CHAPTER

2

DISSECTING CELLULAR ACTIVITY FROM SINGLE GENES TO SINGLE mRNAs

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Fluorescent proteins have revolutionized cellular biology, introducing a time component in the spatial analysis of biological processes. However, it has been only recently that transcribed nucleic acids could be detected in live cells (Bertrand et al., 1998). Single-molecule detection of the constituents of gene expression is currently transforming our vision of molecular biology, allowing the focus to shift from cellular populations to single cells and eventually to the quantum units of single genes, the single mRNAs. Ultimately, we will be able to describe the dynamics of genes within live cells and analyze the synthesis and motion of single mRNAs traveling toward their translation sites.

2.1. LIVE-CELL SINGLE-LOCUS DETECTION OF DNA IN ITS GENOMIC CONTEXT

Chromosomes are the largest molecules present in cells. Human cells contain 46 chromosomes ranging from 58 megabases for chromosome Y (38 megatons/mol) to 243 megabases for chromosome 2 (160 megatons/mol). While most biological processes related to the regulation of genomic DNA have been biochemically or genetically dissected, it is only with the subcellular visualization of chromosomes—and more recently specific loci—that some aspects of replication, transcription, or chromosome segregation could be understood (Cremer and Cremer, 2001). Furthermore, our understanding that the different nuclear components are in constant motion (Phair and Misteli, 2000) has increased the need for the generation of new technical approaches that will enable the rapid imaging of nuclear dynamics in living cell systems. While examples of fluorescent proteins are known and have been routinely used in live cells (Tsien, 1998; Simpson et al., 2001; Zhang et al., 2002), nucleic acids possessing such properties have not been available until recently. Fluorescent thymidine analogs have been microinjected into nuclei of live cells and were incorporated into the cellular DNA. During subsequent cell cycles, the labeled DNA was replicated and segregated several times and the fluorescent nucleotide analogs were segregated to discrete chromosomes by semi-conservative replication. This approach revealed that interphase chromosome territories are dynamic structures, the spatial position of which could be regulated (Zink and Cremer, 1998; Zink et al., 1998). The noninvasive (genetically encoded fluorescence) live cell detection of chromosomes could only be achieved using the affinity of DNA-interacting proteins to chromatin. The general affinity of chromatin-interacting factors has been exploited to visualize

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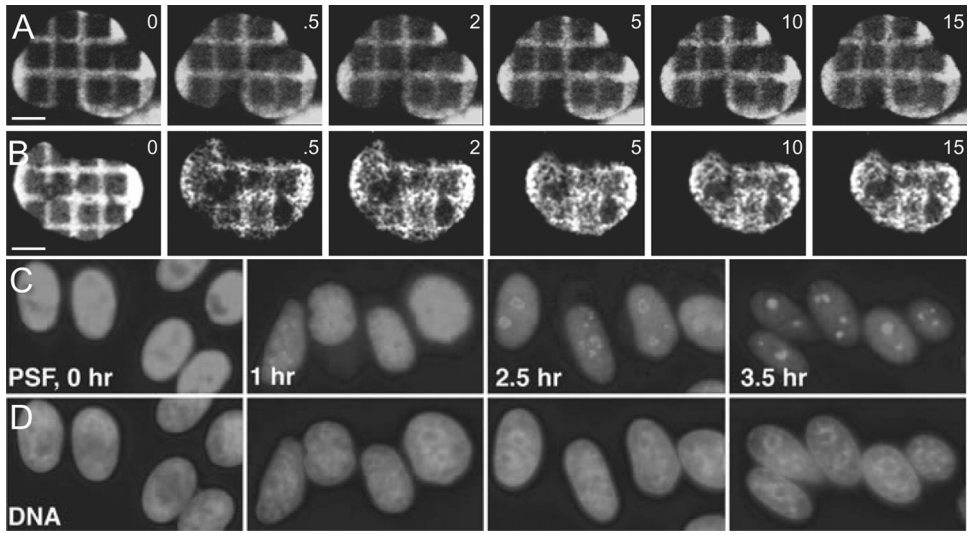

Figure 2.1. Nucleic acid binding proteins as tracers for chromatin organization. (A) Histone H2b fused to YFP was used to mark chromatin. Photobleaching of a grid on the nucleus led to the formation of a nuclear pattern that was used as a tracer for the spatial reorganization of chromatin in normal human cells or (B) in cells that were ATP depleted. (C) The formation of concave nucleolar caps is followed for time after actinomycin D treatment using the protein PSF as a marker. (D) Hoechst DNA staining shows the relationship of these caps to the nucleolar and perinucleolar chromatin. [A and B from (Shav-Tal et al., 2004a); C and D from Shav Tal et al. (2005).] See insert for color representation of this figure.

chromatin dynamics in interphase cells as well as individual chromosomes during mitosis (Lever et al., 2000; Phair and Misteli, 2000; Kimura and Cook, 2001; Forrest and Gavis, 2003; Gerlich et al., 2003; Walter et al., 2003; Phair et al., 2004; Shav-Tal et al., 2004a,b) (see Figure 2.1A, B). Proteins that interact with specific subchromosome regions have been fused to GFP and provided insights into the dynamic redistribution of centromeres (Shelby et al., 1996), nucleoli (Savino et al., 2001; Shav-Tal et al., 2005) (see Figure 2.1C,D), and telomeres (Hediger et al., 2002) within live cells.

