CONTEXT-DEPENDENT FUNCTION OF THE SPLICING FACTOR HNRNP L

APPROVED BY SUPERVISORY COMMITTEE

Kristen W. Lynch, Ph.D.

Wade C. Winkler, Ph.D.

Beatriz Fontoura, Ph.D.

Joseph Albanesi, Ph.D.

DEDICATION

To my mother and father, Juanita Mena Hernandez and Javier Motta Reyes.

CONTEXT-DEPENDENT FUNCTION OF THE SPLICING FACTOR HNRNP L

by

LAURA BEATRIZ MOTTA-MENA

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2010

ACKNOWLEDGEMENTS

I am forever grateful to my mentor, Dr. Kristen Lynch, who has an amazing ability to guide, encourage, and motivate the people in her lab. Her direction and supervision during the past five years has been invaluable and critical to the development of this project. She has a tremendous passion for science that drives the lab and makes me realize there are few mentors quite like her.

Thank you to the members of my thesis committee, Drs. Wade Winkler, Beatriz Fontoura, and Joseph Albanesi, who were always encouraging of my project and offered helpful advice. I am especially grateful to Wade, and members of the Winkler lab, for allowing me to join their lab for past year to finish my thesis work and for making me an honorary member of the lab. Great thanks to Dr. Kevin Gardner, who was an unofficial committee member, for giving us great advice on follow-up experiments and for being a great "foster" mentor to me this past year. Also, the Lynch lab members, past and present, made working in the lab that much more fun, thank you. In particular, I want to thank Florian Heyd whose contributions to this project were critical, and whose friendship I will always cherish. And of course my mentor "numero dos," Amy House, who helped me to get started in the lab, pushed me to try experiments on my own, and made me feel confident in my abilities as a scientist.

Finally, I must thank my mom, Juanita, my "pop", Javier, and my brother, Javi for their support, love, and encouragement; without them I could not have made it this far and accomplished this goal. My husband, Dr. Michael Dellinger, who helped me through the toughest times in graduate school, and who was always excited to talk science with me, and whose love I am most grateful for.

CONTEXT-DEPENDENT FUNCTION OF THE SPLICING FACTOR HNRNP L

Laura Beatriz Motta-Mena, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2010

Kristen W. Lynch, Ph.D.

Based on the number of genes impacted (~95% of humans genes), alternative splicing is one of the most extensively used mechanisms for generating proteomic diversity and cellular complexity. Splicing of pre-mRNAs is carried out by a highly specialized, RNA-based macromolecular enzyme known as the spliceosome. The spliceosome is made up of 5 small nuclear RNP (snRNP) complexes (U1, U2, U4/U6, and U5), all of which consist of a uridine-rich snRNA and multiple proteins. Importantly, the spliceosome is not a pre-formed enzyme but instead forms through the step-wise assembly of the snRNP complexes on the pre-mRNA. Mechanistically, the selection of exons or splice sites during alternative splicing occurs by modulating the assembly of the spliceosome on a pre-mRNA. Ultimately, the decision to include or exclude an exon into the final mRNA is based on the integration of both the synergistic and

antagonistic forces between groups of protein regulators and between protein regulators and the snRNP complexes.

An excellent model system to illustrate the mechanisms of alternative splicing, as well as the physiologic significance of this mode of regulation, is the human CD45 gene. HnRNP L binds to a motif present in both CD45 variable exons 4 and 5 to affect their coordinate repression. Previously, it was shown that hnRNP L regulates exon 4 by stalling the U1 and U2 snRNPs in a non-permissive A-like exon-defined spliceosomal complex. Here, we show that, in contrast to its direct repression of exon 4, hnRNP L represses exon 5 by countering the activity of a neighboring splicing enhancer element. As the splice sites flanking exon 4 and 5 are distinct, we directly examined the effect of varying splice site strength on the mechanism of hnRNP L function. Remarkably, binding of hnRNP L to an exon represses strong splice sites but enhances weak splice sites. A model in which hnRNP L stabilizes snRNP-binding can explain both effects in a manner determined by the inherent snRNP-substrate affinity. Overall, these findings demonstrate that context can fundamentally alter the activity of a splicing regulatory protein and can therefore impact our predictions of splicing patterns and mechanisms of splicing regulation.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Motta-Mena, L.B., and Lynch, K.W. (2010) Alternative Splicing: Regulatory mechanisms and functional impact. Encyclopedia of Biological Chemistry, *accepted*.

Motta-Mena., L.B., Heyd, F., and Lynch, K.W. (2010) Context-dependent regulatory mechanism of the splicing factor hnRNP L. Mol Cell 37, 223-234.

ABSTRACTS

Motta-Mena, L.B., Heyd, F., and Lynch, K.W. "Context-dependent regulatory mechanism of exon-bound hnRNP L is determined by splice site strength." Fourteenth Annual Meeting of the RNA Society, 2009. *Oral presentation*.

Motta-Mena, L.B., and Lynch KW. "Context-dependent function of the ARS motif in exons 4 and 5 of CD45." Gordon Research Conference on The Biology of Post-transcriptional Gene Regulation, 2008. *Poster presentation*.

Motta-Mena, L.B., and Lynch KW. "Regulation of CD45 exon 5: Interplay between a strong ESE and an activation-induced repressor." Cold Spring Harbor Meeting on Eukaryotic mRNA Processing, 2007. *Poster presentation*.

LIST OF FIGURES

LIST OF DEFINITIONS

α – Anti

A – Adenosine

AEC – A-like Exon-Defined Complex

ARS – Activation Responsive Sequence

ATP – Adenosine Triphosphate

bp - Basepair

BPS – Branch Point Sequence

BSA – Bovine Serum Albumin

C – Cytidine

CP – Phosphocreatine

CUGBP – Cytidine-Uridine-Guanosine Binding Protein

DExD/H – family of RNA helicases, containing Asp-Glu-X-Asp/His motif

DMEM – Dulbecco's Modified Eagle's Medium

DTT – Dithiotreitol

EDTA – Ethylenediaminetetraacetic acid

eNOS – Endothelial Nitric Oxide Synthase gene

ESE – Exonic Splicing Enhancer

ESS – Exonic Splicing Silencer

FR – Fold Repression

G – Guanosine

GST – Glutathione-S-Transferase

HnRNP – Heterogeneous Nuclear Ribonuclear Protein

ISE – Intronic Splicing Enhancer

ISS – Intronic Splicing Silencer

JSL1 – Jurkat Splicing Line 1

KCl – Potassium Chloride

kD – kilo Dalton

KH – K Homology RNA Binding Domain

MBP – Maltose Binding Protein

MgCl2 – Magnesium Chloride

mRNA – Messenger RNA

MS2 – Bacteriophage Coat Protein

MBNL – Muscleblind

NaCl – Sodium Chloride

NaOAc – Sodium Acetate

NE – Nuclear Extract

NOVA1/2 – Neuro-Oncological Ventral Antigen 1/2

nt- Nucleotides

oligo – Oligonucleotide

PAGE – Polyacrylamide Gel Electrophoresis

PCA – Phenol Chloroform Isoamyl Alcohol

PCR – Polymerase Chain Reaction

PK – Proteinase K

PMA – Phorbol 12-myristate 13-acetate

PPT – Polypyrimidine Tract

pre-mRNA – Precursor Messenger RNA

PSF – PTB Associated Splicing factor

PTB – Polypyrimidine Tract Binding Protein

PVA – Polyvinyl Alcohol

Py – Pyrimidine

RNA – Ribonucleic Acid

RNAi – RNA Interference Mechanism

RNase – Ribonuclease

RRM – RNA Recognition Motif

RS – Arginine-Serine Rich Domain

RT – Reverse Transcription

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SDS – Sodium Dodecyl Sulfate

SF1- Splicing Factor 1

SF2/ASF – Alternative Splicing Factor/Splicing Factor 2

snRNA – Small Nuclear RNA

snRNP – Small Nuclear Ribonucleoprotein Particle

ss- Splice Site

T – Thymidine

TCR – T Cell Receptor

Tra – Transformer Protein

Tri-snRNP – U5/U4•U6 Small Nuclear Ribonucleoprotein Particle

U – Uridine

U2AF – U2 Auxiliary Factor

CHAPTER ONE

Introduction and Literature Review

Impact of alternative splicing on biology

In higher eukaryotes genes code for the information required to produce many different cell types, and to arrange these cells into specific tissues and organs that will eventually make up a complete organism. In humans this process is accomplished with only ~25,000 protein-coding genes (Venter et al., 2001; Lander et al., 2001), which is a relatively small number if you consider that the small mustard plant *Arabidopsis thaliana* has close to the same number of genes (~21-25,000) (The Arabidopsis Genome Initiative, 2000). It is evident from these kinds of genomic sequencing data that the number of protein-coding genes in an organism does not correlate with the level of biological complexity that organism can hope to achieve.

How then is organismal complexity accomplished? Higher eukaryotes have developed several mechanisms to amplify the informational content of their seemingly limited genomes and generate biological complexity. Some of these include the genetic swapping of protein-domains, post-translational protein modifications, small RNA regulation, and alternative splicing (Black, 2003). Based on the number of genes impacted (~95% of humans genes), alternative splicing is quite possibly the most extensively used mechanism for generating proteomic diversity and cellular complexity (Nilsen and Graveley, 2010).

Pre-mRNA splicing is the process by which non-coding sequences (introns) are removed and the protein-coding regions (exons) are joined together by the splicing

machinery to create a mature mRNA molecule (Black, 2003). The majority of protein-coding genes in metazoans contain multiple exons, which can potentially be spliced together in many different or alternate combinations. This process is referred to as alternative splicing and results in multiple mRNAs being produced from the same gene (Black, 2003). As each unique mRNA can potentially code for a distinct protein, alternative splicing allows a single gene to encode multiple proteins with specific, and sometimes opposing, biological activities (Matlin et al., 2005). Regulated splicing does not simply lead to the static generation of multiple proteins from a single gene. More often that not, alternative exons are regulated in a cell-type and developmental-stage dependent manner, or in some cases, their splicing is controlled in response to signaling cascades triggered by specific extracellular signals (Black, 2003; Blencowe, 2006; Lynch, 2007). This allows cells to fine-tune protein expression in accordance with cell function and/or in response to changes in the extra-cellular environment.

The vast majority of human genes contain numerous regulatory sequences that determine the precise pattern of alternative splicing in any given cell (Black, 2003; Matlin et al., 2005). Such regulation relies on manipulating the intricate network of interactions between the RNA transcript and the splicing machinery that is required for splicing catalysis. Not surprisingly the complexity of this process makes it highly susceptible to mutations (Cooper et al., 2009). Current estimates suggest more than half of known disease causing mutations in humans cause misregulation of splicing (Lopez-Bigas et al., 2005). Therefore, understanding the signal sequences and proteins that control alternative splicing is critical for predicting normal protein expression and for interpreting the pathogenic effects of genetic mutations. Taken together, these findings

highlight the widespread impact of alternative splicing on cellular gene expression and the importance of having a thorough understanding of the basic principles of the splicing reaction.

Splice site sequences and types of alternative splicing patterns

In a pre-mRNA the exon/intron boundaries are marked by specific, conserved sequences called splice sites (ss) (Wahl et al., 2009)(Figure 1-1A). These sequences are specifically recognized by the splicing machinery and direct the removal of introns and joining together of exons to produce a mature transcript. The 5'ss marks the exon/intron junction at the 5'-end of the intron and includes a conserved GU dinucleotide. At the other end of the intron the 3'ss has three conserved sequence elements. The first element, going from 5' to 3' of the intron, is the branch point sequence (BPS), which contains the reactive adenosine that participates in the first-step of splicing. The second element is a pyrimidine-rich sequence called the polypyrimidine tract (PPT) that is 10-40 nucleotides upstream of the third element, a conserved AG dinucleotide at the extreme 3'-end of the intron (Figure 1-1A).

Each pre-mRNA splicing event removes one intron through two sequential transesterification reaction steps (Wahl et al., 2009)(Figure 1-1B). In the first step of the splicing reaction the 2'-OH of the reactive adenosine in the BPS attacks the phosphodiester bond at the 5'ss and cleaves the RNA molecule. The cut 5'-end of the intron becomes covalently linked to the adenosine creating a loop in the RNA in the shape of a lariat. In the second step of splicing, the 3'-OH of the released 5' exon attacks the phosphodiester bond at the 3'ss (start of the 3' exon), thus joining the two exons together and releasing the intron lariat.

While some exons in an RNA messenger transcript are constitutively spliced, that is they are always included in the final message, the splicing of other exons is regulated (Black, 2003; Blencowe, 2006; Graveley, 2001)(Figure 1-2). The most common type of regulated

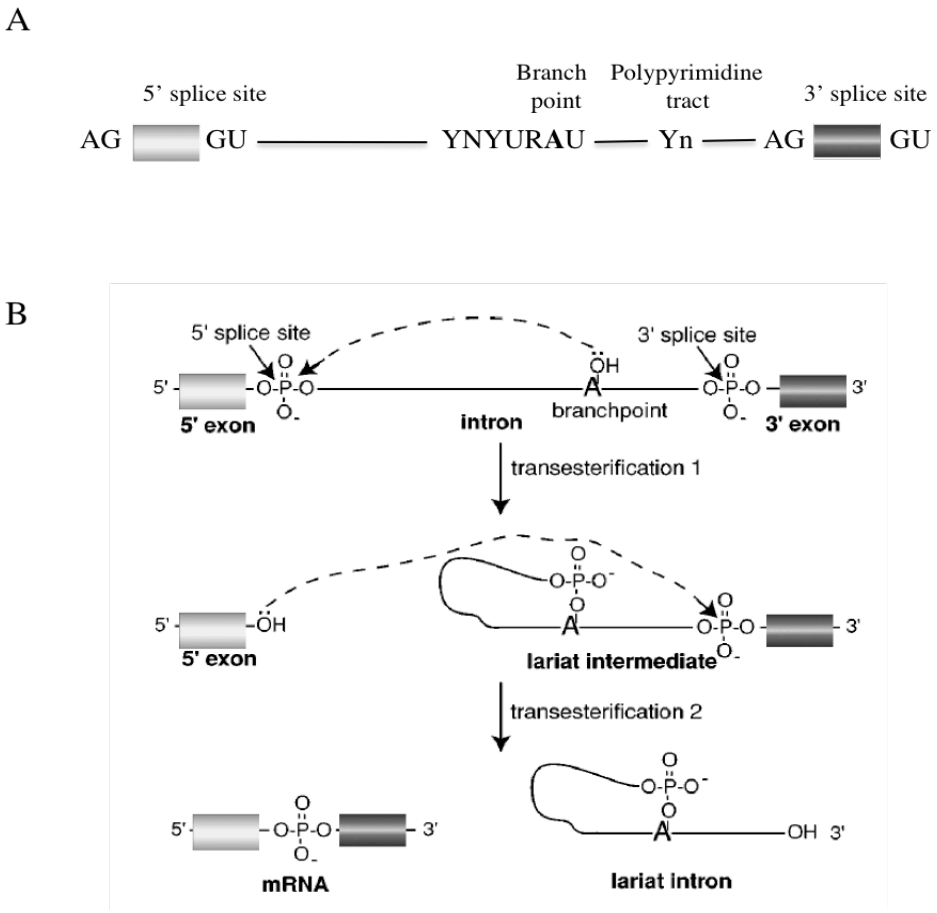

Figure 1-1. Splicing proceeds in two transesterification steps. (A) The exon and intron boundaries in a pre-mRNA are defined by conserved sequence elements: the 5' splice site, the branch point sequence, a pyrimidines-rich track, and the 3' splice site. Y is a pyrimidine, R is a purine, and N is any nucleotide. (B) First the 2'OH of the reactive adenosine in the BP attacks the phosphodiester bond at the 5'ss and cleaves the RNA molecule. Next, the 3'OH of the released 5'exon attacks the phosphodiester bond at the 3'ss, joining the 2 exons together and releasing the intron in lariat form. (Modified from Brow, 2002).

splicing event is what is called a cassette exon, which is an exon that is sometimes included and sometimes excluded from the mature mRNA. In some cases, cassette exons

are mutually exclusive in which case only one exon from a group of two or more variants is selected. There are also instances where alternate 5' or 3' splice sites, located in tandem, can be used to produce different mRNA isoforms. In addition, by combining the use of alternative promoters and alternative splicing the 5'-end of a transcript can be altered giving rise to different mRNAs. Similarly, the 3'-end of the mRNA can be changed by pairing the use of alternate polyadenylation-sites and alternative splicing. Lastly, in some cases the failure to remove a particular intron (i.e., intron retention event) in an mRNA can occur.

Regardless of whether an exon is constitutively or alternatively spliced, its splice sites have to be recognized and paired from within a multitude of similar sequences in the pre-mRNA molecule. These bona-fide splice sites must also be correctly positioned within atomic distance so that catalysis can occur. In cells, this very important process is carried out by a highly specialized and complex RNP (ribonucleoprotein) machine known as the spliceosome (Wahl et al., 2009).

The splicing reaction is catalyzed by the spliceosome

The catalysis of pre-mRNA splicing is performed by the spliceosome, which is a dynamic, macromolecular enzyme that is composed of 5 snRNP (small nuclear RNP) complexes: U1, U2, U4/U6, and U5. Each snRNP consists of a uridine-rich snRNA (or two in the case of U4/U6) and several common and complex-specific proteins (Wahl et al., 2009). The RNA component of the snRNPs interacts directly with the transcript RNA by base-pairing with the splice site sequences. The catalytic spliceosome is not a pre-formed enzyme but instead assembles on the pre-mRNA in a step-wise pathway that involves several intermediates (Wahl et al., 2009). These spliceosome assembly

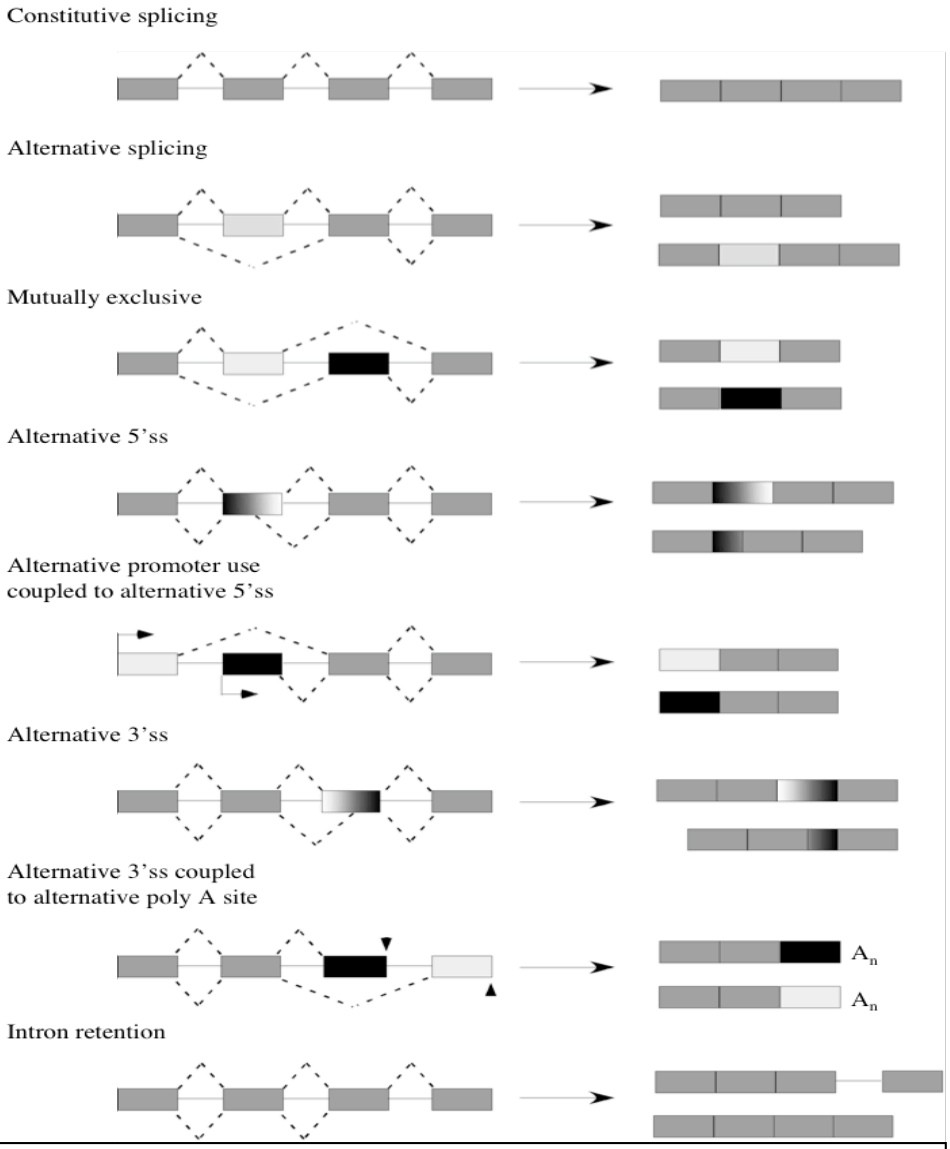

Figure 1-2. Patterns of pre-mRNA alternative splicing. Cases in which alternative promoter sites or alternative polyA sites are coupled with alternative splicing are indicated. Exons are depicted as boxes and introns as lines; dashed lines indicate the splice sites used in the splicing reaction. Constitutive exons are shown in grey and regulated exons are shown as light grey or black boxeszzz.

intermediates are characterized by the association and dissociation of individual or in some cases groups of snRNPs with the pre-mRNA (Figure 1-3).

The first spliceosomal intermediate is called E (or early) complex and is defined by three key molecular interactions (Figure 1-3). First, the snRNA of the U1 snRNP base-pairs with the 5'ss, second, the SF1 protein binds to the BPS, and third, the U2AF (U2 snRNP auxiliary factor) subunits U2AF65 and U2AF35, bind the PPT and the AG dinucleotide at the 3'ss, respectively. Together these interactions are critical for the initial definition of the exon/intron boundaries and promote further assembly. E complex formation is followed by the ATP-dependent binding of the U2 snRNP through base-pairing with the BPS leading to A complex formation (Figure 1-3)(Wahl et al., 2009). In addition, stabilization of the U2-snRNA/pre-mRNA interaction, by protein components of the U2 snRNP, results in displacement of the SF1 protein from the BPS. Subsequent recruitment of the U4/U6•U5 tri-snRNP results in the formation of a B complex (Wahl et al., 2009). At this point, the snRNPs undergo extensive remodeling in order to generate a catalytically active spliceosome. During activation, the base-pairing interactions between the U4/U6 snRNAs are disrupted, allowing the U6 snRNA to bind the U2 snRNA and the 5'ss of the pre-mRNA. In addition, the snRNA component of the U5 snRNP contacts nucleotides of the 5' and 3' exon. As a result of these rearrangements, the U4 and U1 snRNPs are released, along with proteins involved in securing the U5 snRNP to the U4/U6 snRNP, giving rise to an activated spliceosome (B* complex) (Wahl et al., 2009). The activated spliceosome then carries out the first catalytic step of splicing, generating a catalytic or C complex (Figure 1-3). Next, the interaction between the U6 snRNA and the 5'ss is destabilized, allowing the second step of splicing to occur (Konarska et al., 2006). At this point, the spliceosome dissociates from the RNA transcript, and the U2, U5, and U6 snRNPs are free to participate in subsequent rounds of splicing (Figure 1-3).

Many of the conformational rearrangements that occur during spliceosome assembly are facilitated by spliceosome-associated DEXD/H-type RNA helicases (Brow, 2002; Staley and Guthrie, 1998). These RNA helicases, which act at specific steps of the splicing cycle (Wahl et al., 2009), use the energy of ATP hydrolysis to catalyze the snRNA-snRNA and pre-mRNA-snRNA rearrangements (as well as RNP remodeling events) that are essential for the formation of the active site in the spliceosome (Staley and Guthrie, 1998).

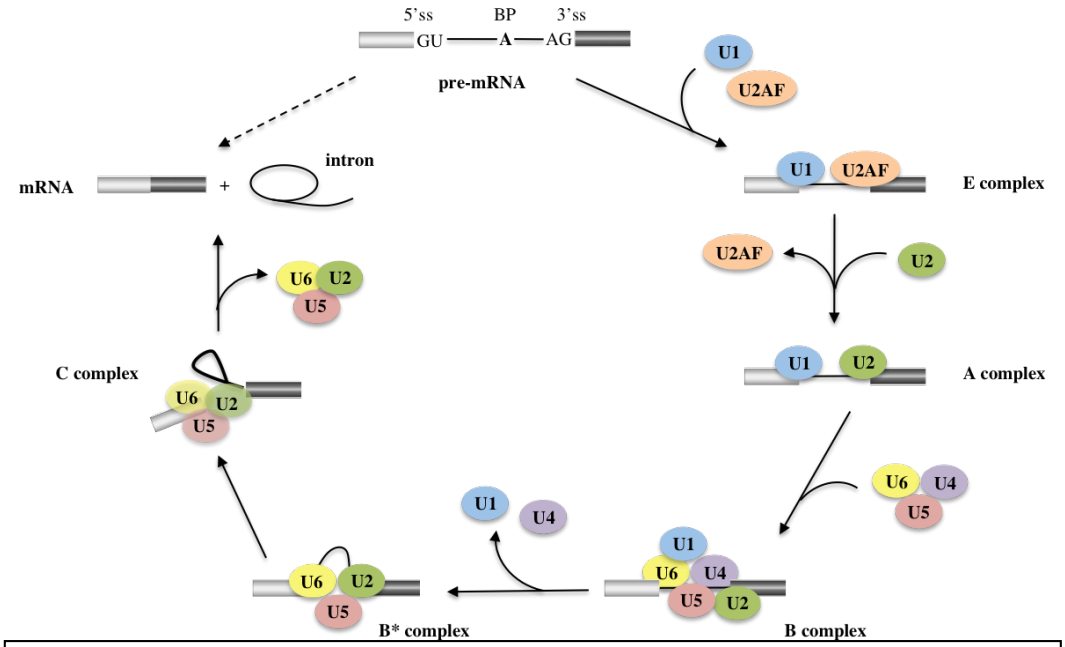

Figure 1-3. Stepwise assembly of the major spliceosome during pre-mRNA splicing. Assembly begins with the addition of the U1 snRNP to the 5'ss and the U2AF (U2 snRNP auxiliary factor) to the BP and PPT, respectively, to yield the E complex. This followed by the ATP-dependent binding of the U2 snRNP to the BP, which displaces U2AF and leads to A complex formation. Subsequent recruitment of the U4/U6/U5 tri-snRNP results in the formation of a B complex. At this point, the snRNPs undergo extensive remodeling that results in the loss of the U1 and U4 snRNPs and the formation of an activated spliceosome (B* complex). The activated spliceosome then undergoes the first catalytic step of splicing, generating the C complex. The catalytic spliceosome then undertakes the second catalytic step of splicing, after which releases the mRNA and the intron in the shape of a lariat. (Modified from Wahl, 2009).

Although spliceosome assembly is commonly described as initially occurring across an intron, in higher eukaryotes where introns can be as many as hundreds to hundreds of thousands of nucleotides long, spliceosome components are thought to first assemble across an exon (which are no more than ~50-250 nucleotides). During this so-called "exon–definition" process, the U2AF that is bound to the upstream 3'ss engages in cross-exon interactions with the U1snRNP that is bound to the downstream 5'ss (Berget, 1995; Reed, 2000). Eventually, cross-exon interactions must transition into cross-intron interactions in order for catalysis to occur; however, precisely when and how this happens is currently not known. Therefore, spliceosome assembly is a highly complex and intricate problem that involves understanding how the many interactions that occur during the splicing cycle can be modified to alter splicing patterns.

Auxiliary sequence elements control splice site recognition

In metazoan organisms the splice site consensus sequences are highly degenerate and are generally not sufficient to efficiently direct spliceosome assembly (Matlin et al., 2005; Black, 2003; Chen and Manley, 2009). Moreover, the majority of metazoan introns are large (usually between hundreds to thousands of nucleotides long) and can contain many "pseudo" splicing signals (Matlin et al., 2005). Thus, pre-mRNA substrates almost always contain additional regulatory sequence elements that aid in the recognition and selection of legitimate splice sites (Black, 2003; Blencowe, 2006; Hertel, 2007; Matlin et al., 2005). These *cis*-acting sequences are found in exons as well as in introns and can have positive or negative effects on spliceosome assembly. Sequence elements that activate splice-site usage and promote spliceosome assembly are called splicing enhancers. In contrast, sequences that block splice-site usage and inhibit assembly of the

splicing machinery are described as splicing silencers. The majority of regulatory sequences exert their effects by acting as binding sites for *trans*-acting protein factors, which in turn engage the snRNP subunits in a way that inhibits or, in the case of positive regulators, promotes assembly on neighboring splice sites (Black, 2003; Matlin et al., 2005).

Exonic splicing enhancers (ESEs) are often bound by members of the SR (serine-arginine) protein family (Figure 1-4A) (Fu, 1995; Graveley, 2000; Long and Caceres, 2009). Importantly, ESEs are found within the majority of constitutive and regulated exons, such that SR-protein-binding to an ESE is often essential for the selection and pairing of genuine splice sites. SR proteins usually contain one or two RNA binding domains near their N-terminal end, followed by an RS-domain that is rich in arginine-serine di-peptides at their C-terminus (Graveley, 2000). The splicing activity of SR proteins is largely attributed to their RS-domains, which mediates many protein-protein interactions and whose phosphorylation-state is key to the splicing activity of the protein (Fu, 1995; Graveley, 2000; Long and Caceres, 2009). Proteins that recognize intronic splicing enhancers (ISEs) do not fall into a specific family of proteins, but instead include of a variety of RNA-binding proteins; some of which include members of the CUGBP and ETR-like factors (CELF) family, the heterogeneous nuclear RNP (hnRNP) family, as well as the tissue-specific splicing regulators NOVA1/2 and FOX1/2 (Ladd et al., 2001; Forch et al, 2000; Ule et al., 2006; Zhou and Lou, 2008; Zhang et al. 2008). All of these proteins will be discussed further below.

