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Review Article

Introns and Their Therapeutic Applications in Biomedical Researches

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Context: Although for a long time, it was thought that intervening sequences (introns) were junk DNA without any function, their critical roles and the underlying molecular mechanisms in genome regulation have only recently come to light. Introns not only carry information for splicing, but they also play many supportive roles in gene regulation at different levels. They are supposed to function as useful tools in various biological processes, particularly in the diagnosis and treatment of diseases. Introns can contribute to numerous biological processes, including gene silencing, gene imprinting, transcription, mRNA metabolism, mRNA nuclear export, mRNA localization, mRNA surveillance, RNA editing, NMD, translation, protein stability, ribosome biogenesis, cell growth, embryonic development, apoptosis, molecular evolution, genome expansion, and proteome diversity through various mechanisms.

Evidence Acquisition: In order to fulfill the objectives of this study, the following databases were searched: Medline, Scopus, Web of Science, EBSCO, Open Access Journals, and Google Scholar. Only articles published in English were included.

Results & Conclusions: The intervening sequences of eukaryotic genes have critical functions in genome regulation, as well as in molecular evolution. Here, we summarize recent advances in our understanding of how introns influence genome regulation, as well as their effects on molecular evolution. Moreover, therapeutic strategies based on intron sequences are discussed. According to the obtained results, a thorough understanding of intron functional mechanisms could lead to new opportunities in disease diagnosis and therapies, as well as in biotechnology applications.

Keywords: Gene regulation, Intron, Junk DNA, Molecular evolution, Therapeutic application.

1. Background

The first use of DNA sequencing in the late 1970s revealed that genes in eukaryotic cells are discontinues, i.e. they have an intron /exon structure (1). More DNA sequencing has revealed that introns make up the majority of sequences in most eukaryotic genes. However, it has also been demonstrated that these regions are removed after transcription, leading to the conclusion that they are unnecessary parts of DNA (2, 3). These features raise some questions: if introns are considered junk, what is the reason for their existence and why haven't they been eliminated through evolution? Moreover, it is known that eukaryotic cells expend significant amounts of energy to enhance and

maintain intron sequences. As a result, a debate about the function of introns emerged shortly after their discovery (1, 4) . So, the early functions related to introns include the simplified development of unique genes and the possibility for the expression of various proteins from a single gene (5). Additionally, introns play a critical role in protecting coding regions in eukaryotic genomes from frequent errors (6). Gradually, more experimental analyses revealed that introns or their derived elements have significant effects on gene expression in a wide range of organisms, including insects, nematodes, and mammals (7). However, their exact mechanisms are still not fully understood. For example, several reports, have demonstrated that certain introns are crucial for the accumulation of specific mRNAs such as rat growth hormone and growth hormone receptor (8, 9), triosephosphate isomerase (10), and human β-globin (11). Moreover, evidence in plants shows that introns act post-transcriptionally to enhance mRNA processing, possibly, by facilitating mRNA maturation or increasing the stability of nascent transcripts (12, 13). These reports indicate that introns and splicing are necessary for mRNA to be transported from the nucleus. However, analyzing the structure of certain genes, such as histones and olfactory receptors, in higher vertebrates has shown that the presence of introns and splicing is not necessary for the functioning of all genes (1). On the other hand, the absence of introns in some genes can result in both reduction and repression of gene expression (14, 15), but it does not have any effect on other genes (1).

Nevertheless, while accepting that gene expression is affected by certain intron sequences, there is still a debate about the mechanisms behind this that needs to be elucidated In addition, analyzing the structure of genes has revealed that many of them contain 10-50 introns (1, 16). Consequently, this raises further questions, such as whether all introns of a gene have an impact on gene regulation. Do they act with the same mechanism? Meanwhile, which intron has a greater impact on the regulation of the corresponding gene? To answer these questions, many investigations have been conducted on factors affecting intron activities. These investigations have found that intron sequence context (ISC) and intron position (17-22) play a significant role. In addition, it has become increasingly clear that intron excision by the spliceosome can affect multiple steps of mRNA metabolism. These steps include primary transcription of the gene, correction and polyadenylation of the pre-mRNA, nuclear export, translation, and degradation of the mRNA product (5, 23, 24). Exon and intron sequence context not only play critical roles in gene regulation but also have essential functions in molecular evolution (1, 23, 25). In this review, we present a brief and up-to-date overview of how introns exert their effects on eukaryotic gene regulation and molecular evolution. Additionally, their application in diagnosis and treatment will be discussed.

2. Splicing and Splicing Control Elements

Precise removal of introns by splicing (**Fig. 1**) before mRNA maturation is a crucial and universal step in higher eukaryotic genes. This process yields suitable mRNAs for gene expression (26, 27). Although the accuracy and complexity of intron removal still amazes even 30 years after the discovery of introns (28), it has been determined that intron excision and splicing includes several step by-step assembly and catalytic processes. These processes comprise exon and intron diagnosis, intron cutting, and exon joining (29, 30), which are controlled by splicing control elements: 1. Classical cis-controlling elements, are weakly conserved intronic cis-elements, which are essential for defining the exon boundaries, including GU and AG dinucleotides at the exon–intron and intron–exon junctions, respectively (5'- and 3'-splice locations), an A nucleotide at the branch point and a polypyrimidine tract (Py)n with variable length upstream of the 3'-splice site. The branch point is usually placed 18–40 nucleotides upstream from the polypyrimidine tract. 2. Basal splicing machinery is an enormous complex macromolecule that is composed of as many as 300 distinct proteins acting as RNA recognition motifs (RRMs) or ATPases and five RNAs (snRNAs) (U1, U2, U4, U5 and U6) which its components bind to classical cis-controlling elements and stimulate gathering of the splicing complex. 3. Cis auxiliary elements are exonic and intronic cis-elements, which are generally necessary for effectual splicing of constitutive and alternative splicing. 4. Trans auxiliary elements can interact with enhancers and silencers (31-34). These elements interact with the basal splicing machinery, specifically the spliceosome complex (**Fig. 1**), which acts as a trans-acting element (32, 35). However, auxiliary elements are additional information that act as auxiliary cis-acting elements, such as exonic/intronic splicing enhancers/silencers (ESE/S, ISE/S), and auxiliary trans-acting factors, such as serine arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs).