Studying transcription and its regulation has proved to be a real challenge in live cells. Punctate nuclear structures are currently observed in fixed cells with phosphorylation site-specific antibodies directed to the RNA polymerase II catalytic subunit (CTD, C-terminal domain). These antibodies can designate the transcriptional state of the polymerase, which is known to correlate with specific sites of phosphorylation. However, this information is not available from live cells where GFP fusions of major components of the transcription apparatus do not carry information regarding transcriptional states (Iborra et al., 1996; Sugaya et al., 2000). In addition, the kinetic behavior of the polymerase alone is not sufficient to determine transcriptional kinetics since genes are dispersed and heterogeneous. What is needed is a specific, transcribing gene in order to interpret the flux of the transcription components. The first live-cell optical resolution of a single specific locus could be performed using the affinity of the lactose operon repressor protein (*lacI*) to its operon DNA sequence (*lacO*). Amplified repeats (256) of the *lacO* were sufficient to detect a single locus in a live cell using a lacI GFP fusion (Robinett et al., 1996) (see Figure 2.2). The *lacO* repeated sequences have been successfully integrated at specific loci by homologous recombination in

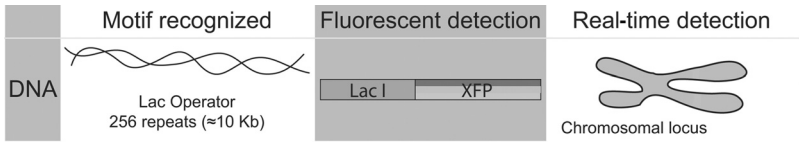

Figure 2.2. Live-cell single DNA locus detection using the *lacO/lacI* system. A tandemly repeated array (256 copies) of the lac operator (*lacO*) sequence is inserted into a chromosome. A fluorescent protein is then artificially tethered to this specific DNA sequence by fusion to the DNA-binding lac-repressor protein (*lacI*), which binds these repeats and thereby allows the detection of the integration locus in living cells. [Adapted from Shav-Tal et al. (2004b).] See insert for color representation of this figure.

yeast (Robinett et al., 1996; Straight et al., 1996; Heun et al., 2001), bacteria (Gordon et al., 1997), and viruses (Fraefel et al., 2004) or have been randomly integrated in mammalian cell genomes providing specific landmarks that can be subsequently mapped (Tsukamoto et al., 2000; Chubb et al., 2002). Other bacterial operons have been used, such as the tetracycline responsive element (TRE), providing the opportunity to label several loci in the same cell using different colors of fusion proteins (Michaelis et al., 1997). Finally, the engineering of an inducible transcription unit flanked by *lacO* repeats could reveal the dynamic interplay of chromatin-binding proteins and chromatin spatial organization during gene activation in a live cell (Tsukamoto et al., 2000; Janicki et al., 2004). The ability to follow transcription in live cells has opened a whole new field of investigation that addresses the time scale and dynamic plasticity of transcription regulatory mechanisms.

2.2. *IN SITU* DETECTION OF SINGLE RNA MOLECULES

2.2.1. Visualization of Single RNAs in Fixed Cells

The distribution of RNA molecules in fixed cells is visualized using the technique of fluorescent *in situ* hybridization (FISH). In this method the RNA of interest is detected and identified by exposing the fixed cell to an oligonucleotide probe consisting of the complementary nucleotide sequence to the RNA of interest. The probe is labeled with a fluorescent moiety and after hybridization of the probe with the RNA, and subsequent washing away of the unhybridized probe, the fluorescent signal can be detected by microscopy. This method can be utilized for quantifying the number of RNA molecules in fixed cells using sensitive quantitative digital microscopy with calibrated reagents (Femino et al., 1998; Fusco et al., 2003).

The RNA quantification procedure requires that a probe bind to only one unique sequence in the mRNA studied. These DNA probes are typically 50 nucleotides long and should contain at least four T positions for linking with fluorescent dyes. Also, a 50% G/C content for consistent hybridization kinetics is preferred. The RNA molecules throughout the cell are quantified by acquiring three-dimensional fluorescent image stacks of a region of interest from a cell that was hybridized with the specific probe, thus collecting the complete light output for each FISH probe, followed by deconvolution for the correction of optical blurring. After collecting an ideal three-dimensional digital volume of the cell, the cell is digitally divided into voxels, or three-dimensional pixels, in order to measure the number

of fluorescent molecules throughout the cell (Femino et al., 2003). In parallel, the total fluorescent intensity (TFI) of one FISH probe is calculated by imaging known quantities of labeled probes. This information is then used to calculate the fluorescent signal in each voxel of the cell, and thus a quantification of the number and distribution of RNA molecules in a cell is obtained.

