

Alternative Splicing and Nonsense Mediated Decay in Mitochondrial Complex-I Biogenesis and its Implication in Human Diseases

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Abstract

In mammals, complex-I (NADH-ubiquinone oxidoreductase) of the mitochondrial respiratory chain has 31 supernumerary subunits in addition to the 14 conserved from prokaryotes to humans. Multiplicity of structural protein components, as well as of biogenesis factors, make complex-I a sensible pace-maker of mitochondrial respiration. The work reviewed here shows that the Alternative Splicing and Nonsense Mediated Decay pathways regulate the transcription products of different nuclear genes encoding subunits of complex I. Complex-I dysfunction has been found to be associated with several human diseases. Involvement of altered pattern of transcription products of complex-I genes in pathogenetic mechanisms of these diseases is examined.

Introduction

Complex-I is the first enzyme of the mitochondrial respiratory chain. The enzyme catalyzes the transfer of electrons from NADH to ubiquinone [1] in the inner mitochondrial membrane and conserves the free energy, so made available, as a transmembrane electrochemical proton gradient ($\Delta\mu\text{H}^+$) which is utilized to make ATP from ADP in the mitochondrial process of oxidative phosphorylation (OXPHOS) [2]. In human cells the ATP produced by OXPHOS covers, under physiological conditions, more than 80% of the ATP that cells need. This requirement is particularly stringent in organs like the human brain. Mammalian complex-I is made up of 38 subunits encoded by nuclear genes and 7 subunits encoded by mitochondrial genes [3,4]. With so many genes involved, one might expect that complex cellular processes are involved in the control of complex-I biogenesis and function whose understanding is of interest given the fundamental role played by the complex in the control of cellular physiopathologic events like cell growth and death, aging, and pathogenesis of tumors and neurological diseases [5]. Dysfunctions of complex-I encompass more than 30% of hereditary mitochondrial encephalopathies. Complex-I defects have also been observed in other neurological disorders like sporadic and familiar Parkinson Disease (PD), Hereditary Spastic Paraplegia, Friedreich Ataxia, as well as in aging [5-8].

Alternative splicing is a critical process in the complexity and function of the eukaryotic genome [9]. A precursor mRNA, transcribed from a single gene, can be processed to generate alternative splicing (AS) transcripts, some of which encoding for different proteins. More than one alternative splice isoforms can be maintained concurrently in the steady-state mRNA pool of a single tissue or cell type, and changes in the isoform ratios have been associated with physiological variation and susceptibility to disease [10-12]. Splice sites are recognized through their consensus sequence which is, however, rather ill defined. Pre-mRNAs usually exhibit cis-acting elements which according to their location and activity are referred to as intronic/exonic splicing enhancers (ISEs/ESEs) or intronic/exonic splicing silencers (ISSs/ESSs). These are typically recognized by the members of two protein families, namely the heterogeneous nuclear ribonucleic particle proteins (hnRNP) and the Serine/Arginine rich protein family [13]. The competition between ESEs/ISEs and ESSs/ISSs ensures authentic splice sites recognition and alternative splicing regulation.

Approximately one third of AS transcripts have the potential to introduce a premature termination codon (PTC), which can elicit nonsense-mediated mRNA decay (NMD) [14]. NMD is a cytoplasmic, post-transcriptional mRNA surveillance system which destabilizes transcripts containing PTCs, thus protecting cells from the production of aberrant truncated proteins [15]. The core of the human NMD machinery is constituted by the hUPF1, hUPF2 and hUPF3 proteins [16]. During pre-mRNA maturation exon-junction complexes (EJCs) are deposited 24 nucleotides upstream of exon-exon junctions (EJs) [17,18]. These EJCs are displaced from the transcript during the first round of translation by the ribosomes [19]. If translation terminates prematurely more than 55 nt upstream of the last exon-exon junction, EJC proteins, including hUPF3 and hUPF2, remain associated with the mRNA. Under such circumstances, the PTC is recognized by the SURF complex, which consists of the phosphoinositide 3-kinase-related protein kinase SMG1, UPF1 and translation termination factors eRF1 and eRF3 [20]. SMG1 and UPF1 constituents of SURF subsequently bind the PTC distal EJC [21]. This binding triggers, only in the case of a PTC, SMG1-mediated phosphorylation of UPF1 with translational repression and mRNA decay [21].

