COMBINATORIAL REGULATION OF SIGNAL-INDUCED CD45 EXON REPRESSION BY HNRNPL AND PSF

APPROVED BY SUPERVISORY COMMITTEE

Kristen W. Lynch, Ph.D.

YuhMin Chook, Ph.D.

Melanie H. Cobb, Ph.D.

Rama Ranganathan, M.D./Ph.D

DEDICATION

In loving memory of my Grandpa, Joseph T. Keith

COMBINATORIAL REGULATION OF SIGNAL-INDUCED CD45 EXON REPRESSION BY HNRNPL AND PSF

by

ALEXIS ALLYSON MELTON

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

June, 2007

ACKNOWLEDGMENTS

First, I would like to thank my mentor, Dr. Kristen Lynch. She has been the driving force for this project and myself though the difficult times and was always a source of encouragement. Her enjoyment of science is contagious. I would also like to thank the members of my committee: Dr. YuhMin Chook, Dr. Melanie Cobb, and Dr. Rama Ranganathan. Their advice and guidance throughout the duration of this project was irreplaceable. I am also appreciative of the support of the Medical Scientist Training Program.

My undergraduate mentor, Dr. Arthur Gutierrez-Hartmann, has provided me with advice and support for many years. I could not have asked for more from my first lab experience as a high school student and am greatly appreciative of his continued involvement in my career. In addition, I would like to thank Dr. Rebecca Schweppe for her teaching and patience during my first years of scientific research.

I could not have made it through this process without the support of Hayden Schwenk, who spent countless hours listening to me vent about my failed experiments and telling me that “at some point Kristen has to let you graduate”.

My parents, Michael and Vicki Melton, have loved and supported me throughout my life and were critical during the hard times. My brother, Austin Melton, has always been there to keep me grounded. I appreciate all of the love and support from my grandma, Lois Keith, who showed a never ending interest in listening to me talk about my experiments. My late grandpa, Joseph Keith, saved me a special place in his heart as his granddaughter, and my late grandmother, Dale Perkins, shared her creative spirit.

COMBINATORIAL REGULATION OF SIGNAL-INDUCED CD45 EXON REPRESSION BY HNRNPL AND PSF

Alexis Allyson Melton, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

Supervising Professor: Kristen W. Lynch, Ph.D.

CD45 is a hematopoetic-specific tyrosine phoshatase. In resting T cells three variable exons are partially repressed, and following antigen challenge, these exons are more highly repressed. Previous work identified the ESS1 silencer element that functions to mediate exon 4 silencing under resting conditions by binding to hnRNP L. ESS1 is also sufficient to confer the activation-induced increase in exon repression, and this document describes two mechanisms responsible for mediating this effect. First, hnRNP L silencing function is slightly increased in activated cells as compared to resting cells. Additionally, PSF binds to the ESS1 complex in a signal-dependent manner and provides a significant increase in repressive activity. Further investigation shows these two mechanisms are largely

independent but show some functional crosstalk, and while neither of these mechanisms is sufficient in isolation, the combination of these two effects accounts for an increase in exon silencing that is of similar magnitude to the total observed change in splicing in response to cellular activation.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Melton, A.A., J. Jackson, J. Wang, and K.W. Lynch. Combinatorial Control of Signal-Induced Exon Repression by hnRNP L and PSF. *Manuscript in Revision.*

Schweppe, R.E., **A.A. Melton**, K.S. Brodsky, L.D. Aveline, K.A. Resing, N.G. Ahn, and A. Gutierrez-Hartmann (2003). Purification and Mass Spectrometric Identification of GA-Binding Protein (GABP) as the Functional Ets Factor Binding to the Basal Transcription Element of the Prolactin Promoter. *JBC*. 278(19):16863-72

ABSTRACTS

Melton, A.A., and K.W. Lynch. Investigation of Signal-Induced Changes Controlling CD45 Exon 4 Repression (poster). CSH Eukaryotic mRNA Processing Meeting, 2005.

Melton, A.A., and K.W. Lynch. Signal-Induced Changes in Protein Composition of an ESS-Bound Complex Results in Increased Exon Repression (oral). RNA Society Meeting, 2006.

Melton, A.A., and K.W. Lynch. PSF Is the Critical Regulator of Signal-Induced CD45 Exon Silencing and Shows Changes in Associated Proteins Following Stimulation (oral). RNA Society Meeting, 2007.

LIST OF FIGURES

LIST OF APPENDICES

LIST OF DEFINITIONS

α – anti

A – Adenosine

ARS – Activation Responsive Sequence

C – Cytiditine

DTT – Dithiotreitol

EDTA – ethylenediaminetetraacetic acid

ERK – Extracellular Signal Regulated Kinase

ESE – Exonic Splicing Enhancer

ESS – Exonic Splicing Silencer

FBS – Fetal Bovine Serum

FR – Fold Repression

G – Guanosine

GST – Glutathione-S-Transferase

HIV – Human Immunodeficiency Virus

hnRNP – Heterogeneous Nuclear Ribonuclear Protein

ISE – Intronic Splicing Enhancer

ISS – Intronic Splicing Silencer

JSL1 – Jurkat Splicing Line 1

kDa – kilo Dalton

KH – K Homology RNA Binding Domain

M – Molar

MAPK – Mitogen Activated Protein Kinase

MEKK1 – MEK Kinase Kinase 1

mRNA – Messenger RNA

NE – Nuclear Extract

NFκB – Nuclear Factor kappa B

nt – Nucleotides

PAGE – Polyacrylamide Gel Electrophorsis

PCR – Polymerase Chain Reaction

PKA – cAMP Dependent Protein Kinase

PKC – Protein Kinase C

PMA – Phorbol 12-myristate 13-acetate

PSF – PTB Associated Splicing Factor

PTB – Polypyrimidine Track Binding Protein

RNA – Ribonucleic Acid

RPMI – Roswell Park Memorial Institute Cell Culture Media

RRM – RNA Recognition Motif

RRMH – RNA Recognition Motif Homology

RS – Arginine-Serine Rich Domain

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SDS – Sodium Dodecyl Sulfate

SELEX – Systematic Evolution of Ligands by Exponential Enrichment

SFK – Src Family Protein Tyrosine Kinase

snRNP – Small Nuclear Ribonucleoprotein

ss – splice site

SUMO – Small Ubiquitin-like Protein

TCR – T Cell Receptor

Tra2 – Transformer 2 Protein

Tri-snRNP – U4/U5/U6 Small Nuclear Ribonucleoproteins

U – Uradine

U2AF – U2 snRNP Auxiliary Factor

CHAPTER ONE

Introduction and Review of Literature

Following the sequencing of the human genome, the surprisingly small number of estimated human genes has increased the emphasis on alternative mechanisms to enhance protein diversity. Some of these alternative mechanisms include: alternative transcription and translation start sites, alternative 3'-end processing, alternative pre-mRNA splicing, and RNA editing (Hui and Bindereif, 2005). Alternative pre-mRNA splicing appears to be the most wide spread of these diversity mechanisms. Recent computational analysis has estimated that 35-95% of all human genes show at least one alternative splice variant (Modrek and Lee, 2002), and exon-exon junction microarray analysis performed on multiple cell lines indicates that at least 74% of human genes are subject to alternative splicing regulation (Johnson et al., 2003).

The CD45 gene has been known to be subject to alternative splicing regulation for many years (Birkeland et al., 1989; Hermiston et al., 2003). The gene contains three variable cassette exons that are subject to exon skipping in resting T cells as well as signal-induced increases in exon skipping following antigen challenge of the T cell (Lynch and Weiss, 2000). Investigation of CD45 splicing mechanisms have identified required splicing regulatory elements as well as splicing regulatory proteins that bind to these sequences and function to repress the splicing of these exons under resting conditions (Rothrock et al., 2003; Rothrock et al., 2005; Tong et al., 2005); however, previous work has not elucidated the mechanisms controlling the signal-induced repression of CD45 variable exons. In this

document I present data demonstrating post-translational modifications to the regulator of basal exon silencing, hnRNP L, correlate with an increase in functional silencing activity of the protein. In addition, the splicing regulatory protein PSF binds specifically to the silencing complex under activated conditions and causes an increase in exon skipping. Importantly, while neither of these mechanisms alone is sufficient to confer the total activation-induced silencing, both mechanisms in combination produce and effect equal to the total increase activity following stimulation. This chapter, however, highlights important previous discoveries and provides the necessary background information for a more complete understanding of the splicing regulation described in future chapters.

Alternative Splicing Patterns

There are five major types of alternative splicing; however, the use of alternative promoters and alternative poly(A) sites could be classified separately and affect either the first or last exon of a transcript respectively. The five major classes of alternative splicing events include: the use of alternative 5' or 3' splice sites, cassette exon skipping, mutually exclusive cassette exons, and intron retention (Black, 2003; Hui and Bindereif, 2005; Figure 1-1). Of the total number of alternative splicing events within the cell, 70-90% of these events have been estimated to alter the coding sequence of the resulting protein (Johnson et al., 2003; Modrek and Lee, 2002). Changes in protein coding can cause changes in localization, protein-protein interactions, ligand binding, enzymatic activity, allosteric regulation, post-translational modifications, and others (Black, 2003; Hui and Bindereif,

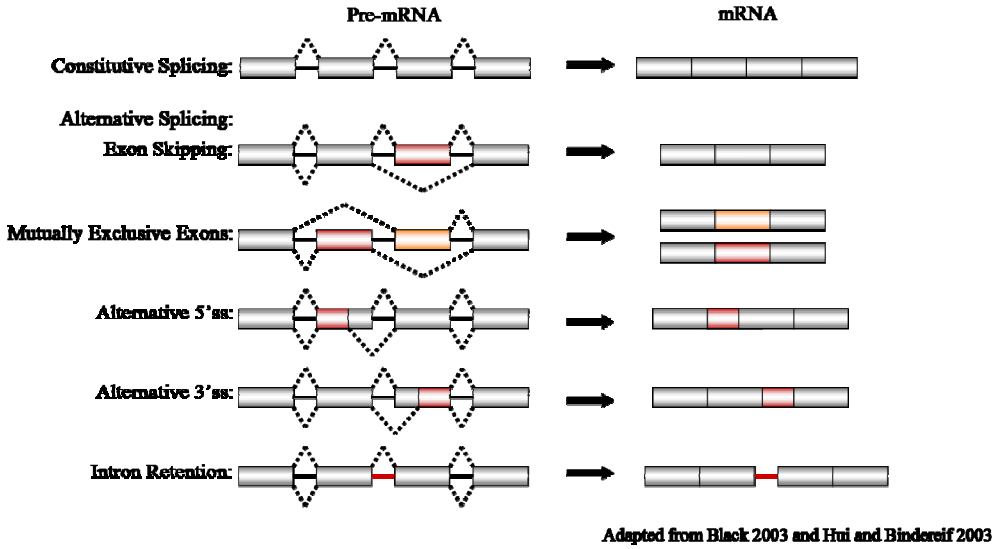

Figure 1-1: Schematic Representation of Alternative Splicing Patterns. Constitutive splicing generates a single mRNA. Alternative splicing of the pre-mRNA generates multiple mRNA transcripts from a single pre-mRNA sequence. Five patterns of alternative splicing are shown above with the resulting change in the mRNA from that generated from constitutive splicing shown at the right. Exons are represented by rectangles and introns by lines. Dashed lines indicate the region to be removed following completion of the splicing reaction.

2005; Johnson et al., 2003), and alterations in splicing can even regulate whether a protein is expressed (Black 2003).

Alterative splicing patterns of different genes are known to be regulated in many ways and affect a myriad of processes. Several splicing patterns are often observed for a single gene within the same cell, and the ratio of these isoforms can be regulated by cell type, tissue type, developmental stage, sex, cellular activation, apoptosis, and many others (Black 2003). Changes in cellular function in response to environmental stimuli and signal transduction within the cell are known to affect the splicing pattern of many genes and provide one mechanism by which cellular activity is altered in response to environmental changes (Tarn, 2007). Signal-regulated alterations in splicing are especially relevant to the

studies described in future chapters and will be discussed in more detail later in this chapter; however, prior to discussing alternative splicing and its regulation, the general aspects of the spliceosome, splicing mechanism, and splicing regulation must be presented.

The Spliceosome and the Splicing Reaction

The process of intron removal and exon ligation takes place in two trransesterification reactions catalyzed by the spliceosome. This process requires several sequences present at the exon/intron boundaries as well as sequences within the introns. These sequences contain several well conserved residues embedded within the context of more variant surrounding sequences. The 5' splice site (5'ss) marks the exon/intron boundary at the 5' end of the

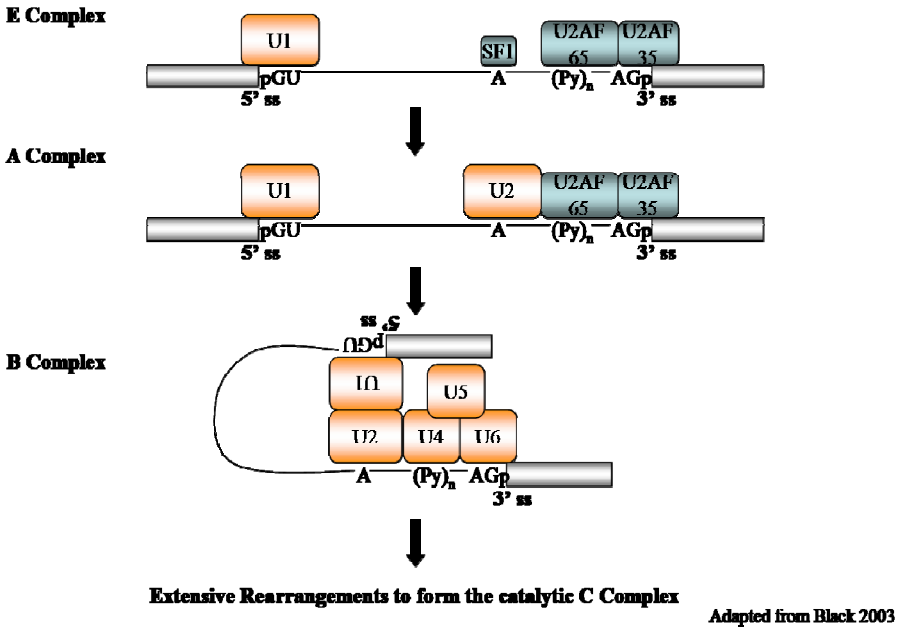

Figure 1-2: Schematic Representation of General Spliceosome Assembly, snRNPs, and Conserved Splice Site Sequences. The conserved 5' and 3' splice site (ss) sequences are indicated with Py indicating a pyrimidine. The five snRNPs are shown bound to the appropriate sequences at the indicated step of spliceosome assembly. E, A, and B assembly complexes are indicated with C complex being formed from the rearrangement of B complex.

intron and contains a conserved GU dinucleotide (Black 2003; Figure 1-2). Similarly, the 3' splice site (3'ss) marks the exon/intron boundary at the 3' end of the intron; however, this region of the intron contains three conserved sequence elements. The terminal 3' region of the intron contains an almost invariant AG dinucleotide at the exon/intron boundary (Black 2003; Figure 1-2), and upstream of this sequence is the branch point sequence which contains an invariant adensosine, referred to as the branch point. The region between the branch point and the 3'ss is a pyrimidine rich region called the polypyrimidine track (Black 2003; Figure 1-2).

The spliceosome is made up of five individual small nuclear ribonucleoprotiens (snRNPs) and multiple accessory proteins that catalyze intron removal and exon ligation (Black 2003). In addition to various protein components, each of the snRNP complexes contains an snRNA (U1, U2, U4, U5, U6), and these snRNPs are assembled onto the pre-mRNA in stepwise fashion, yielding a catalytically active splicing complex. In the initial steps of spliceosomal assembly, the U1 snRNA base pairs to the 5'ss within the transcript and the accessory proteins SF1, U2AF65, and U2AF35 bind to the 3'ss (Das et al., 2000). This complex is considered the first step in spliceosome assembly and is referred to as E complex (Das et al., 2000). The progression from E to A complex requires the binding of the U2 snRNP to the branch point sequence and is the first ATP-dependent step in the pathway (Black 2003; Figure 1-2). Following the addition of tri-snRNP (U4, U5, U6), U2AF65 and U2AF35 are released leading to the formation of B complex (Black 2003; Figure 1-2). At this point in assembly, extensive RNA and protein rearrangements take place within the macromolecular complex, including the dissociation of the U4 snRNA, to form the catalytic

spliceosome (C complex) (Black 2003; Figure 1-2). The functional spliceosome then catalyzes the nucelophilic attack of the branch point A on the 5'ss, leading to the formation of the upstream exon and lariat-exon intermediate (Figure 1-3). The second transesterification reaction takes place when the released upstream exon attacks the phosphate at the 3' end of the intron, allowing for exon ligation and the release of the lariat structure (Black 2003; Figure 1-3).