This technique was used to first detect the nuclear site of transcription of the β-actin gene and then to quantify the RNA molecules made at this locus. The β-actin gene is a serum-responsive gene. In order to detect the transcription site for β-actin mRNA, the gene was activated for increasing amounts of time using serum stimulation (Femino et al., 1998). Probes that hybridized to the 5' end of the gene detected nascent transcripts, whereas probes to the 3' untranslated region (UTR) detected almost completed transcripts, providing a means to address the transcription time and the kinetics of the transcriptional activation. Using the RNA quantification method, it was found that serum-starved cells contain 500 ± 200 β-actin mRNAs per cell. However, following serum induction this mRNA population increases to approximately 1500 copies per cell. As for transcription kinetics, the addition of serum resulted in the synchronous activation of transcription of one of the β-actin alleles in all cells of the population. This occurred in a matter of a few minutes. Some minutes later, both alleles were transcribing. This allowed the measurement of transcription rates, which corresponded to 1.1–1.4 kb/min. It could also be determined that the 3' end of the gene was more packed with polymerases than the 5' end, suggesting that termination was rate-limiting. This approach was therefore capable of producing high-resolution information of the endogenous transcriptional process.

This study was followed by single-cell analysis of gene expression for a number of active genes. Several oligonucleotide probes were conjugated with different fluorescent dyes, and mixtures of probes were designed to comprise a "spectral barcode" for each gene studied. Each designated gene was then labeled with a unique combination of dyes. The simultaneous detection of several transcription sites in single fixed cultured cells showed extensive cellular stochasticity in gene expression profiles (Levsky et al., 2002). This work emphasized the necessity of single-cell approaches to characterize gene expression profiles. For instance, the response of β-actin, γ-actin, c-jun, and c-fos genes to serum induction was readily detected on the single-gene level in single cells, and even minute incremental changes in gene expression could be observed, whereas microarray analysis could only detect a significant change in the c-fos gene.

2.2.2. Visualization of Single RNAs in Living Cells

Identification of RNA molecules in fixed cells can now be extended to the visualization of their travels within a living cell. While fluorescent fusion proteins have proven to be useful tools for following protein dynamics within a living cell, tagging of RNA for real-time studies requires a different approach. A number of protocols for RNA tagging and detection in living cells have been devised. Fluorescent *in vivo* hybridization (FIVH) uses a fluorescently labeled probe that will bind to the RNA of interest, similar to fixed cell FISH (Politz et al., 1999). While this hybridization approach allows the study of endogenous RNA molecules, double-stranded RNA complexes form that might trigger cellular degradation pathways and could affect the translational process. Moreover, the need for introduction of the probe into living cells by techniques such as microinjection or membrane permeabilization also impose stress on the cell and might indirectly affect the processes under study. Finally, since

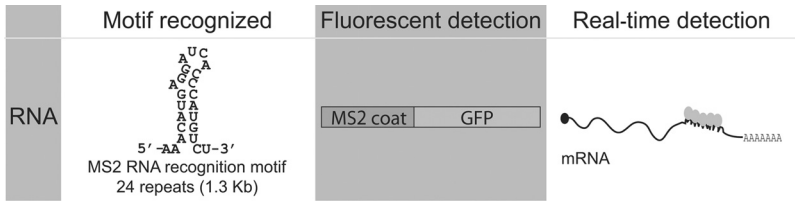

Figure 2.3. Live-cell detection of RNA molecules using the MS2 system. RNA molecules are detected using a fusion of green fluorescent protein (GFP) with the MS2 bacteriophage coat protein,which has an extremely high affinity for a short RNA-recognition motif that is derived from the phage genome. Single mRNA molecules that contain 24 MS2 RNA repeats can be detected. [Adapted from Shav-Tal et al. (2004b).] See insert for color representation of this figure.

one probe binds to one RNA molecule and an excess of probe is used in these techniques, the ability to detect a fluorescent signal from single mRNA molecules in living cells is confounded by the unhybridized probe.

In order to overcome limitations in signal detection, amplification of the fluorescent signal on a single RNA transcript is required. This has been addressed by the addition of specific protein-binding motifs to the RNA of interest. A phage RNA sequence that contains stem-loop binding sites (up to 24 sites) and that is specifically bound by the phage capsid protein MS2 is added to the DNA sequence of the gene of interest. This gene will now encode an RNA molecule with multiple binding sites for fluorescent molecules, which in this system are fluorescently tagged MS2 proteins (e.g., GFP-MS2) (see Figure 2.3). Since the binding of the MS2 protein (or GFP-MS2) to the stem-loop binding site is specific and extremely stable (K_d 39 nM), the GFP-MS2 protein binding occurs by dimerization; this site is multimerized 24 times, a strong signal above the diffuse GFP-MS2 background can be obtained, and this signal can be utilized for following the travels of specific RNAs in living cells.

This mRNA-tagging system was first used to follow the process of *ASH1* mRNA localization occurring in budding yeast (Bertrand et al., 1998). *ASH1* mRNA is one of many RNAs that are translocated from the mother yeast cell to the budding daughter cell and are found to concentrate at the bud tip (Long et al., 1997; Takizawa et al., 1997). The Ash1 protein is a nuclear DNA-binding protein required for control of mating-type switching in yeast (Darzacq et al., 2003). An mRNP complex containing an mRNA consisting of the 3′UTR of *ASH1* and MS2 repeats, bound by GFP-MS2 proteins, was imaged as it traveled from the mother cell to the bud tip. This movement was directional and exhibited speeds that correlated with that of the myosin V motor, known to be one of the required genes for successful localization.

