How introns influence and enhance eukaryotic gene expression☆

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Although it has been known since the late 1970s that intron-containing and intronless versions of otherwise identical genes can exhibit dramatically different expression profiles, the underlying molecular mechanisms have only lately come to light. This review summarizes recent progress in our understanding of how introns and the act of their removal by the spliceosome can influence and enhance almost every step of mRNA metabolism. A rudimentary understanding of these effects can prove invaluable to researchers interested in optimizing transgene expression in eukaryotic systems.

A basic feature of most eukaryotic genes is that they are interrupted by one or more (and sometimes dozens of) introns, which are seemingly unintelligible sequences that must be removed upon transcription to create mRNAs with intact open reading frames. Since their discovery, there has been much debate as to the evolutionary origin and current-day functions of such intervening sequences. Functions that were recognized early on include facilitated evolution of novel genes by exon shuffling or duplication and expression of multiple proteins from a single gene by alternative splicing. Recently, however, it has become increasingly clear that introns and the act of their removal by the spliceosome can influence many other stages of mRNA metabolism, including initial transcription of the gene, editing and polyadenylation of the pre-mRNA, and nuclear export, translation and decay of the mRNA product. Together, these effects can add up to markedly different expression profiles for intron-containing and intronless versions of the same gene [1–3]. Although there is no universal intron requirement for eukaryotic gene expression, in many cases transgene expression can be dramatically increased by addition of just one generic intron to the cDNA [4–6].

Here, we offer a concise and up-to-date overview of how introns exert their positive influences on eukaryotic gene expression. For more in-depth coverage of specific topics, in addition to the primary references, the reader is referred to several excellent reviews that explore the complex web of interconnections that link virtually every step of eukaryotic RNA metabolism [7–10].

Intron-dependent enhancement of transcription

Introns have been shown to increase transcriptional efficiency of numerous genes in a variety of organisms. For example, one early study demonstrated that intronless transgenes in mice are transcribed 10–100 times less efficiently than their intron-containing counterparts [11]. Similarly, transcriptional efficiency of the gene encoding *Drosophila* alcohol dehydrogenase is reduced upon removal of its introns [12].

One way in which introns can affect transcription is by acting as repositories for transcriptional regulatory elements such as enhancers and repressors (Fig. 1). Two well-characterized examples are the immunoglobulin μ and κ intronic enhancers. In both cases, binding sites for multiple transcriptional regulatory proteins exist within the intron immediately upstream of the first constant region exon. V(D)J rearrangement during B-cell development brings these binding sites in close proximity to the promoter, thus enabling transcriptional regulation by the same elements, regardless of the V gene utilized [13]. Alternatively, an intron can regulate transcription by controlling DNA accessibility through modulation of nucleosome position. For example, in both transgenic mice and *in vitro* studies, intronic sequences in the gene encoding rat growth hormone stimulate transcription by promoting assembly of an ordered nucleosome array in the vicinity of the promoter [14].

In contrast to the above effects that occur at the DNA level, it was recently demonstrated that, once transcribed, the splicing signals in an intron can further stimulate transcription by enhancing RNA polymerase II (Pol II) initiation and processivity [15–17] (Fig. 1). In yeast and mammalian cells, promoter-proximal introns can enhance transcription initiation [15,17]. U1 small nuclear RNA (snRNA) – a well-known effector of 5′-splice-site recognition – was recently shown to associate with the general transcription initiation factor TFIIH, and to be required for the stimulatory effects of a promoter-proximal intron upon Pol II re-initiation [18]. The processivity effect is mediated by the spliceosomal U small nuclear ribonucleoproteins (snRNPs) that, in the course of assembling on a newly transcribed intron, recruit the protein Tat-specific factor 1 (TAT-SF1). In turn, TAT-SF1 interacts with positive transcription elongation factor b (pTEFb), a kinase capable of phosphorylating the C-terminal domain (CTD) of the Pol II large subunit [16]. Increased CTD