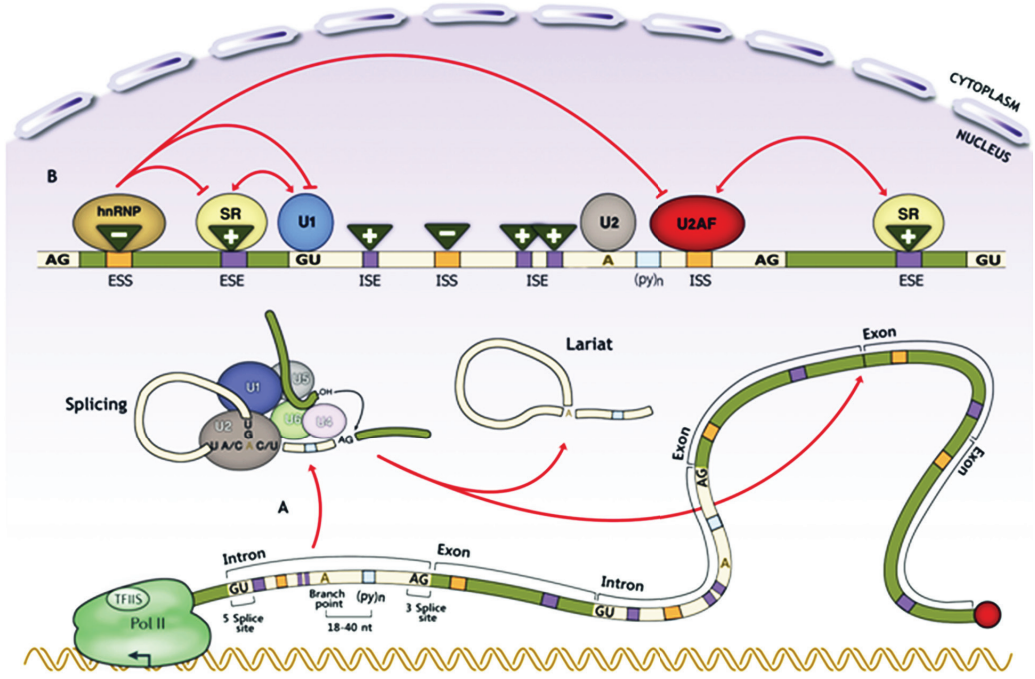

Figure 1. Splicing and controlling splicing elements. A) Schematic representation of splicing which is performed as a co-transcriptional process. **B)** Splicing control elements. Classical cis-regulatory elements are indicated by blue letters and boxes. ESE: exon splicing enhancer; ESS: exon splicing silencer; ISS: intron splicing enhancer; ISS: intron splicing silencer; SR: serine arginine; hnRNPs: heterogeneous nuclear ribonucleoprotein particles.

Overall, SR family binding at ESE and ISE simplifies exon recognition although hnRNPs are inhibitory. These elements contribute to accurate splicing by aiding in the identification of suitable splice sites or by suppressing or increasing the usage of certain splice sites, particularly in alternative splicing (31, 32) (36,37).

2.1. Effects of Splicing Control Elements (SCEs) On Gene Regulation

Regarding, the contribution of SCEs in the regulation of gene expression, it has been determined that various genetic diseases such as hereditary breast and ovarian cancer (caused by the c.4185 + 4105C > T variant in BRCA1, the first reported deep intronic variant in this gene that activates a pre-existing cryptic donor site) and Ataxia-telangiectasia disease (due to the c.2839-581_2839-578del variant in the ATM gene, which creates an ISE), can be resulted from SCEs malfunctioning (23) (38-40). Among spliceosome-associated catalytic entities (SCEs), the spliceosome plays a crucial role in splicing and gene expression regulation, particularly at the transcriptional level (14, 41). Additionally, in addition to its role in RNA processing, the components of the spliceosome complex interact with the TFIIH subunit of RNA polymerase II. This subunit regulates various stages of transcription, both *in vivo* and *in vitro,* including abortive initiation, re-initiation, promoter escape, and early elongation (**Table 1**) (42-47). Moreover, it has been demonstrated that some SCEs can also affect the mRNA nuclear export (**Table 1**) (48-50).

2.2. Splicing Effects on the Gene Expression Regulation

In addition to direct effects of SCEs on gene regulation, it has been demonstrated that SCEs also play a role during the splicing process. During splicing, a protein compound called the Exon Junction Complex (EJC), which consists of a stable heterotetramer core and other factors, is placed 20 to 24 nucleotides upstream of each exon-exon junction by the splicing machinery (**Fig. 2**) (51-54). This complex plays a crucial role in various processes related to mRNA, including its stability, survival, transport, and translation (23, 55-57).

