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Review article

How introns enhance gene expression

Orit Shaul

The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel

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ABSTRACT

In many eukaryotes, including mammals, plants, yeast, and insects, introns can increase gene expression without functioning as a binding site for transcription factors. This phenomenon was termed 'intron-mediated enhancement'. Introns can increase transcript levels by affecting the rate of transcription, nuclear export, and transcript stability. Moreover, introns can also increase the efficiency of mRNA translation. This review discusses the current knowledge about these processes. The role of splicing in IME and the significance of intron position relative to the sites of transcription and translation initiation are elaborated. Particular emphasis is placed on the question why different introns, present at the same location of the same genes and spliced at a similar high efficiency, can have very different impacts on expression − from almost no effect to considerable stimulation. This situation can be at least partly accounted for by the identification of splicing-unrelated intronic elements with a special ability to enhance mRNA accumulation or translational efficiency. The many factors that could lead to the large variation observed between the impact of introns in different genes and experimental systems are highlighted. It is suggested that there is no sole, definite answer to the question "how do introns enhance gene expression". Rather, each intron-gene combination might undergo its own unique mixture of processes that lead to the perceptible outcome.

1. Introduction

The ability of introns to elevate gene expression was identified in a wide range of organisms, including mammals, nematodes, insects, fungi, and plants. In certain cases, introns were shown to function as internal promoters (Furger et al., 2002; Morello et al., 2002; Samadder et al., 2008). This review focuses on introns that do not function as binding sites for transcription factors, and elevate expression only when present in the transcribed region. This phenomenon was termed 'intron-mediated enhancement' (IME) (see Gallegos and Rose, 2015; Laxa, 2017; Moore and Proudfoot, 2009; Nott et al., 2003; Rose, 2008 for earlier reviews of IME). In many cases, the presence of introns increases the steady state levels of mature mRNA in the cytosol (e.g., Akua et al., 2010; Brinster et al., 1988; Callis et al., 1987; Curi et al., 2005; Dean et al., 1989; Morello et al., 2011; Neuberger and Williams, 1988; Nott et al., 2003; Rethmeier et al., 1997; Rose, 2004, 2008). This can result from impacts on the rate of transcription, nuclear export, and transcript stability. Moreover, the presence of introns can also increase the efficiency of mRNA translation (e.g., Akua and Shaul, 2013; Bourdon et al., 2001; Curi et al., 2005; Gudikote et al., 2005; Hoshida et al., 2017; Lu and Cullen, 2003; Mascarenhas et al., 1990; Matsumoto et al., 1998; Nott et al., 2003, 2004; Rose, 2004; Samadder et al., 2008). The mechanisms that underlie these divergent impacts of introns on eukaryotic gene expression are not fully understood. This review discusses the current knowledge about these processes, as well as several open questions in this topic. These questions include the role of splicing in IME, the significance of intron's position relative to the sites of transcription and translation initiation, as well as why different efficiently spliced introns have different impacts on expression.

When discussing the positive influence of introns on gene expression, care should be taken to distinguish between the following possibilities. First, there are splicing-dependent effects (e.g., the deposition on the mRNA of specific proteins that affect its fate). Second, some effects depend on the mere presence of intronic sequences, such as 5′ splice sites (5′ SSs), even in the absence of splicing. And last, some impacts are mediated by isolated splicing regulators, which can also bind mRNAs derived from intronless genes. These possibilities are not mutually exclusive, but their presence should be kept in mind. During this review, an effort will be made to distinguish between these possibilities.

Most of the information described in this review results from work on mammalian and plant systems. Despite differences in length and AU composition between vertebrate and plant introns, the basic mechanism of splicing in these groups is similar in many respects (Simpson et al., 1999). The 5′ SS, 3′ SS, and branchpoint consensus sequences are similar in plants and vertebrates, but plant introns are more UA-rich. Since vertebrate genes are characterized by short exons separated by long introns, their splicing generally depends on exon definition. Plant

E-mail address: orsha@mail.biu.ac.il.

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and yeast introns are shorter in comparison with vertebrate introns and their splicing generally depends on intron definition, but exon-defining interactions can also occur in plants (Simpson et al., 1999, and references therein). There is no mechanistic difference in spliceosomal complex assembly over exons or introns (reviewed by De Conti et al., 2013).

2. Introns can increase the rate of transcription

Transcription is spatially and temporally coupled to splicing (reviewed by Saldi et al., 2016). Nuclear run-on (NRO) experiments showed that promoter-proximal introns can increase the efficiency of transcription in mammalian and plant cells (Brinster et al., 1988; Furger et al., 2002; Samadder et al., 2008). Chromatin immunoprecipitation (ChIP) analysis showed that the abundance of RNA polymerase II (Pol II) binding sites on an intron containing reporter construct was 4-fold higher compared with the intronless construct (Laxa et al., 2016). Interestingly, some of the contribution of introns to transcription is mediated by 5′ SSs (and related sequences) and by splicing-independent functions of splicing factors.

2.1. The contribution of U1 snRNA and 5′ SSs to transcription

The 5′ SSs of introns are recognized by U1 small nuclear RNA (snRNA). The interaction of U1 snRNA with 5′ SSs, or 5′ SS-similar sequences located at other intronic or exonic regions, supports several splicing-independent functions of U1 snRNA. These functions include effects on transcription initiation, reinitiation, elongation, and accuracy. U1 snRNA associates with the general transcription factor II human (TFIIH) complex and stimulates the rate of formation of the first phosphodiester bond by Pol II in human cells (Kwek et al., 2002). The interaction of U1 snRNA with the cyclin H component of TFIIH leads to the phosphorylation of the C-terminal domain (CTD) of Pol II by TFIIH, thereby promoting transcription initiation (Guiro and O'Reilly, 2015; O'Gorman et al., 2005) (Fig. 1A). In addition, promoter-proximal 5′ SSs bound by U1 snRNA recruit TFIIH and Pol II, thereby stimulating transcription reinitiation (Damgaard et al., 2008; Kwek et al., 2002) (Fig. 1B).

The significance of an intact 5′ SS for U1 snRNA-mediated transcription enhancement was demonstrated in several studies. NRO analysis showed that introns enhanced transcription about 3-fold in several mammalian and yeast genes, depending on the presence of an intact 5′ SS (Furger et al., 2002). Point mutations in the 5′ SS reduced transcription, and this effect was partially rescued by a 'suppressor' U1 snRNA that could base pair with a sequence near the mutated 5′ SS (Furger et al., 2002). ChIP experiments in human cells showed enhanced promoter docking of transcription initiation factors on a reporter gene that contained a functional 5′ SS (Damgaard et al., 2008). Mutations that reduced U1 snRNA-5′ SS interaction lowered nascent transcription and splicing, while mutations that stabilized this interaction increased transcription and splicing (Damgaard et al., 2008). The efficiency of reinitiation increased 3-fold in the presence of an intron with a correct 5′ SS, and was reduced after treatment with a U1 snRNA complementary oligonucleotide. At the same time, the efficiency of reinitiation was not affected by mutation of the 3′ SS (Kwek et al., 2002). Importantly, a 5′ SS was able to stimulate transcription even in the absence of splicing, although to a lower level compared with a full intron (Damgaard et al., 2008). Altogether, these data indicate that U1 snRNA enhances transcription independently of its role in splicing, whereas splicing can enhance transcriptional output more than the increase mediated by the splicing-independent roles of U1.

