Dynamics of Single mRNPs in Nuclei of Living Cells

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Understanding gene expression requires the ability to follow the fate of individual molecules. Here we use a cellular system for monitoring messenger RNA (mRNA) expression to characterize the movement in real time of single mRNA-protein complexes (mRNPs) in the nucleus of living mammalian cells. This mobility was not directed but was governed by simple diffusion. Some mRNPs were partially corralled throughout the nonhomogenous nuclear environment, but no accumulation at subnuclear domains was observed. Following energy deprivation, energy-independent motion of mRNPs was observed in a highly ATP-dependent nuclear environment; movements were constrained to chromatin-poor domains and excluded by newly formed chromatin barriers. This observation resolves a controversy, showing that the energetic requirements of nuclear mRNP trafficking are consistent with a diffusional model.

Recent technological developments have facilitated imaging of single RNA molecules in the cytoplasm of living cells (1). We have developed a cellular system in which the expression of a transgene array can be followed sequentially in single living cells, and we have previously analyzed the particular chromatin-related modifications occurring at this specific locus from a silenced state throughout its transcriptional activation (2). Here we use this system to address the mechanism by which individual mRNA transcripts move within the nucleoplasm after release from the transcription site.

In this system, a genetic locus, its transcribed mRNAs, and the translated protein were rendered visible in cells after electroporation with the cyan fluorescent protein (CFP) or the red fluorescent protein (RFP)–lac repressor protein (marks the genomic locus), yellow fluorescent protein (YFP)–MS2 (labels the mRNA), and pTet-On (for transcriptional induction) (3). Transcriptional activation by doxycycline induced the expression of particles imaged (Fig. 1K), although directed translocations were easily detected for cytoplasmic CFP peroxisomes by using our SPT algorithm (3) (fig. S3C). Extremely confined movements were observed for transcription sites and for some mRNPs (less than 1% of detectable mRNPs) (Fig. 11 and fig. S3B). These mRNPs were confined within small nucleoplasmic volumes. Observations of living cells (3) indicated that once mRNPs were released from the transcription site, their movements through the nucleoplasm on the way to the nuclear pore were rarely hindered by stable interactions with nuclear substructures. Altogether, these data indicated that nucleoplasmic mRNP movement was governed by laws of simple diffusion and was not directional.

Fluorescence recovery after photobleaching (FRAP) of the tagged mRNPs was followed over time, and the mean diffusion coefficient \(D_3\) of these mRNPs was calculated to be 0.09 ± 0.006 \(\mu\msec^{-2}\) (Fig. 2, A and B), corroborating the SPT data. As with SPT, an immobile fraction was not observed.

We then addressed the movement of mRNPs present in the vicinity of the transcription site, using a third technique. By use of a photoactivatable form of green fluorescent protein (GFP) movements of nuclear mRNPs were followed by sequential imaging of living cells, and the obtained signal was enhanced by deconvolution (Fig. 1, A to C). Live cells at early times after induction (up to 30 min) presenting a low concentration of mRNPs were used to continuously track individual particles and study their motion (Fig. 1D). Single-particle tracking (SPT) was performed on mRNPs that remained in focus for a minimum of eight consecutive frames (>3 s) (Fig. 1, E to H) [movies S6 to S10 (5)]. Total distances traveled for tracked mRNPs were from 2 to 10 \(\mu\msec\) (mean 5 \(\mu\msec\)); and mean velocities ranged from 0.3 to 0.8 \(\mu\msec\) (Fig. S3A). These mRNPs displayed a diffusive pattern of movement (Fig. 1E) following a simple diffusion model \(<r^2>=4Dt\) (Fig. 1J) (3). The diffusion coefficients at 37°C \(D_3\) ranged from 0.01 to 0.09 \(\mu\msec^2\) (mean 0.04 \(\mu\msec^2\)) (Fig. 1K). The mean square displacement (MSD) plotted over time \(\Delta t\) was linear in 58% of the cases, characteristic of simple diffusion (Fig. 1J); for 42% of the mobile mRNPs, the plots began linearly but reached a plateau (Fig. 1J), characteristic of corralled diffusion (Fig. 1F). This result indicated the presence of barriers hindering the movement of the mRNPs. Directed movements in the nucleus were not seen among the tracked particles or observed in the overall population of particles imaged (Fig. 1K), although directed translocations were easily detected for cytoplasmic CFP peroxisomes by using our SPT algorithm (3) (fig. S3C).