Most predicted PTC-introducing alternative splicing events appear as cellular noises produced at low level by inefficient splicing during the post-transcriptional processing independently of NMD [22,23]. There is evidence that only a relatively small proportion of PTC containing AS gene transcripts are subjected to NMD disruption [24]. It has, in fact, been found that absence of the NMD SMG1 factor induces expression

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changes of only 9% of the predicted PTC-containing AS products, and only 2% of these show level changes higher than 20% [24]. In this case the PTC-containing splice isoforms would physiologically be produced by the gene to control its expression in a constitutive or regulated manner. This process is referred to as hUPF1-dependent AS-coupled NMD [25]. The genes associated with this type of regulation include those encoding splicing factors [26,27]. Recently it has been found that genes with a variety of non-splicing functions are also regulated by UPF1-dependent, AS-coupled NMD. Among these genes there are those associated with intracellular signaling, membrane dynamics, cell death, DNA repair, transcriptional regulation, and metabolism [24,28,29]. These findings demonstrate that, in vivo, AS-coupled NMD controls genes of diverse functional categories and may have additional secondary effects on gene expression.

Different Complex-I Genes Produce Alternative Splicing Isoforms

An extended analysis of all the 38 nuclear genes encoding for the structural subunits of mitochondrial complex-I has revealed that many if not all of them produce in physiopathological conditions different splicing isoforms with or without PTC in the frame [28-30]. In particular our group detected and analyzed alternative splicing isoforms of at least 12 genes (Ndufa3, Ndufa13, Ndufa8, Ndufs2, Ndufs4, Ndufa4, Ndufa12, Ndubf6, Ndufv1, Ndufa5, Ndubf11, Ndufa7) (Table 1). A number of PTC-containing complex-I AS transcripts have been identified as novel NMD substrates [28,29]. On the other hand some genes (Ndufa4, Ndufa5, Ndufa12, Ndubf6, Ndubf11) are able to produce different AS isoforms without PTC in the frame and NMD insensible (Table 1). What is the function of this substantial amount

of alternative splicing products, with or without PTC generated by complex-I gene remains unknown. In some cases, considering the low level of expression this would represent mis-splicing or cellular noise. In other cases as for example the Ndufs4 gene [28], and the Ndubf11 gene [30] the alternative splicing transcripts identified could have a role in the regulation of the complex-I biogenesis and their altered expression could be involved in the pathogenetic mechanism of diseases associated with complex-I defect.

Regulation of the Ndufs4 gene expression by coupling of alternative splicing and NMD

The nuclear Ndufs4 gene encodes for one of the 45 subunits of mitochondrial complex-I [31], which is an essential component for the overall architecture of the complex [32] and hotspot for mutations in complex-I deficiency [33]. The Ndufs4 gene produces a canonical mRNA as well as three PTC-containing alternative transcripts (Figure 1A) [34]. Two, SV1 and SV2, are regulated in the cytoplasm by the NMD process. A third transcript, SV3, NMD insensible appears to be regulated in the nucleus by a hUPF1 dependent process which affects the quantity of the transcript rather than its stability [28]. Interesting in NMD-hUPF1 factor depleted cells up-regulation of three PTC-containing isoforms is associated with significant down-regulation of the canonical transcript free of PTC [34]. These data underscore the possible involvement of the hUPF1 dependent AS-coupled NMD in the post-transcriptional regulation of the Ndufs4 expression. In the simplest case, it is possible that some constant fraction of the gene's pre-mRNA is spliced into an unproductive, NMD-targeted form to regulate the normal protein level produced by the gene. It cannot be excluded, however, that the proportion of the transcripts targeted for degradation might be regulated by an external input. It has to be recalled here that the Ndufs4 gene encoding for a 18kDa subunit is involved in the last step of complex-I assembly [32]. The level of the protein is therefore critical for a functional respiratory complex. A similar regulation was observed also for the Ndufa5 where an up-regulation of a NMD insensible AS#17 isoform as well as of the NMD sensible AS#15 and AS#16 was associated with down-regulation of the canonical transcript (Figure 1B) [29]. It is plausible that AS-coupled NMD regulation of the Ndufa5 gene is constitutive but as underscored with the Ndufs4 gene it cannot be excluded a responsive gene expression. Further studies will be needed to clarify this issue.

