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The timing of pre-mRNA splicing visualized in real-time

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Since it became clear that intervening sequences or introns are spliced out from precursor pre-mRNA molecules in the nucleus before mature mRNAs are exported to the cytoplasm, questions were raised about the timing of splicing. Does splicing start while RNA polymerase II is still transcribing? Is splicing a slow or a fast process? Is timing important to control the splicing reaction? Although our understanding on the mechanism and function of splicing is largely based on data obtained using biochemical and large-scale "omic" approaches, microscopy has been instrumental to address questions related to timing. Experiments done with the electron microscope paved the way to the discovery of splicing and provided unequivocal evidence that splicing can occur co-transcriptionally. More recently, live-cell microscopy introduced a technical breakthrough that allows real-time visualization of splicing dynamics. We discuss here some of the microscopy advances that provided the basis for the current conceptual view of the splicing process, and we outline a most recent development that permits direct measurement, in living cells, of the time it takes to synthesize and excise an intron from individual pre-mRNA molecules.

In the summer of 1977, a set of electron micrographs shown at the annual Cold Spring Harbor Symposium revolutionized the way molecular biologists perceived the structure of genes. Following the development of methodologies to observe isolated RNA and DNA molecules by electron microscopy, it was found that isolated RNA molecules can hybridize to double-stranded DNA by displacing one of the DNA strands.[1] Using this approach, the Roberts and Sharp teams independently realized that adenoviral polysomal RNAs do not hybridize in a continuous manner with the DNA from which they are transcribed. Rather, the mRNA sequence was complementary to multiple noncontiguous regions of the DNA.[2,3] They concluded that adenoviral genes were split into several pieces, and because adenovirus is a mammalian virus, it seemed likely that at least some mammalian genes could have a similar structure. This was soon found to be the case. Although different mechanisms could result in joining together certain segments of genomic sequence into mature mRNA, evidence quickly accumulated supporting a model for mRNA synthesis that involves splicing of a larger precursor molecule or pre-mRNA.

Understanding the splicing mechanism required the development of cell-free systems where conditions could be controlled and the components purified and reconstituted. In vitro splicing assays based on extracts obtained by moderate salt solubilization of lysed cells or crude nuclei were able to accurately splice exogenously added pre-mRNA, indicating that splicing and transcription are independent processes (for a review see ref. 4). Yet, visualizing dispersed chromatin by electron microscopy showed that splicing was coupled to transcription in vivo. Back in 1969, Miller and Beatty reported that hypotonic lysis of cells released chromatin from the nucleus.[5] The released chromatin was then centrifuged onto a grid for

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observation in the electron microscope. This procedure resulted in a loosened two-dimensional array of chromatin fibers that maintained the nucleosomal structure. Nascent transcripts remained attached to the chromatin and appeared as ribonucleoprotein fibers extending from the chromatin backbone. The length of nascent transcripts, which could be precisely measured, increased with increasing distance from the transcription start site. Using this approach, Beyer and Osheim showed that the spliceosome formed shortly after synthesis of the 3′ splice site and that splicing of pre-mRNA often occurred on the nascent transcript.[6] This visual demonstration that introns could be removed prior to transcript release from the template and thus presumably prior to polyadenylation challenged the prevailing textbook view that splicing took place after polyadenylation. Beyer and Osheim assumed an elongation rate of 1500 nucleotides per minute to then deduce that splicing of introns removed cotranscriptionally occurred within 3 min after synthesis of the 3′ splice site. Similar conclusions were obtained when studying actively transcribed genes in *Chironomus tentans* salivary gland polytene chromosomes, which can be isolated by microdissection for direct observation in the electron microscope.[7] In vivo splicing rates in the range of 5 min or less were also measured biochemically for adenovirus and β-globin transcripts.[8-10] Such rates differed substantially from the results obtained in cell-free assays, where a lag period of 15–45 min was observed at the start of the reaction before the onset of splicing, following which spliced RNA accumulated for 2–3 h.[4] Thus, splicing in vivo is at least 10-fold more rapid than in vitro. This discrepancy highlights the importance of nuclear organization: most likely, spliceosome components in the nucleus are organized in a way that favors efficient recognition of splice site sequences as soon as they emerge from RNA polymerase.

