Alternative Splicing and Programmed Cell Death (44346)

ZHI-HONG JIANG AND JANE Y. WU[1]

Department of Pediatrics and Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. **Programmed cell death (PCD) is critical for development and homeostasis of multicellular organisms. Genetic and biochemical studies have revealed that PCD is under complex and delicate regulation. An important level of such regulation may be pre-mRNA splicing as suggested by the observation that a number of PCD regulatory genes are expressed as functionally distinct or even antagonistic isoforms as a result of alternative splicing. Studies on alternative splicing of these genes are reviewed here. Expression and function of a large number of genes involved in PCD are regulated by alternative splicing, including death receptors and intracellular components of the death machinery. Alternative splicing affects not only intracellular distribution but also functional activity of these death regulators, providing a fine-tuning mechanism in modulating a presumably tightly controlled process of cell death.**

[P.S.E.B.M. 1999, Vol 220]

Programmed cell death (PCD), also known as apoptosis, is a form of cell death characterized by cell shrinkage and nuclear condensation, usually without inducing inflammatory responses (1). PCD is a fundamentally important biological process that is essential for development and homeostasis of multicellular organisms (see Refs. 2 and 3 for reviews). For example, PCD is essential for deleting autoreactive cells during lymphoid development and eliminating excessive neurons during neural development. Inappropriate PCD may play a role in the pathogenesis of a variety of human diseases, including cancer, autoimmune diseases, viral infections, and neurodegenerative disorders (4).

Morphological characteristics of PCD were described over 20 years ago (1); however, only in the past few years have genes involved in the genetic pathway of PCD been identified. We have now begun to understand molecular mechanisms underlying PCD. Elegant studies by Horvitz and colleagues on development cell death in *C. elegans* revealed several central players in PCD, *ced* genes (reviewed in Ref. 2). The *ced*-3 and *ced*-4 genes function to kill cells whereas the *ced*-9 gene suppresses PCD. The genetic program controlling PCD is highly conserved through evolution. Vertebrate homologs of *ced*-9 are members of the *bcl*-2 family, capable of inhibiting PCD. Apoptosis protease activating factor 1 (apaf-1) (5) is the likely vertebrate homolog of *ced*-4. The *ced*-3 gene product is a cysteine protease (6) related to members of vertebrate ICE (interleukin-1β-converting enzyme) family now named caspases (cysteine aspases) (7).

A variety of signals including growth factor deprivation, activation of certain cell surface receptors (death receptors), and metabolic or cell cycle perturbation may activate cell death machinery *via* divergent signal transduction pathways. It is currently thought that these divergent death signals converge as a central death signal that leads to activation of caspases (Fig. 1). It is the activation of the caspases that results in the terminal events leading to the ultimate demise of the cell.

Genetic and biochemical studies suggest that expression and function of PCD genes are under complex regulation at multiple levels, from transcription control to post-translational modification of different PCD genes. In this review, we will focus on the potential role of alternative pre-mRNA splicing in regulating PCD. Since the discovery of pre-mRNA splicing, numerous studies have suggested that alternative splicing is an important mechanism for generating functionally distinct products from the same gene (See Refs. 8–11 for recent reviews). Alternative splicing has

[1] To whom requests for reprints should be addressed at Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110. E-mail: jwu@pharmdec.wustl.edu

The authors' work was supported by a grant from the NIH (RO1 GM53945), a special fellowship from the Leukemia Society of America, and an institutional pilot research grant through Washington University from the Howard Hughes Medical Institute (76296-538202).

0037-9727/99/2202-0064$10.50/0

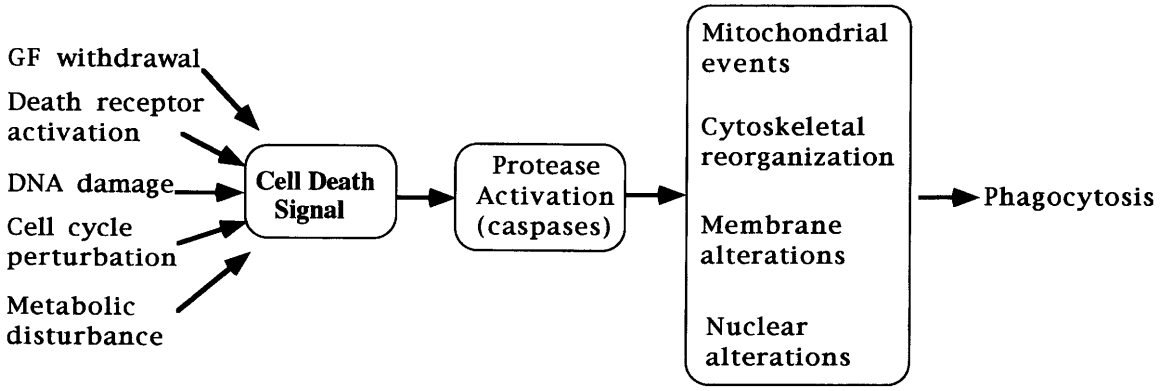

Figure 1. The major events involved in programmed cell death.

been shown to regulate expression and function of genes involved in almost every aspect of cell function including cell proliferation and cell death. The molecular mechanisms controlling pre-mRNA splicing and alternative splicing regulation in general have been covered in several excellent recent reviews (8–14). Here we will review recent studies on alternative splicing of genes involved in regulating PCD. These studies demonstrate that alternative splicing is involved in the processing of mRNA precursors of many genes important for PCD and that expression and function of these PCD genes are regulated by alternative splicing.

Expression of PCD Genes and their Alternative Splicing

Alternative splicing has been found to be involved in processing pre-mRNAs of a large number of genes acting at different steps along the PCD pathway, ranging from membrane-associated death signal receptors to members of the caspase family (Table I). This suggests that alternative splicing may play an important role in regulating PCD.

Multiple proteins and their interactions feed into the PCD pathway at different steps. Binding of death signaling molecules, such as Fas ligand (FasL), tumor necrosis factor (TNF), and TNF-related apoptosis-inducing-ligand (TRAIL), to their receptors leads to activation of death receptors. In the case of Fas (also named APO-1 or CD95) or tumor necrosis factor receptor 1 (TNFR1), oligomerization of the receptors results in transduction of the signal. Following activation of the death receptors, the signal is transduced to activate intracellular components of death machinery resulting in possible dissociation of Apaf-1/procaspase-3 complex from the death-protecting molecules such as members of the *Bcl*-2 gene family (15, 16). Activation of caspases leads to the final degradation events of PCD.

