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Review

Function of alternative splicing

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Abstract

Alternative splicing is one of the most important mechanisms to generate a large number of mRNA and protein isoforms from the surprisingly low number of human genes. Unlike promoter activity, which primarily regulates the amount of transcripts, alternative splicing changes the structure of transcripts and their encoded proteins. Together with nonsense-mediated decay (NMD), at least 25% of all alternative exons are predicted to regulate transcript abundance. Molecular analyses during the last decade demonstrate that alternative splicing determines the binding properties, intracellular localization, enzymatic activity, protein stability and posttranslational modifications of a large number of proteins. The magnitude of the effects range from a complete loss of function or acquisition of a new function to very subtle modulations, which are observed in the majority of cases reported. Alternative splicing factors regulate multiple pre-mRNAs and recent identification of physiological targets shows that a specific splicing factor regulates pre-mRNAs with coherent biological functions. Therefore, evidence is now accumulating that alternative splicing coordinates physiologically meaningful changes in protein isoform expression and is a key mechanism to generate the complex proteome of multicellular organisms.

Keywords: Alternative splicing; Review; Localization; Enzymatic activity; Binding properties

1. Introduction

1.1. Abundance of pre-mRNA splicing

An average human gene contains a mean of 8.8 exons, with a mean size of 145 nt. The mean intron length is 3365 nt, and the 5′ and 3′ UTR are 770 and 300 nt, respectively. As a result, a "standard" gene spans about 27 kbp. After pre-mRNA processing, the average mRNA exported into the cytosol consists of 1340 nt coding sequence, 1070 nt untranslated regions and a poly (A) tail (Lander et al., 2001). This shows that more than 90% of the pre-mRNA is removed as introns and only about 10% of the average pre-mRNA are joined as exonic sequences by pre-mRNA splicing. Human cells are not only capable of accurately recognizing the small exons within the larger intron context, but are also able to recognize exons alternatively. In this

Abbreviations: CGRP, calcitonin-gene-related peptide; DSCAM, Down syndrome cell adhesion molecule; GDNF, glial cell line-derived neurotrophic factor; GMAP-210, Golgi-microtubule-associated-protein of 210 kDa; GnRH, gonadotrophin releasing hormone; HER2, human epidermal growth factor receptor; ICAD, inhibitor of caspase-activated DNAse; IL-4, interleukin 4; LDL, low-density lipoprotein; NMD, nonsense-mediated decay; PECAM-1, platelet/endothelial cell adhesion molecule-1; RUST, regulated unproductive splicing and translation; TSH, thyroid stimulating hormone; VEGF, vascular endothelial growth factor.

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process, an exon is either incorporated into the mRNA, or is excised as a part of an intron. Initially, this was thought to be only a minor processing pathway affecting about 5% of all genes (Sharp, 1994), but over time, it became clear that it is highly abundant. Bioinformatic analysis shows that 59% of the 245 genes present on chromosome 22 are alternatively spliced, and DNA microarray experiments indicate that 74% of all human genes are alternatively spliced (Johnson et al., 2003). The high frequency of alternative splicing in humans is also supported by EST-based database analysis, indicating that 35–60% of all human gene products are alternatively spliced (Mironov et al., 1999; Brett et al., 2000; Kan et al., 2001; Modrek et al., 2001), suggesting that alternative splicing of human genes is the rule and not the exception. On average, a human gene generates two to three transcripts. However, extreme cases exist: The human neurexin3 gene can potentially form 1728 transcripts due to alternative splicing at four different sites. In *Drosophila*, the Down syndrome cell adhesion molecule (DSCAM) can potentially generate 38016 isoforms due to alternative splicing (Celotto and Graveley, 2001). This number is larger than the total number of genes present in *Drosophila*. Alternative splicing is observed in all tissues, but tissue-specific splicing is most prevalent in brain cells (Stamm et al., 2000; Xu et al., 2002). EST data comparison strongly indicates that similar levels of alternative splicing occur in evolutionarily distinct species, such as human, mouse, *Drosophila* and *C. elegans*, emphasizing the importance of alternative splicing throughout evolution (Brett et al., 2002). The increased recognition of alternative splicing is reflected by the steady growth in the number of publications describing alternative splicing. It increased from 16 publications in 1985 to 1073 in 1998. Since then, about 1000 publications per year deal with various aspects of alternative splicing.

1.2. Mechanism of splice-site selection

The mechanism of splicing has been determined in great detail (Jurica and Moore, 2003; Nilsen, 2003). In contrast, it is not yet fully understood how splice sites are selected. The major problem is the degeneracy of splicing regulatory sequences, such as the 5′, 3′ splice sites, branch points and exonic/intronic sequence elements. These can only be

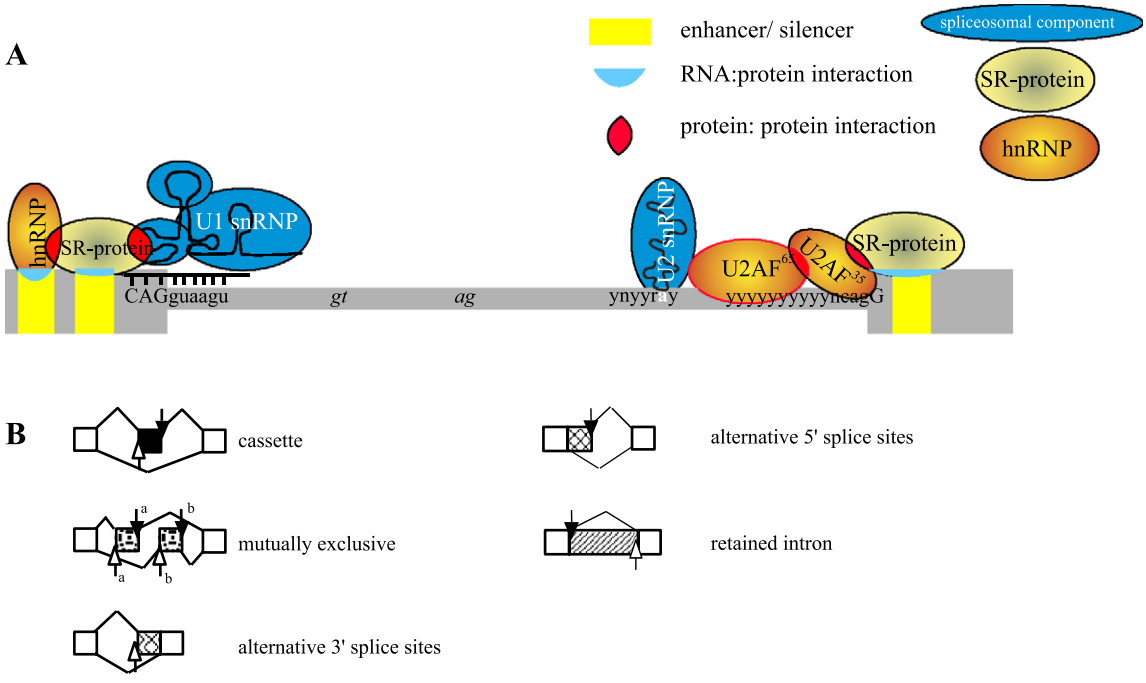

Fig. 1. Splice-site selection. (A) Exons are indicated as boxes, the intron as a thick line. Splicing regulator elements (enhancers or silencers) are shown as yellow boxes in exons or as thin boxes in introns. The 5′ splice site (CAGguaagu) and 3′ splice site $(y)_{10}$ncagG, as well as the branch point (ynyyray), are indicated (*y*=c or u, *n*=a, g, c or u). Upper-case letters refer to nucleotides that remain in the mature mRNA. Two major groups of proteins, hnRNPs (orange) and SR or SR-related proteins (green), bind to splicing regulator elements; the protein–RNA interaction is shown in blue. This protein complex assembles around an exon enhancer, stabilizing the binding of the U1 snRNP close to the 5′ splice site, e.g., due to protein–protein interaction between an SR protein and the RS domain of U170K (shown in red). This allows the hybridization (thick black line with stripes) of the U1 snRNA (black) with the 5′ splice site. The formation of the multiprotein–RNA complex allows discrimination between proper splice sites indicated at exon–intron borders and cryptic splice sites (small *gt ag*) that are frequent in pre-mRNA sequences. Factors at the 3′ splice site include U2AF, which facilitates binding to U2 snRNP to the branchpoint sequence. In exons with weak polypyrimidine tracts, the binding of U2AF is facilitated by the SR proteins binding to exonic enhancers. Green: SR and SR-related proteins; orange: hnRNPs; blue: protein–RNA interaction; red: protein–protein interaction; thick black line with stripes: RNA–RNA hybridization. (B) Types of alternative splicing events: Alternative exons are shown as boxes with different shading. Flanking constitutive exons are shown as white boxes. The open arrow indicates the position of the alternative 3′ splice site analyzed; a closed arrow indicates the position of the 5′ splice sites analyzed.