U1 snRNA also contributes to transcription fidelity in mammalian cells (Fig. 1D). Although Pol II usually initiates transcription at both the sense and antisense directions, productive elongation occurs primarily in the sense orientation in mammalian cells. This selection of promoter directionality partly depends on the enrichment of the U1 snRNA recognition sites (5′ SS-related sequences) near transcription start sites (TSSs) in the sense relative to the antisense direction (Almada et al., 2013). Another way by which U1 snRNA elevates transcriptional fidelity is the protection of sense mRNA from premature cleavage and polyadenylation (PCPA) (Almada et al., 2013; Berg et al., 2012; Kaida et al., 2010). This function of U1 snRNA is also not related to its role in splicing, since the inhibition of splicing, either chemically (using the splicing inhibitor spliceostatin A) or by antisense oligonucleotides against U2 snRNA, did not prevent PCPA suppression by U1 snRNA (Kaida et al., 2010). PCPA suppression depends on the base pairing of U1 snRNA with 5′ SSs or other sites, presumably cryptic 5′ SSs, present in the pre-mRNA, particularly in introns (Berg et al., 2012; Kaida et al., 2010) (Fig. 1D).

When the sites of U1 snRNA interaction with nascent transcripts were experimentally determined by a crosslinking-based method, it was confirmed that U1 hybridizes to both intronic 5′ SSs and to motifs with a similarity to 5′ SSs present throughout introns (Engreitz et al., 2014). The interaction of U1 snRNA with 5′ SS-similar motifs was also identified on intronless transcripts, confirming that this interaction is independent of splicing. Nevertheless, U1 snRNA interaction sites were more enriched in introns than exons (Engreitz et al., 2014).

2.2. The involvement of other splicing factors in transcription

Other interactions with splicing factors were also shown to enhance transcription. The spliceosomal U1 and U2 small nuclear ribonucleoproteins (snRNPs) interact with the human transcription elongation factor TAT-SF1 and stimulate transcription elongation (Fong and Zhou, 2001). Depletion of SRSF1 (SF2/ASF) or SRSF2 (SC35), two splicing regulators of the serine/arginine-rich (SR) protein family, resulted in defective transcription in mammalian cells (Lin et al., 2008). SRSF2 depletion was accompanied by inefficient recruitment of the positive transcription elongation factor b (P-TEFb) to the Pol II complex, and by Pol II accumulation on the body of genes. This suggested that this SR protein plays a critical role in transcriptional elongation (Lin et al., 2008). These data can possibly be explained by the finding that Pol II is frequently paused within 20–40 nt downstream from the TSS, and its release and entry into the elongation phase require the recruitment of P-TEFb. For this, P-TEFb should be released from an inhibitory complex assembled at gene promoters. This inhibitory complex includes, besides P-TEFb, the 7SK noncoding RNA and SRSF2. P-TEFb release is facilitated by SRSF2 binding to the nascent RNA produced by the paused Pol II (Ji et al., 2013, and references therein). The binding sites of SRSF2 on the nascent RNA that account for this release, which are sequences with similarity to exonic splicing enhancers (ESEs), should be located very close to the TSS (less than 60 nt apart) (Ji et al., 2013).

Based on the above data, the function of SRSF2 in the P-TEFb-mediated release of paused Pol II and the activation of transcription seems to be unrelated to the role of SRSF2 in splicing. However, it is relevant to mention here that the inhibition of splicing reduced the phosphorylation of Pol II CTD on Ser2, which is carried out by P-TEFb; the exact mechanism that linked phosphorylation inhibition to splicing inhibition was not clear (Koga et al., 2015). In yeast, the interaction of the splicing factor U2AF with the CTD of Pol II recruits the splicing factor Prp19 complex (PRP19C), leading to enhancement of the Pol II elongation rate (Chanarat et al., 2011). There are puzzling data regarding the impact of introns and splicing on the rate of Pol II elongation. While there are indications that splice sites are pol II pausing signals (Nojima et al., 2015), it was found that the elongation rate is faster in introns than exons (Jonkers et al., 2014) (reviewed by Saldi et al., 2016 and by Wallace and Beggs, 2017). At the same time, kinetic measurements showed that Pol II elongation rates are uncoupled from ongoing splicing (Brody et al., 2011). Thus, there is still much to learn about the interrelations between splicing, splicing factors, and transcription.

