channel kinetics, but that in the α subunit dictates slow-channel kinetics. Thus, the LCPs of four AChR subunits contribute in an asymmetric manner to optimize the activation of AChRs through allosteric links to the channel and to the agonist binding sites (Shen *et al.*, 2005).

The mutation αV285I introduces a bulky amino acid into the M3 transmembrane domain and causes FCCMS (Fig. 3). Kinetic studies demonstrate that the mutation slows the channel opening rate β and speeds the channel closing rate α, resulting in a 15.1-fold reduction in the channel gating equilibrium constant θ (= β/α). On the other hand, the mutation minimally affects affinity for ACh. The probability of channel openings decreased when we introduced Leu, a bulky amino acid, at position V285, but rather increased when we introduced smaller amino acids such as Thr and Ala. We observed similar effects when we introduced similar substitutions into the β, δ, and ε subunits. Thus, introduction of bulky amino acids narrows the channel pore, while introduction of smaller amino acids widens the channel pore. Our analysis thus revealed that the M3 domain backs up the channel-lining pore lined by the M2 transmembrane domains and has stereochemical effects on channel gating kinetics (Wang *et al.*, 1999).

FCCMS can be effectively treated with anticholinesterases and 3,4-diaminopyridine. The pharmacologic effects of these drugs were discussed in the section of endplate AChR deficiency (Section 3.1.2).

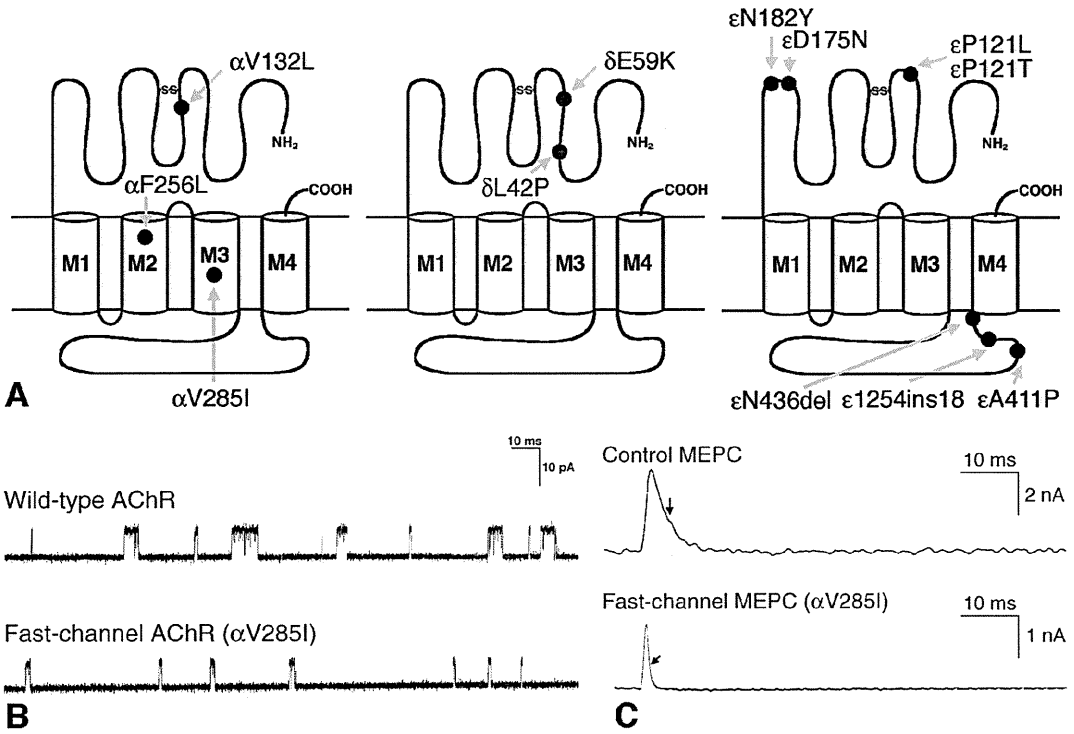

Fig. 3. Fast channel CMS. (A) Schematic diagram of AChR subunits with FCCMS mutations. (B) Single channel currents from wild-type and fast channel (αV285I) AChRs expressed on HEK293 cells. (C) Miniature endplate current (MEPC) recorded from endplates of a control and a patient harboring αV285I. The patient's MEPC decays faster than that of the normal control.