In hUPF1 knockdown cells decreased level of different complex-I canonical transcripts (Ndufa12, Ndubf6 and Ndufa5) with no PTC [29] was observed, consistent with two previous different microarray profiling studies showing that transcripts level of at least 6% and 9% of the human genes, not associated with PTC-introducing AS events, are increased or decreased upon depletion of hUPF1. It is possible that the effects observed by our group on the level of complex-I gene transcripts are a consequence of a role of AS-coupled NMD, UPF1 dependent, in regulating transcription factors [24]. The data presented provide evidence showing that NMD is involved not only in maintaining the transcriptome integrity by removing non-functional and aberrant PTC-bearing transcripts, but also in transcriptional as well as post-transcriptional control of different complex-I genes expression. Preliminary analysis have shown, in fact, that the mis-regulation of complex-I genes, induced by siRNA silencing of hUPF1, is accompanied with depression of complex-I enzymatic activity [29].

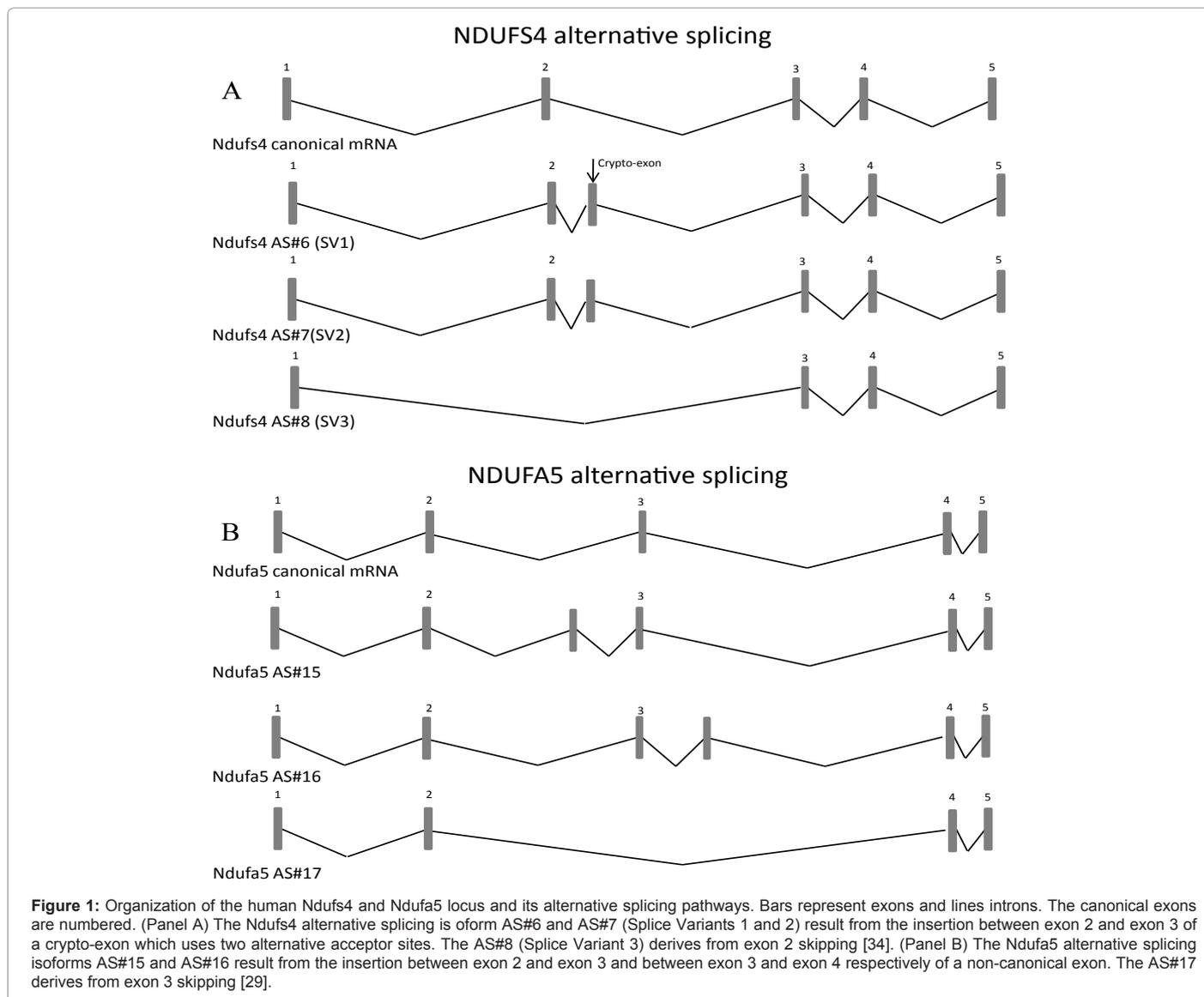
Gene	AS isoform	PTC	NMD sensible	References
NDUFA3	AS#1	+	+	[29]
NDUFA13	AS#2	+	+	[29]
NDUFA8	AS#3	+	+	[29]
NDUFS2	AS#4	+	+	[29]
	AS#5	+	+	
NDUFS4	AS#6 (SV1)	+	+	[28,34]
	AS#7 (SV2)	+	+	
	AS#8 (SV3)	+	-	
NDUFA4	AS#9	-	-	[29]
NDUFA12	AS#10	+	+	[29]
	AS#11	-	-	
NDUBF6	AS#12	-	-	[29]
	AS#13	-	-	
NDUFV1	AS#14	+	+	[29]
NDUFA5	AS#15	+	+	[29]
	AS#16	+	+	
	AS#17	-	-	
NDUBF11	Short 153 aa coding	-	-	[30]
	Long 163 aa coding	-	-	
NDUFA7	//	+	+	[28]