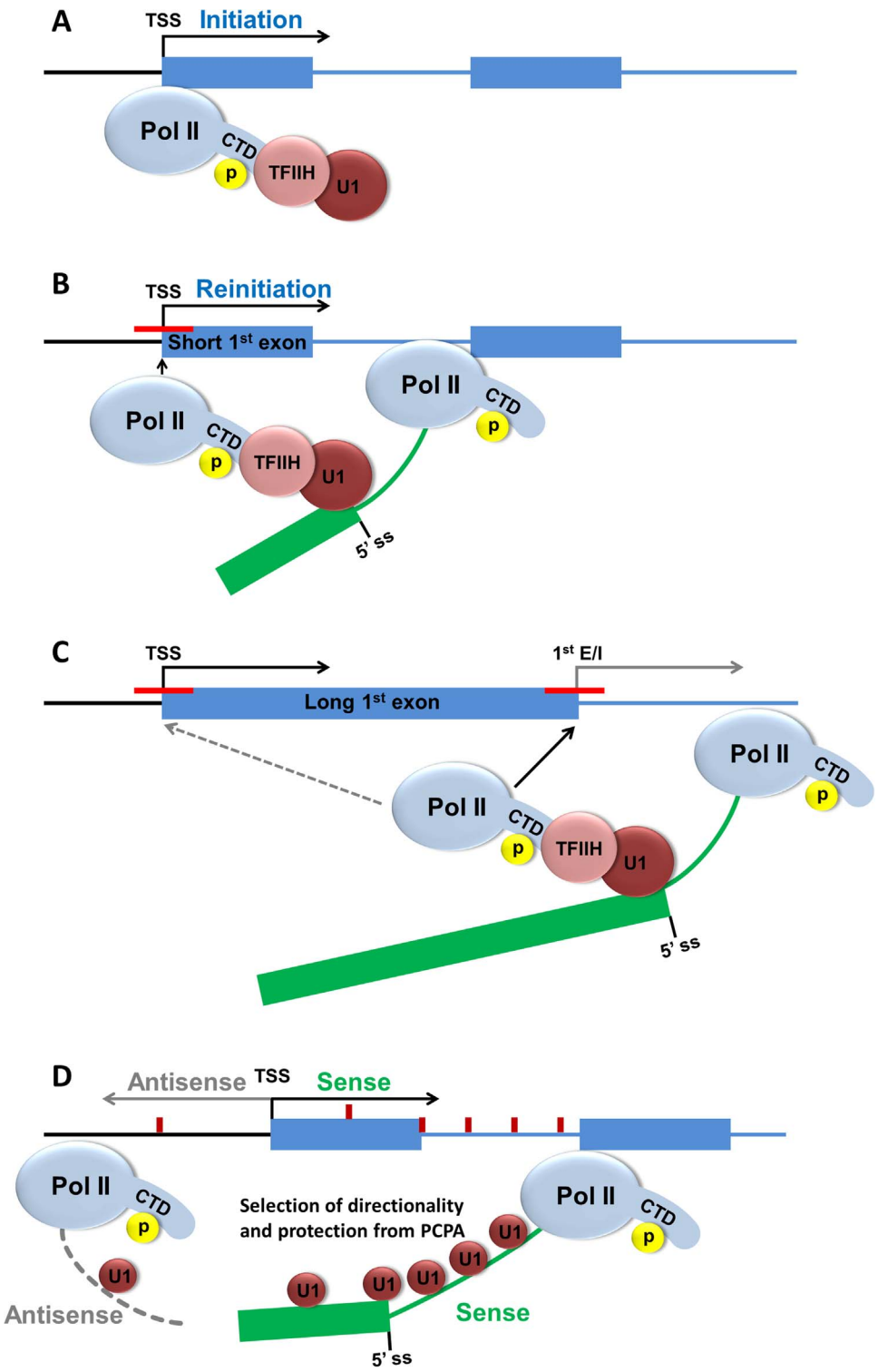

Fig. 1. A model to explain part of the contribution of promoter-proximal introns and U1 snRNA to transcription. (A) During transcription initiation, U1 snRNA associates with TFIIH independently of introns. This leads to phosphorylation of the CTD of Pol II by TFIIH, and stimulation of transcription initiation. (B) U1 snRNA associates with a promoter-proximal 5′ SS on the nascent transcript and recruits TFIIH and Pol II. This brings these factors to the vicinity of the promoter and stimulates transcription reinitiation. Promoter-proximal introns (i.e., short first exons) also correlate with the presence of a single, TSS positioned peak of activating histone modifications (red line). (C) When the first intron is not close to the promoter, U1-TFIIH-Pol II interaction at the 5′ SS is more distant from the TSS, and, thus, less likely to promote reinitiation. Rather, it might contribute to improper initiation near the first exon-intron boundary (1st E/I). The incidence of such additional, improper initiation also correlates with the presence of two peaks of activating histone modifications (red lines) – around the TSS and the first exon-intron boundary. (D) Sequences which, following their transcription, form U1 snRNA recognition sites, are enriched near TSSs in the sense relative to the antisense direction (dark red lines). These sequences are particularly enriched in introns, and support the selection of promoter directionality (the fact that productive elongation occurs primarily in the sense orientation). In addition, U1 snRNA binding to its recognition sites on the nascent transcript protects sense mRNA from premature cleavage and polyadenylation (PCPA). See text for references. Black line – the promoter region; blue and green boxes – exons in the DNA and RNA, respectively; blue and green lines – introns in the DNA and RNA, respectively; P – phosphorylation; TSS – transcription start site.

3. Is intron impact on transcription mediated by chromatin modification?

Compared with genes with long first exons, genes with short first exons (i.e., with promoter-proximal introns) show higher expression levels, accuracy in TSS usage, and a lower frequency of antisense transcription (Bieberstein et al., 2012). Pol II and the general transcription factors TFIID, TFIIB, and TFIIF peak at the correct TSS of all genes. However, genes with long first exons displayed an additional downstream peak of these factors at the 5′ SS, from which transcription was also initiated (Bieberstein et al., 2012) (Fig. 1C). Part of these observations could apparently be attributed to the processes described above. When the intron is not close to the promoter, U1-TFIIH-Pol II interaction at the 5′ SS is more distant from the TSS, and, thus, less likely to promote reinitiation (Fig. 1C; see also Section 9). This reduces the ability of promoter-distal introns to enhance expression. Possibly, this U1-TFIIH-Pol II interaction could even contribute to the improper initiation at the 5′ SS that follows a long first exon (Fig. 1C). However, it was suggested that chromatin modifications can also play a role in these observations (Bieberstein et al., 2012). Interestingly, the length of the

first exon also correlates with the sites of chromatin modifications, including the activating marks histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 9 acetylation (H3K9ac). These marks are enriched at the 5′ SSs of first introns, and changing the length of the first exon altered their position on a reporter gene (Fig. 1B, C). Intron elimination or inhibition of splicing (either chemically or by mutating the 3′ SS) eliminated these methylation peaks (Bieberstein et al., 2012). One possible causal basis for the correlation between the sites in which methylation marks and general transcription factors are enriched is the ability of H3K4me3 to directly bind TFIID, and of H3K9ac to promote this binding (Vermeulen et al., 2007).

During transcription, the SETD2 methyltransferase, which can be recruited by Pol II, generates the histone H3 lysine 36 trimethylation (H3K36me3) mark. Splicing enhances the recruitment of SETD2 and H3K36 trimethylation (de Almeida et al., 2011). This can explain the finding that H3K36me3 levels are higher in intron-containing compared with intronless genes. However, splicing is not indispensable for this modification (de Almeida et al., 2011). The levels of H3K36me3 increase toward the 3′ end of genes (reviewed by Zhang et al., 2015). The inhibition of splicing changes the localization of the H3K36me3 mark, probably by affecting SETD2 recruitment (de Almeida et al., 2011; Kim et al., 2011; Saldi et al., 2016). H3K36me3 recruits histone deacetylases that deacetylate histones over gene bodies, thereby preventing initiation from aberrant TSSs within gene bodies (Zhang et al., 2015).

The above data are in agreement with the possibility that introns affect the sites of histone modifications, thereby assisting the selection of correct TSSs. However, further study is necessary to clarify the causal relationships between introns, chromatin modification, and TSS selection. For example, although the 5′ untranslated-region (5′ UTR) intron of *Arabidopsis* GGT1 aminotransferase enhanced transcription, its insertion did not affect the abundance of activating histone modifications in the reporter constructs (Laxa et al., 2016). Other possible links between introns and chromatin modification were also evaluated. The *Chironomus tentans* HRP65 protein interacts with p2D10, a histone H3 acetyltransferase, thereby increasing transcription (Sjolinder et al., 2005). It was initially thought that HRP65 is a splicing factor, but it became apparent that this is not the case (Kozlova et al., 2006). Mammalian Hu proteins associate with pre-mRNA sequences surrounding alternative exons, and inhibit histone deacetylase 2. The resultant histone hyperacetylation leads to increased local transcriptional elongation rates, which affect the inclusion of alternative exons (Wang et al., 2010; Zhou et al., 2011). However, it is currently unknown whether Hu proteins are effective only in alternative exons, or play a more general role in determining the rate of transcription elongation.

