THE QUEST FOR A MESSAGE: BUDDING YEAST,
A MODEL ORGANISM TO STUDY
THE CONTROL OF pre-mRNA SPLICING

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Abstract

Removal of introns during pre-mRNA splicing is a critical process in gene expression, and understanding its control at both single-gene and genomic levels is one of the great challenges in Biology. Splicing takes place in a dynamic, large ribonucleoprotein complex known as the spliceosome. Combining Genetics and Biochemistry, *Saccharomyces cerevisiae* provides insights into its mechanisms, including its regulation by RNA-protein interactions. Recent genome-wide analyses indicate that regulated splicing is broad and biologically relevant even in organisms with a relatively simple intronic structure, such as yeast. Furthermore, the possibility of coordination in splicing regulation at genomic level is becoming clear in this model organism. This should provide a valuable system to approach the complex problem of the role of regulated splicing in genomic expression.

Introduction

The transcripts of most eukaryotic genes need to be processed to become functional. During the course of pre-mRNA splicing, the nascent transcript sustains the removal of intervening non-coding sequences, termed introns, interspersed within the coding regions of a gene, the exons. RNA splicing is essential and requires great accuracy because the lack thereof produces truncated or aberrant proteins upon translation, which can be deleterious for the cell. The process involves two consecutive transesterification reactions that are carried out by a large dynamic ribonucleoprotein complex termed the spliceosome [1]. The core components of this cellular machinery share a high degree of evolutionary conservation, from yeast to humans. They include five small nuclear RNAs (U snRNAs) that upon binding of a specific set of proteins form small nuclear ribonucleoprotein particles (snRNPs), as well as many additional accessory protein factors [2]. All these components possibly make the spliceosome the largest molecular machine in the cell [3]. The most generally accepted view of the splicing process, in which studies in yeast have contributed significantly, is that the spliceosome assembles in a stepwise manner onto the nascent transcript by recognizing meager cues located within the primary sequence of the pre-mRNA (reviewed in [1]). First, U1 snRNP binds to the beginning of the intron, or 5' splice site (5'ss). The branch site (BS) and downstream sequences are then identified by protein factors (BBP and Mud2 in budding yeast, SF1 and U2AF in metazoans) that participate in the definition of the 3' end of the intron (3'ss). This initial recognition of the intron is followed by stable base pairing of U2 snRNP to the branch site and formation of the active spliceosome upon the engagement of the pre-assembled tri-snRNP U4/U6.U5. The entire assembly process is governed by various rearrangements in RNA-RNA, RNA-protein and protein-protein interactions that are driven by dedicated ATPases known as helicases. Those offer many opportunities for regulation and quality checks (reviewed in [4]). After splicing, the different components of the spliceosome are disassembled. The mature RNA is exported to the cytoplasm to be translated into proteins, the lariat intron is degraded, and the spliceosomal components are recycled for further rounds of splicing.

A problem of definition

Generally the informational content of splice sites is low (GU for the 5' ss, AG for the 3' ss), and does not explain the stunning accuracy of the spliceosome [5]. Decoding the rules that define a particular sequence as an intron is a great scientific challenge, approached both theoretically and experimentally. It is assumed that factors promoting or inhibiting spliceosome assembly play a role in deciding what constitutes a splice site. This inherent flexibility, together with the fact that most eukaryotic genes contain several introns that can be alternatively spliced, provide many organisms with the potential to greatly augment their coding capacity. Indeed, by alternative splicing cells can produce many isoforms with different or even antagonistic functions from a single gene. Alternative splicing thus has a central function in gene expression and can be controlled in a tissue, developmental stage [6]or even sex specific manner [7], as evidenced by microarrays and comparison between the sequences of genomes and ESTs (expressed sequence tag [8,9]). Protein factors involved in the control of alternative splicing bind to sequences either in introns or in exons and direct the spliceosome to enhance or prevent usage of a particular splice site. They achieve this either by direct interactions with the spliceosome or by masking splicing signals (reviewed in [10]). Recently we have proposed that the spliceosome can also be modulated by interference with rearrangements it has to undergo during assembly [11] as discussed further below.

