A journey

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Twenty years of the journal *RNA*, 20 years of RNA research—the journal has witnessed and facilitated an explosive growth of the RNA field, a relatively young field in many ways. Having studied alternative splicing for more than 20 years, it is a great time for me to pause and reflect on what we have learned and still need to learn on this particular topic.

There was heightened excitement when the first issue of *RNA* was making its way to Sue Berget's laboratory at Baylor College of Medicine where I was conducting part of my postdoctoral research. Only, the excitement was not caused by the anticipated arrival of the journal, although the arrival of this new journal was indeed discussed repeatedly in the laboratory, but by another bigger than usual cleanup of a radioactive material spill in our room specifically reserved for generating and using *in vitro* RNA splicing substrates. Sue's was one of the hardcore laboratories that used biochemical means to study the mechanism of splicing, which drew me to her laboratory. When I was a graduate student studying splicing in plants, I was always envious of people who conducted *in vitro* biochemical splicing analysis, as it was not feasible to use such an approach in plant systems due to the difficulty of preparing splicing-competent nuclear extracts from plant cells. In Sue's laboratory, a standing order of the radioisotope ^{32}P -UTP guaranteed that one vial of 1 mCi of ^{32}P -UTP was delivered to the laboratory every week, which could result in 20 *in vitro* transcribed RNA substrates. As such, radioactive RNA substrates were generated almost every day, which unavoidably led to the occasional bigger than usual decontamination buzz. Another buzzing activity that usually involved the whole laboratory, students, postdoctoral fellows, and technicians alike, occurred on the nuclear extract making days, which happened approximately once a month. On those days, 100 L (sometimes twice as much) worth of HeLa cell pellets would arrive at our laboratory from a company in Minnesota. Everybody worked hard as a team on those days for a whole day following Sue while she was shouting out orders. At the end of the day, we would have 100 mL of pure nuclear extracts at 10–15 mg/mL, which, if tested splicing-competent, would last us a couple of months. Those were

some of my most vividly memorable good old days in Sue's laboratory.

When the first issue of *RNA* did arrive, we pored over the articles. Being in a splicing laboratory, we obviously read very carefully the article from Paula Grabowski's laboratory that used *in vitro* splicing analysis to reveal a role of exon enhancers in promoting U2AF binding at the polypyrimidine tract. Looking back, this article represents the state of the approach at the time for mechanistic studies of splicing. The core questions asked by the investigators in the splicing field were what and how the sequence elements located on pre-mRNA and RNA-binding proteins (RBPs) that act in *trans* regulate splicing. To answer these questions, typically, one generated *in vitro* transcribed splicing substrate containing wild type or mutated sequence elements and carried out splicing assays in a test tube in which a potential splicing regulator was added or depleted from the nuclear extracts. In parallel, experiments were carried out using splicing reporters and protein expression vectors through cell transfection techniques. While it was relatively easy to identify sequence elements through deletion/mutation analysis, the bottleneck was identifying the *trans*-acting protein factors that recognize and interact with the sequence elements. The heroic classical biochemical purification approaches, i.e., with investigators spending many hours in cold rooms, led to the identification of a number of splicing factors. These elegant studies, combined with the power of yeast genetics, have built our knowledge foundation of splicing, a process carried out by the spliceosome that is one of the most complex macromolecular machines in eukaryotic cells.

Studies of alternative splicing using *in vitro* biochemical approaches met with more difficulties due to an inherent nature of alternative exons, being surrounded by 3' and 5' splicing signals that can deviate significantly from the consensus sequences, which are recognized and bound by spliceosomal components. The presence of these sub-optimal splicing signals makes the already inefficient *in vitro* splicing system almost not suitable to study alternative splicing events. One had to become a true artist in making "super duper" splicing-competent nuclear extract and extremely "hot" and clean

