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Review

Cyclooxygenase variants: The role of alternative splicing

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Abstract

Alternative splicing of cellular pre-mRNA is responsible for production of multiple mRNAs from individual genes. Splice variants are expressed in cell- and tissue-specific contexts that are important in development and physiology. Alternative splicing can serve as a regulatory mechanism whereby developmental programming and environmental factors/stimuli affect biological activities of translated proteins. Cyclooxygenase (COX)-1 and -2 genes produce splice variants whose biological expression, relevance, and activities have been of significant interest. COX variants are produced by a variety of splicing mechanisms. Four structural domains of the COX proteins (the amino terminal signal peptide, membrane-binding domain, dimerization domain, and catalytic domain) are defined by specific COX exons. COX splice variants may, therefore, result in potential changes in protein subcellular localization, dimerization, and activity. COX variant proteins may act in roles which diverge from those of COX-1 and -2.

Keywords: Alternative splicing; Cyclooxygenase; Exon skipping; Intron retention; Polyadenylation; Prostaglandin; Splice variant

Elucidation of the sequences of eukaryotic genomes revealed that, for any given organism, there are fewer genes than predicted by either the transcriptome or proteome. This disparity is due to transcriptional, posttranscriptional, and posttranslational processes. Of these, alternative splicing of pre-mRNA transcripts plays a major role in generating mRNA and protein diversity. Eukaryotic pre-mRNAs are a combination of exons and introns. In addition to capping and polyadenylation, processing of a pre-mRNA results in the removal of intron sequences and the splicing together of exons in order to form a mature mRNA. Retention of introns, partial or full deletion of exons, or switching exons can produce profound effects on the structure and function of the protein encoded by an mRNA.

Based on expressed sequence tag (EST) analysis, 35–60% of the human transcriptome is alternatively spliced [1]. Because this method looks at only a limited number of cells and tissues, growth states, stimuli, etc., these values for alternatively spliced transcripts may be conservative. Analysis of genes encoding mouse transcription factors indicated that 62% of these genes are alternatively spliced [2]. Therefore, alternative splicing, once considered an anomaly, is in fact important to cellular and organismal homeostasis and profoundly influences the complexity and size of eukaryotic proteomes [3].

Alternative splicing produces variant transcripts by shortening, elongating, deleting or switching exons or by evoking intron retention. Exons can be classified into two types: constitutive and alternative. Constitutive exons are those most commonly present in a fully processed mRNA. Alternative exons are only present in the fully processed mRNA in certain cells or under certain conditions. Alternative exons may be included in a transcript along with constitutive exons (Fig. 1A). Mutually exclusive exon switching may also occur between two alternative exons, where either exon (but not both) is incorporated into a message (Fig. 1B). Additionally, there are splicing events which utilize an alternative 5′ or 3′ splice site within an exon, thereby changing the length of the exon in the processed mRNA