While *ASH1* mRNP probably harbored several mRNA molecules, the behavior of single RNA molecules in living cells has also been studied using this live-cell approach (Fusco et al., 2003; Shav-Tal et al., 2004a). First, the RNA transcripts were quantified in fixed cells by sensitive FISH to confirm that the RNAs of interest traveled as single transcripts (Femino et al., 1998). The next step was visualization of these RNAs in living cells. The single mRNA molecules containing 24 MS2 stem-loop repeats were bound by GFP-MS2 proteins and formed RNA–protein complexes (mRNPs) that were amplified above background and tracked frame by frame in time-lapse movies made of living cells. Cytoplasmic mRNPs exhibited different types of movements that were characterized as diffusive, corralled, directed, or stationary. Switching between the different mobilities could be observed,

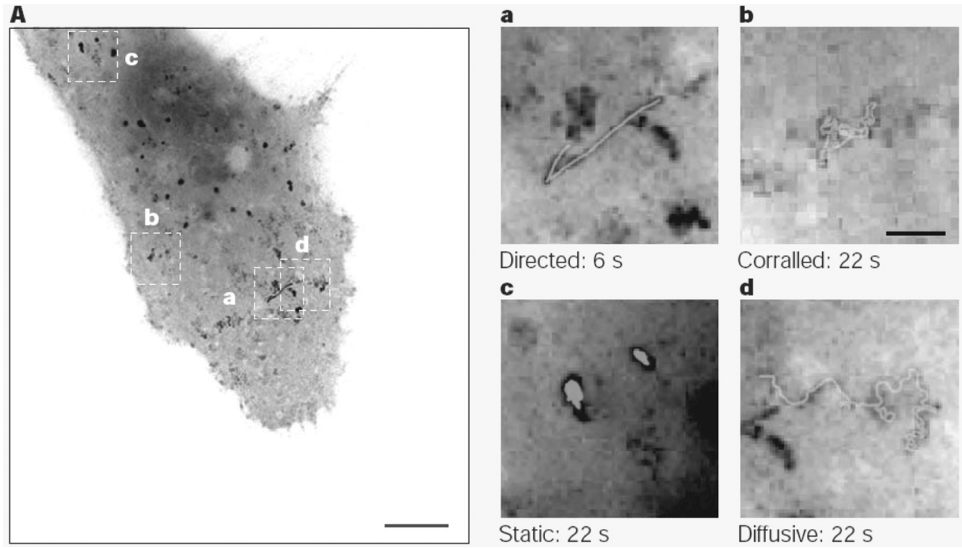

Figure 2.4. Live-cell analysis of cytoplasmic mRNPs. An mRNA transcript that contains the coding sequence of *lacZ*, 24 MS2 stem loops, and the 3′ untranslated region (UTR) of the human growth-hormone gene (hGH) was transiently expressed in COS cells together with the green fluorescent protein (GFP)–MS2 fusion protein. GFP–MS2-tagged RNA particles were followed, and different types of motility were detected in single living cells as indicated by the boxes in part A: directed (Aa), corralled (Ab), static (Ac), and diffusive (Ad). Bar, 2 μm The image in part A is a maximum-intensity image projection of 200 time frames. Bar, 10 μm. [From Fusco et al. (2003).] See insert for color representation of this figure.

and directional movements were demonstrated to follow cytoplasmic filaments (Fusco et al., 2003) (Figure 2.4).

Several studies have addressed the possible mechanisms for the nucleoplasmic travels of mRNAs in fixed cells (Zachar et al., 1993; Singh et al., 1999) and living cells (Politz et al., 1998; Politz et al., 1999; Calapez et al., 2002; Snaar et al., 2002; Molenaar et al., 2004). However, live-cell approaches have been limited to the tagging of the whole mRNA poly(A)$^+$ population in the nucleus, which could not provide a detailed analysis of a specific mRNA or of single mRNA molecules. Using the MS2 system, it is possible to probe the kinetics of single mRNA molecules as they traverse the nuclear environment (Shav-Tal et al., 2004a). In this study the travels of mRNPs containing single mRNAs, as they were released from the site of transcription and traveled to the nuclear pore, were examined in living cells using the MS2 system. Nuclear mRNPs were detected and analyzed by single-particle tracking and were found to exhibit random movements, following rules of simple diffusion (Figure 2.5). In contrast to the cytoplasm, directional movements of mRNPs were not seen in the nucleoplasm. While single-particle tracking proved useful for the analysis of mRNP movements in their nuclear microenvironments, FRAP analysis was used to follow the behavior of the population of these mRNPs and photoactivation was used to address the mobility of subpopulations of mRNPs at the time of their release from the site of transcription. These studies provided biophysical measurements for the diffusion coefficients of the mRNPs and consequently of nucleoplasmic viscosity.