Many factors that regulate spliceosome assembly and thus alternative splicing do not show a degree of sequence specificity that would explain their selectivity [10,12]. Additional unknown mechanisms other than sequence recognition must therefore be taken into account. Relative concentrations of splicing factors, and events related to transcription [13], as well as RNA structure [14] are likely to be involved. Consequently, many intricate variables make accurate prediction of mRNA a hard problem; yet solving it is of critical importance to enable the full understanding of the genetic information contained in genomes.

A reductionist approach

In contrast to the complex metazoan gene structure, the one of the budding yeast *Saccharomyces cerevisiae* is simpler as only about 5% of its roughly 6000 genes contain one intron and only a few have two [15,16]. In addition, splicing signals are clearer in yeast than in metazoans. Finally, yeast lacks obvious homologs of several accessory factors known to control splicing in metazoans [10]. Thus, only a few cases of alternative splice site usage have been reported in this organism [17]. The simplicity

and versatility of *S. cerevisiae* as a working system, together with the fact that many spliceosomal components are evolutionarily conserved, make yeast an ideal model system to study some aspects of splicing. It permits for dissection of a complex mechanism without the further complications encountered in higher organisms. Importantly as well, genomes of several fungi, spanning an evolutionary distance comparable to that of yeast to humans, have been fully sequenced, providing an important tool to explore the evolution of particular spliceosomal features [18].

Interestingly, *S. cerevisiae* introns are not randomly distributed throughout the genome, but are found mostly in highly expressed genes. Indeed, the handful of genes that contain introns produce nearly one third of the total cellular transcripts [19]. In fact, the presence of the intron itself allows for higher expression of a particular gene [20]. Introns are particularly enriched in ribosomal protein genes (RPGs). No less than 102 out of the 139 RPGs encoded in the yeast genome contain an intron, which makes them the most important functional intron-containing group. However, yeast introns also tend to be present in genes related to secretion and meiosis. Likewise, the size distribution of introns is not random, showing a bimodal curve with one peak at 100 nucleotides, and another at 400 nucleotides [15]. With only few exceptions, the non-RPGs account for the short introns, whereas the RPGs contain the longer ones. This particular intron distribution in yeast allows to study the expected role of splicing in the coordination of gene expression, as processes like ribosome biosynthesis or meiosis require an exquisite balance between many components (see for example [21,22]).

Hence, from the point of view of molecular mechanisms, as well as from the perspective of the whole genome, budding yeast constitutes a valuable system to study splicing and its implications for the cell. Here we will briefly review different strategies *S. cerevisiae* uses to control gene expression at a post transcriptional level through interactions of pre-mRNAs with proteins. These examples have been chosen because they represent three very different ways the cell controls expression by taking advantage of spliceosomal characteristics, and should provide insights into the mechanisms of other regulated events that are more complex to approach. They can also provide additional hints in the searches for regulated splicing events in this and other genomes. We will first address the meiosis-specific regulation of Mer1-dependent splicing that occurs through recruitment of the splicing machinery to normally inefficiently spliced

pre-mRNAs. We will then explore the Yra1 dependent splicing regulation of its own pre-mRNA that is shunted towards an alternative route when an excess of the protein is present. Finally we will discuss the regulation of *RPL30* pre-mRNA splicing by blockage at a particular step of splicing when its gene product is in excess.

With these examples we intend to show that in yeast regulated splicing is sophisticated. This is highly relevant because due to the evolutionary conservation of the spliceosome, its regulation is likely to follow strategies analogous to those of more complex organisms; albeit with a reduced set of tools. Given that yeast has proven to be a valuable tool to study spliceosomal function, we argue that this usefulness can be extended to the study of its regulation.