described as consensus sequences that are loosely followed (Black, 2003). As a result, it is not possible to accurately predict splicing patterns from genomic sequence. The accurate recognition of splice sites in vivo is the result of a combinatorial regulatory mechanism (Smith and Valcarcel, 2000). As splice sites are degenerate, additional sequence elements located in the exon or in adjacent intronic elements aid their recognition by binding to regulatory proteins. These proteins can be subdivided into serine-arginine-rich SR proteins and hnRNPs. In general, these proteins bind weakly to RNA. Increased specificity is achieved by the binding of multiple proteins to RNA that is aided by protein–protein interactions (Fig. 1A). Several regulatory proteins bind to the components of the spliceosome, e.g., to the U1 and U2snRNP, which defines the 5′ splice site and branch-point, respectively (Maniatis and Reed, 2002; Maniatis and Tasic, 2002). The formation of a specific protein–RNA complex from several intrinsically weak interactions has several advantages: (i) it allows a high sequence flexibility of exonic regulatory sequences that puts no constraints on coding requirements, (ii) the protein interaction can be influenced by small changes in the concentration of regulatory proteins which allows the alternative usage of exons depending on a tissue and/or developmental-specific concentration of regulatory factors, (iii) phosphorylation of regulatory factors that alter protein–protein interactions can influence splice-site selection, (iv) the regulatory proteins can be exchanged with other proteins after the splicing reaction, allowing a dynamic processing of the RNA. The alternative recognition of splice sites has been extensively reviewed (Graveley, 2001; Hastings and Krainer, 2001; Maniatis and Tasic, 2002; Black, 2003). Since splicing factors bind to numerous weakly conserved sequences, a single factor can regulate multiple target genes. Those target genes have been identified for the neuron-specific splicing factor NOVA-1 (Ule et al., 2003). NOVA-1 target genes are highly related in function. They were associated with the function of inhibitory synapses, post-synaptic and presynaptic structures, as well as signaling and protein synthesis, suggesting that a single splicing factor regulates isoform expression of different genes in inhibitory neurons. It is likely that other cell-type-specific splicing factors also control biologically coherent functions.

Most alternative splicing events can be classified into five basic splicing patterns: cassette exons, alternative 5′ splice sites, alternative 3′ splice sites, mutually exclusive cassette exons and retained introns (Fig. 1B). An estimated 75% of all alternative splicing patterns change the coding sequence (Kan et al., 2001; Okazaki et al., 2002; Zavolan et al., 2003). The alternative usage of internal cassette exons is the most prominent splicing pattern. More complicated alternative splicing patterns consist of combined basic patterns and are frequently observed, e.g., in the simultaneous skipping of multiple exons in the CD44 gene (Screaton et al., 1992), combination of intron retention in cassette exons of the splicing factor 9G8 (Popielarz et al., 1995) and SFRS14 (Sampson and Hewitt, 2003) and multiple alternative 3′ splice sites in the gene encoding SC35 (Sureau and Perbal, 1994). The regulation of alternative pre-mRNA splicing is further complicated by its coupling with other RNA processing steps, such as transcription (Cramer et al., 2001), RNA export (Reed and Hurt, 2002) and polyadenylation (Soller and White, 2003).

1.3. Plasticity of splice-site selection

It is frequently observed that alternative exon usage changes during development or cell differentiation (Stamm et al., 2000), both in vivo and in cell cultures. A growing list of external stimuli has been identified that changes alternative splicing patterns. These stimuli can be sub-grouped into receptor stimulation, cellular stress (pH, temperature, metal ion and osmotic conditions) and changes in neuronal activity (Kaufer et al., 1998; Akker et al., 2001; Stamm, 2002). In most cases, these changes are reversible, indicating that they are part of a normal physiological response (Stamm, 2002). In several cases, the mechanism leading to changes in alternative splicing is, at least partially, understood (Matter et al., 2002; Stamm, 2002) and involves changes in the phosphorylation of splicing factors, which influences their ability to bind to RNA or to other splicing factors. Since splicing factors appear to regulate coherent biological functions (Ule et al., 2003), changing their activity will most likely result in a coordinated response to the stimulus that triggered the initial phosphorylation signal. Using this mechanism, the mRNA expression of different, seemingly unrelated genes can be coordinated. In an attempt to understand the functional differences of isoforms generated by alternative splicing, we review the consequent differences in function reported in the literature. These data are updated and are available in the Internet (http://www.ebi.ac.uk/asd/index.html) as part of the Alternative Splicing Database (ASD; Thanaraj et al., 2004).

2. Function of alternative splicing

Gene regulation through alternative splicing is more versatile than is regulation through promoter activity. Variant transcripts generated through alternative splicing, similar to those initiated from distinct promoters, are often tissue and/or developmental specific, resulting in effects seen only in certain cells or developmental stages. However, changes in promoter activity alter predominantly the expression levels of the mRNA. In contrast, changes in alternative splicing can modulate transcript expression levels by subjecting mRNAs to nonsense-mediated decay (NMD) and alter the structure of the gene product by inserting, or deleting, novel protein parts. The structural changes fall into three categories: introduction of stop codons, changes of the protein structure and changes in the 5′ or 3′ untranslated region. The effects caused by

alternative splicing range from a complete loss of function to subtle effects that are difficult to detect.

2.1. Introduction of stop codons

mRNAs that contain premature stop codons can be degraded by nonsense-mediated decay (NMD). The splicing machinery marks exon–exon junctions with a protein complex. NMD occurs when a stop codon is present more than 50–55 nucleotides upstream of the 3′-most exon–exon junction, in which case polysome associated Upf proteins interact with exon–junction–protein complex to elicit NMD. In contrast, a mRNA appears to be immune to NMD if translation terminates less than 50–55 nucleotides upstream of the 3′-most exon–exon junction or downstream of the junction, in which case translating ribosomes most likely remove proteins bound to the exon–junction (Maquat, 2002). An essential prerequisite for NMD to occur is that proteins are translated. In the absence of translation, a mRNA is not subject to NMD, even when premature stop codons fulfill the NMD criteria (Stoilov et al., 2004). About 25–35% of alternative exons introduce frameshifts or stop codons into the pre-mRNA (Stamm et al., 2000; Lewis et al., 2003). Since approximately 75% of these exons are predicted to be subject to nonsense-mediated decay, an estimated 18–25% of transcripts will be switched off by stop codons caused by alternative splicing and nonsense-mediated decay (Lewis et al., 2003). This process, which has been termed RUST, for regulated unproductive splicing and translation, currently represents the function of alternative splicing with the most obvious biological consequences. The exact number of genes affected by RUST is only a crude estimate, as mRNAs undergoing nonsense-mediated decay will be unstable and underrepresented in cDNA libraries, which would result in an underestimation of RUST. Detailed analyses of the polypyrimidine tract binding protein indicates that RUST can be autoregulated (Wollerton et al., 2004). The rules of nonsense-mediated decay have been understood only in recent years. Older database annotations of protein isoforms did not take this mechanism into account and include protein isoforms that might not be expressed at all in a cell. Therefore, it should be tested whether protein isoforms created by transcripts with premature stop codons really exist as proteins.

2.2. Addition of new protein parts

Approximately 75% of alternative splicing events occur in the translated regions of mRNAs and will affect the protein-coding region (Okazaki et al., 2002; Zavolan et al., 2003). Changes in the protein primary structure can alter the binding properties of proteins, influence their intracellular localization and modify their enzymatic activity and/or protein stability by diverse mechanisms. One commonly found mechanism is the introduction of protein domains that are subject to posttranslational modification, such as phosphorylation. The scale of the changes evoked by alternative splicing range from a complete loss of function to very subtle modulations of function that can be only detected with specialized methods.

2.2.1. Binding properties

Protein isoforms generated by alternative splicing differ in their binding properties, both to small molecular weight ligands, such as hormones, and to macromolecules, such as proteins or nucleic acids. The effect of alternative splicing ranges from a complete loss of binding to a 2- to 10-fold change in binding affinity. Often, isoforms that show a complete loss of binding exert a dominant-negative effect over isoforms that can still bind the ligand. Prominent examples are summarized in Table 1.