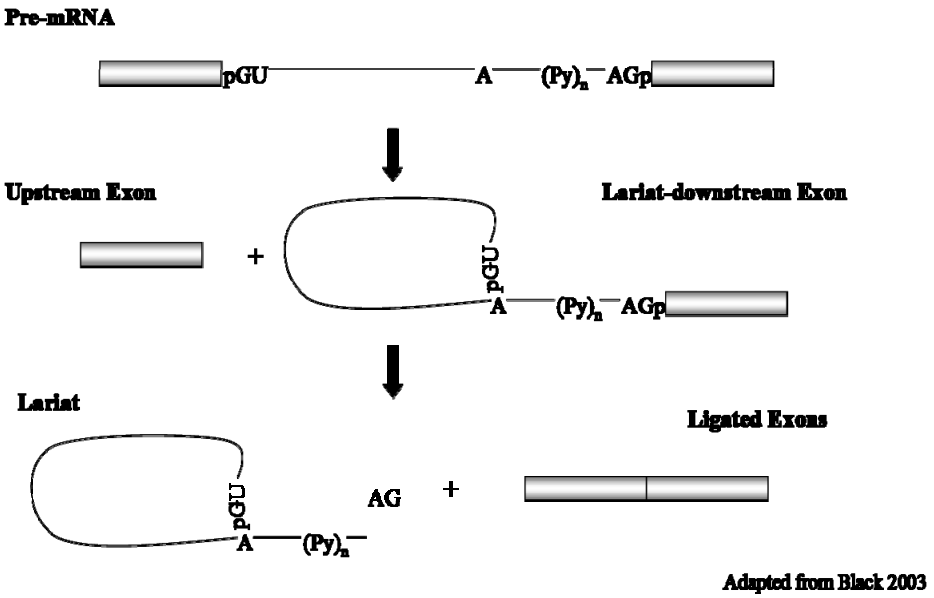

Figure 1-3: Schematic Representation of the Two Transesterification Reactions Resulting in Exon-Exon Ligation. The unspliced pre-mRNA is indicated at the top. Following the first transesterification reaction, the upstream exon is released and a lariat-exon structure is formed. The second transesterification reaction ligates the upstream and downstream exons and releases the lariat structure.

While the splice site sequences are required for splicing to occur these sequences are not sufficient to allow for proper spliceosome assembly and function, especially since introns within mammalian cells are often thousands of nucleotides in length with exons averaging only several hundred nucleotides (Black 2003). Thus, snRNP components have been shown to interact first across an exon (Hoffman and Grabowski, 1992). The interaction of splicing

components across exons is called exon definition and appears to be required for the splicing of most mammalian exons (Robberson et al., 1990). Defining the exon boundaries prior to splicing promotes the proper pairing of the 5' and 3'ss by the spliceosome across the introns, leading to intron removal and exon ligation. Exon definition is affected by additional cis-acting sequences within the RNA that either promote or repress the use of a particular splice site or exon (Pozzoli and Sironi, 2005). These sequences are referred to as either enhancer sequences or silencer sequences and can be present either within the intron or the exon. Further description of these elements is the subject of the next section.

Cis-Acting Sequences and Trans-Acting Factors in Splicing Regulation

As mentioned above, the canonical splice site sequences described in the previous section do not provide sufficient information for the accurate removal of introns (Senapathy et al., 1990), and recent estimates suggest that these sequences provide less than 50% of the required information (Lim and Burge, 2001). Therefore, additional regulatory sequences are of critical importance in both constitutive and alternative splicing events. These sequences are classified based on two criteria. First, sequences are identified by the activity of the sequence with enhancer sequences promoting the inclusion of an exon or use of a splice site and silencer sequences inhibiting the use of an exon or splice site (Black 2003). Second, these sequences are categorized based on their location in either the intron or the exon, leading to descriptions of regulatory sequences as either intronic splicing enhancers (ISE), exon splicing enhancers (ESE), intronic splicing silencers (ISS), or exonic splicing silencers (ESS) (Black 2003).

Splicing regulatory elements have been identified using several methods. Initial identification and characterization of splicing regulatory sequences utilized minigene constructs in which deletion, insertion, and mutational analysis was performed. While this method is still widely used, additional more generalized methods have been introduced. Various ESE elements have been identified using *in vitro* or *in vivo* selection protocols followed by SELEX (systematic evolution of ligands by exponential enrichment) analysis (Coulter et al., 1997; Liu et al., 2000; Liu et al., 1998; Schaal and Maniatis, 1999). SELEX analysis has also been used to identify potential ESE sequences within specific genes (Woerfel and Bindereif, 2001), and several classes of hexameric enhancer sequences have been identified using a bioinformatics approach (Fairbrother and Chasin, 2000). In addition to work performed to identify enhancer sequences, several systematic approaches have focused on the identification of splicing silencing sequences. Recent studies have used bioinformatics approaches in which the frequency of short sequences in true exons were compared to pseudoexons or noncoding exons (Sironi et al., 2004; Zhang and Chasin, 2004), and another study utilized a screening approach in which a 10-mer library was screened *in vivo* using a splicing reporter and several confirmed splicing silencing sequences were identified (Wang et al., 2004). Thus, previous and continuing research efforts have allowed for the systematic identification and evaluation of a growing number of splicing regulatory sequences.

With the identification of cis-acting sequences, the characteriztion of trans-acting regulators that bind to these sequences is also an active area of research. The known splicing regulatory factors can be divided into three major groups: the SR protein family which most

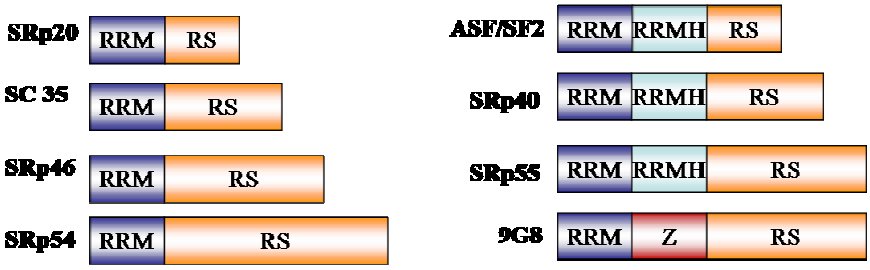

Figure 1-4: Domain Structure of Various SR Proteins. Several members of the SR protein family are represented. The RNA recognition motif (RRM) and serine/arginine rich (RS) domains help define the family. Several SR proteins also contain RRM homology domains (RRMH), and 9G8 contains a zinc knuckle domian.

often bind to splicing enhancer sequences; the hnRNP protein family which most often bind to splicing silencer sequences; and those proteins which have been shown to have splicing regulatory function but are not members of either the SR or hnRNP protein families (Black 2003).

First, the SR protein family has been the most thoroughly studied of the splicing regulatory proteins (Caceres et al., 1997; Graveley, 2000; Manley and Tacke, 1996). The members of this family all share a common domain structure. Each of the proteins contains at least one RNA recognition motif (RRM) for RNA binding and a second domain rich in arginine/serine dipeptides (RS domain). Schematic representation of the domain structure of several SR proteins is shown in Figure 1-4 (Black 2003). Research has demonstrated that at least one member of the SR protein family must be present to allow productive splicing in cellular extracts (Black 2003). This general role for SR proteins in splicing demonstrates functional redundancy *in vitro,* with similar results being obtained following the addition of different SR protein family members; however, SR protein identity appears to be a more important factor *in vivo* (Black 2003).

Aside from their general role in promoting splicing, SR proteins play a significant role in splicing regulation by binding to enhancer (or less often silencer) sequences and functioning to regulate alternative splicing events. Previous research suggests that most if not all exons contain ESE sequences that bind to specific SR proteins, and SR proteins may be part of the general mechanism of exon definition (Black, 2003; Cartegni et al., 2003). The most frequently described enhancer sequences are known as the purine rich GAR enhancers. These enhancers are most commonly bound by the SR proteins ASF/SF2 and Tra2 (Liu et al., 1998; Tacke and Manley, 1999). However, it is important to note that RNA regulatory sequences can demonstrate significant deviation from the "ideal" consensus sequence (Black 2003), and the splicing regulatory proteins which bind to these elements show increased promiscuity as well as functional redundancy to accommodate for increases in sequence variation.

The mechanism by which SR proteins, and specifically the RS domains of these proteins, function to activate the splicing machinery has been extensively studied; although many questions remain. It has been proposed that the RS domain may function as a counter ion to the negatively charged phosphate backbone of the RNA since the unmodified RS domain is highly positively charged; however, studies have clearly shown that phosphorylation of the RS domain is required for splicing function (Black 2003). Given this information, a more likely mechanism involves the RS domain functioning in protein-protein interactions within the cell, forming a large protein complex which enhances spliceosome assembly and splicing of the bound exon. Data supporting this theory comes from several yeast experiments where phosphorylated RS domains were shown to interact with other SR

proteins (Wu and Maniatis, 1993; Yeakley et al., 1999), and protein-protein interactions between U1 70K, a non-SR protein containing an RS domain, and phosphorylated ASF/SF2 have also been shown (Kohtz et al., 1994). *In vivo*, these large SR protein complexes show specificity in their protein-protein interactions; although, the identity of many of the SR protein complex members remains unknown (Black 2003). Many of these complexes are known to require the binding of additional RS domain containing proteins such as U2AF35, SRm160, and SRm300 (Blencowe et al., 1998; Eldridge et al., 1999; Li and Blencowe, 1999; Zuo and Maniatis, 1996); however, the specific protein contacts in these complexes is still an area of investigation.

An additional level of SR protein regulation occurs at the level of RS domain phosphorylation. SR proteins have been shown to be substrates for SR protein kinase (SRPK) 1 and 2 as well as the Clk/Sty family of kinases. SR protein localization, protein interactions, and *in vitro* splicing activity have all been shown to be altered in response to the activity of these kinases (Black 2003). Additionally, alterations in the phosphorylation state of SR proteins correlate with changes in SR protein activity (Sacco-Bubulya and Spector, 2002; Sanford and Bruzik, 2001), and *in vitro* enhancer activity is reduced by both hyper- and hypophosphorylation of the RS domain (Kanopka et al., 1998; Prasad et al., 1999; Sanford and Bruzik, 1999). Therefore, a more detailed understanding of the large SR protein complexes as well as SR protein phosphoryaltion will be required to further elucidate the mechanisms of action.

As a general rule, while SR proteins bind to enhancer sequences and function to activate splicing, hnRNP proteins bind to silencer sequences and function to repress splicing.

The hnRNP family is a diverse family of proteins defined by their association with unspliced RNA and function at multiple steps in RNA metabolism (Dreyfuss et al., 1993). Thus, the hnRNP family is composed of many diverse members with unique domains and structures. While most members are predominantly nuclear, several hnRNP proteins shuttle between the nucleus and cytoplasm (Nakielny and Dreyfuss, 1999). The domain structures of several members of the hnRNP family are outlined in Figure 1-5.

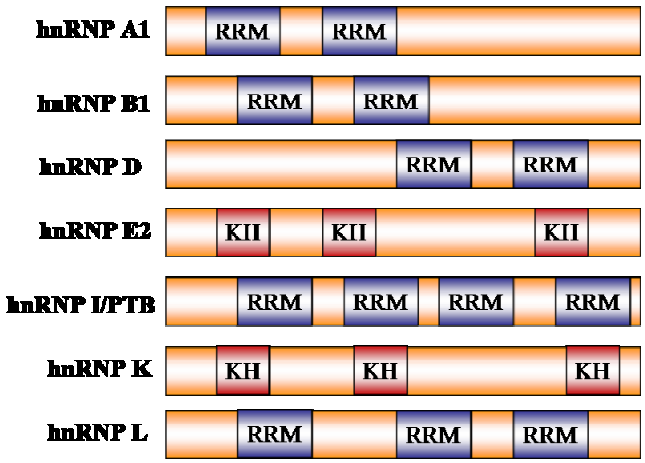

Figure 1-5: Domain Structure of Several Members of the hnRNP Family. The hnRNP family contains a large number of proteins with diverse structures. These proteins contain RNA recognition motifs or KH domains for RNA binding domains as well as a diversity of other domain structures in individual members.

Some of the most abundant and well studied members of the hnRNP family are the A/B type. The A/B type hnRNPs consist of multiple isoforms derived from a combination of multiple genes and alternative splicing (Black, 2003; Pozzoli and Sironi, 2005). The best characterized of the hnRNP A/B class is hnRNP A1 which contains two RRM type RBDs at its amino-terminus and a glycine rich domain at the carboxy-terminus (Black 2003; Figure 1-5). The "ideal" hnRNP A1 binding site was experimentally determined to be UAGGA/U

using SELEX analysis (Burd and Dreyfuss, 1994); however, consistent with the properties of many RNA binding proteins, hnRNP A1 has also been identified bound to RNA sequences divergent from the "ideal" sequence.

The first reports of hnRNP A1 splicing regulation came from its antagonism of SR proteins in *in vitro* splicing assays. In these assays, competing 5'ss showed an hnRNP A1-dependent shift in the splicing pattern (Mayeda et al., 1994). Since its first identified roles in splicing regulation, hnRNP A1 has been shown to bind to and regulate several alternative

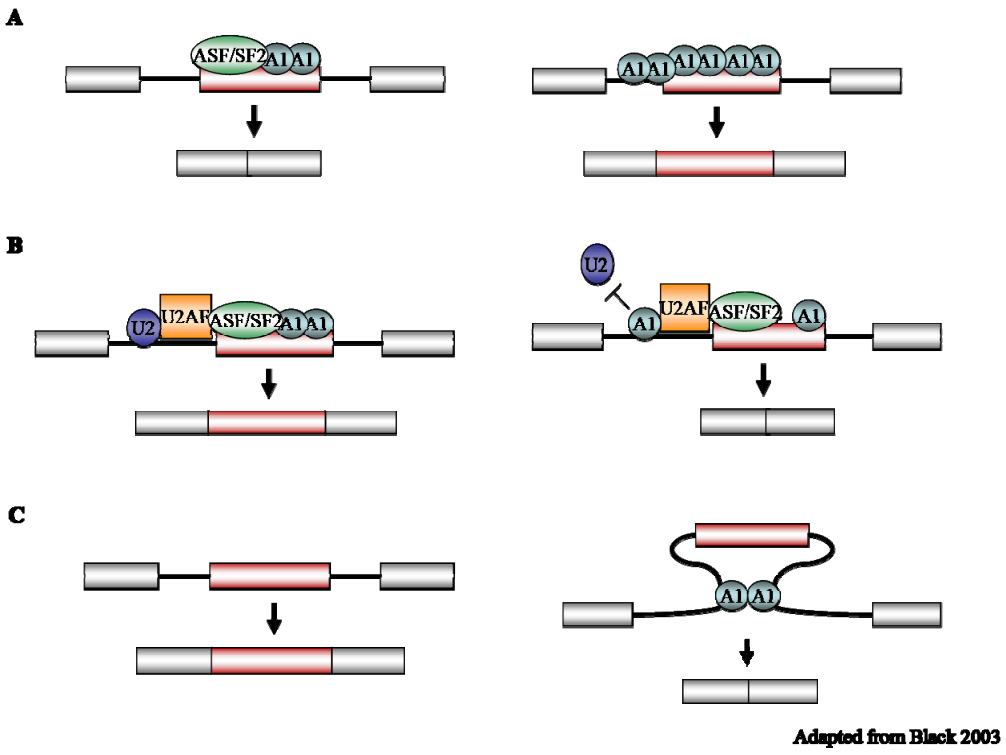

Figure 1-6: Proposed Mechanisms of hnRNP A1 Exon Silencing. (A) HnRNP A1 oligomeriztion model in which hnRNP A1 coats the RNA and inhibits splicing. This function is inhibited by ASF/SF2. (B) Blocked U2 binding to the branch point sequence by hnRNP A1 inhibits exon inclusion. (C) Association of hnRNP A1 molecules in upstream and downstream introns and looping out of the variable exon, causing exon skipping

splicing events, including IDX exon repression in the c-H-ras gene, exon 5 skipping of the CD44 gene, the K-SAM exon in the FGF-2 receptor, exon 7B skipping of its own transcript, and exon 3 of the HIV tat gene (Black, 2003; Pozzoli and Sironi, 2005).