Death Receptors TNF-R1 and Fas are both cysteine-rich cell surface receptors related to the low-affinity nerve growth factor family. Binding of their respective ligands, FasL and TNF, to Fas and TNF-R1 induce apoptosis. The extracellular region of these TNF receptor family members carries two to six repeats of a cysteine-rich motif. These receptors also share homology in their cytoplasmic domain. This domain, which is sufficient to trigger apoptosis when overexpressed alone, has been termed the death domain. Additional members of this family have been identified including lymphocyte-associated receptor of death (LARD, also named DR3, wsl, Apo-3 or TRAMP), death receptor-4 (DR4), and TRAIL receptor inducer of cell killing-2 (TRICK-2) (17–19).

Many members of the death receptor family have multiple isoforms produced as a result of alternative splicing. Human activated peripheral mononuclear cells, EBV infected B cells, and hematopoietic and nonhematopoietic tumor cell lines express several Fas mRNA variants in addition to the predominant Fas mRNA species that generates the membrane-bound form of the functional receptor (20) (Table I). With the exception of FasEx06Del, which is characterized by an in-frame deletion of the exon 6 encoded region, all of these variants of alternative splicing lead to translation in a different reading frame with premature termination codons, resulting in the formation of soluble receptor proteins lacking the transmembrane domain. Interestingly, in FasEX08Del, which was predominantly found in tumor resistant clones, alternative splicing leads to skipping of exon 8, and therefore, a downstream change of the reading frame with a premature termination codon and elimination of the cytoplasmic death domain (21, 22). It is possible that these soluble receptors or the receptor lacking the death domain may function as dominant inhibitory forms of the full-length receptor.

Alternative splicing of LARD pre-mRNA by skipping one or more exon generates at least 11 distinct isoforms (17). The full-length LARD-1, isoforms LARD-1b and LARD-8 encode proteins containing both the transmembrane domain and the death domain, whereas the other isoforms generate secreted molecules without a transmembrane domain. TRICK2 can transduce the cytotoxic signal from TRAIL/APO-2L. Two TRICK2 mRNA isoforms, TRICK2α and TRICK2β, are generated by alternative splicing and differ by 29 amino acid residues extending into the intracellular domain (18). TRICK2α and TRICK2β can both function as receptors of TRAIL together with DR4.

***Bcl*-2 Gene Family.** *Bcl*-2 gene was originally dis-

Table I. Alternatively Spliced Products of Different PCD Genes and their Functional Activities

Gene family	Gene	Isoform	Function
Receptors	Fas (Apo-1, CD95)	Fas	membrane-bound
		FasEX06Del(FasTMDel)	soluble
		FasEX03,4Del	soluble
		FasEX03,4,6Del	soluble
		FasEX04Del	soluble
		FasEX04,6Del	soluble
		FasEX04,7Del	soluble
		FasEX08Del	membrane-bound
	LARD (DR3, Wsl, Apo-3, TRAMP)	LARD-1, 1b, 8	membrane-bound
		LARD-2-7,9-11	soluble
	TRICK2	TRICK2α	active, membrane-bound
		TRICK2β	active, membrane-bound
Bcl-2	Bcl-2	Bcl-2α	membrane-bound, prevent PCD
		Bcl-2ΔTM	inactive, or less active
	Bcl-x	Bcl-x	prevent PCD
		Bcl-xS	promote PCD
		Bcl-xβ	prevent PCD
		Bcl-xΔTM	prevent PCD
		Bcl-xγ	prevent PCD
	Bcl-w	Bcl-w	prevent PCD
		Bcl-w-rox	?
	Bax	Baxα	membrane-bound
		Bax-β	soluble
		Bax-γ	?
	Bim	BimEL	promote PCD
		BimL	promote PCD
		BimS	promote PCD
Ced-4	Ced-4	Ced-4L	prevent PCD
		Ced-4S	promote PCD
Caspases	Caspase-1 (ICE)	ICEα	active
		ICEβ	active
		ICEγ	active
		ICEδ	inactive
		ICEε	inactive
	Caspase-2 (Ich-1)	Ich-1L	active, promote PCD
		Ich-1S	inactive, prevent PCD
		Ich-1β	inactive
	Caspase-3 (CPP32, YAMA, apopain)	CPP32α	active
		CPP32β	active
	Caspase-4 (TX, Ich-2, ICErel-II, Mih-1)	Mih-1α	active
		Mih-1β	active
		Mih-1γ	active
		Mih-1δ	active
	Caspase-6 (Mch-2)	Mch-2α	active
		Mch-2β	inactive
	Caspase-7 (Mch-3, ICE-LAP3, CMH-1)	Mch-3α	active
		Mch-3β	inactive
	Caspase-10 (Mch-4)	Mch-4α	active
		Mch-4β	active

covered as an oncogenic gene at the chromosomal breakpoint in B-cell lymphoma carrying t(14;18) chromosomal translocation (23, 24). Subsequent studies found that *Bcl-2* has potent antiapoptotic activity and is capable of inhibiting apoptotic cell death induced by many different death signals (reviewed in Refs. 25 and 26). The *Bcl-2* gene is essential for lymphoid development (27). A large number of proteins have now been identified that belong to the *Bcl-2* gene family. The *Bcl-2* family of proteins plays a key role in regulation of PCD.