2.2.1.1. Binding between proteins and small ligands. Alternative splicing can delete binding domains or introduce structural changes that abolish binding activity. For example, alternative variants of the thyroid stimulating hormone (TSH) receptor are unable to bind TSH. These variants occur in TSH-secreting tumors and cause insensitivity to TSH (Ando et al., 2001). Other examples of loss of ligand binding due to alternative splicing include the following: latency-associated peptide-binding protein, which binds TGF-beta in an isoform-dependent manner (Koli et al., 2001); the dopamine D3 receptor, where a frameshift introduced by alternative splicing deletes the dopamine binding region (Nagai et al., 1993); the retinoic acid receptor hRXR beta3; where an isoform looses retinoic acid binding capacity and acts in a dominant negative way (Mahajna et al., 1997). Alternative splicing can further determine the ligand specificity of a receptor. A well-studied example is that of the fibroblast growth factor receptor gene FGFR-2, which creates two isoforms that differ by 49 amino acids in the extracellular domain. Depending on the presence of this domain, the receptor binds to both fibroblast and keratinocyte growth factor or only to fibroblast growth factor (Miki et al., 1992). The affinities between the modified protein and its ligand(s) can also be altered. The angiotensin II type 1 receptor isoforms show threefold differences in binding affinities (Martin et al., 2001). The binding of GDP to the olfactory G protein Galpha^{olf} is reduced when 80 amino acids are inserted through alternative splicing, leading to a sixfold lower activation of adenylate cyclase (Liu et al., 2001). In the adenylate cyclase-activating polypeptide type 1 receptor, a 24-amino-acid insertion causes a sixfold increase in ligand binding (Daniel et al., 2001). The binding of gonadotrophin releasing hormone (GnRH) to shorter splice variants of the GnRH receptor is reduced 4- to 10-fold (Wang et al., 2001), which abolishes signaling. The insulin receptor binds insulin with a twofold difference, depending on the inclusion of exon 11 (Sesti et al., 2001). In peptide-hormone systems, alternative splicing can change the peptide-ligand

Table 1
Alternative splicing events that influence binding properties

Gene name	Modulation of binding to (physiological effect)	Reference
4.1.R isoforms	Fodrin and actin	(Kontrogianni-Konstantopoulos et al., 2001)
Acetylcholinesterase	RACK1 (PKCII mobilization)	(Birikh et al., 2003)
Agrin	Achetylcholine receptor	(Hoch et al., 1993; Tseng et al., 2003)
Angiotensin II type 1 receptor	Angiotensin II	(Martin et al., 2001)
Ankyrin	Spectrin and tubulin	(Davis et al., 1992)
ApoE-Receptor2	Alpha macroglobulin	(Brandes et al., 2001)
B cell antigen receptor	Multimerisation	(Indraccolo et al., 2002)
Basic helix-loop-helix/per-arnt-sim	Promoter complex	(Pollenz et al., 1996)
Calcitonin/CGRP	Different hormone receptors	(Leff et al., 1987; Yeakley et al., 1993)
Dystrophin	Syntrophin	(Ahn and Kunkel, 1995)
Fibrinogen-420	AlphaMbeta2 and alphaxbeta2-integrins	(Lishko et al., 2001)
Fibroblast growth factor receptor gene FGFR-2	Keratinocyte growth factor	(Miki et al., 1992)
Fibronectin	Integrin alpha4beta1	(Mostafavi-Pour et al., 2001)
Fos	DNA/promoter	(Nakabeppu and Nathans, 1991)
Golgi-microtubule-associated-protein of 210 kDa	Golgi membranes	(Ramos-Morales et al., 2001)
Gonadotrophin releasing hormone receptor	Affinity to GnRH (no signaling)	(Wang et al., 2001)
Hepatocyte nuclear factor homeoproteins	Promoter complex (modulation or dominant negative)	(Bach and Yaniv, 1993)
Ikaros	DNA/ promoter	(Tonnelle et al., 2001)
Insulin receptor	Insulin	(Sesti et al., 2001)
Interferon regulatory factor-3a	DNA binding	(Karpova et al., 2001)
Latency associated peptide binding protein	TGF-beta	(Koli et al., 2001)
Lymphoid transcription factor LyF-1	DNA/ promoter	(Hahm et al., 1994)
Myosine phosphatase I	cGMP-dependent protein kinase I (type of muscle contraction)	(Khatri et al., 2001)
Neuronal cell adhesion molecule L1	TAX-1 and contactin	(De Angelis et al., 2001)
Norepinephrine transporter	Norepinephrine	(Kitayama et al., 2001)
Nuclear factor I	DNA/ promoter	(Mukhopadhyay et al., 2001)
Oct 2	DNA/promoter	(Lillycrop and Latchman, 1992)
Olfactory G protein golf alpha	Binding to GDP (activation of adenylate cyclase)	(Liu et al., 2001)
P73alpha	Promotor specificity	(Takagi et al., 2001)
PAX-8	Promoter complex	(Kozmik et al., 1993)
Peroxisomal import receptor 5	Type-2 peroxisomal targeting signal	(Dodt et al., 2001)
Pituitary adenylate cyclase-activating polypeptide type 1 receptor	Pituitary adenylate cyclase-activating polypeptide	(Daniel et al., 2001)
Retinoic acid receptor beta 3	Retinoic acid (dominant negative isoforms)	(Mahajna et al., 1997)
Schwannomin	Syntenin (tumor suppression)	(Jannatipour et al., 2001)
Staufen	dsRNA	(Monshausen et al., 2001)
Tau	Microtubule	(Luo et al., 2004)
Tenascin	Fibornectin	(Chiquet et al., 1991)
Tenascin-C	Alpha7beta1integrin (neurite outgrowth)	(Meiners et al., 2001; Mercado et al., 2004)
Thyroid hormone receptor beta	Thyroid hormone (hormone insensivity)	(Ando et al., 2001)
Type XIV collagen	Glycosaminoglycans (cell adherance),	(Imhof and Trueb, 2001)
Type(IV) collagen	Mulitimerisation	(Ball et al., 2001)
Vascular endothelial growth factor	Different receptors	(Park et al., 1993; Robinson and Stringer, 2001)
Wilm's tumor protein suppressor gene	Prostate apoptosis response factor par4	(Richard et al., 2001)

binding properties. The best mechanistically studied example is that of the calcitonin/CGRP gene (Lou and Gagel, 1999), which generates two different hormones from a single gene. From this gene, calcitonin-gene-related peptide (CGRP), a hormone acting as a vasodilator, is produced predominantly in neurons, whereas calcitonin is produced predominantly by the parafollicular cells of the thyroid gland, where it regulates calcium and phosphorus metabolism (Leff et al., 1987; Yeakley et al., 1993). Other well-studied examples are alternative variants of the vascular endothelial growth factor (VEGF), whish binds to isoform-specific receptors (Robinson and Stringer, 2001) and differ in their ability to interact with the extracellular matrix (Park et al., 1993).

2.2.1.2. Binding between proteins. Similar to the interaction between proteins and ligands, protein–protein interactions can be regulated by alternative splicing. Bind-

ing can be completely governed by alternative splicing through deletion or creation of binding domains (Davis et al., 1992; Hoch et al., 1993; Ahn and Kunkel, 1995; Lishko et al., 2001; Birikh et al., 2003; Tseng et al., 2003). In some cases, a novel binding site is created in the minor alternative form. Rather than completely deleting binding domains, they can be disrupted by the insertion of protein sequences (Ball et al., 2001; De Angelis et al., 2001; Mostafavi-Pour et al., 2001). Frequently, binding affinities are modulated. Depending on the alternative exon composition, the interaction between the 4.1R band protein and the fodrin/actin complex can be completely abolished or modulated twofold (Kontrogianni-Konstantopoulos et al., 2001). Often, multiple binding motifs are present in proteins and alternative splicing can control their number. For example, regulated expression of variants with either four or five low-density lipoprotein (LDL)-receptor ligand binding motifs modulates the activity of the ApoE-Receptor2, resulting in variants that can bind to reelin, but not alpha2-macroglobulin (Brandes et al., 2001). In tenascin C, alternative splicing determines the number of fibronectin type III domains (Puente Navazo et al., 2001), which regulates binding to fibronectin (Chiquet et al., 1991).