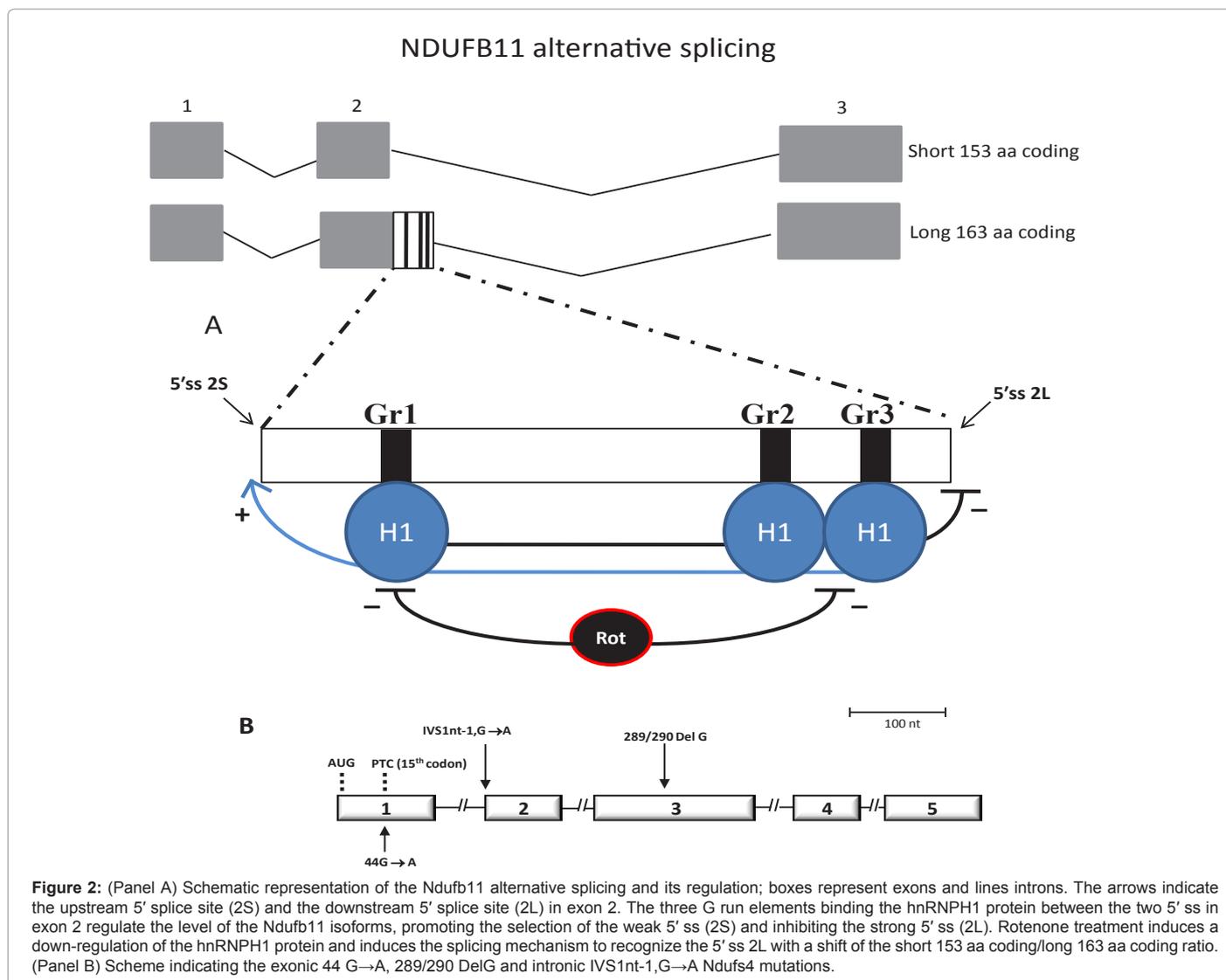
Table 1: Complex-I genes showing alternative splicing isoforms. The presence of PTC and NMD susceptibility are also indicated.