While the mechanism of splicing inhibition for hnRNP A1 remains an issue of debate, several models have been proposed based upon the known activities of hnRNP A1. Given hnRNP A1 will interact with itself and that it binds DNA elements in a tandem fashion, hnRNP A1 could coat the RNA producing a region of the transcript that is inaccessible to the spliceosome (Black 2003; Figure 1-6A). This theory is supported by data from the Krainer lab in which hnRNP A1 was crosslinked to RNA regions outside of the known hnRNP A1 binding element in HIV tat exon 3, and importantly, this observation was dependent upon the presence of the ESS sequence for nucleation of hnRNP A1 oligomeriztion (Zhu et al., 2001). Additional observations have shown that some splicing regulation is dependent upon the relative ratios of ASF/SF2 to hnRNP A1, and tissue specific differences in splicing pattern of these genes correlate with changes in this ratio (Caceres et al., 1994; Hanamura et al., 1998). In the case of HIV tat exon 3, both SC 35 as well as ASF/SF 2 are known to bind to sequence elements within exon 3 and activate splicing; however, in the presence of hnRNP A1, only ASF/SF2 is able to activate splicing. Therefore, in the model of hnRNP A1 oligomerization, ASF/SF2 is able to inhibit the formation of the higher order hnRNP A1 complex while SC35 is not (Zhu et al., 2001; Figure 1-6A).

An additional model exists regarding hnRNP A1 function in the regulation of HIV tat exon 3. HnRNP A1 also binds to an intronic silencer sequence downstream of the branch point sequence of HIV tat exon 3. Binding of hnRNP A1 to this site allows the binding of

U2AF65 and U2AF35 to the 3'ss but blocks the recruitment of the U2 snRNA, inhibiting spliceosome assembly and causing repression of the exon (Tange et al., 2001; Figure 1-6B).

A third possible mechanism for splicing regulation by hnRNP A1 comes from the autoregulation of its own transcript. In this case, exon 7B is skipped in hnRNP A1 and included in hnRNP A1b. The skipping of this exon was shown to be dependent upon intronic regions flanking the variable exon as well as hnRNP A1 protein. Given that both introic sites are necessary for regulation and hnRNP A1 is known to dimerize, the proposed model suggests molecules of hnRNP A1 bound to each of the upstream and downstream ISS regions associate with one another, resulting in looping of the intervening region of RNA and the skipping of exon 7B (Blanchette and Chabot, 1999; Figure 1-6C).

Polypyrimidine tract binding protein (PTB) (also known as hnRNP I) is another well studied member of the hnRNP family. This member of the hnRNP family contains four RRM-type RNA binding domains with an optimal binding site indentifed as the tetranucleotide sequence UCUU embedded in a pyrimidine rich region of RNA (Perez et al., 1997; Valcarcel and Gebauer, 1997). PTB has been shown to bind to the polypyrimidine tracks of many regulated and weak exons, leading to the suggestion that PTB functions as general repressor of these types of exons as well as pseudoexons (Wagner and Garcia-Blanco, 2001). The location of PTB binding suggests a possible model where PTB competes for U2AF binding (Lin and Patton, 1995); however, in many PTB-regulated transcripts additional PTB-binding elements are necessary for appropriate regulation (Wagner and Garcia-Blanco, 2001). In cases where multiple PTB-binding elements are required, it has been suggested that PTB is part of a higher order complex which bridges the multiple

regulatory sites, and the complex functions to inhibit splicing (Wagner and Garcia-Blanco, 2001). Thus, the hnRNP proteins share important functional similarities with the SR protein family in that the mechanisms of regulation are not well understood but often require the formation of large protein complexes that ultimately influence the assembly of the spliceosome.

While the majority of identified splicing regulatory proteins belong to either the SR or hnRNP family, the splicing regulation of several genes has been shown to be dependent upon proteins which do not belong to either of these protein families. For example, variable exon 4 of CD44 contains an AC-rich enhancer sequence whose function is dependent upon the YB-1 and p72 proteins (Honig et al., 2002; Stickeler et al., 2001). Thus, members of the SR and hnRNP family are not the only proteins that function in alternative splicing regulation, and it is likely that in the future many other types of proteins will be found to serve a functional role in the regulation of alternative splicing.

Combinatorial Regulation of Alternative Splicing

One important point regarding the regulation of alternative splicing events in higher eukaryotes is that alternative splicing events are regulated through a combination of various positive and negative sequence elements as well as multiple positive and negative protein regulators. Given that the majority of SR and hnRNP proteins are expressed in most cells and tissues, it follows that most alternative splicing regulation is a balance between positive and negative influences (Black 2003).

One example of combinatorial regulation of alternative splicing comes from the tissue specific splicing pattern of exon N1 in the c-src gene. The 18 nucleotide N1 exon is specifically included in neurons and excluded in all other cells (Black 2003). The N1 exon contains multiple intronic enhancers and silencers both upstream and downstream of the N1 exon as well as an exonic enhancer within the N1 exon itself (Black, 2003; Modafferi and Black, 1997; Modafferi and Black, 1999; Rooke et al., 2003). Insertion of several of the known N1 regulatory sequences is required to confer regulation on a heterologous exon (Modafferi and Black, 1997). Several of the intronic silencer elements are known to bind hnRNP H and PTB, functioning to repress exon inclusion, while the ESE binds ASF/SF2 and the ISE-binding factors have not been identifed (Modafferi and Black, 1997; Rooke et al., 2003). While the specific details of the combined regulation remain interesting questions within the field, there is no doubt that mammalian splicing regulation is governed by the balance of multiple elements and factors with both stimulatory and inhibitory effects.

Signal-Regulated Alternative Splicing

While cell type and tissue specific alternative splicing is one category of alternative splicing, alternative splicing in response to various stimuli has become appreciated as an important mechanism regulating cellular gene expression. Many extracellular signals have been found to induce changes in the splicing pattern of cellular genes. These stimuli include but are not limited to: cell division, apoptosis, neuronal depolarization, environmental stress, and pathogen invasion (Ip et al., 2007; Tarn, 2007). While signal-induced splicing regulation

remains in the early stages of investigation, several systems have been studied and provide the first models of splicing regulation by cellular signaling pathways.

Many of the current descriptions of signal-induced alternative splicing involve the post-translational modification of members of the SR or hnRNP family. As described above, the members of the SR protein family are subject to phosphorylation by both the SRPK family and the Clk/Sty family of protein kinases as well as by Akt and Topoisomerase I (Black, 2003; Tarn, 2007), and changes in the phosphorylation state of SR proteins have been shown to affect their function (Black 2003). While the upstream pathways that activate many of these kinases have yet to be identified, SRPK1 has been shown to localize to the cytoplasm during the majority of the cell cycle and relocate to the nucleus during mitosis, resulting in SR protein aggregation (Ding et al., 2006). While the consequences of the aggregation on splicing regulation remain unknown, it has been hypothesized that SRPK1 phosphorylates the SR proteins to induce aggregation and inhibit splicing during the period of the cell cycle where little pre-mRNA is being transcribed (Ding et al., 2006). Additional splicing regulation during the cell cycle is mediated by the dephosphorylaiton of the highly phosphorylated RS domain of SRp38, causing a potent inhibition of general splicing during mitosis (Shin and Manley, 2004).

Another example of the regulation of splicing through the phosphoylation of an SR protein comes from the insulin regulation of PKCβ splicing. Following insulin stimulation, an intronic enhancer sequence of PKCβ binds to SRp40 upstream of the βII exon and promotes the inclusion of this exon (Chalfant et al., 1995; Patel et al., 2001; Patel et al., 2005). The insulin-induced inclusion of the βII exon is known to require the activation of

PI3K, ultimately leading to the activation of Akt and the phosphorylation of Ser86 of SRp40 (Patel et al., 2005).

The splicing of the EDA exon of fibronectin is also influenced by SR protein phosphorylation. The splicing pattern of the EDA exon was shown to be affected by growth factor stimulation, and these changes in splicing pattern correlate with alterations in the phosphorylation state of the SR proteins 9G8 and ASF/SF2 (Blaustein et al., 2005). Following either the depletion of Akt or the overexpression of either the SRPK or Clk/Sty family of kinases, the changes in 9G8 and ASF/SF2 phosphorylation were blocked (Blaustein et al., 2005), again suggesting that specific phosphorylation events and signaling pathways affect the splicing regulatory function of these RNA binding proteins.

SR proteins are not the only splicing regulatory proteins subject to modification by phosphorylation. Many of the hnRNP proteins have been identified as cellular phosphoproteins and several cellular kinases have been shown to act on individual hnRNPs (Guil et al., 2006; Magistrelli et al., 1999; Mayrand et al., 1993; Xie et al., 2003). One of the most well characterized examples of signal-induced hnRNP regulation is the p38-dependent phosphorylation of hnRNP A1 in response to cellular stress. Caceres and others have shown that in response to activation of the stress kinase p38, hnRNP A1 is targeted for phosphorylation by the Mnk1/Mnk2 cellular kinases (Allemand et al., 2005; Guil et al., 2006). The phosphorylation of hnRNP A1 inhibits the normal nuclear-cytomplamic shuttling of the protein and causes cytoplasmic accumulation of hnRNP A1 (Allemand et al., 2005; Guil et al., 2006). As a result of sequestering hnRNP A1 in the cytoplasm, nuclear hnRNP A1 levels decrease, and the decreased nuclear hnRNP A1 levels correlate with alterations in

the splicing pattern of the adenoviral E1A splicing reporter (van der Houven van Oordt et al., 2000). In parallel to the regulation of hnRNP A1 localization, PTB localization within the cell is also dependent upon phosphorylation at serine 16 (Xie et al., 2003). In response to PKA activation, serine 16 of PTB becomes phosphorylated and causes the accumulation of PTB in the cytoplasm (Black, 2003; Xie et al., 2003).

The signal-induced changes in splicing pattern of CD44 exon 5 provide an additional example of splicing regulation by signal transduction pathways. Antigen challenge of a T cell induces the inclusion of variable exon 5 in the CD44 gene (Konig et al., 1998), and the inclusion of this exon is known to be regulated by activation of the Ras-Raf-MEK-Erk pathway (Konig et al., 1998; Weg-Remers et al., 2001). At the RNA level, an ESE within the exon binds to Sam68 (a STAR family protein) in combination with SRm160 (Cheng and Sharp, 2006; Matter et al., 2002). Erk has been shown to phosphorylate Sam68 directly; however, mutation of this site has only small effects on the CD44 splicing pattern (Matter et al., 2002). Additional links therefore must exist between the Ras-Raf-MEK-Erk pathway and the RNA binding complex, and additional work has demonstrated that Swi/SNF chromatin remodeling complex serves as an additional component of regulation within this system (Batsche et al., 2006). Thus, while there are still remaining questions regarding the regulation of CD44 exon 5, the signaling pathway and post-translational modification of RNA binding factors is critical to the proper splicing of the CD44 gene in activated T cells.

While the previous examples have focused on phosphorylation events, additional post-translational modifications have been identified on splicing regulatory proteins. For example, Sam68 has been shown to be subject to tyrosine phosphorylation, serine/threonine

phosphorylation, methylation, and acetylation (Lukong and Richard, 2003; Najib et al., 2005). Additionally, several hnRNP family members have been identified in PRMT methyltransferase complexes that induce the methylation of RGG boxes within these hnRNPs (Bedford and Richard, 2005). Sumolyation, ubiquitination, and acetylation have also been identified on individual hnRNP proteins (Kim et al., 2006; Krecic and Swanson, 1999; Vassileva and Matunis, 2004). Therefore, these types of modifications are just as likely to participate in the regulation of protein function in response to cellular signaling pathways as the phosphorylation events described above.

CD45 Signal-Induced Alternative Splicing

The CD45 gene encodes a hematopoetic-specific transmembrane protein tyrosine phosphatase that provides an excellent model for studying signal-induced alternative splicing. Three variable exons of the CD45 gene are inducibly skipped upon T cell activation, leading to decreased tyrosine phosphatase activity and maintenance of T cell homeostasis (Hermiston et al., 2002; Lynch, 2004). Importantly, while T cell activation results in only a 3-5 fold change in the ratio of CD45 isoforms, the physiologic importance of this change is evidenced by the fact that naturally occurring polymorphisms within the CD45 gene that disrupt signal-induced alternative splicing correlate with susceptibility to a wide range of autoimmune diseases and viral infections in humans (Dawes et al., 2006; Jacobsen et al., 2000; Tackenberg et al., 2003; Tchilian et al., 2001).

The CD45 gene is highly expressed on the cell surface of all nucleated hematopoetic cells and contains several domains outlined in Figure 1-7 (Hermiston et al., 2003). The intracellular domain functions as a tyrosine phosphatase and modulates immune cell signaling while the extracellular domain contains three fibronectin repeats and a cyosine rich domain which are all subject to N-linked glycoslylation. The three variable exons (4, 5, and 6) comprise the N-terminal region of the extracellular domain and are subject to O-liked glycosylation (Hermiston et al., 2003).

While the stimulatory and inhibitory influences of CD45 protein in immune cell regulation are still debated, the involvement of CD45 in this process is well documented. CD45 was initially characterized as a positive regulator of T cell function because the lack of CD45 gene expression caused severe-combined immunodeficiency in humans (SCID) and mice (Byth et al., 1996; Kishihara et al., 1993; Kung et al., 2000; Mee et al., 1999); however, while the deletion of the CD45 gene causes thymic defects and dramatically reduced numbers of T cells, the few T cells that can be found in the periphery are self-reactive but do not produce a normal autoimmune T cell response following activation (Hermiston et al., 2003; Trop et al., 2000).

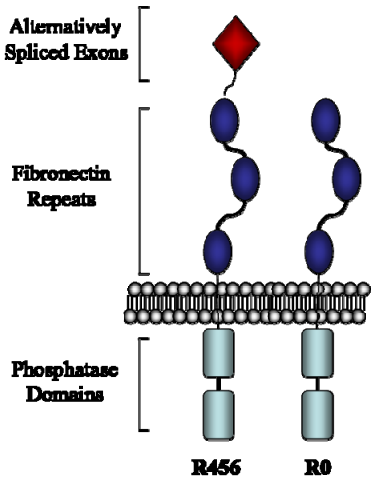

Figure 1-7: Domain Structure of CD45. CD45 contains two intracellular phosphatase domains and three fibronectin repeats. The alternatively spliced exons encode the N-terminal region of the extracellular domain.

The phosphatase activity of CD45 has been studied for many years, and during that time, numerous substrates have been identified. The Src family protein tyrosine kinases (SFKs) serve as the primary substrate for CD45 tyrosine phosphatase activity (Hermiston et al., 2003). Lyk and Fyn are two members of the SFK family that are activated at the earliest time points following antigen recognition by the T cell receptor (TCR). These kinases then phosphorylate multiple subunits of the TCR, leading to the recruitment and activation of ZAP-70 (a protein tyrosine kinase). Following ZAP-70 activation, many different cellular proteins are phosphorylated, activating a multitude of intracellular signaling pathways and causing a myriad of cellular changes (Hermiston et al., 2003). CD45 has been shown to act on the inhibitory phosphorylation sites of both Lck and Fyn, suggesting CD45 may function to stimulate TCR signaling by counteracting the inhibitory signals; however, deficiency of CD45 has also been observed to cause in increase in SFK activity (Hermiston et al., 2003). Thus, the functioning of CD45 in the regulation of the immune response is highly complicated and not well understood.