2.2.1.3. Binding between proteins and nucleic acids. Interaction of transcription factors with DNA can be modified by alternative splicing, which contributes to transcriptional regulation (reviewed by Lopez, 1995). The loss of binding between a transcription factor isoform and DNA can inhibit transactivation in a dominant-negative way if the binding-negative isoform can replace the binding-competent isoform in the transactivation complex. Examples include fosB (Nakabeppu and Nathans, 1991; Wisdom et al., 1992), hepatocyte nuclear factor homeoproteins (Bach and Yaniv, 1993), Oct-2, (Lillycrop and Latchman, 1992), basic helix-loop-helix/per-arnt-sim(Pollenz et al., 1996), p53 (Wolkowicz et al., 1995), AML-1 (Tanaka et al., 1995), ikaros (Tonnelle et al., 2001) and interferon regulatory factor-3a (Karpova et al., 2001). In addition to acting in a dominant negative fashion, alternative splice variants can also modulate transactivation (Hahm et al., 1994). Isoform-specific differences can vary between promoters (Mukhopadhyay et al., 2001; Takagi et al., 2001) or manifest only in certain tissues, as in the case of the prostate apoptosis response factor par4/WT1 system (Richard et al., 2001), which contributes to establishing cell-specific mRNA expression patterns. Frequently, alternative splicing does not directly affect DNA binding but modulates the formation of complexes between various transcription factors. This, in turn, regulates the affinity between transcription factor complexes and DNA (Ormondroyd et al., 1995; Kozmik et al., 1993). Similar to DNA, the binding properties of RNA binding proteins can be modulated by alternative splicing, e.g., of the staufen RNA binding protein (Monshausen et al., 2001).

2.2.2. Intracellular localization

Alternative splicing determines the intracellular localization of numerous proteins, usually by influencing localization signals or regulating the interaction of proteins with membranes. Protein isoforms that lack membrane binding properties can either accumulate in the cytosol or are secreted into the extracellular space in an extreme form of localization regulated by alternative splicing.

2.2.2.1. Insertion into membranes. An important property of proteins regulated by alternative splicing is their insertion into membranes (Table 2A). In most cases, localization in the membrane is an obvious property of a protein, since transmembrane domains can be accurately predicted from the primary structure. By deleting or interrupting transmembrane or membrane-association domains, non-membrane-bound isoforms are generated by alternative splicing. These soluble isoforms can be released from the cell, e.g., into the blood or the extracellular space, or they translocate into a different intercellular compartment. The soluble isoforms can lose the function of the membrane bound form, acquire new functions, exert dominant negative effects over the membrane bound forms or modulate the function of the membrane-bound form. Soluble isoforms can lose the ability to transduce signals (Kestler et al., 1995; Tone et al., 2001), they can be less stable (Garrison et al., 2001) or have a different effect on immune system modulation (Riteau et al., 2001). In numerous cases, the exact function of the soluble isoform has not been

Table 2A
Regulation of membrane binding through alternative splicing

Gene name	Reference
Acetylcholinesterase	(Meshorer et al., 2004)
Asialogly coprotein receptor	(Spiess and Lodish, 1986)
Attractin	(Kuramoto et al., 2001)
CD1	(Woolfson and Milstein, 1994)
CEPU-1/Neurotrimin	(Lodge et al., 2001)
Cyclo-oxygenase	(Chandrasekharan et al., 2002)
Fas	(Cascino et al., 1995)
Flt3 ligand	(Lyman et al., 1995)
Growth hormone receptor	(Rosenfeld, 1994)
HLA-G	(Riteau et al., 2001)
Immunoglobulin epsilon	(Zhang et al., 1994; Anand et al., 1997)
Inhibin binding protein	(Bernard and Woodruff, 2001)
Insulinoma-associated tyrosine-phosphatase-like protein	(Diez et al., 2001)
Interleukin-2 receptor	(Horiuchi et al., 1997)
Interleukin-4 receptor	(Blum et al., 1996)
Neurexin III alpha	(Ushkaryov and Sudhof, 1993)
Peptidylglycine alpha-amidating monooxygenase	(Eipper et al., 1993)
Qa-2	(Tabaczewski et al., 1994)
Soluble D-factor/LIF receptor	(Tomida, 1997)
ST2	(Tago et al., 2001)
Steel factor	(Miyazawa et al., 1995)
Vascular endothelial growth factor-1	(Shibuya, 2001)

All alternative splicing events listed change a membrane bound form into a soluble form.

determined (Eipper et al., 1993; Ushkaryov and Sudhof, 1993; Tabaczewski et al., 1994; Woolfson and Milstein, 1994; Zhang et al., 1994; Cascino et al., 1995; Anand et al., 1997; Horiuchi et al., 1997; Shibuya, 2001; Tago et al., 2001; Walker et al., 2001). If the soluble isoform retains the ability to bind a ligand, it can regulate the concentration and bioactivity of that ligand, which indirectly interferes with the function of the membrane-bound form. Such a regulation has been described for the interleukin 4 (IL-4) receptor and the growth hormone binding protein (Rosenfeld, 1994; Blum et al., 1996). Membrane-bound proteins often form multimers. The soluble forms can influence this multimerization. Neurite outgrowth (Lodge et al., 2001) and inhibin actions are influenced by this mechanism (Bernard and Woodruff, 2001). Some transmembrane proteins, e.g., the flt3 ligand are activated by proteolytic cleavage. By deleting the transmembrane region, alternative splicing can form constitutively active molecules that do not require the activation by proteases (Lyman et al., 1995). Several cases have been described where the majority of protein isoforms are soluble and membrane-bound forms are created by modifying the signal peptide into a transmembrane domain (Spiess and Lodish, 1986; Chandrasekharan et al., 2002; Meshorer et al., 2004). As in other cases of alternative splicing, the interaction between protein and membrane can be modulated and not completely abolished. For example, binding of the Golgi-microtubule-associated-protein of 210 kDa (GMAP-210) to Golgi membranes can be modulated twofold by alternative splicing (Ramos-Morales et al., 2001), and small changes in the phosphorylation kinetics evoked by the steel factor (Miyazawa et al., 1995) depend on membrane associations, which are modulated by alternative splicing.

The production of membrane-bound isoforms can be tissue-specific, which can lead to tissue-specific membrane bound epitopes causing specific autoimmunity reactions, as in the case of the insulinoma-associated tyrosine-phosphatase like protein (Diez et al., 2001).

2.2.2.2. Localization in different cellular compartments.

Receptors. Alternative splicing can determine the localization of proteins in various subcellular sites and organelles (Table 2B). Proteins can be sequestered into compartments, where they perform no function. This mechanism is widely used for receptor molecules, and alternative splicing can regulate their retention in membrane-enclosed compartments. For example, the inclusion of an endoplasmatic reticulum retention signal in the glutamate receptor 1B reduces the cell surface expression of this receptor and restricts its trafficking (Chan et al., 2001). One splice variant of the dopamine D2 receptor is retained more efficiently in the endoplasmatic reticulum than the other (Prou et al., 2001), which influences the overall dopamine D2 activity. Receptors are often internalized after binding to their respective ligand. This ligand-dependent internalization can be modulated by alternative splicing, as in the case of

Table 2B
Alternative splicing events that change the intracellular localization of proteins

Gene name	Change in localization	Reference
μ-Opioid-receptor	Isoform dependent internalization after opioid stimulation	(Koch et al., 2001)
Acetylcholinesterase	Nucleus/cytosol/membrane	(Soreq and Seidman, 2001; Perry et al., 2002)
Bach1	Nucleus/cytosol	(Kanezaki et al., 2001)
B-cell antigen receptor	Accumulation in the endoplasmatic reticulum rather than cell membrane, reduction of functional, cell-membrane-bound B-cell antigen receptore	(Indraccolo et al., 2002)
Beta-adrenergic receptor	Endocytosis and down-regulation of the receptor	(Wang and Ross, 1995)
CD40	Transmembrane domain, lack of signaling capacity	(Tone et al., 2001)
Dopamine D2 receptor	Retention in the endoplasmatic reticulum	(Prou et al., 2001)
E2F	Nucleus/cytosol	(De la luna et al., 1996)
Estrogen receptor	Internuclear, nuclear/cytosolic	(Pasqualini et al., 2001)
Glutamate receptor 1B	Endoplasmatic reticulum retention signal	(Chan et al., 2001)
Interleukin-6	Intracellular signaling domain	(Kestler et al., 1995)
Lens epithelium-derived growth factor	Internuclear	(Nishizawa et al., 2001)
Metabotrophic glutamate receptor 5	Accumulation in lamellipodia/filopodia in undifferentiated cells	(Mion et al., 2001)
Mouse spermine oxidase	Nucleus/cytosol	(Cervelli et al., 2004)
NF-kappa B	Nucleus/cytosol	(Grumont and Gerondakis, 1994)
Nitric oxide synthase	Membrane bound	(Brenman et al., 1997)
Pactolus	Unstable secreted form	(Garrison et al., 2001)
Pre-T cell receptor alpha chain	Surface expression of an mature signaling complex	(Ramiro et al., 2001)
SpSHR2	Nuclear/cytoplasmatic localisation	(Kontrogianni-Konstantopoulos and Flytzanis, 2001)
Thromboxane A2 receptor	Agonist-induced internalisation	(Parent et al., 2001)
TPIP	Membrane/cytosolic localization	(Walker et al., 2001)
Tyrosine phosphatase dPTP61F	Nucleus/cytosol	(McLaughlin and Dixon, 1993)
Wilson-disease protein	Golgi/cytosol	(Yang et al., 1997)
x-II ORF, HTLV-I	Nuclear/mitochondrial localization	(D'Agostino et al., 2001)

the μ-opioid receptor isoforms. These differ in opioid-induced desensitization, because they differ in their opioid-induced internalization, which might contribute to tolerance towards morphine (Koch et al., 2001). A similar mechanism takes place in the beta-1 adrenergic receptor (Wang and Ross, 1995) and thromboxane A2 receptor isoforms (Parent et al., 2001). B-cell antigen receptors lacking the extracellular domain are unable to heterodimerize, which results in their accumulation in the endoplasmatic reticulum rather then their expression in the plasma membrane (Indraccolo et al., 2002). As a result, these isoforms are unable to signal from the cell surface. Finally, alternative splicing changes the properties of receptors that import proteins into membrane-enclosed organelles. For example, only one isoform of the peroxisomal import receptor 5 (PEX5L) can interact with proteins containing the type-2 peroxisomal targeting signal (Dodt et al., 2001), which can potentially influence the localization of several proteins.