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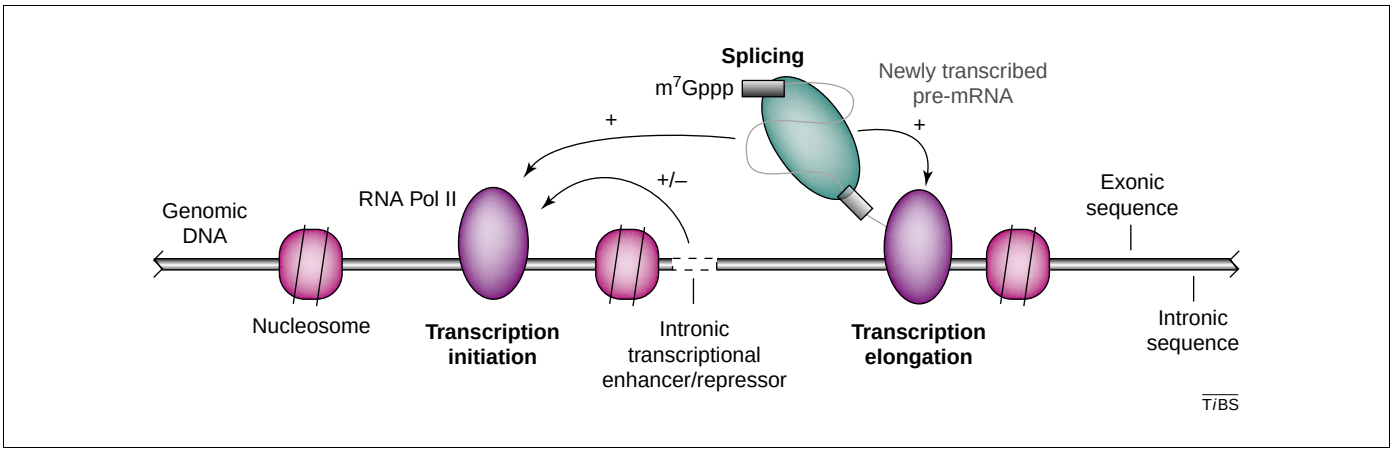

Fig. 1. Introns can affect the efficiency of transcription by several different means. Transcriptional enhancer or repressor, or nucleosome-positioning elements within introns can influence the efficiency of transcription initiation. Spliceosome components (green) assembling on a newly transcribed intron can further enhance transcription at the level of both initiation and elongation.

phosphorylation is necessary for both promoter clearance and efficient transcript elongation. Although it has been known for some time that the CTD functions to recruit pre-mRNA processing factors and thereby stimulate mRNA maturation, these latest studies have demonstrated that the communication actually goes both ways, with the assembling spliceosome providing positive feedback to the polymerase [15–18].

Interactions between splicing and other pre-mRNA processing events

In addition to splicing, other pre-mRNA processing events include 5′-end capping with 7-methyl guanosine (m^7G), 3′-end cleavage and polyadenylation, and sometimes editing and/or modification of internal nucleotides (nts). In intact cells, many of these steps are spatially and temporally linked to the transcriptional machinery and to one another (Fig. 2). The capping reactions are tightly coupled to transcription initiation via interactions between the capping enzymes and the RNA Pol II CTD [19]. Once formed, the cap structure can enhance recognition of the 5′-most intron via interactions between the nuclear cap-binding complex and U1 snRNP [20]. Consistent with cap addition occurring before complete transcription of any intron (i.e. when the transcript is only 20–40 nts), no reciprocal effect of splicing upon cap formation has been observed.