In spite of the difficulties in deciphering the mechanism of CD45 regulation in the immune response, the expression of the various CD45 isoforms has been well characterized and demonstrated to have functional consequences for the phosphatase activity of the protein. As mentioned above, CD45 contains three variable exons (4, 5, and 6 or A, B, and C) that encode for a region of the extracellular domain. Evaluation of CD45 splicing by RT-PCR has shown that in primary lymphocyte cultures, exons 4, 5 and 6 are partially included in resting cells, but following stimulation of these cultures

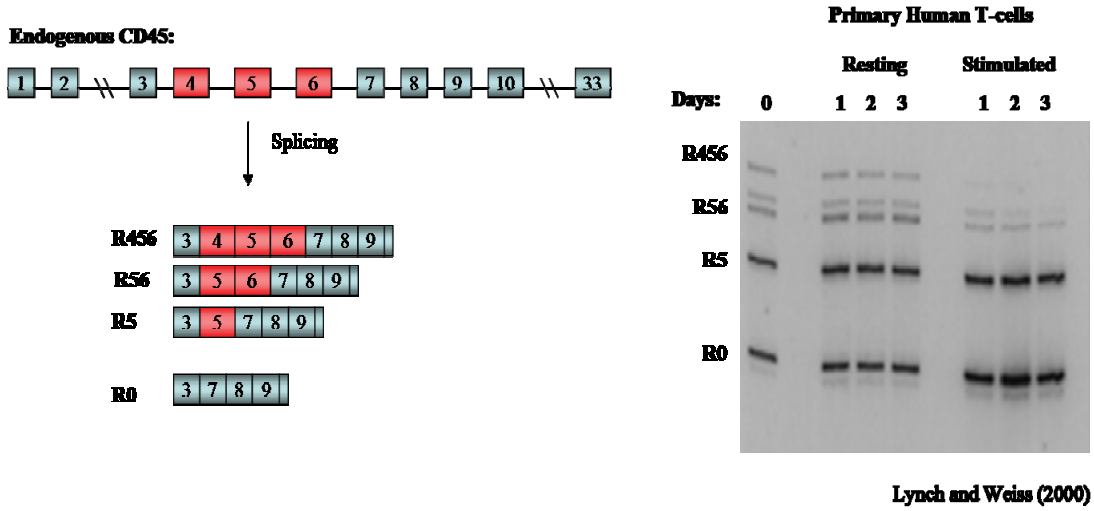

Figure 1-8: Endogenous CD45 Shows Signal-Induced Alterations in Splicing. CD45 variable exons 4, 5, 6 are partially included in resting peripheral lymphocytes, but after activation, these exons are increasingly skipped during the next 72 hours.

with PHA to mimic antigen challenge, these exons are increasingly skipped over the next three days (Lynch and Weiss, 2000; Figure 1-8).

While the combinations of alternative inclusion and exclusion of the three variable exons could generate up to eight different isoforms, studies have provided evidence for five of these isoforms in humans, and the protein isoform expression correlates with the change in splicing behavior (Trowbridge and Thomas, 1994). More specifically, after antigen stimulation expression of the larger isoforms is decreased while expression of the smaller isoforms is increased. The change in isoform expression appears to have significant effects on the activation state of the cell as Lewis rats that express primarily the larger isoforms are at increased risk of developing autoimmune disease where as Brown Norway rats express mostly the smaller isforms of CD45 and show resistance to autoimmune disease (Subra et al., 2001).

Additional biochemical analysis of CD45 has demonstrated that the larger isforms exist predominantly in a monomeric state, and following the increased expression of the small isoforms, a larger percentage of CD45 exists as a dimer (Hermiston et al., 2003). Comparing the phosphatase activity of the monomeric to the dimeric state of CD45 reveals decreased phosphatase activity following dimeriztion (Hermiston et al., 2003). Therefore, in primary T cells the predominant form of CD45 is the monomeric, high-molecular weight isoform with highly active phosphatase. This form of CD45 functions to promote signaling through the T cell receptor, leading to activation of the T cell. After antigen stimulation, cells increases the skipping of the three variable exons and expresse the dimeric, low-molecular weight isoform with decreased phophatase activity, thus decreasing signaling through the TCR and reducing the activation of the T cell several days after the initiation of the immune response (Figure 1-9).

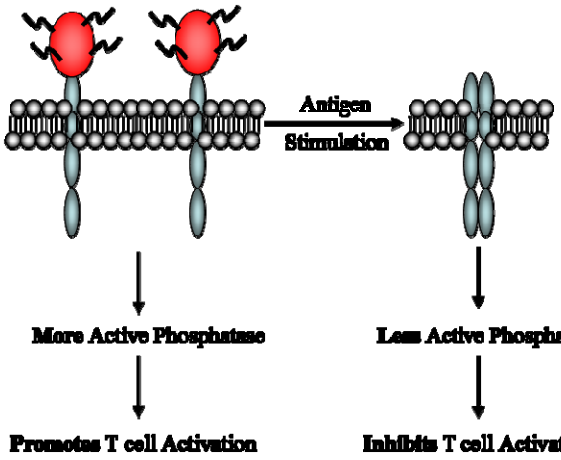

Figure 1-9: Alterations in CD45 Splicing Cause Changes in the Function of the Protein. The larger isoforms of CD45 containing the variable exons are held in the monomeric state with increased phosphatase activity. The smaller isoforms of CD45 dimerize and reduce the phosphatase activity of the protein.

Since alternations in isoform expression occur by means of alterations in pre-mRNA splicing, the mechanisms controlling CD45 splicing are of critical importance and have been the primary focus of the Lynch lab for

several years. The first step in evaluating the splicing regulation of CD45 was the development of the JSL1 cell line. The JSL1 cell line is a Jurkat derived T cell line that mimics the CD45 splicing pattern observed in primary human lymphocytes. Stimulation of JSL1 cells with the phorbol ester PMA to mimic antigen challenge causes an activation-dependent decrease in the level of variable exon inclusion in the endogenous CD45 gene (Lynch and Weiss, 2000). Further investigation provided evidence that the signal-induced change in splicing is dependent upon PKC and Ras activation and requires de novo protein translation (Lynch and Weiss, 2000).

Evaluation of the cis-acting elements within the CD45 variable exons identified several enhancer and silencer sequences within each of the exons (Lynch and Weiss, 2000; Rothrock et al., 2003; Tong et al., 2005). The initial and most complete characterization focused on variable exon 4. Mutational analysis of this exon was performed in the context of a CD45 minigene composed of variable exon 4 flanked by constitutive exons 3 and 7 of CD45. The splicing of this minigene recapitulates the endogenous pattern of CD45 splicing with variable exon 4 showing increased levels of repression after cellular stimulation in JSL1 cells (Rothrock et al., 2003; Figure 1-10). A 60-nucleotide silencer element was identified within variable exon 4. This element was given the designation ESS1 and was found to be both necessary and sufficient to confer basal and signal-induced splicing regulation in this exon as well as a heterologous exon (Rothrock et al., 2003; Figure 1-10). Variable exons 5 and 6 were also found to have

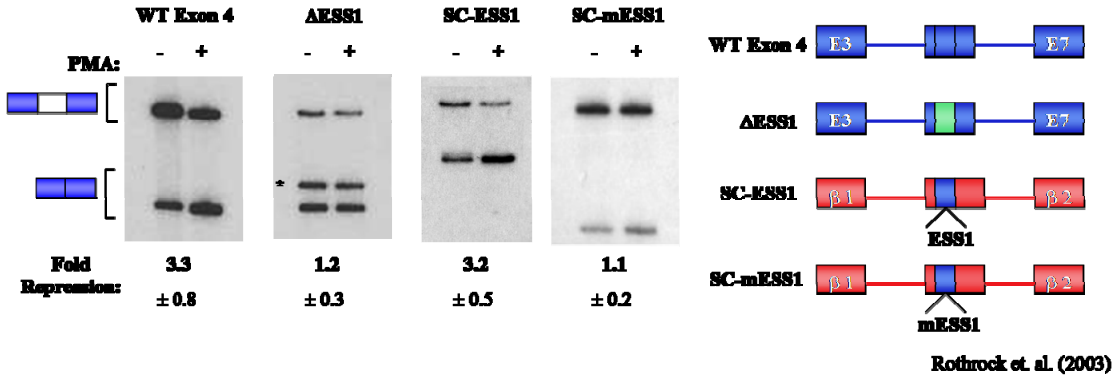

Figure 1-10: Minigene Analysis Show the ESS1 Sequence is both Necessary and Sufficient to Mediate CD45 Exon Silencing. The WT exon 4 and the ΔESS1 minigene are composed of variable exon 4 either with or without the ESS1 sequence flanked by CD45 exons 3 and 7. RT-PCR shows partial exon repression in resting cells with increased exon repression following stimulation in the WT exon. The deletion of the ESS1 element abolishes both basal and activation-induced exon silencing. The SC-ESS1 and SC-mESS1 minigenes contain β-globin exons with the ESS1 or a mutatant ESS1 sequence inserted as shown. The insertion of the ESS1 element is sufficient to cause regulation of the exon and mutations within this sequence reverse this effect. Fold repression is defined as the change in the ratio of exon inclusion to exon skipping between resting and activated cells.

ESS1-like elements which contribute to their regulation following stimulation (Rothrock et al., 2003; Tong et al., 2005), and recent work has identified several more genes both with and without ESS1 elements which are regulated in response to immune cell activation (Ip et al., 2007).

With the identification of the ESS1 element, further studies were conducted to identify the protein factors bound to this sequence under resting conditions. Three members of the hnRNP family were idenfied as ESS1-binding proteins: hnRNP L, PTB, and hnRNP E2 (Rothrock et al., 2005). Functional analysis of these proteins revealed that hnRNP L is the primary mediator of basal exon silencing while PTB showed no activity and hnRNP E2 demonstrated general inhibition of splicing at high concentrations (Rothrock et al., 2005). Despite the improved understanding of basal CD45 exon silencing through the ESS1

element, many unanswed questions remain regarding the regulation of increased exon repression after cellular activation.

The data presented herein provides the first insight into the alternations in the ESS1-binding complex that mediate the increase in CD45 variable exon skipping in activated T cells. First, the primary regulator of basal exon silencing, hnRNP L, was observed to have a differential migration pattern upon two-dimensional gel analysis under resting and stimulated conditions. This result suggests differential post-translational modifications of hnRNP L under these two conditions. Importantly, hnRNP L purified from either resting or stimulated conditions shows slight variations in silencing activity with increased levels of exon repression being observed following the addition of hnRNP L purified under stimulated conditions when compared to hnRNP L purified under resting conditions.

Next, several other hnRNP family members were found to bind to the ESS1 element under both resting and stimulated conditions; however, PSF was identified bound to ESS1 only after cellular activation. Functional evaluation showed the depletion of PSF causes a significant increase in exon inclusion and accounts for the majority of the activation-induced increase in exon repression. Additional investigation into the regulation of PSF binding has shown that PSF is present within the nucleus in distinct protein complexes, and alterations in the protein-protein interactions of PSF may account for the activation-dependent PSF addition to the ESS1-binding complex.

The interplay between the hnRNP L and PSF contributions to the activation-induced exon regulation demonstrated that while neither mechanism in isolation is able to account for the total increase in exon silencing observed following stimulation, the combination of both

mechanisms accounts for a change of similar magnitude to the total activation-induced increase in exon silencing. Thus, while many questions remain to be answered regarding the signal-induced regulation of CD45 splicing, the critical regulators at the level of transcript binding have been identified.

CHAPTER TWO

Analysis of Signaling Pathways Involved in the Regulation of CD45 using Retroviral Infection and Candidate Gene Expression

Introduction

Alternative pre-mRNA splicing is one process known to be regulated by immune cell activation. Signal-induced changes in pre-mRNA splicing generate discrete mRNA transcripts and protein isoforms that often have unique functions, serving as one mechanism by which function may be modulated in response to signaling pathways. Despite the fact that signal-induced changes in splicing serve as an important mechanism of gene regulation, the signaling pathways, binding factors, and RNA sequences which mediate these events are largely unknown and remain a critical area of investigation.

The CD45 gene is subject to activation-dependent changes in response to T cell stimulation. As described in detail in Chapter 1, the alternative splicing of CD45 has been shown to be regulated by a 60 nucleotide silencer sequence which functions to cause basal exon repression by binding hnRNP L as well as several other hnRNP family members (Rothrock et al., 2003; Rothrock et al., 2005). While these advances are critical to the mechanistic understanding of CD45 splicing, many questions remain regarding the activation-dependent increase in exon skipping.

Evaluation of signaling pathways often uses tissue culture systems in which the constitutive active or dominant negative mutant cDNAs are expressed and the effects on the desired process are analyzed. Previous work showed that the jurkat derived JSL1 cell line

mimics the splicing regulation observed in primary human T cells (Lynch and Weiss, 2000), providing a cell culture system for the evaluation of signaling requirements for CD45 alternative splicing.

One technically challenging aspect of the JSL1 cell culture system, however, is the difficult and inefficient transfection of plasmid DNA using traditional methods of plasmid transfection. The low transfection efficiency causes only a small fraction of the cells to express the desired protein product, and any effect on the splicing pattern caused by the transfected product to be masked by the larger, unmodified fraction of the cellular population. In order to address this issue, a retroviral infection system was developed to increase the efficiency of plasmid transduction. The MaRX II retroviral infection system was chosen for evaluation for several reasons. Using this system, both transient expression as well as stable integration of plasmid DNA can be achieved (Hannon et al., 1999). This system also allows for integrated plasmid DNA to be recombined out of the genome for analysis, making this system useful for cDNA library screening (Hannon et al., 1999). Chapter two describes the development and characterization of a MaRX II retroviral infection system, as a potential tool to begin investigation of the signaling pathways required for the regulation of CD45 splicing.

Results

Retroviral Packaging of Plasmid DNA Provides Increased Plasmid Transduction Over Electroporation

Previously, the JSL1 cell line had been adapted for use in the MaRXII system by creating a stable cell line expressing the murine specific viral receptor with the newly derived cell line being called JVR cells. The ecotropic receptor was added to the human JSL1 cell line in order to accommodate the use of the packaging cell line which produces murine specific virus. The use of viral particles with species specificity increases the safety of the system by preventing the virus from infecting the individuals performing these experiments. The viral packaging cell line, a 293T derived cell line, was also present in the lab. The ecotropic viral packaging line, called ϕNX cells, have been modified to stably express the gag, pol, and env retroviral proteins (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html). The gag and pol proteins are expressed off of the CMV promoter while the env protein provides the ecotroptic specificity and is driven off of an RSV promoter (Figure 2-1). These cells are classified as helper virus free and are capable of transiently producing virus without the addition of other factors (Grignani et al., 1998). The MaRX II retroviral vector was used for packaging within the virus (Figure 2-1). The vector contains two mammalian promoters to drive expression in mammalian cells: LTR promoter and PGK promoter. The vector also contains both zeocin and ampicillin resistance cassettes.

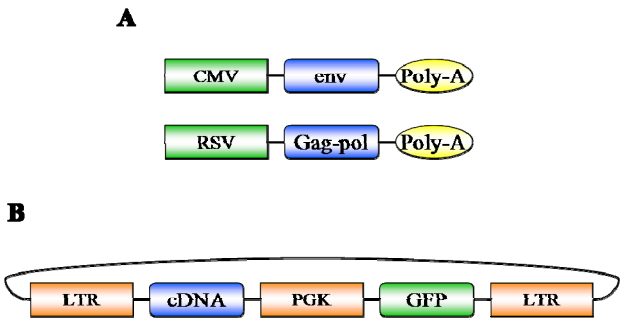

Figure 2-1: Schematic Representation of Plasmids. (A) Schematic representation of the env and gag-pol constructs stably expressed in the ϕNX viral packaging cell line. (B) Schematic representation of the adapted MaRX II vector used for target cDNA expression in JVR cells.

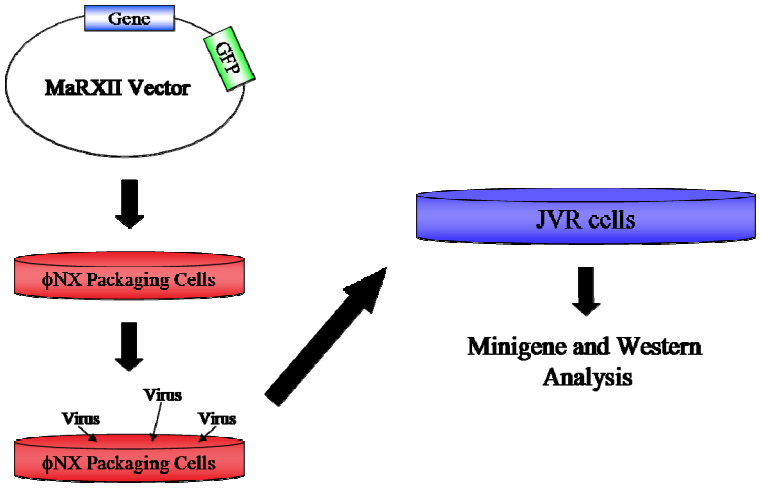

Figure 2-2: Schematic of Retroviral Vector Packaging and Delivery. MaRX II plasmid was transfected into the ϕNX viral packaging cell line and incubated while packaged virus is secreted into the media. Media from the transfected ϕNX cells was mixed with JVR cells to allow for infection. JVR cells were incubated and treated as desired, and protein and RNA were harvested and analyzed.

Viral transduction into JVR cells is achieved by transfecting the ϕNX packaging line with the MaRX II plasmid which is then packaged into viral particles and secreted into the media. JVR cells are infected with the virus and begin to express the cDNAs contained within the MaRX II vector (Figure 2-2). To determine the efficiency of viral transduction into the JVR cells and compare it with the efficiency of electroporation, MaRX II vector containing GFP cDNA driven off the PGK promoter was used. Virally infected and electroporated JVR cells were subjected to analysis by FACS to evaluate GFP expression. As seen in Figure 2-3, the percentage of cells expressing GFP was much greater (70-90%) when cells were infected with MaRX II packaged retrovirus than with the electroporation of the same MaRX II plasmid (30%). Therefore, retroviral infection causes plasmid transduction with higher efficiency than standard electroporation and provides a delivery system that is predicted to be sufficient for the evaluation of candidate proteins.