Other proteins. The nuclear localization and function of transcription factors can be regulated by alternative splicing. The tissue-dependent deletion of a leucine-zipper results in a BACHt transcription factor that is nuclear, whereas the factor containing the leucine-zipper is cytosolic. Alternative splicing-dependent localization regulates the activity of E2F and NF-kappa B in a similar way (Grumont and Gerondakis, 1994; De la luna et al., 1996). Other protein classes are affected as well: casein kinase II isoforms contain a classical nuclear localization signal in their alternative exons, which governs their localization (Fu et al., 2001), HTLV-I proteins derived from the x-II ORF can be either nuclear or mitochondrial (D'Agostino et al., 2001), phosphatases can be either nuclear or cytoplasmatic (McLaughlin and Dixon, 1993). Acetylcholinesterase can adhere to synapses, associate with the red blood cell membrane or be secreted, depending on the alternative usage of a 3′ exon (Soreq and Seidman, 2001) within the cells. Its retained intron in the readthrough-form dictates cytoplasmic rather than nuclear accumulation (Perry et al., 2002, 2004). Due to alternative splicing, cytosolic proteins, such as the spermine oxidase, can accumulate in the nucleus, where they exert novel functions (Cervelli et al., 2004).

Sublocalisation within an organelle. Alternative splicing can regulate the sublocalisation of a protein within an organelle. In the nucleus, the localization of proteins in different nuclear substructures, such as in the nucleoplasma and speckles, can be regulated by alternative splicing (Nishizawa et al., 2001). In the cytosol, MEK5 localization can be either granular or diffuse, depending on the splicing variants (English et al., 1995). Due to alternative splicing, a protein can be targeted to several compartments. Isoforms of the estrogen-receptor alpha can be either nuclear, cytosolic or in both the cytosol and nucleus, depending on which exon is used (Pasqualini et al., 2001). Differences in intracellular localization can occur only in certain cells or during particular developmental stages: the localization of GluR5b and GluR5a isoforms are different in undifferentiated neurons, but identical in differentiated neurons (Mion et al., 2001), where these isoforms differ in their ability to promote neurite outgrowth. Similarly, differences in the localization of SpSHR2 splice variants are obvious only at the 16-cell stage of sea urchin development, whereas both forms are nuclear at the 4-cell stage (Kontrogianni-Konstantopoulos and Flytzanis, 2001). As with most cases of alternative splicing, quantitative effects can be detected. MHC class II invariant chain isoforms are transported into the endocytic pathway. Due to alternative splicing, endoplasmatic retention signals can be introduced that change the speed of endocytosis (Arunachalam et al., 1994). Finally, the intracellular localization of mRNA can be regulated by alternative splicing (Hannan et al., 1995).

2.2.3. Enzymatic and signaling activities

Alternative splicing modulates all aspects of enzymatic activity, such as affinity, substrate specificity, catalytic properties, V_{max} and activity regulation (Table 3A). A frequent mechanism to change enzymatic activity is the inclusion of a stop codon prior to the sequence encoding the active center. This mechanism is found in functionally diverse enzymes (Swaroop et al., 1992; Zheng and Guan, 1993; Wang et al., 1994; Fernandes et al., 1995; Gasdaska et al., 1995; Horiuchi et al., 2000; Li and Koromilas, 2001). The deletion of protein parts that are necessary for catalysis has similar effects, e.g., in the aromatic L-amino acid decarboxylase (O'Malley et al., 1995). Often, these inactive variants have dominant negative effects over the catalytically active forms (Li and Koromilas, 2001; Stasiv et al., 2001).

Table 3A
Regulation of enzymatic and signaling activities by alternative splicing

Gene name	Biological effect	Reference
Aromatic L-amino acid decarboxylase	Generation of an inactive variant	(O'Malley et al., 1995)
Cytochrome *P*450F3	Substrate specificity	(Christmas et al., 2001)
DT-diaphorase	Deletion of substrate binding site	(Gasdaska et al., 1995)
Human granulocyte-macrophage colony-stimulating factor receptor	Different phosphorylation substrate, activation of different signaling pathways	(Lilly et al., 2001)
Interferon-inducible protein kinase PKR	Deletion of kinase domain	(Li and Koromilas, 2001)
Interleukin-1 receptor-associated kinase	Autophosphorylation	(Jensen and Whitehead, 2001)
Nitric-oxide synthase	Shorter variants that act dominant negative	(Stasiv et al., 2001)
Terminal deoxynucleotidyl transferase	Enzymatic less active variants can modulate the more active isoforms	(Benedict et al., 2001)

2.2.3.1. Receptor molecules. Isoforms lacking signalling activity are frequently generated from receptor molecules (Table 3B). In these cases, the intracellular domain is altered, which inhibits ligand-dependent intercellular signaling. For example, the expression of a truncated human epidermal growth factor receptor (HER2; neu/c-erB-2) variant inhibits growth-factor-mediated tumor progression (Aigner et al., 2001), nonfunctional LH-receptors without intracellular signaling domain are generated (Sokka et al., 1992; Kestler et al., 1995), activation of the Tcf/beta-catenin pathway depends on the alternative splicing of the FP prostanoid receptor (Fujino and Regan, 2001), deletion of the intracellular domain of the erythropoietin receptor stimulates apoptosis (Nakamura et al., 1992), and the deletion of the intracellular kinase domain in trkB, trkC and the PDGF-alpha receptor abolishes signaling (Mosselman et al., 1994; Menn et al., 1998; Stoilov et al., 2002a). Similarly, receptor coupling to intracellular molecules can be changed. Coupling of the dopamine D2 and serotonin receptors to specific G proteins is regulated by alternative splicing (Guiramand et al., 1995; Wang et al., 2000). Changes in coupling are also found in other systems: only certain splice variants of the somatostatin receptor 2 and vasopressin receptor V2 couple to adenylate cyclase (Reisine et al., 1993; Firsov et al., 1994). Since receptors are composed of multiple subunits, changes in one subunit can have indirect effects that are difficult to detect (Wagner et al., 2001). Again, alternative splicing can dictate both the complete loss of function as well as its modulation by inserting novel sequences into the intracellular domain, as in the case of CD46 (Purcell et al., 1991) or the prostaglandin E receptor EP3 that differ in their coupling efficiencies to adenylate cyclase (Harazono et al., 1994).

2.2.3.2. Other proteins. Similar to other cases of alternative splicing regulation, subtle changes in activity can be achieved. The terminal deoxynucleotidyl transferase gene produces isoforms with different enzymatic activities. Experiments in transgenic mice show that the less active variant can modulate the activity of the more active variant by an unknown mechanism (Benedict et al., 2001). Isoforms of the granulocyte-macrophage colony-stimulating factor receptor alpha subunit slightly differ in their ability to phosphorylate and activate Jak-2. Both K_m and V_{max} of the norephinephrine transporter can be changed two- to three-fold, depending on the splice variant (Kitayama et al., 2001). Substrate specificity can also be altered by alternative splicing domains. Alternative splice variants of the cytochrome *P*450 systems CYP4F3 prefer either leukotriene B_4 or arachidonic acid as a substrate (Christmas et al., 2001). The molecular mechanism for these more subtle changes is mostly not understood, but the crystal structure of the UDP-*n*-acetylglucosamine pyrophosphorylase demonstrated that modifications near the active site result in modified multimerisation, which influences the activity (Peneff et al., 2001).