Regulation of the Ndufb11 gene expression by Alternative Splicing and its possible involvement in the apoptotic process

The human X-linked Ndufb11 gene encodes for a 153 amino acid (aa) protein which appears to play a role in the assembly of a functional and stable mammalian mitochondrial complex-I [35-37]. The human gene produces, in addition to the transcript encoding the conserved 153 aa protein, another transcript, PTC-free, encoding a protein of 163 aa. The two transcripts differ in the last 30 nucleotides of exon 2 and are the products of alternative splicing at the 5' splice site of exon 2. The short, 153 aa coding, transcript utilizes an upstream 5' splice site (5'ss 2S) while the long, 163 aa coding, transcript utilizes a downstream splice site (5'ss 2L) (Figure 2A) [30]. The short Ndufb11 transcript is predominantly expressed in several tissues with respect to the long transcript [38]. The ability of the Ndufb11 gene to produce high level of the short isoform with respect to the long isoform appears to be due to the presence between the two 5'ss of three G run, ESS elements, apt to bind the hnRNPH1 protein and to prevent the choice of the downstream 5'ss, although this is a better consensus site (Figure

2A) [30]. Rotenone affects the ratio of the Ndufb11 isoforms only in the neuronal SH-SY5Y cells, increasing the relative amounts of the long versus the short transcript [30]. Since rotenone treatment in the SH-SY5Y cells down-regulates the expression of the hnRNPH1 protein it is likely that the rotenone induced shift in the ratio of the short vs. the long Ndufb11 isoforms, is associated with the depressed capacity of hnRNPH1 to regulate the alternative splicing of the gene. Rotenone treatment of SH-SY5Y cells induces apoptosis [39,40]. In humans cells more than 200 proteins are involved in apoptosis [41]. A large number of apoptotic factors are regulated by alternative splicing, a process that allows for the production of protein isoforms, often with distinct functions, from a common mRNA precursor. The abundance of apoptosis genes, that are alternatively spliced, and the frequent antagonistic roles of protein isoforms strongly suggest that alternative splicing is a crucial mechanism in regulating life and death decisions [41]. In the case of the Ndufb11 gene it is possible that its post-transcriptional regulation is involved in the apoptotic process. The two different proteins produced by the Ndufb11 gene might have, in fact, a different impact on complex-I function. Over expression of