Several models have been proposed for the function of *Bcl-2* in PCD. Structural similarity between *Bcl-2* family member *Bcl-xL* and the pore-forming domain of a certain bacterial toxin suggests that *Bcl-2* family proteins can function as channels for transporting ions or proteins across the membrane. Recent biochemical studies suggest that antiapoptotic members of the Bcl-2 family may function in PCD by interacting with *Ced-4*/Apaf-1 proteins to suppress activation of caspases. The *Bcl-2* superfamily is composed of both death-preventing and death-promoting proteins (25, 26, 28, 29). *Bcl-2*, *Bcl-xL*, *Bcl-w*, *Bfl-1*, *Brag-1*, *Mcl-1*, and *A1* inhibit PCD, whereas *Bax, Bak, Bcl-xS, Bad, Bid, Bik,*

Bim, and *Hrk* activate PCD. Many members of this family contain a carboxyl-terminal transmembrane domain that anchors the protein to the mitochondrion and other intracellular membrane structures. Several proteins of this family, including *Bcl*-2, *Bcl*-x and *Bax,* have both membrane-bound and soluble isoforms generated by alternative splicing (Table I). Protein-protein interaction among different *Bcl*-2 family members as well as interactions with other PCD regulatory proteins are an important mechanism for their function. It has been proposed that the relative ratio of death-preventing and death-promoting proteins and selective interactions among different *Bcl*-2 family members determines whether the cell is to survive or to die.

The *Bcl*-2 gene contains at least two exons. Bcl-2α is produced by splicing within the first exon and joining to the second exon, whereas, *Bcl*-2β mRNA is transcribed from the first exon (30). *Bcl*-2α is the predominant isoform containing a carboxyl-terminal transmembrane domain and has potent death-inhibitory activity. *Bcl*-2β lacks the transmembrane domain and has reduced (31) or no (32) antiapoptotic function.

The *Bcl*-x gene plays a critical role in development of both the hematopoietic process and the central nervous system. *Bcl*-x–deficient mice exhibit massive cell death of immature hematopoietic cells and neurons (33). *Bcl*-x has several transcripts with different biological functions. The mouse *Bcl*-x gene exhibits a three-exon structure with the first exon containing a 5′ untranslated region (Fig. 2) (34). The coding region of *Bcl*-xL is generated by ligation of exons II and III through a splicing reaction, whereas *Bcl*-xS is produced by usage of an alternative 5′ splice site located within exon II with the BH1/BH2 domain deleted. *Bcl*-xL is similar to *Bcl*-2 in inhibiting cell death whereas *Bcl*-xS antagonizes the antiapoptotic action of *Bcl*-2 or *Bcl*-xL (35). Three other murine *Bcl*-x isoforms have been identified: *Bcl*-xΔTM (36), *Bcl*-xβ (37, 38), and *Bcl*-xγ (39). *Bcl*-xΔTM deletes the carboxyl terminal transmembrane domain

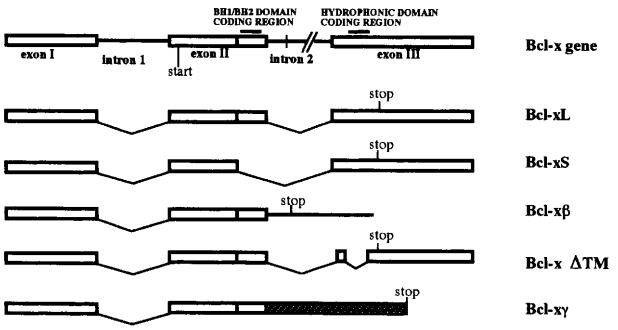

Figure 2. Alternative splicing of the *Bcl*-x gene. The *Bcl*-x gene has three exons with exon 1 encoding the 5′ untranslated region. The translation start site is located in exon 2 in all the identified isoforms. *Bcl*-xS utilizes a 3′ splice site located in exon 2 with an in-frame deletion of the region coding for the BH1/BH2 domain. *Bcl*-xβ is generated by splicing intron 1 with intron 2 retained in the transcript. *Bcl*-xΔTM involves additional splicing using 5′ and 3′ splice sites that reside in exon 3. *Bcl*-xγ is produced by alternative splicing using a different carboxyl-terminal exon.

of *Bcl*-x *via* alternative splicing. The involved 5′ splice donor site is in exon 3 and located 12 nucleotides downstream of the 3′ splice acceptor site for *Bcl*-xs. *Bcl*-xΔTM is possibly a product of an intraexonic splicing using noncanonical splice donor and acceptor sites because both the 5′ and 3′ splice sites reside in exon 3 of the *Bcl*-x gene. *Bcl*-xΔTM can inhibit apoptosis in B cells. The *Bcl*-xβ mRNA is generated by ligation of exon 1 and exon 2 with the intron 2 retained. *Bcl*-xβ is expressed in embryonic and postnatal tissues and can inhibit apoptosis in neurons. *Bcl*-xγ, which carries a unique carboxyl terminus, is expressed primarily in thymocytes and mature T cells (39). Expression of *Bcl*-xγ in mature T cells is associated with ligation of the T-cell receptor (TCR). Unlike other *Bcl*-x isoforms, Bcl-xγ is specifically associated with TCR ligation and is important for resistance to TCR-dependent apoptosis. Activated T cells, which express *Bcl*-xγ, are resistant to PCD after CD3 ligation of TCR. Generation of *Bcl*-xγ involves alternative splicing, which ligates exon 1 and exon 2 with exon 2 spliced to an exon different from exon 3 (Fig. 2). The exon specific to *Bcl*-xγ has not been well characterized.

Similar to *Bcl*-2 and *Bcl*-xL, the *Bcl*-w protein promotes cell survival (40). The *Bcl*-w gene contains at least four closely spaced exons with the coding region split between exon 3 and exon 4. *Bcl*-w-rox corresponds to transcripts spliced from exon 3 of the *Bcl*-w gene to an exon of the adjacent *rox* gene. The location of the remainder of the *rox* gene and the significance of *bcl*-w-*rox* transcripts is unclear (40).