3.4 CMS due to defects in skeletal muscle sodium channel, $Na_V1.4$

Another class of postsynaptic CMS is due to mutations in skeletal muscle sodium channel, $Na_V1.4$, encoded by *SCN4A* (Tsujino *et al.*, 2003). Dominant gain-of-function mutations in this gene cause hyperkalemic periodic paralysis (Ptacek *et al.*, 1991), paramyotonia congenita (McClatchey *et al.*, 1992; Ptacek *et al.*, 1992), potassium-aggravated myotonia (Lerche *et al.*, 1993), and hypokalemic periodic paralysis type 2 (Bulman *et al.*, 1999). On the other hand, loss-of-function mutations cause a CMS.

Failure of normal-amplitude endplate potential depolarizing the resting potential to -40 mV in intercostal muscle of a CMS patient with episodes of apnea and myasthenic symptoms since birth prompted us to search for mutations in *SCN4A*. We identified two heteroallelic missense mutations, S246L and V1442E (Tsujino *et al.*, 2003). Activation kinetics of the mutant $Na_V1.4$ was normal for both S246L and V1442E, but the fast inactivation curves were shifted to hyperpolarization by 7.3 mV for S246L and 33.2 mV for V1442E, indicating that both mutations enhance fast inactivation of the $Na_V1.4$ immediately after it is activated. Moreover, a high proportion of the V1442 channel was in the inactivated state even at a normal resting membrane potential. Recovery from the fast-inactivated state was slowed for both mutations. This was in contrast to gain-of-function mutations in other diseases, which shift the fast inactivation curves to depolarization. Neither S246L nor V1442E affected slow inactivation. Analysis of use-dependent inactivation in HEK293 cells by stimulating at 50 Hz for 3 ms revealed that wild-type and S246L channels decreased the peak current only by 5% and V1442E channel decreased it by 30% during the first few pulses and suggested that the S246L mutation is relatively benign.

4. Synaptic CMS

Defects in three components of the synaptic basal lamina, AChE, β2 laminin and neural agrin, are associated with CMS. The CMS caused by mutations in agrin was discussed above under the postsynaptic CMS (Section 3.1.3) because the site of action of agrin is the LRP4/MuSK complex at the endplate.

4.1 Endplate AChE deficiency due to defects in collagen Q

Three tetramers of catalytic AChE subunits are linked by a triple helical collagen Q (ColQ) to constitute an asymmetric ColQ-tailed AChE (Krejci *et al.*, 1997). ColQ carries three domains (i) an N-terminal proline-rich attachment domain (PRAD) that organizes the catalytic AChE subunits into a tetramer, (ii) a collagenic domain that forms a triple helix, and (iii) a C-terminal domain enriched in charged residues and cysteines. ColQ-tailed AChE is organized in the secretory pathway, excreted, and anchored into the synaptic basal lamina using two domains of ColQ (Fig. 4). First, the collagen domain harbors two heparan sulfate proteoglycan (HSPG) binding domains (Deprez *et al.*, 2003) that bind to HSPG, such as perlecan (Peng *et al.*, 1999). Second, the C-terminal domain binds to MuSK (Cartaud *et al.*, 2004).