Exonic and intronic splicing silencers (ESSs and ISSs) are negative regulatory elements that are commonly recognized by members of the hnRNP family of RNA-

binding proteins (Figure 1-4B). Historically, hnRNPs were defined solely by their ability to co-purify with nascent pre-mRNAs, as a result there is remarkable structural and functional diversity among family members (Dreyfuss et al., 1993). However, more than half of the major hnRNP proteins have been ascribed diverse and specific roles in

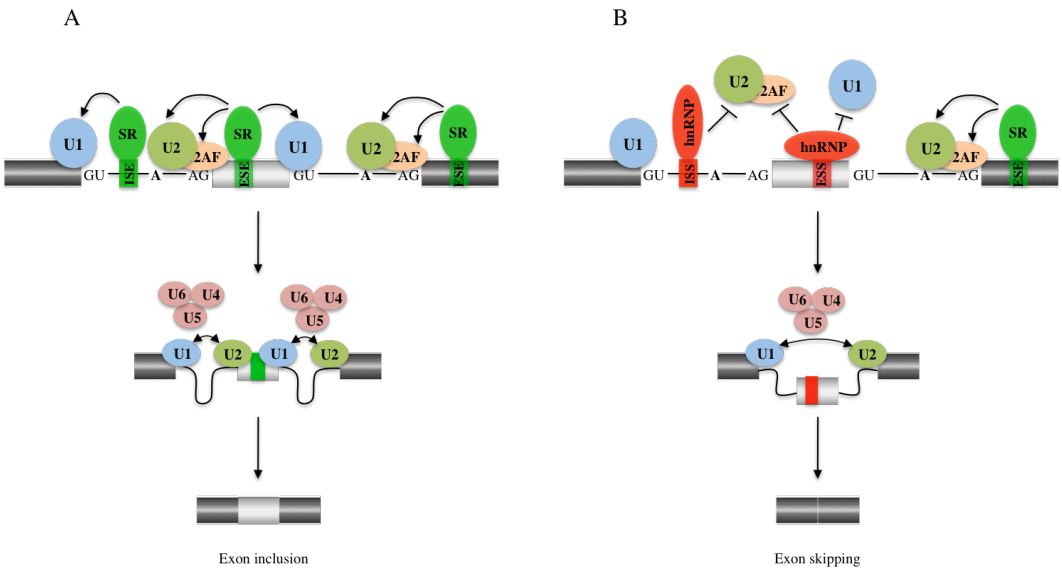

Figure 1-4. *Cis*-acting regulatory elements and their cognate binding proteins control pre-mRNA splicing. (A) Exonic and intronic splicing enhancer (ESE and ISE) elements are typically bound by SR proteins. SR proteins can promote and/or stabilize the interactions of the U1 snRNP to the 5'ss and of the U2 snRNP and U2AF to the 3'ss across the exon or the intron. Therefore, assembly is able to proceed and U4/U6/U5 tri-snRNP is recruited resulting in exon inclusion. (B) Exonic and intronic splicing silencers (ESSs and ISSs) are negative regulatory elements that are commonly recognized by hnRNP proteins. HnRNPs can occlude or antagonize the binding of the spliceosomal subunits to canonical splicing signals, thereby repressing the use of an exon. Constitutive and alternative exons are depicted as black and grey boxes, respectively.

splicing, where their binding to the pre-mRNA often blocks the interaction of spliceosomal subunits to an overlapping or adjacent splice site (Martinez-Contreras et al. 2007). HnRNPs frequently contain one or several RNA recognition domains that is often followed by a glycine-rich or an arginine rich domain, which is believed to be important for protein-protein interactions (Dreyfuss et al., 1993; Martinez-Contreras et al. 2007).

Interestingly, there is evidence of phosphorylation, SUMO-lation, and arginine methylation of some hnRNPs, however precisely how these modifications regulate protein activity is not entirely known (van der Houven van Oordt et al., 2000; Mikula et al., 2006; Habelhah et al., 2001; Xie et al., 2003; Vassileva and Matunis, 2004; Li et al., 2004). Nevertheless, hyper-phosphorylation of hnRNP A1 under stress conditions was found to correlate with the increased accumulation of this protein in the cytoplasm (van der Houven van Oordt et al., 2000). Many other hnRNPs are known to shuttle continuously between the nuclear and cytoplasmic compartments (Pinol-Roman and Dreyfuss, 1992; Michael et al., 2002). It is possible that post-transcriptional modification of some these proteins could control their intracellular localization and in that way regulate their splicing activity. Alternatively, protein modifications could modulate the binding of hnRNPs to the pre-mRNA and/or to specific protein partners.

It is clear that these two families of RNA-binding proteins, the hnRNP and SR proteins, play essential roles in splicing, among other steps of mRNA processing. Nonetheless, additional RNA-binding regulatory proteins have been identified that possess splicing activity similar to SR proteins (activators) and hnRNPs (repressors) but do not fall neatly into one family or the other (Black, 2003). Some of these include the neuron-specific RNA-binding proteins NOVA-1 and -2, the neuron- and muscle-specific proteins FOX-1 and -2, and members of the CELF and muscle-blind like (MBNL) family of proteins (Jensen et al., 2000; Ule et al., 2005; Underwood et al., 2005; Ladd, 2001).

As a common rule, SR proteins bound to ESEs function as general splicing activators during exon-definition, and the function of hnRNPs is to antagonize this enhancing activity of SR proteins (Black, 2003; Matlin et al., 2005). For the most part,

SR proteins and hnRNPs recognize short degenerate RNA sequences with low to moderate binding affinity (Long and Caceres 2009; Martinez-Contreras et al., 2007). Nevertheless, by combining multiple "weak" interactions (like between SR or hnRNP proteins and ESEs or ESSs, respectively), splicing regulatory proteins are able to achieve the high binding and functional selectivity that is needed in order to regulate distinct sets of alternative splicing events (Black, 2003; Hertel, 2008). The combinatorial control of splicing by splicing activators and repressors is discussed in more detail below.

Mechanisms of alternative splicing regulation

Both hnRNP and SR proteins (as well as other related splicing factors) draw on a variety of strategies to direct the selection and pairing of specific splice sites and regulate splicing patterns (House and Lynch, 2008; Chen and Manley, 2009). Specific examples of these mechanisms of action will be discussed in more detail below.

Activation and inhibition of splice sites recognition

The best characterized mechanism of splice site selection involves the enhancement or inhibition of the binding of the U1 or U2 snRNPs to their corresponding splice sites (House and Lynch, 2008; Chen and Manley, 2009). As was alluded to previously, SR proteins have important roles in splice site activation. During the initial recognition of an exon ("exon definition"), ESE-bound SR proteins can recruit and stabilize the interactions between the U1 snRNP and the 5'ss and between the U2AF complex and the U2 snRNP and the 3'ss (Figure 1-4A) (Long and Caceres, 2009). The function of SR proteins in this process is dependent on their RS-domain, which serves as a protein-protein interaction module that interacts with and helps recruit components of the spliceosome (Wu and Maniatis, 1993; Kohtz et al., 1994). The RS domain of an ESE-

bound SR protein can also directly contact the RNA substrate at the branchpoint sequence and in this way promote assembly (Shen et al., 2004). Apart from interacting directly with the pre-mRNA and other components of the splicing machinery, SR proteins can promote exon inclusion by associating with other SR proteins and SR-like proteins (SRm160/300), through their RS domains, to form larger splicing-enhancing complexes that heavily recruit the splicing machinery (Blencowe et al., 1998; Li and Blencowe, 1999; Eldridge, 1999). It should be noted that the phosphorylation state of the RS-domain can often influence the splicing activity and cellular localization of the protein (Xiao and Manley, 1997; Misteli et al., 1998). Together these results emphasize the importance and diverse functional roles of SR proteins during splice site activation. Non-SR proteins that bind to intronic enhancer sequences instead of ESEs can also facilitate activation of splice site recognition. For example, the CELF family member ETR3 (also called CUGBP2) binds to a GU-rich intronic enhancer element downstream of exon 5 of the chicken and human cardiac troponin T (TnT) pre-mRNA and recruits the U1 snRNP to the 5'ss to promote splicing (Ladd and Cooper, 2001; Gromak et al., 2003; Faustino and Cooper, 2005).

The inhibition of splice site selection often relies on the participation of hnRNP proteins, which can repress splice site recognition in a number of ways (Martinez-Contreras et al., 2007). First, the binding of hnRNPs to silencer elements that overlap or are located in close proximity to canonical splicing signals can occlude the binding of the spliceosomal subunits to these sites (Figure 1-4B). For example, the polypyrimidine tract binding protein (PTB, also called hnRNP I) can sterically block the binding of U2AF to the PPT in the 3'ss region upstream of β-tropomyosin exon 6B and other regulated exons

(Sauliere et al., 2006; Singh et al., 1995; Spellman and Smith, 2006). In a similar fashion, the interaction of the U2 snRNP is sterically blocked by hnRNP A1 binding to an ISS that overlaps with one of three alternative branch point sequences found upstream of exon 3 of the HIV Tat transcript (Tange et al., 2001). Splicing factors other than hnRNPs can

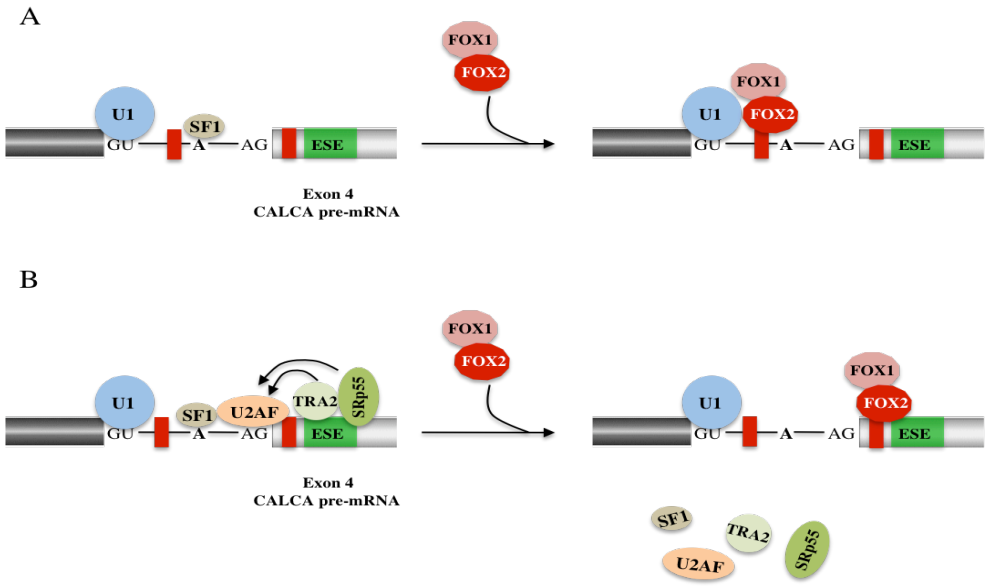

Figure 1-5. Mechanisms of alternative splicing regulation by FOX1/2. (A) FOX1/2 bind to an intronic silencer (red box) and inhibit the inclusion of CALCA exon 4 by sterically blocking the binding of SF1 to the branch point (**A=** reactive adenosine). (B) Binding of SR-55 and Tra2 to an enhancer element (ESE) recruits U2AF to the 3'ss upstream of CALCA exon 4; however, FOX1/2 binding to an adjacent exonic silencer (red box) antagonizes the activity of enhancer proteins, resulting in destabilization of U2AF and exon exclusion. (Modified from Chen and Manley, 2009).

also physically occlude the entry of early spliceosomal components, as is the case of the neuron-specific splicing factors FOX1 and FOX 2. Binding of these two proteins causes skipping of exon 4 of the calcitonin-related polypeptide-α (CALCA) pre-mRNA by binding to an ISS to prevent SF1 from interacting with the neighboring branch site (Figure 1-5A)(Zhou and Lou, 2008). In addition to inhibiting exon recognition by blocking 3'ss usage, repressor proteins also prevent the recognition of the 5'ss by the U1

snRNP (Figure 1-4B). For instance hnRNP H can bind to a sequence that overlaps the exon/intron junction at the 5'ss in exon 3 and exon 2 of the NF-1 and TSHβ transcripts, respectively, thus restricting the accessibility of the U1snRNP and causing exon skipping (Buratti et al., 2004).

When the binding sites for splicing inhibitors do not overlap with the splice sites, the "bind-and-block model" cannot explain their repressive effects. In this case, a different model has been proposed to explain the activity of splicing repressors that involves the propagation of hnRNPs along an exon, and into the upstream intron (3'ss region), to occlude the binding of general splicing factors and SR proteins (see below) (Martinez-Contreras et al., 2007). Protein multimerization is initiated by the binding of an hnRNP to a high-affinity ESS that then promotes cooperative binding of more hnRNP molecules to adjacent low-affinity silencer elements (Zhu et al., 2001). An alternate model proposes that a regulated exon can be "looped-out" as a result of protein-protein interactions between splicing inhibitor proteins bound to sites flanking the regulated exon (Damgaard et al., 2002; Nasim, et al., 2002). As a result, the accessibility of spliceosome components for the regulated exon is restricted and the flanking constitutive exons in the pre-mRNA are brought in close proximity facilitating intron definition between the two exons (Martinez-Contreras et al., 2007). The "looping-out" model has been proposed to explain the repressive effect of hnRNP A1 on exon 7B its own pre-mRNA (Figure 1-6A) (Hutchison et al., 2002) and exon 7 of the survival of motor neuron 2 (SMN2) pre-mRNA (Kashima et al., 2007a), as well as the inhibitory function of PTB on c-scr exon N1 (Sharma et al., 2005).

Antagonism among splicing activators and repressors

Functional antagonism and competitive binding between hnRNPs and SR proteins are two additional mechanisms shown to modulate splice site choice. In the first mechanism, ESE-bound SR proteins can promote splicing by counteracting the repressive activity of hnRNPs bound to silencer elements, and vice versa. In the second, hnRNPs

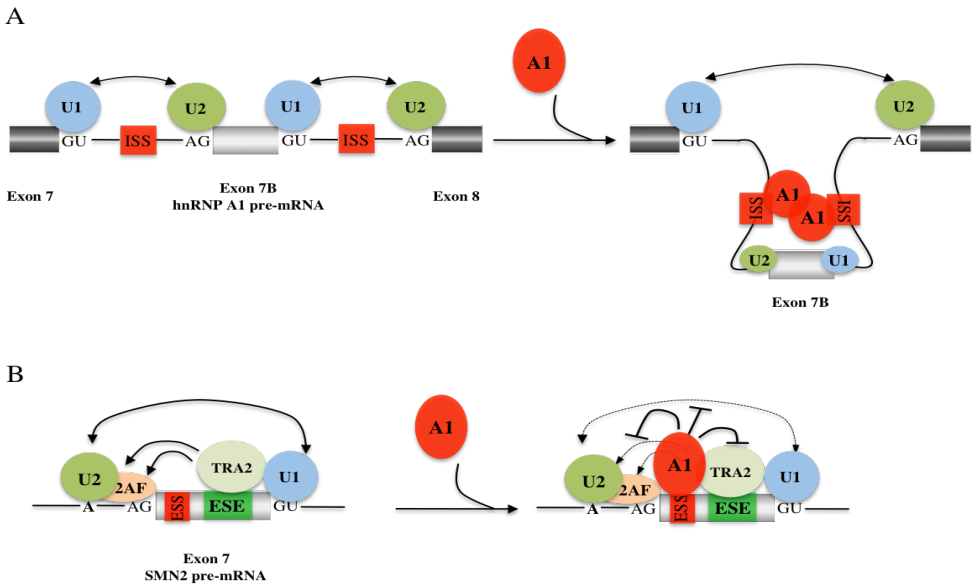

Figure 1-6. Different mechanisms of splicing control used by hnRNP A1. (A) HnRNP A1 molecules bound to intronic splicing silencers (ISSs) in the introns flanking alternative exon 7B of the HnRNP A1 pre-mRNA interact with each other to loop out exon 7B and simultaneously bring the splice sites exons 7 and 8 close together to stimulate their splicing. (B) On SMN2 exon 7, hnRNP A1 binds to exonic silencer element (ESS) and blocks the activity of a downstream Tra2-dependent ESE, this in turn inhibits the recruitment and/or stabilization of the U2 snRNP on the upstream 3'ss (Modified from Chen and Manley, 2009).

can block the binding of SR proteins, and vice versa, to a substrate by competing for access to a proximal or overlapping binding site. Functional antagonism between activators and inhibitors of splicing is critical to the regulation of exon 7 of the SMN2 pre-mRNA. HnRNP A1 binds to an ESS within exon 7 and antagonizes the ability of the SR-like protein transformer 2 (Tra2) bound to a distant ESE downstream in exon 7 to

promote and/or stabilize the binding of the U2 snRNP to the upstream 3'ss region (Figure 1-6B)(Kashima and Manley, 2003; Martins de Araujo et al., 2009; Kashima et al., 2007b).

On the other hand, an example where the competitive effects between splicing factors regulates exon recognition comes from the regulated splicing of exon 4 of the CALCA gene. Binding of the repressor proteins FOX1 and FOX2 to exon 4 prevents the splicing activators Tra2 and SRp55 from binding to an ESE that is the adjacent to the FOX-binding sites. This in turn, inhibits the ability of the SR activators to recruit the U2AF heterodimer to the 3'ss upstream of exon 4 (Figure 1-5B)(Zhou and Lou, 2008). Similarly, the regulation of exon 2 of the α-tropomyosin pre-RNA is determined by a competition between the SR protein 9G8 (exon inclusion) and the hnRNP F and H proteins (exon skipping) for the binding to the same sequence element (Crawford and Patton, 2006). From the examples discusses so far, it easy to see how the relative levels of individual SR proteins and hnRNPs can contribute to the developmental- and tissue-specific regulation of alternative splicing (see below).

Context-dependent function of splicing regulators

Studies into the identity and function of the sequences and proteins involved in regulating splice site choices have clearly established that some proteins activate and some repress splice site usage. However, these same studies also reveal examples where often times the activity of a regulatory effector protein in splicing depends on the context of the sequence to which it binds (Chen and Manley, 2009). Theoretically, any given regulatory protein could potentially have dual effects on splicing, that is, in one context a protein may function as an enhancer of splicing and as a repressor of splicing in another.

The most frequent type of determinant of context-dependent function of protein regulators appears to be the location of their binding site (exonic or intronic) relative to the regulated exon (Chen and Manley, 2009; Singh and Valcarcel, 2005). Other variables like strength of binding site (binding affinity of protein for that sequence), proximity to canonical splice sites, and splice site strength have been proposed to influence the function of regulatory proteins in splicing (Feng et al., 2008; Mayeda and Krainer, 1992; Eperon et al., 2000; Hui et al., 2005; Caceres et al., 1994; Motta-Mena et al., 2010).

Some of the proteins whose activity is dependent on the location of their cognate binding site include NOVA1, NOVA2, hnRNP L, SF2/ASF, hnRNP H, among others. For example, NOVA1 and NOVA 2 regulate a specific set of mRNA precursors in the brain, where these proteins are specifically expressed (Ule et al., 2003 and 2005). Binding of NOVA1 and NOVA2 to a well-defined YCAY element present in an exon results in exon skipping, while binding of these proteins to a the same YCAY sequence in the intron downstream of a regulated exon promotes exon inclusion (Figure 1-7)(Ule et al., 2006; Licatalosi et al., 2008). In a similar fashion, hnRNP L binding to an ESS within exon 4, 5 and 6 of the CD45 transcript was shown to lead to the repression of these exons (Rothrock et al., 2005; Tong et al., 2005); while in a separate example binding of hnRNP L to a sequence in the intron 13 (similar to the CA-rich motif within the CD45 exons) of the endothelial NO synthase (eNOS) pre-mRNA was shown to activate splicing of the intron (Hui et al., 2003 and 2005). In an analogous example, the binding of hnRNP H to an intronic G-rich sequence downstream of a 5'ss enhances spliceosome assembly on that intron, but it inhibits splicing when the G-rich element is located in an exon (Caputi and Zahler, 2001).

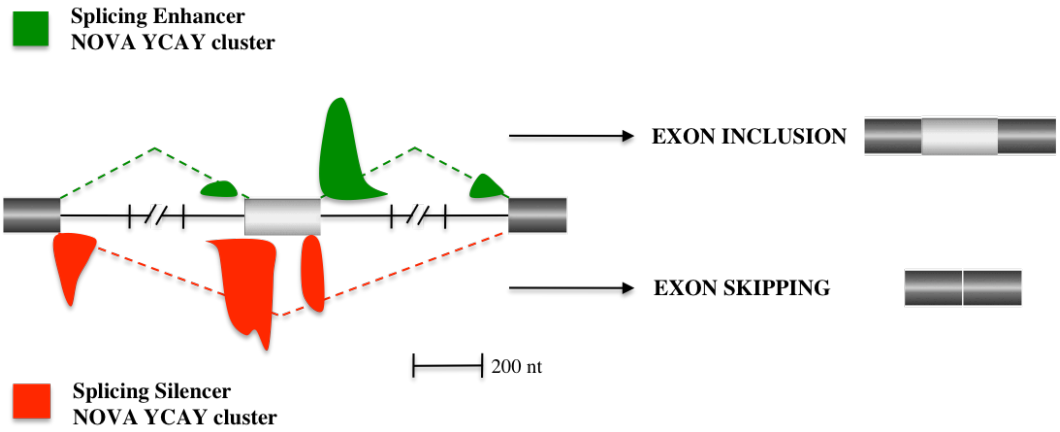

Figure 1-7. NOVA-RNA interaction map. High-throughput sequencing following *in vivo* cross-linking and immunoprecipitation (HITS-CLIP) was used to provide a high-resolution map of NOVA-2 binding in mouse neocortex tissue. Close to 75% of HITS-CLIP tags mapped to known NOVA-regulated genes and exhibited a significant enrichment of YCAY clusters (NOVA preferred binding sites). By compiling data from 1085 HITS-CLIP tags identified from 71 NOVA-2-regulated cassette exons and mapping these onto a "composite" pre-mRNA, Licatalosi et al. (2008) generated a map that shows that the position of NOVA-binding sites (YCAY clusters) determines the outcome of regulation by NOVA (Blencowe et al., 2009). Specifically, NOVA-binding to YCAY clusters within intronic regions promotes exon inclusion (top panel; green), while exonic YCAY-clusters bound by NOVA promoted exon exclusion (bottom panel; red). (Modified from Ule et al. .2006 and Licatalosi et al.. 2008)z.

In contrast to the previous examples, the SR protein SF2/ASF can function as a negative regulator of splicing when bound to an intron, which is the case when SF2/ASF binds to the adenovirus major late region L1 pre-mRNA and prevents the recruitment of the U2 snRNP to the 3'ss of exon IIIa (Kanopka et al., 1996). However, when bound to ESEs SF2/ASF effectively functions as an activator of splicing by promoting exon-definition (Krainer et al., 1990a and 1990b; Ge and Manley, 1990). Studies aimed at determining the effects of binding location on the mechanism of NOVA1/2 function found that NOVA1/2 bound to an ESS directly inhibits the binding of the U1 snRNP (Ule et al., 2006). However, the mechanisms underlying the position-dependent effects of other splicing regulatory proteins are not yet known. Nevertheless, the observation that sequence context can fundamentally alter the directionality of protein function greatly

impacts our predictions of mechanisms of splicing regulation and of alternative splicing patterns.

Regulation at later stages of the splicing reaction

As is evident from the splicing mechanisms depicted above, many alternative splice site choices are made during the very early stages of spliceosome assembly, that is to say during the initial binding of the U1 and U2 snRNPs to the pre-mRNA. Recent data however have demonstrated that regulation can also occur later on in the assembly pathway, after the stabilization of U1 and U2 snRNP-binding (Bonnal et al., 2008; House and Lynch, 2006; Sharma et al., 2008). In three of the four examples discovered so far the block in splicing occurs in the transition from exon-definition to intron-definition and is mediated by a different, yet specific protein factor in each case: hnRNP L, PTB and RBM5. In the case of hnRNP L, its binding to a ESS in CD45 exon 4 (see above) hyper-stabilizes the binding of the U1 and U2 snRNPs across the exon, thereby inhibiting the ability of the snRNPs to participate in the cross-intron interactions necessary for catalysis (Figure 1-8)(House and Lynch, 2006). In the second example, PTB regulates the skipping of exon N1 in the c-src RNA precursor, specifically in non-neuronal cells, by binding to intronic sequences flanking the N1 exon, instead of inhibiting by binding to the exon (as in exon 4 of CD45) (Sharma et al., 2008). PTB binding to the upstream and downstream introns of the N1 exon prevents the U1 and U2 snRNPs on each intron from interacting with one another (intron-definition) thus excluding the N1 exon (Sharma et al. 2005 and 2008). One model suggests that the intron-bound PTB molecules interact with one another and "loop-out" the N1 exon so it is blocked form further assembly (Sharma et al. 2005, see above). Another model proposes that the PTB-repressed complex prevents the

recruitment of specific intron-bridging factor(s) that are necessary for the transition to the intron-defined spliceosomal complex (Sharma et al., 2008). Given that additional

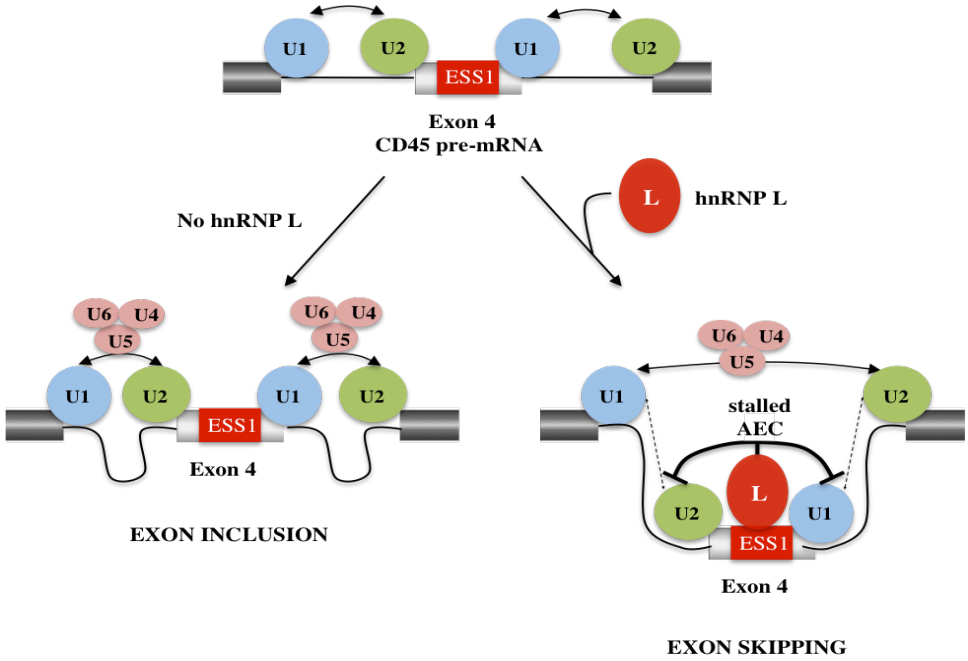

Figure 1-8. HnRNP L represses CD45 exon 4 by hyperstabilizing an AEC. (Left panel) In the absence of hnRNP L binding, U1 and U2 snRNPs flanking exon 4 first interact across the exon and subsequently move to cross-intron interactions. Later the U4/U6/U5 tri-snRNP is recruited to each intron flanking exon 4, resulting in the removal of each intron and the inclusion of exon 4 in the final mRNA. (Right panel) Binding of hnRNP L to the ESS1 element within CD45 exon 4 inhibits the pairing of the U1 and U2 snRNPs across an intron and traps the exon in a A-like exon-defined complex (AEC), thus preventing the progression from an A to B complex along the spliceosome assembly pathway.

experiments showed that PTB acts specifically on the exon N1 5'ss complex, a third model was proposed that suggests PTB could prevent a conformational change in the U1snRNP or block contacts on the U1 snRNP that are needed to create/promote cross-intron interactions between the snRNPs (Sharma et al., 2008; personal communication Sharma and Black). Lastly, RBM5 (RNA-binding protein 5) was shown to inhibit inclusion of exon 6 of the CD95 gene by blocking the recruitment of the U4/U6•U5 tri-snRNP to growing complexes in the two introns flanking exon 7; at the same time,

RBM5 promotes the addition of the tri-snRNP to the intron-defined spliceosomal complex between exons 5 and 7 (Bonnal et al., 2008).

Regulatory decisions can also be made even after at the first catalytic step of splicing, at the second catalytic step (Lallena et al., 2002). Binding of the spliceosomal protein SPF45 to an AG-dinucleotide at the 3'-end of an intron marks that site as the one participating in the second step of the cleavage reaction. In the *Drosophila* Sex-lethal transcript, SPF45 binds to a proximal 3' AG upstream of a distal 3' AG that is initially bound by U2AF35. Binding of PSF45 activates the proximal AG for the second catalytic step of the splicing reaction and promotes exon inclusion. However, when the Sex-lethal (Sxl) protein is present it interacts with SPF45 at the proximal 3'AG and stalls catalysis, resulting in exon skipping (Lallena et al., 2002).

The results discussed above demonstrate that the commitment to splicing of some alternative exons can occur during splice site pairing in the transition from exon-definition to intron-definition, or during the selection of the 3'ss AG at the second catalytic step. Given the dynamic nature of the spliceosome, due at least in part to its remarkable stepwise assembly on pre-mRNAs, the potential for regulation at multiple points in assembly is incredibly high; as such we would expect many more spliceosome intermediates to be identified in the future. Taken together these studies ultimately provide a better mechanistic understanding of the regulation of alternative splicing.