4. Intron-mediated transcriptional enhancement correlates with gene looping

A looped conformation of genes, which brings together their promoter and terminator regions, might enhance transcription, possibly by facilitating Pol II recycling and reinitiation (Moabbi et al., 2012; O'Sullivan et al., 2004). Gene looping favors transcription in the sense direction over upstream, antisense transcription (Agarwal and Ansari, 2016). Insertion of an intron with an intact 5′ SS into the *INO1* gene increased its transcription (as shown by NRO), and was accompanied by gene looping in yeast (Moabbi et al., 2012). Interaction of the promoter and terminator regions of this gene with the 5′ and 3′ SSs of the intron, respectively, supported a model for intron-dependent gene looping (Moabbi et al., 2012). The intron was not indispensable for looping, since an activator-dependent increase in transcription of the intronless *INO1* gene increased its looping as well (El Kaderi et al., 2009). Both activator- and intron- dependent transcriptional increase were reduced in the looping defective *sua7-1* strain, which has a mutated TFIIB. Still, it cannot be ruled out that the TFIIB mutation (sua7-1) affects intron-mediated transcriptional activation through an aspect of transcription other than gene looping (Moabbi et al., 2012). Thus, it cannot be excluded that, similar to activation-mediated transcriptional enhancement, intron-mediated transcriptional enhancement leads to gene looping. This looping could, in turn, facilitate further transcriptional enhancement in a positive feedback loop.

5. The exon junction complex facilitates mRNA export to the cytosol in a cap-dependent manner

Several studies indicated that, despite some controversy, splicing can facilitate mRNA export to the cytosol, although it is not essential for this process (reviewed by Dimaano and Ullman, 2004; Reed, 2003); (Valencia et al., 2008, and references therein). Fluorescence in situ hybridization (FISH) analysis showed that the rate of mRNA export is 6- to 10-fold higher for spliced mRNAs compared with their cDNA counterparts (Valencia et al., 2008). It was indicated that the export of mRNA from the nucleus to the cytosol is facilitated by the exon junction complex (EJC) (Le Hir et al., 2001). Upon splicing, the spliceosome deposits multiple proteins, known as the EJC, 20–24 nucleotides upstream of exon-exon junctions (EEJs). The core of the EJC is composed of four proteins: eukaryotic initiation factor 4A3 (eIF4A3), Magoh, Y14, and MLN51 (also known as Barentsz) (reviewed by Boehm and Gehring, 2016; Le Hir et al., 2016). EJCs are part of the messenger ribonucleoprotein (mRNP) complex that shuttles from the nucleus to the cytosol. ALYREF is one of the components of the mammalian TREX (transcription-export) complex. A short motif in ALYREF directs its interaction with both the eIF4A3 component of the EJC and the cap-binding complex (CBC) (Cheng et al., 2006; Gromadzka et al., 2016, and references therein) (Fig. 2). In mRNPs containing several EJCs, the export complexes are located only between the 5′ cap and the first EJC (Cheng et al., 2006). This cap- and EJC- dependent interaction of the TREX complex with spliced mRNAs can explain the positive contribution of splicing to mRNA export (Fig. 2). Besides supporting translocation, the EJC can also affect the site of mRNA localization in the cytosol (Hachet and Ephrussi, 2004).

6. The correlation between introns and mRNA stability

In genome-wide analyses, mRNA stability showed a positive correlation with the number of introns in human, mouse, and *Arabidopsis* genes (Duan et al., 2013; Narsai et al., 2007; Sharova et al., 2009; Wang et al., 2007). Insertion of an intron into cDNA constructs containing instability determinants increased their stability in human cells (Zhao and Hamilton, 2007). It was also shown that introns can enhance pre-mRNA 3′ end processing and polyadenylation. This effect was mediated by the splicing regulator polypyrimidine tract-binding protein (PTB), and resulted in a significant increase in the half-life of the affected transcripts (Lu and Cullen, 2003; Millevoi et al., 2009). In addition, U1 snRNA bound to a 5′ SS suppresses premature cleavage and polyadenylation, and determines mRNA length (Berg et al., 2012; Kaida et al., 2010). This might also affect transcript stability. However, in several studies carried out on mammalian and plant cells, introns that

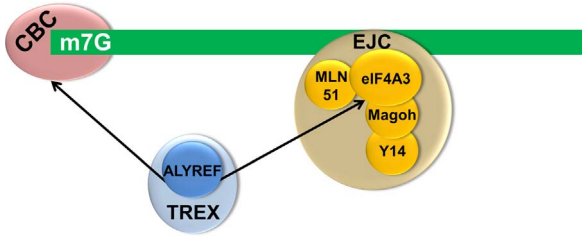

Fig. 2. The EJC-mediated contribution of introns to nuclear export. The ALYREF component of the TREX (transcription-export) complex interacts with both the eIF4A3 component of the EJC and the CBC (Cheng et al., 2006; Gromadzka et al., 2016). This interaction can explain the positive contribution of splicing to mRNA export. green line – mRNA. Only the 5′ region of the transcript is shown.

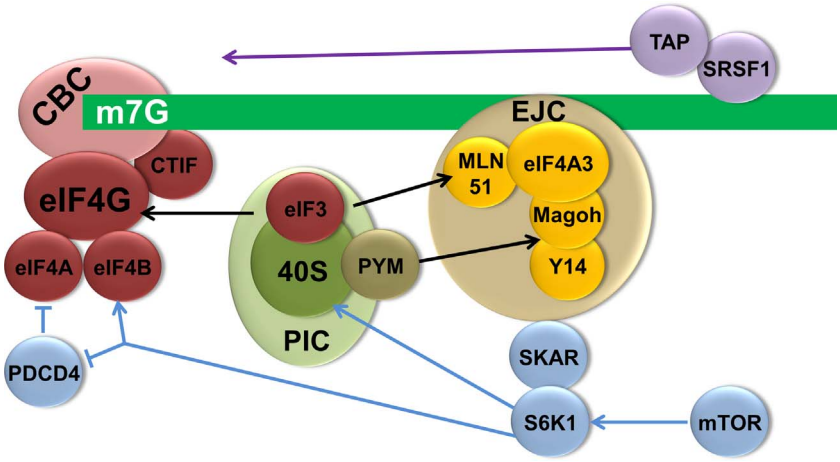

Fig. 3. The contribution of introns to translation. This figure presents only the contribution of EJC and SR proteins assembled on the mRNP following splicing (and not their contribution as free components). MLN51 embedded in the EJC interacts with eIF3 and the preinitiation complex (PIC). eIF3 facilitates PIC recruitment to the 5′ cap by eIF4G, followed by initiation of scanning. PYM, which can interact with both the PIC (through the 40S ribosomal subunit) and Y14-Magoh, facilitates PIC recruitment to the mRNA. During mTOR signaling, mTOR activates S6K1. The EJC-associated protein SKAR binds the activated S6K1, which phosphorylates the 40S ribosomal subunit protein rpS6, the translation initiation factor eIF4B, and the eIF4A inhibitor PDCD4 (PDCD4 phosphorylation leads to its degradation). These phosphorylation events result in eIF4B recruitment to eIF4A and the enhancement of eIF4A activity, which facilitates scanning. The SR splicing regulator SRSF1 promotes the first round of translation by recruiting the translational activator TAP. See text for references. green line − mRNA. Only the 5′ region of the transcript is shown.

enhanced expression did not increase mRNA stability (Brinster et al., 1988; Nott et al., 2003; Rethmeier et al., 1997).

