Preface

Cells possess a wealth of posttranscriptional control mechanisms that impact on every conceivable aspect of the life of an mRNA. These processes are intimately intertwined in an almost baroque manner, where promoter context influences the recruitment of splicing factors, where the majority of pre-mRNAs undergo alternative splicing, and where proteins deposited during nuclear processing impact distal cytoplasmic processing, translation, and decay. If there is a unifying theme to *mRNA Processing and Metabolism: Methods and Protocols*, it is that mRNA processing and metabolism are integrated processes.

Many of the techniques used to study mRNA have been described in a previous volume of this series (*RNA–Protein Interaction Protocols*, Susan Haynes, ed.) and specialized methods journals. In selecting topics for *mRNA Processing and Metabolism: Methods and Protocols*, I sought input on new and novel techniques and approaches that build on this foundation using technological advances in microscopy, whole genome sequencing, microarrays, mass spectrometry, fluorescent detection methodologies, and RNA interference. I have tried not to bias this book toward any single model organism, and approaches described in the various chapters use yeast, *Drosophila*, *Xenopus*, mice, plants, and cultured mammalian cells.

One needs some form of organizational context, and the most logical was to start with events closest to transcription and finish with mRNA degradation. In Chapter 1, Keogh and Buratowski describe the application of chromatin immunoprecipitation to study cotranscriptional pre-mRNA processing events in yeast. Chromatin immunoprecipitation has proved to be particularly powerful for studying the recruitment of mRNA processing components to the "RNA Factory." The C-terminal domain of the large subunit of RNA polymerase II is perhaps the key integrator of nuclear pre-mRNA processing, and its function is modulated by hyperphosphorylation of the YSPTSPS heptad repeat. Different kinases function in this capacity, and presumably generate different patterns of phosphorylation. These differentially phosphorylated CTDs, in turn, recruit phosphoCTD-associating proteins, or PCAPs, that play a central role in coordinating transcription and pre-mRNA processing. In Chapter 2, Phatnani and Greenleaf describe approaches for identifying PCAPs that are applicable to both yeast and mammalian cells.

One of the major outcomes of genome sequencing is the realization of the extent of the role alternative splicing plays in the diversification of the

proteome. Therefore, understanding the regulation of splice site selection is, and will remain, an active area of study for understanding cellular differentiation, cell stress, and malignancy. It is also an area ripe for the development of novel therapeutics. Four chapters of this book are devoted to new approaches to study alternative splicing. Splicing is typically studied using nuclear extracts, yet to be able to manipulate splicing in a meaningful manner one needs a way to visualize this in intact cells. Several approaches to this problem are described by Wagner and coworkers in Chapter 3. Numerous approaches for recovering functional spliceosomes have been described previously. A new and particularly successful way to accomplish this using a tobramycin-binding affinity tag is described by Hartmuth and coworkers in Chapter 4.

Although one usually thinks of alternative splicing in terms of a single exon, the splicing of some pre-mRNAs is enormously complex. A case in point is the *Drosophila Dscam* gene. The *Dscam* protein is involved in axonal targeting and contains a cluster of 48 mutually exclusive exons. This single gene can generate more than 38,000 alternatively spliced forms, a number that is more than twofold greater than the total number of genes in the *Drosophila* genome. As with most genes containing mutually exclusive exons, the exons within three of the alternatively spliced *Dscam* cassettes are approximately the same size. How, then, can one distinguish between them to characterize mechanisms involved in alternative splicing? In Chapter 5, Celotto and Graveley describe how this can be addressed using single-strand conformational polymorphism (SSCP) gel electrophoresis.

With the completion of numerous genomes, we have become increasingly aware of the importance of RNA editing. In Chapter 6, Zhang and Carmichael describe various methods for studying adenosine-to-inosine editing in RNA. RNA export has emerged as a key regulatory step in mRNA metabolism, and, with the advent of RNAi, its study has moved from earlier work in *Xenopus* oocytes to cultured cells. Numerous approaches to study mRNA export are described by Cullen in Chapter 7. Helicases play critical roles in all aspects of mRNA processing and metabolism. For example, the DEAD-box RNA helicase Dbp5, which is localized to the perinuclear region of the cell, is required for mRNA export. In Chapter 8, Tseng-Rogenski and Chang describe assays for RNA unwinding by this family of enzymes.

The last aspect of nuclear processing covered in this book is the most controversial: nuclear translation. The concept of nuclear translation has been around for some time, but it reached the forefront with two major observations. The first was the mandatory involvement of translation in scanning for premature termination codons in the nucleus-associated degradation of

nonsense-containing mRNA. The second was the observation by Iborra and coworkers of the transcription-dependent incorporation of amino acids into some product in the nucleus. In Chapter 9, these authors describe approaches they developed to study translation in the nucleus.