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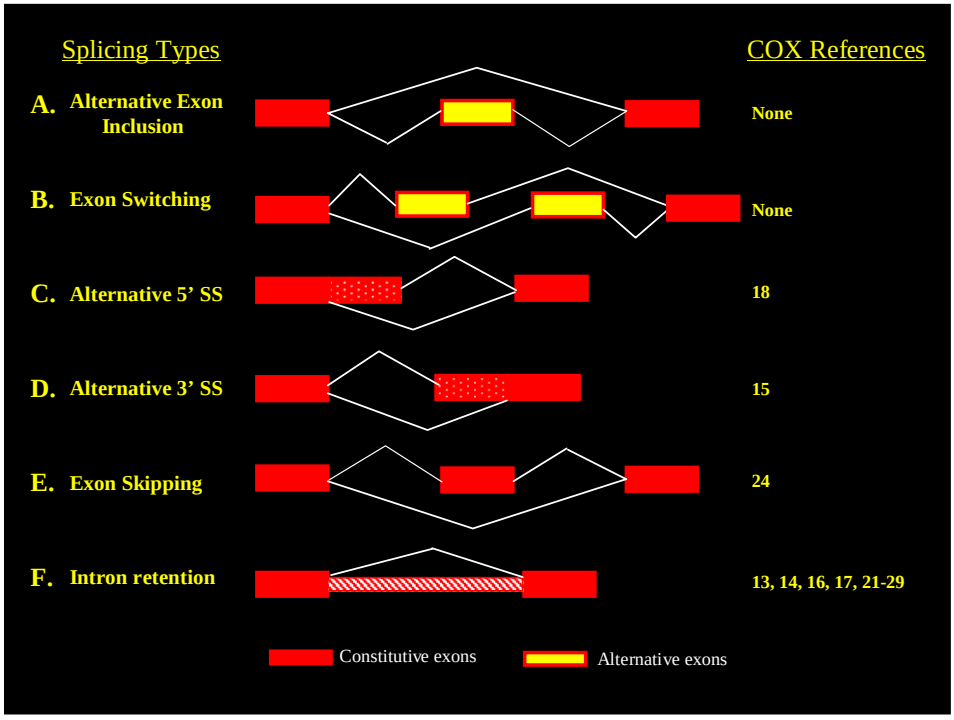

Fig. 1. Alternative splicing in eukaryotes. (A,B) Constitutive exons are shown as dark boxes, alternative exon cassettes are shown as light boxes. (C,D) Regions of exonic sequence which are removed at alternative 5′ or 3′ splice sites are indicated by white dots. (E) Alternative skipping of exonic sequence. (F) The retained intron is indicated by boxed diagonal lines. Splicing is indicated by thin lines, the upper set indicating traditional splicing and the lower set indicating alternative splicing. References to examples of each type of splicing in COX isoforms are indicated to the right.

(Figs. 1C and D). Exons may also be skipped entirely (Fig. 1E). Finally, retention of intron sequences (Fig. 1F) significantly affects transcript function. Intron retention requires specific *cis*- and *trans*-acting regulators to suppress intron excision, because, despite being frequently large, e.g., >10,000 bp, introns are efficiently removed [4].

Retention of introns occurs to regulate genes or produce new proteins. In this process, an entire intron or only a part of an intron may be retained. In known cases of intron retention, the size of intronic sequence that is retained is generally less than 1000 nucleotides. Retained introns may be in the 5′, 3′, or middle regions of otherwise fully processed mRNAs. Partial retention of an intron is achieved through splicing to alternative splice donor or acceptor sites in the intron. Occasionally, sequence from the central region of an intron is retained and, in this case, the retained intron portion resembles an alternative exon.

Alternative splicing of exons and/or introns always affects a change in the primary sequence of the mRNA transcript. However, not every alternative splicing event changes the open reading frame and instead changes only the 5′ or 3′ UTR. Although these changes do not alter the protein coding sequence, they may alter the translation efficiency or half-life of the mRNA.

Changes in coding sequences due to alternative splicing can have profound consequences on the function and activity of proteins. For example, two BCL-x proteins with opposite biological roles are formed by alternative splicing of the BCL-x pre-mRNA. Through partial exon deletion, either BCL-x (short) or BCL-x (long) is formed, which have, respectively, pro-apoptotic and anti-apoptotic activity [5]. In another example, CD44, a cell surface glycoprotein important in the immune system, is synthesized in cells in many functionally distinct variant forms as a result of alternative splicing. The transcripts share a constitutive set of exons and then differ from each other in the choice of 10 alternative exons included in the pre-mRNA's variable region. Multiple activities have been characterized for the translated CD44 proteins in lymphocyte activation, homing, and ligand binding, providing further evidence for biological relevance of splice variants [6]. As recently reviewed by Grabowski and Black [7], mammalian brain is a rich source of alternative splicing events and characterized splice variants. Variants from this system may provide a new understanding about the mechanisms and importance of alternative splicing.