The EJC core component Y14 was indicated as increasing mRNA stability by directly interacting with the 5′ cap and the decapping factor Dcp2, and inhibiting the mRNA-decapping activity of this enzyme (Chuang et al., 2013). Overexpression of Y14 prolonged the half-life of a reporter mRNA. However, it was indicated that this function of Y14 is independent of its presence in the EJC. It can be hypothesized that Y14 might be more readily recruited to the 5′ cap of spliced mRNA due to its presence in the microenvironment following EJC displacement. Still, it remains to be determined whether there is a difference between Y14 impact on the half-life of mRNAs derived from intron-containing or intronless genes.

It should be noted that if introns are located more than 50–55 nt downstream of termination codons (TCs), they can lower mRNA stability through the nonsense-mediated mRNA decay (NMD) pathway (Zhang et al., 1998). These TCs can be either those of the main open reading frame (ORF), or they can be derived from alternative splicing, mutations, or upstream ORFs (uORFs) (reviewed by Shaul, 2015).

This review focuses on the impact of efficiently spliced introns on gene expression. However, it should be noted that inefficient splicing can lower mRNA stability by both the NMD and the nuclear exosome pathways (Danin-Kreiselman et al., 2003; Kramer et al., 2016; Sayani et al., 2008; Sayani and Chanfreau, 2012).

7. Introns can increase the efficiency of mRNA translation

Besides increasing mRNA content by affecting transcription, export, and stability, the presence of introns was also shown to increase the efficiency of mRNA translation in mammals, plants, yeast, and *Xenopus* (Akua and Shaul, 2013; Bourdon et al., 2001; Curi et al., 2005; Gudikote et al., 2005; Hoshida et al., 2017; Lu and Cullen, 2003; Mascarenhas et al., 1990; Matsumoto et al., 1998; Nott et al., 2003, 2004; Rose, 2004; Samadder et al., 2008). The ability of introns, which are spliced in the nucleus, to affect translation in the cytosol is also attributed to proteins in the mRNP complex that reaches the cytosol. It was shown that the EJC can affect translation (Lee et al., 2009; Nott et al., 2004; Wiegand et al., 2003) (reviewed by Le Hir et al., 2003, 2016; Le Hir and Seraphin, 2008; Moore and Proudfoot, 2009). The four core EJC proteins are associated by other proteins, called EJC peripheral factors, whose composition can vary under different conditions (reviewed by Le Hir et al., 2016). Translation can also be affected by such EJC peripheral proteins, and by SR proteins (see below).

Several studies provided some clues about possible mechanisms by which the EJC can increase translation efficiency. Interestingly, some studies demonstrated that EJC proteins can affect translation in an intron-independent manner, and this possibility should also be borne in mind. Tethering to intronless reporter mRNAs the EJC core components Magoh, Y14, or MLN51 (but not eIF4A3), or the peripheral EJC protein RNPS1, increased reporter translation in human cells (Nott et al., 2004; Tange et al., 2005; Wiegand et al., 2003). Upon overexpression in human cells, only MLN51, but not eIF4A3 or Magoh and Y14, affected translation efficiency (Chazal et al., 2013). MLN51 and eIF4A3 can associate independently of the EJC, and, together or separately, they interact with the translation factors eIF3, eIF4A1, eIF4E, and components of the small (rpS14) and large (rpL26) ribosomal subunits. MLN51 can interact with the RNA in a low affinity as an isolated component, or with a high affinity when embedded within the EJC. Under both conditions, MLN51 can stably interact with eIF3. Consequently, MLN51 ability to link eIF3 with the RNA is higher when it is part of the EJC (Chazal et al., 2013). eIF3 is a component of the 43S preinitiation complex (PIC), and facilitates 43S PIC recruitment to the 5′ cap by eIF4G (Fig. 3). This is followed by scanning initiation. These data can explain the following observations. First, overexpressing MLN51 enhanced 2- and 5-fold the translation yields of intronless or intron-containing reporters, respectively. A mutated MLN51 that could not incorporate into EJCs increased the translation of both reporters by 2-fold. Overexpression of MLN51 also stimulated global cellular translation, to a larger extent than did the mutated MLN51. Altogether, these data indicate that MLN51 can enhance translation as an isolated protein, but it does so with higher efficiency when embedded in the EJC (Chazal et al., 2013) (Fig. 3).

In agreement with the idea that EJC core proteins can play roles independent of their deposition in the EJC, *Drosophila* eIF4A3, Y14, and Magoh can bind nascent transcripts of not only intron-containing but also intronless genes (Choudhury et al., 2016). Besides its deposition in EJCs of intron-containing genes, human eIF4A3 is recruited to the 5′-end of CBC-bound mRNAs of both intron-containing and intronless genes by directly interacting with the CBC-dependent translation initiation factor (CTIF). eIF4A3 enhances the translation of CBC-bound mRNAs by promoting the efficient unwinding of secondary structures in the 5′ UTR (Choe et al., 2014). Since eIF4A3 facilitates the first round of translation of CBC-bound mRNAs, its recruitment by CTIF should occur before EJC displacement by the ribosome, and before the transition to translation of eIF4E-bound mRNA, which depends on eIF4A1 and eIF4A2 for disrupting 5′ UTR secondary structures. Thus, this role of eIF4A3 is apparently independent of its deposition in the EJC.

The human EJC-associated protein partner of Y14-Magoh (PYM) dissociates the EJC from the mRNA, and is essential for the efficient recycling of EJC components (Gehring et al., 2009b). PYM, which binds to Y14–Magoh, can also bind via a separate domain to the small (40S) ribosomal subunit and the 48S PIC (Diem et al., 2007) (Fig. 3). PYM binding to the EJC is much more efficient in the presence of these ribosomal subunits, ensuring that EJC displacement will mainly occur

during translation (Gehring et al., 2009b). Thus, PYM can help to direct translation preferentially to EJC-bound mRNAs, and to recycle the EJC components necessary for incorporation into new EJCs (Diem et al., 2007; Gehring et al., 2009b). Overexpression of *Arabidopsis* homologs of PYM and the EJC components Y14 and Magoh increased the expression of intron-containing reporter constructs in plants (Mufarrege et al., 2011). However, PYM did not interact with ribosomal subunits or components of the translation initiation complex in *Drosophila*, excluding a major role of PYM in translation regulation in this organism (Ghosh et al., 2014).

The EJC-associated protein SKAR binds the kinase S6K1 following S6K1 activation during mechanistic target-of-rapamycin (mTOR) signaling (Fig. 3). Subsequently, S6K1 phosphorylates the 40S ribosomal subunit protein rpS6, the eukaryotic translation factor eIF4B, as well as the eIF4A inhibitor PDCD4 (leading to PDCD4 degradation). This results in eIF4B recruitment to eIF4A and the enhancement of eIF4A helicase activity, which facilitates 40S ribosomal subunit scanning to the initiation codon (Max et al., 2008, and references therein). However, it is not known whether SKAR-S6K1 interaction is limited to mTOR signaling.