Within the cell, mRNA is always complexed with proteins in messenger ribonucleoprotein particles (mRNPs), and every step in processing and metabolism occurs in the context of the mRNP. Although some mRNA-binding proteins are specific to particular sequence elements (e.g., AU-rich elements that impact on mRNA decay), others like eIF4E and poly(A)-binding protein are present on all mRNAs [with the exception of histone mRNAs for poly(A)-binding protein]. Key to understanding the dynamics of mRNA processing and metabolism is the ability to recover mRNP complexes in their native state. Detailed methods for the immunoprecipitation of both nuclear and cytoplasmic mRNk/ are described by Lejeune and Maquat in Chapter 10. In much the way that bacterial genes are organized into structural operons, mRNPs may organize mRNAs that function in related pathways into functional operons. In Chapter 11, Penalva and coworkers describe the application of expression profiling to RNP complexes sharing one or more RNA-binding proteins. This approach has been used already to identify mRNAs associated with such proteins as FMRP, the protein encoded by the Fragile X gene. The complement to recovering mRNPs by immunoprecipitation is recovery based on a sequence or structural feature of a particular target mRNA. The binding of MS2 coat protein to the MS2 stem-loop structure has been used successfully for numerous mRNAs. In Chapter 12, Baron-Benhamou and coworkers describe an alternative method that instead uses a small peptide of the bacteriophage lambda N protein binding to the small Box B RNA sequence for recovery of mRNP complexes.

Translation of the processed mRNA is controlled primarily at the level of initiation, and eukaryotic cells (and viruses) employ a rich variety of mechanisms to affect this process. Although many simple experiments may still be accomplished using the standard rabbit reticulocyte lysate, this system does not demonstrate the synergy between the cap and the poly(A) tail that is the hallmark of eukaryotic mRNA translation. Systems using yeast or *Drosophila* embryo extracts have been described, but for many laboratories these can be challenging to implement. Also, they may not be compatible with mammalian or viral proteins under study. Chapters 13 and 14 describe two in vitro translation systems from mammalian cells that retain the characteristics of in vivo translation. The system described in Chapter 13 by Svitkin and Sonenberg uses ascites cells and includes approaches for depleting translation extracts of key proteins such as poly(A)-binding protein. An

alternative system using cultured HeLa cells is described by Thoma et al. in Chapter 14. Both systems display dependence on the cap and poly(A) for translational activity, in addition to which the HeLa cell system shows synergy between an IRES and the poly(A) tail.

Translation and mRNA decay are intimately linked, and results from numerous different approaches suggest that mRNAs may be targeted for decay once they are no longer being actively translated. In yeast and mammals, mRNA decay can proceed through decapping followed by 5'–3' exonuclease-mediated degradation, or 3'–5' exonuclease-mediated degradation, both of which are preceded by loss of the poly(A) tail. Two chapters by Bergman et al. and Mukherjee et al. present techniques to address these processes in vitro. Chapter 15 describes several approaches to study decapping, both by decapping pyrophosphatases like Dcp2, and by the scavenger decapping enzyme DcpS. Chapter 16 provides detailed protocols for preparing various modified mRNA substrates and experimental approaches using these substrates to study exonucleolytic degradation of both the poly(A) tail and the mRNA body in vitro. mRNA decay can also be catalyzed by sequence-selective endonucleases. This appears to be a secondary pathway that targets select mRNA populations, frequently in response to activation of a particular signal transduction pathway. It is particularly difficult to demonstrate endonuclease-mediated mRNA decay in vivo, because the products of this reaction are rapidly degraded. In Chapter 17, Hanson and Schoenberg describe a very sensitive ligation-mediated RT-PCR assay for detecting labile endonuclease cleavage products.

The list of processes in which siRNAs and miRNAs are involved is growing exponentially, and this book concludes with three chapters that address approaches to studying the biochemistry of RNA interference (RNAi), its application to studying pre-mRNA processing, and new approaches for stable gene inactivation in both cultured cells and animals. The biochemistry of RNAi is addressed by Tang and Zamore in Chapter 18, where they discuss various approaches to study both the generation of siRNAs and degradation of target mRNAs by the RISC complex in plants. In Chapter 19, Celotto and Graveley show how to take advantage of the ease of performing RNA interference in *Drosophila* tissue culture cells to study the roles of individual proteins in alternative splicing. What makes this approach so powerful is that, by using *Drosophila* cells, one need not prepare siRNAs, optimize transfection efficiency, or develop the expression vectors required for mammalian cells. Rather, all one needs is to prepare a double-stranded RNA greater than 100 bp, and add this directly to the culture medium to affect the efficient knockout of any individual splicing factor. Lastly, many readers of *mRNA Processing*

and Metabolism: Methods and Approaches will be interested in applying RNA interference to studying their favorite gene or pathway in mammalian cells. Although the use of transiently transfected siRNAs has proven effective for short-term studies, a bigger challenge for the long term is the ability to stably inactivate a particular gene. This is addressed in Chapter 20, where Hannon and Conklin present detailed protocols for using vectors expressing short hairpin RNAs to affect stable RNAi of targeted genes in mammalian cells and in transgenic animals.

In closing, I wish to thank the contributing authors of *mRNA Processing and Metabolism: Methods and Approaches* for their time and effort in making this a timely text that will prove useful for researchers working on many different aspects of mRNA processing and metabolism.

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