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RNA splicing substrates to observe a trace amount of splicing from a substrate that contains an alternative exon. Added to this difficulty is the need to prepare splicing-competent nuclear extracts from physiologically relevant cell lines or tissues in studies of a particular alternative splicing event, which is often impossible (the best nuclear extracts were made using HeLa cells). Fortunately, significant advances in our understanding of alternative splicing regulation have been made through studies of human diseases, in which the disease-related protein turned out to be an RBP. For example, in the CTG triplet expansion disease myotonic dystrophy, the muscleblind-like (MBNL) protein is sequestered at the CUG triplet-containing RNA foci in the nucleus, while the CELF1 (formerly known as CUGBP1) protein level is increased. Both MBNL and CELF1 have been demonstrated to regulate alternative splicing of a large number of genes. Another excellent example is the paraneoplastic neurodegenerative disorder autoimmune antigen NOVA1/2 proteins. After the identification of these proteins as the targeted autoimmune antigens, significant efforts have been made to understand the function of these proteins. The successful combined use of SELEX, CLIP, and mouse knockout models led to the discovery that these proteins regulate alternative splicing of many synapse-related proteins in the central nervous system. To date, a large number of mouse knockout, including conditional knockout, models as well as transgenic mouse models have been generated. The genome-wide RNA target binding and transcriptome analyses using these models are continuing to reveal the secrets of alternative splicing regulation.

While I was learning to become an expert in preparing splicing-competent nuclear extract from a variety of different cell types, a realization started to dawn on us that perhaps the low *in vitro* splicing efficiency was due to the fact that these assays were conducted in a test tube away from the natural splicing environment, the nucleus, and more precisely, the site of transcription. In 1997, two important papers were published that changed the thinking of the field. The Bentley laboratory published a *Nature* paper demonstrating the role of the CTD of RNAPII in pre-mRNA splicing and polyadenylation, while the Kornblihtt laboratory published a *Proc Natl Acad Sci* paper describing a provocative result: The same alternative splicing event showed a different outcome when its transcription was driven by different promoters. These studies pointed to an intimate link between transcription and splicing. So many light bulbs flickered on (and off) by these publications, and new hypotheses and theories

were drawn out almost weekly if not daily in Sue's laboratory for a period of time, many by Sue, and some of which were proven years later.

With the completion of the human genome project in 2000, as well as the development of many new experimental tools to examine splicing and chromatin at the genome level, many connections have been made between chromatin structure, nucleosome positioning, histone modifications, transcription, RNAPII, and alternative splicing. Today, we have a very integrated view of gene expression, and it is generally accepted that changes occurring at the chromatin or transcriptional level can lead to changes of alternative splicing outcomes. Obviously, the more we know, the less surprised we will be by any additional hidden layers of connections.

Looking back, I cannot help but marvel at the long way the field has come in terms of techniques, approaches, and conceptual advances. Looking forward, I feel the excitement of new discoveries in at least two areas of alternative splicing research. First, we will gain an in-depth understanding of the bidirectional relationship between splicing/alternative splicing and chromatin structure involving DNA modifications, histone modifications, and nucleosome positioning. Second, we will have a much better appreciation and understanding of the dynamic nature of alternative splicing. In the past years, significant efforts have been put forward to decipher the code of cell-type specific regulation of alternative splicing. We have only seen the tip of the iceberg of the dynamic regulation of alternative splicing within a given cell type, such as those illustrated by work from the Black (depolarized neurons) and Lynch (activated T-cells) laboratories.

So, is *in vitro* splicing assay a lost art, like Southern and Northern blot analysis, as well as manual Sanger sequencing? It does feel that way when fewer and fewer publications include *in vitro* splicing assays. However, I do believe that *in vitro* biochemical analysis, such as those examining interactions between RNA and proteins, occupies an important place in the research of alternative splicing. The best studies, in my view, are still those that combine *in vivo* and *in vitro* approaches. While *in vivo* studies investigate the biological functions and regulatory mechanisms of a given alternative splicing event, they cannot replace the *in vitro* studies that probe the direct effects of protein–RNA, RNA–RNA, or protein–protein interactions. Importantly, in days when more and more research efforts are devoted to study the contribution of RNA processing in human diseases, these detailed interactions may serve as potential diagnostic and therapeutic targets in disease diagnosis and intervention.

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