Besides EJC core and peripheral proteins, the mRNP includes other proteins that might also affect its fate. The human SRSF1 (SF2/ASF) protein is a splicing regulator of the SR family, which shuttles with the mRNP to the cytosol. SRSF1 stimulated the translation of reporter mRNAs in human cells, in cell-free translation systems, and upon tethering to a reporter mRNA (Sanford et al., 2004). Although SRSF1 is associated only with CBC- but not eIF4E-bound mRNA, it enhances translation during both the first (CBC-dependent) and steady state (eIF4E-dependent) rounds of translation (Sato et al., 2008). SRSF1 promotes the first round of translation by recruiting the translational activator TAP (Fig. 3). It was indicated that the efficiency of the first round of translation influences subsequent translation (Sato et al., 2008) (see below). It was also shown that SRSF1 enhances eIF4E-dependent translation during mTOR signaling (Michlewski et al., 2008). The activity of the cytoplasmic cap-binding protein, eIF4E, which is important for translation initiation, is antagonized by the eIF4E-binding protein (4E-BP). SRSF1 leads to phosphorylation and inhibition of 4E-BP by recruiting the mTOR kinase, and/or by leading to inhibition of the PP2A phosphatase (Michlewski et al., 2008). Since SRSF1 was not detectable on eIF4E-bound mRNA (Sato et al., 2008), it is not clear whether the later function of SRSF1 is related to its splicing-dependent deposition on the mRNA.

Although EJCs or SRSF1 are not detectable on eIF4E-bound mRNA, the efficiency of the first round of translation influences subsequent steady-state rounds of translation (Lejeune et al., 2002; Sato et al., 2008). This might be due to the fact that during steady-state translation, interaction between eIF4G and the poly(A)-binding protein cytoplasmic 1 (PABPC1) leads to mRNA circularization, which increases the efficiency of ribosome recycling. It is also possible that some of the mRNP proteins displaced during the first round of translation remain in the mRNA microenvironment and contribute to subsequent rounds of translation. It was suggested that after EJC disassembly, MLN51 might remain associated with the mRNA, with which it can interact also in the absence of the EJC (Chazal et al., 2013). Thus, the contribution of introns to translation is not limited to the first round of translation. However, the data presented above indicate that some interactions with the mRNA, including that of MLN51, are more efficient in the presence of assembled EJCs. Considering that MLN51 contributes to the recruitment of translation initiation factors, which have to be accessed during each round of translation, it is possible that the assembled EJCs might provide a surplus benefit to mRNAs with regard to translational efficiency. In this respect, it is relevant to indicate that it was suggested that the enhancement of translational efficiency by introns might increase the likelihood that newly exported mRNAs will be preferentially translated over older transcripts, and that this might be particularly important for signal transduction and regulatory pathways (Gehring et al., 2009b; Moore and Proudfoot, 2009). Interestingly, genes with regulatory roles are particularly enriched with 5′ UTR introns (Cenik et al., 2010). As discussed later in this review, 5′ UTR introns might have a particular contribution to translational efficiency (Akua and Shaul, 2013).

8. The role of splicing in the enhancement of gene expression by introns

There is an ongoing discussion in the IME literature whether splicing itself is essential for the ability of introns to elevate gene expression. This issue is discussed here in light of the above-presented data. Several studies showed that the presence of introns was sufficient to enhance expression even when splicing was prevented (e.g., Damgaard et al., 2008; Kaida et al., 2010; Kwek et al., 2002). These data can be at least partially explained by the splicing-independent functions of U1 snRNA, including enhancement of transcription, suppression of PCPA, and supporting the selection of proper promoter directionality. These functions are facilitated by U1 interaction with 5′ SS-related sequences located at either the 5′ end of introns or at other sites, preferentially enriched on introns rather than exons (Almada et al., 2013; Damgaard et al., 2008; Engreitz et al., 2014; Kaida et al., 2010; Kwek et al., 2002).

To study whether splicing can contribute to expression more than the impacts described above, which depend on the mere presence of intronic sequences, several experimental requisites should be met. It should be demonstrated that the investigated intron does not include transcription factor binding sites, by showing that it cannot enhance expression when localized outside the transcribed region. It should also be verified that there is no cryptic splicing, and that the unspliced intron does not introduce TCs or uORFs, which can impair translation and elicit NMD. The studies described below were selected based on fulfillment of at least part of these requirements.

It was found that in mammalian cells, splicing can elevate mRNA levels and transcription more than the elevation obtained by the mere presence of an intact 5′ SS. Insertion into an intronless construct of a 5′ SS or a full intron enhanced mRNA levels by 2- and 8-fold, respectively, compared with the intronless construct (Damgaard et al., 2008). It was verified that the 5′ SS alone did not mediate cryptic splicing, and that the observations were not due to changes in mRNA stability. It should however be considered that, compared with an isolated 5′ SS, a full-length intron may include additional 5′ SS-related sequences. In plants, derivates of the 5′ UTR intron of the maize *Sh1* gene mediated a 25–44-fold enhancement of expression, but only a 2-fold elevation when their splice sites were mutated (Clancy and Hannah, 2002). Internal AUG codons were eliminated, and the intron did not enhance expression from outside the transcribed region (Clancy and Hannah, 2002; Clancy et al., 1994). An intron of the *Arabidopsis TRP1* (*PAT1*) gene mediated about 5- and 2.5-fold enhancement with spliceable or 5′ SS mutated introns, respectively (Rose, 2002; Rose and Beliakoff, 2000). Potential TCs were eliminated from the retained intron, which was located in the coding sequence, and it was verified that the 5′ SS mutated intron was not spliced. The 5′ UTR intron of the *Arabidopsis AtMHX* gene considerably enhanced gene expression (David-Assael et al., 2006). The native intron, or the intron with both 5′ and 3′ SSs mutated, mediated 270- and 5-fold enhancement of expression, respectively, compared with an intronless construct (Akua et al., 2010). Internal AUG codons were eliminated from both the native and mutated 5′ UTR introns. It was also verified that the mutated intron did not undergo cryptic splicing, and that the intron was unable to enhance expression from outside the transcribed region (when placed 78 or 572 nt upstream the TSS) (Akua et al., 2010). Altogether, these data suggest that in mammalian cells, as well as monocot (maize) and dicot (*Arabidopsis*) plants, splicing is essential for substantial enhancement, but in the absence of splicing, low-level enhancement can be obtained by intronic sequences.

Some of the impact of unspliced introns can be attributed to the above-discussed contribution of 5′ SSs, or, when they are mutated, to 5′

SS related sequences. However, other intronic sequences are apparently also able to mediate this impact. While the full, 416 nt long *AtMHX* intron whose 5′ SS and 3′ SS were mutated mediated 5-fold enhancement of expression, a similar construct with an internal deletion of 320 nt lost its ability to mediate this low-level enhancement (Akua et al., 2010). The deleted internal sequence did not include motifs with considerable similarity to 5′ SSs. Thus, much remains to be studied regarding the contribution of specific intronic sequences to the enhancement of gene expression.