Promoting splice site recognition: Meiosis specific splicing enhancement by Mer1

Meiosis is the result of a sophisticated pattern of gene expression mostly controlled by an elaborate transcriptional program (reviewed in [23]). However, some meiosis-specific genes are transcribed to some extent during vegetative growth, and post-transcriptional regulation plays an important role in their correct expression [24]. For instance, *MER2* and *MER3* are very inefficiently spliced during vegetative growth due in part to their non canonical 5'ss which is poorly recognized by the splicing machinery [25,26]. *AMA1* constitutes another example and its splicing is hindered by the presence of an inhibitory sequence just after its 5'ss [27]. The situation dramatically changes during meiosis when the three pre-mRNAs are spliced and their protein product is expressed. For all three, this change is due to the expression of the meiosis specific protein Mer1 [17,28,29]. This protein binds a specific 8 nucleotide sequence within close proximity of the 5'ss in all three pre-mRNAs but also interacts with the U1 snRNP as well as spliceosomal factors bound to the 3' region of the intron, the U2 snRNP, and components of the machinery involved in the nuclear retention of transcripts [27,30,31]. The model emanating from these observations is that in vegetative growth the spliceosome cannot be formed due to the poor recruitment of the U1 snRNP to the pre-mRNA. In contrast, during meiosis, Mer1 is expressed and helps the recruitment of the U1 snRNP thus leading to the start of spliceosome formation, which is further promoted by more interactions with downstream elements, resulting in enhanced splicing. Similarly, several metazoan splicing factors such as TIA-1, involved in the control of apoptosis, also employ the strategy of promoting U1 snRNP recruitment to the pre-mRNA [32].

Escaping splicing: Yra1 autoregulation

Yra1 is an essential component of the RNA export machinery from the nucleus to the cytoplasm and is loaded onto mRNAs cotranscriptionally [33,34]. It then accompanies the transcripts throughout splicing and export. Yra1 protein overexpression blocks mRNA export [35,36] and inhibits growth [37]. Therefore its expression has to be tightly regulated, which is achieved by an autoregulatory feedback loop based on the excess Yra1 inhibiting its own further expression [35]. The *YRA1* gene structure is unusual because it contains a large intron (776 nt) located after a long first exon (at 300 nt from AUG) and contains a non-consensus branchpoint sequence (gACUAAC instead of the conserved UACUAAC). These three features are highly unusual in *S. cerevisiae* and render the *YRA1* pre-mRNA a suboptimal substrate for splicing, which is required for autoregulation [38]. In case of an excess of Yra1 protein, the transcript is exported from the nucleus rather than being spliced and degraded by the Xrn1 dependent 5' to 3' RNA turnover pathway in the cytoplasm [38,39]. The current model emerging from the aforementioned observations is that the splicing and the export machineries compete for the *YRA1* transcript, but that when Yra1 protein is in excess, the latter is favoured. The molecular mechanism is not clear to date, but it has been proposed that Yra1 protein could be a negative regulator of the splicing of its intron by hindering a secondary structure bringing the 5' ss and branch site closer together [38], or by favouring export. Such strategies of interference with the link between RNA splicing and transport are also used by the HIV virus to control the export of its genetic material from the host's nucleus [40].

Blocking spliceosomal rearrangements: L30 autoregulation

The L30 protein is one of the roughly 80 gene products that constitute the ribosome [21,41], and is toxic to cells when present in excess [42]. Its expression levels therefore have to be tightly controlled, which is in part achieved through a mechanism of autoregulation. Indeed, when in excess, the L30 protein binds its own pre-mRNA in a structure that mimics its binding site on the 25S rRNA where it normally serves its function in the ribosome [43]. Through its binding to this structure, L30 is able to inhibit splicing [44,45] and translation [46] of its own transcript thus preventing accumulation of the protein. The RNA fold bound by L30 in its transcript includes the 5'ss, but surprisingly splicing inhibition of L30-bound *RPL30* does not occur at the

onset of splicing. The intron is recognized by the early spliceosomal components U1 snRNP, BBP, and Mud2 [11]. Instead, splicing is blocked at the point of recognition of the *RPL30* branch site by U2 snRNP. No direct interactions have been found between L30 and splicing factors, and it is thought that L30 inhibits splicing by impeding an essential remodelling step that allows the nascent spliceosome to recruit U2 snRNP. By an as of yet undefined mechanism, the inhibited complex that still binds L30 is stripped of U1 snRNP and is then exported to the cytoplasm. At this point it is degraded by the nonsense mediated decay (NMD) pathway [47] that is in charge of protecting the cell from messages bearing premature stop codons [48]. Additional roles for other RNA structures in splicing have been described [49], including connections with disease [50], and it is conceivable that secondary structures can promote selection of alternate splice sites [51], contributing to evolutionary diversity.