Another approach for tagging of mRNA in living cells is by the use of a human U1A tag in experiments conducted in yeast cells. The U1A protein binds with high specificity

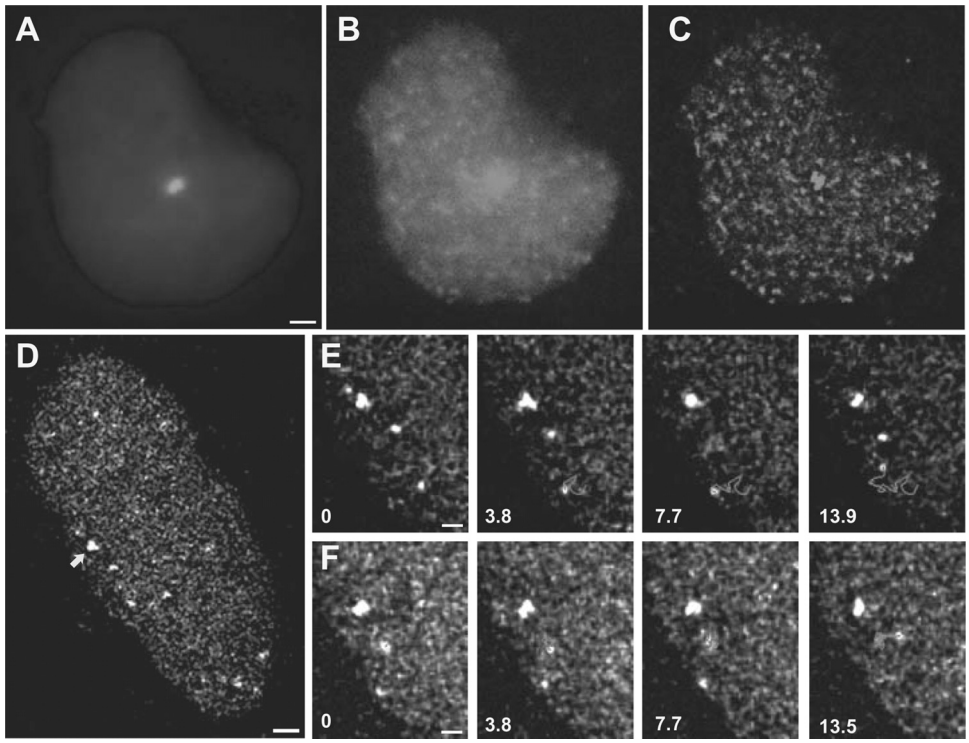

Figure 2.5. Live-cell analysis of nucleoplasmic mRNPs. (A) Image from a time-lapse movie acquired from transcriptionally induced cells containing a *lacO* cassette co-transfected with CFP-lac repressor (see Figure 2.2). (B) An inducible transcript tagged with MS2 repeats cotransfected with YFP-MS2 (see Figure 2.3). (C) Reduction of noise for tracking of RNA particles was obtained by deconvolution (Huygens software). Bar = 5 μm. (D) Tracking of particles in a transcriptionally active cell (arrow = transcription site) showed (E) diffusing particles, and (F) corralled particles. [From Shav-Tal et al. (2004a).] See insert for color representation of this figure.

and affinity to a small RNA hairpin, which does not exist in yeast. This tag is useful only in yeast since mammalian cells also have the endogenous U1A protein. In this system a GFP-U1A protein was used to follow the export pathway of different mRNAs that were engineered to contain the U1A hairpin (Brodsky and Silver, 2000). This approach was used for the analysis of the effects of UTRs, introns, RNA processing factors, nucleoporins, and transport factors on the export of the mRNAs and provided important insights on the connection between pathways of mRNA processing and mRNA export.

Most studies utilizing the MS2 or U1A RNA-tagging systems have been performed with exogenous genes since this strategy requires site-specific insertion of the hairpins into the gene of interest. Tracking RNAs produced from endogenous genes has been approached usually by indirect labeling of RNA with GFP-tagged RNA-binding proteins. Two examples are GFP-exuperantia, used to follow *bicoid* mRNA (Theurkauf and Hazelrigg, 1998) or GFP-polyA-binding protein II (PABP2) (Calapez et al., 2002). The former labels a specific RNA molecule, while the latter tags any RNA molecules transcribed by RNA polymerase II. However, the high abundance of these proteins and their lower affinity for RNA permits exchange and the few molecules of GFP bound per RNA do not allow single-molecule or single-mRNP studies. The introduction of multiple RNA-binding sites to a specific

transcript is therefore an essential step in obtaining high-quality fluorescent signal over the background fluorescence.

The development of techniques that can provide a platform for the detection of mRNAs in living cells is of key interest when attempting to study mRNA kinetics of real-time processes occurring in living organisms. A perfect example in which the MS2 system has been utilized in an organism is the process of mRNA localization (Shav-Tal and Singer, 2005). Localized translation is controlled spatially and temporally in specified areas in *Drosophila* oocytes and embryos by the differential localization of a variety of mRNAs necessary for development. For instance, the nanos protein is required for the formation of the anterior–posterior body axis in the developing *Drosophila* oocyte (Tautz, 1988; Gavis and Lehmann, 1992). Some of *nanos* mRNA is localized to the posterior end of the oocyte during development, where it is stable and translated. This localization of *nanos* mRNA occurs in late stages of oogenesis. By tagging the nanos mRNA with a GFP-MS2 protein, it was possible to examine the dynamic aspects of the localization process. It was found that in contrast to other transport systems that require motor proteins for mRNA localization, *nanos* mRNA diffuses and reaches the posterior region through microtubule-dependent cytoplasmic movements, where it remains anchored by the actin cytoskeleton (Forrest and Gavis, 2003). Studies are now underway to introduce the MS2-binding sites into the genome of various organisms. This will ultimately allow the detection of the transcriptional activity of any endogenous gene and its mRNA complement in living cells and tissues.