Alternative splicing of COX: correlation of COX protein domains and gene structure

Studies in the field of COX splice variants have provided insight into the evolutionarily conserved structure

of COX genes. Recent reviews [8,9] address the evolution of COX genes. COX-1 and -2 are paralogous proteins that arose from an ancient gene duplication event occurring early in or prior to vertebrate speciation [8]. In human, the COX-1 gene is located on chromosome 9q32–q33.3 and COX-2 is on chromosome 1q25.2–q25.3. COXs are peroxidases that additionally oxygenate fatty acids. They are distantly related to other fatty acid oxygenases, including pathogen inducible oxidases and linoleate diol synthase, in plants and lower organisms.

COX-1 and COX-2 represent potential paradigms of "domain shuffling" because their four structural domains (signal peptide, dimerization domain, membrane-binding domain, and catalytic domain) are defined by specific exons (Fig. 2). All exons of these genes encode at least some protein coding region (none encode only 5′ or 3′ UTR sequence), thus complete skipping of any COX exon, or retention of any intron, is predicted to have an effect on the protein.

Human COX-1 consists of 11 exons and 10 introns, whereas COX-2 has only 10 exons and 9 introns. The difference between the two genes is the presence of an additional small intron (intron 1) at the 5′ end of the COX -1 gene. This "extra" intron of 94 nucleotides in the human COX-1 gene is located in the sequence encoding the N-terminal signal peptide of the protein. Other than this difference, the intron/exon boundaries of the COX-1 gene are the same as in the COX-2 gene, strongly supporting the hypothesis that they have descended from an ancestral gene via gene duplication (Fig. 2).

Because of intron/exon conservation in COX-1 and COX-2, the following description of gene and protein structure will use COX-1 as reference. Beginning at the COX-1 gene's 5′ end, exons 1 and 2 encode the signal peptide. Exon 3 encodes the dimerization domain (Fig. 3), and contains one of four consensus N-linked glycosylation sites in COX-1, three of which are conserved in COX-2. The EGF-like domain of this module

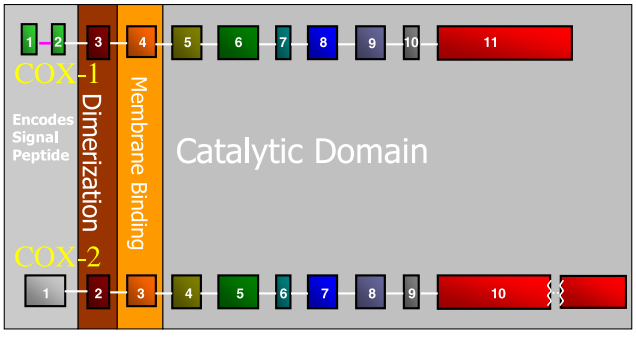

Fig. 2. Correlation of COX exons with COX protein domains. Evolutionary conservation of COX-1 and COX-2 genes and protein domains encoded by specific exons.

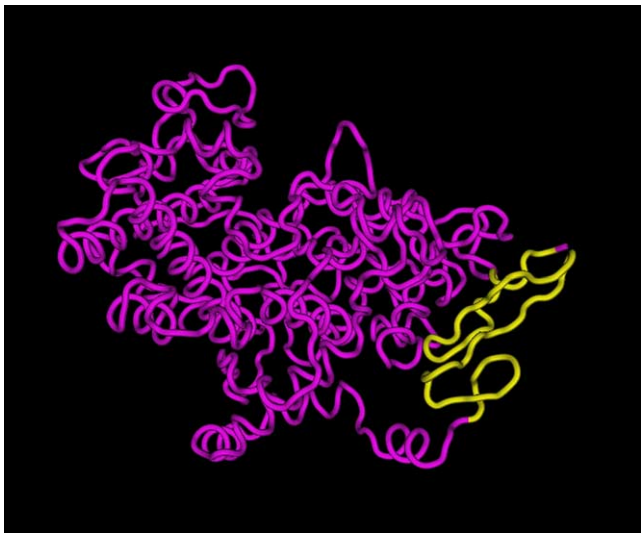

Fig. 3. Effect of exon 3 skipping on COX-1 structure. Ovine COX-1 crystallographic structure from the NCBI molecular modeling database [36] is used. Amino acids encoded by exon 3 are indicated in yellow and constitute the majority of the dimerization domain. An analogous sequence in COX-2 is encoded by exon 2.

contains multiple disulfide bonds. For both isoforms, homodimer formation occurs through interactions of this domain, but no heterodimers of COX-1 and COX-2 occur.