Endplate AChE deficiency is caused by congenital defects of ColQ (Donger *et al.*, 1998; Ohno *et al.*, 1998; Ohno *et al.*, 2000). Congenital defects of ColQ cause endplate AChE deficiency. No mutations have been detected in a gene encoding the catalytic subunit of AChE in CMS

or in any other disease. There are three classes of ColQ mutations. First, mutations in the proline-rich attachment domain (PRAD) hinder binding of ColQ to AChE. Sedimentation analysis of AChE species of the patient muscle and transfected cells shows complete lack of ColQ-tailed AChE. Second, mutations in the collagen domain, most of which are truncation mutations, hinder formation of triple helix of ColQ. Sedimentation analysis of muscle and transfected cells demonstrate a truncated single-stranded ColQ associated with a homotetramer of AChE. Third, the mutations in the C-terminal domain have no deleterious effect on formation of the asymmetric ColQ-tailed AChE, but they compromise anchoring of ColQ-tailed AChE to the synaptic basal lamina as elegantly shown in vitro overlay binding of mutant and wild-type human recombinant ColQ-tailed AChE to the frog endplate (Kimbell *et al.*, 2004).

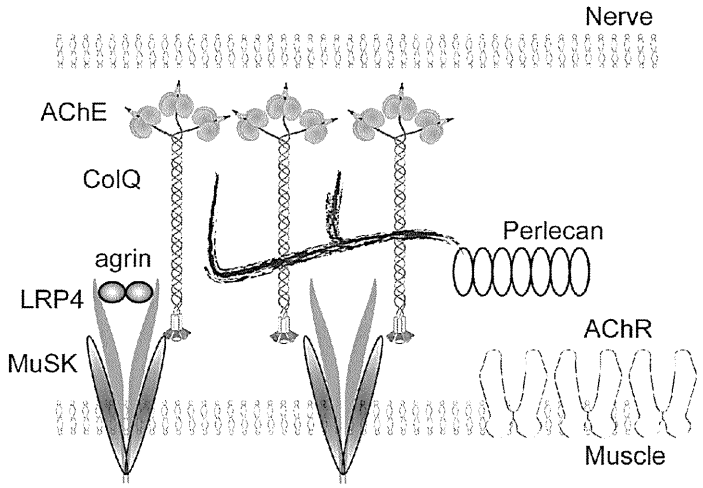

Fig. 4. ColQ anchors to the synaptic basal lamina by binding to perlecan and MuSK.

EMG studies show a decremental response as in other CMS. In addition, most patients have a repetitive CMAP response on a single nerve stimulus. The repetitive CMAP decrements faster than the primary CMAP. It can be overlooked unless a well rested muscle is tested by single nerve stimuli. The prolonged dwell time of unhydrolyzed ACh in the synaptic space prolongs the endplate potential; when this exceeds the absolute refractory period of the muscle fiber action potential, it elicits a repetitive CMAP. As mentioned above, a repetitive CMAP also occurs in slow channel syndrome.

Some aspects of the pathophysiology of endplate AChE deficiency resemble those of the SCCMS. As in the SCCMS, neuromuscular transmission is compromised by three distinct mechanisms. First, staircase summation of endplate potentials causes a depolarization block, which inactivates a proportion the voltage-gated skeletal sodium channel, $Na_V1.4$. (Maselli & Soliven, 1991). Second, prolonged exposure of AChR to ACh during physiologic activity desensitizes a fraction of the available AChRs (Milone *et al.*, 1997). Third, repeated openings of AChR cause calcium overloading to the endplate, which culminates in an endplate myopathy (Groshong *et al.*, 2007). Unlike in the SCCMS, the nerve terminals are abnormally small and often encased by Schwann cells. This decreases the quantal content and hence the amplitude of the endplate potential (Engel *et al.*, 1977).

Anticholinesterase medications have no effect on neuromuscular transmission and can cause excessive muscarinic side effects. Quinidine (Fukudome *et al.*, 1997; Harper & Engel, 1997) and fluoxetine (Harper *et al.*, 2003), which shorten the open duration of the AChR channel and benefit the slow-channel syndrome, can increase muscle weakness. A respirator dependent infant with severe endplate AChE deficiency was improved by intermittent blockade of AChR by atracurium, an agent that protects AChR from overexposure to ACh (Breningstall *et al.*, 1996). Ephedrine sulfate at a dose of 150 to 200 mg per day in adults is effective for myasthenic symptoms (Bestue-Cardiel *et al.*, 2005; Mihaylova *et al.*, 2008). Although high concentrations of ephedrine are able to block AChR openings (Milone & Engel, 1996), molecular bases of ephedrine effects in clinical practice remain elusive. As an alternative to ephedrine, albuterol sulfate 8 to 16 mg per day also shows benefit (Liewluck *et al.*, in press).