Table 1. Possible mechanisms by which intron can regulate expression of

Pathways	Regulatory elements	Activities	Mechanisms	Regulation levels	Sample of biological processes	Reference
SCEs	U1	Transcription	Interaction with TFIIH	Transcription and Post transcription	Embryonic development, Cell growth, Apoptosis	(31, 32, 41, 45, 179)
		Splicing	Interaction with SCEs			
	Other elements	Splicing				
		alternative splicing				
		mRNA nuclear transport	Interaction with nuclear transport			
EJCs	Upf2, Upf3 Y14, RNPS1, Magoh, eIF4AIII, MLN51 RNPS1, ACINUS	NMD	Distinction between PTC and NTC	Translation	mRNA quality control	(49, 53, 60, 67, 138, 180, 181)
	Y14; Magoh	mRNA localization	Imprints some of the information	Post transcription	Regulation of sub-cytoplasmic regions	
	Yap56; Aly; REF SRm160 Tap-p15; PININ	mRNA nuclear transport	Splicing-coupled mechanisms	Post transcription	Overcome the nuclear retention	
IDREs	SnoRNAs and scaRNAs	rRNA modifications	Guide for nucleoside modifications	Translation	Ribosome biogenesis	(66, 81, 85, 182)
		snRNA modifications		Post transcription	Splicing	
		tRNA modifications		Transcription	Translation	
		mRNA modifications		Post transcription	mRNA transport	
		Orphan	?	?	?	
	MiRNAs	Cleared undesired mRNA	Inhibition of transcription	Transcription	Cell transitions quickly Developmental timing Apoptosis and tissue growth	(68, 69, 76)
			Suppression of translation	Translation		
			?	?		
	nmRNAs	Regulate HLTFG genes	As activator	Transcription	Developmental stage	(88, 90, 91, 111)
	TFs	Transcription Factor	Contribute in transcription	Transcription	Numerous	(41, 183)
	IREs	Providing regulatory motifs	Transposition	Transcription	Molecular evolution RNA editing Splicing and apoptosis Cell growth and differentiation	(41, 98, 126, 171)
				Post transcription		
				Translation		
				?		
ISCS	PAs	Contribution to polyadenylation	Polyadenylation coupled mechanisms	Post transcription	mRNA Transport mRNA Survival mRNA stability	(18, 41, 184)
				Translation		
				Post translation		
	TFBs	Providing sites for TFs	Contribute in transcription	Transcription	Numerous	(23, 41, 183)
	CpG island	Contribution to transcription	Transcription	Transcription	Numerous	(23, 41, 183)
	Enhancer	RNA polymerase assembly	Increase transcription	Transcription	Numerous	(23, 41, 183)
	Promoter	RNA polymerase assembly	Transcription	Transcription	Numerous	(23, 41, 183)
	GR	Targets for gene regulation	Contribute in transcription Contribute in polyadenylation	Transcription	Gene expression, Genome stability, Evolution	(20, 95, 108, 109)
				Post transcription		
				Translation		