Exon 4 encodes a membrane-binding domain containing four tandem amphipathic helices. In addition to functioning in membrane attachment, the four helices frame the entry to the cyclooxygenase active site. This domain also contains a consensus N-linked glycosylation site, which is not conserved in COX-2, on the third helix. Crystal structure determination and other analyses have failed to find this site to be glycosylated in COX-1.

Exons 6–11 encode the catalytic domain of the enzyme with its spatially separated peroxidase and cyclooxygenase active sites. Although many residues encoded by these exons are important in defining these active sites, some residues encoded by exons 6 and 9 are particularly critical. Histidines that serve as the distal and proximal axial ligands to heme are encoded in exons 6 and 9, respectively. Moreover, exon 9 is rich in other functionally important amino acids, including an active site tyrosine (Tyr 385) that forms a tyrosyl radical essential for catalysis and an evolutionarily conserved consensus N-linked glycosylation site [8,10]. Glycosylation at this site has been shown to be essential to protein folding.

Exon 11 additionally encodes specific residues that are important to non-steroidal anti-inflammatory drug (NSAID) pharmacology. Isoleucine 523 (valine in COX-2), which governs selectivity of many COX-2 selective NSAIDs, as well as a serine residue (Ser 516),

which is the acetylation site by aspirin, are encoded by this exon. The equivalent exon in COX-2 also contains an N-glycosylation site not found in COX-1 that has unknown function but is glycosylated in vivo. The relationship between COX protein structure, catalytic domain, and mechanism has recently been reviewed elsewhere [10–12].

The correlation of specific exons with domains of COX isoforms allows the following predictions regarding the effects of alternative splicing on COX protein: (1) skipping (individually or in tandem) of COX-1 exons 2 though 5 or exon 7, or COX-2 exons 2, 3, 4, or 6 yields in-frame deletions affecting the signal peptide, dimerization domain, membrane-binding domain, or catalytic domain as shown in Fig. 2; (2) skipping of COX-1 exons 6, 8, 9, 10, or 11 individually or COX-2 exons 7, 8, 9, or 10 individually will introduce a frameshift; (3) simultaneous skipping of COX-1 exons 6 and 8 or COX-2 exons 5 and 7 yields in-frame deletions removing portions of the catalytic domain; (4) skipping of COX-1 exon 9 or COX-2 exon 8 prevents heme binding and eliminates the potential of enzymatic activity; (5) retention of COX-1 intron 1 allows interruption of the signal peptide that is not possible in COX-2.

Known COX splice variants

Intron retention, exon skipping, and alternative splice site selection have been described for COX isoforms (Table 1). However, alternative exon inclusion and mutually exclusive alternative exon splicing events (Fig. 1) have not been observed for COX variants, and the sequence of the COX genes suggests that alternative exons do not exist.

The first COX splice variant published was from the COX-2 gene [13]. This variant retained intron 1 and constituted 70% of COX-2 mRNA in non-proliferating chicken embryo fibroblasts as determined by Northern blot analysis. Interestingly, this intron completely lacks a 3′ splice acceptor site and is an example of AG-independent splicing. Removal of intron 1 occurred immediately upon initiation of *src*-mediated signal transduction in dividing cells, or through treatment with phorbol ester or serum. The fact that neoplastic transformation and mitogenic stimulation by v-*src* results in rapid removal of the intron and an increase in fully spliced COX-2 mRNA indicates that splicing out of intron 1 is regulated by signal transduction and may be a method of regulating expression of COX-2 in these cells by introducing a frameshift. Retention of this intron was also observed during NSAID-induced apoptosis, where very large amounts of the intron 1-retaining transcript are induced [14].