the 163 aa subunit has been found to induce a down-regulation of the complex-I enzymatic activity and an overproduction of ROS. In a preliminary analysis we have found that higher levels of the 163 aa expression compared to the 153 aa protein, resulted in decreased cell viability. However, the 163 aa subunit transfectants maintained viability and did not undergo apoptosis. It has been demonstrated in other systems that overexpression of pro-apoptotic protein is unable to induced apoptosis. For example overexpression of the Bcl-xs protein sensitized the cells to drugs-induced apoptosis but was unable alone to induced by itself cell death [42]. In the same manner overexpression of the 163 aa subunit could sensitize the SHSY-5Y cells to rotenone-induced apoptosis. Mitochondrial disruption is a hallmark of caspase-dependent apoptosis [43]. The mitochondrial outer membrane (MOM) is disrupted, causing the release of proapoptotic molecules such as cytochrome c, AIF, HtrA2/Omi, Smac/Diablo, and endoG. Released cytochrome c activates caspase-9. At the same time, damaged mitochondria generate excessive reactive oxygen species (ROS) and the mitochondrial transmembrane potential ($\Delta\Psi_m$) dissipates. Complex-I function is a determinant in the apoptosis process since different death's signals affect the activity of the complex [44,45]. In addition

proteins, like AIF (Apoptosis induced factor) involved in apoptosis are also found in purified complex-I [46]. The killer lymphocyte protease granzyme A (GzmA) triggers caspase-independent target cell death, with morphological features of apoptosis. GzmA reaches the mitochondrial matrix where it cleaves the NDUFS3 protein, an iron-sulfur subunit of the NADH: ubiquinone oxidoreductase complex I, inducing deficit of NADH oxidation and overproduction of the superoxide anion [47]. It is possible that the alternative splicing regulation of the Ndufb11 genes might enter in this circuit. Normally the AS isoform encoding the 163 aa protein is maintained to low level with respect the 153 aa protein. Apoptosis signals might modulate the alternative splicing of the Ndufb11 gene and shift the 153/163 ratio; in this contest the 163 aa protein might compete with the 153 aa protein, inducing production of ROS by complex-I and facilitating apoptosis.

Pathological Implication

Alternative splicing and NMD variation in neurological disease

Approximately 33% of inherited and acquired diseases are associated with premature termination codons (PTCs), which truncate

the ORF and preclude synthesis of full-length proteins [48]. PTCs can originate as a result of various types of alterations in germline or somatic DNA. A frame shift mutation can result in a PTC. A single-base pair nonsense mutation that converts a sense codon to a nonsense codon also generates a PTC. A mutation within either an intron or an exon that results in inefficient or inaccurate intron removal from pre-mRNA can create an intron-derived PTC or a shift in the ORF and a PTC downstream of the shift [13,49]. Most PTCs elicit nonsense-mediated mRNA decay (NMD). NMD reduces the level of PTC-containing mRNA to 5-25% of the normal (PTC-free) level and, thus, reduces synthesis of the encoded truncated protein. Indeed, the phenotypic severity of a number of diseases caused by nonsense mutations correlates with the extent of reduction in the level of mRNA from the mutant allele [50-52].