4.2 CMS due to a defect in β2 laminin

Laminins are cruciform heterotrimeric glycoproteins composed of α, β, and γ chains and are assembled from products of five α, four β, and three γ genes. The laminin molecules are named according to their chain composition. For example, laminin-321 contains α3, β2, and γ1 chains (Aumailley *et al.*, 2005). Three laminins are present at the synaptic basal lamina, laminin-221, laminin-421, and laminin-521. Each contains the β2 subunit. Laminin-421 is restricted to the primary synaptic cleft and promotes the precise alignment of pre- and postsynaptic specializations. Laminin-521 lines the primary and secondary clefts, promotes presynaptic differentiation, and prevents Schwann cells from entering the synaptic cleft. The synaptic laminins provide a stop signal for axons at developing endplates and organize presynaptic differentiation (Sanes, 1997). Mice deficient for *Lamb2* that encodes β2 laminin show reduced terminal branching of presynaptic motor axons, with a decreased number of active zones, no clustering of the synaptic vesicles above the active zones, and extension of Schwann cell processes into the primary synaptic cleft, and decreased spontaneous and evoked quantal release (Noakes *et al.*, 1995; Patton *et al.*, 1998). In addition to its presence at the endplate, β2-laminin is also highly expressed in renal glomeruli and the eye. *LAMB2* mutations in humans cause Pierson syndrome characterized by ocular malformation including small non-reactive pupils, loss of accommodation, and abnormalities of the lens, cornea and retina and by fatal nephrotic syndrome that requires renal transplantation (Zenker *et al.*, 2004).

Maselli and coworkers reported a 20-year-old woman with Pierson syndrome caused by two heteroallelic frameshifting mutations (1478delG and 4804delC) in *LAMB2* who also had a severe CMS (Maselli *et al.*, 2009). The nephrotic syndrome was corrected by a renal transplant at age 15 months. The patient had respiratory distress in infancy, delayed motor milestones, a decremental EMG response, limited ocular ductions, bilateral ptosis, severe proximal limb weakness, scoliosis, and required assisted ventilation at night and sometimes during the day. AChE activity was spared at the NMJ. Electron microscopy of the NMJ showed small axon terminal size and encasement of nerve endings by the Schwann cell, widening of the primary synaptic clefts with invasion of the synaptic space by processes of Schwann cells, moderate simplification of postsynaptic membranes, and decreased number of synaptic vesicles. Both morphological and microelectrode studies were similar to those observed in *Lamb2*-mice (Noakes *et al.*, 1995). Notably, symptoms were worsened by pyridostigmine but were improved by ephedrine.

5. Presynaptic CMS

Choline acetyltransferase (ChAT) is the only presynaptic molecule that is known to be defective in CMS.

5.1 CMS with episodic apnea due to defects in choline acetyltransferase (ChAT)

ACh released from the nerve terminal is hydrolyzed into choline and acetate by AChE at the synaptic basal lamina. Choline is taken up by the nerve terminal by a high-affinity choline transporter on the presynaptic membrane (Apparsundaram *et al.*, 2000; Okuda *et al.*, 2000). ChAT resynthesizes ACh from choline and acetyl-CoA (Oda *et al.*, 1992). After the synaptic vesicles are acidified by the vesicular proton pump (Reimer *et al.*, 1998), the resynthesized cationic ACh is packed into a synaptic vesicle by the vesicular ACh transporter (vAChT) in exchange for protons (Erickson *et al.*, 1994).