Recently, two human COX-2 variants exhibiting alternative 3′ splice site selection and intron retention have been identified. In one variant, named COX-2a, 110 nucleotides are skipped at the 5′ end of exon 5 through the use of an alternative splice acceptor [15]. Translation of this message from the initiation codon in exon 1 is predicted to terminate within the non-deleted portion of the exon 5 sequence due to an introduced frameshift. This splice variant has been reported to be upregulated in human platelets following coronary artery bypass grafting (CABG). A second splice variant, COX-2b, was identified by PCR amplification of rat macrophage mRNA [16] and subsequently in human myometrium from women in labor [17]. This splice variant completely retains intron 7 sequence (282 nts). As a result, 16 novel amino acid residues are encoded beyond exon 7 before the predicted protein terminates at a stop codon within the intron. The observed levels of COX-2b mRNA are higher in myometrium from women in labor. Neither COX-2a nor COX-2b has been characterized at the protein level and neither protein could possess COX activity because of the heme-binding sequence removal.

The first identification of a COX-1 splice variant was from a human lung cDNA containing an in-frame deletion of the final 111 nucleotides (37 amino acids) of exon 9 [18]. This was due to the use of an alternative splice donor site in exon 9. Human COX-2 exon 8 does not contain an analogous splice site and could not produce this variant. The region deleted in exon 9 includes the N-linked glycosylation site characterized as being essential for folding and activity of COX-1 [19]. The deletion

Table 1
Summary of published COX splice variants

	Reference(s)	Type of alternative splicing (species observed)
COX-2 variant description		
Retention of entire intron 1 sequence	[13,14]	Intron retention (chicken)
110 nts deleted from exon 5	[15]	Alternative 3′ splice site (human)
Retention of entire intron 7 sequence	[16,17]	Intron retention (rat and human)
Alternative poly(A) site selection	[32–34]	Polyadenylation variant (vertebrates)
COX-1 variant description		
111 nts deleted from exon 9	[18]	Alternative 5′ splice site (humans)
Retention of partial intron 2 sequence	[21–23]	Intron retention (rat)
Retention of entire intron 1 sequence	[24–29]	Intron retention (dog, mouse rat, human)
Alternative poly(A) site selection	[30,31]	Polyadenylation variant (vertebrates)

does not remove the active site tyrosine residue (Tyr 385) or the proximal histidine heme ligand encoded by this important exon. PCR amplification of human tissues and cells indicated that this variant was differentially expressed compared to COX-1. Studies of normal lung fibroblasts in vitro demonstrated it to be differentially regulated by IL-1β and other inducers of prostaglandin production. The level of the variant mRNA is unchanged in cancerous versus normal colon tissue. In every cellular/tissue context thus far studied, expression of this variant is much lower than COX-1. The combination of differential regulation and low, but easily detectable, levels of expression relative to COX-1 suggests that this variant is not a result of aberrant pre-mRNA processing.

Ectopic expression and analysis of the partial exon 9 deletion protein was recently accomplished in mammalian cells and insect cells which expressed robust levels of the protein [20]. However, no COX products were formed by the variant protein in these cell systems. Peroxidase assays using 2,2′-azino-bis[3-ethyl benzthiazoline-6-sulfonic acid] (ABTS) as reducing co-substrate failed to detect activity in H_2O_2-treated microsomes of COS-7 cells expressing the variant. Analyses of human brain, colon, and tonsil RNA indicated that the variant is present at levels 5–10% of the COX-1 transcript, but immunoprecipitation of brain COX-1 protein failed to detect the variant by Western blot analysis, leading the authors to conclude that the protein was either not made in vivo or was rapidly degraded [20].