2.2.4. Protein stability

The inclusion of alternate protein domains can regulate the half-life of proteins (Table 4). In the alternative form of human thyroperoxidase (TPOzanelli), the half-life is reduced from 11 to 7 h. Interestingly, this form accumulates in Grave's disease (Niccoli-Sire et al., 2001), but the molecular mechanisms leading to disease are unclear. Protein stability can be altered due to autophosphorylation that signals the degradation of receptor molecules. For the interleukin-1 receptor-associated kinase, this autophosphorylation-dependent degradation is isoform specific, leading to a molecule that is not down-regulated by its ligand (Jensen and Whitehead, 2001). Stability signals can be introduced by sequences that are subsequently cleaved by proteases, as in the case of the soluble secreted endopeptidase. If the cleavage site is present, the protein is cleaved in the endoplasmatic reticulum and exported, whereas the splice variant resides in the endoplasmatic reticulum (Raharjo et al., 2001). Since endoproteolytic sites are

Table 3B
Alternative splicing-induced changes of intracellular receptor domains

Gene name	Biological effect	Reference
Dopamine D2 receptor	Coupling to G proteins	(Guiramand et al., 1995)
Erythropoietin receptor	Deletion of the intracellular domain, apoptosis	(Nakamura et al., 1992)
FP prostanoid receptor	Coupling to signal transduction pathways	(Fujino and Regan, 2001)
Granulocyte-macrophage colony stimulating factor (GM-CSF), beta-subunit	Control of cell proliferation	(Wagner et al., 2001)
HER2 (neu/c-erB-2)	(In)sensitivity towards growth factors	(Aigner et al., 2001)
MEK1	Autophosphorylation activity	(Zheng and Guan, 1993)
Membrane cofactor protein, CD46	Addition of sequences	(Purcell et al., 1991)
PDGF-alpha receptor	Tyrosine phosphorylation activity	(Mosselman et al., 1994)
Prostaglandin E receptor EP3	Coupling to adenylate cyclase	(Harazono et al., 1994)
Serotonin 2C receptor	Coupling to G proteins	(Wang et al., 2000)
Somatostatin 2 receptor	Coupling to adenylate cyclase	(Reisine et al., 1993)
TrkB	Deletion of the intracellular kinase domain	(Stoilov et al., 2002a)
TrkC	Deletion of the intracellular kinase domain	(Menn et al., 1998)
Vasopressin V2 receptor	Coupling to adenylate cyclase	(Firsov et al., 1994)

Table 4
Change of protein stability by alternative splicing

Gene name	Biological effect	Reference
C-Fos	Increased half-life	(Nestler et al., 1999)
Interleukin-1 receptor-associated kinase	Influences ligand dependent half-life	(Jensen and Whitehead, 2001)
Peptidylglycine alpha-amidating monooxygenase	Blockage of a endoproteolytic site necessary for function	(Eipper et al., 1993)
Protein kinase C delta	Proteolytic cleavage site	(Sakurai et al., 2001)
Soluble secreted endopeptidase	Proteolytic cleavage site	(Raharjo et al., 2001)
Thyroperoxidase	35% reduction of half-life	(Niccoli-Sire et al., 2001)

required for protein function, their modulation through alternative splicing can control the activity of a protein, as in the case of the peptidylglycine alpha-amidating monooxygenase (Eipper et al., 1993). The destruction of a cleavage-sensitive site through alternative splicing is also possible: A 78 bp insertion into a caspase-3-sensitive site generates a protease-insensitive protein kinase C delta isoform (Sakurai et al., 2001). The effect of alternative-dependent protein stability has best been studied for the c-*fos* gene, that generates a shorter isoform that is more stable than the full length protein. This isoform accumulates under chronic behavioral changes in the brain, which could be part of a molecular memory process (Nestler et al., 1999).

2.2.5. Insertion of domains that are subject to posttranslational modification

Posttranslational modifications can be dictated by alternative splicing, usually by generating consensus sites for phosphorylation, glycosylation, palmitoylation or sulfatation (Table 5). In addition, binding between a kinase and its substrate can be regulated by alternative splicing, e.g., binding of the SR-protein kinase 1 to scaffold attachment factor B (Nikolakaki et al., 2001). The biological role of these posttranslational modifications has been understood in several systems. For example, isoform-dependent phosphorylation of the potassium channel Kv4.3 allows the modulation of outward currents by the alpha-adrenergic system via protein kinase C (Po et al., 2001). Similarly, isoform-specific attachment of acetylcholinesterase isoforms to membranes involves covalent interaction with a structural collagen-like subunit for the synaptic isoforms, glycophosphoinositide interaction of the "erythrocytic" isoforms or no C-terminal interaction at all for the different 3′ splice variants (Soreq and Seidman, 2001).

2.2.6. Change of ion-channel properties

The majority of changes evoked by alternative splicing are subtle and often difficult to detect. Exceptions are those changes that occur in transcripts encoding ion channels. These are rather frequent, because the properties of ion channels can be precisely measured by electrophysiological methods, which allow the detection of even small changes (Table 6). Furthermore, alternative splicing is abundantly used in the brain (Lander et al., 2001; Modrek and Lee, 2002). Almost all aspects of ion channel functions can be altered by alternative splicing, including channel inactivation, steady-state kinetics, voltage dependency, desensitization time and ligand binding (Iverson et al., 1997; Chemin et al., 2001a; Decher et al., 2001; Tian et al., 2001a,b). As in other cases, the effects can range from complete loss of function to subtle effects. The permeability of ions can be completely abolished, as in the case of the acid-sensing ion channel (Bassler et al., 2001), or it can be modulated, as in the case of the KCNQ2 channel, the activation time of which is reduced by two- to fivefold (Pan et al., 2001). Effects mediated by alternative splicing are often small: The desensitization time of glutamate receptors is altered fourfold, depending on the usage of a mutually exclusive exon (Mosbacher et al., 1994), and the conductance of slowpoke, a calcium-activated potassium channel, is reduced by 30% through alternative splicing (Lagrutta et al., 1994). Regulation through alternative splicing can be

Table 5
Insertion of posttranslational modification sites through alternative splicing

Gene name	Biological effect	Reference
Acetylcholinesterase	Membrane attachment	(Soreq and Seidman, 2001)
BACE-1	Glycosylation	(Tanahashi and Tabira, 2001)
Fibroblast growth factor receptor	Protein kinase C site	(Gillespie et al., 1995)
Kv4.3	Protein kinase C site	(Puente Navazo et al., 2001)
Myosin light chain kinase	Src-phosphorylation site, regulates enzymatic activity	(Birukov et al., 2001)
Nucleoside transporter ENT1	Casein kinase site	(Handa et al., 2001)
P53	Casein kinase II site	(Han and Kulesz-Martin, 1992)
Protein kinase B gamma	Ser-phosphorylation site, regulates enzymatic activity in an insulin dependent manner	(Brodbeck et al., 2001)
SNAP-25	Palmitylation	(Bark, 1993)

Table 6
Changes in ion-channel properties through alternative splicing

Gene name	Biological effect	Reference
Acid-sensing ion channel	Ca permeability	(Bassler et al., 2001)
Alpha-Bungarotoxin-sensitive nicotinic receptors	Splice variant inhibits function of the full length channel	(Garcia et al., 1995)
Glutamate receptor	Fourfold change in desensitization time	(Mosbacher et al., 1994)
KchIP	Onset of current activation, recovery from inactivation	(Bahring et al., 2001)
KchIP2	Half-maximal inactivating voltage	(Decher et al., 2001)
KCNQ2	Deactivation time changed by two- to fivefold	(Pan et al., 2001)
Large-conductance Ca^{2+}-activated voltage-dependent K^+ channel	Modulation of sensitivity towards Ca^{2+} and voltage	(Korovkina et al., 2001)
Large-conductance Ca^{2+}- and voltage-activated K^+ channel (BK)	Glucocorticoid and phosphorylation sensitivity	(Tian et al., 2001a,b)
Slowpoke	Conductance changed by approx. 30%	(Lagrutta et al., 1994)
T-type Ca channel	Kinetics, voltage dependency	(Chemin et al., 2001a)
Tuca1	Developmentally controlled Ca permeability	(Okagaki et al., 2001)

indirect, involving channel interacting proteins with isoform-specific modulation activities (Bahring et al., 2001). Alternative splicing of channel isoforms is often subject to developmental control, leading, e.g., to developmental-specific calcium influxes (Okagaki et al., 2001). The observed changes caused by alternative splicing are cell-type specific in several cases, which reflects the cell-type specificity of the alternative exon (Chemin et al., 2001b; Korovkina et al., 2001).