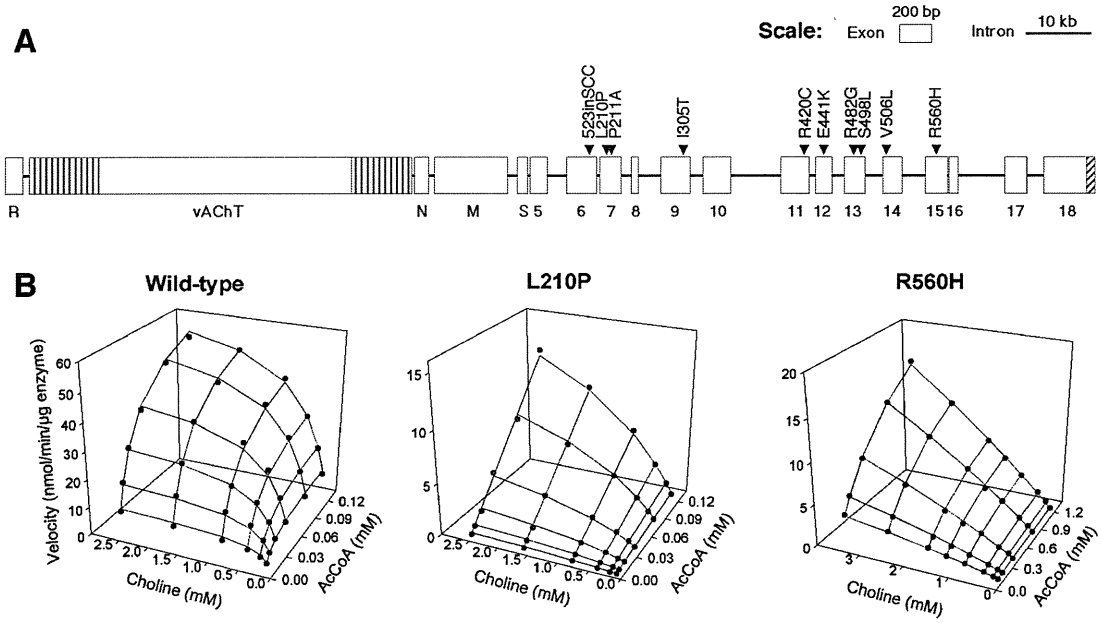

Fig. 5. Choline acetyltransferase (ChAT). (A) Genomic structure of *CHAT* and identified mutations. A gene for vesicular acetylcholine transporter (vAChT) is in the first intron of *CHAT*. (B) Kinetics of wild-type and mutant ChAT enzymes. ChAT synthesizes acetylcholine using choline and acetyl-CoA. L210P abrogates an affinity of ChAT for acetyl-CoA (AcCoA), and R560H abolishes an affinity of ChAT for choline.

We determined the complete genomic structure of *CHAT* encoding ChAT, and identified ten mutations in five CMS patients with the characteristic clinical features of sudden episodes of apnea associated with variable myasthenic symptoms (Ohno *et al.*, 2001). Additional *CHAT* mutations were later reported by other groups (Maselli *et al.*, 2003; Schmidt *et al.*, 2003; Barisic *et al.*, 2005; Mallory *et al.*, 2009; Yeung *et al.*, 2009; Schara *et al.*, 2010). All of our patients showed a marked decrease of the endplate potential after subtetanic stimulation that recovered slowly over 5 to 10 min, which pointed to a defect in the resynthesis or

vesicular packaging of ACh at the nerve terminal. Kinetic studies of mutant ChAT enzymes disclosed variable decreases in affinity for choline and/or acetyl-CoA, as well as variable reduction the catalytic rate (Ohno *et al.*, 2001) (Fig. 5). Moreover, some recombinant mutants expressed at a reduced level in COS cells. Two patients carried a functionally null mutation on one allele, but ChAT encoded on the other allele was partially functional. Heterozygous parents that carried the null allele were asymptomatic indicating that humans can tolerate up to but not exceeding 50% reduction of presynaptic ChAT activity. None of our patients has autonomic symptoms or signs of central nervous system involvement other than that attributed to anoxic episodes. This suggests that the ChAT activity and/or substrate availability are rate limiting for ACh synthesis at the motor nerve but not at other cholinergic synapses. Indeed, stimulated quantal release at the endplate is higher than at other cholinergic synapses, which points to selective vulnerability of the NMJ to reduced ACh resynthesis. Crystal structure of ChAT resolved at 2.2 Å revealed that some of the reported *CHAT* mutations in CMS patients are not at the substrate-binding or the catalytic site of ChAT. Hence these mutation exert their effect by an allosteric mechanism or render the enzyme structurally unstable (Cai *et al.*, 2004).