In contrast to capping, 3′-end formation is reciprocally linked to splicing [8]. When studied *in vitro*, an upstream 3′-splice site can significantly enhance use of a downstream polyadenylation site, and a downstream polyadenylation site can, likewise, increase excision of the 3′-most intron. The molecular basis for these effects involves direct contacts between the splicing and polyadenylation machineries. For example, both the snRNP protein U1A and SRm160 (SR-related nuclear matrix protein of 160 kD) – a splicing co-activator – interact with the cleavage-polyadenylation specificity factor CPSF 160 [21,22], and U1A also contacts poly(A) polymerase [23]. Furthermore, interactions between the C terminus of poly(A) polymerase and the splicing factor $U2AF^{65}$ (U2 snRNP auxiliary factor of 65 kD) can enhance upstream 3′-splice-site recognition [24].

Introns are also required for specific modification of some exon sequences by RNA editing [25]. Because RNA editing can reprogram individual codons, this process can be used to subtly modulate the activity of the encoded protein. For example, cells alter the gating and ion-conductance properties of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propanate) receptors by a specific glutamine to arginine substitution. This change at the protein level is brought about by an adenosine to inosine conversion at the RNA level. In many cases, such editing events require intramolecular base pairing between the

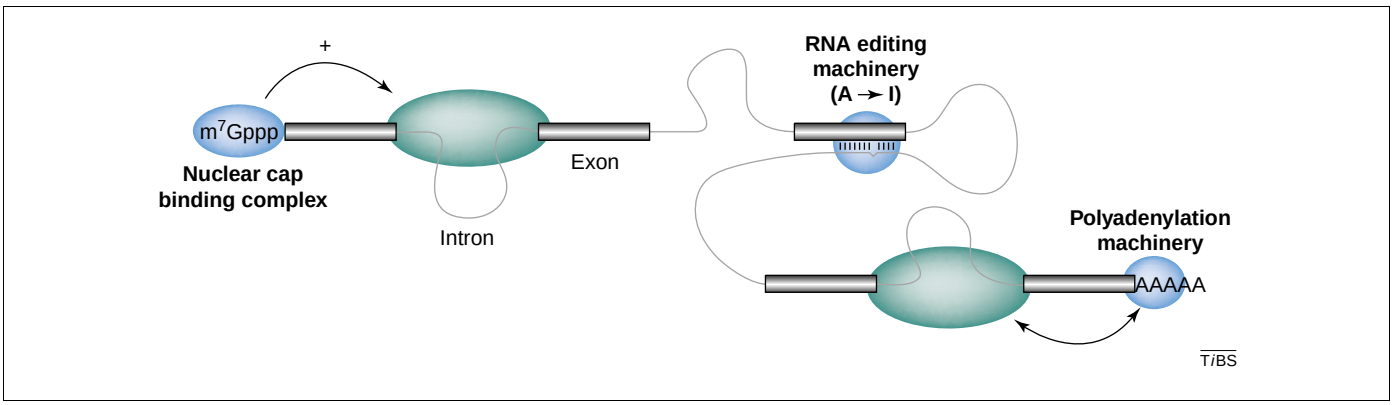

Fig. 2. Interactions between pre-mRNA processing events. The nuclear cap-binding complex promotes the excision of the 5′-most intron, whereas interactions between the spliceosome (green) and polyadenylation machinery promote excision of the 3′-most intron and proper 3′-end formation. In many cases, sequences in introns serve as guides for the chemical alteration of exonic nucleotides by RNA editing.

exon to be edited and a complementary sequence in the downstream intron; thus, editing must occur before splicing. Recent data suggest that the utilization efficiency of a cytidine to uracyl editing-site in apolipoprotein B pre-mRNA is dependent on some sort of direct communication between the splicing and editing machineries [26]. Finally, editing can also affect splicing by creating new splice sites in pre-mRNAs [27].

Effects of splicing on downstream mRNA metabolism

Splicing and mRNA export

Once processed, mature mRNAs must interact with export receptors to be translocated to the cytoplasm via the nuclear pore complex (NPC) [28,29]. Functions ascribed to the splicing machinery in the export process include retention of unspliced transcripts within the nucleus and promotion of mature mRNA export. Nuclear retention is caused by interactions of splicing factors with the splice-site consensus sequences in nascent and partially processed transcripts [30]. This retention role serves to ensure that cytoplasmic translation only occurs on mature mRNAs. Many retroviruses, however, require expression of proteins from unspliced or partially spliced transcripts, as well as from fully spliced mRNA. Such viral genes often contain *cis*-acting elements within introns that recruit specific viral or cellular export factors to the RNA and thereby overcome the nuclear retention [31].