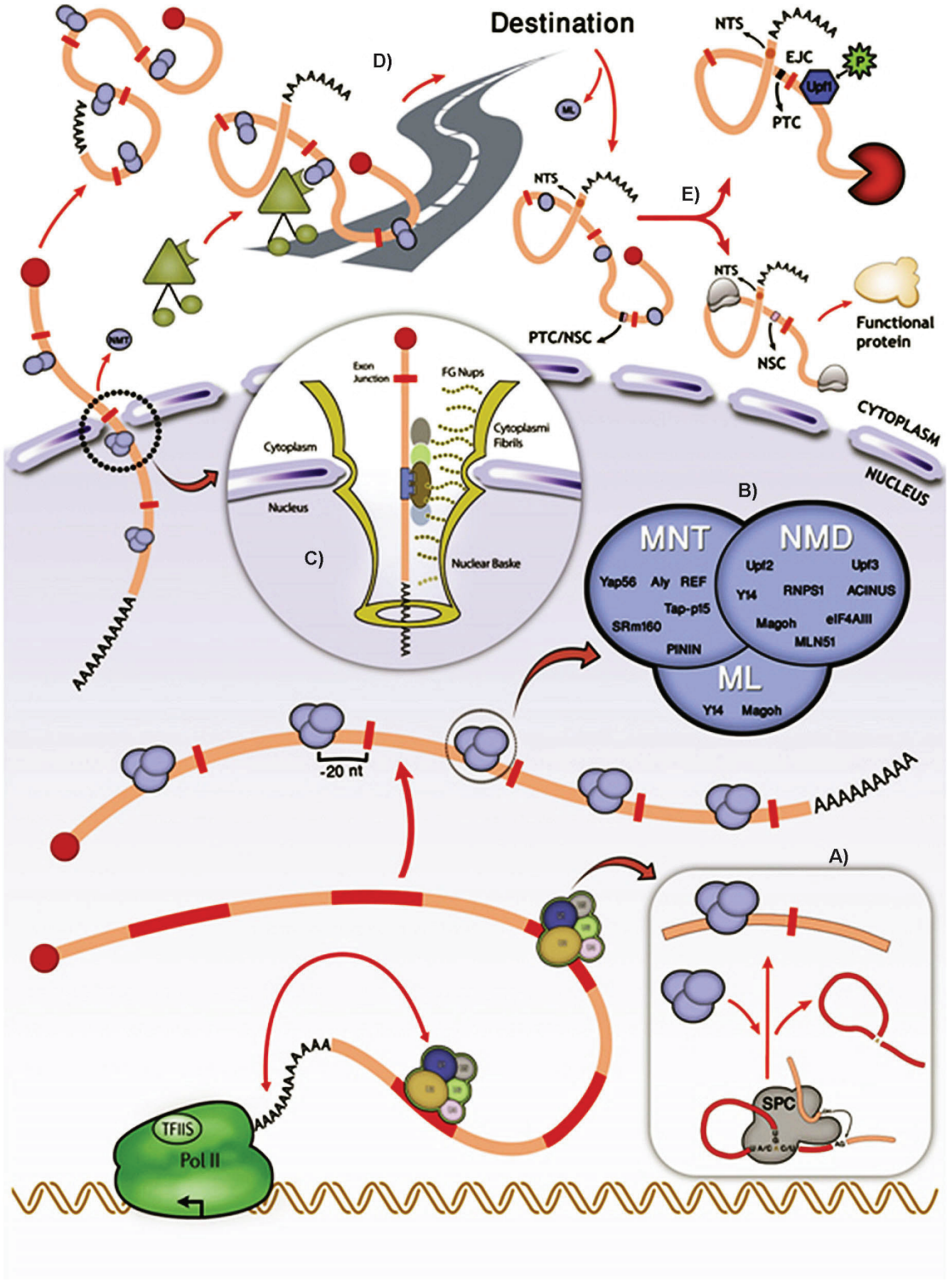

Figure 2. Formation, components and effects of exon junction complexes. A) EJC formation. **B)** EJC composition. **C)** A model of mRNA nuclear transport. **D)** Motor-based active mRNA localization. **E)** Translation or NMD. PTCs are typically followed by minimum one EJC (curved arrows in **E**). Motor protein needed in mRNA nuclear transport is indicated by green triangle. MNT: mRNA nucleolar transport; ML: mRNA localization; NMD: nonsense mediated mRNA decay.

It is involved in post-transcriptional processes such as mRNA nuclear transport and translational regulation. The complex is formed through a splicing-coupled model, where components of EJC bind to mRNA and nuclear pore elements. EJCs are made of three types of proteins based on their functions, including MNT, ML and NMD proteins, which are involved in mRNA nucleolar transport, mRNA localization and nonsense mediated mRNA decay, respectively. These proteins based on their function have different domains such as RNA-binding domain (RBD), nuclear transport domain (NTD) and mRNA localization domain (MLD) and after doing their function, they dissociate from EJC (**Fig. 2**) (49, 52, 58-60). Additionally, it is responsible for mRNA localization using imprinting information, which is necessary for proper cytoplasmic localization. Splicing-coupled model, mRNA transport using the channel needs binding of the mRNA to solvable transport receptors, which include MNT proteins of the EJC in this model. These receptors mediate transport in an energy independent manner by interacting with the phenylalanine-glycine repeats, a group of nucleoporins that line the channel of the nuclear pore complex, and detach as of mRNP after transfer to the cytoplasm and other components of EJCs, which are involved in ML and NMD remain attached to the mRNP. For additional details, refer to **Table 1**. Active transport of mRNAs from the nucleus to peripheral cytoplasmic target positions, needs ML factors, which act as a memory and imprint through splicing. In this model, mRNP particles movement along cytoskeletal filaments is associated with a motor protein, most probable with adaptor proteins, and is transported to the aim locate as dormant. Finally, at destination sites the ML factors and other RNPs such as those that repress translation during transport are released from the mRNA and are fastened to the select locate using particular proteins and recruit ribosomes and other translational components (**Fig. 2**) (23, 61-63). The complex also participates in nonsense-mediated mRNA decay (NMD), mRNA quality control, and translation by distinguishing between premature and normal termination stop codons (PTC and NTC) (**Fig. 2D**) (23, 53, 64-67).

2.3. Intronic-Derived Regulatory Elements

Major effects of the introns on gene regulation and molecular evolution, whether direct or indirect, are associated with regulatory elements that arise from intronic sequences. These elements are involved in the regulation of transcription or post-transcriptional processes. MicroRNAs (miRNAs) act as trans-vehicles in gene regulation, preventing transcription and/or repressing translation (68-72). They can be derived from intronic regions. These elements, which are diverse in structure and function, are widely distributed and abundant in eukaryotic cells. They are divided into four classes based on their origins, including intergenic (In), intronic (Id), palindromic (P), and exonic (E). Id-miRNAs are the only class of miRNAs that are derived from introns (68, 69, 73-75). Id-miRNAs are transcribed by the Pol-II promoters of the encoding genes and are co-expressed in the intron areas of the gene transcriptions (pre-miRNA). After RNA splicing and additional treating, the spliced intron might function as a pri-miRNA for Id-miRNA generation. In the nucleus, the pri-miRNA is excised by Drosha RNase to create a hairpin-like pre-miRNA pattern, which is later transferred to the cytoplasm for more treating by Dicer to make mature and active miRNA (**Fig. 3B**) (76). The Id-miRNAs which can be considered gene expression products, have the ability to interfere with the expression of other genes (76, 77). Hence, it has been suggested that these elements are capable of rapidly triggering cell transitions in response to external stimuli without the need for time-consuming protein synthesis (69). They also play critical roles in various biological functions, including developmental timing, apoptosis, and tissue development (76, 78, 79). Moreover, small nucleolar RNAs (snoRNAs) are another type of intronic-derived regulatory elements (IDREs) that originate only from intronic sequences. They are located in the nucleoli and act as cofactors in ribosome biogenesis (**Fig. 3C**) (66, 80-83). Meanwhile, the roles of snoRNAs are not restricted to ribosome synthesis. They are also used as guides for modifying other cellular RNAs, including snRNAs, tRNAs, and mRNAs (66, 81, 84). Nonetheless, the functions of a large number of these "orphan" snoRNAs remain unknown, and there are likely even more snoRNAs waiting to be discovered. In wholly stated cases, vertebrate snoRNAs initiate from introns of either protein coding/noncoding RNA polymerase II-transcribed genes. Here the pathways of snoRNA derivation from protein-coding gene are demonstrated. The main pathway is similar to id-miRNA biogenesis, whereby after splicing lariat undergoes several processing including lariat debranching, 5′ and