In most patients, anticholinesterase medications are of benefit in ameliorating the myasthenic symptoms and preventing the apneic crises but few patients fail to respond to cholinergic therapy remaining permanently paralyzed and remain respirator dependent. Prophylactic anticholinesterase therapy is advocated even for patients asymptomatic between crises. Parents of affected children must be indoctrinated to anticipate sudden worsening of the weakness and possible apnea with febrile illnesses, excitement, or overexertion. Long-term nocturnal apnea monitoring is indicated in any patient in whom ChAT deficiency is proven or suspected (Byring *et al.*, 2002).

6. Conclusions

We reviewed the clinical and molecular consequences of defects in 11 genes associated with CMS. Molecular studies of CMS began with identification of a missense mutation in the AChR ε subunit in a SCCM patient (Ohno *et al.*, 1995). Since then, mutations in seven postsynaptic, three synaptic, and one presynaptic proteins have been discovered. In some CMS the disease gene has been elusive and await discovery. Resequencing analysis with the next generation sequencers may speed this effort.

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RNA Pathologies in Neurological Disorders

Kinji Ohno and Akio Masuda

Abstract RNA is not a simple intermediate linking DNA and protein. RNA is widely transcribed from a variety of genomic regions, and extensive studies on the functional roles and regulations of noncoding RNAs including antisense RNAs and small RNAs are in progress. In addition, the human genome project revealed that we humans carry as few as ~22,000 genes. Humans exploit tissue-specific and developmental stage-specific alternative splicing to generate a large variety of molecules in specific cells at specific developmental stages. Neurological disorders are also subject to aberrations of the splicing mechanisms. This review focuses mostly on splicing abnormalities due to pathological alterations of splicing *cis*-elements and *trans*-factors. Pathomechanisms associated with disrupted splicing *cis*-elements can be applied to any human diseases, and we did not restrict the descriptions to neurological diseases. On the other hand, we limited the descriptions of dysregulated splicing *trans*-factors to neurological disorders. Neurological diseases covered in this review include congenital myasthenic syndromes, spinal muscular atrophy, myotonic dystrophy, Alzheimer's disease, frontotemporal dementia with Parkinsonism linked to chromosome 17, facioscapulohumeral muscular dystrophy, fragile X-associated tremor/ataxia syndrome, Prader–Willi syndrome, Rett syndrome, spinocerebellar atrophy type 8, and paraneoplastic neurological disorders.

Keywords The RNA world · Pre-mRNA splicing · Splicing *cis*-elements · Splicing *trans*-factors · Branch point sequence (BPS) · Exonic splicing enhancer (ESE) · Exonic splicing silencer (ESS) · Intronic splicing enhancer (ISE) · Intronic splicing silencer (ISS) · Nonsense-mediated mRNA decay (NMD) · Nonsense-associated skipping of a remote exon (NASRE) · Congenital myasthenic syndromes · Spinal muscular atrophy (SMA) · Myotonic dystrophy (DM1, DM2) · Alzheimer's disease· Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) ·

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Facioscapulohumeral muscular dystrophy (FSHD) · Fragile X-associated tremor/ataxia syndrome (FXTAS) · Prader–Willi syndrome, Rett syndrome · Spinocerebellar atrophy type 8 (SCA8) · Paraneoplastic neurological disorders (PND)

Contents

1 Introduction

The central dogma first enunciated by Francis Crick depicts RNA as an intermediate that links DNA and protein (Crick, 1970). The beginning of life, however, was the RNA world where there were no DNA or proteins (Gilbert, 1986). In the RNA world, RNA was the only carrier of genetic information that DNA currently serves as, and the only functional molecule that proteins currently serve as. Although the RNA transmits no genetic information to progeny and constitutes a limited number of functional molecules in our human body, the RNA world is still in effect in our body. Humans transcribe more than half of our entire genome including noncoding regions. The transcripts work as *antisense RNAs, microRNAs, and snoRNAs*. Researchers are now working to disclose the functional significance of these noncoding RNAs.