Additional determinants that contribute to splicing control

After reviewing the many strategies that splicing regulators use to direct splicing, additional considerations must also be discussed in order to fully understand how alternative splicing is/can be regulated. Often times in splicing (and in other biological

processes), there is more than one level of regulation governing a given splicing event. For example, the availability and/or activity of *trans*-acting splicing proteins can be dependent on tissue type, developmental stage, or extracellular stimulus (Black, 2003; Blencowe, 2006; Blaustein et al., 2007; Chen and Manley, 2009). Moreover, the function of splicing proteins can also be regulated by post-transcriptional modifications and by their relative localization inside the cell (Martinez-Contreras et al., 2007; Long and Caceres, 2009; Chen and Manley, 2009).

A significant number of tissue-specific (e.g., NOVA1/2, FOX1/2, nSR100) and developmental stage-specific [e.g., CUGBP1/2, MBNL1] splicing regulators, and their corresponding target mRNA transcripts, have now been identified (Underwood et al., 2005; Calarco et al., 2009; Buckanovich et al., 1993). While the vast majority of these proteins regulate either neural- or muscle-specific splicing events; recently, two newly identified epithelial cell-specific splicing factors, ESRP1 and ESRP2, were shown to control expression of a number of epithelial cell-specific exons in several mRNA transcripts (Warzecha et al., 2009). The levels of ESRP1 and ESRP2 were found to correlate with changes in the splicing patterns seen during the epithelial-to-mesenchymal transition (Warzecha et al., 2009). Another recent study looked at alternative splicing events that occur specifically during the postnatal heart development (Kalsotra et al., 2008). Using splicing microarrays coupled to computational and expression analysis, the authors demonstrated that a number of critical fetal-to-adult splicing transitions are regulated by postnatal changes in CUGBP1 and MBNL1 protein expression levels (Kalsotra et al., 2008).

The repertoire of splicing factors, like the SR and hnRNP proteins, is unique to each cell type (Smith and Valcarcel, 2000). Thus, the regulation of the expression and activity of these proteins is essential to the maintenance of specific alternative splicing patters that dictate cell-type or tissue function. Importantly, changes in the ratios of splicing proteins can have important consequences on the splicing outcomes for any number of genes in a specific cell or tissue (Smith and Valcarcel, 2000). Data supporting this notion comes from a study that found that the protein levels of SF2/ASF and hnRNP A1 (that is their relative ratios) vary naturally in different rat tissues and in various immortalized and transformed cell lines (Hanamura at al., 1998). As previously discussed, antagonism between these two proteins has long been known to regulate splicing of a number of pre-mRNAs.

Activation of several signaling pathways has also been implicated in alternative splicing regulation (Blaustein et al., 2007). Recently, splicing microarray studies have identified several genes that undergo changes in alternative splicing in T cells in response to antigen challenge and immune response activation (Ip et al., 2007). Other studies have aimed to identify the molecular mechanisms by which extracellular cues are transduced from the cell surface (where a lot of the initial signaling cascades are turned on) to the splicing machinery inside the nucleus. One such study has used CD45, a gene long known to be alternatively spliced in response to T cell activation, to examine these mechanisms. This work demonstrated that in resting T cells the splicing factor PSF is directly phosphorylated by the Ser/Thr kinase GSK3 (glycogen synthase kinase 3) and that this phosphorylation promotes the interaction of PSF with a protein called TRAP150. The interaction of PSF with TRAP150 prevents PSF from binding to the CD45 pre-

mRNA (Figure 1-9A). Upon T cell activation, GSK3 activity is strongly downregulated (Diehn et al., 2002) which results in reduced phosphorylation of PSF. This in turn releases PSF from TRAP150 and allows it to bind the splicing regulatory elements in CD45 to repress variable exon inclusion (Figure 1-9B)(Heyd and Lynch submitted).

Alternative splicing can also be regulated by the rate and pausing of

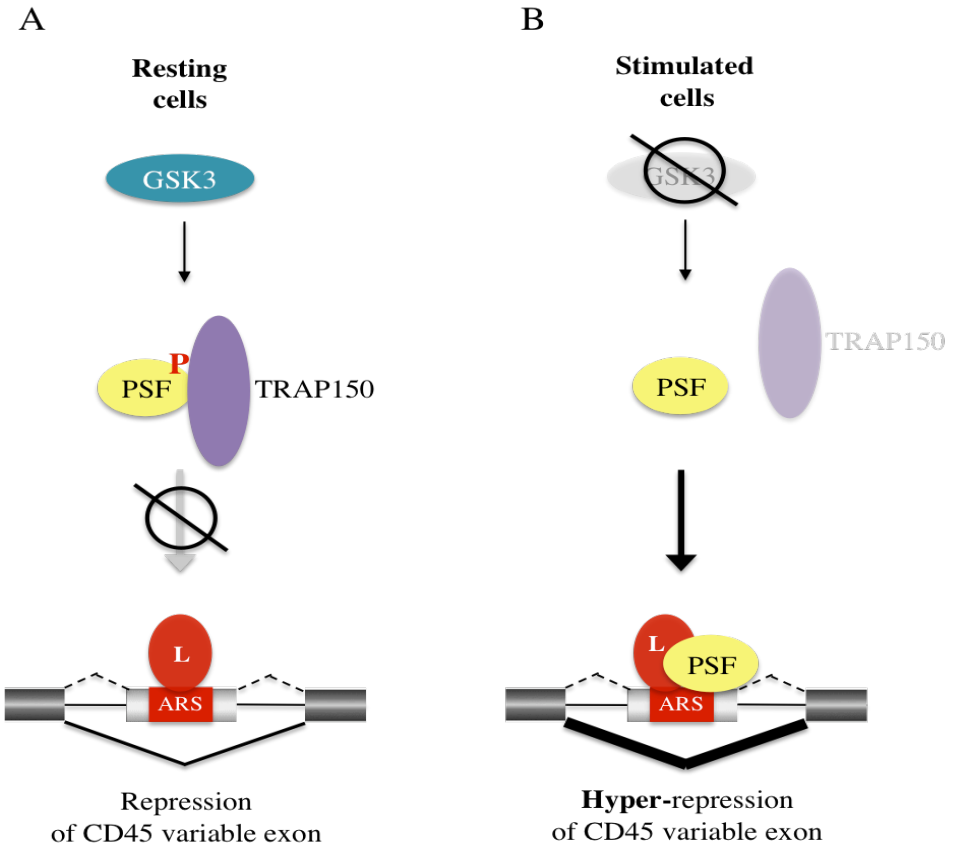

Figure 1-9. Regulation of PSF activity on CD45 alternative splicing. (A) In resting T cells GSK3 is active and phosphorylates PSF, promoting the interaction of PSF with TRAP150 which in turn blocks PSF from binding the activation-responsive sequence (ARS) element within the CD45 variable exons. (B) Upon stimulation, the activity of GSK3 is downregulated, resulting in decreased levels of phosphorylated PSF, unphosphorylated PSF is no longer bound by TRAP150, allowing PSF to bind to the exon 4 ESS1 element and function in the signal-induced hyper-repression of the CD45 variable exons. (Modified from Heyd and Lynch, *submitted*).

transcriptional elongation. Introducing transcription pause sites into a gene or using a mutant RNA polymerase II with a lower elongation rates results in higher inclusion of alternative exons in the mature mRNA (Roberts et al., 1998; de la Mata et al 2003; Howe et al., 2003). One explanation for the effects of RNA polymerase II rates on splicing is that as RNA synthesis is going on and the spliceosome is assembling on the pre-mRNA it is presented with several potential splice sites. More often than not, the "strongest" signal sequences will outcompete the rest. However, if the polymerase slows down or pauses, and the "stronger" splice site is not synthesized yet, then the "weaker" splice site which is available, will be used by the spliceosome (Kornblihtt, 2006). An alternative model to explain how transcription influences splicing has been proposed that is based on the finding that several splicing regulator proteins can interact with C-terminal domain (CTD) of RNA polymerase II (Yuryev et al., 1996; Morris and Greenleaf, 2000; Rosonina et al., 2005; de la Mata and Kornblihtt, 2006; Misteli and Spector, 1999). The CTD can be differentially phosphorylated during the transcription cycle and specific phosphorylations can confer different elongation properties on the enzyme (Dahmus, 1996; Corden and Patturajan, 1997; Buratowski, 2003). In general the CTD is thought to act as a platform for pre-mRNA splicing factors to be recruited and that way increase the local concentration of available splicing factors at sites of active transcription (Kornblihtt, 2006).

The control of the splice site choice can also be influenced by secondary structures in the pre-mRNA molecule. For example, a stem loop structure within exon 6 B of the chicken β-tropomyosin pre-mRNA was shown to sequester this exon and cause its exclusion from the mature transcript (Fiszman et al., 1992). In other cases RNA

secondary structures can affect alternative splicing by masking either splice site or binding sites for splicing regulatory proteins (Grover et al., 1999; Hiller et al., 2007; Camats et al., 2008).

Combinatorial control of the splicing reaction

Fundamentally, alternative splicing occurs through the enhancing or silencing of exons or splice sites by modulating the assembly of the spliceosome on a pre-mRNA. Ultimately, the decision to include or exclude an exon into the final mRNA is based on the combination and/or integration of both of the synergistic and antagonistic forces between groups of protein regulators and between protein regulators and the spliceosomal subunits (Smith and Valcarcel, 2000; Hertel, 2007). Indeed, combinatorial regulation of splicing makes sense because the majority of the RNA-RNA, RNA-protein, and protein-protein interactions in the spliceosome are "weak" (that is low-binding affinity). In fact, having these types of flexible intermolecular interactions enables the spliceosome to be highly responsive to regulation. In conclusion, the control of splice site choice by the spliceosome does not depend on any one single parameter, instead it relies on the relative contributions of various parameters (such as splice site strength, the presence and strength of *cis*-acting sequence elements, RNA secondary structures, exon/intron length, the rate of transcription, and others) to control when and how an exon is effectively included or excluded from the final RNA message.

CD45: a model gene to study signal-induced alternative splicing

The human CD45 gene encodes a transmembrane protein tyrosine phosphatase that is expressed on the surface of all nucleated haematopoietic cells and plays central roles in antigen receptor signal transduction and lymphocyte development (Hermiston et

al., 2003). In recent years, the contributions of CD45 studies to the understanding of signal-induced alternative splicing, as well as the mechanisms of splicing regulation in general (see below), have proven that this gene is an excellent model system for studying pre-mRNA splicing.

CD45 protein has two main structural features (Figure 1-10)(Hermiston et al., 2003). The first (going from the N- to C-terminus) is an extracellular domain that is heavily N- and O-linked glycosylated and contains a cysteine-rich region followed by three fibronectin type III repeats. The second (which follows the single transmembrane domain) is the cytoplasmic tail, which contains two PTPase homology domains, D1 and D2, however only D1 has enzymatic activity (Desai et al., 1994). Importantly, several isoforms of CD45 are expressed as a result of alternative splicing of three exons (E4, E5 and E6) in the N-terminal extracellular region (Trowbridge and Thomas, 1994). Because the three variable exons encode multiples sites of O-linked glycosylation and sialic acid modification, the various protein isoforms differ significantly in size, shape, and negative charge (Hermiston et al., 2003). These changes in protein composition and architecture ultimately have an impact on the activity of the protein (McCall, 1992) and will be discussed later.

As mentioned above, the extracellular domain of CD45 is subject to extensive regulation, most notably at the level of pre-mRNA splicing (Birkeland et al., 1989; Hermiston et al., 2002; Hermiston et al., 2003). The expression of specific CD45 variants is dependent on the developmental stage and activation state, as well as the lineage, of the lymphocyte studied (Fukuhara et al., 2002; Trowbridge and Thomas, 1994). For the purposes of this study we will specifically examine the isoforms of CD45 that are

expressed in T cells during the naïve and activated states. In naïve T cells five CD45 isoforms can be detected at the mRNA and protein level (Thomas, 1989; Trowbridge and Thomas, 1994). When the T-cell receptor (TCR) is engaged and the cell is activated a switch in the splicing of the three variable exons occurs, wherein the three exons are

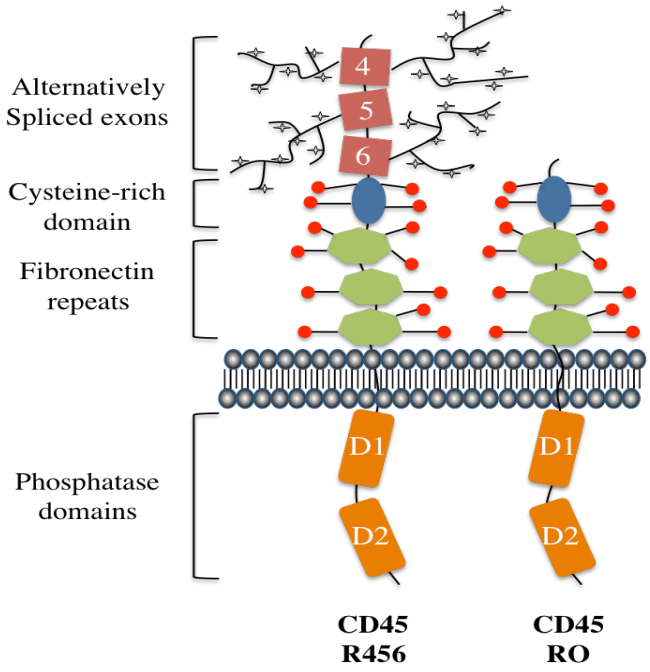

Figure 1-10. Domain structure of CD45 protein isoforms. Alternative splicing of the CD45 variable exons (4, 5, and 6) changes the N-terminal region of the protein. The three exons encode multiple sites of both O-linked glycosylation and sialic acid modification; therefore, alternate CD45 isoforms (R456 and R0) differ in their glycosylation patterns. This region is followed by a glycine-rich domain and three fibronectin type III repeats that are both N-glycosylated, a single transmembrane domain, and two intracellular phosphatase domains (D1 and D2). (Modified from Hermiston et al. 2003).

preferentially excluded from the final mRNA transcript (Trowbridge, 1991; Trowbridge and Thomas, 1994). This event results in a 3 to 5 fold change in isoform expression from the higher molecular weight isoforms (R456, R45, R56, R5), which contain the three exons in various combinations, to the lower molecular weight isoform (R0) that contains none (Lynch and Weiss, 2000).

The change in isoform expression upon T cell activation, results in a shift in the relative levels of the five CD45 protein variants expressed on the cell surface, as well as an effect on the enzymatic activity of the protein (Trowbridge, 1991; Trowbridge and

Thomas, 1994). Previously it was shown that the larger CD45 isoforms (see above) exist mostly as monomers and have high phosphatase activity (Hermiston et al., 2003, Xu and Weiss, 2002). Conversely, the smaller RO isoform of CD45 dimerizes more frequently and efficiently than the larger isoforms, and as a result of dimerization its phosphatase

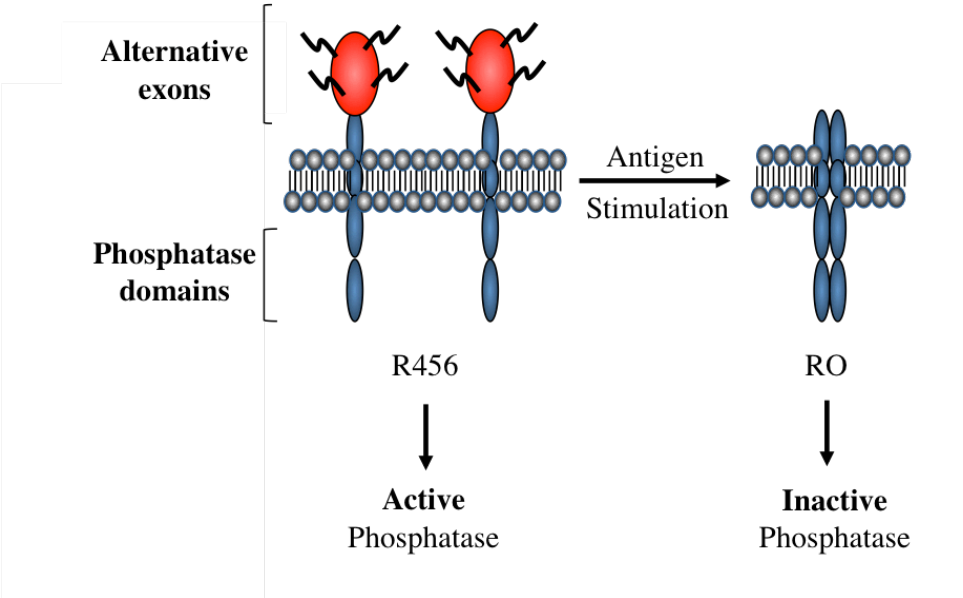

Figure 1-11. Alternative splicing of the CD45 pre-mRNA determines the activity of the encoded protein. The larger CD45 isoform (R456), which contain the region encoded by the variable exons, exist predominantly as monomers and are catalytically active. In comparison, the smaller isoform (R0), which lacks the variable exons, dimerizes more efficiently resulting in steric inhibition of the catalytic site.

activity is inhibited. Additional studies revealed that sialylation and O-glycosylation of the alternatively spliced exons inhibits protein dimerization (Xu and Weiss, 2002). From these data a model for CD45 regulation in T cells was derived (Figure 1-11). Naïve or resting T cells express more of the larger CD45 isoforms, which because they contain the variable exons are glycosylated and sialylated. Given the charge and bulkyness of the extracellular domain of these protein variants, CD45 predominantly exists as a monomer, which is the catalytically active state. Upon cellular activation, the switch in alternative

splicing results in more of the RO isoform present on the T cell surface. Since in RO the variable exons are missing, the protein is not modified and it is therefore able to dimerize efficiently and as result the protein is less active (Xu and Weiss, 2002).

Physiologically the differential dimerization of the CD45 isoforms is thought to regulate the "priming" of naïve T cells in preparation for TCR activation and the attenuation of prolonged TCR signaling (Hermiston et al., 2003). CD45 participates in the earliest stage of TCR activation by dephosphorylating the negative regulatory sites on two Src family protein tyrosine kinases (SFKs), Lck and Fyn (Saunders and Johnson, 2010). The "CD45-activated" forms of Lck and Fyn are then capable of acting directly on the TCR if antigen is encountered (Saunders and Johnson, 2010). On the contrary, the loss of CD45 activity upon cellular stimulation has an opposing effect on the activation of Lck and Fyn, which over time contributes to the cessation of the previously elicited immune response. It is important to note that naturally occurring mutations in the CD45 gene, some of which disrupt splicing sequences, have been implicated in the susceptibility to autoimmune diseases and viral infections (Tchilian et al., 2001; Tackenberg et al., 2003; Lynch and Weiss, 2001; Dawes et al., 2006, Cale et al., 1997; Kung et al., 2000). In addition, loss of CD45 has frequently been reported in patients with acute lymphoblastic leukemia, Hodgkin's lymphoma and multiple myelomas (Ratei et al., 1998; Ozdemirli et al., 1996; Ishikawa et al., 2000).

Given that CD45 function in lymphocyte development and TCR activation depends on the regulation of the expression of its different splice variants, the study of the mechanisms that govern alternative splicing of the variable exons of CD45 is extremely important and therefore has been the primary focus of the Lynch laboratory for

the past several years. In order to study the splicing of CD45 in a laboratory setting, a T-

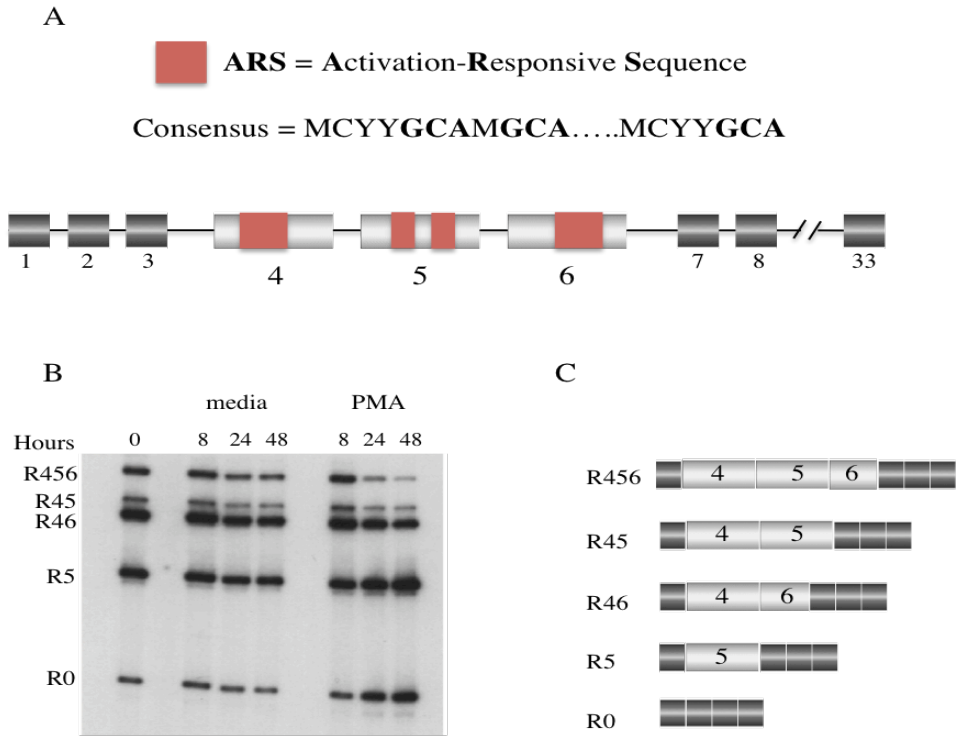

Figure 1-12. Signal-induced alternative splicing of the CD45 variable exons is mediated by an ARS. (A) Schematic of the CD45 gene showing that location of the ARS consensus element within each variable exon (4, 5, and 6); of note, in exons 4 and 6 the ARS is contained within a single region while in exon 5 it is divided into two regions. (B) RT-PCR analysis of CD45 mRNA isoforms expressed in JSL1 cells under resting and PMA-activated conditions. Variable exons are partially skipped under resting conditions, but are preferentially skipped upon stimulation. (C) Schematic of the five CD45 isoforms that are expressed at both the RNA and protein level.

cell derived, immortalized cell line was generated. The JSL1 cell line recapitulates the CD45 splicing pattern seen in primary human T lymphocytes under resting conditions (Figure 1-12B, C). Also, treatment of JSL1 cells with the phorbol ester PMA is able to mimic the cellular activation observed during TCR activation (as seen in primary lymphocytes) and triggers the activation-induced increased skipping of the regulated CD45 exons (Figure 1-12B, C)(Lynch and Weiss, 2000).

Initial studies into the splicing regulatory sequences within the CD45 variable exons identified a conserved sequence motif we call the activation-responsive sequence (ARS) in each variable exon (Figure 1-12A)(Lynch and Weiss, 2000; Rothrock et al., 2003; Tong et al., 2005). The ARS motif consists of tandem GCA repeats that when mutated or deleted abolish the basal and activation-induced repression of each of variable exons (Lynch and Weiss, 2000; Rothrock et al., 2003 and 2005; Tong et al. 2005). For exons 4 and 6 the ARS motif is embedded within a 60 nt exonic splicing silencer (ESS1) element that is both necessary and sufficient for regulation (Figure 1-12A)(Rothrock et al., 2003; Tong, et al., 2005). In contrast, the ARS motif in exon 5 is split across two regions (S1 and S2) that are separated by an exonic splicing enhancer sequence (ESE) (Figure 1-12A and 1-14B)(Tong et al., 2005).

The ARS-containing ESS1 regulatory element from CD45 exon 4 associates, in resting cells, with several members of the hnRNP family of RNA binding proteins, including hnRNPs L, E2, K, D and PTB ((Figure 1-13A)(Rothrock et al., 2005; Melton et al., 2007). Of these multiple hnRNPs, the binding of hnRNP L is most sensitive to mutations of the ARS core motif. Moreover, both *in vitro* and *in vivo* studies have confirmed that hnRNP L is the primary mediator of ESS1-dependent repression in resting cells, with the other hnRNPs having much less if any functional effect (Rothrock et al., 2005; Melton et al., 2007). Upon cellular stimulation, hnRNP L-like (hnRNP LL) and the hnRNP-related protein PSF join the exon 4 ESS1-associated complex and function in combination with hnRNP L to achieve maximal exon repression (Figure 1-13B)(Melton et al., 2007; Oberdoerffer et al., 2008, Topp et al., 2008). HnRNP L was shown to block exon 4 inclusion by directly stalling spliceosome assembly after the ATP-dependent

addition of the U1 and U2 snRNPs in an A-like exon-defined complex (AEC) (Figure 1-8)(House and Lynch, 2006).

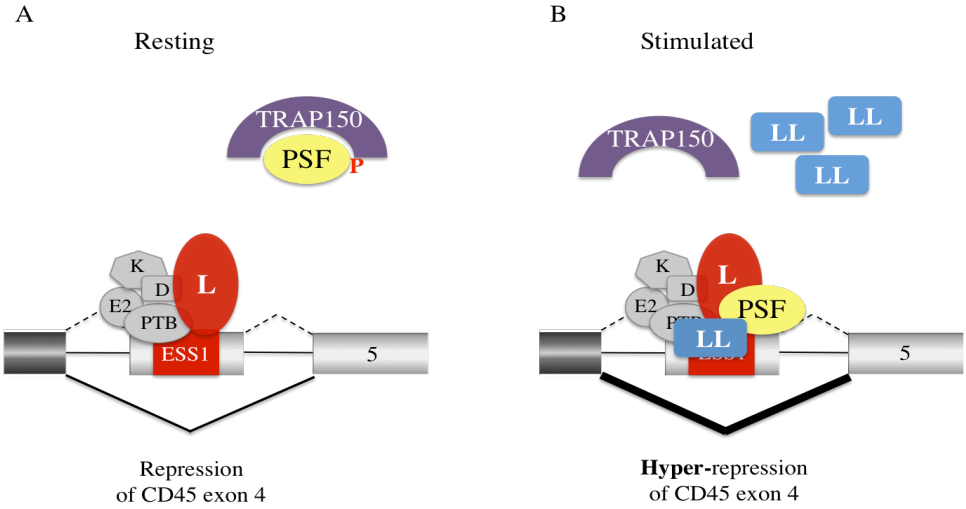

Figure 1-13. HnRNP L, LL and PSF are RNA-binding proteins that function in the regulated splicing of CD45 exon 4. (A) In resting cells, skipping of CD45 exon 4 is mediated by the binding of hnRNP L to an exonic splicing silencer (ESS1). Although other proteins associate with the ESS1 element (hnRNPs E2, K, D, and PTB), they have little to no repressive activity; thus, hnRNP L is the primary mediator of exon 4 skipping. Also, under resting conditions PSF is phosphorylated and interacts with TRAP150. The interaction of PSF with TRAP150 prevents PSF from binding to the ESS in CD45 exons 4 and 5. (B) Upon cellular activation, the phosphorylation of PSF is reduced and it is now able to bind and, together with hnRNP L and hnRNP LL, further repress exons 4 and 5. The differential recruitment of hnRNP LL to exon 4 under resting and activated conditions is due, at least in part, to an increase in hnRNP LL protein levels upon stimulation.

Binding of hnRNP L to the activation-responsive sequence (ARS) in exons 5 and 6 also regulates the skipping of these exons (Tong et al., 2005; Motta-Mena et al., 2010). Given the similar layout of the ARS motifs in exons 4 and 6, it seemed likely that the regulation of exon 6 most resembled that which was established previously for exon 4. However, the unique arrangement of the ARS in exon 5 made it a valuable model for determining whether the broader sequence context of an exon can influence the mechanisms by which a particular regulatory element and/or associated proteins functions (Figure 1-14B). We know that the enhancer sequence in exon 5 is also

important for the recognition and regulation of this exon (Figure 1-14A)(Tong et al., 2005), and so far the *trans*-acting factors that bind to and function on the exon 5 ESE are not known. Moreover, the mechanism by which hnRNP L represses exon 5, and whether it is different from the mechanism of repression on exon 4, is also not known.

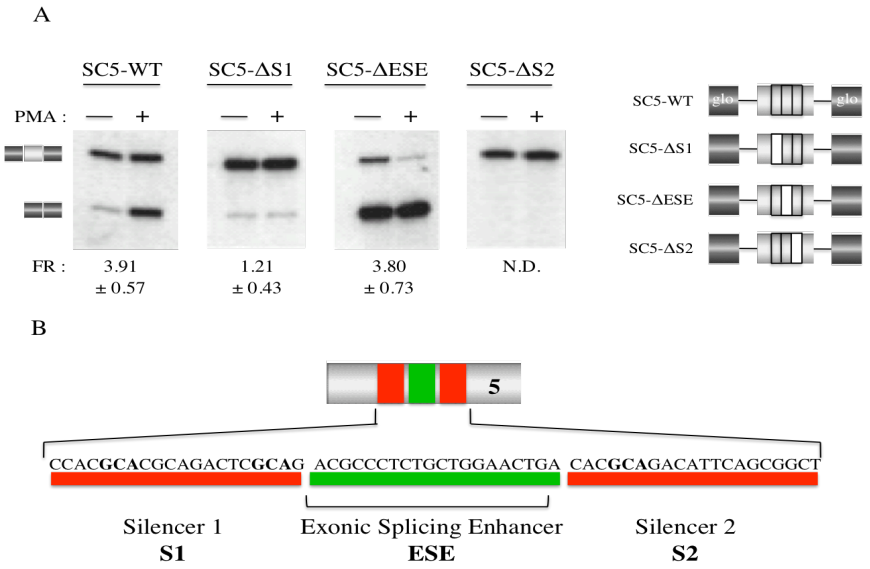

Figure 1-14. Systematic mutagenesis of exon 5 identified three regions important for regulation of this exon: S1, ESE, S2. (A) RT-PCR analysis of minigenes that were stably expressed in JSL1 cells under resting (-PMA) or activated (+PMA) conditions. The SC5-WT minigene contains variable exon 5 and surrounding intron flanked by intron and exon sequences from the human β-globin gene. The other three minigenes are identical except a region of 20 nt was substituted with heterologous sequence previously shown to have no splicing-regulatory activity (Schaal and Maniatis, 1999)(white box). The change in isoform ratio upon cellular activation is expressed as an FR value (calculated as the change in the ratio of 3-exon product to 2-exon product between resting and activated conditions), and where FR = 1 indicates no change in splicing between the two conditions. Quantification shown is the result of at least four independent clones in at least two separate stimulations (Modified from Tong et al. 2005). (B) Sequence of the regions important for regulation of exon 5: the two ARS-containing sequences, S1 and S2, and the enhancer element (ESE). The ARS core motifs if in bold in both the S1 and S2 elements.