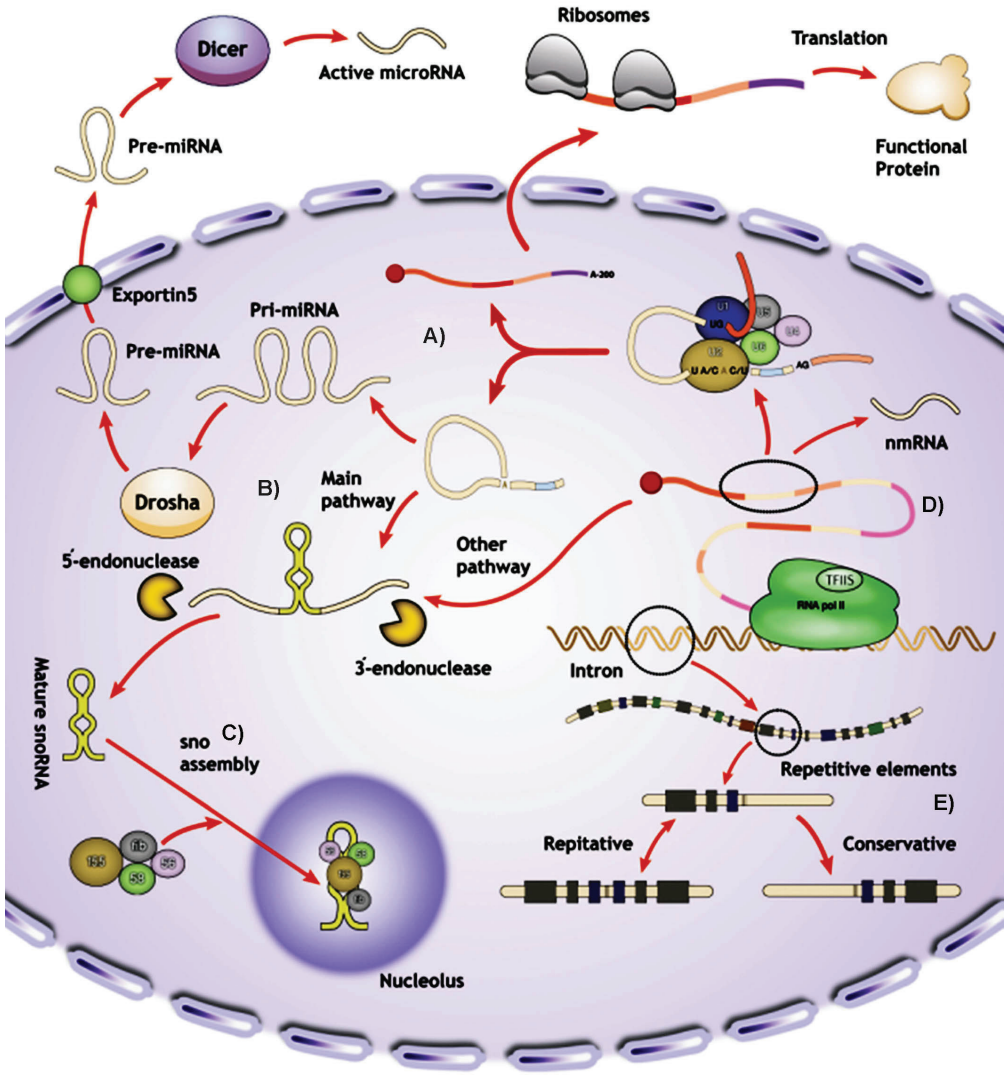

Figure 3. Schematic diagram of biogenesis of the intronic derived regulatory elements. A) Transcription and splicing processes. **B)** Biogenesis of Id-miRNA. **C)** snoRNA synthesis. **D)** nmRNA biogenesis. **E)** Intronic repetitive elements. The effects of introns on gene regulation and molecular evolution might be the result of intronic repetitive elements (IREs) by providing regulatory motifs throughout mobilization as replicative and conservative forms.

3′ exonucleation olising; at continue mature snoRNA assembly by accessory proteins and then transported from the nucleoplasm into the nucleolus as snoRNP complex. In the other pathway snoRNA is produced by endonucleolytic processing as directly from pre-mRNA (66, 85). Nuclear messenger RNAs (nmRNAs), a group of small non-coding RNAs (sncRNAs), are involved in the developmental program by regulating the expression of high-level transcription factor genes (HLTFGs) such as Hox genes, as well as some other protein-coding genes. These nmRNAs, along with certain types of IDREs, function similarly to miRNAs and snoRNAs (86-92). Interestingly, the introns of these genes may represent elements of the generation-specific control keys (GSCK) that act as non-coding mRNA (**Fig. 3D**) (91). Other types of sncRNAs, such as small cajal body-specific RNAs (scaRNAs), also originated from introns (93). Perhaps many other types of these IDREs remain to be discovered. For additional details, refer to **Table 1.**