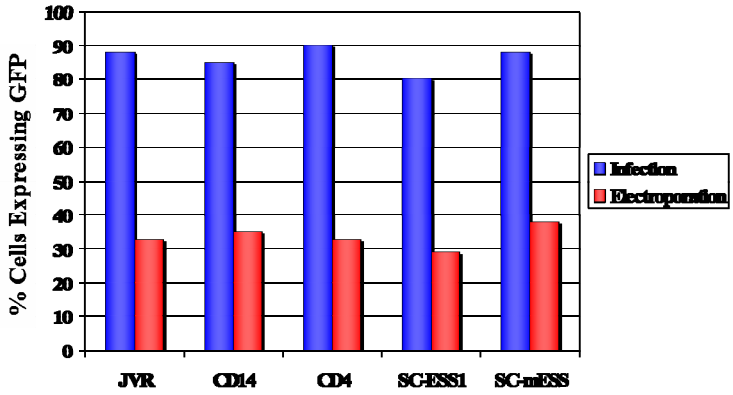

Figure 2-3: Infection Leads to Increased Levels of Plasmid Transduction Over Eletroporation in JVR cells. Percentage of Cells expressing GFP following infection or eletroporation of the MaRX II plasmid expressing GFP off of the PGK promoter as determined by FACS analysis. CD14, CD4, SC-ESS, and SC-mESS represent JVR derived cell lines stably expressing the indicated minigene.

The LTR Promoter Shows Expression in JSL1 Cells Under Resting and Stimulated Conditions

In the above experiments the GFP cDNA was driven off of the PGK promoter; however, the LTR promoter will be used to drive expression of the target cDNA while the PGK promoter drives expression of GFP which will be used to monitor plasmid transduction efficiency. To evaluate expression from the LTR promoter and its possible responsiveness to PMA, JVR cells were infected with a MaRX II vector containing a GFP cDNA driven by the LTR promoter in place of GFP driven by the PGK promoter. FACS analysis of these cells shows reasonable GFP expression from the LTR promoter (Figure 2-4; Blue vs. green and pink) that shows some increase in expression following PMA treatment (Figure 2-4, green vs. pink). Given that reasonable GFP expression is observed following infection, the LTR promoter is likely to allow for sufficient candidate gene expression for evaluation of its involvement in CD45 signal-induced splicing regulation.

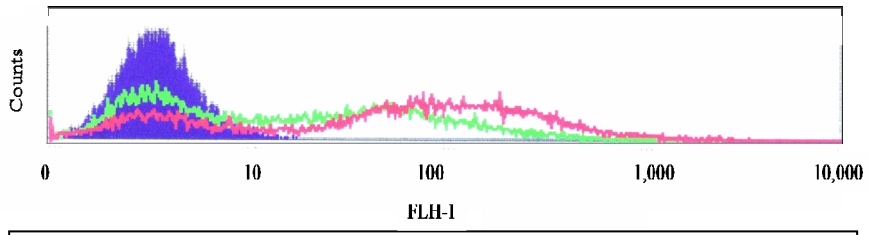

Figure 2-4: GFP Expression Driven by the LTR Promoter in the Presence and Absence of PMA Treatment. The solid blue peak represents uninfected JVR cells (not expressing GFP) while the green and pink lines represent JVR cells infected with LTR driven GFP either untreated (green) or treated with PMA (pink). Increased expression of GFP is seen in the LTR driven GFP infected JVR samples, represented by the increased forward scatter (rightward shift). PMA treatment (pink) shows only slightly increased GFP expression as compared to untreated samples (green).

Of note, however, is the increase in expression from the LTR promoter after PMA treatment. While the effect appears small, differences in expression level in treated and untreated samples may represent a concern for future experiments.

Infection with Activated Ras Shows No Change in CD45 Splicing

In previous studies, Lynch and Weiss demonstrated that the transfection of the constitutively active mutant of the small G protein Ras (Ras R12, T59) was sufficient to cause at least a partial decrease in the level of variable exon inclusion in the absence of PMA treatment (Lynch and Weiss, 2000). Thus, the previously used Ras R12 as well as the more widely used Ras V12 mutants were cloned into the MaRX II vector downstream of the LTR promoter with GFP expression being driven by the PGK promoter. The expression of GFP was used to monitor viral transduction levels using FACS analysis of the JVR cells. Splicing analysis was performed using RT-PCR of both a minigene that recapitulates the splicing pattern of the endogenous CD45 gene (see Chapter 1) and the endogenous CD45 gene to see if the expression of active Ras was sufficient to cause an increase in exon repression or an "activated phenotype" when compared to levels of exon inclusion in cells infected with empty vector. While Western blot analysis shows increased levels of Ras V12 expression

over empty vector infected samples, little difference is observed following infection of the Ras R12 mutant (Figure 2-5, B).

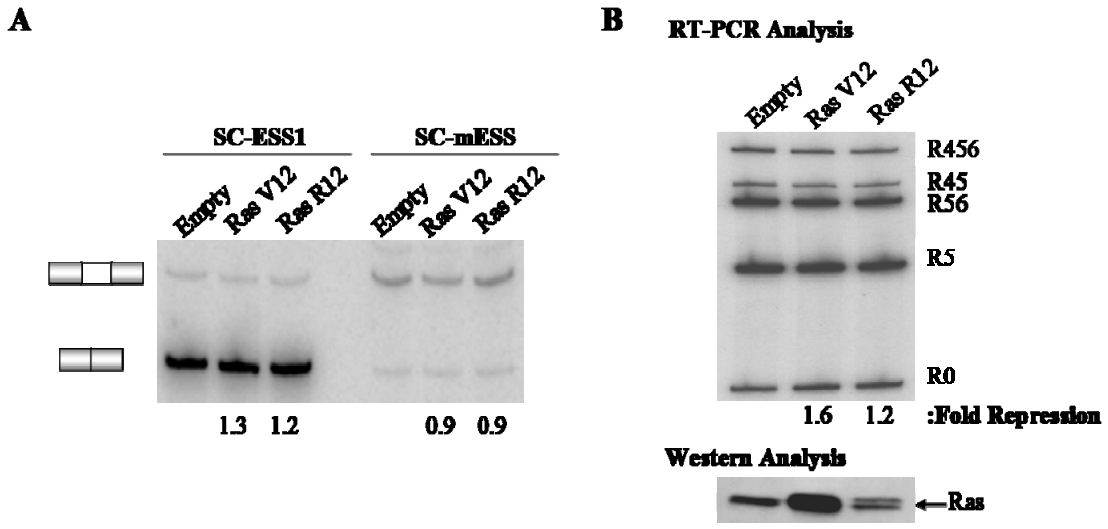

Figure 2-5: Active Ras Does Not Cause a Change in CD45 Splicing. (A) RT-PCR analysis of the SC-ESS1 and SC-mESS (control) after infection with empty vector, Ras V12, or Ras R12. Fold repession values for Ras infected mutants were calculated by comparing the level of exon inclusion after active Ras infection to the levels of exon inclusion in empty vector controls. Values show no significant difference in variable exon inclusion. (B) RT-PCR analysis of the endogenous CD45 gene following expression of active Ras mutants. Western blot shows total expression of Ras in each of the indicated samples.

The splicing of both the SC-ESS1 minigene as well as the endogenous CD45 gene show little if any change in the level of exon inclusion (Figure 2-5, A & B). However, the overall activation state of Ras pathways in these cells is unknown; therefore, it could be that Ras V12 is being over expressed but is not activating the downstream pathways required to observe changes in the splicing of CD45. Additionally, Ras R12 does not show significant levels of overexpression, possibly accounting for the discrepancy between the previously obtained results and the results reported here.

Infection with Dominant Negative IκBα Shows No Change in Fold Repression of CD45 Splicing

Evaluation of another signaling pathway known to be activated by PMA in T cells was also evaluated using the MaRX II retroviral infection system. A dominant negative mutant of IκBα (DN-IκBα), containing two alanine substitutions for two critical serines, was cloned into the MaRX II vector similar to the active Ras constructs described above. This mutant is unable to be phosphorylated by IκB kinase which normally signals the degradation of IκBα (Karin and Ben-Neriah, 2000). The degradation of IκBα allows NFκB to be released from inhibition, translocate into the nucleus, and become an active transcription factor (Karin and Ben-Neriah, 2000). To evaluate a possible role for the NFκB pathway in the regulation of CD45 splicing, JVR cells were infected with a dominant negative mutant of IκBα and treated with PMA. Following treatment with PMA, the IκBα mutant construct

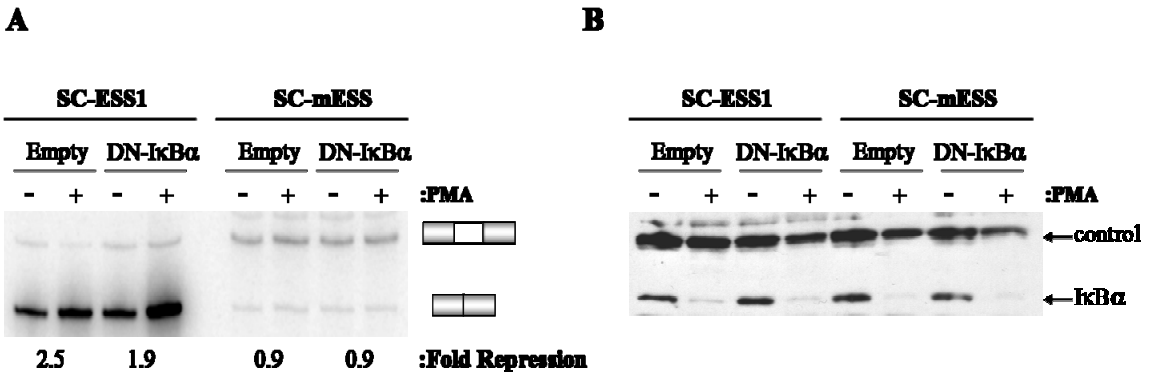

Figure 2-6: DN-IκBα Does Not Affect the Fold Repression of CD45 Exon 4. (A) RT-PCR minigene analysis 60 hours following PMA treatment of either empty vector infected or DN-Iκbα infected JVR cells. Fold repression values after DN-IκBα infection are not significantly different from empty vector infected cells. (B) Western blot analysis using anti-IκBα antibody, showing a decrease in IκBα protein levels 30 minutes following stimulation with PMA in both empty vector and DN-IκBα infected cells. Control arrow indicates a non-specific background band used as a loading control.

should block the activation of the NFκB pathway and prevent the normally observed increase in exon silencing, assuming activation of the NFκB pathway is required to mediate the change in splicing. RT-PCR analysis of JVR cells, however, shows no significant change in the repression CD45 variable exon 4 between empty vector and DN-IκBα (Figure 2-6, A). However, Western blot analysis for IκBα shows degradation of the protein in both PMA treated samples (Figure 2-6, B, empty vector and DN-IκBα); thus, the degradation of IκBα is not being blocked by expression of the DN-IκBα mutant. Two possible explanations for this observation exist: the mutant protein is not being expressed in the JVR cells or the mutant protein is also being degraded following PMA treatment. With either explanation, the NFκB pathway is not being inhibited as desired, so no conclusions can be made about the requirement for the pathway's activity in the alternative splicing of CD45.

Infection with hnRNP Family Members Causes No Change in Splicing with Variable Protein Expression

Additional evaluation of the system was performed using myc-tagged versions of several members of the hnRNP family. HnRNP L and PTB are known to bind to the ESS1 sequence under resting conditions and hnRNP L has been shown to mediate basal exon repression (Rothrock et al., 2005); thus, overexpression of at least hnRNP L should cause an increase in exon repression in resting cells. However, RT-PCR analysis from the SC-ESS1 and SC-mESS minigenes show no reproducible change in the level of variable exon inclusion in the presence of either hnRNP L or PTB (Figure 2-7 and Figure 2-8, RT-PCR Analysis). Another member of the hnRNP family, hnRNP A1, was also used as a control construct since

it does not bind with high affinity to the ESS1 sequence (see Chapter 3), and this construct also does not induce any change in the level of exon silencing (Figure 2-7, RT-PCR Analysis). Although the lack of exon silencing upon hnRNP L infection was initially surprising, Western blot analysis for the tagged protein shows little or no expression of hnRNP L in JVR cells (Figure 2-7, Western Analysis) and is the most likely protein to function in exon repression. HnRNP A1 and PTB show some expression in the JVR cells (Figure 2-7, Western Analysis) but are unlikely to affect the splicing pattern because hnRNP A1 does not bind to the ESS1 sequence with high affinity and PTB has not shown activity in other assays (Rothrock et al., 2005).

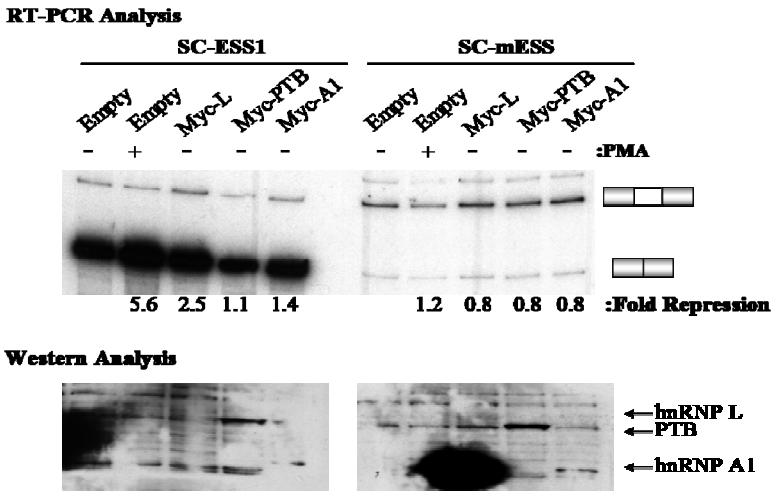

Figure 2-7: Infection by hnRNP Family Members Does Not Affect CD45 Splicing and Shows Variable Protein Expression. RT-PCR analysis of the indicated minigene showing little change in the fold repression value following infection by the indicated hnRNP protein. Fold Repression was calculated by comparing the levels of exon inclusion after hnRNP infection to levels of exon inclusion after empty vector infection. Western analysis was performed using anti-myc antibody. Bands corresponding to PTB and hnRNP A1 are observed after infection by the corresponding vector. Expression of hnRNP L is not observed.

The initial analysis of the JVR cells was performed approximately 60 hours post-infection. It is possible that the splicing was affected, but is not being observed due to the

time point at which the analysis was performed. Therefore, JVR cells were infected and harvested at 24, 48, and 72 hours post-infection. RT-PCR analysis of the SC-ESS1 minigene again shown no change in the level of exon silencing after infection with either myc-hnRNP L or myc-hnRNP A1 (Figure 2-8, RT-PCR Analysis). Western analysis again reveals little or

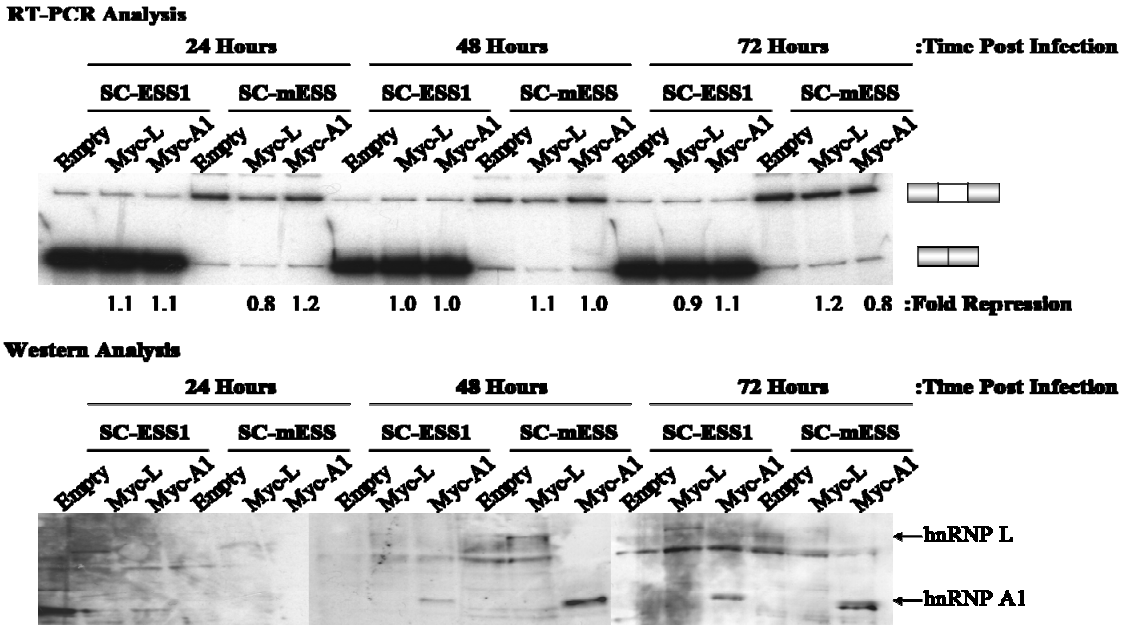

Figure 2-8: Time Course of hnRNP Infection Does Not Change CD45 Splicing. RT-PCR splicing analysis after various times post-infection. Calculation of fold repression was determined as described in Figure 2-7 and shows no change at any time point following hnRNP infection. Western blot analysis using anti-myc antibody shows hnRNP A1 expression at 48 hours and 72 hours post-infection. Slight expression of hnRNP L is seen at 48 and 72 hours.

no expression of myc-tagged hnRNP L and shows reasonable expression of hnRNP A1 (Figure 2-8, Western Analysis). The lack of expression of hnRNP L does not appear to be caused by the addition of the N-terminal myc-tag or a mutation within the vector or cDNA sequence, as it is expressed in the ϕNX packaging cells at similar levels to those seen for PTB and hnRNP A1 in these cells (Figure 2-9).