In addition to alleviating nuclear retention by removing introns, splicing can actively promote RNA export. Early on, it was reported that certain mRNAs transcribed from cDNAs failed to exit the nucleus and, therefore, did not express protein, whereas the same mRNAs expressed from intron-containing constructs could enter the cytoplasm and be efficiently translated [32,33]. More recently, it was discovered that spliced RNAs can be more efficiently exported from *Xenopus* oocyte nuclei than identical RNAs transcribed from cDNAs [34]. The mechanistic basis of this effect is the specific recruitment of export factors to spliced RNAs as part of the exon junction complex (EJC; Fig. 3) [35–38]. It should be noted, however, that although splicing can enhance RNA export from intron-containing genes, it is not an absolute requirement because many export factors can interact with RNA independently of splicing [39]. Furthermore, naturally intronless transcripts (e.g. histones) often contain specific sequences that recruit export factors independently of splicing [40,41]. For a more complete discussion of the interconnections between splicing and mRNA export see [38,42].

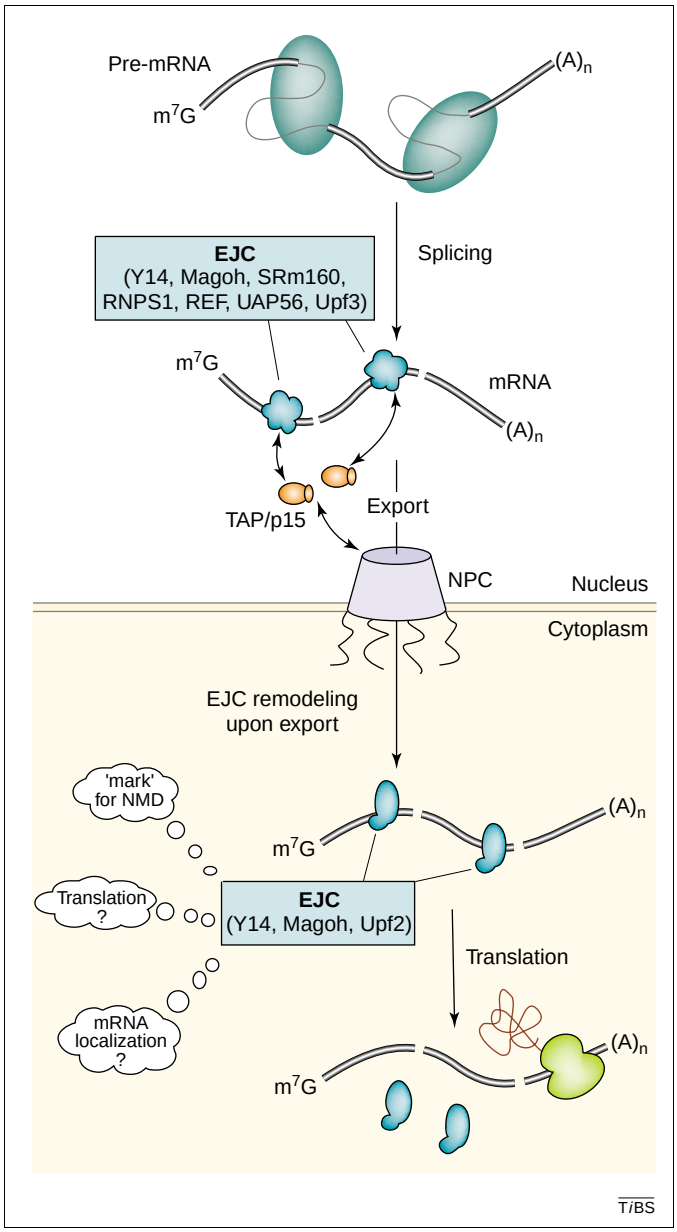

Fig. 3. Formation, structural evolution and removal of exon junction complexes (EJCs). EJCs are deposited on mRNAs by splicing at a fixed position 20–24 nucleotides upstream of exon–exon junctions. Proteins thus far identified as nuclear EJC components are indicated. Interactions between EJCs, TAP/p15 and components of the nuclear pore complex (NPC) might facilitate mRNA export. Upon export, the composition of the EJC changes or is remodeled, the proteins known to comprise cytoplasmic EJCs are indicated. Known and potential (?) roles for the EJC in the cytoplasm are indicated. EJCs are removed by ribosomes (light green) during the first round of translation.

Splicing and mRNA localization

An efficient mechanism for regulation of sub-cytoplasmic protein localization is to localize the mRNA. During *Drosophila* oogenesis, establishment of polarity in the future embryo requires *oskar* mRNA to be restricted to the posterior pole. Two proteins involved in *oskar* mRNA localization are *Mago Nashi* and *Tsunagi* [43–46]. Interestingly, the human homologs of these proteins, Magoh and Y14, are recruited to mRNAs in the nucleus as a consequence of splicing, and then accompany spliced mRNAs to the cytoplasm [37,47–49]. Thus, pre-mRNA splicing in the nucleus probably imprints some of the information required for proper cytoplasmic localization of spatially restricted mRNAs.

Splicing and translation