Bax shows extensive amino acid homology with *Bcl*-2 and forms homodimers and heterodimers with *Bcl*-2 *in vivo.* When *Bax* predominates, PCD is accelerated, and the death repressor activity of *Bcl*-2 is inhibited (41). The *Bax* gene consists of six exons (Fig. 3). Protein-encoding information is contributed by all six exons. Several transcripts are generated by alternative splicing (Fig. 3). *Bax*-α encodes a protein containing a transmembrane domain. The retention of intron 5 results in the *Bax*-β mRNA that generates a product lacking the predicted transmembrane domain. The predicted

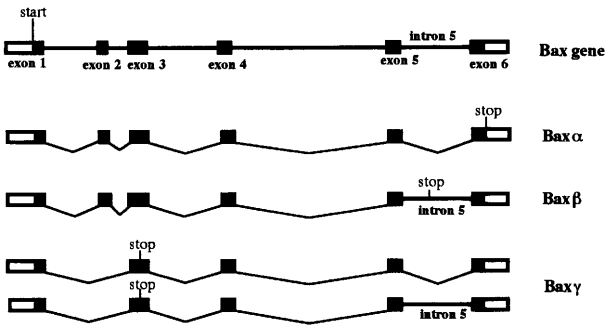

Figure 3. The genomic structure of the *Bax* gene. The *Bax* gene has six exons. The translation start codon is in exon 1. The translation stop codons for different isoforms are as indicated. *Bax*β is the product of alternative splicing with intron 5 retained and the transmembrane domain, which is present in *Bax*α, deleted. Skipping of exon 2 leads to production of *Bax*γ which has two forms, with intron 5 either retained or removed.

membrane form α and cytosolic β forms of *Bax* are paralleled to the α integral membrane form of *Bcl*-2 and predicted β cytosolic form. The γ forms of *Bax* mRNA species lack the small exon 2, shifting the reading frame in exon 3. As a result, *Bax*-γ utilizes a stop codon in exon 3 and is a much smaller peptide as compared to *Bax*-α and Bax-β. The function of Baxγ is uncertain yet.

Bim (*Bcl*-2 interacting mediator of cell death) is a new member of the *Bcl*-2 family, which can promote apoptosis (42). It has a BH3 region that is the only domain with sequence homology to *Bcl*-2 family members. This BH3 region is required for its *Bcl*-2 binding and for most of its cytotoxicity. Three *Bim* isoforms, *Bim*EL, *Bim*L and *Bim*S, generated by alternative splicing, all have a BH3 region and can induce apoptosis. All three isoforms interact *in vivo* with *Bcl*-2 and *Bcl*-xL; however, the short form is the most potent inducer of cell death and suppresser of colony formation (42).

***Ced*-4 Gene.** Recent studies reveal that the *Ced*-4 family can physically interact with *Bcl*-2 proteins and cell-death machinery (43–47). *Ced*-4 serves as an adapter protein bridging *Bcl*-2/*Bcl*-xL to caspases. In the worm, two forms of *Ced*-4, *Ced*-4L and *Ced*-4S, are generated by alternative selection of the two 3′ splice sites (48). *Ced*-4S is expressed as a predominant form. The *Ced*-4L transcript contains an in-frame insertion of 72 nucleotides and encodes a protein with a 24-amino acid insertion relative to *Ced*-4S. Ced-4S induces cell death, and *Ced*-4L protects cells from programmed cell death. Supporting the hypothesis that alternative splicing plays a role in regulating PCD is the fact that in animals carrying the *Ced*-4 n2273 mutant allele, which has a mutation at the *Ced*-4S 3′ splice site (AG to AA), the *Ced*-4L level is increased, and the anterior pharynx of the mutant animals has extra cells that fail to undergo PCD (48).

A human protein Apaf-1 (apoptotic protease activating factor-1) has been identified as a possible vertebrate homolog of *Ced*-4 (5). It appears that human Apaf-1 also has more than one transcript; however, the exact nature of Apaf-1 alternative splicing is not clear yet (Wang X, personal communication).

Caspase Family. Caspases are at the heart of the conserved cell-death pathway. They act as the executive killer during apoptosis. To date, at least 11 homologs of human origin have been published and have now been named caspases 1–11 (7, 49, 50). Further adding to the complexity, many members of this family have different isoforms generated by alternative splicing.

Several alternatively spliced isoforms of ICE (caspase-1) have been described (51). All the alternative splicing events are within the coding sequence of ICE. Four ICE isoforms maintain open reading frames and are designated ICEβ, γ, δ, ε. ICEα and ICEβ have partial intramolecular autoprocessing activity. However, most of the propeptide is deleted in ICEγ, and the region containing the cleavage sites between the p20 and p10 subunits of ICE is deleted in ICEδ. ICEε, homologous to the P10 subunit of active ICE, can form a heterodimer with P20 and may regulate its activity *in vivo* by forming an inactive complex. Only the ICEα, β, and γ isoforms can induce apoptosis.

Alternative splicing of *Ich*-1 (caspase-2) gives rise to at least three transcripts, *Ich*-1L, *Ich*-1S, and *Ich*-1β. A previous study (52) suggested that production of *Ich*-1L and *Ich*-1S resulted from alternative usage of two competing 5′ splice sites. However, our analyses of the genomic DNA and cDNA sequences indicate that production of *Ich*-1L and *Ich*-1S transcripts results from alternative exclusion or inclusion of a 61-base-pair (bp) exon, respectively (Fig. 4). Inclusion of the 61-bp exon results in translation termination using a more upstream stop codon in *Ich*-1S. Therefore, the longer *Ich*-1S transcript encodes a shorter protein product. The sequence of the 61-bp exon, which is present in *Ich*-1S but absent from *Ich*-1L, is identical between human and mouse *Ich*-1 sequences (52). *Ich*-1L contains a sequence homologous to both P20 and P10 subunits of ICE, whereas *Ich*-1s is a truncated version of *Ich*-1L, containing only the P20 region. Recently, another *Ich*-1 isoform, *Ich*-1β, has been identified (53). *Ich*-1β contains a deletion downstream of the first potential aspartic proteolytic cleavage site between the large and small subunits. The deletion alters the coding frame and causes a termination 10 amino acid residues downstream of the aspartic acid residue, essentially generating a functional large subunit. Unlike *Ich*-1S, *Ich*-1β may act as a positive regulator of *Ich*-1 activity (53). The genomic structure of *Ich*-1 has not been reported, and the exact splicing events that generate *Ich*-1β are not clear yet.

