76]. In general, localizing mRNAs are shuttled to specific areas of the cell or the oocyte along cytoskeletal elements such as microtubules or actin filaments. They seem to be actively translocated by motor proteins of the myosin, kinesin and dynein families. A corollary of localization is that the mRNA must be translationally repressed during its movement. A number of *trans*-acting factors mediate translational repression by binding the RNA directly (ZBP1, [77]; Puf6p, [78]; Khd1p, [79, 80]).

8.4.2.1 Some Examples of Localization in Mammalian Cells and Drosophila

Localization is particularly important during development. The most characterized cellular systems to study RNA localization in mammalian cells are migrating fibroblasts, oligodendrocytes and neurons. In fibroblasts β-actin mRNAs are localized at the leading edge of the cell, a fact that correlates with the requirement of high protein levels for actin polymerization during cell movement. The complex of mRNA, ZBP1 and ZBP2 assembled in the nucleus [81] moves in the cytoplasm along actin filaments probably carried by a myosin motor [82, 83] to be anchored at the leading edge possibly by EF1α [84] where it is finally translated.

Neurons and oligodendrocytes are also a class of highly "polarized" cells since many mRNAs typically travel from the cell body to the extremities in dendrites and axons. RNAs travel in granules that may contain many copies of an mRNA or several types of mRNA. All this trafficking moves on cytoskeleton elements by motors: MBP mRNA is probably associated with microtubules through a kinesin [85]. The same motor is also responsible for the CamKIIα targeting in hippocampal dendrites [86] and tau mRNA in axons [87]. Moreover in neurons, β-actin is localized in the growth cone by ZBP1 along microtubules. Since the same motor can drive the movement of different RNAs, the recruitment of specific adaptors and RNA binding proteins in the locasome will allow the selection of the final "address" for the specific cargo in the complex.

During development, localization mechanisms are also used by *Drosophila* cells to create mRNA gradients, and consequently protein gradients, indispensable for generating specific patterns of expression essential for development of the oocyte and the embryo. One of the first determinants breaking the initial symmetry of the oocyte is *gurken* mRNA. It is involved in the specification of both the anterior–posterior and the dorsal–ventral axis by two rounds of signals at different times

during oogenesis [88]. At the beginning *gurken* is localized at the future posterior pole of the oocyte, sending a signal back to the oocyte to initiate the formation of the anteroposterior axis. The signal leads to the repolarization of the oocyte microtubules and the migration of the oocyte nucleus to the dorsoanterior corner of the oocyte [89]. When *gurken* is localized in an anterodorsal cap near the oocyte nucleus the second round of signaling initiates the formation of the dorsoventral axis. The overlying follicle cells acquire dorsal fates, leading later to secretion of correct eggshell structures [88, 90]. The *gurken* mRNA first moves across the internal oocyte to the anterior and then turns towards the nucleus in the anterodorsal position. Both steps require dynein and microtubules, but they rely on different microtubule networks [91]. RNA binding proteins such as Squid and Hrp48 are involved in *gurken* dorsal movements [92] and its localized translation is restricted to the dorsal anterior region [91, 93]. After the initial signal from *gurken,* the further development of the anterior–posterior symmetry of the oocyte involves several other localized transcripts in addition to *gurken*: *bicoid* for anterior specification and *oskar* and *nanos* mRNAs, both localized in the posterior. This axial polarity is established by opposite gradients of these proteins maintained in the oocyte by the maternal determinants (from ovarian nurse cells), transported on cytoskeletal networks to their destination and then anchored and translated. At the anterior pole *bicoid* is recruited in two phases: an earlier phase in mid-oogenesis when microtubules are polarized towards the anterior pole, and a later phase, after nurse cell dumping, when ooplasmic streaming is thought to facilitate the mixing of the incoming material [94]. In the first phase, microtubules and the binding of the *trans*-acting factor Exuperentia are essential for the localization at the anterior pole [15]. Also in the late phase *bicoid* is localized by active transport [94] instead of diffusion and trapping as is the case with *nanos* (see below). This involves the binding of Staufen protein to *bicoid,* before nurse cell dumping, and transport of the complex on a subset of microtubules that originates at the anterior pole. Microtubules and actin filaments are responsible for the enrichment of *bicoid* at this pole not through anchoring, but instead by a continuous active dynein-driven transport [94].

At the posterior pole *oskar* is one of the first mRNAs recruited, probably by kinesin I. Interestingly, proteins in the exon-junction-complex (EJC) and the splicing reaction *per se* seem to be involved in its localization [95, 96]. Oskar protein in turn is required for *nanos* mRNA localization. The peculiarity of *nanos* localization is how the specific expression in the posterior pole is achieved. Indeed, *nanos* enters the oocyte during nurse cell dumping and is dispersed in the ooplasm by streaming movements and by diffusion in the whole oocyte [97]. Once at the posterior, it is anchored to the actin cytoskeleton and translated [97]. In contrast, outside this region *nanos* is translationally repressed by Glorund in the oocyte [98] and in the embryo by Smaug [99, 100] or also degraded [101]. Another mRNA which becomes localized at the posterior pole by degradation outside its target region is *hsp83* [102]. Both these posterior enrichments require two distinct *cis*-acting elements in the 3′ untranslated region (UTR) of the RNA: a degradation element that targets the mRNA for destruction in all regions of the egg or embryo, and a protection element that stabilizes the mRNA at the posterior [101].

In the early stages of *Drosophila* embryonic development, initial nuclear cleavages are not accompanied by cell division, creating a large multinucleate syncytium in a broad band of cortical cytoplasm (periplasm) where zygotic transcription begins at the blastoderm stage. The nuclei form a layer subdividing the periplasm into two compartments: the apical, above the nuclei, and the inner, basal periplasm below the nuclei. The pair-rule mRNAs, essential for the further segmentation of the embryo, are restricted in the apical compartment. Their localization mechanism requires specific sequences in the 3′UTR of the transcripts [103], Squid protein to promote apical transport [104] and dynein-mediated transport on microtubules [105, 106].

8.5 Conclusion

We now know much about how RNA travels from its birth place to its functional sites. However much more needs to be known and new tools need to be developed to fully understand the process. The study of single molecules in live cells is becoming essential to discover the connections between different pathways and the actual mechanisms for regulating gene expression in the cell.

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