Removal of an intron from a pre-mRNA can also affect the translational efficiency of the resultant mRNA. Such effects – positive and negative – have been most clearly documented in *Xenopus* oocytes [50,51], wherein splicing can influence translational efficiency without significantly altering steady-state cytoplasmic mRNA levels. Braddock *et al.* [50] found that when a mature mRNA is injected directly into oocyte nuclei, it is translationally repressed

after export to the cytoplasm. This repression could be overcome either by injecting antibodies against the FRGY2 family of DNA- and RNA-binding proteins or by including a spliceable intron in the 3′ untranslated region (UTR). Whereas, binding of FRGY2 proteins in the nucleus translationally inactivates or 'masks' mRNPs [52]; splicing can apparently enable an mRNA to escape such masking and to actively engage ribosomes. Matsumoto *et al.* [51] found these translational effects to be highly dependent on intron position. In their study, an intron placed in the 5′ UTR was highly stimulatory, whereas the same intron placed in the 3′ UTR repressed translation to below the level of the corresponding intronless mRNA. However, the introns used in this study were different from that used by Braddock *et al.* [50]. The molecular mechanisms underlying these intron-identity and position-dependent differences, as well as the mechanisms behind the effects of splicing on translation, remain to be elucidated. Nonetheless, for researchers interested in optimizing the expression of transgenes, it is important to note that intron position is an important variable. In addition to potentially inhibiting translation, introns in the 3′ UTR can trigger nonsense-mediated decay (NMD) of the mRNA as described below, resulting in even lower protein expression.

Splicing and NMD

NMD is the quality control mechanism by which mRNAs containing premature-termination codons are selectively eliminated by eukaryotic cells [10,53]. If not degraded, such aberrant mRNAs – which can arise from mutations in DNA or through faulty pre-mRNA processing – would encode potentially harmful truncated proteins. A crucial step in NMD is the distinction between premature and normal termination codons. In mammalian cells, stop codons are generally identified as premature when they are located >50–55 nts upstream of an exon–exon junction. Thus, almost all authentic termination codons are located in the terminal exon of mammalian genes. This observation led to a proposal that the act of splicing somehow marks the exon–exon junctions in mRNAs, and that these marks then accompany the mRNA to the cytoplasm where they can be detected by the translation-termination machinery [10,53]. The nature of these marks and their functions in other stages of mRNA metabolism are discussed.