The data presented in this thesis demonstrates that unique features of the sequence context of the ARS element in exon 5 results in hnRNP L associating in

isolation on this exon without the additional binding proteins that were previously found to associate with the ESS1 sequence from exon 4 under resting conditions. (Rothrock et al., 2005). Similarly, binding and functional data demonstrated that PSF participates with hnRNP L in the stimulation-induced silencing of CD45 exon 5, as it does for exon 4, however, hnRNP LL has little or no effect on the regulation of CD45 exon 5 (Motta-Mena et al., 2010). In addition, using computational methods and validation studies *in vitro*, the SR protein SF2/ASF was found to specifically bind to the ESE element within exon 5 and function to enhance CD45 exon 5 splicing (Motta-Mena et al., 2010).

Moreover, binding and functional studies demonstrated that hnRNP L bound to the silencers in exon 5 directly competes with SF2/ASF bound to the ESE, inhibiting the ability of the ESE-complex to recruit the U2snRNP to the upstream 3'ss (Motta-Mena et al., 2010). Surprisingly, this mechanism is markedly distinct from the previously reported mechanism of direct repression of exon 4 by hnRNP L (House and Lynch, 2006). Because the splice sites flanking exon 5 are weak compared to those of exon 4, we examined the effect of hnRNP L binding to exons with varying splice site strengths. Remarkably, in multiple distinct exon contexts we found that hnRNP L represses exons flanked by strong splice sites but enhances those flanked by weak splice sites (Motta-Mena et al., 2010). Thus, hnRNP L can repress or activate exon inclusion by distinct mechanisms due, at least in part, to differences in splice site strength. Taken together, our results provide evidence that a given splicing regulatory protein can function through different mechanisms in a manner independent of location but constrained by the local sequence context.

CHAPTER TWO

HnRNP L Binds to the ARS Motif of CD45 Exons 4 and 5 with Different Coassociated Proteins and Represses Each Exon by Distinct Mechanisms

Introduction

Pre-mRNA splicing is a critically important step during pre-mRNA processing in which the non-coding intronic sequences are removed and the exonic coding sequences are joined together. The catalysis of pre-mRNA splicing is mediated by the spliceosome, a large macromolecular machine composed of five small nuclear ribonucleoprotein (snRNP) complexes (U1-, U2-, U4/U6-, U5-snRNP) (Wahl et al., 2009). The spliceosomal snRNPs assemble on the pre-mRNA in a coordinated fashion and recognize specific signal sequences at the exon and intron boundaries (called splice sites) to direct the excision of introns and ligation of exons. In higher eukaryotes, the majority of pre-mRNAs contain multiple exons, which can potentially be spliced together in many different combinations by the process of alternative splicing (Motta-Mena and Lynch, 2010). Importantly, the regulated inclusion or exclusion of an exon by the spliceosome is typically controlled by various proteins bound to splicing enhancer and silencer sequences located within the exon or the flanking introns (Motta-Mena and Lynch, 2010). Mechanistically, these *trans*-acting protein factors function by engaging the spliceosomal snRNPs in such a way that promotes or, in the case of negative regulators, inhibits assembly on neighboring splice sites.

In general, both constitutive and regulated exons contain exonic splicing enhancers (ESEs) that are bound by members of the SR (serine-arginine) family of

proteins; therefore, SR protein-binding to ESEs is essential for the recognition and inclusion of constitutive and alternative exons (Long and Caceres, 2009). Similarly, exonic splicing silencers (ESSs) are thought to play a key role in defining constitutive exons by suppressing neighboring pseudo splice sites and in modulating the level of inclusion of alternative exons (Pozzoli and Sironi, 2005). These negative regulatory elements are commonly recognized by members of the hnRNP family of RNA-binding proteins, a loosely defined family of structurally-diverse RNA-binding proteins that share overlapping functions in RNA splicing, packaging, export and stability (Martinez-Contreras et al., 2007).

An emerging theme in alternative splicing is that of networks of coregulated splicing events wherein the same regulatory protein, bound to its defined consensus sequence element, coordinates the inclusion or exclusion of exons in multiple pre-mRNAs. Coordinate regulation has been demonstrated for a number of genes involved in controlling synaptic plasticity via the binding the neural-specific protein Nova to YCAY motifs embedded in the pre-mRNAs (Ule et al., 2006). Similarly, the neural- and muscle-specific proteins FOX1/2 regulate the splicing of multiple genes involved in neuromuscular function that contain a UGCAUG RNA element (Zhang et al., 2008). Of interest, in these and other studies, the location (intronic or exonic) of the protein-binding site relative to the regulated exon was found to affect the directionality of the protein's activity (positive or negative) on splicing.

An excellent model system to illustrate the mechanisms of regulated alternative splicing, as well as the physiologic significance of this mode of regulation, is the human CD45 gene. CD45 encodes a tyrosine phosphatase protein that is expressed on the surface

of T cells (and other lymphocytes) where it plays essential roles in antigen receptor signaling and lymphocyte maturation (Hermiston et al., 2003). CD45 has three variable exons (exons 4, 5, and 6) that are partially skipped under resting condition, but are coordinately hyper-repressed upon antigen-induced activation of T cells. The extent of inclusion has been shown to alter the dimerization of the CD45 protein, which in turn affects it ability to act on its target substrates (Xu and Weiss, 2002).

Previously it was shown that in resting cells, skipping of the CD45 variable exons was regulated by the binding of the hnRNP L protein to an activation-responsive sequence (ARS) that is embedded within each variable exon (Figure 1-13A and 1-14A)(Rothrock et al., 2003 and 2005; Tong et al., 2005). For exons 4 and 6, the ARS motif is contained within a single silencer element (termed ESS1) that is both necessary and sufficient for regulation. The ARS motif in exon 5 however is split across two silencer regions (termed S1 and S2) that are separated by an ESE (Figure 1-14B)(Tong et al., 2005). Interestingly, several regulated exons from a number of other genes that undergo activation-responsive alternative splicing were found to also contain the ARS motif (Rothrock et al., 2003; Ip et al., 2007). Therefore, it is possible that hnRNP L may also regulate the splicing of these novel ARS-containing exons in addition to the CD45 variable exons. It should be noted that while some of these ARS-containing alternative exons show a splicing pattern similar to the CD45 exons (increased skipping upon stimulation), others display opposite regulation (increased inclusion upon activation) (Ip et al., 2007); thus, it appears that hnRNP L can have dual effects on splicing. Moreover, other studies have shown that location of hnRNP L binding (intronic vs. exonic) can strongly influence the type of effect the protein has on splicing (enhancer or repressor)

(Hui et al., 2005; Hung et al., 2008). Based on these results, and the findings from the Nova and FOX1/2 studies described above, it is possible that the differential activity of hnRNP L is due to context differences of the ARS motif in these exons.

The differential arrangement of the ARS regulatory element in CD45 exons 4 and 5 provides a powerful system, and a great first start, to study how the broader sequence context of an exon can influence the mechanisms by which the ARS motif and hnRNP L functions. In this chapter we demonstrate that the differential arrangement of the ARS regulatory element in exon 5 causes hnRNP L to bind to this exon in the absence of other coassociated proteins previously observed on exon 4 (PTB and hnRNPs E2, K, D in resting conditions; hnRNP LL in activated conditions). Moreover, we show that the SR protein SF2/ASF binds to the ESE in exon 5 and functions to enhance the inclusion of this exon. Finally, we also demonstrate that hnRNP L represses exon 5 by directly competing with SF2/ASF for binding to the exon, thus inhibiting the ability of the SF2-containing ESE complex to recruit the U2 snRNP and promote the formation of a spliceosomal A complex. This mechanism is surprisingly distinct from the mechanism of repression of exon 4, in which hnRNP L blocks inclusion by directly stalling spliceosome assembly after the ATP-addition of the U1 and U2 snRNPs (House and Lynch, 2006).

Results

HnRNP L binds to the exon 5 ARS in isolation under resting conditions and together with PSF upon stimulation

Previously, a group of hnRNP proteins (hnRNP L, E2, K, D and PTB) were found to associate with the ARS-containing ESS1 element from CD45 exon 4 in resting cells (Figure 1-13A). Importantly, hnRNP L is the only one of these ESS1-associated

proteins whose affinity is significantly decreased by mutations that abolish both the basal and signal-induced activity of the ARS motif (Rothrock et al., 2005). Also, modulating the levels of hnRNP L protein both *in vitro* and *in vivo* results in a decrease in the ARS-dependent silencing of exon 4 (Rothrock et al., 2005; Melton et al., 2007). Therefore, hnRNP L is the primary protein responsible for CD45 exon 4 repression through the ARS motif in resting cells (Rothrock et al. 2005; Melton et al. 2007). Cellular activation results in the recruitment of two additional proteins, hnRNP L-like (hnRNP LL) and PSF, to the ESS1 in exon 4, which together with hnRNP L are responsible for the activation-induced increase in exon repression (Figure 1-13B)(Melton et al., 2007; Topp et al., 2008; Oberdoerffer et al., 2008).

As a first step to comparing the function of the ARS motif in CD45 exons 4 and 5, we performed RNA-affinity experiments to determine whether the ARS motif in exon 5 recruits a similar or related set of proteins as compared to exon 4. We first characterized the proteins that bound to the wild type exon 5 RNA sequence (E5-WT) compared to RNAs that contained substitutions in the ESE (E5-ΔESE) or ARS motifs (E5-ΔS1S2). Additional controls included the ESS1 element from exon 4 (E4-ESS1) and a nonspecific RNA (NS) that was previously shown to have no splicing-regulatory activity (Rothrock et al., 2003). The RNAs were chemically coupled to beads and incubated in nuclear extract from resting JSL1 cells, afterwards the beads were washed extensively, and the RNA-associated proteins were eluted and visualized by silver stain. The JSL1 nuclear extract recapitulates the ARS-mediated exon repression in *in vitro* splicing assays and therefore contains all functionally relevant repressor proteins (Rothrock et al., 2005).

Consistent with previous studies, we observed binding of hnRNP L, PTB and hnRNP E2 to E4-ESS1 by silver stain and western blot analysis (Figure 2-1A). We also detected a strong signal for hnRNP L binding to the E5-WT RNA (Figure 2-1A).

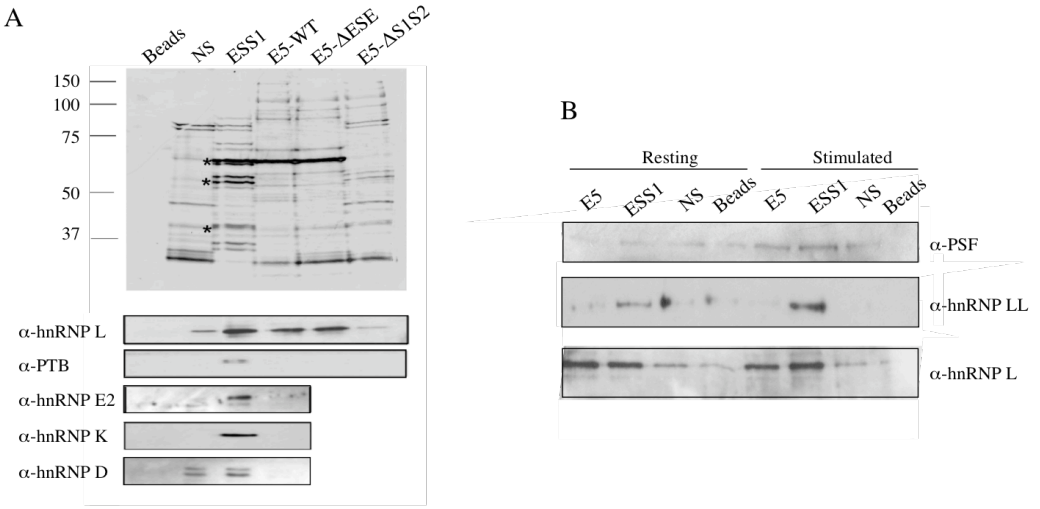

Figure 2-1. HnRNP L binds to the ARS core of exons 4 and 5 with different coassociated proteins. (A) Top, silver stain of RNA-affinity pulldowns done resting nuclear extract with exon 4 (ESS1) and exon 5 (WT, ΔESE, ΔS1S2) probes. Asterisk indicates hnRNP L, PTB, and hnRNP E2. Bottom, Western blot analysis of same RNA-pulldown samples using antibodies against previously characterized ESS1-binding proteins. (B) Western blot analysis of RNA-pulldown samples done in resting (left) and stimulated (right) nuclear extract.

Importantly, replacing the ARS motifs in exon 5 abolished binding of hnRNP L, whereas mutation of the enhancer element had little to no effect on the association of hnRNP L with the RNA (Figure 2-1A). Interestingly, however, neither PTB nor hnRNP E2 were observed to associate with the E5-WT RNA by either silver stain or western blot, nor were the more weakly ESS1-associated protein hnRNP L and D (Figure 2-1A).

These preliminary results were further confirmed by RNA mobility shift assays, wherein increasing amounts of a particular purified recombinant protein are incubated with a given ^{32}P-labeled RNA, the resultant RNA-protein complexes are resolved on a native polyacrylamide gel and detected by autoradiography. Titration of recombinant

GST-tagged hnRNP L with E4-ESS1, E5-WT or E5-ΔESE demonstrated that the inherent affinity of this protein is similar for all three RNAs; however, the affinity of hnRNP L for exon 5 was reduced by ~10-fold upon mutation of the ARS elements (E5-ΔS1S2)(Figure

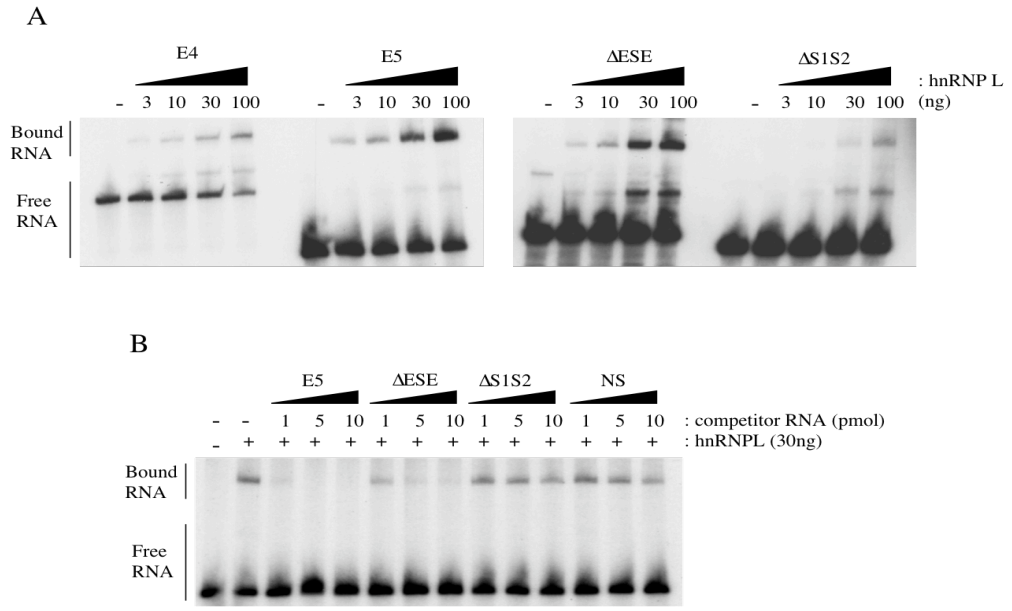

Figure 2-2. Binding of hnRNP L to exon 5 is sensitive to mutation of the ARS elements. (A) RNA mobility-shift experiments of radiolabeled versions of the probes from Figure 2-1, incubated with increasing amounts of recombinant hnRNP L. (B) RNA mobility-shift experiment using 1 pmol E5-WT probe incubated with recombinant hnRNP L in the absence or presence increasing amounts of the indicated competitor RNAs.

2-2A). The specificity of hnRNP L for the ARS in exon 5 was further confirmed by competition native gel shift assays, which showed competition with unlabeled E5-ΔS1S2 RNA had little to no affect on the association of hnRNP L with ^{32}P-labeled E5-WT, while addition of unlabeled E5-WT or E5-ΔESE RNA almost completely abolished hnRNP L binding (Figure 2-2B). Of the other ESS1-associated hnRNPs, only GST-tagged PTB showed any ability to bind exon 5 in mobility shift assays, but even in this case, the affinity of PTB for exon 5 was at least 10-fold lower than for exon 4 (Figure 2-3A).

MBP-tagged hnRNP E2 protein showed little to no binding for the E5-WT or E5-ΔESE (Figure 2-3B). Thus, we conclude that hnRNP L is the major, if not only, protein bound to the exon 5 ARS motif in resting cells.

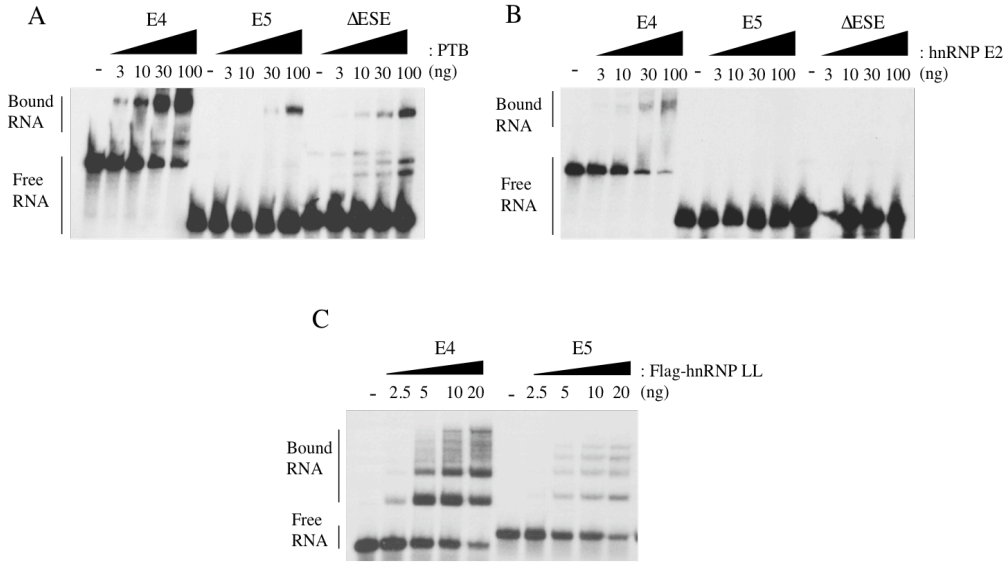

Figure 2-3. E4-ESS1-associated proteins PTB, hnRNP E2 and LL have decreased affinity for exon 5. RNA mobility-shift of radiolabeled ESS1, E5-WT and E5-ΔESE probes incubated with increasing amounts of recombinant PTB (A), hnRNP E2 (B), or hnRNP LL (C).

To determine whether PSF and/or hnRNP LL bind to exon 5 under activated conditions, as was previously shown for exon 4, we carried out a similar RNA affinity experiment in nuclear extract from stimulated JSL1 cells. Neither PSF nor hnRNP LL are readily detected by silver stain (data not shown; Melton et al., 2007). Nevertheless, western blot analysis confirmed that both PSF and hnRNP LL associate with E4-ESS1 preferentially under activated conditions (Figure 2-1B). Remarkably, PSF also associated with the E5-WT RNA in nuclear extract from activated JSL1 cells; however, there was no detectable association of hnRNP LL with E5-WT in either resting or stimulated nuclear extracts (Figure 2-1B). RNA mobility shift assays done with Flag-tagged hnRNP

LL protein purified from JSL1 cells further confirmed that this protein has markedly reduced affinity for exon 5 relative to exon 4 (Figure 2-3C). Taken together, these results demonstrate that cellular activation triggers the recruitment of PSF to the ARS motif in exon 5, without the additional association of hnRNP LL.

Recombinant hnRNP L and PSF cause ARS-dependent exon silencing of exon 5 in vitro

To confirm that the binding of hnRNP L and PSF to E5-WT RNA is functionally related to the regulated repression of CD45 exon 5, we performed *in vitro* splicing assays. For this assay we transcribed pre-mRNA *in vitro* from a minigene containing exon 5 or exon 4 (used as control) flanked by CD45 constitutive exons 3 and 7 and incubated it in JSL1 nuclear extract under conditions permissive to splicing, the resulting spliced product were detected and quantified by RT-PCR. In the absence of exogenous recombinant protein, splicing of both the exon 4 and exon 5 pre-mRNAs in nuclear extract showed a low but detectable level of inclusion (Figure 2-4A; left). Addition of hnRNP L to the reaction resulted in a decrease in exon 5 inclusion levels, as well as exon 4 inclusion (Figure 2-3A; left). Importantly, mutation of the ARS-containing S1 and S2 silencers within exon 5 abolished any effect of hnRNP L on exon 5 inclusion (Figure 2-4A; right). Furthermore, the repressive effect of hnRNP L is specific, since addition of recombinant PTB protein did not decrease inclusion of exon 5 (Figure 2-4B).

Previously, it was shown that PSF represses exon 4 only when purified from stimulated cells (Melton et al., 2007). The same was found to be true for exon 5, where recombinant PSF purified from stimulated, but not resting, cells repressed inclusion of this exon (Figure 2-4D). Notably, the extent of PSF repression was similar to that observed in total nuclear extract derived from stimulated cells. In contrast, addition of

recombinant hnRNP LL had little to no effect on exon 5 inclusion, even though the same concentrations of protein strongly repressed exon 4 (Figure 2-4C). Taken together, the

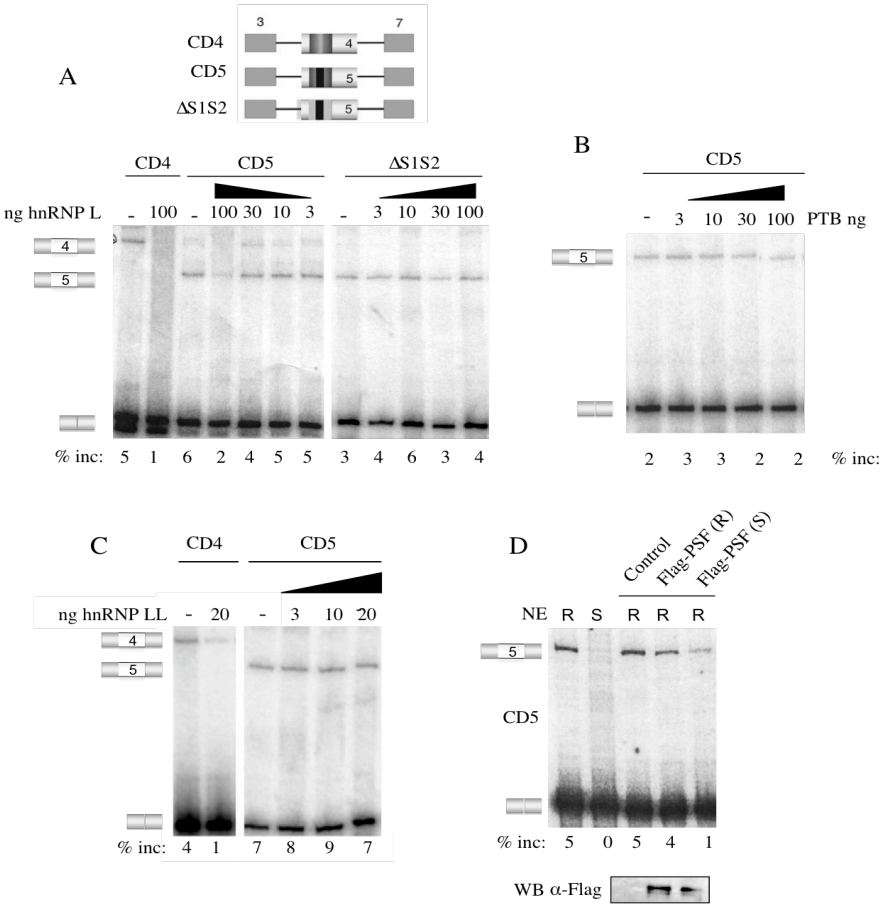

Figure 2-4. HnRNP L and PSF, but hnRNP LL, induce repression of exon 5 in *in vitro* splicing assays. RT-PCR of *in vitro* splicing reactions in resting JSL1 nuclear extract supplemented with recombinant hnRNP L (A), PTB (B), or hnRNP LL (C). Schematics of the minigenes used in these experiments are shown at the top. Hatched boxes correspond to substitution mutation of regulatory sequences. (D) RT-PCR of *in vitro* splicing reactions of CD5-derived RNA incubated in JSL1 nuclear extract supplemented with Flag-tagged PSF protein purified from resting (R) or stimulated (S) JSL1 cells. Western blot with anti-Flag antibody of protein fractions added to the reactions above.

binding and functional data suggest that hnRNP L binding to the ARS is the primary mediator of repression of CD45 exon 5 in resting cells, as it is for exon 4. Upon activation, PSF participates with hnRNP L in the stimulation-induced silencing of exon 5,

as it does for exon 4. In contrast, hnRNP LL, the other exon 4 repressor protein functional in stimulated cells, has little or no effect on the regulation of CD45 exon 5.

SF2/ASF is a candidate ESE-binding protein of CD45 exon 5

The regulation of CD45 exon 5 is further under the control of an enhancer element, whose activity is crucial for the recognition and inclusion of this exon by the spliceosome. Hence, identifying the trans-acting factor(s) that bind to and function on the exon 5 ESE is/are a top priority if we are to understand how hnRNP L represses inclusion of exon 5. Initially, we tried to identify the ESE-binding protein(s) by RNA affinity methods. Unfortunately, the inclusion efficiency of exon 5 was significantly lower *in vitro* as compared to *in vivo* (6% to ~85% respectively; Rothrock et al., 2003, Tong et al., 2005), suggesting that the exon 5 enhancer activity was limiting in our nuclear extracts. Therefore, given the low abundance of the ESE-specific activator proteins in extracts we were unsuccessful in detecting ESE-specific proteins by RNA affinity assays coupled to mass spectrometry analysis (data not shown). Subsequently, we used computational methods to identify candidate ESE-binding protein(s). The input sequence used was the 60 nt E5-WT RNA and as control we used the sequence for the E5-ΔESE RNA. Strikingly, we found that the strongest enhancer motif within exon 5 predicted by RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/; Fairbrother et al., 2002) overlapped with a binding site for a known SR protein SF2/ASF predicted by ESEfinder (http://rulai.cshl.edu/tools/ESE2/; Cartegni et al., 2003).

The ability of SF2/ASF to bind to the ESE element of CD45 exon 5 was independently confirmed using western blot analysis of the E5-WT, ΔESE, ΔS1S2-affinity purified proteins. In this assay, endogenous SF2/ASF in JSL1 nuclear extract

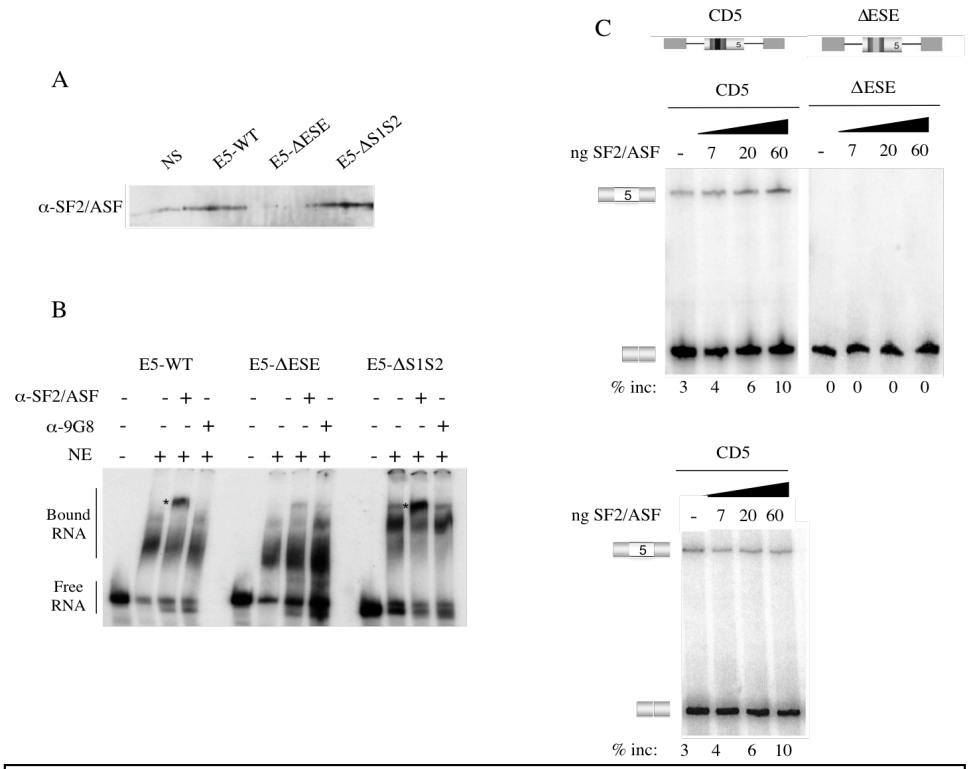

Figure 2-5. SF2/ASF is a candidate ESE-binding protein of CD45 exon 5. (A) Western blot with anit-SF2/ASF of RNA-affinity pulldowns done with nonspecific (NS) and exon 5 (E5, ΔESE, ΔS1S2) probes as in Figure 2A. (B) RNA mobility shift assay done with indicated RNAs in JSL1 nuclear extract, in the absence (-) or presence (+) of anti-SF2/ASF (α-SF2/ASF) or anti-9G8 (α-9G8) antibody. Super-shifted complexes are indicated with asterisk. (C) RT-PCR of *in vitro* splicing reactions done with indicated RNAs in JSL1 nuclear extract supplemented with recombinant SF2/ASF (top) or 9G8 (bottom). The numbers shown below each panel represent the mean exon inclusion, n=3.

effectively binds to the E5-WT and E5-ΔS1S2 RNAs, but substitution of the ESE (E5-ΔESE) abolished binding almost entirely (Figure 2-5A). We were also able to detect endogenous SF2/ASF binding to exon 5 by antibody-induced super-shift analysis of RNA mobility shifts assays (Figure 2-5B). Consistently, the ability of the anti-SF2/ASF antibody to retard exon 5 RNA-associated complexes was dependent on the presence of the ESE within exon 5. Addition of an antibody against a related SR protein, 9G8, did not

super-shift either one of the exon 5 RNAs; therefore, binding of SF2/ASF to the exon 5 ESE is specific.