There are several reasons why splicing could enhance mRNA level and/or the efficiency of translation to a higher extent compared with the mere presence of intronic sequences. First, the contribution of introns to nuclear export and translation enhancement was attributed to EJC core and peripheral proteins, as well as splicing regulators of the SR family, which accompany the mRNP to the cytosol (Figs. 2 and 3). The deposition of EJC and SR proteins on the mRNP depends on splicing. In addition, the inhibition of splicing changes the localization of chromatin modifications that may contribute to the selection of correct TSSs (de Almeida et al., 2011). The inhibition of splicing also reduces Pol II CTD phosphorylation on Ser2, which plays a role in the recruitment of cleavage and polyadenylation factors (Koga et al., 2015).

9. The significance of intron's position relative to the sites of transcription and translation initiation

Experiments in which the same intron was inserted in different positions of the same gene indicated that the stimulatory impact of introns on transcription depends on their proximity to the promoter (Furger et al., 2002; Rose, 2004). There are several possible reasons for these findings. It is reasonable that the splicing-independent contribution of U1 snRNA to transcription initiation and selection of proper promoter directionality can benefit from proximity of U1 snRNA recognition sites to TSSs. As illustrated in Fig. 1B, a promoter-proximal 5′ SS can position the interacting factors U1-TFIIH-Pol II in the vicinity of the TSS, thereby supporting transcription reinitiation. When the intron is not close to the promoter, U1-TFIIH-Pol II interaction at the 5′ SS occurs at a greater distance from the TSS, and is, thus, less likely to promote efficient reinitiation at the correct TSS (Fig. 1C). Promoter-proximal introns also position U1 snRNA interaction sites, which are more enriched in introns than exons, near TSSs, thereby facilitating the selection of proper promoter directionality (Almada et al., 2013; Engreitz et al., 2014) (Fig. 1D). Bieberstein et al. (2012) suggested that the special contribution of promoter-proximal introns is due to increased levels of H3K4me3 and H3K9ac chromatin modifications on promoters of genes with short first exons (i.e., with promoter-proximal introns), which result in higher expression levels, and accuracy in the selection of the TSS and transcription directionality.

A study in *Arabidopsis* showed that when the first intron of a reporter gene was moved from its position 52 nt downstream the main TSS to a new position 19 nt downstream this TSS, most accumulated transcripts initiated from a secondary, further upstream TSS (Gallegos and Rose, 2017). This supports the idea that intron position can affect TSS selection. Deleting the 303 proximal nt of the reporter gene promoter, including the two TSSs, resulted in utilization of novel TSSs in normally untranscribed sequences. However, the remaining of some promoter region was essential for transcription initiation, indicating that introns cannot replace promoters (Gallegos and Rose, 2017).

The positive contribution of introns to mRNP export and translational efficiency is partially mediated by the processes illustrated in Figs. 2 and 3. These processes involve interaction of certain factors (ALYREF, or eIF3 and the 43S PIC) with both 5′ cap and EJC-components or binding proteins. Although this interaction can possibly be achieved through secondary structures of the mRNP, proximity of the EJC to the 5′ cap can probably facilitate it. Perhaps some of the contrasting findings regarding the contribution of introns to nuclear export (Valencia et al., 2008, and references therein) resulted from different distances of the intron (and, hence, the EJC) from the 5′ cap, and/or from differences in the secondary structure of the mRNP, which affected the practical distance between these elements. The same rationale may hold true for cases in which different impacts of introns on translation were observed.

There are indications that intron localization in the 5′ UTR could be particularly beneficial for its ability to enhance translation (Akua and Shaul, 2013). An intron with a strong ability to enhance translation (27-fold) when localized in the −67 position relative to the AUG, mediated only a 3-fold enhancement in the +12 position, despite being spliced at a similar high efficiency. This small (82 nt) downstream shift in the position of the investigated introns from the 5′ UTR into the coding sequence resulted in only a minor reduction in their ability to elevate mRNA levels. Thus, the precise localization of an intron in the 5′ UTR, or perhaps in a certain distance from the CAP and/or the AUG, is apparently more important for its ability to enhance translation than for its impact on mRNA levels (Akua and Shaul, 2013). Bioinformatics analysis indicated that the distance between 5′ UTR introns and AUG codons shows a conserved trend in *Arabidopsis*, supporting the idea that 5′ UTR introns may play a role in translation (Chung et al., 2006).

About 30–40% of eukaryotic mRNAs contain uORFs, which, if translated, might impair the translation of the main ORF and lead to NMD (reviewed by Kozak, 2002; Shaul, 2015). Both inhibitory processes can be at least partially prohibited by reinitiation of translation at the AUG of the main ORF (Kozak, 2002; Zhang and Maquat, 1997). The initiation factor eIF3 can increase the efficiency of reinitiation. It was, therefore, suggested that the presence of eIF3 bound to EJCs downstream of uORFs (through its interaction with MLN51) could facilitate reinitiation (Chazal et al., 2013, and references therein). This scenario could also benefit from EJC proximity to the translation initiation site.

10. Why aren't all introns equal?

Perhaps the most mysterious issue concerning intron ability to enhance expression is why different introns, present at the same location of the same genes and spliced at a similar high efficiency, can have very different impacts on expression − from almost no effect to considerable stimulation (Rose, 2002). Although some of this variability could possibly be attributed to different densities of potential U1 snRNA binding sites in introns, it was investigated whether there are other sequence elements that affect intron ability to enhance expression. Since most introns reported to enhance expression were first introns, a bioinformatic approach was used to identify pentamer motifs that are more abundant in first compared with other *Arabidopsis* introns. This resulted in the assignment to introns of an 'IMEter score', which reflects the abundance of the indicated pentamer motifs in individual introns (Parra et al., 2011; Rose et al., 2008); (reviewed by Gallegos and Rose, 2015). The IMEter motifs were found to be redundant and dispersed throughout enhancing introns, although they were more concentrated towards the 5′ end of introns. The IMEter motifs identified in *Arabidopsis* are conserved in the first introns of most plant species (Parra et al., 2011). Interestingly, there was a good correlation between the abundance of the IMEter motifs in introns (as reflected by their IMEter scores) and the ability of different introns to enhance mRNA accumulation in *Arabidopsis* (Gallegos and Rose, 2015; Parra et al., 2011; Rose et al., 2008). The IMEter algorithm identified two sequence motifs (C-GATT and TTNGATYTG) that were candidates for special involvement in the enhancement of mRNA levels in *Arabidopsis*. The impact of these motifs was tested by introducing 11 copies of these motifs into an intron with a small influence on expression. To preserve the length and nucleotide composition of the intron, the motifs were created by rearranging nucleotide order in stretches of pre-existing contiguous nucleotides with the necessary composition. Introducing 11 copies of the first or second motif indicated above into the intron, increased its ability to elevate mRNA levels by 4- and 13-fold, respectively (Parra

et al., 2011; Rose et al., 2016). These motifs do not show similarity to U1 snRNA or known SR protein recognition sites, and are unlikely to function as binding sites for transcription factors. The way by which these motifs increase mRNA content remains a mystery, and mechanisms operating at the DNA level have also been suggested (Rose et al., 2011, 2016; Gallegos and Rose, 2017).