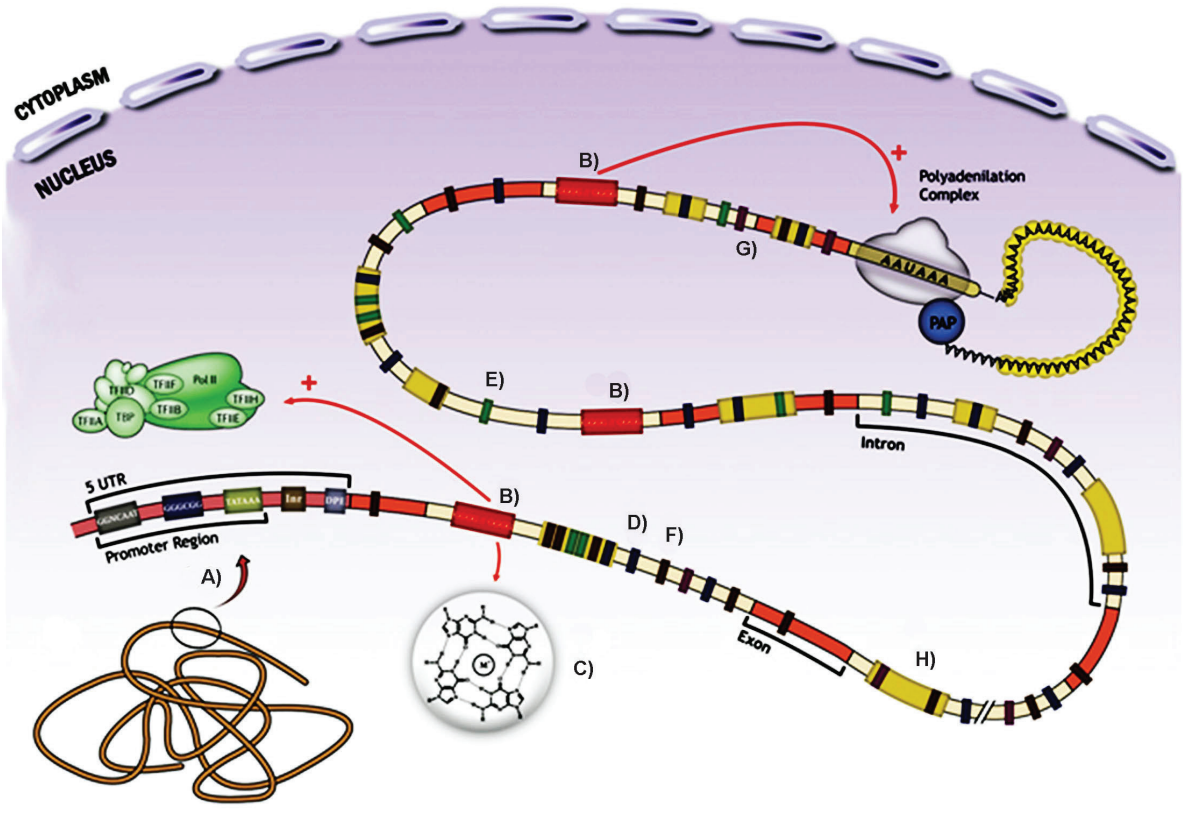

Figure 4. Sequence context and structure of the introns. A) Schematic diagram of the complexity of the genome and a model of eukaryotic gene structure. **B)** G-rich regions (red box). **C)** Schematic diagram of a G-quadruplex with four guanines arranged around a central monovalent cation. **D)** Intronic and exonic promoter (blue line), these structures with variant LDF can be distributed as random throughout a gene including intron and exons, nonetheless some of them can have significant distribution in correlation with other regulatory motifs including CpG islands (green lines, **E**), TFBs (brown line, **F**), splicing and polyadenylation sites (violet lines, **G**) and REs (yellow area, **H**).

2.4. Sequence Context and Structure of the Introns asRegulatory Elements

Major sections of the intronic regions of the eukaryotic genome are tightly regulated, which can be attributed to the presence of regulatory elements in these regions (94). In this context, changes in certain sequences, including the intron regions, may result in biological malfunction (68, 69). Apart from trans-acting regulatory elements derived from introns, many regulatory functions are attributed to the intron sequence context and structures (ISCSs), which are thought to function as cis-acting regulatory elements (20, 95). The intron sequence context can contribute to gene regulation through various stages of mRNA-related processes. It provides regulatory elements such as promoter- and enhancer-like structures (**Fig. 4 D**), transcription factor binding sites (**Fig. 4E**), CpG islands (**Fig. 4F**) as well as splicing and polyadenylation sites (14, 41, 44, 96-99).

While intron remains mainly in the double-stranded (Watson-Crick base pair) form, it can also form other complex structures, such as wobble base pairs, Hoogsteen triplexes, and G-quadruplexes. These structures are distributed non-randomly and may contribute to diverse biological activities during gene expression, as well as in genome stability and evolution (20, 95, 100-102). Nonetheless, very little is known about how their non-randomness contributes to biological functions. In the middle of the most interesting non-random sequences of the genome are G-rich sections (**Fig. 4B**), which have the potential to

form G-quadruplex DNA or G-tetraplex DNA (**Fig. 4C**) (20, 103-107). G-quadruplex DNA provides targets for the regulation of gene expression by binding agents that are crucial for transcription or splicing (20). These structures are distributed throughout both intra- and inter-regions of genes, with a positional bias towards the 5'- and 3'-ends of the gene. This bias suggests a specific function, particularly in the initiation and termination of gene transcription, respectively (20, 95). In humanoid genomics, the number of locations with potential for the formation of G-quadruplex structures is estimated to exceed 300,000 (108, 109). Some of the potential G-quadruplex regions are located within introns and are correlated with the function of the corresponding protein. In particular, proto-oncogenes are rich in G nucleotides, while tumor suppressor genes have a lower frequency of G-runs compared to the genomic average (20). Promoter regions are also G-rich areas (110-113). Interestingly, positional biases of G-rich areas at the 5'-ends of the first introns of genes from frogs to humans have been established. These biases may provide structural targets for regulating gene expression at the transcription or RNA processing levels (20, 100-102). In our previous research, we have provided evidence supporting the existence of promoter- and enhancer-like structures near CpG islands in the first intron of the human factor VIII gene (114).