2.2.3. Perspectives

Studying the kinetics of gene expression—from the locus of transcription, through nucleoplasmic travels, nuclear export, translation, and degradation—is of key importance for the understanding of fundamental processes taking place along the gene expression pathway. Major breakthroughs in the field of bioimaging have advanced our capability to probe these processes in living cells. However, at this time our technical abilities are mainly confined to addressing real-time processes occurring on exogenous gene constructs. We foresee a necessary step forward in the development of tools for the detection and analysis of endogenous gene expression. Such approaches will allow us to study the regulation of gene expression in the *in vivo* environment and ultimately even in the living organism.

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REFERENCES

Bertrand, E, Chartrand, P, Schaefer, M, Shenoy, SM, Singer, RH, and Long, RM (1998). Localization of ASH1 mRNA particles in living yeast. *Mol Cell* **2**:437–445.

Brodsky, AS, and Silver, PA (2000): Pre-mRNA processing factors are required for nuclear export. *RNA* **6**:1737–1749.

Calapez, A, Pereira, HM, Calado, A, Braga, J, Rino, J, Carvalho, C, Tavanez, JP, Wahle, E, Rosa, AC, and Carmo-Fonseca, M (2002). The intranuclear mobility of messenger RNA binding proteins is ATP dependent and temperature sensitive. *J Cell Biol* **159**:795–805.

Chubb, JR, Boyle, S, Perry, P, and Bickmore, WA (2002). Chromatin motion is constrained by association with nuclear compartments in human cells. *Curr Biol* **12**:439–445.

Cremer, T, and Cremer, C (2001). Chromosome territories, nuclear architecture and gene regulation in mammalian cells. *Nat Rev Genet* **2**:292–301.

Darzacq, X, Powrie, E, Gu, W, Singer, RH, and Zenklusen, D (2003). RNA asymmetric distribution and daughter/mother differentiation in yeast. *Curr Opin Microbiol* **6**:614–620.

Femino, AM, Fay, FS, Fogarty, K, and Singer, RH (1998). Visualization of single RNA transcripts *in situ*. *Science* **280**:585–590.

Femino, AM, Fogarty, K, Lifshitz, LM, Carrington, W, and Singer, RH (2003). Visualization of single molecules of mRNA *in situ*. *Methods Enzymol* **361**:245–304.

Forrest, KM, and Gavis, ER (2003). Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*. *Curr Biol* **13**:1159–1168.

Fraefel, C, Bittermann, AG, Bueler, H, Heid, I, Bachi, T, and Ackermann, M (2004). Spatial and temporal organization of adeno-associated virus DNA replication in live cells. *J Virol* **78**:389–398.

Fusco, D, Accornero, N, Lavoie, B, Shenoy, SM, Blanchard, JM, Singer, RH, and Bertrand, E (2003). Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr Biol* **13**:161–167.

Gavis, ER, and Lehmann, R (1992). Localization of nanos RNA controls embryonic polarity. *Cell* **71**:301–313.

Gerlich, D, Beaudouin, J, Kalbfuss, B, Daigle, N, Eils, R, and Ellenberg, J (2003). Global chromosome positions are transmitted through mitosis in mammalian cells. *Cell* **112**:751–764.

Gordon, GS, Sitnikov, D, Webb, CD, Teleman, A, Straight, A, Losick, R, Murray, AW, and Wright, A (1997). Chromosome and low copy plasmid segregation in E. coli: visual evidence for distinct mechanisms. *Cell* **90**:1113–1121.

Hediger, F, Neumann, FR, Van Houwe, G, Dubrana, K, and Gasser, SM (2002). Live imaging of telomeres: yKu and Sir proteins define redundant telomere-anchoring pathways in yeast. *Curr Biol* **12**:2076–2089.

Heun, P, Laroche, T, Shimada, K, Furrer, P, and Gasser, SM (2001). Chromosome dynamics in the yeast interphase nucleus. *Science* **294**:2181–2186.

Iborra, FJ, Pombo, A, Jackson, DA, and Cook, PR (1996). Active RNA polymerases are localized within discrete transcription 'factories' in human nuclei. *J Cell Sci* **109**(Pt 6):1427–1436.

Janicki, SM, Tsukamoto, T, Salghetti, SE, Tansey, WP, Sachidanandam, R, Prasanth, KV, Ried, T, Shav-Tal, Y, Bertrand, E, Singer, RH, and Spector, DL (2004). From silencing to gene expression; real-time analysis in single cells. *Cell* **116**:683–698.

Kimura, H, and Cook, PR (2001). Kinetics of core histones in living human cells: little exchange of H3 and H4 and some rapid exchange of H2B. *J Cell Biol* **153**:1341–1353.

Lever, MA, Th'ng, JP, Sun, X, and Hendzel, MJ (2000). Rapid exchange of histone H1.1 on chromatin in living human cells. *Nature* **408**:873–876.

Levsky, JM, Shenoy, SM, Pezo, RC, and Singer, RH (2002). Single-cell gene expression profiling. *Science* **297**:836–840.