Besides these motifs, deletion analysis identified a short (35 nt) motif in the first intron of the maize *Sh1* gene, which increased expression without altering splicing efficiency, and only when present in the transcribed (but not the promoter) region (Clancy and Hannah, 2002). The vital feature of this motif was U-richness rather than the specific sequence. The influence of this motif was tested at the level of reporter gene activity, thus its relative contribution to the enhancement of mRNA accumulation and/or translational efficiency is unknown.

In vertebrates, first introns include a larger number of evolutionarily conserved blocks compared with downstream introns (Keightley and Gaffney, 2003; Park et al., 2014). This analysis was based on a block size of 100–200 nt. Conservation in first introns had a positive correlation with activating chromatin marks, including H3K4me3, and with the expression level of human genes, as estimated by RNA-seq (Park et al., 2014). The density of transposable elements in long human introns is particularly low in the 5′ portion of first introns, compared with the 3′ portion of first introns and with the 5′ or 3′ portion of the remaining introns. This suggests that the 5′ ends of mammalian first introns are particularly functionally important (Majewski and Ott, 2002). The functional significance of the conserved elements in mammalian first introns still has to be clarified.

It was also identified that there are intronic elements with a special ability to positively or negatively influence translation (Akua and Shaul, 2013). The IMEter scores of several introns tested correlated with their ability to enhance mRNA levels, but not with their influence on translational efficiency. This indicated that the ability to enhance translation is not mediated by the IMEter motifs. Deletion analysis identified in the 416 nt long 5′ UTR intron of the *Arabidopsis AtMHX* gene an internal 118 nt element with a special ability to enhance the efficiency of translation, without affecting splicing (Akua and Shaul, 2013). Insertion of this element into another intron that had a low ability to affect translation resulted in a 19-fold increase in translational efficiency compared with a construct that included the same intron without this element. This effect did not result from the change in the total length of the intron (as demonstrated by other constructs examined) or from a change in splicing efficiency (which was high in all constructs). The ability of this element to enhance translation was, to a large extent, dependent on localization of introns containing it in the 5′ UTR (Akua and Shaul, 2013) (see also above). Thus, 5′ UTR introns might provide preferable platforms over downstream introns for elements that enhance translation. Interestingly, the 5′ UTR intron of *AtMHX* also included a short U-rich (73% U residues) element which, despite having a positive contribution to splicing, lowered the ability of the former element to enhance translation (Akua and Shaul, 2013).

The fact that different introns can have different impacts on translation, and the identification of intronic elements with a special impact on translation, raise the interesting question of whether specific introns, or intronic elements, can differentially affect the deposition, localization and/or composition of mRNP proteins. There is some flexibility in the site of exon junction complex deposition (Mishler et al., 2008; Sauliere et al., 2010, 2012; Singh et al., 2012; reviewed by Muhlemann, 2012). It was found that EJCs are present in most (80%), but not all, EEJs in human cells, and that 40–50% of the EJCs are noncanonical, i.e., are not localized to the canonical positions 24 nt upstream of EEJs. In addition, peripheral EJC proteins can differ under different conditions (reviewed by Bono and Gehring, 2011). The majority of EJC components are recruited and interact with the pre-mRNA prior to exon ligation (Gehring et al., 2009a; Jurica et al., 2002; Makarov et al., 2002; Merz et al., 2007; Reichert et al., 2002; Zhang and Krainer, 2007). Introns themselves play a role in this recruitment − some EJC components are first recruited by intron-associated proteins, and are subsequently translocated to the EJC (Hirose et al., 2006; Ideue et al., 2007). It will be interesting to learn whether specific intronic elements can recruit specific mRNP proteins with a special ability to enhance translational efficiency (or can interfere with such recruitment, in the case of inhibitory elements).

11. Conclusions

When the impact of different introns was tested in the context of different genes, very different impacts on expression were observed, both quantitatively and qualitatively. Different levels of enhancement were observed, and some introns did not affect expression at all, or enhanced expression by influencing only some of the processes reviewed here. For example, some enhancing introns did not affect transcription (Dean et al., 1989; Rose and Beliakoff, 2000; Rose and Last, 1997; Yuan et al., 2013), nuclear export (Nott et al., 2003; Yuan et al., 2013; Zhao and Hamilton, 2007), transcript stability (Nott et al., 2003), or translation efficiency (Rethmeier et al., 1997; Zhao and Hamilton, 2007). In addition, it was shown that the same intron, despite being efficiently spliced, could make very different contributions in the context of different promoters (Akua et al., 2010; Emami et al., 2013; Laxa et al., 2016; Neuberger and Williams, 1988; Yuan et al., 2013), different coding sequences (Rethmeier et al., 1997), or different cells (Yuan et al., 2013).

Considering this variability, as well as the many ways by which introns can affect gene expression, it is very possible that there is no sole, definite answer to the question “how do introns enhance gene expression”. Rather, each intron-gene combination might undergo its own unique mixture of processes that lead to the perceptible outcome. Introns can differ in the strength, density, and position of U1 snRNA binding sites, as well as the presence and position of other intronic elements that positively or negatively affect mRNA content or the efficiency of translation. The presence, location and composition of mRNP proteins, including SR proteins as well as EJC core and peripheral proteins, could vary in different intron-gene combinations. Chromatin modifications might differ in different genes, chromosomal locations, and cells. The position of introns relative to the sites of transcription and translation initiation, which plays such a crucial role, could also vary. Moreover, different transcripts could attain different secondary or higher order structures, depending on their nucleotide sequence as well as the occupation of mRNP proteins, which might also differ in different cells and/or physiological conditions. These structures might, in turn, affect the practical distance between the interacting mRNP elements illustrated in Figs. 1–3, and, hence, the impact on expression.

It is also possible that enhancing introns advance genes towards an optimized expression efficiency, but when the expression is *a priori* close to optimal it cannot be considerably exceeded. This can explain cases in which the same intron elevates the expression of a weak promoter to a much larger extent compared with its impact on a strong promoter (e.g., Akua et al., 2010). Similarly, if the transcript has a 5′ UTR with a strong ability to enhance translation, the impact of mRNP proteins on translation might be secondary, less noticeable.

Thus, perhaps rather than asking, for example, “do introns contribute to nuclear exit”, we should put more emphasis on questions such as “what combination of intron, gene, intron position, chromosomal position, cell type, and physiological conditions favors a contribution of introns to nuclear exit”. The same rationale is valid for other processes by which introns enhance expression. It would be particularly very interesting to gain more knowledge about the contribution of specific intronic elements, besides the canonical splicing-related motifs, to intron ability to elevate mRNA content and translational efficiency.

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