To demonstrate that the binding of SF2/ASF to the ESE within exon 5 was functionally significant we used *in vitro* splicing assays. Titration of purified recombinant SF2/ASF into *in vitro* splicing reactions using the CD45 exon minigene increased the levels of exon 5 inclusion in a dose dependent manner (Figure 2-5C; top left). Importantly, SF/ASF had no enhancement activity on an exon 5 substrate that lacks the ESE (Figure 2-5C; top right). In addition, recombinant 9G8 protein had no effect on the splicing efficiency of exon 5 (Figure 2-5C; bottom). Thus, although we cannot completely rule out the presence or importance of other proteins on the exon 5 ESE, these results demonstrate that SF2/ASF binds specifically to the ESE element within exon 5 and functions to enhance CD45 exon 5 splicing.

The repressive activity of the ARS motifs in exon 5 is dependent on the presence of the ESE

A hallmark of the activity of the ESS1 element from CD45 exon 4 is that this ARS-containing sequence can confer hnRNP L-dependent repression on a heterologous exon (Rothrock et al., 2003 and 2005). However, previous studies had suggested that the ARS motifs from exon 5 were unable to function efficiently when removed from their native context (Tong et al., 2005). Importantly, we know that inclusion of exon 5 is dependent on the activity of a critical element that is bound by SF2/ASF. Thus, we wanted to understand how context affects the mechanism by which hnRNP L, via the ARS, functions to repress exon 5 and whether the interplay between the ESE and the ARS motifs plays a role in this process.

To this end, we carried out a systematic mutational analysis of the exon 5 regulatory sequences (ESE and S1S2) alone or in combination. In these cell-based assays we used minigenes in which exon 5, or derivatives thereof, is flanked by constitutive exons from the human β-globin gene. To make the mutant minigenes we substituted the target

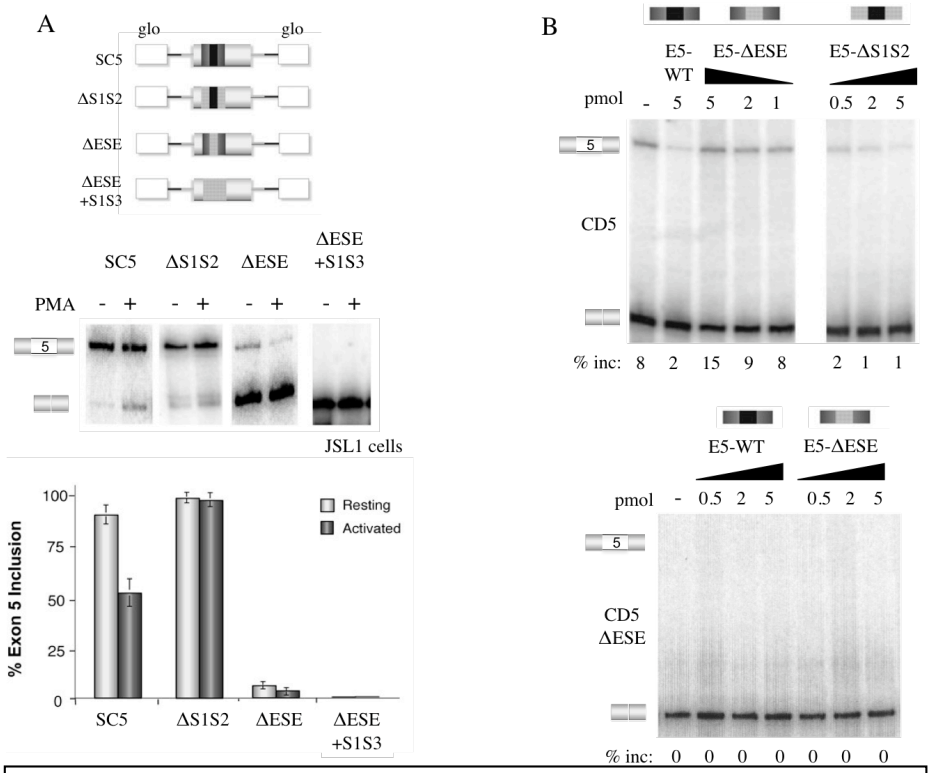

Figure 2-6. The ARS motifs in exon 5 repress the exon by antagonizing the activity of the ESE. (A) RT-PCR analysis of RNA derived from resting (-PMA) or stimulated (+PMA) JSL1 clones that stably express WT (SC5) and mutant (ΔS1S2, ΔESE, ΔESE+S1S2) exon 5 minigenes, schematics of which are shown at the top. White boxes and black lines correspond to sequence from the human β-globin gene. Rest of coloration is consistent with Figures 2-1. Bottom, mean percent inclusion of exon 5 +/- SD, n>6. (B) RT-PCR of *in vitro* splicing reactions using WT (top) or ΔESE (bottom) CD5 substrate in the absence (-) or presence of increasing amounts of various exogenous RNA competitors. Mean % inclusion is shown below, n>3

sequence with a sequence of equal length that we previously demonstrate has no splicing activity (Tong et al., 2005). These minigenes were stably expressed in our JSL1 cell line,

RNA was isolated from resting (-PMA) or activated (+PMA) cells, and the spliced mRNA products were assayed by RT-PCR. Deletion the ARS-containing S1S2 silencers increased the level of exon inclusion, albeit marginally, compared to that seen with the wild type minigene (Figure 2-6A; SC5 8.5% versus S1S2 1.4% skipping). Importantly, the signal-induced decrease in exon inclusion was also dependent on the presence of the silencers within exon 5 (Figure 2-6A). In contrast, substitution of the ESE in exon 5 almost entirely abolished exon inclusion in resting cells (Figure 2-6A; ~85% to ~10% inclusion; Tong et al., 2005). Interestingly, deletion of the ARS-containing S1S2 sequences in the background of the ΔESE minigene resulted in no increase in the inclusion of exon 5 (Figure 2-6A). Thus, the drop in exon 5 inclusion upon substitution of the ESE is due to the loss of the enhancer element, and not the S1S2 silencers directly repressing exon 5. This result suggests that in the absence of the ESE the ARS motifs (contained in S1S2) have no silencer activity on their own, but rather they function to directly counter the ESE activity.

HnRNP L represses CD45 exon 5 by sterically blocking the activity of an SF2/ASF-containing ESE-complex

The functional dependence of the ARS-containing S1S2 sequences on the ESE, and the inherent arrangement of the three regulatory sequences (Figure 1-14B), suggests a mechanism wherein hnRNP L bound on either side of the ESE interferes with binding of SF2/ASF. To test this prediction, the *in vitro* splicing assay was used to determine the functional effect of titrating various exogenous competitors RNAs on repression of CD45 exon 5. Addition of exogenous E5-WT RNA resulted in a notable decrease in the level of exon 5 inclusion (Figure 2-6B), suggesting that the E5-WT RNA titrates more

SF2/ASF than hnRNP L away from the substrate RNA, which leads to a loss of exon enhancement. Addition of a competitor that lacks the ARS motifs (E5-ΔS1S2) reduced exon

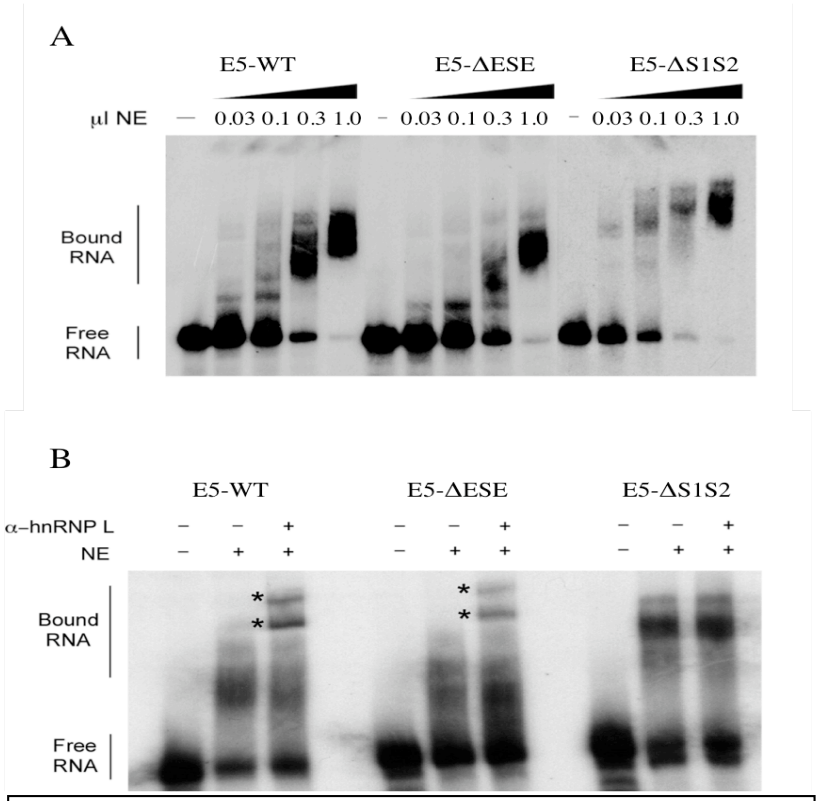

Figure 2-7. An enhancer and a silencer complex specifically associate with the ESE and S1S2, respectively, in exon 5. (A) RNA-mobility shift experiment done with radiolabeled exon 5 probes (WT, ΔESE, ΔS1S2) and increasing amounts of JSL1 nuclear extract. (B) Same as in panel A except reactions were incubated in the absence (-) or presence (+) of an antibody against hnRNP L (α-hnRNP L). Super-shifted complexes are indicated by asterisk.

inclusion at even lower concentration that the E5-WT competitor (Figure 2-6B), consistent with the lack of hnRNP L binding to the E5-ΔS1S2 RNA allowing more efficient recruitment and sequestration of SF2/ASF. In contrast, addition of the E5-ΔESE competitor increases the level of exon inclusion in a dose-dependent manner (Figure 2-6B), as we would expect if the E5-ΔESE RNA primarily sequesters the repressive hnRNP

L (via the remaining S1S2 sequences) away from the pre-mRNA substrate, thereby alleviating repression. Importantly, in control experiments the E5-ΔESE competitor does not increase exon use in a pre-mRNA substrate that lacks the ESE (Figure 2-6C), confirming the prediction from Figure 2-6A that binding of hnRNP L to the ARS motifs in exon 5 has no inherent silencing activity in the absence of the ESE.

Taken together, these data strongly argue that there is direct competition between an AF2-ASF-containing ESE complex and hnRNP L to bind the exon 5 and that the balance of these competing activities ultimately determines the extent of exon inclusion. This model is also supported by RNA mobility shift assays in which the E5-WT complex migrates at a diffuse midpoint between that observed for the E5-ESE and E5-S1S2 RNAs, suggesting that the E5-WT RNA binds a mixture of the "enhancer complex" and the "silencer complex" (Figure 2-7A). We further characterized these complexes by supershifting with anti-hnRNP L and anti-SF2/ASF antibodies. As predicted, the complex assembled on the E5-WT RNA contains both proteins (Figure 2-5B and 2-7B). In contrast, mutation of the ESE abolished binding of SF2/ASF (Figure 2-5B), whereas mutation of the ARS-containing S1S2 sequences abolished binding of hnRNP L (Figure 2-7B).

To directly demonstrate competitive binding, we carried out UV crosslinking assays. Crosslinking with JSL1 nuclear extract showed that a 70 kDa protein, which was previously confirmed by immunoprecipitation to be hnRNP L (Tong et al., 2005), associates more strongly with the exon 5 RNA when the ESE is mutated (Figure 2-8; left). In contrast, two additional bands are markedly reduced upon mutation of the ESE, demonstrating there is a differential association of these ESE-binding proteins versus

hnRNP L for exon 5 (Figure 2-8; left). Given the molecular weight of SF2/ASF (~30

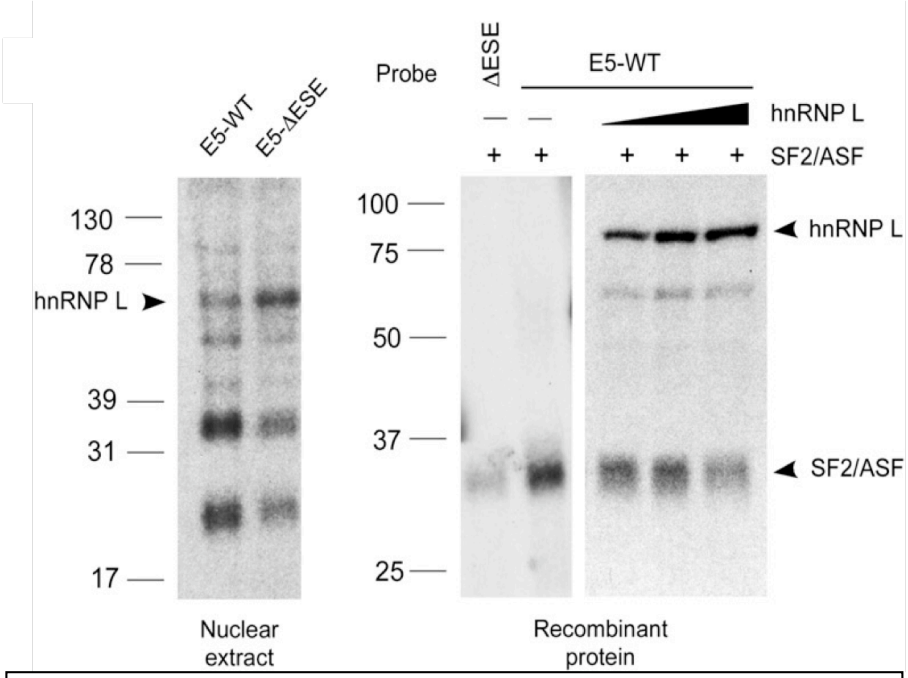

Figure 2-8. HnRNP L and SF2 compete for binding to Exon 5. U.V. crosslinking of radiolabeled exon 5 probes (WT and ESE) with JSL1 nuclear extract (left) or recombinant proteins (right) as indicated.

kDa), we expected to migrate close to the identified ESE-binding proteins; however, because our SF2-ASF antibody is not amenable to UV crosslinking assays, we could not conclusively demonstrate that SF2/ASF is among the proteins that associate in an ESE-specific manner. Nevertheless, we carried out UV crosslinking assays with recombinant SF2/ASF protein. In these assays, we confirmed that SF2/ASF crosslinks to exon 5 in a largely ESE-dependent manner (Figure 2-8; right). More importantly, titration of recombinant hnRNP L protein to the reaction resulted in reduced SF2/ASF binding to the E5-WT substrate (Figure 2-8; right), thus confirming direct competition between hnRNP L and SF2/ASF for binding to CD45 exon 5.

The ESE within exon 5 activates the formation of a spliceosomal A complex on the upstream intron

The data presented above provide strong evidence for a competition model where hnRNP L bound to the ARS motifs in exon 5 represses inclusion by directly competing with the activity of a critical enhancer element bound by SF2/ASF. Surprisingly, this mechanism is unlike the mechanism of repression of exon 4, where the binding of hnRNP L to the ARS motifs hyper-stabilizes the binding of the U1 and U2 snRNPs to the splice sites flanking the exon in such a way that inhibits their ability to participate in cross-intron interactions (House and Lynch, 2006). Therefore, to further characterize the mechanism by which hnRNP L functions on exon 5, we investigated what step in spliceosome assembly is regulated by the ESE and the S1S2 silencers. To facilitate this analysis, we made single intron minigenes that consist of the upstream exon 3 and exon 5 or exon 5 (E3-E5) and the downstream exon 7 (E5-E7) and examined their splicing using *in vitro* splicing assays. Splicing of the WT E3-E5 RNA was efficient in the JSL1 nuclear extract. Deletion of the ESE, however, greatly diminished the efficiency of splicing relative to the wild type substrate, demonstrating that the ESE is functional in the single-intron minigene (Figure 2-9A; top). Furthermore, much like the complete exon 5 minigene, mutation of the S1S2 sequences increased splicing efficiency, but only when the enhancer was present (Figure 2-9A; top).

Remarkably, however, substitution of the ESE in the E5-E7 construct did not decrease splicing efficiency in either the presence or absence of the silencers (Figure 2-9A; bottom). Instead, removal of the ESE increased splicing efficiency; nevertheless, this seems to be due to a spurious context effect since this is counter to the effect of deleting

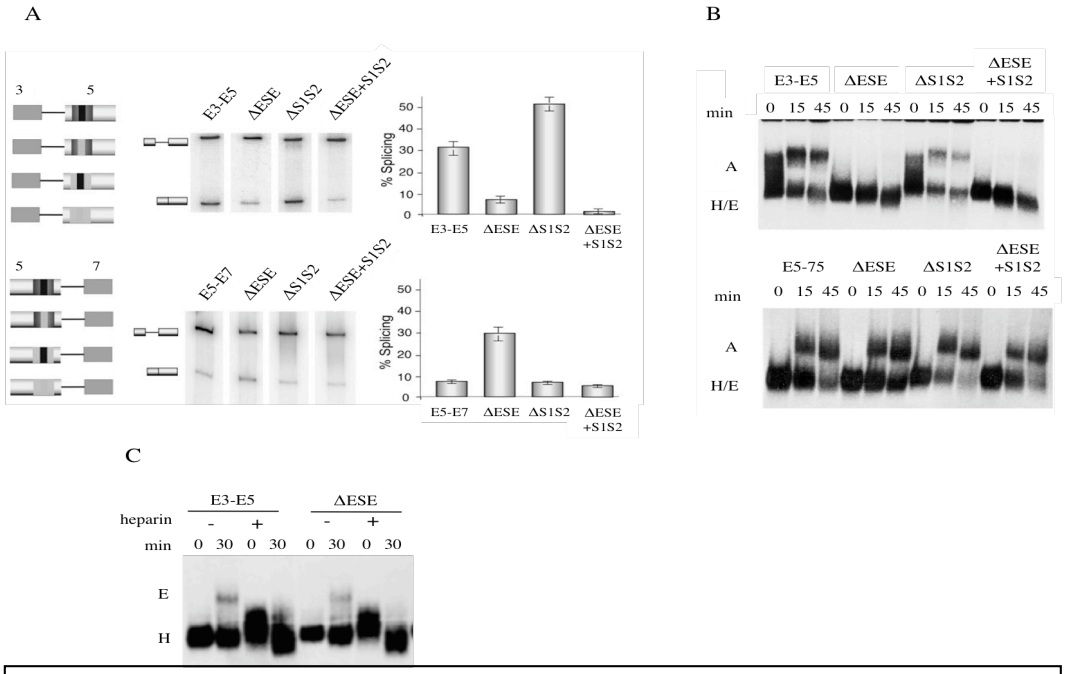

Figure 2-9. The ESE in exon 5 activates the formation of A-complex on its upstream intron. (A) RT-PCR of *in vitro* splicing reactions. Schematics of each of the minigenes used are shown on the left. Graph represents mean +/- SD from 3 independent experiments. (B) Radiolabeled RNA substrates derived from each of the minigenes shown in panel A were incubated in nuclear extract for the times indicated and the resulting spliceosome complexes were resolved on native agarose gels. (C) Assembly done as in panel B except nuclear extract was depleted of ATP prior to use.

the ESE in the full minigene. In addition, deletion of the silencer sequence also had no effect on the splicing efficiency of the E5-E7 substrate (Figure 2-9; bottom). These data thus suggest that the intron downstream of exon 5 is refractory to control by the regulatory sequences in this exon and that the ESE, and so also the S1S2 silencers, function primarily on the upstream intron to regulate inclusion of exon 5.

Next, we analyzed the E3-E5 splicing reactions on nondenaturing agarose gels to separate the different spliceosome intermediates. Spliceosome assembly progressed efficiently in resting JSL1 nuclear extract, as a complex, confirmed to be the spliceosomal A complex by its dependence on ATP and the U2 snRNP (data not shown), was readily detected after a 30 minute incubation. Consistent with the significant

decrease in splicing observed upon deletion of the ESE, no detectable A complex was formed on the E3-E5 ΔESE substrate (Figure 2-9B; top). On the other hand, the efficiency of A complex formation on the E3-E5 RNA that lacks the ARS motifs (ΔS1S2) was the same or greater than for the wild type E3-E5 (Figure 2-9B; top). Importantly, substitution of the silencers in the background of the ESE mutation (E3-E5 ΔESE+S1S2) did not restore A complex formation (Figure 2-9B; top), demonstrating that the loss of A complex formation on the E3-E5 ΔESE RNA is again a direct result of loss of the enhancer activity and not a result of a residual silencer activity. We also examined the efficiency of A complex formation on the E5-E7 substrates, and consistent with the splicing of these minigenes, we found that formation of this complex is not dependent on the presence of the ESE or S1S2 silencers alone or in combination (Figure 2-9B; bottom).

To directly assess whether loss of A complex in the absence of ESE in E3-E5 is due to a direct block in A complex formation or to inhibition of the earlier ATP-independent E complex, the E3-E5 wild type and ΔESE substrate RNAs were incubated in the absence of ATP and the assembly reactions were resolved on agarose gels. The identity of E complex was confirmed by its dependence on the U1 snRNA (data not shown) and sensitivity to heparin. Strikingly, there was no significant difference in the efficiency of E complex formation in the absence or presence of the ESE in exon 5 (Figure 2-9C); thus, the ESE is not required during E complex formation. We conclude then that the ESE within exon 5 promotes A complex formation specifically on the intron upstream of exon 5, presumably by recruiting by the U2 snRNP to the 3'ss.

HnRNP L represses A complex formation on the intron upstream of exon 5

If the competition model of exon 5 regulation, which says that S1S2 silencers in exon 5 function to directly counter the activity of the ESE, is correct, then hnRNP L should inhibit the ability of the enhancer complex to activate formation of A complex on the upstream intron. To test this prediction, we varied the levels of hnRNP L and monitored the effect on A complex formation on E3-E5 using agarose gels and *in vitro* splicing assays. Remarkably, addition of recombinant hnRNP L to an assembly reaction, at levels that effectively repressed E3-E5 splicing, also specifically inhibited A complex formation (Figure 2-10A). Importantly, hnRNP L had no effect on the formation of A

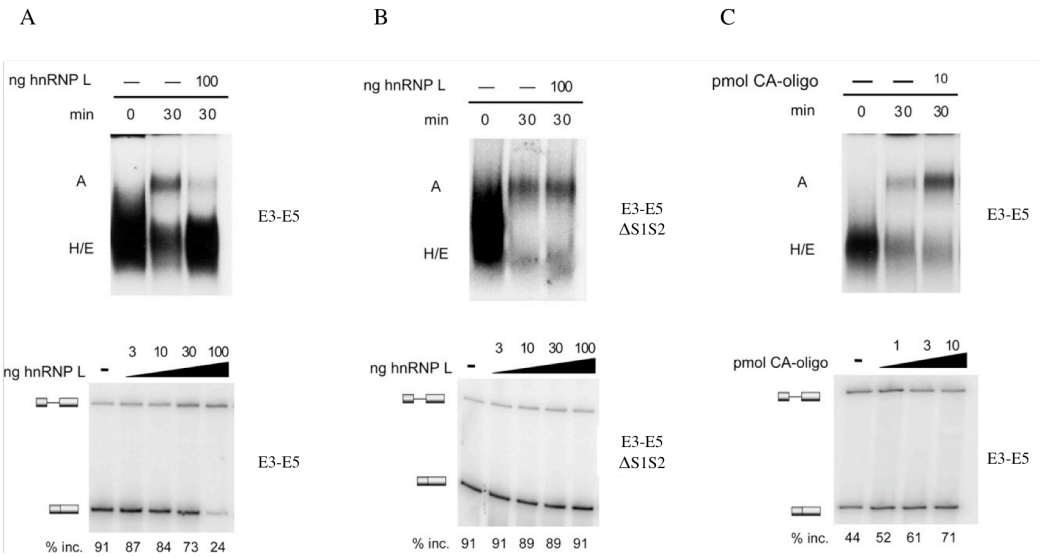

Figure 2-10. HnRNP L specifically inhibits A-complex formation on exon 5. (A) Assembly and RT-PCR analysis done in the absence (-) or presence (+) of 100 ng of recombinant hnRNP L protein. (B) Same as panel A except with ΔS1S2 substrate (C) Same as in panel A, except reactions were incubated in the absence (-) or presence (+) of 10 pmol of CA-oligo.

complex or the splicing of the E3-E5 substrate that lacks the silencers (ΔS1S2) (Figure 2-10B). Furthermore, depleting hnRNP L from the nuclear extract, as was done in previous studies using a poly-CA oligo (Hui et al., 2003), resulted in an increase in both splicing

and A complex formation (Figure 2-10C). Taken together, these data demonstrate that hnRNP L binding to the ARS motifs represses A complex formation on the intron upstream of exon 5.

Discussion

Previously it was shown that the CD45 variable exons 4 and 5 are independently repressed through the activity of the ARS core motif sequence (Rothrock et al. 2003; Tong et al., 2005). Also, a group of hnRNP proteins (hnRNP L, E2, K, D and PTB) were identified that bind to the regulated ARS-containing ESS1 element within exon 4 (Rothrock et al., 2005). Moreover, several lines of evidence confirmed that hnRNP L was the primary repressor protein through which the function of the ARS was mediated, while the other hnRNPs had little functional effect (Rothrock et al., 2005; Melton et al., 2007). Interestingly, while in exons 4 and 6 the ARS motifs are contained within a single 60 nt region (ESS1), the ARS motifs in exon 5 are split across two silencer regions (S1 and S2) by a strong exonic splicing enhancer (ESE) element (Figure 1-14B)(Tong et al., 2005). In this chapter we demonstrate that hnRNP L binding to the ARS motifs in exon 5 is the primary mediator of basal repression, as it is for exon 4 (Figure 2-1 to 2-4). However, neither PTB nor hnRNP E2 were observed to associate with exon 5 by either silver stain, Western blot or RNA mobility shift assays, nor were the more weakly ESS1-associated proteins hnRNP K and D (Figure 2-1 and 2-3). Earlier studies also established that the increase in skipping of exon 4 upon cellular activation was due to the activity hnRNP L plus the additional recruitment of PSF and hnRNP LL to this exon (Melton et al., 2007; Topp et al., 2008). Strikingly, *in vitro* binding and functional assays showed that PSF also associates with and functions to represses CD45 exon 5 in a signal-dependent manner;

however, there was no detectable association or activity of hnRNP LL on exon 5 under either resting or stimulated conditions (Figure 2-1B, 2-3C, 2-4C, and 2-4D). Thus, it appears that subtle differences in the arrangement of the ARS motifs in CD45 exons 4 and 5, results in hnRNP L binding to this sequence with a different set of coassociated proteins under both resting and activated conditions.

The inherent arrangement of the three regulatory sequences in exon 5, the ESE flanked by the S1 and S2 ARS-containing sequences, suggested a mechanism of regulation wherein hnRNP L bound on either side of the ESE would interfere with binding of the enhancer protein(s) (Figure 1-14B). Such a model is supported by cell-based functional assays (Figure 2-6A), described in this chapter, that show that under resting conditions the S1S2 sequence elements in exon 5 have no repressive activity in the absence of the ESE, suggesting that the silencers do not directly repress inclusion of this exon, but rather function by directly countering the ESE activity. Moreover, altering the relative spatial arrangement of S1, ESE and S2, such that the ESE is to one side of the silencers, also results in a loss of normal silencing (data not shown). However, hnRNP L binding to the silencers in this configuration is also somewhat reduced, thus we cannot strictly conclude that the configuration of the silencers flanking the enhancer is required for inhibition of the enhancer versus optimal recruitment of hnRNP L. Nevertheless, competitive binding between hnRNP L and ESE-specific proteins, including the SR protein SF2/ASF, was confirmed using competition *in vitro* splicing assays, antibody-induced supershift RNA mobility shift assays, and UV crosslinking (Figure 2-6B, 2-5B, 2-7B, and 2-8).

SF2/ASF was identified as an ESE-binding protein of exon 5 using computational prediction programs, and its functional relevance was demonstrated by RNA affinity pulldown assays coupled to western blot analysis and *in vitro* splicing assays. However, because the ESE activity is limiting in our nuclear extracts, as determined by the inefficient inclusion of exon 5 *in vitro* relative to cell-based assays, this precluded our efforts to identify additional ESE-binding proteins. Moreover, although SF2/ASF clearly enhances splicing of exon 5 (Figure 2-5C; top left), it does not increase splicing as much as we would predict or as high as the levels seen in JSL1 cells. Therefore, we cannot rule out the contribution of other ESE-specific proteins, alone or in combination with SF2/ASF, to the inclusion of CD45 exon 5.