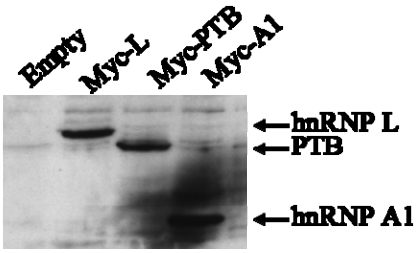

Figure 2-9: Expression of hnRNP Family Members in ϕNX Cells. Western blot analysis using anti-myc antibody of transfected ϕNX cell lysates show similar levels of expression of myc-tagged hnRNP L, PTB, and hnRNP A1.

Lack of hnRNP L Protein Expression is Not Caused by Lack of Vector Derived mRNA

In order to determine if the lack of protein expression in JVR cells was due to lack vector derived hnRNP L mRNA, RT-PCR was performed using primers to the C-terminal portion of the hnRNP L cDNA sequence and continuing on into the MaRX II vector. As seen in Figure 2-10, hnRNP L mRNA is being transcribed off the MaRX II plasmid and appears to be full length since the C-terminal region of the protein was targeted for amplification. Primers were also added to amplify β-actin as an internal control (Figure 2-10).

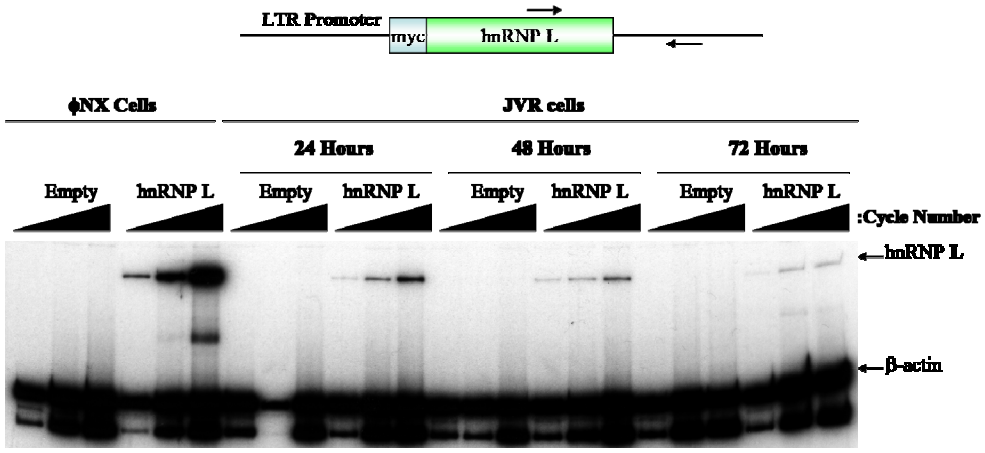

Figure 2-10: Vector Derived hnRNP L mRNA is Transcribed in JVR and ϕNX Cells. RT-PCR analysis of hnRNP L mRNA showed vector derived hnRNP L transcript present at all time points in hnRNP L infected JVR cells as well as ϕNX cells. Primer locations are indicated in the above schematic. Primers directed against β-actin were used to account for differences in input RNA.

In order to evaluate the total levels of hnRNP L transcript within the cells, RT-PCR analysis was repeated using internal hnRNP L sequences. Using these primers, it appears that there is no increase in transcript level in hnRNP L infected cells as compared to vector treated controls (Figure 2-11). Additional analysis of the myc-tag region of the MaRX II vector was performed to determine if there was decreased levels of transcription initiation which could account for the lack of protein expression; however, RT-PCR

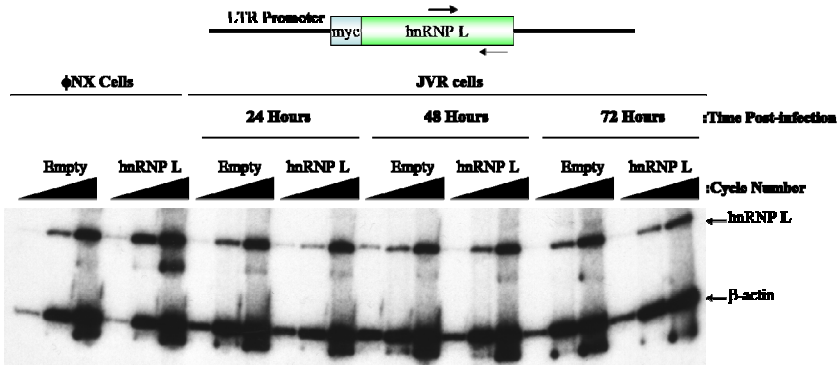

Figure 2-11: Endogenous hnRNP L mRNA Levels Are Unchanged After hnRNP L Infection. RT-PCR analysis of both vector derived and endogenously expressed hnRNP L mRNA show equal mRNA levels in empty vector and hnRNP L infected JVR cells as well as ϕNX cells. HnRNP L primer locations are indicated by the arrows in the above schematic. Primers directed against β-actin were used to account for differences in input RNA.

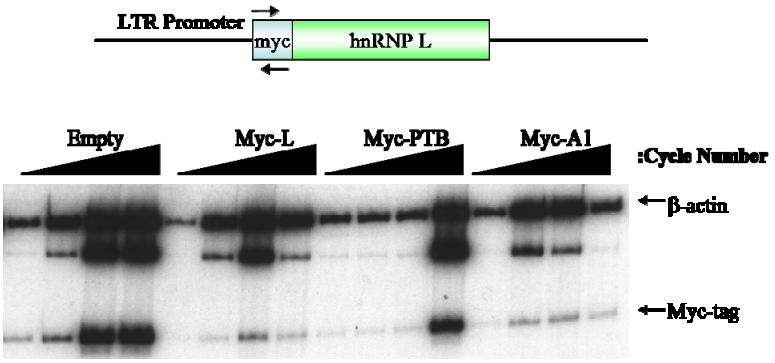

Figure 2-12: Levels of N-terminal Myc-Tag RNA Are Unchanged in hnRNP Vectors. RT-PCR analysis of myc-tag region of MaRX II vector in JVR cells shows no reproducible difference between empty vector, hnRNP L, PTB, or hnRNP A1. Primer locations are indicated by arrows in the above schematic and β-actin was analyzed as an input control.

analysis again shows no significant change in the levels of transcription of the myc-tag region of the vector in hnRNP L plasmids compared to an empty vector, myc-PTB, or myc-hnRNP A1 (Figure 2-12). Given the analysis of hnRNP L mRNA, the levels of hnRNP L expression within the cell appear to be tightly regulated as its expression is specifically repressed following infection by an hnRNP L containing plasmid.

Discussion

While PMA is sufficient to induce a change in CD45 splicing, the required intracellular signaling cascades are unknown. This chapter describes the initial development and characterization of a viral infection system to facilitate the identification of the required signaling pathways using a candidate gene approach. In these studies, a system was developed in which plasmid DNA can be delivered to JVR cells with high efficiency using a retroviral vector. However, while the transduction of this plasmid appears to be efficient, various levels of gene expression were observed following infection and the splicing pattern of CD45 was not altered by infection of any of the above candidate genes.

Infection with active Ras has previously been shown to cause a change in the splicing pattern of CD45, causing the variable exons to be more highly skipped (Lynch and Weiss, 2000); therefore, it was used as a positive control for the development of the system. Given that no effect on CD45 splicing was observed in the above experiments and that the Ras protein appears to be expressed in those cells, it is possible that the protein is not sufficiently expressed to cause activation of downstream pathways, ultimately leaving CD45 splicing

unaffected. Further experiments evaluating the activation of downstream Ras targets would be required to determine if the expressed protein is, in fact, expressed and active.

Similar to the inconclusive results following infection with activated Ras, infection with DN-IκBα showed degradation of the IκBα protein in empty vector as well as DN-IκBα infected samples after treatment with PMA. Because the DN-IκBα protein was untagged, it is unclear if the DN-IκBα is not being expressed following plasmid transduction or the mutant version of the protein is not regulating the activity of the pathway as anticipated. Future experiments will be required to conclusively determine if the NFκB pathway plays a role in CD45 alternative splicing.

Additional infections using members of the hnRNP family were also used to evaluate the system, and while PTB and hnRNP A1 were able to be expressed from the plasmid, myc-tagged hnRNP L protein was undetectable. The lack of protein expression was not due to a flaw in the vector, nor was it caused by a lack of mRNA transcription. The JVR cells appear to precisely and specifically regulate cellular levels the hnRNP L protein. The tight regulation of hnRNP L expression could be anticipated given its multiple regulatory roles in RNA metabolism. In this case, the lack of change in splicing pattern is expected given that the levels of protein expression are unchanged.

Together the studies described above indicate the level of expression from this vector may be variable and insufficient to evaluate the effects of overexpression of candidate proteins, especially for highly regulated and abundant proteins such as members of the hnRNP family. Another complication could also be caused by the induction of LTR driven transcription following treatment with PMA. While this was tested and shown to be a small

effect for the expression of GFP, further studies not described here suggest that the PMA inducibility of the LTR promoter may be more significant. Therefore, this system has overcome a few of the technical hurdles preventing the identification of required signaling pathways; however, future work should address the problems in this system such as the variable levels of protein expression. I, however, did not pursue such experiments due exciting advancements regarding alterations in the ESS1-binding complex described below.

CHAPTER THREE

Modifications to Members of the Basal ESS1 Silencing Complex Following Cellular Activation

Introduction

Alternative splicing is typically controlled by the binding of splicing regulatory proteins to sequences within or near the variable exon that, in turn, control the recognition and use of the exon by the splicing machinery, or spliceosome (Black, 2003; Matlin et al., 2005). Generally, splicing regulatory proteins fall into one of two categories: SR (serine-arginine-rich) proteins generally enhance exon inclusion whereas hnRNP proteins typically promote exon skipping (Black, 2003).

Despite the prevalence of signal-induced splicing regulation, little is known as to how the substrate-specific binding or activity of splicing regulatory proteins is influenced by signal transduction pathways. A few studies have described changes in the phosphorylation of SR or hnRNP proteins in response to cellular stress or stimulation, leading to altered activity and/or subcellular localization of these proteins (Allemand et al., 2005; Blaustein et al., 2005; Patel et al., 2001; Patel et al., 2005; van der Houven van Oordt et al., 2000). However, there has been little direct demonstration that altered phosphorylation of an SR/hnRNP protein confers a specific change in known signal-responsive alternative splicing events.

In previous work, Rothrock et. al. identified an exonic splicing silencer (ESS1) within CD45 variable exon 4 that mediates both partial exon repression in resting cells and further exon skipping upon cellular stimulation (Rothrock et al., 2003). In addition, the Lynch

laboratory has determined that the partial exon repression in resting cells is conferred via the binding of hnRNP L to ESS1 (Rothrock et al., 2005). Given these observations regarding the basal activity of the ESS1 silencer, further evaluation of the members of the basal ESS1-binding complex was pursued. In this chapter I report the evaluation of changes in the expression level, modification state, or protein interactions of the members of the basal ESS1-binding complex. These experiments showed no significant changes in the nuclear expression level of these proteins; however, hnRNP L was shown to be differentially modified in resting versus stimulated cells. While still unidentified, this change in modification state of hnRNP L correlates with a small increase in repressive activity of the protein following stimulation. Therefore, post-translational modifications to hnRNP L serve as one mechanism by which the silencing activity of the ESS1 sequence is enhanced after cellular activation.

Results

In Vitro Splicing Recapitulates the Signal-Responsive Splicing Pattern of CD45

As a first step toward characterizing the changes in splicing regulation that occur upon cellular activation, evaluation of JSL1 nuclear extracts was performed to determine if nuclear extracts from resting and stimulated cells contain the minimal factors necessary to confer the differences in isoform expression observed *in vivo*. Importantly, the signal-induced alternative splicing of *CD45* exon 4 can be fully recapitulated *in vitro* using splicing competent nuclear extracts derived from JSL1 cells cultured in the absence (resting) or presence (stimulated) of PMA (See Chapter 7). As shown in Figure 3-1, exon 4 is included

in ~4% of products derived from *in vitro* splicing of the WT minigene (see chapter 1) in extract from resting cells (R-NE), while incubation of the same construct in extract from PMA-stimulated cells (S-NE) results in only ~1% inclusion of exon 4. This difference in exon 4 inclusion between resting and stimulated extracts correlates to the same 3-5 fold increase in exon repression that is observed *in vivo* in both minigenes and the endogenous CD45 pre-mRNA. Importantly, this differential processing of exon 4 in extract from resting and activated cells is dependent on the ESS1 regulatory motif, since substitution of the ESS1 element with

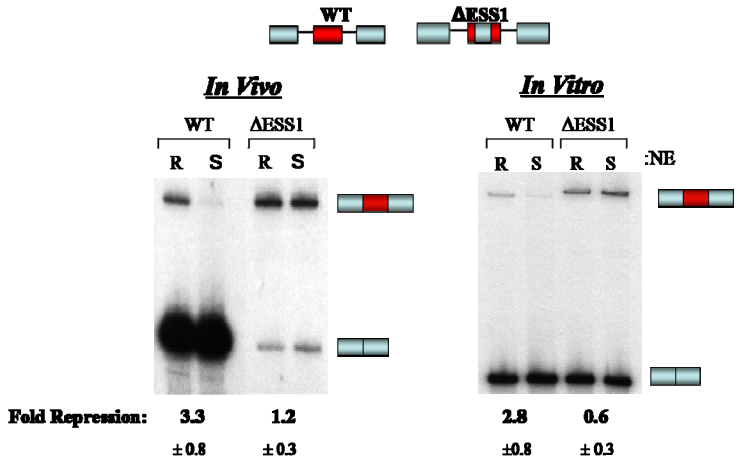

Figure 3-1: Nuclear Extracts Recapitulate the *In Vivo* Splicing Pattern *In Vitro*. RT-PCR analysis of CD45 minigenes from JSL1 cells show partial variable exon 4 repression under resting conditions with an increase in exon repression following treatment of JSL1 cells with PMA. *In vitro* splicing using JSL1 derived nuclear extract demonstrates the same pattern as observed in vivo with partial exon repression in resting extract and an increase in exon repression in stimulated extract. Deletion of the ESS1

unrelated sequence of similar length (Fig. 3-1, ΔESS1) leads to increased inclusion of exon 4 in extract from resting cells, and no change in the level of exon inclusion between resting and stimulated extract. Thus, nuclear extracts derived from resting and stimulated JSL1 cells support ESS1-dependent signal-induced changes in splicing to the same extent as observed *in*

vivo. Importantly, these results demonstrate that the change in isoform expression of CD45 observed upon T cell activation *in vivo* can be entirely accounted for by direct regulation of splicing, and allow us to rule out alternative mechanisms of regulation by coordinated events such as transcription or mRNA export. In addition, these extracts provide a valuable tool for dissecting the changes in the ESS1-bound splicing regulatory complex that are induced upon activation to cause the signal-dependent silencing of exon 4.

