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Genetics and biochemistry remain essential in the structural era of the spliceosome

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ABSTRACT

The spliceosome is not a single macromolecular machine. Rather it is a collection of dynamic heterogeneous subcomplexes that rapidly interconvert throughout the course of a typical splicing cycle. Because of this, for many years the only high resolution structures of the spliceosome available were of smaller, isolated protein or RNA components. Consequently much of our current understanding of the spliceosome derives from biochemical and genetic techniques. Now with the publication of multiple, high resolution structures of the spliceosome, some question the relevance of traditional biochemical and genetic techniques to the splicing field. We argue such techniques are not only relevant, but vital for an in depth mechanistic understanding of pre-mRNA splicing.

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1. Introduction

Eukaryotic genes contain introns that must be accurately and efficiently removed to ensure fidelity in gene expression. Our understanding of the mechanisms and regulation of pre-mRNA splicing predominantly results from the clever combination of genetics and biochemistry. Recently the splicing field has been treated to high-resolution structures of human and *S. cerevesiae* tri-snRNP (PDB: 5GAN, 3JCM, 3JCR) [1–3], *S. cerevesiae* B^{act} (PDB: 5GM6, 5LQW) [4,5], C (PDB: 5LJ3, 5GMK) [6,7], and C^* [8] complexes, as well as human C^* [9], and the *S. pombe* Intron-Lariat Spliceosome (ILS) (PDB: 3JB9) [10]. These structures provide invaluable insights into spliceosome architecture, assembly, and function, and more spliceosome structures should be pursued. However, structural data are not without limitations. Here we review how genetic and biochemical approaches have traditionally informed our understanding of the spliceosome and discuss how these techniques remain essential in the structural era of the spliceosome.

The spliceosome is unique among cellular machines. Unlike the ribosome, which is assembled and then translates many cellular mRNAs, the spliceosome must assemble anew on each intron to be spliced. This elaborate assembly pathway allows for multiple points of regulation throughout the splicing cycle. Many assembly steps correspond to interconversions between mutually exclusive RNA:RNA interactions, and are driven by DExD/H box ATPases (reviewed in [11]). These same DExD/H box ATPases also serve to regulate splicing fidelity, selectively promoting the advancement

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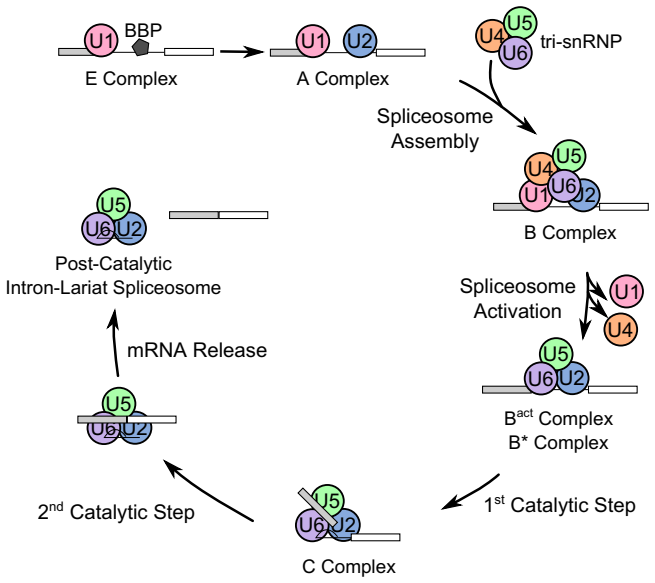

Fig. 1. The splicing cycle. SnRNPs are indicated as colored circles, the pre-mRNA exons are gray and white boxes connected by a line representing the intron. The transitions between key spliceosomal subcomplexes (E, A, B, B^{act}, C, ILS) are illustrated, and the two catalytic steps of splicing are labeled.

of "correct" substrates through consecutive splicing steps (reviewed in [12]).

The spliceosome (reviewed in [13]) catalyzes the removal of introns from nascent pre-mRNA transcripts via two transesterification reactions. The first transesterification reaction links the pre-mRNA 5′ splice site (5′SS) and the branch site (BS) to form lariat-3′exon intermediate. The second reaction between the 5′ and 3′ splice sites forms spliced exon product. The spliceosome consists of five individual snRNAs and over one hundred unique proteins. These snRNAs and proteins are associated into a set of functional subcomplexes: the U1, U2, U4, U5, and U6 snRNPs, and the Nineteen Complex (NTC). These subcomplexes, in conjunction with associated proteins, proceed through the ordered series of compositional and conformational rearrangements required to splice each intron (Fig. 1). Therefore, the spliceosome should not be thought of as a single entity, but rather as a dynamic, heterogeneous ensemble of subcomplexes that must interconvert throughout the splicing cycle.