Imaging the spatial organization of genes, nascent RNAs, and spliceosome components in the intact cell nucleus was made possible through successive optimizations of another microscopic technique termed in situ hybridization. The pioneer method relied on synthesis of radioactive RNA complementary to the DNA of interest. Cells were made permeable and incubated with the radioactive probe, resulting in formation of RNA-DNA hybrids. The location of these hybrids was then detected at the cytological level by autoradiography.[11,12] Over the next 30 years, this technology progressed from a laborious, time-consuming method limited to the detection of highly abundant nucleic acid species with low cytological resolution, to a relatively fast, highly precise, and sensitive imaging of single genes and RNA molecules in the cell. An important advance was the optimization of the in situ hybridization procedure for fluorescent detection of nuclear RNA using biotin-labeled DNA probes and fluorophore-conjugated avidin.[13] Next, the method was further optimized for the simultaneous detection of DNA, unspliced precursors, and spliced mRNA. Using these tools, several laboratories showed that spliced mRNA localized in close proximity to the DNA from which it was transcribed, consistent with the view that many introns are excised while the pre-mRNA is still tethered to the gene locus via RNA polymerase.[14-16] There are, however, situations where splicing is uncoupled from transcription: in the case of regulated alternative splicing events, intron removal can be delayed until after release of the pre-mRNA from the site of transcription.[17] In situ hybridization procedures were also optimized to localize the small nuclear RNAs that form the building blocks of the spliceosome.[18] By combining in situ hybridization and immuno-labeling for fluorescent and electron microscopic visualization, it became evident that spliceosomal small nuclear ribonucleoproteins and splicing protein factors accumulated in dedicated sub-nuclear compartments termed Cajal bodies and nuclear speckles (for a review see ref. 19). Unlike other RNA-based macromolecular machines in the cell such as the ribosome, spliceosomes form anew on each nascent intron and disassemble after each splicing event.[20] As most cells contain a surplus of long-lived spliceosomal components in the nucleus, these molecules are dynamically oscillating between two states: being part of a spliceosome or waiting for the opportunity to assemble a new spliceosome. Since actively spliced genes are intimately associated with the periphery of nuclear speckles, one possibility is that clustering of spliceosomal components in dedicated compartments increases their local concentration in the neighborhood of nascent transcripts, thus enhancing spliceosome assembly on newly synthesized introns.[21] This could be a reason why splicing is so much faster in vivo than in vitro.

An imaging breakthrough was introduced by the development of genetically encoded fluorescent tags that combined with fluorescence-based microscopic approaches of increasingly higher spatial and temporal resolution, making it possible to analyze protein movement in living cells. Time-series recordings of cells expressing the green fluorescent protein (GFP) fused to an essential splicing protein provided direct evidence that nuclear speckles supply spliceosomal components to nearby activated sites of transcription.[22] Subsequent developments of methods such as FRAP (fluorescence recovery after photobleaching) unravelled the kinetic properties of splicing proteins in the nucleus of live cells.[23] FRAP revealed that spliceosomal proteins are continuously moving in the nucleus, shuttling in and out of nuclear speckles within seconds. Surprisingly, trafficking kinetics was independent of ongoing transcription and splicing, challenging the view that spliceosomal components are stored in nuclear speckles until a signal triggers their recruitment to nascent introns; rather, it is more likely that the building blocks of the spliceosome are constantly roaming the nuclear space until they collide and transiently interact with either a nascent pre-mRNA, to form a spliceosome, or with other splicing proteins localized in nuclear speckles.[24] FRAP analysis further revealed that core spliceosomal snRNP proteins have a residence time of 15–30 s in the nucleoplasm, where spliceosomal snRNPs are thought to interact predominantly with pre-mRNA.[25] Based on these results it was suggested that splicing can be accomplished within 30 s, which is significantly more rapid than previously reported.[25]