2.3. Influence on mRNA function

Few examples have been described where alternative splicing functions by influencing the properties of the mRNA. Alternative splicing events occur in 5′ and 3′ UTRs. A common theme there is the stability of the RNA. For example, alternative exons in the 5′ UTR of the HIV-1 virus can either promote or inhibit the nuclear degradation of their surrounding mRNA, which regulates HIV-1 gene expression (Krummheuer et al., 2001), and alternative splicing of SC35 regulates its mRNA stability (Sureau et al., 2001). Splice variant transcripts can be targeted to specific subcellular sites, such as axons or dendrites (Meshorer et al., 2004). Such targeting may further depend on cellular activities, e.g., neuronal activation under stress (Meshorer et al., 2002).

2.4. Examples of coordinated changes in biological systems

2.4.1. Isoform differences

The previous examples demonstrate the influence of alternative splicing on the molecular properties of proteins. In numerous cases, biological assays demonstrated the dependence of complex biological systems on alternative splicing. For example, tonic and phasic muscle contractions depend on the interaction between myosin phosphatase and cGMP-dependent protein kinase I, which is regulated by an alternative exon in a leucine-zipper (Khatri et al., 2001). The neurite-growth promoting activity of NCAM depends on a single alternative exon (VASE; Doherty et al., 1992). Ca^{2+}-dependent cell aggregation mediated by platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) depends on a binding motif encoded by an alternative exon (Yan et al., 1995). Studies in transgenic mice showed that small differences between isoforms can have dramatic effects. For example, the loss of one of the c-*ret* protooncogene kinases isoforms leads to kidney malformation and loss of ganglia in the enteric nervous system, whereas loss of other isoforms has no effect (de Graaff et al., 2001). Overexpressed synaptic acetylcholinesterase induced progressive accumulation of neuronal hallmarks of stress (e.g., HSP70), whereas the "readthrough" 3′-variant acetylcholinesterase attenuated such phenomena in transgenic mice (Sternfeld et al., 2000). Protein isoforms generated by alternative splicing can vary significantly between the members of different populations, where the expression of isoforms is correlated with a specific phenotype. Dragonflies from ponds separated by 16 km differ largely in their troponin T transcript variations. The composition of troponin T variants is correlated with complex physiological parameters, like the Ca^{2+} sensitivity of skinned fibers and the power output of flight muscles. Since dragonflies depend on their flight performance for hunting and mating, alternative splicing can be used to optimize the aerial performance and energetic costs, depending on the current habitat (Marden et al., 2001). These examples show that alternative splicing controls complex biological features. The best understood example for the role of alternative splicing in an organism is the determination of the sex of somatic cells in *Drosophila* that has been comprehensively reviewed (Baker, 1989; Schutt and Nothiger, 2000; Forch and Valcarcel, 2003). Another well-characterized system that demonstrates the importance of alternative splicing is programmed cell death, apoptosis, which also has been recently reviewed in detail (Jiang and Wu, 1999; Wu et al., 2003).

2.4.2. Missplicing events in disease

Since alternative splicing regulates isoform formation and controls numerous cellular functions, it is not surprising that missplicing events can cause or contribute to human diseases (Philips and Cooper, 2000; Faustino and Cooper, 2003). Diseases can be either caused by mutations in regulatory pre-mRNA sequences and regulatory factors (Krawczak et al., 1992; Cooper and Mattox,

Table 7
List of database resources on alternative splicing

Database	Entry mode and notes	Species covered	Reference	URL
(I) Databases on alternative splicing				
AEDB—Alternative exon database	Manually created from literature	All animals	(Stamm et al., 2000; Thanaraj et al., 2004)	http://www.ebi.ac.uk/asd/aedb/index.html
ASD—Altsplice (and AltExtron)	Computationally generated (delineating from gene-EST/mRNA alignments)	Human and other model species	(Clark and Thanaraj, 2002; Thanaraj et al., 2004)	http://www.ebi.ac.uk/asd//
AltRefSeq	Computationally generated (RefSeq genes with detected alternative splicing patterns)	Human	(Kan et al., 2001)	http://sapiens.wustl.edu/~zkan/TAP/ALTSEQ.html
ASDB: database of alternatively spliced genes	Computationally generated (from SwissProt protein and EMBL nucleotide sequence databases)	All animals	(Gelfand et al., 1999; Dralyuk et al., 2000)	http://cbcg.nersc.gov/asdb
EASED	Computationally generated (by examining high-scoring ESTs to mRNA alignments)	Human and other species	(Pospisil et al., 2004)	http://eased.bioinf.mdc-berlin.de//
AsMAMDB	Computationally generated (by examining EST/mRNA alignments with gene sequences)	Mammalian	(Ji et al., 2001)	http://166.111.30.65:100/
ASAP	Computationally generated (through mapping of unigene clusters of EST sequences to genes)	Human	(Lee et al., 2003)	http://www.bioinformatics.ucla.edu/ASAP/
MouSDB-Splice variants in mouse transcriptome	Computationally generated (through mapping of RIKEN's full length mouse cDNA sequences and dbEST mouse sequences to genes)	Mouse	(Zavolan et al., 2003)	http://genomes.rockefeller.edu/MouSDB/
Intronerator-Alt-Splicing Catalog	Computationally generated (through mapping of EST/cDNAs with genes)	*C. elegans*	(Kent and Zahler, 2000)	http://www.cse.ucsc.edu/~kent/intronerator/altsplice.html
EnsEMBL	Computationally generated (data presented as alternate transcripts identified through a series of computational tools	Human and other species	(Birney et al., 2004)	http://www.ensembl.org/
VARSPLICE records in Swiss-Prot	Manual (through submissions by experimentalists) and Computational. (through similarity searches)	Human and other species	(Birney et al., 2004)	http://www.ebi.ac.uk/swissprot/
EMBL-BANK nucleotide sequence database	Manual (through submissions by experimentalists), Annotation of Alternative exons or Alternative splicing feature lines in	Human and other species	(Kulikova et al., 2004)	http://www.ebi.ac.uk/embl/
(II) Databases that are used often in the derivation of data on alternative splicing				
SANBI STACK	EST clusters and consensus sequences	Human	(Christoffels et al., 2001)	http://www.sanbi.ac.za/Dbases.html
TIGR gene indices	EST clusters and contigs	Human and other species	(Quackenbush et al., 2001)	http://www.tigr.org/tdb/tgi/
SANBI's eVOC	Set of detailed human terms/vocabularies that describe the sample source of human experimental material such as cDNA and SAGE libraries	Human	(Kelso et al., 2003)	http://www.sanbi.ac.za/evoc/
(III) Data sets on splice regulatory proteins, sequences and diseases				
Human splicing factor database for	Manually created-collection of splice variants from various resources. Presentation of data for array oligo design	Human	(Stamm et al., unpublished)	To be made available soon at http://www.ebi.ac.uk/asd
Drosophila splicing protein database	Manually created. Information on gene sequences and comparisons with other species	*Drosophila*	(Mount and Salz, 2000)	http://www.wam.umd.edu/~smount/DmRNAfactors/table.html
RRM-containing proteins	Computer generated-collection of proteins containing RNA recognition motifs	Metazoan splicing factors	(Bateman et al., 2004)	http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00076
Splice-site mutations	Manual	Human	(Nakai and Sakamoto, 1994)	http://www.hgc.ims.u-tokyo.ac.jp/~knakai/

1997) or by changes in the relative concentration of regulatory factors, which depends on the allelic composition (Nissim-Rafinia and Kerem, 2002). In many disorders, such as cancer or Alzheimer's disease, alternative splicing patterns of functionally distinct genes are altered, raising the question whether changes in alternative splicing are the cause or consequence of a disease (Stoilov et al., 2002b). Aging events, in particular, are associated with alternative splicing modulations (Meshorer and Soreq, 2002).