Future studies of this COX-1 deletion variant need to clarify whether this variant is truly absent in vivo and further address its potential catalytic activity. The mRNA for this variant clearly exists in many human tissues analyzed thus far. Further support for the importance of this variant is evident in the conservation of the human COX-1 alternative splice site across mammalian species (Fig. 4). This nearly consensus alternative splice donor site sequence in mammals is agGUGGGY, where the lowercase letters represent the final 2 nucleotides of exon 9 that are retained in the variant and GUGGGY represents the 5′ end of deleted exonic sequence. Conservation of this alternative splice site indicates that the partial exon 9 splice variant may also exist in other species.

Consensus eukaryotic 5′splice site	**agGURAGU**
Human	agGUGGGC
Mouse	aaGUGGGC
Rat	aaGUGGGC
Rabbit	agGUGGGC
Sheep	ggGUGGGC
Dog	ggGUGGGU
Alternative splice site	**agGUGGGY**

Fig. 4. Cross-species alignment of potential alternative 5′ splice site nucleotides in COX-1 exon 9 corresponding to the alternative splice site reported by Diaz et al. in exon 9 of human COX-1 variant mRNA. A consensus eukaryotic 5′ splice site sequence is indicated in bold above the alignment, with lowercase letters representing exon nucleotides and uppercase letters representing intron nucleotides. Below the alignment, in bold, is a consensus sequence of the alternative splice site in COX-1 exon 9 derived from the alignment shown.

A second COX-1 splice variant was described in 1995 which lacks exons 1 and 2, but contains an insertion from within intron 2 [21]. TPA treatment of rat tracheal epithelial cells resulted in identification of the partial-intron retaining variant. Northern blot analysis indicated that this variant was surprisingly abundant, constituting more than 90% of the total COX-1 mRNA population in the transformed, immortalized cell line from which it was isolated. TPA treatment of these cells did not change the expression level of variant mRNA, but did increase the expression of fully spliced COX-1 mRNA to levels that equaled those of the variant. No protein was detected from this variant mRNA. If there were protein expressed, however, its detection may have been hampered because the COX-1 antibodies used in the study cross-reacted with COX-2. This variant would require recognition of an internal ribosome entry site for synthesis of any COX-related protein. Even then, the transcript would not encode a signal peptide domain needed for translocation of a putative nascent peptide into the ER. Later studies of this variant in rat identified differential expression in aging stomach, and elevated levels of the transcript in colorectal tumors which decreased following NSAID treatment [22,23].

Finally, studies of dogs have yielded three additional COX-1 splice variants [24]. One retains intron 1 and was termed COX-3 because it retained COX activity. The remaining two variants are alternatively spliced to skip exons 5 through 8 and one of these variants additionally retains intron 1 sequence. These two in-frame deletion variants are partial COX sequence variants, and thus were named PCOX proteins. PCOX-1a retains intron 1 sequence and PCOX-1b does not. Intron 1 retention in two of these splice variants, COX-3 and PCOX-1a, inserts 30 amino acids into the N-terminal signal peptide of the proteins, and when expressed ectopically in insect cells, both variants retain their signal peptide. Insect cell expression of all three canine variants results in easily detectable levels of glycosylated protein. Furthermore, glycosylation-dependent prostaglandin production in Sf9 cells is detected for COX-3, albeit at levels lower than those observed for COX-1 protein. PCOX proteins do not carry out prostaglandin synthesis in insect cells, which is consistent with their lacking a large portion of catalytic domain sequence. However, the proximal heme ligand is not lost from the PCOX proteins, thus they may potentially exhibit peroxidase activity or other heme-dependent enzymatic activities. Importantly, an antibody directed toward the sequence encoded by in-

tron 1 detects a protein in dog brain of the correct size to be PCOX-1a, indicating that this variant is expressed in vivo.

Intron 1 retention in COX-1 produces an in-frame insertion in dog. In rat, mouse, and human mRNA, where intron 1 contains 98 (rat and mouse) or 94 (human) nucleotides of sequence, this is not the case and a frameshift is introduced. However, many studies have identified the intron-1 containing mRNA [10,25–27] in human tissues and cells, as well as in rat [28] and mouse [29]. The question as to the identity of human COX-3 protein remains unanswered.