Mechanism of post-splicing effects

Over the past three years, several studies have yielded key information as to the biochemical basis by which splicing influences downstream mRNA metabolism. It has now been well established that splicing alters the complement of proteins that is stably associated with mRNAs in both the nuclear and the cytoplasmic compartments. This splicing-dependent alteration of mRNP composition was first observed *in vitro*, where it was shown that an mRNP generated by splicing migrated more slowly in native gels than an mRNP not produced by splicing [34]. Subsequent crosslinking analyses with photoactivable groups incorporated near exon–exon junctions revealed an association between specific proteins and spliced mRNAs [54].

More recently, splicing was shown to deposit a complex of proteins – now known as the exon junction complex (EJC) – on spliced mRNAs 20–24 nts upstream of exon–exon junctions [36]. Because it contains a host of proteins that function at multiple stages of RNA metabolism, the EJC is undoubtedly a major effector of spliced mRNA function. When formed *in vitro*, the EJC contains at least six proteins: SRm160, RNPS1, Y14, Magoh, UAP56 and REF/Aly [36,47,49,55]. *In vivo*, the EJC additionally associates with the heterodimeric mRNA export receptor TAP-p15 in the nucleus, as well as the NMD factors Upf3 and Upf2 in the nucleus and cytoplasm, respectively [37,56].

SRm160 and RNPS1 are both facilitators of pre-mRNA splicing [57,58]. Y14 and Magoh are RNA-binding proteins that shuttle between the nucleus and cytoplasm, and both are essential for correct localization of *oskar* mRNA in *Drosophila* oocytes [43–46]. UAP56 is a putative RNA helicase implicated in early spliceosome formation, and was recently shown to be crucial for recruitment of REF/Aly to spliced mRNAs [38]. REF/Aly is a nuclear mRNA export adaptor that binds to mRNAs and facilitates their productive interaction with the NPC. REF/Aly does this by contacting the export receptor TAP, which in turn, with its partner p15, directly contacts the NPC [28]. Direct evidence that the EJC plays a role in export of spliced mRNA came from analysis of truncated mRNAs that either contained a 5′ exon just long enough to accept the EJC (>33 nts) or a 5′ exon too short for EJC deposition (<19 nts). When both mRNAs were generated by splicing in *Xenopus* oocytes, the mRNA containing the longer 5′ exon was efficiently exported, whereas that containing the shorter 5′ exon was not. Furthermore, the longer mRNA associated with all of the previously identified EJC components, but the shorter mRNA did not [37]. Thus, by serving as a strong and specific binding platform for proteins that interact with the NPC, the EJC clearly provides the functional link between splicing and mRNA export [35,37,38].

Upon export to the cytoplasm, EJC composition is significantly altered (Fig. 3). SRm160 and UAP56, which are not known to shuttle, are probably removed before export, although co-immunoprecipitations have suggested that SRm160 can associate weakly with spliced mRNA in the cytoplasm [37,59]. REF/Aly, TAP-p15, RNPS1 and Upf3 – all known shuttling proteins predominantly found in the nucleus – probably dissociate from spliced mRNPs during, or soon after export [37,56,59]. Of the known nuclear EJC components, only Y14 and Magoh have been shown to associate with spliced mRNA in the cytoplasm [37,47,49,60], where they are joined by at least one new protein, Upf2 [47]. Finally, EJCs are displaced upon transit of ribosomes across the exon–exon junctions during the first round of translation [61,62].

Upf2 and Upf3 are essential NMD factors in both yeast and metazoans [10,63]. Consistent with the idea that the EJC is the 'mark' that defines the positions of exon–exon junctions during mRNA export, tethering of a Upf2- or Upf3-MS2 fusion protein downstream of a translation-termination codon is sufficient to trigger NMD of the bound mRNA [64]. Two other EJC proteins, RNPS1 and Y14, also exhibit this activity [59]. Moreover, the conserved position of the EJC (20–24 nts upstream of exon–exon junctions) places it in an appropriate position so that

the '50–55 nt rule' for recognition of premature termination codons can be explained. Taken together, these observations clearly establish the EJC as the splicing-dependent mark required for NMD in mammalian cells.