2.5. Bioinformatic resources

With the realization that alternative splicing acts as an important regulatory mechanism of gene expression, a number of large-scale efforts are emerging to create resources on splice variants and alternate transcript structures. The resources are of two types: value-added data on alternative splice events and computational tools to decipher the splice signals. Table 7 presents the list of data resources on alternative splicing and those that are used in the

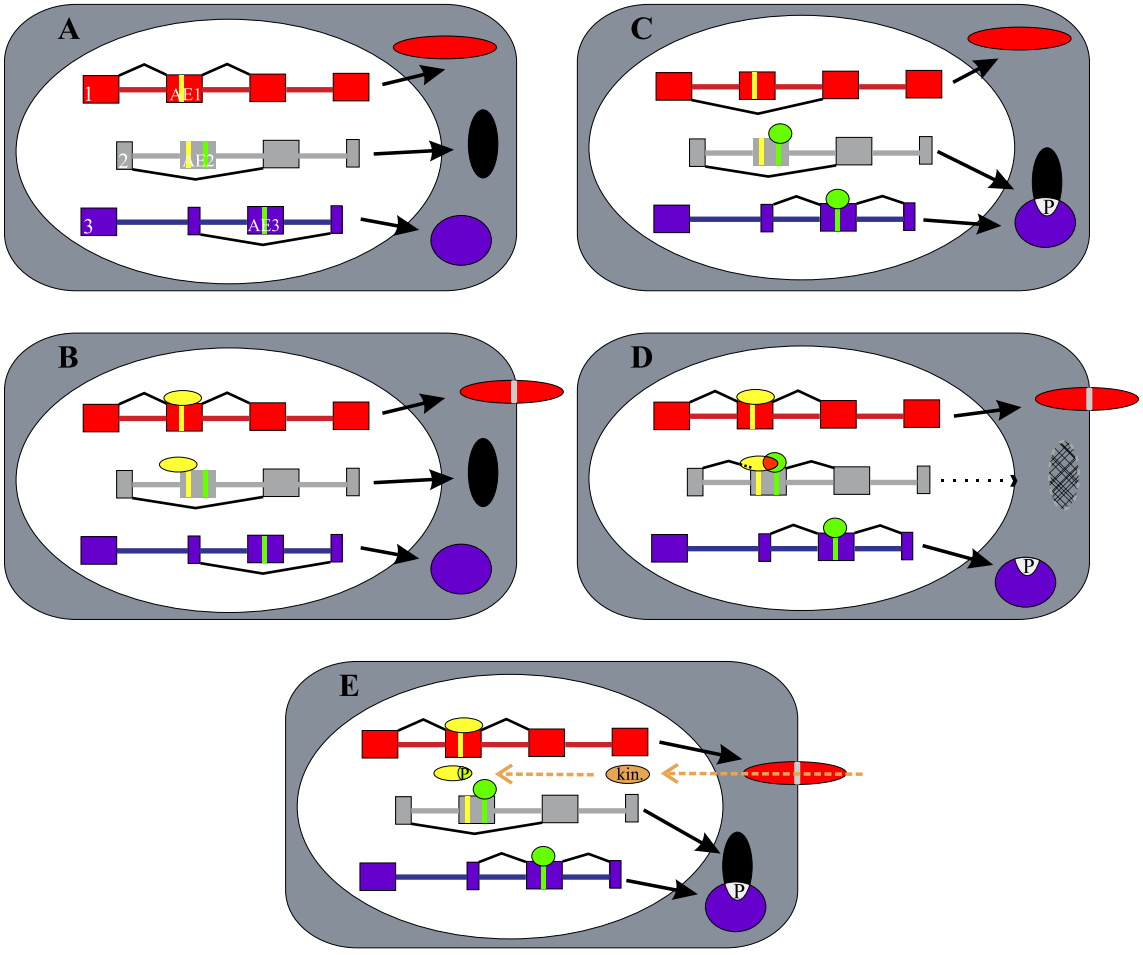

Fig. 2. Alternative splicing contributes to cell identity by generating complex protein expression patterns from a limited number of genes. Cells are indicated as large squares; the cell nucleus is indicated as a large circle. Three genes are shown in the nucleus and are indicated by different colors. Proteins generated by these genes are shown in the same color on the right of each cell. Boxes indicate exons, horizontal lines show introns. Small ellipses indicate proteins. Each gene has an alternative spliced exon (AE1, AE2 and AE3). In this oversimplification, splicing regulation is achieved by regulatory proteins 1 (yellow ellipse) and 2 (green circle) that activate an exon after binding to the appropriate enhancer (yellow or green square in the exon). The splicing pattern of the alternative exon in each case is indicated. Exon AE1 encodes a transmembrane domain, AE2, a premature stop codon, and AE3, a phosphorylation site. (A) In cell type 1, none of the splicing regulatory proteins is expressed, leading to skipping of all alternative exons. Only noninteracting intracellular proteins are expressed. (B) In cell type 2, only the splicing regulatory protein 1 is expressed, resulting in the inclusion of AE1 and the expression of transmembrane protein (red). Binding to exon AE2 cannot activate the inclusion of this exon, as additional factors are needed (nonoccupied exon enhancer space). The factor does not influence gene 3, as it does not contain the appropriate enhancers. (C) In cell type 3, only the splicing regulatory protein 2 is expressed (green). It does not influence gene 1, because this gene does not contain its enhancer. As a result, exon AE1 is skipped, and a soluble cytoplasmic protein is produced (red). The factor cannot activate AE2 because additional factors are needed. It induces the alternative exon AE3 that encodes a phosphorylation site, which results in the binding of the protein encoded by gene 2 to the one made by gene 3. (D) In cell type 4, both splicing factors 1 and 2 are expressed, leading to the activation of all alternative exons. AE2 is activated because the binding of both factors is stabilized by the protein–protein interaction (red area between the splicing factors). Since exon AE2 encodes a premature stop codon, no protein is made (dashed circle). (E) External signals can change splice-site selection. Cell type 4 was stimulated, resulting in an ion influx into the cell (orange dashed line), which activates a kinase (kin) that phosphorylates the yellow splicing factor 2. As a result, exon AE2 is skipped and protein is made.

computational pipelines to predict splice events. Data on splice events are derived through three approaches: (i) manual collection of experimentally reported alternative exons from peer-reviewed journals; (ii) computational delineation of alternative exons on gene sequences by using the transcript resources, such as ESTs and full-length mRNA sequences; (iii) high-throughput techniques, such as DNA microarrays, and transcriptome projects.

Computational tools include general tools that are used in the pipelines that predict splice events, tools that predict splice regulatory sequences. The ab initio computational prediction of alternative splice events is still at the developmental stages. However, comparative sequence data analysis is emerging as a major approach to predict human splice variants from the knowledge of splice variants from other species. The functional data presented here are collected at the ASD website, where new entries can be made (http://www.ebi.ac.uk/asd/).

3. Conclusion and perspective: alternative splicing is an important mechanism to generate cell-specific protein patterns

Alternative splicing emerges as one of the most important mechanisms regulating gene expression in multicellular organisms. Transcriptional regulation of the promoter primarily modulates the amount of RNA and generates N-terminal protein variants through different transcriptional start sites. In contrast, regulation through alternative splicing is much more versatile. Due to the insertion of premature stop codons, mRNA isoforms can be efficiently eliminated, which regulates the amount of mRNA. In contrast to transcriptional control, alternative splicing changes the structure of the mRNAs and their encoded proteins. As summarized previously, changes in protein sequence can influence almost all aspects of protein function, such as binding properties, enzymatic activity, intracellular localization, protein stability, phosphorylation and glycosylation patterns. Alternative splice-site regulation is achieved through the combination of multiple weak interactions between regulatory proteins and signals on the pre-mRNA. Since most interactions of regulatory factors with pre-mRNA are weak, the regulation of target pre-mRNAs by a specific factor also depends on the presence of other regulatory proteins that form specific multiprotein pre-mRNA complexes. Through these combinations, a small number of splicing factors regulate a large number of pre-mRNAs that encode protein isoforms with different biological properties. Fig. 2 illustrates how a few splicing regulatory proteins can establish cell-specific expression patterns. It is assumed that two factors regulate three genes. Depending on the expression of regulatory factors, five different cell 'types' expressing various protein isoforms with different properties can be formed. In this example, the binding to a membrane, the generation of the protein and the binding to other proteins are regulated by alternative splicing (Fig. 2, examples in Tables 2A and 1). Since phosphorylation events can influence the assembly of regulatory complexes, the formation of alternative protein isoforms can be regulated by external stimuli that influence phosphorylation patterns (Fig. 2E), which further increases the number of cell-type-specific protein combinations. At first glance, the expression of different protein isoforms appears completely unrelated, but can be traced down to a limited set of regulatory factors, once the regulatory mechanism is understood (Fig. 2). The emerging determination of target genes showed that a biological meaningful network of genes is regulated by a tissue-specific splicing factor (Ule et al., 2003), resulting in coordinated responses of protein expression patterns to a single regulatory factor. Although most splicing factors are not tissue specific, but a have characteristic concentration in a certain tissue (Hanamura et al., 1998), it is possible that similar biological, meaningful responses exist to specific concentrations of factors.

To learn more about these mechanistically and functionally intriguing gene networks, we need to determine the physiological target genes of more splicing factors, create model systems that predict alternative exon regulation and assess the physiological role of the coordinated change in protein isoforms. Since alternative splicing pathways change continuously during physiological processes, it will be important to work out the signal transduction pathways leading to the spliceosome and determine their mechanisms of action.

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