Importantly, the fact that hnRNP L causes skipping of exon 5 by binding to the ARS in such a way that blocks the association of SF2/ASF with the intervening enhancer element, demonstrates that the silencing of exon 5 is mechanistically distinct from that of exon 4. By extending the analysis of the interplay between the ESE and ARS motif in exon 5, we found that the ESE promotes A complex formation, specifically on the intron upstream of exon 5 (Figure 2-9), and that hnRNP L specifically counters the ESE activity to block A complex formation (Figure 2-10). Because a well-established hallmark of A complex is the ATP-dependent addition of the U2 snRNP to the branch point sequence in the 3'ss, it follows that hnRNP L most likely functions by inhibiting the ability of the ESE complex to recruit the U2snRNP to the upstream 3'ss of exon 5 (Figure 2-11, top). Remarkably, in contrast to exon 5, on exon 4 hnRNP L stalls the U1 and U2 snRNPs after binding to the substrate and locks them in an A-like exon-defined complex (AEC) that is non-permissive to splicing (House and Lynch, 2006)(Figure 2-11, bottom). Taken

together, these data demonstrate that the differential configuration of the ARS motifs in CD45 exons 4 and 5 can also influence the mechanism by which hnRNP L functions to repress these exons.

A remaining question that we have not addressed in this study is how the recruitment of PSF to exon 5 alters the mechanism of repression upon cellular stimulation. Interestingly, whereas the basal silencing activity of the ARS motifs in exon 5 requires the presence of the ESE, activation-induced silencing of exon 5 still occurs in the absence of this enhancer (Figure 2-6A), indicating that the mechanism of activation-induced repression of exon 5 is distinct from the basal repression by hnRNP L. One

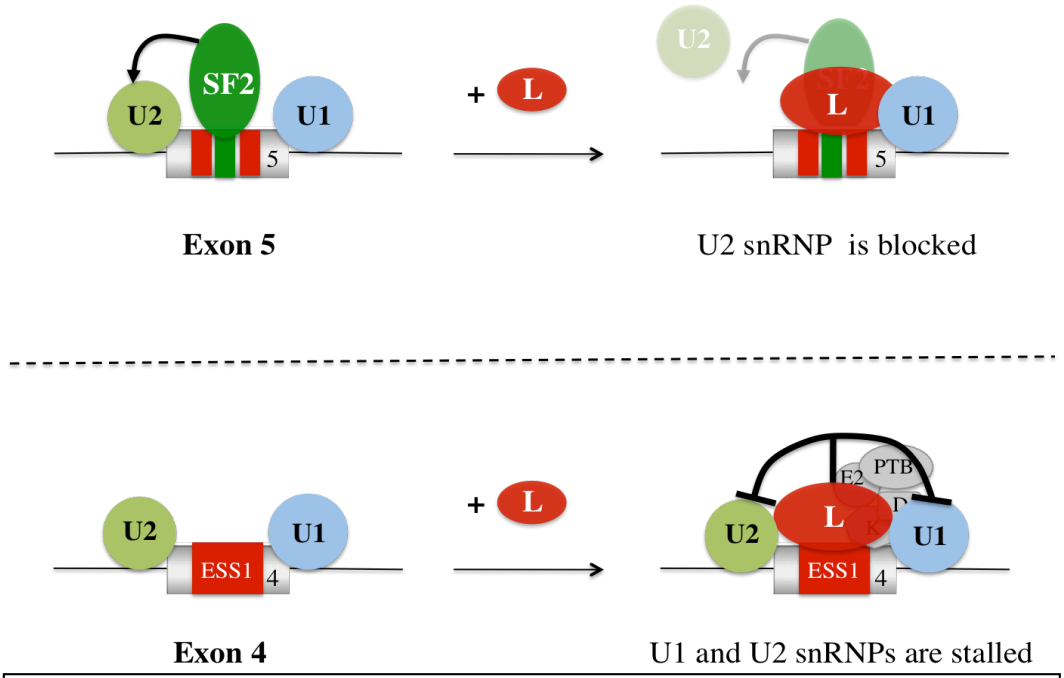

Figure 2-11. HnRNP L regulates CD45 exons 4 and 5 by distinct mechanisms. HnRNP L represses exon 5 by blocking the ability of ESE- bound SF2/ASF to recruit the U2 snRNP and promote A-complex formation (top). This is in stark contrast to the mechanism of repression of exon 4, where hnRNP L stalls spliceosome assembly after the binding of the U2 and U2 snRNPs (bottom) (House and Lynch, 2006).

explanation for these results is that PSF could be directly antagonizing spliceosome assembly formation on exon 5. This latter possibility is consistent with the fact that addition of PSF to the exon 4 silencer complex does not change the mechanism of repression (A.E.H. and K.W.L., unpublished data), suggesting that on exon 4 PSF helps hnRNP L further trap and prevent the AEC complex from progressing on in assembly. However, further studies will be required to understand how the addition of PSF upon cellular activation alters the mechanism of exon 5 silencing.

Perhaps the most interesting question to come out of this study is: Why does hnRNP L use different mechanisms to regulate exon 4 and exon 5? We propose that because in the absence of the ESE activity the U2 snRNP cannot be efficiently recruited to the 3'ss upstream of exon 5 to form an A complex (Figure 2-6A, 2-9B), then hnRNP L preventing the association of the U2 snRNP by inhibiting the enhancer activity is a more-efficient mechanism of regulation than is repressing spliceosome assembly after U2 snRNP association, which is what occurs on exon 4 (House and Lynch, 2006). In other words, for exon 5 the rate-limiting step in assembly is the binding/stabilization of U2snRNP to the flanking 3'ss, and on exon 4, because the U1 and U2 snRNPs are already stably bound to the flanking splice sites, the rate-limiting step is making the transition from an exon-defined to an intron-defined A complex. Based on this idea one would predict a model wherein the mechanism of hnRNP L repression is determined by the inherent snRNP-substrate affinity of each exon. Additional studies that test and confirm this model are presented in Chapter 3. Taken together, the results presented in this chapter provide strong evidence that a given protein can function through different

mechanisms, and that for any given exon the mechanism is constrained by the local sequence context and the rate-limiting step in spliceosome assembly.

CHAPTER THREE

Context-dependent Regulatory Mechanism of Exon-bound HnRNP L is Determined by Splice Site Strength

Introduction

Most precursor messenger RNA transcripts are composed of protein-coding segments (exons) that are interspersed between non-coding sequences (introns) along the RNA. A pre-mRNA must be spliced to remove intronic sequences and join exons to generate a mature, export-ready mRNA molecule. This critical process is carried out by a highly specialized, macromolecular enzyme known as the spliceosome. It is composed of 5 snRNP (small nuclear RNP) complexes (U1, U2, U4/U6, and U5), all of which consist of a uridine-rich snRNA (or two in the case of U4/U6) and multiple proteins (Wahl et al., 2009). Importantly, the spliceosome is not a pre-formed enzyme, but instead forms through the assembly of the snRNP complexes on the pre-mRNA in a step-wise pathway that involves several intermediate complexes (E-, A-, B- and C-complex) (Wahl et al., 2009). While some exons are constitutively spliced, that is they are always included in the final message, the splicing of other exons is regulated (Motta-Mena and Lynch, 2010). The regulated inclusion or exclusion of exons, a process known as alternative splicing, is mediated by the action of a number of regulatory splicing proteins bound to *cis*-acting control sequences embedded in the pre-mRNA. In particular, exonic splicing enhancers (ESEs) and splicing silencers (ESSs) act to promote or inhibit recognition of a regulated exon by the spliceosome through the activity of SR proteins and hnRNPs, respectively (Motta-Mena and Lynch, 2010).

Although, for the most part, hnRNPs function as repressors and SR proteins function as activators of splicing, the activity of these two families of proteins in some cases depends on the context of the sequence to which it binds (Chen and Manley, 2009). Meaning that the same protein, hnRNP or SR, may function as an enhancer of splicing in one context and as a repressor of splicing in another. Of note, several regulatory splicing proteins have been shown to act as either repressor or activator depending on the location (exonic or intronic) of their binding site relative to the regulated exon (Ule et al., 2006; Zhang et al., 2008; Licatalosi et al., 2008; Xue et al., 2009; Yeo et al., 2009). Moreover, the proximity of the regulatory protein's binding site(s) to canonical splicing signals can also play a role in determining the activity of the protein (Mayeda and Krainer, 1992; Eperon, 2000; Hui et al., 2005; Caceres et al., 1994). Other aspects of context like the binding of additional proteins to flanking regulatory elements, neighboring RNA motifs and/or structure, and the relative affinity of a regulatory protein for its cognate binding site have been proposed to also influence the exact function of a protein (Singh and Valcarcel, 2005; Yu et al., 2008; Matlin et al., 2005). However, the mechanisms by which such dual effects are conferred on protein regulators remain poorly understood in most cases. Moreover, these observations do not rule out the existence of other aspects of context that can influence how a particular regulatory protein functions.

The CD45 gene is an excellent model system for studying the regulation and mechanisms of alternative splicing events. The skipping of the three CD45 variable exons (4, 5, and 6) is mediated by a conserved activation responsive sequence (ARS) silencer element that is located within each of the exons (Rothrock et al., 2003). For exons 4 and 6 the ARS motif is contiguous (Rothrock et al., 2003; Tong et al., 2005). In contrast, in

exon 5 the ARS sequence is bisected by an ESE element (Tong et al., 2005). The protein hnRNP L is the primary ARS-binding protein and mediates repression of each of the variable exons (Rothrock et al., 2005; Tong et al., 2005). Interestingly, though bound to a similar sequence in each exon, the mechanisms by which hnRNP L functions to repress CD45 exons 4 and 5 are distinct. Inclusion of exon 5 is completely dependent on the presence of the SR protein SF2/ASF bound to the ESE, which promotes association of the U2 snRNP. In exon 5, hnRNP L causes skipping by binding to the ARS motifs in such a way that blocks the association of SF2/ASF with the intervening ESE, thereby preventing SR-dependent recruitment of the U2 snRNP (Chapter 2)(Motta-Mena et al., 2010). By contrast, in CD45 exon 4 the binding of hnRNP L to the ARS-containing ESS1 element hyper-stabilizes the binding of the U1 and U2 snRNPs to the splicing sites flanking the exon and inhibits their ability to participate in the cross-intron interactions necessary for catalysis (House and Lynch, 2006).

In this chapter, we show that hnRNP L regulates exon 5 by a distinct mechanism from that of exon 4 due to inherent differences in the efficiency of snRNP assembly on the flanking splice sites. Furthermore, we find that in the absence of the enhancer in exon 5 the binding of hnRNP L to the ARS motifs unexpectedly activates exon inclusion; however, when placed in the background of exon 4, the ARS-bound hnRNP L represses exon inclusion. As the strength of the splice sites flanking exons 4 and 5 differ significantly, we examined the effect of varying splice strength on the directionality of hnRNP function. Remarkably, binding of hnRNP L to an exon represses strong splice sites but enhances weak splice sites. Together these data demonstrate that a given

regulatory protein can function through different mechanisms in a manner independent of location but determined by splice site strength.

Results

The ARS motifs from exon 5 can have a positive or negative effect on exon inclusion depending on the context

Previously it was demonstrated that a 60 nt exonic silencer sequence (ESS1) is necessary and sufficient for the basal repression of CD45 variable exon 4 (Rothrock et al., 2003). Later studies showed that the protein hnRNP L is the primary mediator of ESS1-dependent exon silencing *in vivo* as well as *in vitro* (Rothrock et al., 2005). Subsequently, it was found that ESS1-bound hnRNP L represses exon 4 inclusion by stalling spliceosome assembly after the binding of the U1 and U2 snRNPs to the substrate, thereby trapping the repressed exon in an A-like exon-defined complex or AEC (House and Lynch, 2006). Closer inspection of the ESS1 element from exon 4 revealed the presence of an imperfect repeat that is conserved between the ESS1, exon 5, and exon 6, which was termed the activation responsive sequence (ARS) consensus motif (Rothrock et al., 2003). Importantly, mutations in or deletion of the ARS motifs from each of the variable exons disrupts both basal and activation-induced exon silencing (Rothrock et al., 2003; Tong et al., 2004; Melton et al., 2007). Similar to exon 4, the ARS motifs in exon 6 are contained within a single 60 nt silencer element (ESS1); however, in exon 5 the ARS motifs are split into two regions by an exonic splicing enhancer (ESE) element (Tong et al., 2005). Recently, it was demonstrated that hnRNP L specifically binds to the ARS elements in exon 5 and is the primary repressor of exon 5 basal splicing; also, SF2/ASF was identified as an ESE-binding protein that functions to

enhance exon 5 inclusion (Motta-Mena et al., 2010)(Chapter 2). In contrast to exon 4, hnRNP L binding to exon 5 does not directly repress the exon, but rather functions by inhibiting the ability of SF2/ASF to recruit the U2 snRNP to form a canonical spliceosomal A complex (Motta-Mena et al., 2010)(Chapter 2). Therefore, despite the fact that hnRNP L binds to a similar sequence motif (the ARS) within exon 4 and exon 5, hnRNP L uses different mechanisms to regulate each exon.

Closer inspection of the exon 4 and exon 5 sequences pointed to a difference in flanking 3' splice site strength (Figure 3-1). We noticed that, compared to exon 4, exon 5 has a noticeably shorter polypyrimidine tract (exon 4 Yn= 27 versus exon 5 Yn= 15), making it an intrinsically weaker 3' splice site. This initial observation offered a rationale for the differential mechanism of regulation of exons 4 and 5, wherein we would predict that hnRNP L can only stall an AEC complex when the binding of the U1 and/or U2 snRNPs to the flanking splice sites is inherently strong (Figure 2-11). In exon 5, because the 3'ss is inherently weak, the binding of the U2 snRNP is entirely dependent on the presence of the ESE; thus, hnRNP L blocking the activity of the SF2/ASF bound to the ESE is a more efficient mechanism of repression. It appears then that the function of hnRNP L is determined by the inherent snRNP-substrate affinity of each exon.

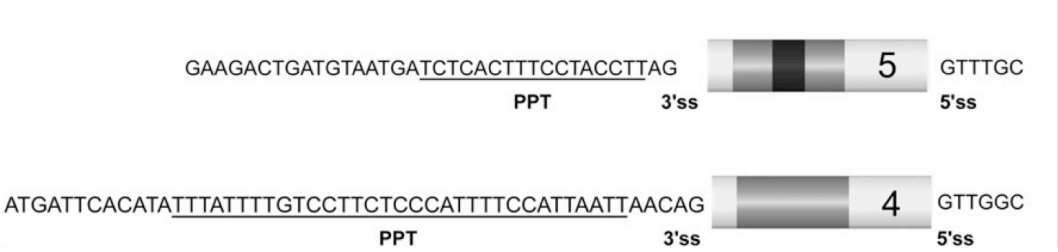

Figure 3-1. The 3' splice site flanking CD45 exon 5 is weak relative to exon 4. Comparison of intronic sequence flanking exons 4 and 5, with polypyrimidine tract (PPT) underlined.

Previously a deletion analysis of the exon 5 regulatory sequences was done, in which a minigene that contained exon 5 and native intronic sequence on either side flanked by constitutive CD45 exons 3 and 7 was used (Motta-Mena et al., 2010)(Chapter 2; Figure 2-6A). These studies revealed that in the absence of all three regulatory elements (SC5 ΔESE+S1S2) exon 5 is for the most part not used, but that addition of the S1S2 sequences by themselves (SC5 ΔESE) has a significant enhancing effect on the otherwise unused exon (Figure 3-2). By contrast, the same experiment done with exon 4 showed that in the absence of both the enhancer and the silencer (ESS1) activities exon 4 is still efficiently recognized by the spliceosome (WT 43% vs. C77G+ΔESE 58% inclusion); moreover, in contrast to exon 5, addition of the ARS-containing ESS1 element alone effectively repressed exon inclusion (C77G+ΔESE 58% vs. ΔESE 14% inclusion) (Lynch and Weiss, 2001). Because, as mentioned above, the strength of the 3' splice site varies significantly between exons 5 and 4, we created a chimeric minigene that consisted of exon 5 flanked by the splice sites and intron sequence from exon 4 to determine if the nature of the activity of the ARS element is dependent of splice site strength. Strikingly, in the background of exon 4, the exon 5 S1S2 sequences have a strong repressive effect on exon inclusion, consistent with the idea that splice site strength might dictate the mechanism of hnRNP L function (Figure 3-2). These data demonstrate that the hnRNP L-binding sequence from exon 5 can have either a positive or negative effect on exon inclusion, depending on context. The most notable distinction between the wild type and exon 4 backgrounds is the overall level of exon 5 inclusion in the absence of all three regulatory sequences (Figure 3-2; SC5-ΔESE+S1S2 vs. SC4-E5-ΔESE+S1S2). In particular, in the background of exon 4 the ΔESE+S1S2 exon is highly included, meaning

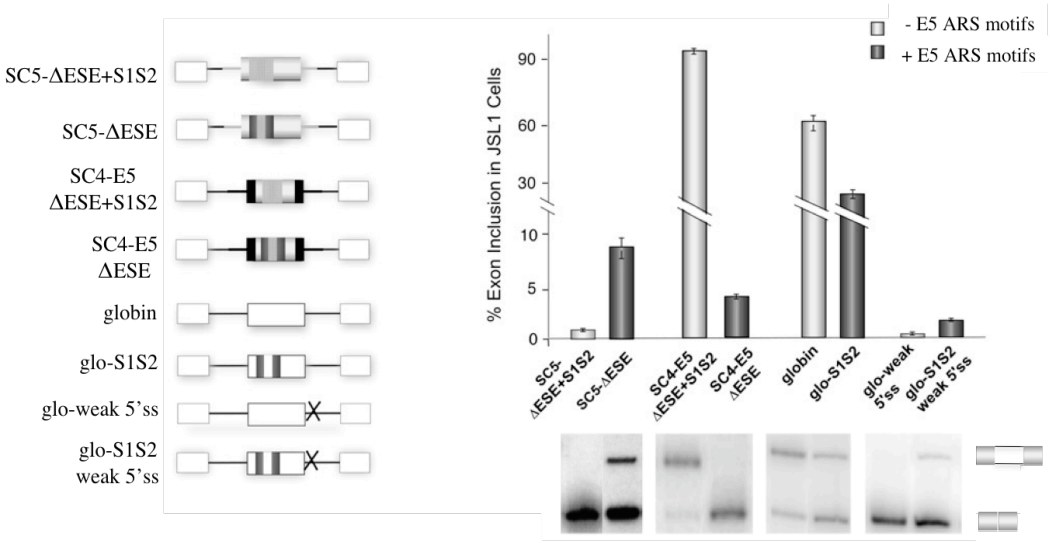

Figure 3-2. HnRNP L represses strong splice sites but activates weak splice sites. Mean exon inclusion +/- SD from RT-PCR of stable cell lines expressing the minigenes shown, done in triplicate. Black boxes and bold black lines represent exonic and intronic sequence from CD45 exon 4 respectively. Glo-weak and glo-weak S1S2 minigenes carry mutations in the 5'ss downstream of the central exon.

that, even without any auxiliary sequences the splice sites are sufficiently strong to be recognized by the spliceosome. However, as mentioned before, the exon 5 splice sites deviate so much from the optimal consensus sequence that, in absence of all regulatory sequences, the exon is essentially not recognized by the spliceosome. This differential inclusion observed for the SC5 ΔESE+S1S2 and SC4-E5ΔESE+S1S2 exons is consistent with the marked difference in the strength of the 5'ss and the length of the polypyrimidine track flanking the two exons.

To determine whether splice site strength is a determinant in the S1S2 sequences functioning as an enhancer or silencer of splicing, we inserted them into a β-globin test exon and then weakened the 5'ss. This minigene consists entirely of β-globin-derived sequences, with the exception of the internal ARS-containing S1S2 sequences of exon 5 that were inserted between the β-globin splice sites in the central exon. The globin

minigene alone (glo) has a relatively high level of exon inclusion, as expected because the splicing sites flanking this exon are strong, and the presence of the S1S2 sequences cause exon repression (Figure 3-2). Remarkably, however, we find that, when the 5'ss flanking the β-globin central exon is weakened, the presence of the S1S2 sequences enhanced exon inclusion by ~6-fold (Figure 3-2; glo-weak 5'ss 0.3% vs glo-S1S2 weak 5'ss 1.9%). Taken together, these data suggest that the nature of the activity of hnRNP L on splicing is determined by the identity and strength of the flanking splice sites. Specifically, hnRNP L binding to an exon, via the ARS motif, represses strong splice sites but enhances weak splice sites.

HnRNP L binding represses exons with strong splice sites but activates weak splice sites

To test whether the above correlation between directionality of hnRNP L function and splice site strength is a general phenomena and to isolate effects of hnRNP L away from potential cobinding proteins, an MS2 tethering experiment was performed in collaboration with another lab member, Florian Heyd. First, we engineered a single-hairpin binding site for the MS2 coat protein into a chimeric exon consisting of β-globin splice sites fused to a splicing-inert sequence from CD45 exon 9 used to lengthen the exon to ~200 nt (Rothrock et al., 2003; F.H.). Next, *in vitro* splicing assays were done with this heterologous minigene in the absence or presence of chimeric MS2-hnRNP L protein. Inclusion of this MS2 text exon was highly efficient, as expected because the splicing sites flanking this exon are strong (F.H.; Figure 3-3). Of note, addition of partially purified MS2-hnRNP L protein to the *in vitro* splicing reaction markedly repressed exon inclusion, consistent with hnRNP L functioning as a silencer of an exon with strong splice sites (F.H.; Figure 3-3). Importantly, MS2-hnRNP L had no effect on

the splicing of a related minigene lacking the MS2 binding site (F.H.;Figure 3-4B), and addition of MS2 protein alone had no silencing activity on the MS2 hairpin-containing

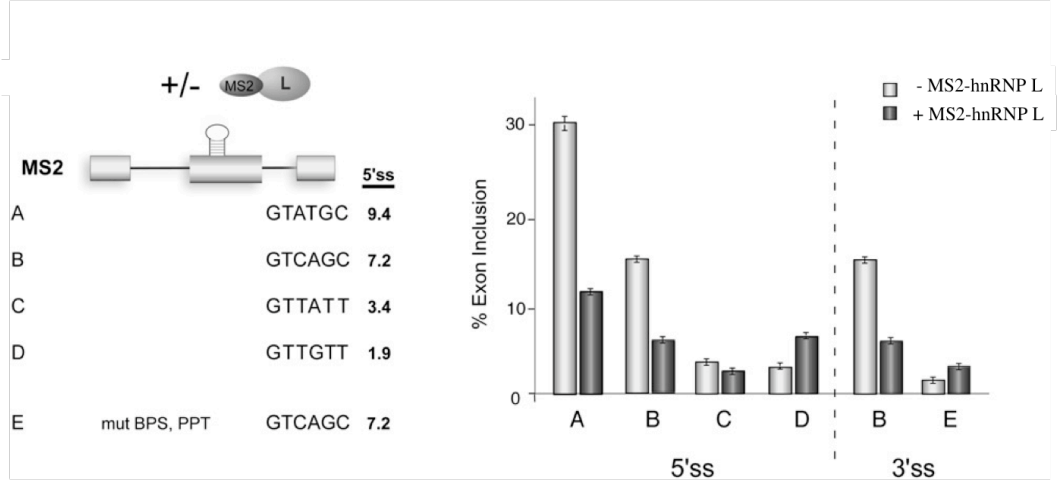

Figure 3-3. MS2-hnRNP L specifically regulates alternative splicing when tethered to RNA. Mean exon inclusion +/- SD from triplicate *in vitro* splicing reactions, done in the absence or presence of MS2-hRNP L, using RNAs transcribed from minigenes shown. Numbers shown for 5'ss represent score for 5' splice site strength (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html).

substrate, confirming that repression is due to the hnRNP L component of the fusion protein and that exon binding is required for repression (F.H.; Figure 3-4A).

We next systematically weakened the 5' or 3' splice site signals flanking the MS2 text exon to further test the correlation between splice sites and activity of hnRNP L. Partial weakening of exon efficiency results in a loss of responsiveness to MS2-hnRNP L binding (Figure 3-3; construct C). Strikingly, however, when the 5'ss was rendered weakest, binding of MS2-hnRNP L actually enhanced exon inclusion by 2- to 3-fold, which represents a significant fold change (F.H.; Figure 3-3; construct D: – MS2-L 2.6% vs. +MS2-L 6.9%). This result was not solely specific for weak 5' splice sites, as weakening of the upstream branch point sequence and polypyrimidine tract also results in MS2-hnRNP L functioning a an activator of weak splice sites (F.H.; Figure 3-3; construct

E: – MS2-L 1.6% vs. +MS2-L 3.4%). Therefore, taken together our results demonstrate that whereas hnRNP L functions as a repressor of efficient exons; however, once the absolute level of exon inclusion is less than some threshold value of ~2-3%, hnRNP L functions as an enhancer. Importantly, these data strongly suggest that splice site strength, independently of binding-site location, plays a significant role in determining

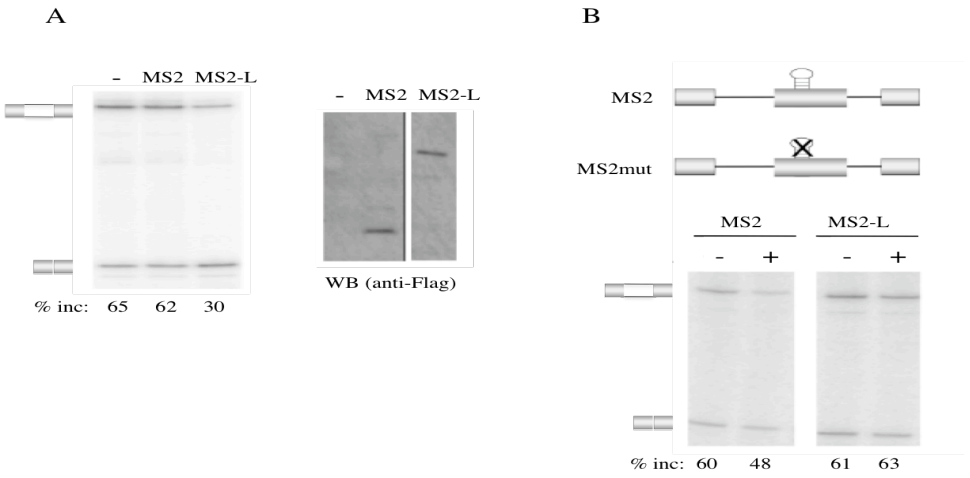

Figure 3-4. The splicing activity of MS2-hnRNP L is due specifically to the hnRNP L component. (A) *In vitro* splicing assays comparing the effects of nuclear extract alone, nuclear extract containing MS2, or nuclear containing MS2-hnRNP L. MS2 alone does not influence alternative splicing; numbers represent mean % inclusion of 3 experiments (left). Western blot analysis shows similar expression of MS2 and MS2-hnRNP L (right). (B) Substrates containing either the correct MS2 stem loop sequence or a mutated (reversed) version (MS2mut) were used in *in vitro* splicing assays in the presence or absence of MS2-hnRNP L fusion protein. Resulting products were quantified using phosphoimager analysis; a result representative of 3 experiments is shown.

the type of effect a given regulatory protein has on the assembly pathway of the spliceosome.

Discussion

Recently, genome-wide protein-RNA interaction studies have introduced the notion of regulatory maps that predict the effect of a regulatory protein on alternative pre-mRNA splicing based on location of binding (Ule et al., 2006; Zhang et al., 2008;

Licatalosi et al., 2008). However, there are other examples of regulated splicing where the binding location of a regulatory protein cannot fully explain activity (Motta-Mena et al., 2010)(Chapter 2). Therefore, other variables within the broader sequence context of the regulated exon must also influence the activity of a given regulatory protein during splicing. Here we show that in three distinct systems (exon 4 vs. exon 5 native splice sites, globin strong vs. weak, and MS2 strong vs. weak), remarkably, hnRNP L bound exclusively to an exon can have dual effects on splicing (activator or repressor) in a manner that is determined by the strength of the flanking splice sites. Critically, this study, among others (Mayeda and Krainer, 1992; Eperon et al., 200; Hui et al., 2005; Caceres et al., 1994; Yu et al., 2008), shows that context can fundamentally alter the activity of a splicing regulatory protein and adds splice site strength to the growing list of "splicing rules" that govern splice site choice during mRNA processing.

The results presented in this chapter, together with Chapter 2, argue that the difference in splice site strength between exons 4 and 5 necessitates the distinct mechanisms by which hnRNP L affects coordinated repression of these exons. Specifically, because the 3'ss upstream of exon 5 is weak the U2snRNP cannot efficiently recruited and so the inclusion of this exon relies heavily on the activity of the ESE (Figure 2-11, top). Therefore, instead of trapping a bound U2 snRNP as occurs on exon 4 (Figure 2-11, bottom), hnRNP L bound to the ARS motifs in exon 5 blocks the ability of SF2/ASF bound to ESE to promote binding of the U2 snRNP to the weak upstream 3'ss. Moreover, by extending the analysis of the role of splice site strength in determining regulatory mechanism, we found that weakening of splice site strength can

flip the activity of exon bound hnRNP L binding from a repressor to an activator in three distinct systems/substrates.

Figure 3-5 shows a model for hnRNP L function based on our data which correlates splice site strength with mechanism of regulation. We propose that during spliceosome assembly hnRNP L stabilizes the inherent snRNP-substrate affinity to inhibit or promote exon recognition, via direct or indirect interactions with the snRNPs. According to this model, when hnRNP L is bound to an exon flanked by strong splice sites, the sum total of the interactions between U1/hnRNP L/U2 and the pre-RNA sequesters the snRNPs in an inactive conformation that cannot progress further on in the assembly pathway (Figure 3-5, top left). Consistent with this interpretation, we find that the stalled AEC that forms on CD45 exon 4 is more resistant to salt and heparin destabilization than is a canonical A complex (House and Lynch, 2006; A.E.H and K.W.L., unpublished data). Moreover, this model is consistent with recent data from the Nilsen group, demonstrating that subtle perturbations in snRNP-splice site interactions can alter the efficiency of subsequent assembly steps (Yu et al., 2008). In contrast, if the splice sites flanking an exon are weak then the interaction of exon-bound hnRNP L with the snRNPs likely stabilizes the otherwise transient recruitment of the snRNPs to the flanking splice site(s) (Figure 3-5, top right). In cases of intermediate strength splice sites, we predict that the snRNP-substrate interactions would be within a range in which they are sufficient enough to not be helped by hnRNP L, but they are also not so strong that the snRNPs can be "trapped" by hnRNP L (Figure 3-3; construct C).