Long, RM, Singer, RH, Meng, X, Gonzalez, I, Nasmyth, K, and Jansen, RP (1997). Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. *Science* **277**:383–387.

Michaelis, C, Ciosk, R, and Nasmyth, K (1997). Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**:35–45.

Molenaar, C, Abdulle, A, Gena, A, Tanke, HJ, and Dirks, RW (2004). Poly(A)+ RNAs roam the cell nucleus and pass through speckle domains in transcriptionally active and inactive cells. *J Cell Biol* **165**:191–202.

Phair, RD, and Misteli, T (2000). High mobility of proteins in the mammalian cell nucleus. *Nature* **404**:604–609.

Phair, RD, Gorski, SA, and Misteli, T (2004). Measurement of dynamic protein binding to chromatin *in vivo*, using photobleaching microscopy. *Methods Enzymol* **375**:393–414.

Politz, JC, Browne, ES, Wolf, DE, and Pederson, T (1998). Intranuclear diffusion and hybridization state of oligonucleotides measured by fluorescence correlation spectroscopy in living cells. *Proc Natl Acad Sci USA* **95**:6043–6048.

Politz, JC, Tuft, RA, Pederson, T, and Singer, RH (1999). Movement of nuclear poly(A) RNA throughout the interchromatin space in living cells. *Curr Biol* **9**:285–291.

Robinett, CC, Straight, A, Li, G, Willhelm, C, Sudlow, G, Murray, A, and Belmont, AS (1996). *In vivo* localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. *J Cell Biol* **135**:1685–1700.

Savino, TM, Gebrane-Younes, J, De Mey, J, Sibarita, JB, and Hernandez-Verdun, D (2001). Nucleolar assembly of the rRNA processing machinery in living cells. *J Cell Biol* **153**:1097–1110.

Shav-Tal, Y, and Singer, RH (2005). RNA localization. *J Cell Sci* **118**:4077–4081.

Shav-Tal, Y, Darzacq, X, Shenoy, SM, Fusco, D, Janicki, SM, Spector, DL, and Singer, RH (2004a). Dynamics of single mRNPs in nuclei of living cells. *Science* **304**:1797–1800.

Shav-Tal, Y, Singer, RH, and Darzacq, X (2004b). Imaging gene expression in single living cells. *Nat Rev Mol Cell Biol* **5**:855–861.

Shav-Tal, Y, Blechman, J, Darzacq, X, Montagna, C, Dye, BT, Patton, JG, Singer, RH, and Zipori, D (2005). Dynamic sorting of nuclear components into distinct nucleolar caps during transcriptional inhibition. *Mol Biol Cell* **16**:2395–2413.

Shelby, RD, Hahn, KM, and Sullivan, KF (1996). Dynamic elastic behavior of alpha-satellite DNA domains visualized *in situ* in living human cells. *J Cell Biol* **135**:545–557.

Simpson, JC, Neubrand, VE, Wiemann, S, and Pepperkok, R (2001). Illuminating the human genome. *Histochem Cell Biol* **115**:23–29.

Singh, OP, Bjorkroth, B, Masich, S, Wieslander, L, and Daneholt, B (1999). The intranuclear movement of Balbiani ring premessenger ribonucleoprotein particles. *Exp Cell Res* **251**:135–146.

Snaar, SP, Verdijk, P, Tanke, HJ, and Dirks, RW (2002). Kinetics of HCMV immediate early mRNA expression in stably transfected fibroblasts. *J Cell Sci* **115**:321–328.

Straight, AF, Belmont, AS, Robinett, CC, and Murray, AW (1996). GFP tagging of budding yeast chromosomes reveals that protein–protein interactions can mediate sister chromatid cohesion. *Curr Biol* **6**:1599–1608.

Sugaya, K, Vigneron, M, and Cook, PR (2000). Mammalian cell lines expressing functional RNA polymerase II tagged with the green fluorescent protein. *J Cell Sci* **113**(Pt 15):2679–2683.

Takizawa, PA, Sil, A, Swedlow, JR, Herskowitz, I, and Vale, RD (1997). Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. *Nature* **389**:90–93.

Tautz, D (1988). Regulation of the *Drosophila* segmentation gene hunchback by two maternal morphogenetic centres. *Nature* **332**:281–284.

Theurkauf, WE, and Hazelrigg, TI (1998). *In vivo* analyses of cytoplasmic transport and cytoskeletal organization during *Drosophila* oogenesis: Characterization of a multi-step anterior localization pathway. *Development* **125**:3655–3666.

Tsien, RY (1998). The green fluorescent protein. *Annu Rev Biochem* **67**:509–544.

Tsukamoto, T, Hashiguchi, N, Janicki, SM, Tumbar, T, Belmont, AS, and Spector, DL (2000). Visualization of gene activity in living cells. *Nat Cell Biol* **2**:871–878.

Walter, J, Schermelleh, L, Cremer, M, Tashiro, S, and Cremer, T (2003). Chromosome order in HeLa cells changes during mitosis and early G1, but is stably maintained during subsequent interphase stages. *J Cell Biol* **160**:685–697.

Zachar, Z, Kramer, J, Mims, IP, and Bingham, PM (1993). Evidence for channeled diffusion of pre-mRNAs during nuclear RNA transport in metazoans. *J Cell Biol* **121**:729–742.