Nuclear Protein Levels and ESS1 Affinity of the ESS1-Binding Proteins are Unchanged Following Stimulation

Previously, Rothrock et. al. identified hnRNP L as the main functional component of the ESS1-binding complex present in resting cells, although this complex also contains PTB and hnRNP E2 (Rothrock et al., 2005). Given the activity of this hnRNP L-PTB-E2 complex in resting cells, I first sought to determine if the increase in exon repression in activated cells correlates with an increase in expression of some or all of these proteins upon activation.

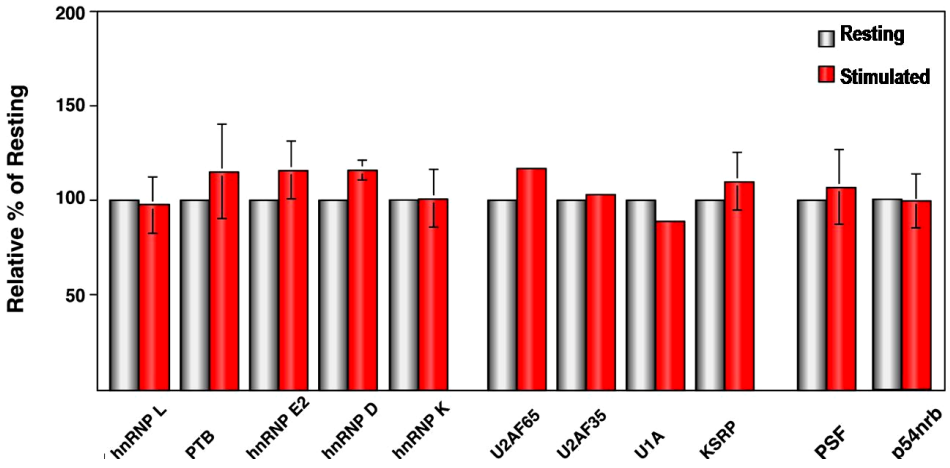

Figure 3-2: Nuclear Expression Levels of ESS1 Binding Proteins Are Unchanged. Nuclear extract prepared from either resting or stimulated JSL1 cells was subjected to Western blot analysis for the indicated protein. Quantitation was perfomed using densitometry and expression level was normalized to levels observed in resting nuclear extract (set to 100%).

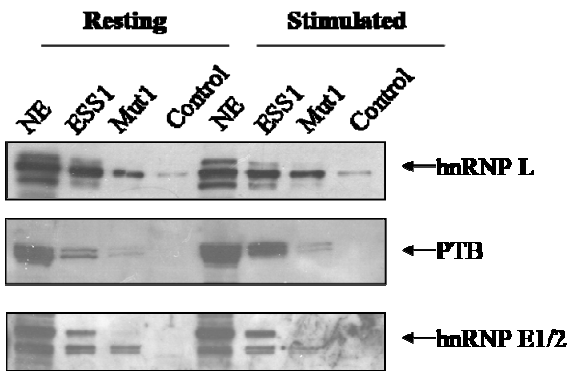

Figure 3-3: Resting and Stimulated Affinity Purification of Previously Identified ESS1-Binding Proteins Show Equal Affinity. Biotinylated-RNA was incubated with the indicated nuclear extract (NE) and protein binding was analyzed by Western blot analysis. As demonstrated previously in Rothrock et. al. (2005), hnRNP L, PTB, and hnRNP E2 bind to the ESS1 sequence with higher affinity than to either a mutant (Mut1) or a non-specific control RNA sequence. Comparison of binding affinity from resting and stimulated NE shows no significant or reproducible change in levels of protein binding following cellular activation.

However, Western blotting of various concentrations of nuclear extract followed by quantitation using densitometry provided careful evaluation of the nuclear expression of hnRNP L, PTB, hnRNP E2, as well as several other splicing regulatory proteins. Results from these experiments showed no significant change in nuclear protein level following stimulation (Figure 3-2). Additional analysis of binding to ESS1 RNA under both resting and stimulated conditions shows no significant increase in affinity for hnRNP L, PTB, or hnRNP E2 following stimulation (Figure 3-3). Therefore, neither an increase in expression or in ESS1 affinity account for the increase in exon repression after cellular activation.

HnRNP L Demonstrates Differential Post-Translational Modification by Two-Dimensional Gel Analysis

Since the levels of the ESS1-binding proteins do not change significantly, the modification state of these proteins was evaluated by two-dimensional (2D) gel analysis to determine if differential post-translational modification might affect the silencing activity of

these proteins. Total nuclear extract from either resting or stimulated conditions was separated first by isoelectric point followed by separation by SDS-PAGE (see Chapter 7). After separation the samples were transferred to nitrocellulose and specific proteins were evaluated by Western blot analysis. While hnRNP E2 and PTB show no significant changes in migration pattern in resting versus stimulated nuclear extract (Figure 3-4 and Figure 3-5), Western blotting of 2D gels for hnRNP L reveals a differential pattern of migration (Figure 3-6). In nuclear extract prepared from resting cells, the bulk of hnRNP L migrates close to its predicted pI of 6.7,

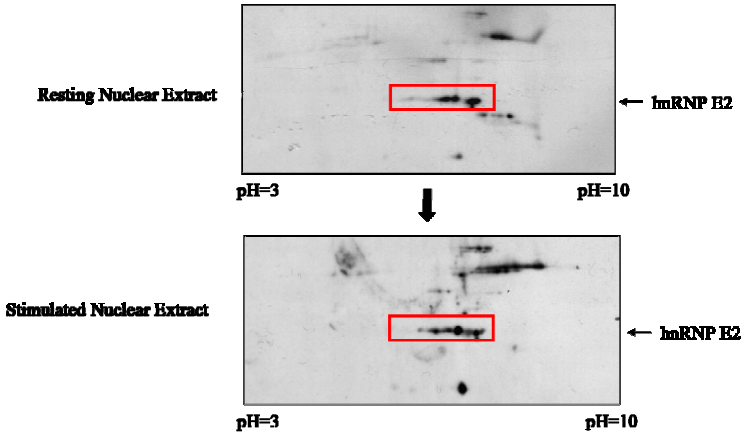

Figure 3-4: Two-Dimensional Gel Analysis of hnRNP E2 Shows No Change in Isoelectric Point. Following two-dimensional gel electrophoresis and Western blot analysis, hnRNP E2 shows no significant shift in migration when comparing resting to stimulated conditions (red boxes).

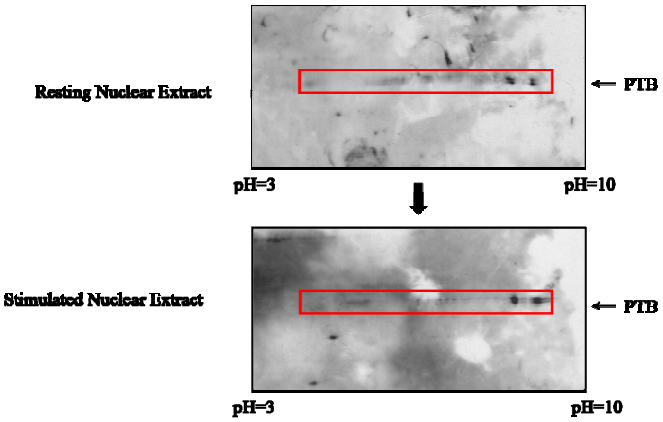

Figure 3-5: Two-Dimensional Gel Analysis of PTB Shows No Change in Isoelectric Point. Following two-dimensional gel electrophoresis and Western blot analysis, PTB shows no significant shift in migration when comparing resting to stimulated conditions (red boxes).

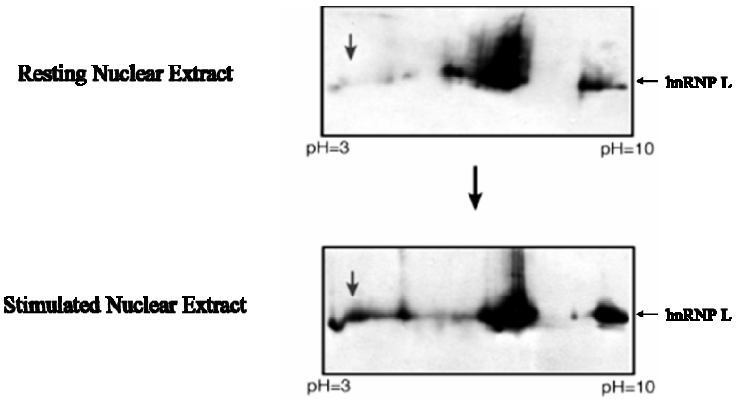

Figure 3-6: Two-Dimensional Gel Analysis of hnRNP L Shows an Acidic Shift in Isoelectric Point. Following two-dimensional gel electrophoresis and Western blot analysis, hnRNP L shows a shift in migration toward acidic pI when comparing resting and stimulated samples (arrows).

with only a minor population migrating at a more acidic pI. In contrast, in extract from stimulated cells, a larger population of hnRNP L shifts to the acidic population (Figure 3-6). The difference in the pI profile of hnRNP L in resting and stimulated extracts suggests differential post-translational modification of at least a portion of the nuclear hnRNP L pool in activated cells.

Initial experiments were conducted to evaluate the nature of the differential modification. In general, an acidic shift from the predicted pI of a protein is consistent with an increase in the phorphoryaltion status; thus, the phosphorylation state of hnRNP L was evaluated using several different methods. Treatment of hnRNP L with a nonspecific phosphatase (CIP) caused an increase in mobility when separated on a one-dimensional gel (Figure 3-7), consistent with previous reports

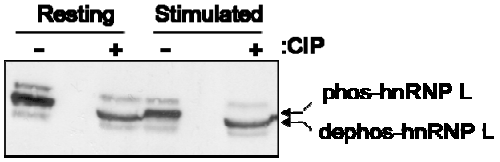

Figure 3-7: CIP Treatment Causes a Shift in hnRNP L on One-Dimensional Gel. Treatment of NE with Calf Intestinal Phosphatase (CIP) followed by SDS-PAGE and Western blot analysis for hnRNP L shows an increase in hnRNP L migration, consistent with dephosphorylation under both resting and stimulated conditions. (Data contributed by Caryn Rothrock)

characterizing hnRNP L as a phosphoprotein within the cell (Navakauskiene et al., 2004); however, the shift is observed in both resting and stimulated samples. Thus, hnRNP L may exist in a partially phosphorylated state under resting conditions and increase in phosphorylation following stimulation. Attempts to treat hnRNP L with phosphatase (CIP) and separate using 2D analysis resulted in a general loss of isoelectric focusing in both resting and stimulated samples (Figure 3-8); thus, the phosphorylation status of

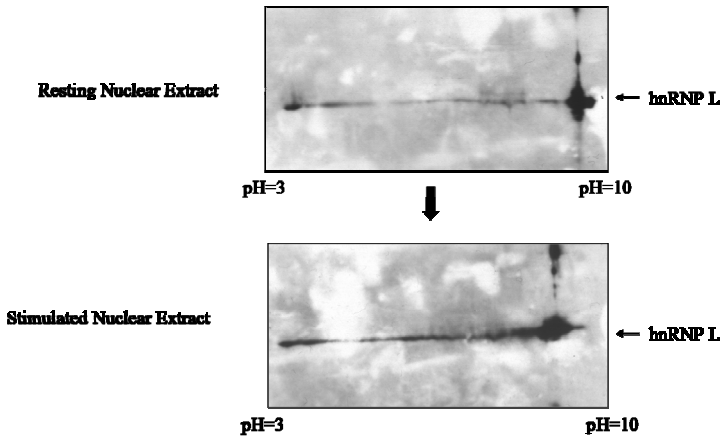

Figure 3-8: CIP Treatment Inhibits Isoelectric Focusing of hnRNP L in Two-Dimensional Gel Analysis. CIP treatment of nuclear extract prior to two-dimensional electrophoresis and Western blot analysis of hnRNP L leads to lack of separation in the first dimension as seen by hnRNP L detection across all pH values.

hnRNP L was also analyzed using phospho-spcific antibodies. Immunoprecipitation of hnRNP L followed by Western blot analysis using either phospho-tyrosine or phospho-threonine specific antibodies showed no band at the predicted size of hnRNP L in either resting or stimulated immunoprecipitations, leaving these experiments largely inconclusive (Figure 3-9).

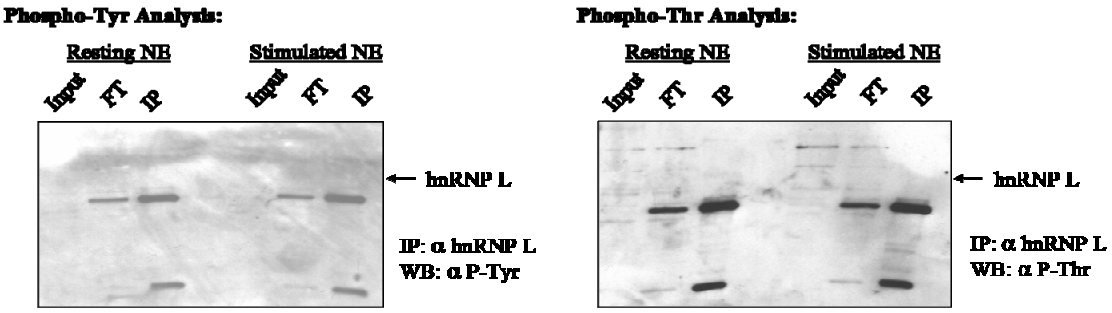

Figure 3-9: Phospho-Tyrosine and Phospho-Threonine Analysis of hnRNP L Shows No Tyrosine or Threonine Phosphorylation. Following immunoprecipitation (IP) of hnRNP L from nuclear extract (NE), Western blot analysis with phopho-tyr or phospho-thr specific antibodies show no signal at the expected size of hnRNP L (indicated by arrowhead). Western blot of these samples for hnRNP L shows significant enrichment of hnRNP L in immunoprecipitated samples (data not shown).

Given the uncertain results presented above, hnRNP L was analyzed for additional post-translational modifications. One other modification known to affect the migration of a protein by 2D gel analysis is acetylation, where a decrease in acetylation causes an acidic shift in pI. Using immunoprecipitation and Western analysis similar to that used for the phospho-specific antibodies described above, a band is observed migrating at the appropriate size for hnRNP L following Western blot analysis using an α-acetyl-lysine antibody (Figure 3-10). The acetylated-lysine band is

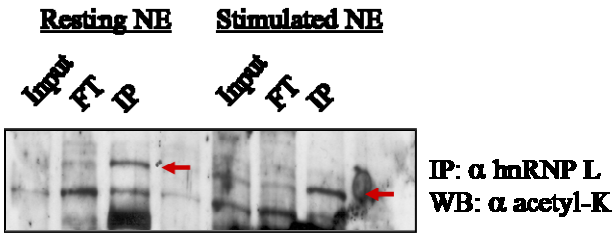

Figure 3-10: HnRNP L Shows Acetylation on Lysine Residues. Following immunoprecipitation (IP) of hnRNP L from nuclear extract (NE) and Western blot analysis using an antibody specific for acetylated-lysine residues, a band is observed at the expected size of hnRNP L (red arrows), suggesting hnRNP L is acetylated under both resting and stimulated conditions. Western blot analysis of hnRNP L shows the expected enrichment of hnRNP L following immunoprecipitation (data not shown).

observed following purification of hnRNP L from both resting and stimulated conditions (Figure 3-10), suggesting again that this modification may be present under both conditions and the change in migration pattern is due to a change in the modification state and not to the presence of absence of any single modification.