Coordinated regulation of splicing

Most knowledge about splicing emerges from research carried out on a small set of extensively studied pre-mRNAs. The question arising from this observation is whether the rest of the transcripts behave in a similar fashion as these models. Similarly, the effect of mutations or deletions of specific splicing factors have been studied on very few transcripts, but are other transcripts affected in the same way? Finally, and no less importantly, it was not clear whether the splicesome could identify different types of transcripts depending on cellular conditions. Such selectivity could provide a quick and powerful response to external stimuli, such as stress conditions, thus adding a critical layer in the coordination of gene expression. To answer these questions it has become pressing to analyze splicing on a more global scale than on a one-gene-at-a-time basis. Microarray technologies have made this possible, and quite manageable in yeast due to the presence of few introns and a simple gene structure. In this section we will discuss recent findings in this area. We will see that many splicing factor mutations or deletions affect certain groups of genes differently than others, but also that there is a link between the spliceosome and the environment that differentially affects splicing of different classes of transcripts.

There are several microarray platforms that enable splicing analysis [52]. The exon-splice junction design includes, for each intron containing gene, an intronic probe to detect the pre-mRNA, a probe spanning two exons for detection of mRNAs, and a probe

that hybridizes in the last exon to evidence the total amount of transcript [53]. The last probe enables to account for changes in transcription levels or RNA stability of a particular transcript. Armed with this tool, several laboratories have set out to study the global effect on splicing of a large set of splicing factors (see [52] for a review). In yeast, a certain number of them can be easily deleted from the genome, with only minor effects on cell viability in standard laboratory conditions [53]. Other splicing factors however are essential, and cannot be readily deleted. Over the years, conditional loss-of-function mutants have been generated for many of those, which enable transient deactivation of a gene under a particular condition such as a restrictive temperature. Global splicing changes have thus been monitored with splicing-specific microarrays in spliceosome component deletion or mutation strains [53,54]. The outcome of these studies is that, as expected, factors involved in similar processes have similar global effects. This should help in characterizing novel factors and functions in the future. Another important conclusion of these experiments is that all transcripts do not respond in the same way to alterations in the spliceosome. To exemplify this, a set of mutations that have been shown to block the first chemical step of splicing have been analysed by microarrays [54]. Whereas some transcripts are equally affected by all the mutations tested, others are only affected in a subset of strains. In addition, a set that is composed essentially of ribosome protein gene transcripts is affected in a manner that correlates with the severity of the mutations, as assessed by the growth defect they produce. Oddly, it has been impossible to date to attribute this array of responses to single features in the primary sequences of the differently affected pre-mRNAs. It will therefore be interesting to determine why there are transcript-specific changes in response to mutations that should affect all transcripts similarly.

The fact that the budding yeast has only a handful of introns triggered the long standing question of why it has any and why they cluster to certain families of genes. Two opposing hypothesis have been brought forward to explain their presence ([55] and references therein). On the one hand, the intron-in model proposes that introns have been introduced relatively recently in *S. cerevisiae* and have not had time to evolve to become a general pattern of yeast gene expression. The intron-out model on the other hand, is better supported by the literature and suggests that introns are being lost from yeast genomes [56-58]. Strikingly, a multitude of introns can be deleted from the *S. cerevisae* genome without causing any apparent deficiency in growth under laboratory