Mih-1 (caspase-4, also known as TX, ICErel-II or *Ich*-2), CPP32 (caspase-3, also named Yama or apopain), and *Mch*-4 (caspase-10) have isoforms that contain deletions

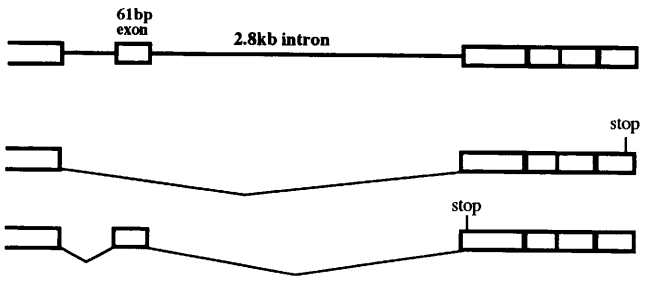

Figure 4. Alternative splicing involved in production of *Ich*-1L and *Ich*-1S (Jiang ZH and Wu J, unpublished data). Transcripts encoding *Ich*-1S are identical to *Ich*-1L in the upstream and downstream exons except that *Ich*-1S contains and *Ich*-1L lacks a 61-base-pair exon in the region immediately upstream to the region coding for the caspase QACRG pentapeptide sequence. As a result, the longer *Ich*-1S transcript produces a shorter and truncated peptide.

in their propeptide domain or 5′ nontranslated region (53, 54). Presumably these isoforms can be processed to active enzymes.

The *Mch*-2 (caspase-6) gene has two transcripts, *Mch*-2α and *Mch*-2β. *Mch*-2β has one-half of its P20 subunit coding region deleted and therefore encodes an inactive enzyme (55). Alternative splicing of *Mch*-3 (caspase-7) pre-mRNA generates two isoforms, *Mch*-3α and *Mch*-3β. *Mch*-3α encodes a peptide that can be processed into an active caspase, whereas *Mch*-3β is produced by alternative splicing that leads to deletion of the region encoding the caspase active site QACRG pentapeptide sequence (56).

The Role of Alternative Splicing in Regulation of PCD

Alternative splicing allows a high degree of protein diversity at low genetic cost. It is clear that alternative splicing plays an important role in expression of many genes in the PCD pathway. The mechanism by which alternative splicing may regulate function of these PCD genes can be summarized in at least three aspects: 1) regulating subcellular localization of PCD gene products; 2) modulating functional activity of PCD gene products; and 3) possibly influencing stability of mRNA and/or translational control.

Several members of the death receptor family and of the *Bcl*-2 superfamily have both membrane-associated as well as soluble forms (Table I). Cellular and subcellular localization of these death regulatory molecules are determined by including or excluding transmembrane domains as a result of alternative splicing. These different isoforms may very likely have distinct functions in PCD. Expression of two isoforms of TRICK2 that have different sequences between the transmembrane domain and the TRAIL-binding domain may be involved in regulation of responses to ligand binding. Several human Fas-splicing variant mRNAs encode soluble proteins. By using *in vitro* apoptosis inhibition assays, it has been shown that the soluble Fas isoforms block Fas-mediated apoptosis induced by agonistic antibody (21, 57) and by its natural ligand (58). These soluble Fas isoforms are still able to trimerize with Fas, but they are not able to form active trimers because they lack other domains including the death domain. As a consequence, the signal provided by FasL or by Fas antibody is prevented from reaching the inside of the cell and results in inhibition of apoptosis. Another Fas isoform, FasEX08Del, is an alternative splicing product that skips exon 8; therefore, it has a downstream change of the reading frame with a premature termination codon and elimination of the whole intracytoplasmic death domain, although it has the transmembrane domain. FasEX08Del is only expressed in tumor resistant clones (22). It is possible that heterotrimers containing the FasEX08Del isoform may render the receptor unable to couple with relevant death domain–binding effectors or unable to displace death domain–binding effectors. The inability of the receptor to couple with relevant transducers may silence a specific pathway. These studies suggest that different alternative splicing products of the death receptor may have distinct roles in the propagation of the apoptotic signal.

Cells may also require two separate intracellular pools of *Bcl*-2 family members, one membrane-bound and the other cytosolic. The cytosolic *Bcl*-2 family members may provide an additional level of regulation by homodimerizing or heterodimerizing with the integral membrane forms.

Many members of the *Bcl*-2 superfamily have multiple alternatively spliced transcripts encoding isoforms with different peptide sequences outside of the transmembrane domain. Because protein-protein interaction has been demonstrated as an important mechanism for function of several *Bcl*-2 family members, it is likely that different isoforms have different properties in interacting with other proteins involved in PCD regulation, therefore, they have different roles in cell death. For example, a simple alternative usage of two competing 5′ splice sites (Fig. 2) results in formation of *Bcl*-xL and *Bcl*-xS, which have different protein sequences. *Bcl*-xL contains a BH1/BH2 domain that is deleted in *Bcl*-xS. Association of pro-survival with pro-apoptotic proteins, such as *Bcl*-2/*Bcl*-xL with *Bax*/*Bad,* requires the BH1 and BH2 domains of the former and the BH3 domain of the later. *Bcl*-xS contains only a BH3 domain and lacks the conserved BH1/BH2 domain. The stable expression of *Bcl*-xS had no effect on cell growth in the presence of growth factor or on the rate of apoptotic cell death following growth factor deprivation (35). However, *Bcl*-xS could prevent the ability of the stably expressed *Bcl*-2/*Bcl*-xL to inhibit apoptosis upon growth factor withdrawal. *Bcl*-xS may act as a dominant inhibitor of *Bcl*-2/*Bcl*-xL by binding the BH3 domain. Therefore, the two alternative splicing products, *Bcl*-xL and *Bcl*-xS, have antagonistic activities in cell death. The delicate balance of different pro-apoptotic and antiapoptotic products of the members of the *Bcl*-2 superfamily is likely to play a critical role in determining the cell's susceptibility to death signals.

Alternative splicing that generates truncated peptides may provide an additional mechanism to quantitatively regulate gene expression. Production of *Bax*γ might represent a splicing strategy to avoid making the full-length *Bax* product, which activates cell death.

The isoforms in the *Bcl*-2 and *Ced*-4 families generated by alternative splicing may also regulate interactions between members in these two families. Some *Bcl*-2 family members prevent apoptosis induced by a variety of stimuli, whereas others promote apoptosis. Competitive dimerization between *Bcl*-2 and *Ced*-4 family members is thought to regulate their function. Recent findings suggest that *Bcl*-2/*Ced*-9 proteins can physically interact with *Ced*-4, which itself binds directly to the *Ced*-3 family of caspases. *Ced*-4 acts as an adaptor molecule between *Ced*-9 and *Ced*-3. The two splicing variants of *Ced*-4, *Ced*-4L and *Ced*-4S, have opposite functions in PCD, suggesting the important role of

alternative splicing regulating PCD. Consistent with this, in the worm carrying a mutation at the *Ced*-4S 3′ splice site, *Ced*-4L expression was increased, and extra cells were found due to decreased cell death (48).