Future challenges

Although we have described numerous examples of how the presence of introns in eukaryotic genes can influence gene expression, in most cases it is not yet known which influences predominate to enhance or repress the expression of any particular intron-responsive gene; undoubtedly, there are more connections yet to be discovered. As more and more genomes are sequenced and compared, we predict that many introns will contain conserved sequence elements distinct from those that define the splice sites. Most of these elements are likely to regulate transcription, splicing and/or editing as described above, or represent novel stable RNA species, which are sometimes derived from pre-mRNA introns [65]. Others, however, might reveal new roles for introns not yet reported, such as alterations in pre-mRNA stability, intranuclear RNA trafficking or even the fate of the encoded protein.

Many questions remain unanswered regarding the molecular mechanisms responsible for the effects of introns already observed. For example, it is currently unclear why different introns can have such divergent effects on protein expression. Is this merely owing to intrinsic differences in their processing efficiencies, or can it be explained by intron-specific alterations in EJC composition? To date, the full complement of EJC proteins is unknown for any exon–exon junction. Some progress has been made toward understanding how and when during splicing the EJC is assembled and which EJC proteins actually contact the mRNA [66], but how the EJC remains so tightly bound to a fixed position without apparent sequence specificity remains unresolved. Furthermore, it is unknown whether the changes to EJC composition during its transit from nucleus to cytoplasm occur passively by simple dissociation of nuclear factors or involve active remodeling. Finally, although it is presumed that an EJC is deposited at every exon–exon junction of mRNAs originating from pre-mRNAs with multiple introns, this has not yet been formally proven.

Further mystery surrounds the effects of introns on translational efficiency. As EJCs are removed by the first round of translation [61,62], how can introns influence the translational efficiency of the product mRNA? Perhaps, in the same way that splicing imprints pre-translational mRNPs with EJCs, interactions between EJC components and other mRNP proteins (e.g. 5′ cap- and/or polyA-tail-binding factors) lead to higher-order structural alterations that maintain cytoplasmic mRNPs in a translationally 'unmasked' state even after EJC removal. In addition, it is unclear whether the effects of EJCs on translation and NMD are entirely separate functions, or whether they are simply alternate outcomes of the same intermolecular interactions contingent on EJC position relative to the open reading frame.

Why do introns and the act of their removal affect such a diverse array of cellular processes? Spatial and temporal coordination between transcription and pre-mRNA-processing events that occur near the site of transcription probably serves to enhance the efficiency of each process as well as to ensure mRNA integrity. How, and why, pre-mRNA splicing evolved to affect so many stages of downstream mRNA metabolism is harder to rationalize. In this regard, it would be of great value to know the evolutionary origin of the EJC. Perhaps the EJC originated from one or more residual proteins that were inadvertently left at exon–exon junctions by the process of pre-mRNA splicing. In this model, other cellular processes could have learned to take advantage of the information inherent in these marks to, for example, distinguish pre-mRNAs from mature mRNAs and exert quality control on mRNA export and reading-frame integrity. Alternatively, in early eukaryotes, pre-mRNA splicing might have been an intrinsically inefficient process. In this scenario, there would have been a strong selective pressure for tagging RNAs as spliced, so that only these RNAs would be efficiently translated. Interestingly, in *Saccharomyces cerevisiae*, the ~4% of genes that contain introns express a disproportionately large percentage of the total cellular protein. So far, it is unknown whether anything akin to the metazoan EJC is present in *S. cerevisiae*. Although many of the known EJC components (Y14, Magoh, RNPS1 and SRm160) are not conserved in budding yeast, REF, UAP56, TAP, Upf2 and Upf3 are. In the future, it would be of great interest to determine whether coupling between splicing and other steps in gene expression occurs in lower eukaryotes, and to what extent these interactions contribute to the evolutionary fitness of the organism.

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