Relevant to this study, hnRNP L has been shown to increase inclusion of at least five exons that contain ARS motifs that might act as putative hnRNP L-binding sites

(MYL6, FAM48, and PAPOLA [Hung et al., 2003]; ERK1 and GCK [K.W.L., unpublished data]), however the mechanism of enhancement has not been investigated.

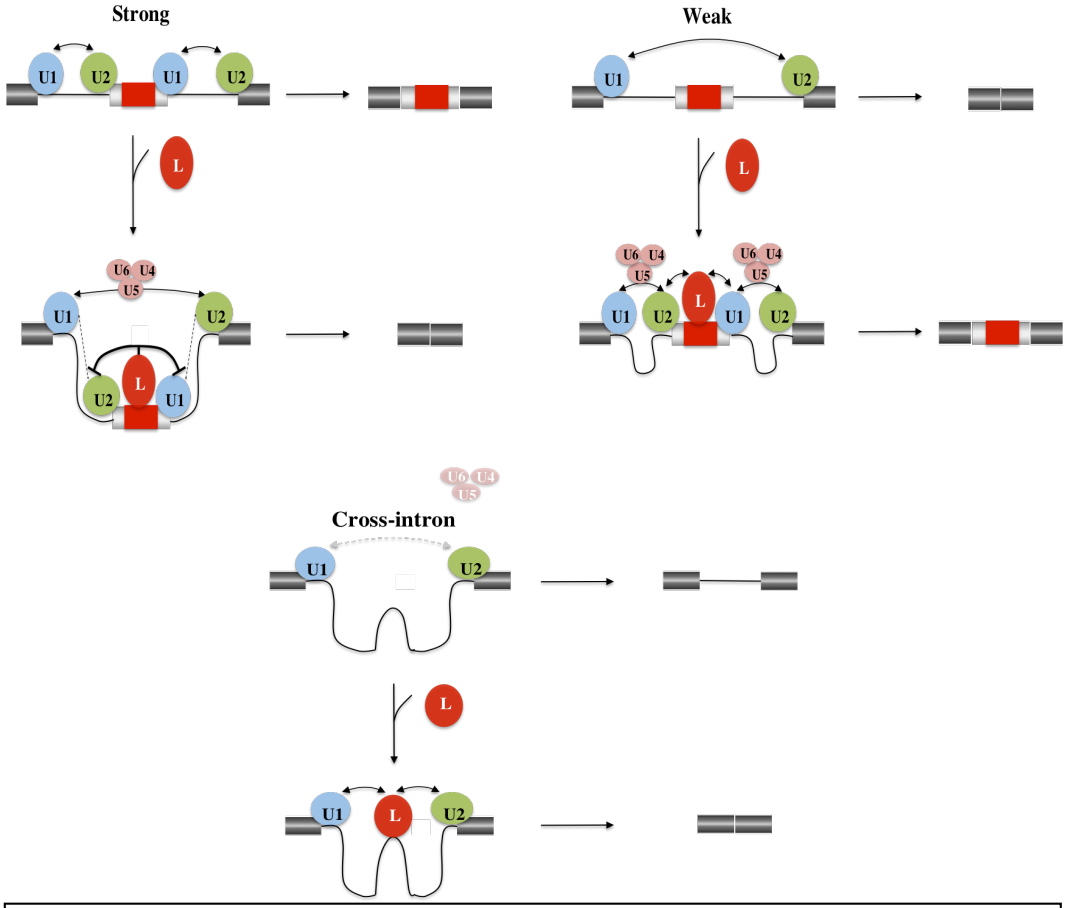

Figure 3-5. Model for hnRNP L function. (A) Interaction of hnRNP L with U1 and U2 snRNPs bound to strong flanking splice sites sequesters them in an inactive conformation that cannot progress further in the spliceosome assembly pathway. (B) However, if an ARS-containing exon is flanked by weak splice sites, then the interaction between U1 and U2 snRNPs and the exon-intron boundary is highly inefficient. In such a case interaction of hnRNP L with U1 and U2 may stabilize their interaction with the splice sites thus promoting progression through assembly pathway. (C) If the hnRNP L-binding sites are located within an intron then the interaction of U1 and/or U2 with hnRNP L would be predicted to bring these snRNPs together in a productive complex.

Interestingly, all five of these exons are flanked by short polypyrimidine tracts and/or suboptimal 5' splice sites, consistent with our model which predicts that hnRNP L promotes inclusion of exons flanked by weak splice sites. However, further investigation

will be required to determine whether splice site strength is the sole determinant of directionality of hnRNP L on these exons. Our model also accommodates the enhancing effect of hnRNP L that has been observed for several genes when this protein is bound within an intron (Hui et al., 2005). It is easy to imagine that coassociation of U1, hnRNP L, and U2 across an intron would promote cross-intron pairing of the snRNPs, thereby promoting subsequent spliceosome assembly (Figure 3-5, bottom). Taken together, the data presented in this study indicate that the same protein, through the same molecular interactions, can differentially influence spliceosome assembly in a manner that is determined at least in part by the strength of the flanking splice sites.

CHAPTER FOUR

Discussion

In previous work, the Lynch laboratory demonstrated that the basal and signal-induced skipping of CD45 exons 4, 5, and 6 is mediated by a consensus sequence motif known as the ARS (Rothrock et al., 2003; Tong, et al., 2005). At least for exon 4, the ARS binds the heterogeneous ribonucleoprotein hnRNP L which causes skipping of this exon by stalling spliceosome assembly at an A-like exon-defined complex (AEC) (Rothrock et al., 2005; House and Lynch, 2006). Preliminary studies suggested that the regulation of exon 5 by the ARS might be distinct from the other two CD45 exons. First, unlike in exons 4 and 6, the ARS element in exon 5 is not contiguous but instead is separated into two regions by an ESE (Tong et al., 2005). Second, in contrast to exons 4 and 6, the ARS-containing regions on their own are not sufficient to induce the levels of exon repression observed in the full exon (Tong et al., 2005). The precise reason(s) for the differential ARS activity in exon 5 has/have been unclear; however, these initial findings pointed to additional contextual effects that were yet to be defined.

In Chapter 2, I show that under resting conditions hnRNP L, which specifically binds to the ARS elements, acts to promote exon 5 repression in the absence of other coassociated proteins previously reported to associate with the ARS-containing ESS1 element in exon 4 (Rothrock et al., 2005; Motta-Mena et al., 2010). Also, I report that PSF, but not hnRNP LL (the other exon 4 repressor protein), functions in the ARS-mediated activation-induced skipping of exon 5. Subsequently, I focused on understanding the mechanism of exon 5 repression by hnRNP L under resting conditions.

In this study, I show that hnRNP L sterically blocks the binding of SF2/ASF to a splicing enhancer element in exon 5, thereby inhibiting SF2/ASF's ability to recruit the U2 snRNP and promote A complex formation (Motta-Mena et al., 2010). Remarkably, this mechanism is entirely distinct from the mechanism of hnRNP L repression on exon 4 (House and Lynch, 2006).

In Chapter 3, I demonstrate that the coordinate regulation of exons 4 and 5 by hnRNP L occurs by different mechanisms due, at least in part, to the inherent snRNP-substrate affinity of each exon. By further testing the correlation between splice site strength and hnRNP L function, I show that in three different systems/substrates hnRNP L can have enhancer or repressor activities when bound exclusively to an exon and that weakening of splice site strength flips the function of hnRNP L from an exon repressor to an activator (Figure 3-2, exon 4 vs. exon 5 native splice sites, globin strong vs. weak, and Figure 3-3 [F.H.]; MS2 strong vs. weak)(Motta-Mena et al., 2010). These data emphasize that variables in addition to binding location can alter the effect a given regulatory protein has on the assembly pathway of the spliceosome.

Differential protein association between exons 4 and 5 reveals diversity of ARS specificity and function

The ARS motif in exon 5 is in effect a simplified regulatory element that recruits only the core repressor proteins. In exon 4, by contrast, the ARS core motif is embedded within a larger silencer element (ESS1). Even though hnRNP L is the primary mediator of basal repression, additional hnRNPs (hnRNP E2, K, D and PTB) have been shown to bind to the exon 4 ESS1 element with similar affinity to hnRNP L although their functional significance is minor (Rothrock et al., 2005). In contrast, under resting

conditions, hnRNP L is the only protein that appears to associate with the exon 5 ARS sequence (Motta-Mena et al., 2010). Moreover, upon activation, repression of exon 5 is increased by the addition of PSF; whereas both PSF and hnRNP LL contribute to the signal-induced skipping of exon 4 (Motta-Mena et al., 2010; Melton et al., 2007; Topp et al., 2008; Oberdoerffer et al., 2008). Despite the differences in coassociated proteins bound with hnRNP L to exon 4 and exon 5, these are unlikely to contribute to the mechanistic distinctions between these two exons because in both exons the ARS element functions largely through binding of hnRNP L. However, the difference in coassociated protein binding to exons 4 and 5 does demonstrate that the ARS core motif is able to recruit hnRNP L as part of a variety of protein complexes that could, perhaps under certain conditions, influence mechanism or the recruitment of specific coassociated proteins.

The differential protein association on exons 4 and 5 also points to specificity differences among the functionally important silencer proteins. In particular, the data highlight differences in binding specificity between hnRNP L and hnRNP LL for ARS in exons 4 and 5. While both of these proteins effectively bind the ARS core in exon 4 and repress splicing of this exon, the affinity of hnRNP LL for exon 5, relative to exon 4, is dramatically reduced in comparison to the binding of hnRNP L to this exon (Figure 2-3C). This differential specificity is consistent with previous reports that describe specific mutations within the exon 4 ARS that abolish binding to hnRNP LL without affecting hnRNP L (Topp et al., 2008). Furthermore, the lack of repression of exon 5 by hnRNP LL is consistent with an array study that suggested no change in the inclusion of CD45 exon 5 in cells depleted of hnRNP LL (Oberdoerffer et al., 2008). In contrast to the

differential role of hnRNP LL, PSF does mediate the signal-induced silencing of both CD45 exons 4 and 5, although whether this is by a common mechanism or not remains to be determined (see *Future experiment*s below).

Implications for predictions of splicing patterns and mechanisms

An important long-term goal in the splicing field is to define a set of rules or ''code'' for splicing that will enable us to predict the splicing pattern of any primary transcript based solely on its sequence (Wang and Burge, 2008). Assignment of specific binding proteins to individual regulatory sequence elements is essential for deciphering the splicing "code." Importantly, the activities of *cis*-regulatory sequences and their related binding effectors have long been known to be fairly context-dependent (Wang and Burge, 2008). In particular, it is well established that the same regulatory sequence and its cognate protein factor(s) act as positive or negative splicing effectors depending on their location within the pre-mRNA (Mayeda and Krainer, 1992; Ule et al., 2006; Zhang et al., 2008; Yeo et al., 2009; Xue et al., 2009). It is also well documented that splicing outcomes are often the result of a balance between regulatory sequences and their cognate binding proteins (Hertel, 2007). Critically, this study addressed two long-held assumptions of the current splicing "code": 1) location is the sole determinant of mechanism, and 2) a given splicing protein uses the same mechanism to activate or repress an exon. The data presented here strongly argue that binding location is not the sole determinant of protein function, but that the antagonistic effects of hnRNP L on splicing (repressor and activator) can be displayed from the same exonic location and similar binding sequence in a manner that is determined on the relative strength of the

flanking splice sites. In addition, even though hnRNP L acts to promote repression of both CD45 exon 4 and exon 5, it does so through two distinct and separate mechanisms.

Nevertheless, this is not to say that position effects cannot also play a significant role in determining mechanism. Indeed, the data alluded to in Figure 3-5 demonstrate that location of hnRNP L binding (intronic versus exonic) can strongly influence regulatory outcome, and numerous other examples of location-dependent mechanism have been well characterized (see above). Furthermore, splice site strength is unlikely to be the only aspect of context that influences splicing mechanism. Binding of additional proteins to flanking regulatory elements (Matlin et al., 2005) and neighboring RNA motifs and/or structure (Yu et al., 2008) are just two other examples of additional context differences that have been shown to alter the susceptibility of an pre-mRNA to a particular regulatory protein. Therefore, we conclude that the mechanism by which a particular protein regulates any given exon cannot be solely attributed to either location or context but, rather, relates to how that protein impinges on the rate-limiting step in assembly of the spliceosome on that exon and how this relates to the efficiency of competing assembly pathways on the same transcript. In terms of a global understanding of the splicing "code," this study adds to the growing list of rules that determine the activity of splicing protein effectors, which will be integrated into future simulation algorithms that predict the splicing pattern of a particular gene, these in turn will help decipher the precise expression of a protein and interpret the pathogenic effects of genetic mutations.

Future experiments

In this study I have shown that hnRNP L silences exon 5 by inhibiting the intervening ESE activity and that the mechanism of hnRNP L activity is determined by

the identity and strength of the flanking splice sites. However, many questions remain regarding the identity of any additional ESE-binding proteins and the mechanistic implications of the splice site-dependent differential activity of hnRNP L. Some of the most immediate questions are discussed below.

First, further RNA-affinity experiments should be performed to identify and characterize other proteins that bind to and function in the inclusion of exon 5. This information will shed further light on the mechanisms of exon enhancement and how this activity is countered by hnRNP L. Although initial studies showed that SF2/ASF promotes inclusion of exon 5 in an ESE-dependent manner (Figure 2-5), SF2/ASF is not sufficient to induce a level of exon inclusion similar to that observed in JSL1 cells; thus, there might be other ESE-specific enhancer proteins. The main problem with the RNA-affinity approach has been that the ESE activity is limiting in nuclear extracts. While scaling up the total input did improve the final yield of the pulldown reaction to some extent, preliminary experiments where three E5-ΔS1S2 pulldown reactions were pulled together and ran on a large polyacrylamide gel (data not shown) revealed only a small increase in the number ESE-specific bands compared to the E5-ΔESE probe. Also, subsequent mass spectrometry analysis yielded few viable protein candidates, and western blot analysis revealed these candidates were false-positive hits (data not shown). Nevertheless, there are additional steps that can be included in the current RNA-affinity protocol to further improve recovery. For example, immunodepleting hnRNP L from the nuclear extract increases the probability that the ESE-binding proteins will interact with exon 5. Initial RNA-affinity experiments using hnRNP L-depleted nuclear extract looked promising because the intensity of ESE-specific bands by silver stain was much improved

from previous experiments (data not shown). Similarly, using the RNase elution methods, instead of SDS buffer alone, to release the RNA-protein complexes from the beads significantly enhanced the appearance of ESE-specific bands on silver stained gel (data not shown). Perhaps combining the use of hnRNP L-depleted nuclear extract, the RNase elution method, and pulling three or more reactions together, might serve to improve the overall isolation of ESE-binding enhancer proteins. There is also reason to believe that the ESE-activity in nuclear extracts decreases over time (LB.M.M. and K.W.L. observations); thus, using fresh nuclear extract preparations in the new RNA-affinity purification scheme is likely to give the best results.

Alternatively, a candidate approach might be useful for identifying the protein(s) that act to promote exon 5 inclusion. Instead of using computational methods, as was used to identify SF2/ASF, a biochemical approach where a panel of different known SR proteins are expressed and purified from cells and then added into *in vitro* splicing assays might be used. One concern with this method is that because SR proteins are known to be promiscuous (Shepard and Hertel, 2009), any splicing effects we see might not be entirely specific to the ESE. For this reason, the panel of SR protein must tested on a minigene lacking the ESE side by side with the wild type minigene. Related to this, results from knockdown experiments targeting specific SR proteins might be difficult to interpret. Also, it might be useful to try different combinations of SR proteins in the same *in vitro* splicing reaction, as the activity of some SR proteins has been shown to be dependent on the interaction with other SR protein partners (Lynch and Maniatis, 1996).

A second unresolved issue is whether MS2-hnRNP L blocks the transition from A to B complex in substrates containing strong splice sites (as seen in exon 4), and it

enhances A complex formation to substrates containing weak splice sites (as seen in exon 5). In chapter 3 it was reported that that the mechanism of hnRNP L repression is determined by the identity and strength of the flanking splice sites. However, spliceosome assembly experiments that confirm this effect directly were shown only for exon 5 (Figure 2-9), and previously for exon 4 (House and Lynch, 2006). An analysis of spliceosome assembly using the MS2 constructs (Figure 3-3) in the presence and absence of MS2-hnRNP L is missing. Previous attempts to use the MS2 substrates from Figure 3-3 for assembly experiments proved that these constructs are not suitable for such experiments, as clear assembly intermediates are not readily visualized in the gel for any of the substrates (with or without MS2-hnRNP L) (F.H., data not shown). In particular, although the enhancement of splicing by hnRNP L on the constructs with weak splice sites represents a significant fold change (Figure 3-3, constructs D and E), the splicing efficiency overall is too small to able to observe complexes on an assembly gel.

Subsequently, a chimeric minigene was made that consisted of the central exon 5 flanked by the splice sites and intron sequence from exon 4 with the CD45 exons 3 and 7 on either side (similar to SC4-E5, Figure 3-2; now called CD4-E5). This CD4-E5 minigene would allow us to determine whether changing the native exon 5 splice sites, which are weak, to the inherently stronger exon 4 splice site would change the mechanism from a block at A complex to a stall at an AEC. Also, the 5'ss of the first exon (CD45 exon 3) was inactivated so that splicing and spliceosome assembly occur only on the downstream intron (CD4-mutE3ss-E5), that way repression of the central chimeric exon can be observed without complications from the exon 3-7 splicing pathway that occurs in the CD4-E5 construct. Preliminary *in vitro* splicing experiments

using these minigenes showed that the splicing efficiency of CD4-mutE3ss-E5-WT is high (~34%) and mutating the ESE decreases the level of splicing (~12%) as expected; however, surprisingly deletion of the S1S2 sequences also decreases splicing efficiency (~21%) (data not shown). Furthermore, deletion of all three regulatory sequences (ΔESE+S1S2; ~9%) decreased splicing to levels similar to the ΔESE construct (data not shown). Since these minigenes contained strong exon 4 splice sites, we expected the silencer sequences in the ΔESE minigene would repress inclusion compared to the null minigene (ΔESE+S1S2), which would be consistent with our model where hnRNP L represses strong splice sites; however, this was not the case (ΔESE+S1S2 9% vs. ΔESE 12%). Thus, it appears that the exon 5 regulatory sequences, in the CD background and flanked by strong splice sites, have some spurious effects that cannot be explained at this point in this study, and that make these minigenes inadequate for further spliceosome assembly experiments. Of interest, the use of an adenovirus background has been successfully employed to study assembly of CD45 exon 4 (N.T.C. and K.W.L.); perhaps moving the E5/E4 chimeric exon to this adenovirus vector might server to improve the overall study (splicing and assembly experiments) of E5/E4 chimeric minigenes.

My work has primarily focused on the mechanism of splicing regulation of exon 5 under resting conditions. Thus, as an initial step toward understanding the mechanism of exon 5 repression upon cellular activation, *in vitro* splicing experiments were done with the E3-E5 minigenes in nuclear extract from resting and stimulated JSL1 cells. Preliminary results from these studies showed that the fold repression (FR) value—which is a measure for the efficiency with which the inclusion of an exon is repressed upon activation—for the E3-E5 wild type construct was ~2 fold less than the FR for the

full length minigene CD5 (E3-E5 FR= 2.7 vs. CD5 FR= 6.3; data not shown). Therefore, while under resting conditions the E3-E5 minigene is regulated in a manner consistent with the full CD5 minigene, under stimulated conditions the single-intron E3-E5 minigene is not. Consequently, the mechanism responsible for increased exon repression upon activation might be different from the mechanism observed under resting conditions. Of note, previous work showed that single-intron constructs do not recapitulate the silencing of exon 4 because the exon-defined complex necessary for repression requires that the exon be flanked by intron sequence on both sides (House and Lynch, 2006). It is possible that the mechanism of repression for exon 5 upon activation involves a similar non-permissive exon-defined complex as seen on exon 4. To test this hypothesis, additional constructs could be made that included the ~110 nt of intronic sequence downstream of exon 5. If an exon-defined complex is responsible then there should be a larger decrease in the levels of exon 5 inclusion in stimulated nuclear extract, giving a larger FR value similar to that seen with the full CD5 minigene. In addition, spliceosome assembly experiments where purified PSF protein is titrated in the reaction might help to define the point of regulation. For example, PSF when added might decrease A complex formation (similar to the effect of hnRNP L addition; Figure 2-10), or it might increase the levels of A-like repressed complex (similar to A.E.C for exon 4; House and Lynch, 2006). *In vitro* splicing experiments where the exogenous PSF is added to the reaction must also be done side by side with assembly experiments to confirm the functional effect of the protein on splicing.

Conclusion

In conclusion, I show that unique features of the sequence context of the ARS element in exon 5 result in hnRNP L functioning to repress this exon by a distinct mechanism from that described for exon 4. In addition, by extending the analysis of the role of splice site strength in determining regulatory mechanism, I find that weakening of splice site strength can flip the effect of hnRNP L binding from a repressor to an activator. Together, these data demonstrate that a given protein can exert different effects on the assembly pathway of the spliceosome. Future characterization of other ARS-regulated exons (Ip et al., 2007; Hung et al., 2008) with 3' and 5' splice sites of varying strengths will provide a broader insight as to the role of splice site strength in the directionality of hnRNP L function on these exons.

CHAPTER FIVE

Materials and Methods

Minigenes and RNAs.

Construct SC5 was previously described in Tong et al., 2005. Briefly, the minigene consists of CD45 exon 5 and surrounding intron flanked by intron and exon sequence from the human β–globin gene. SC5-ΔESE, SC5-ΔS1S2, and SC5-ΔESE+S1S2 were made using PCR to replace the corresponding sequence within exon 5 to TCAGTATGACTCTCAGTATG. This sequence was originally identified as a sequence with no splicing regulatory activity (Schaal and Maniatis, 1999), moreover, we have used it extensively in previous studies with no discernable effects on splicing in any context (Tong et al., 2005; Lynch and Weiss, 2001; Rothrock et al., 2003). Oligonucleotides encoding the 100 nt E5-WT, -ΔESE, and -ΔS1S2 were cloned directly downstream of a T7 polymerase promoter and served as minigene templates for transcription of competitor RNAs and RNA probes. The RNAs were transcribed with T7 polymerase (Promega) in the absence or presence of ^{32}P-CTP to radioactively label probes. The CD4 and CD5 minigenes, used for *in vitro* splicing, were previously described in Rothrock et al. (2003) and Tong et al. (2005). Single-intron constructs (E3-E5 and E5-E7) were cloned into CD5 using MluI and Hind III. The exon 4/exon 5 chimeras (SC4-E5 ΔESE and SC4-E5 ΔESE+S1S2) were generated by inserting the 100 nt regulatory region of exon 5 into the PstI site of SC4 (Rothrock et al., 2003), resulting in exon 5 flanked by the splice sites and intron sequence from exon 4. Substrates for *in vitro* splicing that contained the MS2 binding site were generated by inserting a single

MS2 hairpin into the MluI site of SCglo (Rothrock et al., 2003). A fragment of this construct with the MS2 containing exon and 110 bp intron on both sides was PCR amplified with primers generating XhoI and HindIII sites and ligated into an XhoI/HindIII cut AdML vector (kind gift of K. Hertel) yielding a glo-MS2 exon flanked by 2 AdML exons. Splice site mutations were introduced by PCR and confirmed by sequencing.

Nuclear extract and recombinant proteins.

Nuclear extract was purified from JSL1 cells using a standard protocol previously described in Lynch and Weiss, 2001. Recombinant hnRNP L and PTB were expressed as a GST fusion proteins in SF9 cells and were purified using glutathione sepharose 4B resin (GE Biosciences) as described previously in (Rothrock et al., 2005). MBP-hnRNP E2 was expressed and purified from *Escherichia coli* using a protocol previously described by Gamarnik and Andino, 1997. PSF and hnRNP LL cDNAs were cloned directly downstream of a EF promoter and modified with an N-terminal Flag tag. JSL1 cells stably expressing either Flag-PSF or Flag-hnRNP LL were grown under resting or stimulated conditions and then lysed to prepare nuclear extract. Tagged proteins were purified from nuclear extract with EZ-View Red FLAG-conjugated resin (Sigma) in GFB100 (20 mM Tris-Cl, pH 7.5, 100 mM KCl, and 0.2 mM EDTA, pH 8.0). Following extensive washing in GFB100, the proteins were eluted with 500 ng/ul of 3X Flag peptide (Sigma). SF2/ASF protein was expresses in SF9 cells and was purified as previously described by Lynch and Maniatis, 1996.

Cell culture.

JSL1 cells (Lynch and Weiss, 2000) were cultured in RPMI+ 5% fetal calf serum

at 37°C in 5% CO2. Sub-lines of JSL1 cells that stably express the minigenes described were created by transfecting 10 million cells with 10 ug of minigene plasmid by electroporation and grown under drug selection as described by (Rothrock et al., 2003). For splicing analysis, three independent clones of each minigene were either left untreated or treated with 20 ng/ml of PMA for 60 h, after which cells were harvested and total RNA extracted using RNABee (Tel-Test). Minigene derived spliced products were analyzed by RT PCR using vector-specific primers (see below).

RT-PCR.

RT-PCR and analysis was carried out as previously described in detail by Rothrock et al. 2003. In brief, a low-cycle PCR protocol was used, such that the signal detected is linear with respect to input RNA. Minigenes were analyzed using the vector-specific primers ACT and GE3R (sequence published in Rothrock et al., 2003) for SC5 constructs and T7Mlu and E7R1 primers for CD4 and CD5 minigenes (sequence published in House and Lynch, 2006). Quantitation was done by densitometry using a Typhoon Phosphoimager (Amersham Biosciences).

RNA affinity purification.

500 pmol of *in vitro* transcribed RNA was incubated with sodium periodate and coupled to adipic acid beads overnight using a protocol adapted from that previously described by (Caputi and Zahler, 2002). Beads were incubated with ~200 mg of JSL1 nuclear extract in a 500 ml binding reaction containing (final concentrations): 3.2 mM MgCl2, 20 mM phosphocreatine, 1 mM ATP, 1.3% polyvinyl alcohol, 25 ng of yeast tRNA, 75 mM KCl, 10mM Tris, pH 7.5, 0.1 mM EDTA, 10% glycerol. Binding reactions were incubated with gentle agitation for 30 min at 30°C. The protein-RNA-bead

complexes were washed with GFB100+ 4mM MgCl2, resuspended in 2X SDS loading buffer, denatured for 5 min at 95°C, analyzed under denaturing conditions on a 10% gel (Acrylamide/Bis 37.5:1, BioRad), and detected by silver staining (BioRad) or western blot.

Western blotting.

Western blotting was carried out as previously described in Lynch and Weiss (2000). Antibodies for Western blots were as follows: anti-hnRNP L (4D11, Abcam), anti-PTB N-term (rabbit polyclonal, a gift from D. Black), anti-hnRNP E2 (rabbit polyclonal, a gift from R. Andino), anti-hnRNP K/J (3C2, Immunoquest), anti-hnRNP D (rabbit polyclonal; BioLegend), anti-PSF (6D7, Abnova), and anti-hnRNP LL (Aviva).

RNA mobility shift.

In vitro transcribed RNAs were gel-purified and adjusted to 104 cpm/ml specific activity. Each RNA was incubated with JSL1 nuclear extract in a total volume of 10 ml and under standard binding conditions similar to that described for the RNA-affinity assays, but with the addition of (final concentration) 0.1 ml of RNasin (Promega, 40U/ul) and 0.8 mg of BSA. Reactions were incubated for 20 min at 30°C, after which heparin was added to a final concentration of 5 mg/ml and incubated for an additional 5 min at 30°C. Reactions were analyzed on a 4.5% native gel (Acrylamide/Bis 29:1 BioRad) and visualized by autoradiography.

***In vitro* splicing.**

In vitro splicing reactions were carried out as detailed by Rothrock et al. (2005). In brief, 1 fmol of transcribed pre-mRNA template was incubated in 30% JSL1 nuclear extract in a total volume of 12.5 ml under standard binding conditions similar to those

described above and in Rothrock et al. (2005). Reactions were incubated for 2 h at 30°C, after which the RNA was recovered by proteinase K treatment followed by phenol-chloroform extraction and precipitation. Analysis of the resulting spliced products was done by RT PCR as described above. For competition studies, the specified amount of competitor (E5-WT, -M3, or –M2M4) was added to splicing reactions before addition of RNA substrate and reactions were processed as described above. For in vitro splicing experiments with MS2-tagged hnRNP L, reactions as above were supplemented with nuclear extract from 293 cells that were transiently transfected with an MS2-hnRNP L expression construct, or an empty vector control. Reactions were processed and analyzed as above.

UV Crosslinking.

Radiolabeled RNA was incubated in JSL1 nuclear extract or with recombinant hnRNP L or SF2/ASF under similar conditions described for the RNA affinity purification assays. Reactions were incubated for 20 min at 30°C, crosslinked using UV light (254 nm) for 20 min on ice, and digested with 2 ug (final concentration) of RNase T1 and RNase A each for 20 min at 37°C. Reactions were analyzed under denaturing conditions on a 12% gel (Acrylamide/Bis 37.5:1, BioRad), and visualized by autoradiography.

Spliceosome assembly.

Spliceosome assembly on our pre-mRNA substrates was analyzed using standard protocols (Das and Reed, 1999; Konarska and Sharp, 1986). Radiolabeled pre-mRNA substrates were adjusted to 10^5 cpm/ml and incubated in JSL1 nuclear extract under splicing conditions for the indicated amount of time. For ATP-depleted reactions, ATP

was depleted from nuclear extract by incubating at 25°C for 1 hr. RNase H inactivated extract was used in place of untreated nuclear extract to test requirement for snRNPs in assembly (see below). Spliceosome complexes were analyzed on nondenaturing polyacrylamide (4%; Acrylamide/Bis 40:1) or on agarose (1.5%) gels as specified in each case. Gels were run at 250 V for 5-6 h at room temperature for acrylamide gels and at 70 V for 3-3.5 h at 4°C for agarose gels.

snRNA Inactivation.