Alternative splicing also appears to regulate the enzymatic activity of caspases. Active caspases consist of a p10 plus p20 heterodimer produced by cleavage of the precursor protein at aspartate residues. *Mch*2β, *Mch*3β, *Ich*-1S all encode inactive enzymes because of truncation in the peptide sequence as a result of alternative splicing. In the various spliced products of ICE, only ICEα and isoforms β and γ can induce apoptosis. However, inactive isoforms may be capable of forming a heterodimer with active enzymes and regulating their activity *in vivo*.

Alternative splicing is a mechanism for generating mRNAs with differential turnover rates or with differential properties in translational control (8, 59). For example, genes encoding growth factors such as colony-stimulating factor-1 have alternatively spliced transcripts that have different stability (60, 61). Although there has been no specific report yet on the stability of the alternatively spliced transcripts of above mentioned PCD genes, it is conceivable that mRNAs encoding these PCD gene isoforms may have different stability and different properties in translation. For example, CPP32β contains a deletion of most of its 5′ untranslated region. CPP32α and CPP32β transcripts may have differences in their stability and/or ability to regulate translation. Further studies will help in understanding why so many different isoforms are generated and how they function *in vivo* to control PCD.

Molecular Mechanism for Regulating the Alternative Splicing of PCD Genes

Regulation of alternative splicing is indicated by tissue specificity and developmental stage specificity of the various spliced products involved in apoptosis. For example, naive B and T cells express very little LARD-1 but express combinations of the other isoforms. Upon T-cell activation, a programmed change in alternative splicing occurs so that the full-length, membrane-band LARD-1 predominates. Mature neural tissue constitutively expresses the *Bcl*-xL mRNA, whereas immature thymocytes that are in the process of undergoing selection in the thymus express a high level of *Bcl*-xS message. The highest levels of *Bcl*-xΔTM transcripts were found in two pre-B cell lines and a mastocytoma cell line P815. *Bcl*-xγ isoform in murine tissues is restricted: it was only detected in thymus, lymph node, and eye, but not brain, heart, intestine, kidney, or liver. Expression of *Ich*-1S is highest in embryonic brain, and only *Ich*-1L is expressed in adult thymus.

Very little is known about molecular mechanisms that regulate alternative splicing of these important PCD genes. Since the discovery of the split gene more than 20 years ago, tremendous effort has been expended to identify the molecular components of splicing machinery and try to understand regulatory mechanisms controlling alternative splicing (8–14, 61, 62). *cis*-Elements essential for intron removal most frequently are found at the exon/intron boundaries although some splicing enhancer or repressor sequences have been identified within the exon and intron. As to the transacting factors involved in splicing, biochemical studies have revealed a large number of protein factors in addition to small nuclear ribonucleoproteins (snRNPs) (12, 13).

A critical issue in pre-mRNA splicing and alternative splicing regulation is specific recognition of splice sites and proper association between appropriate 5′ and 3′ splice sites. Biochemical studies using vertebrate splicing substrates as well as genetic studies using yeast systems provide compelling evidence that snRNPs including U1, U2, and U5 play essential roles in splice-site recognition and association (reviewed in Refs. 8–14, 61, 63). Recent work using mammalian and viral splicing substrates indicates that several families of proteins play essential roles in assembling spliceosomes and regulating alternative splicing. These include the heteronuclear ribonucleoproteins family (hnRNP proteins), proteins containing serine-arginine–rich sequences (SR proteins), and specific RNA-binding proteins (such as proteins binding to the polypyrimidine tract and branch site) (13).

Splicing factors of the serine-arginine–rich (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) families are emerging as important alternative splicing regulators (8–14, 64, 65). Several studies have suggested that SR proteins and an hnRNP protein, hnRNPA1, have antagonistic effects on splice site selection (66–68).

To understand the molecular mechanism underlying alternative splicing regulation of the genes involved in PCD, we have established a model system using a murine *Ich*-1 minigene. In transfected cells, *Ich*-1 minigene underwent alternative splicing that was similar to what was observed *in vivo*. Using this *Ich*-1 system, we have demonstrated that several splicing factors can regulate *Ich*-1 alternative splicing of both the cotransfected minigene and the endogenous gene (Jiang ZH *et al.*, unpublished data). SR proteins including SC35 and ASF/SF2 promote exon skipping to produce *Ich*-1L, whereas hnRNPA1 facilitates exon inclusion to produce *Ich*-1S. Most interestingly, we have found that these splicing factors can regulate apoptosis in a manner consistent with their regulation of *Ich*-1 pre-mRNA splicing. The opposite effects of SC35 and hnRNPA1 on apoptosis suggest that the relative concentrations and activities of such splicing factors may provide a previously unsuspected regulatory mechanism for controlling cell death and survival.

In addition to relative concentrations of SR proteins and their antagonistic hnRNP proteins, other mechanisms could regulate functional activities through modification of these proteins such as reversible protein phosphorylation. Several kinases capable of phosphorylating SR proteins have been identified. Some phosphatase activities associated with spliceosome have also been reported (65). Another potential link between SR proteins and apoptosis regulation is the

association of phosphorylated SR proteins including SC35 with the U1-snRNP complex in cells undergoing apoptotic cell death (69).

Our recent work on dissection of molecular components of the splicing machinery involved in alternative splicing of the PCD gene suggest that multiple factors contribute to the complex and dynamic alternative splicing pattern of these PCD genes (Jiang and Wu, unpublished data). Further study is necessary to elucidate mechanisms underlying alternative splicing regulation during PCD.

Summary

Numerous genes involved in PCD undergo alternative splicing to produce multiple isoforms with distinct function in cell death. Alternative splicing not only generates products with different subcellular localization (membrane-associated versus soluble proteins) but also produce proteins with different functions. Molecular analyses of expression and regulation of these genes have suggested a potentially very important role of alternative splicing in regulating PCD. So far, very little is known about molecular mechanisms controlling alternative splicing of these PCD genes. Our work using *Ich*-1 as a model system has initiated molecular dissection of the link between alternative splicing and PCD regulation. Further study is necessary to elucidate the role of alternative splicing in modulation of PCD and mechanisms underlying this important alternative splicing regulation.