The human genome project and the subsequent annotation efforts revealed that we humans carry as few as 22,000 genes. Tissue-specific and developmental stage-specific splicing enables to us to generate more than 100,000 molecules from a limited number of genes (Black, 2003; Licatalosi and Darnell, 2006). Small RNA molecules and RNA splicing mechanisms potentially become targets of neurological diseases (Ranum and Cooper, 2006). This review focuses mostly on splicing aberrations associated with neurological disorders.

2 Physiology of Splicing Mechanisms

In higher eukaryotes, pre-mRNA splicing is mediated by degenerative splicing *cis*-elements comprised of the branch point sequence (BPS), the polypyrimidine tract (PPT), the 5′ and 3′ splice sites, and exonic/intronic splicing enhancers/silencers (Fig. 1). Stepwise assembly of the spliceosome starts from recruitment of *U1 snRNP* to the 5′ splice site, *SF1* to the BPS, *U2AF65* to the PPT, and *U2AF35* to the 3′ end of an intron to form a spliceosome complex E (Sperling et al., 2008). SF1, a 75 kDa protein, is a mammalian homologue of yeast BBP (branch point-binding protein). U2AF65 and U2AF35 bring *U2 snRNP* to the BPS in place of SF1 (Wu et al., 1999; Zorio and Blumenthal, 1999). The BPS establishes base pairing interactions with a stretch of "GUAGUA" of U2 snRNA (Arning et al., 1996; Abovich and Rosbash, 1997), which then bulges out the branch site nucleotide, usually an adenosine to form a spliceosome complex A (Query et al., 1994). Thereafter, pre-mRNAs are spliced in two sequential transesterification reactions mediated by the spliceosome. In the first step, the 2′-OH moiety of the branch site nucleotide carries out a nucleophilic attack against a phosphate at the 5′ splice site, generating a free upstream exon, as well as a lariat carrying the intron and the downstream exon. In the second step, the 3′-OH moiety of the upstream exon attacks the 3′ splice site of the

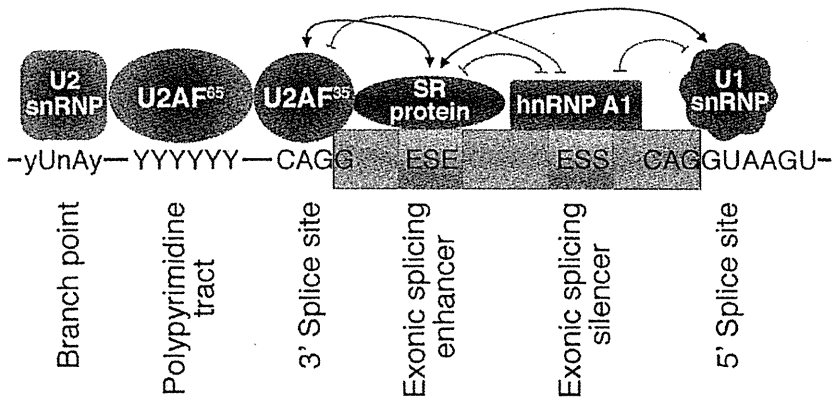

Fig. 1 Representative splicing *cis*-elements and *trans*-factors. Tissue-specific and developmental stage-specific expressions of splicing *trans*-factors including SR proteins and hnRNP A1 enable precise regulations of alternative splicing. ISE and ISS have similar activities as ESE and ESS, but are omitted from the figure