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Following the advent of genetically encoded fluorescent protein tags, Belmont and colleagues pioneered a method to image chromatin dynamics in vivo. They introduced bacterial lac operator repeats into the genome of yeast and mammalian cells that expressed a GFP-lac repressor fusion protein.[26] Binding of the fluorescent repressor to its target sequence makes that particular region of chromatin visible in the nucleus. A similar approach was later developed to visualize RNAs in living cells by genetically inserting the binding sites for the MS2 bacteriophage coat protein in the RNA of interest.[27] The resulting reporter gene was then integrated in the genome of cells that expressed the MS2 coat protein fused to GFP. Insertion of the MS2 binding sites in the terminal exon of reporter genes revealed kinetic properties of the entire mRNA life cycle, from transcription to transport in the nucleus and export to the cytoplasm.[28,29] Based on these results, a prediction was that an intronic insertion of MS2 binding sites should suffice to track splicing in real-time. Bertrand and colleagues combined such an approach with FRAP to measure co-transcriptional splicing kinetics.[30] They analyzed an ensemble population of pre-mRNAs synthesized from a gene cluster comprising ~20 copies of a reporter gene that contains MS2 binding sites in an artificial short intron derived from the adenovirus genome. Upon bleaching the MS2-GFP fluorescence associated with introns at the transcription site, they measured a half-life of 105 s for fluorescence recovery and estimated a mean splicing time of 162 s. This value was in good agreement with the splicing rates previously deduced from electron microscopy[6,7] and biochemical pulse-chase experiments.[31] However, inferring the splicing rate from FRAP curves required the use of numerical models because fluorescence recovery is influenced by many variables in addition to splicing. These include rate of transcription by RNA polymerase II, diffusion and binding of MS2-GFP, and diffusion or degradation of excised introns. Modeling of these results is further complicated by the simultaneous presence, in the region analyzed, of multiple asynchronous transcripts. To circumvent these significant limitations and the potential problems introduced into the interpretation of the data, we directly tracked in time the fluorescence emitted by single pre-mRNAs labeled with MS2-GFP in the intron.[32] We integrated a single reporter gene in the genome of human cells and used a spinning-disk confocal microscope. Spinning disk confocal microscopy combines high sensitivity with high speed optical sectioning with minimal photobleaching, making it one of the methods of choice to analyze intracellular dynamics of single molecules with high spatial and temporal resolution. The fluorescence associated with a single transcription site in the nucleus was detected as a diffraction-limited object and its intensity was determined as a function of time. Increments in fluorescence intensity were due to de novo transcription of binding sites for MS2-GFP, while reductions reflected intron excision followed by degradation or diffusion. These fluorescence intensity fluctuations were used to determine the intron lifetime, defined as the time it takes for an intron to be transcribed, spliced, and degraded (or released from the transcription site). The fluorescence intensities at any given time point varied from cell to cell, presumably reflecting variation in the rate of transcription initiation: the higher the transcription rate the more nascent pre-mRNAs are simultaneously present at the transgene locus, resulting in higher fluorescence intensity. To determine the fluorescence intensity emitted by a single pre-mRNA molecule, we treated cells with spliceostatin A (SSA), a potent splicing inhibitor that causes release of unspliced pre-mRNAs to the nucleoplasm.[33] After release from the transcription site, pre-mRNAs diffused throughout the nucleus making it possible to resolve individual transcripts. Having determined the fluorescence intensity of a single pre-mRNA, we then searched for cells that synthesized one reporter transcript at the time, i.e., cells with fluorescence fluctuations that started at background level, increased to a value in the range corresponding to a single pre-mRNA, and then returned again to background. As the MS2 binding sites were inserted very close to the 5′ splice site, the duration of these fluorescence cycles provided, for the first time, a direct measure of the intron lifetime. We found that transcription and excision of short introns (1.3–1.4 kb) occurs in 20–30 s, which implies a splicing rate much faster than previously reported for the adenovirus-derived short intron based on FRAP experiments.[30] We believe the reason for this discrepancy derives from the higher resolution of our single-molecule analysis: the rapid fluorescence fluctuations that we observed for a single pre-mRNA molecule are probably hidden in the bulk measurement of fluorescence recovery from a multitude of molecules at different stages of the splicing cycle. We then addressed the longstanding question of whether some introns are spliced faster than others. First, we compared the lifetimes of the first and second introns of the β-globin gene and detected a 1.5-fold difference: the most frequent lifetime value was 20 s for the first intron and 30 s for the second; to rule out the influence of transcription, the second intron was shortened by an internal deletion so that both introns have approximately the same length.[32] Next, we compared the lifetimes of two introns derived from the mouse immunoglobulin μ gene. The two introns had exactly the same length but differed in the polypyrimidine tract sequence. We found that the lifetime of an intron with a weak splice site sequence was 1.4-fold longer than the lifetime of an intron with a strong splice site sequence.[32] Finally, we analyzed the influence of transcription on intron lifetime by comparing two introns with the same splice site sequences but differing in length by ~1000 nucleotides inserted downstream of the MS2 binding sites. We found that the cycles of fluorescence gain and loss were ~18 s longer for the longer intron. We also estimated that the array of MS2 binding sites is transcribed at a rate of approximately 4 kb per minute. At this rate, transcription of the extra 1000 nucleotides present in the long intron is expected to take ~15 s, an interval very close to the difference in duration of the fluorescence cycles for the long and short introns (~18 s). This suggests that longer introns take longer to be spliced not because splicing is slower but rather because they

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take longer to be transcribed. We could also infer that splicing is a much more rapid process than transcription.[32] To conclude, we developed an experimental system that we hope will prove valuable to further reveal the kinetic properties of splicing, particularly in the context of alternative splicing decisions. This system could also be useful to study in real-time how delays in splicing may regulate the timing of expression of specific genes, as recently proposed for the sequential appearance of distinct mRNAs in response to TNF induction.[34]

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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