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References and Notes

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Materials and Methods

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(7) fused to the MS2 protein, we followed the mRNPs in a "visible pulse-chase" experiment. A short pulse of activating light (405 nm) confined only to the transcription site fluorescently labeled those RNP particles being actively transcribed or just released (Fig. 2, C to E). This generated an initial pool of fluorescent mRNAs that, upon release from the transcriptional machinery, diffused away from the transcription site in all directions (Fig. 2, F and G) (3) [movie S11 (5)]. Measurements of the area in which these mRNAs moved over time (Fig. 2G) showed a mean diffusion coefficient \( D_{22} \) of 0.034 ± 0.006 \( \mu m^2/sec \), consistent with the range of measurements obtained by the previous two approaches (FRAP yields a \( D_{22} \) of 0.049 ± 0.002 \( \mu m^2/sec \), as discussed below and in fig. S4H). In summary, a similar range of diffusion coefficients for nuclear mRNPs was measured with three techniques.

Analysis of the SPT data did not show any characteristics of anomalous diffusion (8), expected if the mRNPs were to exhibit energy-dependent binding, and SPT performed at room temperature showed \( D_s \) of 0.02 to 0.09 \( \mu m^2/s \) (mean 0.044 \( \mu m^2/s \)) (fig. S3D). According to the Stokes-Einstein equation (3), the diffusion of mRNPs should be affected by the absolute temperature, the radius of the particle, and the viscosity of the nucleoplasm. Because the nucleus is basically an aqueous particle, and the viscosity of the nucleoplasm.

Because the nucleus is basically an aqueous solution (9), nuclear viscosity decreases in a temperature-dependent manner and should lead in turn to a drop in diffusion coefficients. FRAP experiments (3) (fig. S4) revealed that the \( D_s \) of mRNPs and free YFP-MS2 protein were affected in a linear manner by a reduction in temperature. The linear relationship between \( D \) and temperature argues against an energy-driven mechanism for translocation of mRNPs in the nucleus (10). There has been persistent speculation concerning the role of actin and myosin in RNA transport in the nucleus (11, 12); however, inhibitors to known motors did not affect the motion of the nuclear mRNPs (3).

Whether nuclear mRNP movement is an energy-dependent process is controversial (13, 14), and thus far no study has provided an explanation for any energy-dependent effects. To determine whether diffusion was independent of cellular metabolism, we treated cells with the general metabolic inhibitors 2-deoxyglucose (2-DG) and sodium azide. Unexpectedly, the majority of the mRNPs showed measurable decreased mobility throughout the nucleoplasmic space and concentrated in subnuclear regions surrounded by chromatin-dense domains (Fig. 3, A to C) (3) [movies S12 to S14 (5)]. Further analysis showed that the mobility was reduced as a result of the constraints imposed by small subnuclear volumes, as determined by the notable decrease in the corralled radius observed by SPT (Fig. 3D). Replenishment of energy levels restored mRNP movement (3). When energy-depleted cells were subjected to mild permeabilization with Triton X-100, mRNPs remained trapped throughout the nucleoplasm, in contrast to undepleted cells where mRNPs rapidly dispersed (Fig. 3, E to H). This observation confirmed that under conditions of energy depletion, intranuclear structures constrained mRNP mobility. FRAP of energy-depleted cells showed that 46% of the mRNPs were trapped in the bleached area (Fig. 2A). Direct visualization of the mRNPs (3) [movies S12 and S14 (5)] showed that, although constrained, the mRNPs moved within areas of confinement. Particles that moved in and out of a bleached area had an apparent diffusion coefficient of 0.036 \( \mu m^2/s \) (Fig. 2B). This \( D \) was lower than observed for nondepleted cells; however, the extremely corralled nature of the mRNPs under these conditions could explain this difference.

The stalled mRNPs localized in DNA-poor regions, and the reduction in corral size strongly implied that a change in nuclear substructure obstructed free diffusion of mRNPs under conditions of energy deprivation. Chromatin structure in living cells after energy depletion was then examined. Cells transiently expressing histone H2B-YFP, together with a brief exposure to

**Fig. 1.** Live-cell imaging and single-particle tracking of individual mRNPs. Images from time-lapse movies acquired from a cell cotransfected with (A) CFP-lac repressor and (B) YFP-MS2 and induced for more than 30 min. (C) Reduction of noise for tracking of mRNPs was obtained by deconvolution. Bar, 2 \( \mu m \). (D) Tracking of mRNP particles in a transcriptionally active cell induced for only 20 min (arrow, transcription site) [bar, 2 \( \mu m \)] [Movie S6 (5)] showed (E) diffusing particles [Movie S7 (5)], (F) corralled particles [Movie S8 (5)], (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 appears in each frame. Bars, 1 \( \mu m \). (I) Plot of the area per frame in which each of the tracked particles from this particular cell traveled throughout the tracking period. Diffusive particles are in blue, corralled in green, stationary in yellow, and transcription site in red. Particle 1, particle seen in E; 2, F; 5, G; 6, H. (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; plotted from data of tracked directed cytoplasmic particles). (K) Table summarizing the mean velocities and diffusion coefficients of tracked particles at 37°C. %, percent of tracked particles. D, diffusion coefficient.
To quantify the movement of the mRNPs, we measured concentric arcs at various distances (signal wave from the transcription site, as indicated in red, and plotted the normalized to preactivation calculated diffusion rates (3)