2. Before the structures

Here we highlight the genetic and biochemical approaches that have informed our understanding of spliceosome assembly and function.

2.1. In vitro splicing and the assembly of the spliceosome

The spliceosome was first identified over 30 years ago [14]. Since then, *in vitro* splicing reactions have been used to define the canonical and alternative assembly pathways of the spliceosome and the components of the spliceosome present for each individual assembly step. In a typical *in vitro* splicing reaction, a labeled, *in vitro* transcribed pre-mRNA is incubated with ATP and yeast or human cell extract. This is followed by denaturing gel electrophoresis to visualize lariat-3′ exon intermediate, free 5′ exon, spliced mRNA product, and the excised lariat [15–17]. Nondenaturing gel electrophoresis of such splicing reactions allows detection of larger spliceosomal subcomplexes. In addition to the pre-mRNA, at least four distinct subcomplexes that differ in their snRNP complement and order of appearance can be resolved: the commitment complex (U1 and the Branchpoint Binding Protein (BBP), Complex E), pre-spliceosome (U1 and U2, Complex A), complete spliceosome (U1, U2, U4, U5, U6, Complex B), and active spliceosome (U2, U5, U6 and NTC, Complexes B^{act}, B^{*}, and C), define the canonical assembly pathway (Fig. 1) [18–20], which has been verified *in vivo* [21,22].

Both steps of splicing can be reconstituted *in vitro* using a mixture of compositionally defined subcomplexes and recombinant proteins [23,24]. Such assays have served as a basis for other more complex *in vitro* assays monitoring conformational rearrangements within the spliceosome [25], splicing fidelity and discard (release of the pre-mRNA from the spliceosome) [26], pre-mRNA conformation at the single-molecule level [27], and reversibility of the splicing reaction [28] (discussed in Section 2.4).

In vitro splicing reactions have also been exploited to understand the function of individual components of the spliceosome. Endogenous U1, U2, U4, U5, and U6 snRNA can be depleted from yeast or mammalian cell extracts by DNA oligonucleotide-directed RNaseH cleavage, or by affinity selection with 2′-O-methyl RNA oligos complementary to the snRNA. Extracts treated in this way will, when supplied with an *in vitro* transcribed snRNA, process and then incorporate it into snRNPs and form functional spliceosomes [29–33]. This technique has been used to study the effects of otherwise lethal snRNA mutants, as well as to incorporate snRNAs containing fluorophores or other nucleotide modifications into the spliceosome for further study [32,34,35]. For example, metal ion rescue experiments, which required sulfur substitution at specific nucleotides in U6 snRNA, proved that RNA alone was the catalytic component of the spliceosome [35].

Conceptually similar immunodepletion-reconstitution assays have been exploited to study the protein components of the spliceosome (see [36–38] for examples). Such techniques can be adapted to isolate spliceosome subcomplexes through the immunoprecipitation of protein components, which have been studied in depth by mass spectrometry, crosslinking, and other biochemical techniques [39]. Studies of this type have produced an extensive list of "parts" and have been compiled into a searchable spliceosome database (http://spliceosomedb.ucsc.edu/) [40].

Decades of research into *in vitro* splicing and spliceosome purification techniques have yielded detailed purification protocols for the isolation of spliceosome A, B, B^{act}, B^{*}, C, and ILS subcomplexes (Fig. 1), as well as the individual snRNPs, the tri-snRNP, and the NTC (reviewed in [41,42]). These provide an important starting point for structural studies of the spliceosome, as spliceosome subcomplexes isolated in this manner represent particularly stable intermediate forms of the spliceosome, and are thus excellent targets for structural determination.

2.2. Mutually exclusive RNA interactions in the spliceosome

At the molecular level, many spliceosome assembly steps correspond to changes in mutually exclusive basepairing interactions between RNAs and between RNA and protein (reviewed in [43]). Three main techniques have contributed to our understanding of these structural switches in the spliceosome: phylogenetics, genetics, and photochemical crosslinking. These disparate approaches have yielded highly complementary data, establishing that direct physical interactions correspond to functional interactions and vice versa.

The experiments that led to our understanding of how the 5′SS is recognized by the spliceosome provide an elegant example of how structural switches in the spliceosome are identified and characterized (Fig. 2A). Basepairing between U1 snRNA and the pre-mRNA 5′SS was initially proposed based on sequences complementarity between the 5′ end of U1 snRNA and the 5′SS consensus