Zhang, J, Campbell, RE, Ting, AY, and Tsien, RY (2002). Creating new fluorescent probes for cell biology. *Nat Rev Mol Cell Biol* **3**:906–918.

Zink, D, and Cremer, T (1998). Cell nucleus: Chromosome dynamics in nuclei of living cells. *Curr Biol* **8**:R321–R324.

Zink, D, Cremer, T, Saffrich, R, Fischer, R, Trendelenburg, MF, Ansorge, W, and Stelzer, EH (1998). Structure and dynamics of human interphase chromosome territories *in vivo*. *Hum Genet* **102**:241–251.

Figure 2.1

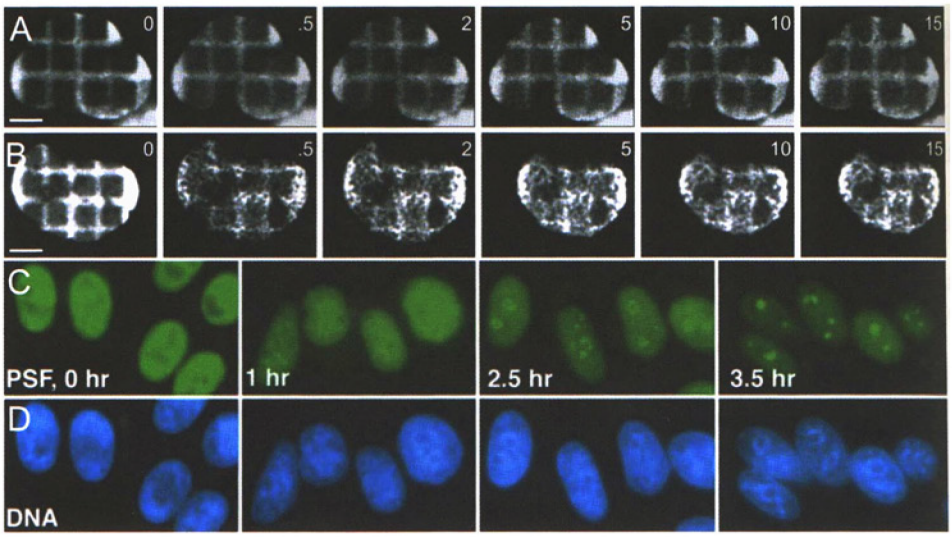

Figure 2.2

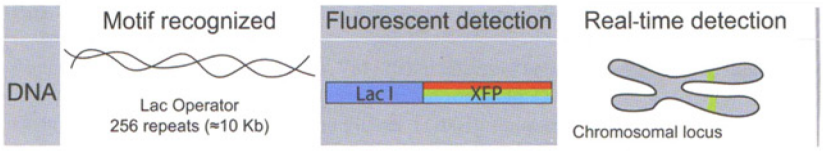

Figure 2.3

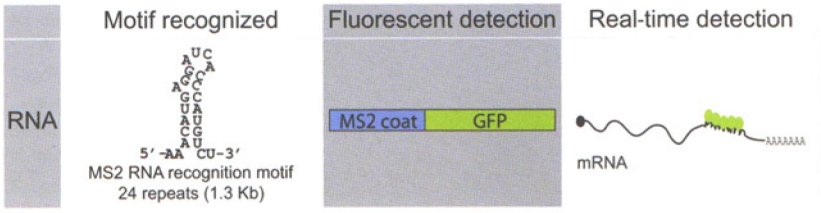

Figure 2.4

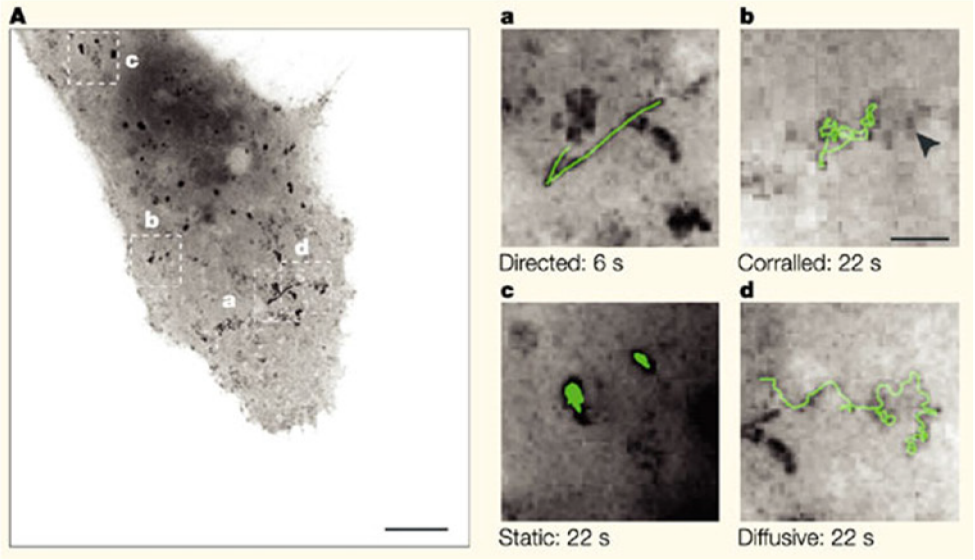

Figure 2.5