Leigh Syndrome is an infantile neurodegenerative disease due to mutations in nuclear or mitochondrial genes involved in mitochondrial energy metabolism. In particular it has been observed that the Ndufs4 gene of complex-I is a hotspot of mutations in the Leigh syndrome [33] resulting in the depletion of the gene product, disassembly of complex-I and reduction of its enzymatic activity [32]. A study of the Ndufs4 transcripts in the fibroblasts from three patients affected by Leigh syndrome harboring three different mutations, introducing a PTC in the gene (Figure 2B), revealed pathological implication of the AS-coupling NMD mis-regulation. In a patient with a point deletion at position 289/290 in exon 3 introducing a PTC, the transcript level was much reduced as compared to control cells [32]. The introduction of the PTC in the middle of the transcript elicited its degradation by NMD [32]. This explains the disappearance of the 18 kDa (AQQD) protein from the patient's cells [32]. In another patient with a 44G→A non-sense mutation in the first exon, disappearance of the mature protein was expected. This mutation which introduced a PTC very close to the canonical AUG start codon, rather than eliciting NMD degradation of the canonical mRNA, up-regulated the three PTC containing alternative transcripts generated by the gene and described in the introduction [34]. The 44G→A non-sense mutation, in fact, inactivated in the patient fibroblasts NMD degradation of SV1 and SV2 and nuclear down regulation of SV3 [28,34]. In a patient with a homozygous splice acceptor site mutation in intron 1 (IV Snt-1, G→A) of the Ndufs4 gene only a mRNA transcript, in which exon 2 was skipped, was detected [53]. Amplification of this transcript and sequencing showed that it corresponded exactly to the PTC containing SV3 isoform detected in the patient with 44G→A nonsense exonic mutation in the Ndufs4 gene [28]. Also in this case the SV3 isoform was insensible to the NMD degradation. The accumulation of the aberrant alternative transcripts, caused by the exonic or the intronic mutation in the Ndufs4 gene, can represent another deleterious event contributing to the pathogenetic mechanism of the mutations in neurological diseases and this correlation underscores the possibility that the NMD pathway may be an attractive target for therapeutic manipulations of genetic defects introducing a PTC.

Alternative splicing isoform ratio variation in neurological disease

Parkinson's disease (PD) is a neurodegenerative disorder resulting from the death of dopamine-generating cells in the substantia nigra [6]. Different genes have been implicated in the familiar form of PD [54]. However the mechanisms that underlie the neuronal degeneration in the majority of cases, sporadic PD, are still unknown. Neuronal cells exhibit particularly high levels of alternative RNA splicing products

[55,56]. A large scale study of human tissues showed, indeed, that the brain presents the highest degree of alternative splicing among 11 tested tissues [57]. These observations suggest that alternative splicing might play a crucial role in the control of gene expression in neuronal cells [58,59]. The importance of alternative splicing in regulating gene expression in human brain is illustrated by a growing number of neurological diseases associated with abnormal mRNA patterns. Massive splicing changes in PD blood cells [60], as well as alteration in the expression of parkin splice variants, in sporadic Parkinson disease (PD) [61,62] and in dementia with Lewy bodies (DLB) [63,64] have been observed. Gene-array studies have demonstrated that cells overexpressing LRRK2 wild-type protein or G2019S mutant protein, which cause 7% of familial PD cases, have a different effect on alternative splicing, associating the G2019S mutation with altered splicing of key neurodegenerative genes including a gene of mitochondrial complex-I [65]. A link between environmental insults and alternative splicing has been identified. The treatment of neuronal cells with rotenone, a pesticide to generate models of PD, has been shown to decrease the exon inclusion in a number of genes [66]. Indeed, AS alteration of complex-I Ndufb11 or Ndufs4 genes has been observed in models of PD [30,67]. These data suggest that in PD there may be problems associated with mRNA splicing of key genes, including genes of complex-I subunits. Different research groups work on identification of biomarker transcripts associated with risk of PD. For example it has been proposed to consider alternative splicing of the SRRM2 protein as a biosignature for PD [68] as well as a network centered on the transcription factors HNF4A and TNF [69]. Since of numerous AS transcripts, complex-I genes could represent a considerable source of biomarkers in PD.

Conclusions

What presented provides an appraisal of the regulation by Alternative Splicing and NMD pathways of the expression of different complex-I genes in human cells. The control of complex-I genes by these two biochemical pathways may have far reaching implications in human pathophysiology. Complex-I is apace-maker of the overall activity of mitochondrial oxidative phosphorylation and with its abundance of structural and ancillary proteins offers the prerequisites to respond to a variety of gene expression mechanisms. Regulation of complex-I by Alternative splicing and NMD pathways is a new emergent area of the complex network of regulatory processes governing complex-I function and biogenesis, contributing to its vulnerability to gene mutations, as well as to sporadic, endogenous and exogenous factors.

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