For RNase H reactions, nuclear extract was incubated in a reaction containing (final concentrations): 30% JSL1 NE, 0.8 mM ATP, 20 mM CP, 4.4 mM $MgCl_2$, 30 units RNasin (Promega), 1 unit RNase H (Roche), 90 mM KCl in the presence or absence of 5 pmol oligonucleotide that is complementary to the target snRNA. The following oligonucleotides were used to target inactivation of the indicated snRNA: U1 and U2 were inactivated by 5'C and E15 respectively (Black et al., 1985), while U6f was used to inactivate U6 (Konforti and Konarska, 1994). Reactions were incubated at 30°C for 1 hour and RNA was extracted from 10 μl of the reaction for analysis by primer extension to determine the specificity and amount of inactivation.

BIBLIOGRAPHY

Berget, S.M. (1995) Exon recognition in vertebrate splicing. J Biol Chem 270, 2411-2414.

Birkeland, M.L., Johnson, P., Trowbridge, I.S., and Pure, E. (1989) Changes in CD45 isoform expression accompany antigen-induced murine T-cell activation. Proc Natl Acad Sci U S A 86, 6734-6738.

Black., D.L. (2003) Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem 72, 291-336.

Blaustein, M., Pelisch, F., and Srebrow, A. (2007) Signals, pathways and splicing regulation. Int J Biochem Cell Biol 39, 2031-2048.

Blencowe, B.J., Issner, R., Nickerson, J.A., and Sharp, P.A. (1998) A coactivator of pre-mRNA splicing. Genes Dev 12, 996-1009.

Blencowe, B.J. (2006) Alternative splicing: new insights from global analysese. Cell 126, 37-47.

Blencowe B.J., Ahmad, S., and Lee, L.J. (2009) Current-generation high-throughput sequencing: deepening insights into mammalian transcriptomes. Genes Dev 23, 1379-1386.

Bonnal, S., Martinez, C., Forch, P., Bachi, A., Wilm, M., and Valcarcel, J. (2008) RBM5/Luca-15/H37 regulates Fas alternative splice site pairing after exon definition. Mol Cell 32, 81-95.

Brow, D.A. (2002) Allosteric cascade of spliceosome activation. Annu Rev Genet 36, 333-360.

Buckanovich RJ, Posner JB, Darnell RB. (1993) Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. Neuron 11, 657–672.

Buratowski, S. (2003) The CTD code. Nat Struct Biol 10, 679-680.

Buratti, E., Baralle, M., De Conti L, Baralle, D., Romano, M., Ayala, Y.M., and Baralle, F.E. (2004) hnRNP H binding at the 5' splice site correlates with the pathological

effect of two intronic mutations in the NF-1 and TSHbeta genes. Nucleic Acids Res 32, 4224-4236.

Caceres, J.F., Stamm, S., Helfman, D.M., and Krainer, A.R. (1994) Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science 265, 1706-1709.

Calarco, J.A., Superina, S., O'Hanlon, D., Gabut, M, Raj, B., Pan, Q., Skalska, U., Clarke, L., Gelinas, D., van der Kooy, D., et al. (2009) Regulation of vertebrate nervous system alternative splicing and development by an SR-related protein. Cell 138, 898-910.

Cale., C.M., Klein, N.J., Novelli, V., Veys, P., Jones, A.M., and Morgan, G. (1997) Severe combined immunodeficiency with abnormalities in expression of the common leucocyte antigen, CD45. Arch Dis Child 76, 163-164.

Camats, M., Guil, S., Kokolo, M., and Bach-Elias, M. (2008) P68 RNA helicase (DDX5) alters activity of cis- and trans-acting factors of the alternative splicing of H-Ras. PLoS One 3, e2926.

Caputi, M., and Zahler, A.M. (2001) Determination of the RNA binding specificity of the heterogeneous nuclear ribonucleoprotein (hnRNP) H/H'/F/2H9 family. J Biol Chem 276, 43850-43859.

Caputi, M., and Zahler, A.M. (2001) SR proteins and hnRNP H regulate the splicing of the HIV-1 tev-specific exon 6D. EMBO J 21, 845-855.

Cartegni, L., Wang, J., Zhu, Z., Zhang, M.Q., and Krainer, A.R. (2003) ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic Acids Res 31, 3568-3571.

Chen, M., and Manley, J.L. (2009) Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nat Rev Mol Cell Biol 10, 741-754.

Cooper, T.A., Wan., L., and and Dreyfuss, G. (2009) RNA and disease. Cell 135, 777-793.

Corden, J.L., and Patturajan, M. (1997) A CTD function linking transcription to splicing.

Trends Biochem Sci 22, 413-416.

Crawford, J.B., and Patton, J.G. (2006) Activation of alpha-tropomyosin exon 2 is regulated by the SR protein 9G8 and heterogeneous nuclear ribonucleoproteins H and F. Mol Cell Biol 26, 8791-8802.

Dahmus, M.E. (1996) Reversible phosphorylation of the C-terminal domain of RNA polymerase II. J Biol Chem 271, 19009, 19012.

Damgaard, C.K., Tange, T.O., and Kjems, J. (2002) hnRNP A1 controls HIV-1 mRNA splicing through cooperative binding to intron and exon splicing silencers in the context of a conserved secondary structure. RNA 8, 1401-1415.

Dawes, R., Hennig, B., Irving, W., Petrova, S., Boxall, S., Ward, V., Wallace, D., Macallan, D.C., Thursz, M., Hill, A., et al. (2006). Altered CD45 expression in C77G carriers influences immune function and outcome of hepatitis C infection. J Med Genet 43, 678-684.

de la Mata, M., Alonso, C.R., Kadener, S., Fededa, J.P., Blaustein, M., Pelisch. F., Cramer, P., Bentley, D., and Kornblihtt, A.R. (2003) A slow RNA polymerase II affects alternative splicing in vivo. Mol Cell 12, 525-532.

de la Mata, M., and Kornblihtt, A.R. (2006) RNA polymerase II C-terminal domain mediates regulation of alternative splicing by SRp20. Nat Struct Mol Biol 13, 973-980.

Desai, D.M., Sap, J., Silvennoinen, O. Schlessinger, J., and Weiss, A. (1994) The catalytic activity of the CD45 membrane-proximal phosphatase domain is required for TCR signaling and regulation. EMBO J 13, 4002-4010.

Diehn, M., Alizadeh, A.A., Rando, O.J., Liu, C.L., Stankunas, K., Botstein, D., Crabtree, G.R., and Brown P.O. (2002) Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. Proc Natl Acad Sci U S A 99, 11796-11801.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., and Burd, C.G. (1993) hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem 62, 289-321.

Eldridge, A.G., Li, Y., Sharp, P.A., and Blencowe, B.J. (1999) The SRm160/300 splicing coactivator is required for exon-enhancer function. Proc Natl Acad Sci U S A 96, 6125-6130.

Eperon, I.C., Makarova, O.V., Mayeda, A., Munroe, S.H., Caceres, J.F., Hayward, D.G., and Krainer, A.R. (2000) Selection of alternative 5' splice sites: role of U1 snRNP and models for the antagonistic effects of SF2/ASF and hnRNP A1. Mol Cell Biol 20, 8303-8318.

Fairbrother, W.G., Yeh, R., F., Sharp, P.A., and Burge, C.B. (2002) Predictive identification of exonic splicing enhancers in human genes. Science 297, 1007-1013.

Faustino, N.A., and Cooper, T.A. (2005) Identification of putative new splicing targets for ETR-3 using sequences identified by systematic evolution of ligands by exponential enrichment. Mol Cell Biol 25, 879-887.

Feng, Y., Chen, M., and Manley, J.L. (2008) Phosphorylation switches the general splicing repressor SRp38 to a sequence-specific activator. Nat Struct Mol Biol 15, 1040.1048.

Fiszman, M.Y., Libri, D., and Balvay, L. (1992) Cis regulating elements which control in vivo alternative splicing of the chicken beta tropomyosin primary transcript. Sym Soc Exp Biol 46, 355-362.

Forch, P., Puig, O., Kedersha, N., Martinez, C., Granneman, S., Seraphin, B., Anderson, P., and Valcarcel, J. (2000) The apoptosis-promoting factor TIA-1 is a regulator of alternative pre-mRNA splicing. Mol Cell 6, 1089-1098.

Fu, X.D. (1995) The superfamily of arginine/serine-rich splicing factors. RNA 1, 663-680.

Fukuhara, K., Okumura, M., Shiono, H., Inoue, M., Kadota, Y., Miyoshi, S., and Matsuda, H. (2002) A study on CD45 isoform expression during T-cell development and selection events in the human thymus. Hum Immunol 63, 394-404.

Ge, H., and Manley, J.L. (1990) A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. Cell 62, 25-34.

Graveley, B.R. (2000) Sorting out the complexity of SR protein functions. RNA 6, 1197-1211.

Graveley, B.R. (2001) Alternative splicing: increasing diversity in the proteomic world. Trends Genet 17, 100-107.

Gromak, N, Matlin, A.J., Coopter, T.A., and Smith, C.W. (2003) Antagonistic regulation of alpha-actinin alternative splicing by CELF proteins and polypyrimidine tract binding protein. RNA 9, 443-456.

Grover, A., Houlden, H., Baker, M., Adamson, J., Lewis, J., Prihar, G., Pickering-Brown, S., Duff, K., and Hutton, M. (1999) 5' splice site mutations in tau associated with the inherited dementia FTDP-17 affect a stem-loop structure that regulates alternative splicing of exon 10. J Biol Chem 274, 15134-15143.

Habelhah, H., Shah, K, Huang, L., Ostareck-Lederer, A., Burlingame, A.L., Shokat, K.M., Hentze, M.W., and Ronai, Z. (2001) ERK phosphorylation drives cytoplasmic accumulation of hnRNP-K and inhibition of mRNA translation. Nat Cell Biol 3, 325-330.

Hanamura, A., Caceres, J.F., Mayeda, A., Franza, B.R. Jr., and Krainer, A.R. (1998) Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. RNA 4, 430-444.

Hermiston, M.L., Xu, Z., Majeti, R., and Weiss, A. (2002) Reciprocal regulation of lymphocyte activation by tyrosine kinases and phosphatases. J clin Invest 109, 9-14.

Hermiston, M.L., Xu, A., and Weiss, A. (2003) CD45: a critical regulator of signaling thresholds in immune cells. Annu Rev Immunol 21, 107-137.

Hertel, K.J. (2008) Combinatorial control of exon recognition. J Biol Chem 283, 1211-1215.

Hiller, M., Zhang, Z., Backofen, R., and Stamm, S. (2007) Pre-mRNA secondary structures influence exon recognition. PLoS Genet 3, e204.

House, A.E., and Lynch, K.W. (2006) An exonic splicing silencer represses spliceosome assembly after ATP-dependent exon recognition. Nat Struct Mol Biol 13, 937-944.

House, A.E., and Lynch, K.W. (2008) Regulation of alternative splicing: more than just the ABCs. J Biol Chem 283, 1217-1221.

Howe, K.J., Kane, M., and Ares, M. Jr. (2003) Perturbation of transcription elongation influences the fidelity of internal exon inclusion in Saccharomyces cerevisiae. RNA 9, 993-1006.

Hui, J., Stangl, K., Lane, W.S., and Bindereif, A. (2003) HnRNP L stimulates splicing of the eNOS gene by binding to variable-length CA repeats. Nat Struct Biol 10, 33-37.

Hui, J., Hung, L.H., Heiner, M, Schreiner, S., Neumuller, N., Reither, G., Haas, S.A., and Bindereif, A. (2005) Intronic CA-repeat and CA-rich elements: a new class of regulators of mammalian alternative splicing. EMBO J 24, 1988-1998.

Hung, L.H., Heiner, M., Hui, J., Schreiner, S., Benes, V., and Bindereif, A. (2008) Diverse roles of hnRNP L in mammalian mRNA processing: a combined microarray and RNAi analysis. RNA 14, 284-296.

Hutchison, S., LeBel, C., Blanchette, M., and Chabot, B. (2002) Distinct sets of adjacent heterogeneous nuclear ribonucleoprotein (hnRNP) A1/A2 binding sites control 5' splice site selection in the hnRNP A1 mRNA precursor. J Biol Chem 277, 29745-29752.

Ip, J.Y., Tong, A., Pan, Q., Topp, J.D., Blencowe, B.J., and Lynch, K.W. (2007) Global analysis of alternative splicing during T-cell activation. RNA 13, 563-572.

Ishikawa, H., Mahmoud, M.S., Fujii, R., Abroun, S., and Kawano, M.M. (2000)

Proliferation of immature myeloma cells by interleukin-6 is associated with CD45 expression in human multiple myeloma. Leuk Lymphoma 39, 51-55.

Jensen, K.B., Dredge, B.K., Stefani, G., Zhong, R., Buckanovich, R.J., Okano, H.J., Yang, Y.Y., and Darnell, R.B. (2000) Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. Neuron 25, 359-371.

Kalsotra, A., Xiao, X., Ward, A.J., Castle, J.C., Johnson, J.M., Burge, C.B., and Cooper, T.A. (2008) A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. Proc Natl Acad Sci U S A 105, 20333-20338.

Kanopka, A., Muhlemann, O., and Akusjarvi, G. (1996) Inhibition by SR proteins of splicing of a regulated adenovirus pre-mRNA. Nature 381, 535-538.

Kashima, T., and Manley, J.L. (2003) A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nat Genet 34, 460-463.

Kashima, T., Rao, N., and Manley, J.L. (2007a) An intronic element contributes to splicing repression in spinal muscular atrophy. Proc Natl Acad Sci U S A 104, 34-26-3431.

Kashima T., Rao, N., David, C.J., and Manley, J.L. (2007b) hnRNP A1 functions with specificity in repression of SMN2 exon 7 splicing. Hum Mol Genet 16, 3149-3159.

Konarska, M.M., Vilardell, J., and Query, C.C. (2006) Repositioning of the reaction intermediate within the catalytic center of the spliceosome. Mol Cell 21, 543-553.

Kornblihtt, A.R. (2006) Chromatin, transcript elongation and alternative splicing. Nat Struct Mol Biol 13, 5-7.

Krainer, A.R., Conway, G.C., and Kozak, D. (1990a) Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. Genes Dev 4, 1158-1171.

Krainer, A.R., Conway, G.C., and Kozak, D. (1990b) The essential pre-mRNA splicing factor SF2 influences 5' splice site selection by activating proximal sites. Cell 62, 35-42.

Kung, C., Pingel, J.T., Heikinheimo, M., Klemola, T., Varkila, K., Yoo, L.I., Voupala, K., Poyhonen, M., Uhari, M., Roger, M., et al. (2000) Mutations in the tyrosine phosphatase CD45 gene in a child with severe combined immunodeficiency disease. Nat Med 6, 343-345.

Ladd, A.N., Charlet, N., and Cooper, T.A. (2001) The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Mol Cell Biol 21, 1285-1296.

Lallena, M.J., Chalmers, K.J., Llamazares, S., Lamond, A.I., and Valcarcel, J. (2002) Splicing regulation at the second catalytic step by Sex-lethal involves 3' splice site recognition by SPF45. Cell 109, 285-296.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., Fitzhugh, W., et al. (2001) Initial sequencing and analysis of the human genome. Nature 409, 860-921.

Li, T., Evdokimov E., Shen, R.F., Chao, C.C., Tekle, E., Wang, T., Stadtman, E.R., Yang, D.C., and Chock, P.B. (2004) Sumoylation of heterogeneous nuclear ribonucleoproteins, zinc finger proteins, and nuclear pore complex proteins: a proteomic analysis. Proc Natl Acad Sci U S A 101, 8551-8556.

Li, Y., and Blencowe, B.J. (1999) Distinct factor requirements for exonic splicing enhancer function and binding of U2AF to the polypyrimidine tract. J Biol Chem 274, 35074-35079.

Licatalosi, D.D., Mele, A., Fak, J.J., Ule, J., Kayikci, M., Chi, S.W., Clark, T.A., Schweitzer, A.C., Blume, J.E., Wang, X., et al. (2008) HITS-CLIP yields genome-wide insights into brain alternative RNA processing. Nature 456, 464-469.

Long, J.C., and Caceres, J.F. (2009) The SR protein family of splicing factors: master regulators of gene expression. Biochem J 417, 15-27.

Lopez-Bigas, N., Audit, B., Ouzounis, C., Parra, G., and Guigo R. (2005) Are splicing

mutations the most frequent cause of hereditary disease? FEBS Lett 579, 1900-1903.

Lynch, K.W., and Maniatis, T. (1996) Assembly of specific SR protein complexes on distinct regulatory elements of the Drosophila doublesex splicing enhancer. Genes Dev 10, 2089-2101.

Lynch, K.W., and Weiss, A. (2000) A Model System for Activation-Induced Alternative Splicing of CD45 Pre-mRNA in T Cells Implicates Protein Kinase C and Ras. Mol Cell Biol 20, 70-80.

Lynch, K.W., and Weiss, A. (2001)A CD45 polymorphism associated with multiple sclerosis disrupts an exonic splicing silencer. J Biol Chem 276, 24341-24347.

Lynch, K.W. (2007) Regulation of alternative splicing by signal transduction pathways. Adv Exp Med Biol 623, 161-174.

Martinez-Contreras, R., Cloutier, P., Shkreta, L., Fisette, K.F., Revil, T., and Chabot, B. (2007) hnRNP proteins and splicing control. Adv Exp Med Biol 623, 123-147.

Martins de Araujo, M., Bonnal, S., Hastings, M.L., Krainer, A.R., and Valcarcel, J. (2009) Differential 3' splice site recognition of SMN1 and SMN2 transcripts by U2AF and U2 snRNP. RNA 15, 515-523.

Matlin, A.J., Clark, F., and Smith, C.W. (2005) Understanding alternative splicing: towards a cellular code. Nat Rev Mol Cell Biol 6, 386-398.

Mayeda, A., and Krainer, A.R. (1992) Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell 68, 365-375.

McCall, M.N., Shotton, D.M., and Barclay, A.N. (1992) Expression of soluble isoforms of rat CD45. Analysis by electron microscopy and use in epitope mapping of anti-CD45R monoclonal antibodies. Immunology 76, 310-317.

Melton, A.A., Jackson, J., Wang, J., and Lynch, K.W. (2007) Combinatorial control of signal-induced exon repression by hnRNP L and PSF. Mol Cell Biol 27, 6972-6984.

Michael, W.M., Choi, M., and Dreyfuss G. (1995) A nuclear export signal in hnRNP A1:

a signal-mediated, temperature-dependent nuclear protein export pathway. Cell 83, 415-422.

Mikula, M., Karczmarski, J., Dzwonek, A., Ruber, T., Henning, E., Dadlez, M., Bujnicki, J.M., Bomsztyk, and K., Ostrowski, J. (2006) Casein kinases phosphorylate multiple residues spanning the entire hnRNP K length. Biochim Biophys Acta 1764, 299-306.

Misteli, T., Caceres, J.F., Clement, J.Q., Krainer, A.R., Wilkinson, M.F., and Spector, D.L. (1998) Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo. J Cell Biol 143, 297-307.

Misteli, T., and Spector, D.L. (1999) RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. Mol Cell 3, 697-705.

Morris, D.P., and Greenleaf, A.L. (2000) The splicing factor, Prp40, binds the phosphorylated carboxyl-terminal domain of RNA polymerase II. J Biol Chem 275, 39935-39943.

Motta-Mena, L.B., Heyd, F., and Lynch K.W. (2010) Context-dependent regulatory mechanism of the splicing factor hnRNP L. Mol Cell 37, 223-234.

Motta-Mena, L.B., and Lynch, K.W. (2010) Alternative Splicing: Regulatory mechanisms and functional impact. Encyclopedia of Biological Chemistry, accepted.

Nasim, F.U., Hutchison, S., Cordeau, M., and Chabot B. (2002) High-affinity hnRNP A1 binding sites and duplex-forming inverted repeats have similar effects on 5' splice site selection in support of a common looping out and repression mechanism. RNA 8, 1078-1089.

Nilsen, T.W., and Graveley, B.R. (2010) Expansion of the eukaryotic proteome by alternative splicing. Nature 463, 457-463.

Oberdoerffer, S., Moita, L.F., Neems, D., Freitas, R.P., Hacohen, N., and Rao, A. (2008) Regulation of CD45 alternative splicing by heterogeneous ribonucleoprotein, hnRNPLL. Science 321, 686-691.

Ozdemirli, M., Mankin, H.J., Aisenberg, A.C., and Harris, N.L. (1996) Hodgkin's disease presenting as a solitary bone tumor. A report of four cases and review of the literature. Cancer 77, 79-88.

Pinol-Roma, S., and Dreyfuss, G. (1992) Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature 355, 730-732.

Pozzoli, U., and Sironi, M. (2005) Silencers regulate both constitutive and alternative splicing events in mammals. Cell Mol Life Sci 62, 1579-1604.

Ratei, R., Sperling, C., Karawajew, L., Schott, G., Schrappe, M., Harbott, J., Riehm, H., and Ludwig, W.D. (1998) Immunophenotype and clinical characteristics of CD45-negative and CD45-positive childhood acute lymphoblastic leukemia. Ann Hematol 77, 107-114.

Reed, R. (2000) Mechanisms of fidelity in pre-mRNA splicing. Curr Opin Cell Biol 12, 340-345.

Roberts, G.C., Gooding, C., Mak, H.Y., Proudfoot, N.J., and Smith, C.W. (1998) Co-transcriptional commitment to alternative splice site selection. Nucleic Acids Res 26, 5568-5572.

Rosonina, E., Ip, J.Y., Calarco, J.A., Bakowski, M.A., Emili, A., McCracken, S., Tucker, P., Ingles, C.J., and Blencowe, B.J. (2005) Role for PSF in mediating transcriptional activator-dependent stimulation of pre-mRNA processing in vivo. Mol Cell Biol 25, 6734-6746.

Rothrock, C., Cannon, B., Hahm, B., and Lynch, K.W. (2003) A conserved signal-responsive sequence mediates activation-induced alternative splicing of CD45. Mol Cell 12, 1317-1324.

Rothrock, C.R., House, A.E., and Lynch, K.W. (2005) HnRNP L represses exon splicing via a regulated exonic splicing silencer. EMBO J 24, 2792-2802.

Sauliere, J., Sureau, A., Expert-Bezancon, A., and Marie J. (2006) The polypyrimidine tract binding protein (PTB) represses splicing of exon 6B from the beta-tropomyosin pre-mRNA by directly interfering with the binding of the U2AF65 subunit. Mol Cell Biol 26, 8755-8769.

Saunders, A.E., and Johnson, P. (2010) Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. Cell Signal 22, 339-348.

Sharma, S., Falick, A.M., and Black, D.L. (2005) Polypyrimidine tract binding protein blocks the 5' splice site-dependent assembly of U2AF and the prespliceosomal E complex. Mol Cell 19, 485-496.

Sharma, S., Kohlstaedt, L.A., Damianov, A., Rio, D.C., and Black, D.L. (2008) Polypyrimidine tract binding protein controls the transition from exon definition to an intron defined spliceosome. Nat Struct Mol Biol 15, 183-191.

Shen, H., Kan, J.L., and Green M.R. (2004) Arginine-serine-rich domains bound at splicing enhancers contact the brachpoint to promote prespliceosome assembly. Mol Cell 13, 367-376.

Shepard, P.J., and Hertel, K.J. (2009) The SR protein family. Genome Biol 10, 242.

Singh, R., Valcarcel, J., and Green, M.R. (1995) Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. Science 268, 1173-1176.

Singh, R., and Valcarcel, J. (2005) Building specificity with nonspecific RNA-binding proteins. Nat Struct Mol Biol 12, 645-653.

Smith, C.W., and Valcarcel, J. (2000) Alternative pre-mRNA splicing: the logic of combinatorial control. Trends Biochem Sci 25, 381-388.

Spellman, R., and Smith, C.W. (2006) Novel modes of splicing repression by PTB. Trends Biochem Sci 31, 73-76.

Staley, J.P., and Guthrie, C. (1998) Mechanical devices of the spliceosome: motors, clocks, and things. Cell 92, 315-326.

Tackenberg, B., Nitschke, M., Willcox, N., Ziegler, A., Nessler, S., Schumm, F., Oertel, W.H., Hemmer, B., and Sommer, N. (2003). CD45 isoform expression in autoimmune myasthenia gravis. Autoimmunity 36, 117-121.

Tange, T.O., Damgaard, C.K., Guth, S., Valcarcel, J., and Kjems, J. (2001) The hnRNP A1 protein regulates HIV-1 tat splicing via a novel intron silencer element. EMBO J 20, 5748-5758.

Tchilian, E.Z., Wallace, D.L., Dawes, R., Imami, N., Burton, C., Gotch, F., and Beverley, P.C. (2001). A point mutation in CD45 may be associated with an increased risk of HIV-1 infection. AIDS 15, 1892-1894.

The Arabidopsis Genome Initiative. (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408, 796-815.

Thomas, M.L. (1989) The leukocyte common antigen family. Annu Rev Immunol 7, 339-369.

Tong, A., Nguyen, J., and Lynch, K.W. (2005) Differential expression of CD45 isoforms is controlled by the combined activity of basal and inducible splicing-regulatory elements in each of the variable exons. J Biol Chem 280, 38297-38304.

Topp, J.D., Jackson, J., Melton, A.A., and Lynch, K.W. (2008) A cell-based screen for splicing regulators identifies hnRNP LL as a distinct signal-induced repressor of CD45 variable exon 4. RNA 14, 2038-2049.

Trowbridge I.S. (1991) CD45. A prototype for transmembrane protein tyrosine phosphatases. J Biol Chem 266, 23517-23520.

Trowbridge, I.S., and Thomas, M.L. (1994) CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. Annu Rev Immnuol 12, 85-116.

Ule J, Jensen KB, Ruggiu M, Mele A, Ule A, and Darnell, R.B. (2003) CLIP identifies Nova-regulated RNA networks in the brain. Science 302,1212–1215.

Ule, J., Ule, A., Spencer, J., Williams, A., Hu, J.S., Cline, M., Wang, H., Clark, T., Fraser C., Ruggie., M, et al. (2005) Nova regulates brain-specific splicing to shape the synapse. Nat Genet 37, 844-852.

Ule, J., Stefani, G., Mele, A., Ruggiu, M., Wang, X., Taneri, B., Gaasterland, T.,

Blencowe, B.J., and Darnell, R.B. (2006) An RNA map predicting Nova-dependent splicing regulation. Nature 444, 580-586.

Underwood, J.G., Boutz, P.L., Dougherty, J.D., Stoilov, P., and Black, D.L. (2005) Homologues of the Caenorhabditis elegans Fox-1 Protein Are Neuronal Splicing Regulators in Mammals. Mol Cell Biol 25, 10005-10016.

Van der Houven van Oordt, W., Diaz-Meco, M.T., Lozano, J., Krainer, A.R., Moscat, J., and Caceres, J.F. (2000) The MKK(3/6)-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. J Cell Biol 149, 301-316.

Vassileva, M.T., and Matunis, M.J. (2004) SUMO modification of heterogeneous nuclear ribonucleoproteins. Mol Cell Biol 24, 3623-3632.

Venter, J. C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandel, M., Evans, C.A., Holt, R.A., Gocayne., J.D, et al. (2001) The sequence of the human genome. Science 291, 1304–1351.

Wahl, M.C., Will, C.L., and Luhrmann, R. (2009) The spliceosome: design principles of a dynamic RNP machine. Cell 136, 701-718.

Wang, Z., and Burge, C.B. (2008) Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. RNA 14, 802-813.

Warzecha, C.C., Sato, T.K., Nabet, B., Hogenesch, J.B., and Carstens, R.P. (2009) ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. Mol Cell 33, 591-601.

Will, C.L., and Luhrmann, R. (2006) Spliceosome structure and function. In the RNA world. Third Edition, R.F. Gesteland, T.R., and J.F. Atkins, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), 369-400.

Xiao, S.H., and Manley, J.L. (1997) Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. Genes Dev 11, 334-344.

Xie, J., Lee, J.A., Kress, T.L., Mowry, K.L., and Black, D.L. (2003) Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein.

Proc Natl Acad Scie U S A 100, 8776-8881.

Xu, A., and Weiss, A. (2002) Negative regulation of CD45 by differential homodimerization of the alternatively spliced isoforms. Nat Immunol 3, 764-771.

Xue, Y., Zhou, Y., Wu, T., Ji, X., Kwon, Y.S., Zhang, C., Yeo, G., Black, D.L., Sun, H., Fu, X.D., and Zhang, Y. (2009) Genome-wide analysis of PTB-RNA interactions reveals a strategy used by the general splicing repressor to modulate exon inclusion or skipping. Mol Cell 36, 996-1006.

Yeo, G.W., Coufal, N.G., Liang, T.Y., Peng, G.E., Fu, X.D., and Gage, F.H. (2009) An RNA code for the FOX2 splicing regulator revealed by mapping RNA-protein interactions in stem cells. Nat Struct Mol Biol 16, 130-137.

Yuryev, A., Patturajan, M., Litingtung, Y., Joshi, R.V., Gentile, C., Gebara, M., and Corden, J.L. The C-terminal domain of the largest subunit of RNA polymerase II interacts with a novel set of serine/arginine-rich proteins. Proc Natl Acad Sci U S A 93, 6975-6980.

Zhou, H.L., and Lou, H. (2008) Repression of prespliceosome complex formation at two distinct steps by Fox-1/Fox-2 proteins. Mol Cell Biol 28, 5507-5516.

Zhu, J., Mayeda, A., and Krainer, A.R. (2001) Exon identity established through differential antagonism between exonic splicing silencer-bound hnRNP A1 and enhancer-bound SR proteins. Mol Cell 8, 1351-136.