Hoechst DNA stain, showed the rapid formation and B). The nucleoplasmic signal was diffusely served by electron microscopy (EM) (fig. S5, A and S16, S15, S19, and S20) for free YFP-NLS, free YFP-MS2, mRNPs at different temperatures, and mRNPs in ATP-depleted cells. (C to E) A cell in which the photoactivatable MS2-GFP (MS2-paGFP) gene was stably integrated (2-3-6-PA) was cotransfected with RFP-lac repressor and pTet-On. Transcription was induced for 30 min by doxycycline. The locus was detected (red) prior to photoactivation (C), and the image in GFP before activation was recorded (D). The 405-nm laser was directed at the boxed region of interest (yellow), and the MS2-paGFP was detected at the transcription site 1.635 s after activation (E). Bar, 2 μm. (F) The RNP signal emanating from the transcription site was followed for 262 s (bar, 2 μm) [Movie S11 (5)]. (G) To quantify the movement of the mRNPs, we measured concentric arcs at various distances (r) from the transcription site, as indicated in red, and plotted the normalized (to preactivation value) average intensities per pixel for each arc. The three plots represent three examples of time points (green, 1.635 s; black, 21.255 s; blue, 31.065 s). The distances between the peaks of the signal wave (Δr) were calculated over time (Δt) and the diffusion coefficients determined.

that nuclear reorganization observed under energy stress is a general effect influencing both chromatin and interchromatin domains.

A model for free diffusion of nuclear mRNPs was proposed more than a decade ago (15). Other studies using a variety of methods—labeled antisense probes (16-18), GFP-tagged RNA-binding proteins (19), or EM analysis of the distribution of mRNPs (20)—have proposed that a population of RNPs move by diffusion through the nucleoplasmic space and have estimated a wide range of diffusion coefficients for these labeled RNPs [for an extended discussion, see (3)]. In most of these studies, a diffuse fluorescent signal attributed to populations of mRNPs was followed, but the possibility could not be excluded that an exchange of the fluorescent tag was contributing to the measured mobility rates. In contrast, the specific MS2 protein amplifies only on mRNAs containing the MS2 aptamer, thereby forming single mRNPs that can be individually and agnostically tracked. Observations of thousands of mRNPs translocations confirmed a strictly diffusion-based model revealing subsets of free diffusing and heretofore undetected corralled and, on rare occasions, constrained states. There were no stable attachments outside the transcription site, nor noticeable accumulations in subnuclear domains. These movements were ATP independent, and the major nuclear reorganization we observed during energy stress may explain the previously described changes in the mobility of various nuclear components [see discussion in (3)]. While the reporter RNP is a bona fide mRNA, it is possible that specific mRNPs or other cell types could have alternative means of nuclear egress.

In summary, this approach allows direct observation of the primary transcript of gene expression and accesses the statistical variance and diversity of molecular events within the population. It provides a means for testing microenvironmental models that act on each molecule. One such model, that of a system directing these transcripts, can be eliminated. Clearly, the complexity of RNA trafficking requires further investigation.

References and Notes
3. Materials, methods, and additional data are available as supporting material on Science Online.

Protein Kinase G from Pathogenic Mycobacteria Promotes Survival Within Macrophages

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Pathogenic mycobacteria resist lysosomal delivery after uptake into macrophages, allowing them to survive intracellularly. We found that the eukaryotic-like serine/threonine protein kinase G from pathogenic mycobacteria was secreted within macrophage phagosomes, inhibiting phagosome-lysosome fusion and mediating intracellular survival of mycobacteria. Inactivation of protein kinase G by gene disruption or chemical inhibition resulted in lysosomal localization and mycobacterial cell death in infected macrophages. Besides identifying a target for the control of mycobacterial infections, these findings suggest that pathogenic mycobacteria have evolved eukaryotic-like signal transduction mechanisms capable of modulating host cell trafficking pathways. Pathogenicity of mycobacteria is tightly linked to their survival in host macrophages. Normally, phagocytosed microorganisms are rapidly transferred from phagosomes to lysosomes and are then degraded. However, pathogenic mycobacteria resist lysosomal delivery and survive within macrophages inside mycobacterial phagosomes (1, 2). Mycobacterial phagosomes are characterized by the absence of lysosomal markers such as lysosomal associated membrane proteins (LAMP) and mature lysosomal hydrolases (3–5). Phagosomes harboring mycobacteria also contain markers for the early endosomal pathway (6, 7), have a reduced amount of the vacular proton-translocating adenosine triphosphatase, and retain the Trp-Asp (WD) repeat–containing protein coronin 1 (also known as TACO or P57) that prevents fusion of phagosomes with lysosomes (8–11).

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