We would like to thank members of the Wu lab for their critical reading of the manuscript.

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer **26:**239–257, 1972.
2. Horvitz HR, Shaham S, Hengartner MO. The genetics of programmed cell death in the nematode *Caenorhabditis elegans.* Cold Spring Harbor Symp Quat Biol **59:**377–385, 1994.
3. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell **88:**347–354, 1997.
4. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science **267:**1456–1462, 1995.
5. Zou H, Henzel WJ, Liu XS, Lutschg A, Wang XD. Apaf-1, a human protein homologous to *C. elegans Ced*-4, participates in cytochrome c–dependent activation of caspase-3. Cell **90:**405–413, 1997.
6. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The *C. elegans* cell death gene *ced*-3 encodes a protein similar to mammalian interleukin-1 β-converting enzyme. Cell **75:**641–652, 1993.
7. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. Human ICE/*Ced*-3 protease nomenclature. Cell **87:**171, 1996.
8. Smith CWJ, Patton JG, Nadal-Ginard B. Alternative splicing control of gene expression. Annu Rev Genet **23:**527, 1989.
9. Maniatis T. Mechanisms of alternative pre-mRNA splicing. Science **251:**33–34, 1991.
10. Rio DC. Splicing of pre-mRNA: Mechanism, regulation, and role in development. Curr Opin Genet Dev **3:**574–584, 1993.
11. Black DL. Finding splice sites within a wilderness of RNA. RNA **1:**763–771, 1995.
12. Moore JM, Query CC, Sharp PA. Splicing of precursor to messenger RNAs by the spliceosome. In: The RNA World. Gesteland RF, Atkins JF, Eds. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp303–358, 1993.
13. Krämer A. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu Rev Biochem **65:**367–409, 1996.
14. Reed R. Initial splice site recognition and base pairing during pre-mRNA splicing. Curr Opin Genet Dev **6:**215–220, 1996.
15. Hengartner MO. Apoptosis: *Ced*-4 is a stranger no more. Nature **388:**714–715, 1997.
16. Hengartner MO. Apoptosis: Death cycle and Swiss army knife. Nature **391:**441–442, 1998.
17. Screaton GR, Xu XN, Olsen AL, Cowper AE, Tan R, McMichael AJ, Bell JL. LARD: A new lymphoid-specific death domain containing receptor regulated by alternative pre-mRNA splicing. Proc Natl Acad Sci USA **94:**4615–4619, 1997.
18. Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI. TRICK2: A new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr Biol **7:**693–696, 1997.
19. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM. The receptor for the cytotoxic ligand TRAIL. Science **276:**111–113, 1997.
20. Ruberti G, Cascino I, Papoff G, Eramo A. Fas splicing, variants and their effect on apoptosis. Adv Exp Med Biol **406:**125–134, 1996.
21. Cascino I, Fiucci G, Papoff G, Ruberti G. Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. J Immunol **154:**2706–2713, 1995.
22. Cascino I, Papoff G, Maria D, Testi R, Ruberti G. Fas/Apo-1/CD95 receptor lacking the intracytoplasmic signaling domain protects tumor cells from Fas-mediated apoptosis. J Immunol **156:**13, 1996.
23. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with t(14; 18) chromosome translocation. Science **226:**1097–1099, 1984.
24. Tsujimoto Y, Jaffe E, Cossman J, Gorham J, Nowell PC, Croce CM. Clustering of breakpoint on chromosome 11 in human B-cell neoplasms with the t(11; 14) chromosome translocation. Nature **315:**340–343, 1985.
25. Kroemer G. The proto-oncogene *Bcl*-2 and its role in regulating apoptosis. Nat Med **3:**614–620, 1997.
26. Reed JC. Double identity for proteins of the *Bcl*-2 family. Nature **387:**773–776, 1997.
27. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. *Bcl*-2 deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidney, and hypopigmented hair. Cell **75:**229–240, 1993.
28. Farrow SN, Brown R. New members of the *Bcl*-2 family and their protein partner. Curr Opin Genet Dev **6:**45–49, 1996.
29. Hegde R, Srinivasula SM, Ahmad M, Fermandes-Alnemri T, Alnemri ES. Blk, a BH3-containing mouse protein that interacts with *Bcl*-2 and *Bcl*-xL, is a potent death agonist. J Biol Chem **273:**7783–7786, 1998.
30. Tsujimoto Y, Croce C. Analysis of the structure, transcripts, and protein products of *Bcl*-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA **83:**5214–5218, 1986.
31. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. *Bcl*-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature **348:**334–336, 1990.
32. Tanaka S, Saito K, Reed JC. Structure-function analysis of the *Bcl*-2 oncoprotein; addition of heterologous transmembrane domain portions of the *Bcl*-2b protein restores function as a regulator of cell survival. J Biol Chem **268:**10920–10926, 1993.
33. Motoyama N, Wang F, Roth KA, Sawa H, Nakayama KI, Negishi I, Senju S, Zhang Q, Fujii S, Loh Z. Massive cell death of immature hematopoietic cells and neurons in *Bcl*-x deficient mice. Science **267:**1506–1510, 1995.
34. Grillot DAM, González-García M, Ekhterae D, Duan L, Inohara N, Ohta S, Seldin MF, Núñez G. Genomic organization, promoter region analysis, and chromosome localization of the mouse *bcl*-x gene. J Immunol **158:**4750–4757, 1997.

35. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. *Bcl*-x, a *Bcl*-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell **74**(4):597–608, 1993.
36. Fang W, Rivard JJ, Mueller DL, Behrens TW. Cloning and molecular characterization of mouse *Bcl*-x in B and T lymphocytes. J Immunol **153**(10):4388–4398, 1994.
37. González-García M, Perez-Ballestero R, Ding LY, Duan L, Boise LH, Thompson CB, Núñez G. *Bcl*-xL is the major *Bcl*-x mRNA form expressed during murine development, and its product localizes to mitochondria. Development **120:**3033–3042, 1994.
38. Shiraiwa N, Inohara N, Okaka S, Yuzaki M, Shoji S, Ohta S. An additional form of rat *Bcl*-x, *Bcl*-xβ, generated by an unspliced RNA, promotes apoptosis in promyeloid cells. J Biol Chem **271:**13258, 1996.
39. Yang XF, Weber GF, Cantor H. A novel *Bcl*-x isoform connected to the T-cell receptor regulates apoptosis in T cells. Immunity **7:**629–639, 1997.
40. Gibson L, Holmgreen SP, Huang DCS, Bernard O, Copeland NG, Jenkins NA, Sutherland GR, Baker E, Adams JM, Cory S. *Bcl*-w, a novel member of the *Bcl*-2 family, promotes cell survival. Oncogene **13:**665–675, 1996.
41. Oltvai ZN, Milliman CL, Korsmeyer SJ. *Bcl*-2 heterodimerizes *in vivo* with a conserved homolog, *Bax*, that accelerates programmed cell death. Cell **74:**609–619, 1993.
42. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DCS. Bim: A novel member of the *Bcl*-2 family that promotes apoptosis. EMBO J **17:**384–395, 1998.
43. Chinnaiyan AM, O'Rourke K, Lane BR, Dixit VM. Interaction of *Ced*-4 with *Ced*-3 and *Ced*-9: A molecular framework for cell death. Science **275:**1122–1126, 1997.
44. James C, Gschmeissner S, Fraser A, Evan GL. *Ced*-4 induces chromatin condensation in *S. pombe* and is inhibited by direct physical association with *Ced*-9. Curr Biol **7:**246–252, 1997.
45. Spector MS, Desnoyers S, Hoeppner DJ, Hengartner MO. Interaction between the *C. elegans* cell-death regulators *Ced*-9 and *Ced*-4. Nature **385:**653–656, 1997.
46. Wu D, Wallen HD, Nunez G. Interaction and regulation of subcellular localization of *Ced*-4 by *Ced*-9. Science **275:**1126–1129, 1997.
47. Wu W, Wallen HD, Inohara N, Nunez G. Interaction and regulation of the *Caenorhabditis elegans* death protease *Ced*-3 by *Ced*-4 and *Ced*-9. J Biol Chem **272:**21449–21454, 1997.
48. Shaham S, Horvitz HR. An alternatively spliced *C. elegans Ced*-4 RNA encodes a novel cell death inhibitor. Cell **86:**201–208, 1996.
49. Nicholson DW, Thornberry NA. Caspases: Killer proteases. Trends Biochem Sci **22:**299–306, 1997.
50. Wang S, Miura M, Jung YK, Zhu H, Li E, Yuan T. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell **92:**501–509, 1998.
51. Alnemri ES, Fernandes-Alnemri T, Litwack G. Cloning and expression of four novel isoforms of human interleukin-1 β converting enzyme with different apoptotic activities. J Biol Chem **270:**4312–4317, 1995.
52. Wang L, Miura M, Bergeron L, Zhu H, Yuan J. *Ich*-1, an Ice/*Ced*-3-related gene, encodes both positive and negative regulators of programmed cell death. Cell **78**(5):739–750, 1994.
53. Alnemri ES. Mammalian cell death proteases: A family of highly conserved aspartate specific cysteine proteases. J Cell Biochem **64:**33–42, 1997.
54. Fernandes-Alnemri T, Litwack G, Alnemri ES. CPP32: A novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein *Ced*-3 and mammalian interleukin-1 β-converting enzyme. J Biol Chem **269:**30761–30764, 1984.
55. Fernandes-Alnemri T, Litwack G, Alnemri ES. *Mch*2, a new member of the apoptotic *Ced*-3/Ice cysteine protease gene family. Cancer Res **55**(13):2737–2742, 1995a.
56. Fernandes-Alnemri T, Takahashi A, Armstrong R, Krebs J, Fritz L, Tomaselli KJ, Wang L, Yu Z, Croce CM, Salveson G, Earnshaw WC, Litwack G, Alnemri ES. *Mch*3: A novel human apoptotic cysteine protease highly related to CPP32. Cancer Res **55**(24):6045–6052, 1995b.
57. Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, Barr PJ, Mountz JD. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science **263:**1759, 1994.
58. Papoff G, Cascino I, Eramo A, Starace G, Lynch DH, Ruberti G. An N-terminal domain shared by Fas/Apo-1 (CD95) soluble variants prevents cell death *in vitro*. J Immunol **156**(12):4622–4630, 1996.
59. Curtis D, Lehmann R, Zamore PD. Translational regulation in development. Cell **81:**171–178, 1995.
60. Shaw G, Kamen R. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selection mRNA degradation. Cell **46:**659–667, 1986.
61. Ladner MB, Martin GA, Noble JA, Nikoloff DM, Tal R. Human CSF-1: Gene structure and alternative splicing of mRNA precursors. EMBO J **6:**2693–2698, 1987.
62. Bingham PM, Chou TB, Mims I, Zachar Z. On/off regulation of gene expression at the level of splicing. Trends Genet **4:**134, 1988.
63. Madhani H, Guthrie C. Dynamic RNA-RNA interactions in the spliceosome. Annu Rev Genet **28:**1–26, 1994.
64. Berget SM. Exon recognition in vertebrate splicing. J Biol Chem **2:**2411–2414, 1995.
65. Fu X-D. The superfamily of arginine/serine rich splicing factors. RNA **1:**663–680, 1995.
66. Cáceres JF, Stamm S, Helfman DM, Krainer AR. Regulation of alternative splicing *in vivo* by overexpression of antagonistic splicing factors. Science **265:**1706–1709, 1994.
67. Mayeda A, Krainer AR. Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell **68:**365–375, 1992.
68. Mayeda A, Helfman DM, Krainer AR. Modulation of exon skipping and inclusion by heterogeneous nuclear ribonucleoprotein A1 and pre-mRNA splicing factor SF2/ASF. Mol Cell Biol **13:**2993–3001, 1993.
69. Utz PJ, Hottelet M, Venrooij WJV, Anderson P. Association of phosphorylated serine/arginine (SR) splicing factors with the U1-small ribonucleoprotein (snRNP) autoantigen complex accompanies apoptotic cell death. J Exp Med **187:**547–560, 1998.