Ectopic expression of a rat COX-1 message containing intron 1 sequence has recently been published [28]. A protein encoded by this mRNA, termed COX-1b protein, begins at the initiation codon of COX-1 and terminates after a sequence of 127 amino acids due to the frameshift introduced by intron retention. Immunoblot analysis detects a protein of the appropriate size in rat tissues. Only ~9 amino acids are conserved between this protein and canine COX-3, and the protein has little similarity to COX-1. COX-1b protein does not possess COX or peroxidase activity. Human and mouse COX-1 mRNA containing intron 1 can also produce proteins similar to rat COX-1b, although the human variant is predicted to be ~4 kDa smaller.

Polyadenylation variants

In addition to alternative splicing, alternative polyadenylation occurs for both COX-1 and COX-2. Polyadenylation variants of both isoforms are found expressed as three transcripts. Human endothelial cells have been shown to express 2.8, 4.5, and 5.2 kb COX-1 transcripts, with the most abundant transcript being the 2.8 kb form [30]. The 5.2 kb transcript is also prominent in human tissues from brain, heart, and muscle, and is found expressed at higher levels than the 2.8 kb transcript in bladder and colon tissues. Interestingly, the 5.2 kb transcript is also associated with partial or entire retention of intron 1 sequence in several human tissues [24]. Human megakaryocytes express all three transcripts in varying amounts and mitogen treatments affect their relative levels [31].

COX-2 polyadenylation variants have been characterized in various organisms. Three variants are observed at 2.2, 3.8, and 4.2 kb in size [32,33]. The two smaller sizes of the variants are derived from cryptic non-consensus polyadenylation sites within exon 10 [33,34].

The biological relevance of COX splice variants

An important question to be answered in the future is the relevance and role of COX splice variants. We propose the following criteria in evaluating the biological significance of COX splice variants: (1) The variant should be present in amounts that are physiologically relevant. Spurious PCR amplification of negligible populations of incomplete or aberrantly spliced transcripts is possible and these amplifications must be eliminated from consideration. (2) Expression of variants should be cell-specific or regulated in a manner which would distinguish the expression of the variant from random incomplete splicing events. (3) A splice variant should either represent a novel way of regulating COX translation (such as retention of COX-2 intron 1 in quiescent chicken embryo fibroblasts) or the splice variant should encode a protein. (4) Expression of the protein should demonstrate a function, such as enzymatic activity, heterodimerization or regulation of COX isoforms. (5) Expression of a variant should be evolutionarily conserved. (6) Finally, a variant should be clearly detectable at the protein level in vivo. Many of the COX splice variants exhibit a majority of these properties. However, unequivocal (e.g., by mass spectrometry) demonstration of the presence of variant COX proteins in vivo has not been reported for any variant.

It has long been known that COX-1 and COX-2-like proteins can exist outside of the lumen of the endoplasmic reticulum, including in the nucleus [26]. Alternative splicing may provide a mechanism by which this can occur. Because specific exons are associated with COX functional domains, skipping of an exon eliminates or alters the function associated with that corresponding domain. For example, skipping of exon 2 (Fig. 2) is predicted to direct synthesis of COX-1 into the cytosolic/nuclear continuum. Also, skipping of exon 4 would prevent membrane binding, allowing COX proteins to associate with nuclear matrix as has been reported [35].

Alternative splicing may play different roles for COX-1 and COX-2. As described above, there are more known alternative COX-1 transcripts than COX-2 transcripts. Despite intron/exon conservation in COX-1 and COX-2, skipping of complete exons has not been found in COX-2 transcripts. Moreover, most of the COX-1 splice variants encode heme-binding proteins whereas none of the COX-2 variants are predicted to do so. Continued investigations will elucidate differences in alternative splicing between COX-1 and COX-2, and may expand the roles of COX-like proteins in physiology, pharmacology, and disease.

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