conditions [59]. It therefore appears that in non-competitive conditions, many introns are dispensable. Even the combined deletion of the introns contained in the fifteen cytoskeleton-related genes does not substantially affect cell growth. In addition, although some intron deletions do cause sever growth defects, they can all be rescued by expression of the intronless mRNA from a heterologous promoter. However, in the presence of drugs and under different stress conditions the presence of certain introns becomes critical [20,59], and regulated splicing of *RPL30* is essential for optimal biological fitness [42]. This suggests that introns are required mostly when versatility in the control of gene expression is needed, as for growth in a competitive environment. It is also consistent with the fact that in many instances the changes in transcript levels due to splicing modulation are less dramatic than those caused by other major regulatory switches such as transcription. Therefore, splicing offers a further means of connecting environmental cues to gene expression, as seen in the following examples. When yeast cells are starved for amino acids, transcription and translation are inhibited at a general level, except for a set of genes involved for example in amino acid biosynthesis, which are upregulated [60-62]. This regulation can now be extended to splicing, because splicing specific microarrays of cells grown in amino acid starvation conditions versus mock treated cells show that there is also regulation at this level [63]. Splicing of most ribosomal protein pre-mRNAs is downregulated, whereas splicing of genes that do not fall into this category are largely unaffected in those conditions. Similarly, when yeast cells are exposed to toxic levels of ethanol, splicing is affected, but in a different way than in the amino acid starved cells. In this condition, the group of genes for which splicing changes is not the same as in amino acid starved cells. Unexpectedly, splicing efficiency of certain genes is increased, indicating that their splicing may not always be thorough under normal conditions, and that splicing can be regulated either positively or negatively. It will be interesting to learn how these specific sets of genes are regulated, since so far no common features have been reported in their primary sequences. Another fascinating question mark also stemming from these observations is how the spliceosome is connected to external cues that allow for this specific and fast regulation. At any rate these data clearly indicate that a sophisticated level of regulation in splicing can be achieved in systems with a reduced set of splicing regulators, and suggest that more exciting findings will be made in an organism where regulated splicing was thought to be far less relevant.

As we have discussed in the first part, the presence of introns can offer a means for tight regulation. Although only few instances of such regulated splicing have been found and studied in the past, tiling microarrays revealed that there may be more instances of regulated splicing than previously thought. Indeed, it seems that splicing of all 13 intron-containing meiotic genes is inhibited during vegetative growth [24]. Furthermore, the panel of regulated genes may not yet be complete because inactivation of NMD shows that there is an accumulation of unspliced pre-mRNAs for roughly one third of all intron-containing genes, indicating that they may be subject to some form of regulation, and degraded when unspliced pre-mRNAs escape to the nucleus [64,65]. This is also known to happen in metazoans as a result of alternative splicing that introduce in frame stop codons [66].

Perspectives

S. cerevisiae has provided a wealth of information on the mechanisms of the spliceosome. In spite of having a reduced intron set, regulated splicing plays a critical role in the life of the cell and its study contributes to our understanding of how the spliceosome can be regulated. Although important aspects of splicing, such as tissue specificity, developmental control, or multiple-choice alternate splicing cannot be approached in this simple organism, the strategies employed for regulation must rely on similar strategies in higher and lower organisms. Thus, having a simplified working model adds an important tool. This is also particularly important in the genomic studies, which are much more manageable in yeast than in metazoans. In this regard, even though the splicing-specific microarrays can generate enormous amounts of data, they entail certain inherent limitations, mainly specificity and sensitivity. In order to overcome these, new methods are now being created, that enable analysis of the whole transcriptome. Probably the most promising of those is the RNA-Seq method which consists of a high throughput quantitative sequencing of cDNA fragments that are then mapped to the genome [5]. One of the advantages of this method is that its dynamic range is greater than in microarrays, which makes it more quantitative. It also avoids the problem of cross-hybridisation that can be encountered with microarrays, making it more specific. This method should generate very solid data that will require a lot of analytical power in order to make sense of the huge amount of information created. It will also be used to analyse the transcriptome of higher eukaryotes, which will bring valuable information about alternative splicing.

Acknowledgments

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Key points

- Pre-mRNA splicing is carried out in a complex, dynamic, evolutionarily conserved ribonucleoprotein machine, the spliceosome. A significant contribution to our knowledge about the spliceosome comes from studies in yeast.
- Accurate prediction of mRNAs is a complex problem because of difficulties in identifying splice sites.
- The rules of alternate splicing are still obscure, with many variables involved. Systems with simplified splicing will provide insights into these rules.
- *S. cerevisiae* has few introns and a reduced set of factors that control splicing, yet regulated splicing is biologically relevant in this organism. Understanding the mechanisms involved will be relevant to more complex systems.
- In budding yeast changes in the overall pattern of splicing at genomic level can be detected in response to cellular and external stimuli. This should provide important cues on the coordination of cellular splicing.

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