3. Introns and Gene Regulation Pathways

In general, introns and their associated processes can modulate any step of gene regulation. However, transcription, post-transcriptional modification, translation, and post-translational modifications are the main steps in gene expression where gene regulation could occur. These steps can be directly and/or indirectly influenced by introns (41, 45, 115, 116). Although many questions regarding the regulatory mechanisms and their related effector elements have been answered so far, a large number of them remain unknown and await discovery. In **Table 1**, a list of different gene regulation pathways is presented, in which either introns or intronic elements are involved. Some of the biological processes controlled by these pathways and elements are indicated.

3.1. Effects of Introns on Proteome Expansion

In higher eukaryotes, comparative analysis between genes and their corresponding expressed sequence forms indicates a highly intricate process of gene regulation. This process leads to the expression of different proteins with various and sometimes even antagonistic functions from a single gene (23, 117-121). Alternative splicing, trans-splicing, and RNA editing are three major processes responsible for proteome complexity. These processes are modulated by cell type, developmental phase, gender, and/or in response to external stimuli (32, 118, 119, 122-125). So, introns, which can be involved in alternative splicing, trans-splicing, and RNA editing, play significant roles in proteome complexity by affecting transcription, mRNA localization, mRNA stability, and translation (23, 120, 126, 127). Moreover, RNA editing and splicing can also be influenced by intronic repetitive elements (IREs), which can result in transcriptome expansion (126, 128, 129). Altogether IREs influence transcriptional, post-transcriptional, and translational levels (98, 126, 130) by providing regulatory motifs throughout the process of mobilization (126, 131).

3.2. The Effects of Introns on Molecular Evolution

Current advances in genomics, including whole-genome sequencing, high-throughput protein characterization, and bioinformatics, have led to a significant improvement in studies on molecular evolution. The evolution (birth, death, and maintenance) of introns in eukaryotic species is a highly debated topic with many unresolved questions. In an interesting study, Li *et al*. unravel some of the key aspects of intron evolution. They studied a species of microcrustaceans, Daphnia pulex, which has been shown to exhibit high intron dynamics. They sequenced and assembled nine complete individual genomes from distinct natural populations. Using a dedicated bioinformatics pipeline, they identified 90 recently gained introns. They reported contrasting conclusions compared to previous studies based on more ancient intron gains. In the species under study, they found that (i) intron gains are rather common and serve as an important source of genetic variation, (ii) intron gains are more frequent than intron losses, and (iii) parallel intron gains occur frequently. The authors reported that intron gains are, on average, slightly deleterious. All intron gains reported in this study result from the repair of double-strand breaks (DSBs), either a single blunt DSB or a single staggered DSB. The authors demonstrated that these mechanisms can

lead to multiple rounds of double-strand break (DSB) repair, which can increase the length and complexity of introns. They also proposed the hypothesis that certain intron sequences may arise from the de novo synthesis of DNA caused by DNA polymerase errors (132).

Here, we emphasize the central role of introns in evolutionary processes. We argue that the effects of introns, specifically through IDREs (Intron-Derived Regulatory Elements) and ISCSs (Intron Splicing Control Sequences), could generate different sets of RNAs. These RNAs are then subject to natural selection based on the phenotypes they produce. IREs, as a type of IDREs and ISCSs (98, 133, 134), have critical roles in molecular evolution. They influence transcription, post-transcription, and translational levels of gene regulation by providing regulatory motifs throughout mobilization (98, 126). Moreover, RNA editing and splicing, which lead to transcriptome expansion, can be influenced by IREs (126, 128, 135). The effects of introns on molecular evolution are not restricted to IREs; they are also influenced by RNA editing-based introns, which serve as a guide for modification (5, 136). On the other hand, since intronic sequences are more variable than exons, these changes lead to the formation of new double-stranded RNA (dsRNA) editing substrates. Due to alternative splicing and RNA editing, the amino acid sequence of proteins can be altered, resulting in the creation of new phenotypes that may undergo natural selection. Thus, RNA editing based on introns plays an important role in molecular evolution (5). Besides ontogeny, which refers to the development of a zygote into a complex multi-cellular organism, the process can also be seen as a miniature of evolution and is influenced by introns (91). In this regard, although alternative splicing plays critical roles in developmental stages (120, 137), recent reports have demonstrated that intron-derived regulatory elements, such as non-messenger RNAs (nmRNAs), also contribute to this process (91).

3.3. The Effects of Introns on Disease Development, Diagnosis, and Healing

Due to the assumed effects on gene regulation and its role in biological processes, any changes in introns and related elements may result in hereditary diseases. In this regard, several disorders, such as myotonic dystrophy, fragile X mental retardation, and dominant β-thalassemia, are caused by dysregulation of Id-miRNAs and NMD, respectively (51, 69, 138). So, this association can be used for both diagnostic and therapeutic purposes. In this regard, alternative splicing patterns of genes have been suggested as tools for diagnosis and treatment (139, 140). The splicing design of a gene can be altered by both extracellular stimuli and mutations in splicing control elements (32, 139, 141). In contrast to extracellular stimuli such as hormones, immune response, neuronal depolarization, and cellular stress, which typically have temporary effects during normal development, splicing mutations often lead to hereditary diseases (119, 120, 142). Moreover, it has been shown that these mutations might be linked to cancer (119, 143-145). In this regard, the alternative splicing of genes involved in apoptosis, angiogenesis, adhesion and metastasis, invasion, propagation, and hormone signaling is now well-recognized in cancer (119, 144, 146, 147). For example, the CD44 gene comprises 10 variable exons (v1–v10) and expresses a family of hundreds of diverse CD44 isoforms. These isoforms are cell-surface glycoproteins that function in cell adhesion, migration, and matrix interactions through alternative splicing. This includes both standard and variable forms (139, 148). Although the standard isoform of CD44 is predominant in healthy human adult tissues, variable isoforms are expressed in some tissues during development and T-cell activation (139, 148). Meanwhile, a group of variable isoforms of CD44 with metastatic potential are expressed in numerous human malignancies (60, 120, 123, 139). Moreover, several genes involved in apoptosis, such as Bcl-x and caspase 9, can express antagonistic pro- and anti-apoptotic isoforms, as identified by alternative splicing (32, 149-151). Consequently, the patterns of alternative splicing of certain genes can provide a tool for determining cellular states, including developmental stages and disease (152). Based on this information, novel therapeutic strategies for correcting or circumventing splicing abnormalities are now emerging. These methodologies include over-expression of proteins that alter the splicing of the affected exons (153-155), the use of oligonucleotides to obstruct the usage of improper splice sites and force the use of functional splice sites (139, 156-159), the use of compounds that influence the phosphorylation of splicing agents such as hnRNPs and SR proteins (139, 160, 161), or stabilize putative secondary structures (162), and a trans-splicing method to substitute mutated exons with wild-type exons (124, 125, 163).

3.4. Intron Applications in Gene and Protein Therapy

Two main approaches for managing genetic disorders are replacement therapy with recombinant proteins and gene therapy. To generate an effective protein, an efficient expression vector with appropriate regulatory factors is necessary for both systems (41). Based on the effects of introns and splicing processes on gene regulation, utilizing introns could be a valuable approach for modifying expression vectors with appropriate regulatory elements. In this regard, it has been stated that some mRNAs transcribed from cDNA failed to exit the nucleus and hence did not produce a protein. However, the same mRNAs expressed from intron-containing constructs were able to enter the cytoplasm and be effectively translated, even up to 500-fold (18, 41, 114, 164, 165).In our previous study, we introduced hBG intron-II in the second intronic position of the hFIXcDNA and observed an increase in the expression level compared to that of the intron-less hFIX-cDNA *in vitro* (41). Nonetheless, each regulator of spliceosome catalytic elements (SCEs), exon junction complexes (EJCs), intron-defined regulatory elements (IDREs), and intron splicing control sequences (ISCSs) has a specific function that modulates gene expression at different levels (**Table 1**). Therefore, harnessing this potential relies on having precise knowledge about introns and their regulatory elements, as well as understanding their functions. To achieve efficient expression, particularly in protein therapy, it is important to consider the use of either a truncated intron in a construct to recruit ISCSs (such as a promoter and enhancer), or a full-length intron in the appropriate position to facilitate processes involving SCEs, EJC, and IDREs. Alternatively, a strategy that combines both of these methods should be considered.

The main challenges in gene therapy are safety and efficiency (166-168). Therefore, both self-regulatory elements and potential regulatory elements derived from the introns are considered safe tools to address these issues (2, 169, 170). It has been revealed that the transposition of the L1 factor results in the activation of the p53-mediated apoptotic pathway in human cancer cells that have a wild-type p53 (171). So, IREs, especially L1 as a transgene, could act as a new approach for cancer therapy by stimulating apoptosis (171). Moreover, RNA reprogramming is an alternative method for gene therapy that involves correcting the sequence of specific transcripts through spliceosome-mediated RNA trans-splicing (SmaRT). This approach is applicable to genetic disorders caused by mutations in large genes or dominant-negative mutations (125, 172-178).

4. Conclusions and Perspective

In summary, the intervening sequences of eukaryotic genes have critical functions in genomic regulation, and molecular evolution. In this regard, this paper briefly reviewed various known and potential functions of the intervening sequences. Based on various known and possible functions of the intervening sequences, as reviewed in this paper, introns not only carry information for splicing, but they also play many supportive roles in gene regulation at different levels. These roles are believed to function as useful tools in applications for various biological processes, particularly in the diagnosis and treatment of diseases. The effects of introns are related to splicing, SCEs (spliceosome catalytic elements), EJC (exon junction complex), IDREs (intron-derived regulatory elements), and ISCSs (intron splicing control sequences). Mutations in these elements that disrupt their functions play a significant role in disease development. Introns can contribute to numerous biological processes, including gene silencing, gene imprinting, transcription, mRNA metabolism, mRNA nuclear export, mRNA localization, mRNA surveillance, RNA editing, NMD, translation, protein stability, ribosome biogenesis, cell growth, embryonic development, apoptosis, molecular evolution, genome expansion, and proteome diversity through various mechanisms. Although the exact molecular mechanisms of some of the mentioned effects are determined, more basic research in this area is required. A major challenge is to unambiguously define the exact roles of each intron of a gene and their mechanisms on gene regulation. In general, having precise knowledge about these mechanisms may open new avenues for diagnosis and therapies against diseases, and it would also be advantageous in biotechnology applications.

Ethics Approval and Consent to Participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no

datasets were generated during the current study.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

A. H-M., M. M., and ME. TY. conceived of the existing idea, document extraction, and analysis. N.G-F. and A.B. draw the shapes and developed the contents. A. Z., M. Q., M. MM., and N. S. authors contributed to the final version of the manuscript. A. H-M. supervised the study. All authors discussed the consequences and contributed to the final manuscript.

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