Two other post-translational modifications were also evaluated with regards to hnRNP L. First, sumoylation of hnRNP L was evaluated by lysing either resting or stimulated JSL1 cells with an inhibitor of desumoylation (NEM; Dadke et al., 2007) and analyzing hnRNP L by Western blot. Sumoylation of hnRNP L would be observed as a decrease in mobility of the protein or smearing upwards of hnRNP L on the gel; however, no decrease in mobility was observed following lysis in NEM (Figure 3-11), suggesting hnRNP L is not sumoylated under these conditions. Secondly, recent reports have begun to elucidate a functional role for the addition of the monosaccharide, N-acetylglucosamine (Vosseller et al., 2002; Vosseller et al., 2001; Wells et al., 2001). This modification is the O-linked addition of β-N-acetylglucosamine (O-GlcNAc) to serine or threonine residues of nucleocytoplasmic proteins (Vosseller et al., 2002; Vosseller et al., 2001; Wells et al., 2001). Importantly, the O-GlcNAc modification has been reported to

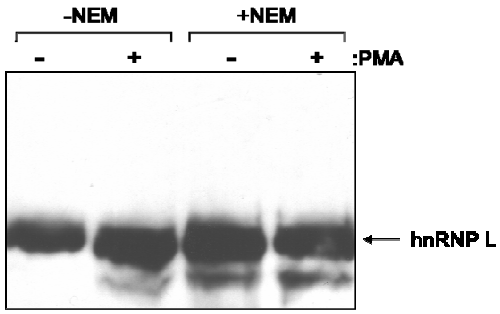

Figure 3-11: Analysis of hnRNP L for Modification by SUMO Shows No SUMO Addition. Following treatment of JSL1 cells with PMA, cells were analyzed in the presence of a desumoylation inhibitor (NEM). Western blot analysis of cell extracts for hnRNP L show no decrease in migration (upward shift) in the presence NEM as would be expected if hnRNP L were subject to modification by SUMO.

change in a protein specific manner in response to T cell activation (Kearse and Hart, 1991), and recent reports have provided some evidence for an antagonistic role of this modification with phosphorylation of serine and threonine residues (Wells et al., 2001). Following treatment of JSL1 cells with an inhibitor to prevent removal of this modification (PUGNAc; Vosseller et al., 2002), Western blot analysis with an O-Glc-NAc specific antibody (Comer et al., 2001) showed no band migrating at the size of hnRNP L after immunoprecipitation (Figure 3-12), suggesting hnRNP L may not be subject to O-GlcNAc modification.

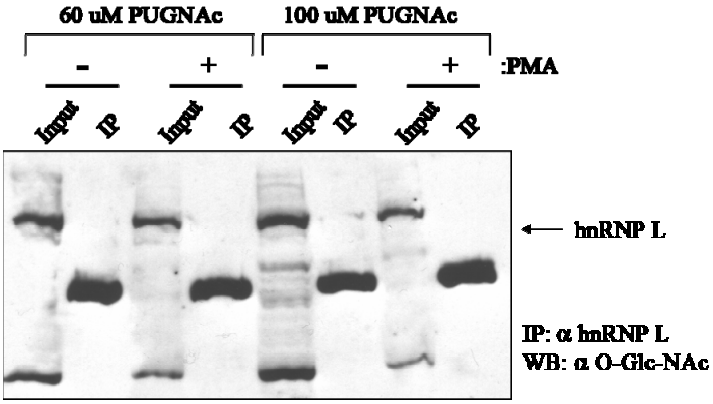

Figure 3-12: HnRNP L Does Not Show Modification by O-Glc-NAc. Analysis of O-Glc-NAc modification using an O-Glc-NAc specific antibody and treatment with an deglycosylation inhibitor (PUGNAc) shows no band at the expected size of hnRNP L following immunoprecipitation (IP). Western blot analysis using hnRNP L specific antibody shows the expected enrichment of hnRNP L following immunoprecipitation (data not shown).

One final method used to analyze the modification status of hnRNP L was performed in collaboration with YingMing Zhao's laboratory (UT Southwestern, Department of Biochemistry). HnRNP L was purified from either resting or stimulated nuclear extracts using a biotinylated-$(CA)_{20}$ RNA sequence (see Chapter 7) and analyzed using mass spectrometry to identify modified peptides. Using this approach, the location and identification of almost any modification can be identified based upon the change in

molecular mass. Unfortunately, the mass spectrometry analysis did not identify any modified peptides in either resting or stimulated samples of purified hnRNP L, despite almost complete coverage of the full-length protein.

Differential Post-Translational Modifications of hnRNP L Correlate with a Modest Increase in Silencing Activity

To determine if the change in modification of hnRNP L correlates with altered silencing activity of this protein, endogenous hnRNP L was purified from resting and stimulated cells using RNA affinity (see chapter 7), and the relative activity of these proteins was tested using *in vitro* splicing assays. As reported previously, addition of bacculovirus-produced recombinant hnRNP L to resting nuclear extract increases repression of the WT

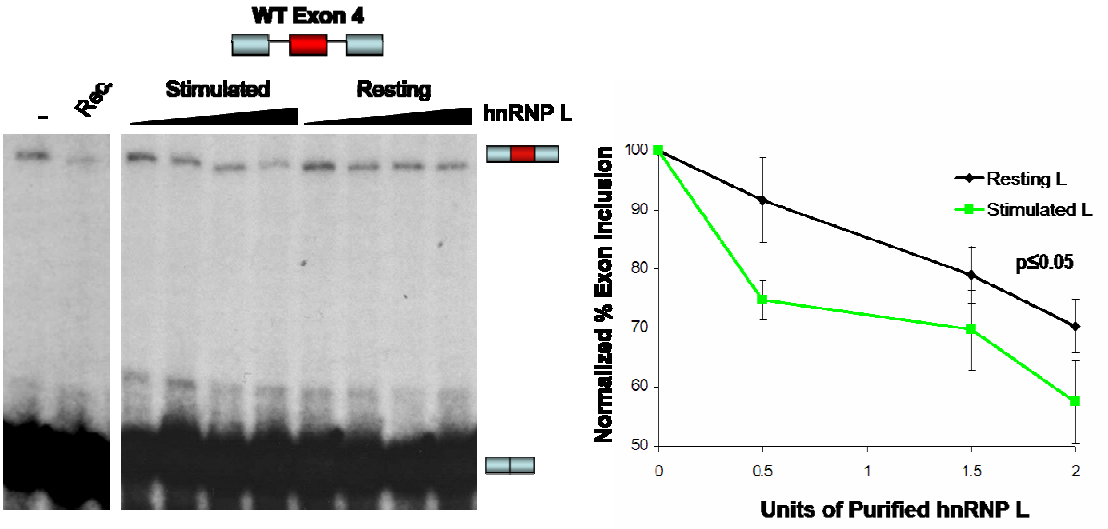

Figure 3-13: Stimulated hnRNP L Shows Increased Silencing Activity Compared to Resting hnRNP L. Equal amounts of hnRNP L purified from either resting or stimulated NE were titrated into *in vitro* splicing reactions containing the WT CD45 minigene and NE prepared from resting JSL1 cells (see Chapter 1). HnRNP L purified from stimulated NE causes increased exon silencing when compared to hnRNP L purified from resting NE. Addition of 100ng of recombinant GST-hnRNP L (rec.) is shown as a positive control. Graph represents quantitation of *in vitro* splicing with all values being normalized to exon inclusion in the absence of hnRNP L addition (set to 100%). Statistical analysis reveals the difference between the activity of resting hnRNP L and stimulated hnRNP L is significant ($p<0.05$).

CD45 exon 4, resulting in a decrease in three-exon product (Rothrock et al., 2005; Figure 3-13). Similar results were obtained upon addition of endogenous hnRNP L purified from JSL1 cells (Figure 3-13). Notably, hnRNP L purified from stimulated cells exhibits an increase in repressive activity compared to equivalent levels of protein purified from resting JSL1 cells (Figure 3-13, resting vs. stimulated). Quantification of the effect of hnRNP L titration over multiple independent experiments confirms that the difference in repressive activity of hnRNP L from resting and activated cells, while relatively modest, is statistically significant (Figure 3-13, $p<0.05$). Thus, the change in migration of hnRNP L by 2D gel analysis correlates with an increase in the silencing activity of the hnRNP L protein in *in vitro* splicing assays, suggesting that differential modifications of hnRNP L influence its repressive activity.

Increase in Silencing Activity of hnRNP L is Unlikely to Account for the Total Increase in CD45 Exon Repression Following Stimulation

Importantly, however, though the change in hnRNP L activity in activated cells is consistent with increased repression of CD45 exon 4, it is sufficiently modest that it is unlikely to account for more than a small portion of the 3-5 fold increase in exon repression observed upon stimulation. HnRNP L knock-down experiments in JSL1 cells further suggest that additional mechanisms contribute to the increase in exon silencing following stimulation. Previously, Rothrock et. al. have shown that a 50% reduction in hnRNP L by RNAi in 293T cells results in a significant reduction in the skipping of CD45 variable exon 4 (Rothrock et al., 2005). JSL1 cells have been previously reported to be refractory to protein knock-down using RNAi techniques (Rothrock et al., 2005); however, using morpholino antisense

oligonucleotides targeted to block the translation initiation complex, one can achieve a similar level of hnRNP L knock-down in JSL1 cells resulting in a dose-dependent reduction in the silencing of exon 4 in both the wild-type minigene as well as the endogenous CD45 gene in resting cells (Figure 3-14, R and data not shown). The effect of hnRNP L depletion on splicing is ESS1-dependent, as no change is observed in the splicing of a construct lacking a functional ESS1 sequence (Figure 3-14, Mut1). In activated cells there is also a decrease in WT exon skipping when comparing morpholino-treated cells to mock-treated controls (Figure 3-14, WT, 0 vs. 5 nmol MO, S), consistent with hnRNP L contributing to the overall

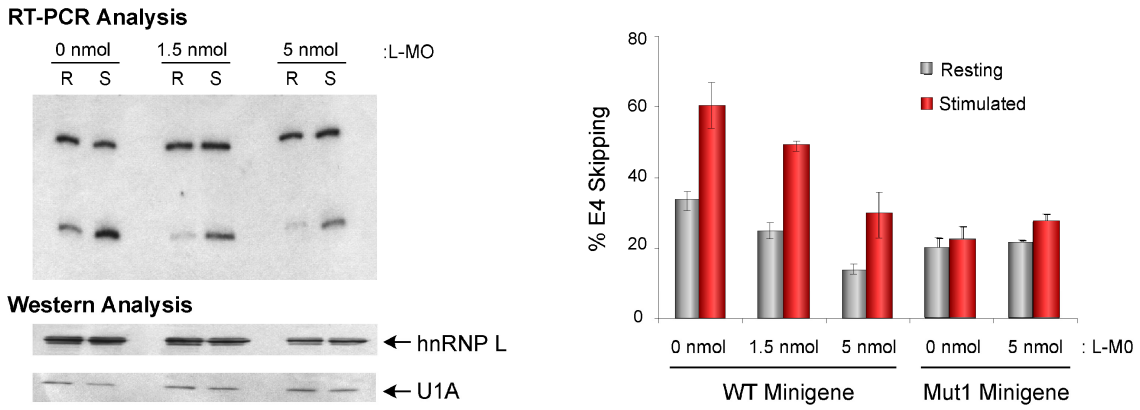

Figure 3-14: HnRNP L Depletion Causes a Decrease in Exon Skipping In JSL1 Cells. JSL1 cells transfected with the indicated amount of morpholino oligo show decreased levels of exon repression in resting and stimulated cells as determined by RT-PCR analysis 48 hours following treatment with PMA. Graph represents the quantitation of the splicing analysis of the wild-type as well as the Mut1 minigene. Protein knock-down was confirmed by Western blot. (Data contributed by Jason Jackson)

exon repression in both resting and activated conditions. In contrast, knock-down of PTB in JSL1 cells with a morpholino oligo has no effect on the basal or activation-induced splicing pattern (data not shown), in agreement with our previous *in vitro* studies (Rothrock et al., 2005). Nevertheless, while the absolute level of exon 4 skipping is decreased upon hnRNP L

knock-down, the morpholino-treated cells retain the ability to promote further exon skipping upon cellular activation, as evidenced by the difference in splicing in the absence and presence of PMA (Figure 3-14, 5 nmol MO, WT, R vs. S). The ability of cells to support signal-induced changes in CD45 splicing under conditions of limited hnRNP L is consistent with the modest contribution of hnRNP L to hyper-exon repression in activated cells. Together, the results presented in Chapter 3 suggest that while hnRNP L plays a major role in basal exon skipping in resting cells, and does contribute to exon 4 skipping in activated cells, additional proteins or mechanisms must exist which confer the majority of the increase in exon repression observed after cellular activation.

Discussion

This chapter describes the characterization of changes to the previously identified members of the ESS1 silencing complex following stimulation. Initial evaluation of nuclear expression level and ESS1 binding affinity of PTB and hnRNP E2 revealed no changes in protein expression after cellular activation. Evaluation of post-translational modifications of PTB and hnRNP E2 using 2D gel analysis showed no shift in migration pattern of these proteins when comparing resting and stimulated samples. While these results do not eliminate the possibility of altered post-translational modifications to PTB and hnRNP E2, these observations, in addition to work done previously demonstrating no specific functional role for either of these two hnRNP family members in the regulation of basal CD45 exon silencing (Rothrock et al., 2005), make PTB and hnRNP E2 unlikely mediators of ESS1-dependent activation-induced silencing.

Similar to PTB and hnRNP E2, hnRNP L showed no change in the level of nuclear protein expression or affinity for the ESS1 sequence following cellular stimulation. However, unlike PTB and hnRNP E2, a population of the nuclear hnRNP L showed an acidic shift in pI, indicating a change in the post-translational modification state of the protein. While several attempts were made at determining the nature and location of the activation-induced modifications of hnRNP L, no definitive conclusions regarding the nature and location of the modification can be made at this time. Much of the data evaluating specific post-translational modifications provided negative observations; however, as with the majority of negative observations, these results could be true negative observations indicating the absence of the specific modification being evaluated or could simply indicate lack of detection. The lack of identified modifications following mass spectrometry analysis is most likely caused by the lack of detection since post-translational modification of hnRNP L has been previously reported in the literature (Navakauskiene et al., 2004). As observed in the 2D gel analysis, the largest population of nuclear hnRNP L appears to be unmodified in both the resting and stimulated state; therefore, in the mass spectrometry analysis the most abundant, unmodified peptides may have been detected while the less abundant, modified peptides were missed. Thus, the mass spectrometry analysis would likely provide positive results regarding the nature and location of various modifications to hnRNP L following enrichment for the modified population of hnRNP L within the cell.

Despite the unknown nature of the modification, the activation-induced change in the modification state of hnRNP L correlates with an increase in the repressive activity of this protein, producing a modest increase in the skipping of CD45 exon 4. While the change in

the activity of hnRNP L serves as one mechanism by which cells cause activation-induced exon silencing, the magnitude of this effect suggests this is not the only regulation occurring in this system after stimulation. These observations are supported by the knock-down of hnRNP L in JSL1 cells, where the depletion causes an increase in the absolute level of exon inclusion under both resting and stimulated conditions but these cells maintain the ability to cause a decrease in the relative level of exon inclusion after PMA treatment. Thus, hnRNP L plays a major role in the basal level of exon repression under resting conditions as well as contributing to the signal-induced exon skipping following stimulation, but the data in this chapter also suggests that additional mechanisms exist which also contribute to the total increase in signal-dependent repression.

CHAPTER FOUR

Changes in Protein Composition of the ESS1-Binding Complex Following Stimulation

Introduction

Signal-regulated alternative splicing has become appreciated as one important mechanism regulating cellular gene expression in response to environmental stimuli. A recent paper identified several previously unknown alternative splicing events in T cells in response to antigen stimulation, expanding upon the previously reported alternative splicing events during the immune response (Ip et al., 2007). The regulation of most alternative splicing events is mediated by the binding of either SR or hnRNP proteins to cis-acting regulatory elements within the pre-mRNA transcript (Black, 2003); however, little characterization has been performed to identify changes in the RNA-binding complexes in response to signal transduction.

CD45 is an excellent model to study signal-regulated alternative splicing with its three variable exons being inducibly skipped in response to antigen stimulation of T cells (Lynch and Weiss, 2000). The splicing regulation of variable exon 4 is dependent upon a 60-nucleotide exonic splicing silencer (ESS1) that exhibits both basal and signal-induced silencing activity (Rothrock et al., 2003). HnRNP L binds to this sequence element and causes basal exon repression as well as making a small contribution to the increase in exon silencing after cellular activation (Rothrock et al., 2005); however, the magnitude of this increase in activity over that present in resting T cells is not sufficient to account for the total increase in exon skipping in activtated T cells.

In the current chapter, I describe the search for additional mechanisms regulating the activation-induced exon repression of CD45 vaiable exon 4. These studies identified several additional ESS1-binding components present under both resting and stimulated conditions, but functional evaluations have not shown these additional members of the hnRNP family to be required. Importantly, PSF and the highly related protein $p54^{nrb}$ were identified bound to the ESS1 complex only under stimulated conditions, and PSF is required to mediate a significant increase in exon repression under these conditions. While PSF is present in the nucleus under resting conditions, it does not bind or function, and it is currently unclear what regulates the signal-induced binding and function. Preliminary experiments show differential PSF-protein associations between resting and stimulated conditions that may play a role in PSF regulation in this system.

Results

In Vitro Splicing Recapitulates the Signal-Responsive Splicing Pattern of CD45

Previously, the lab has shown that the regulated splicing of the CD45 variable exons can be mimicked using minigenes expressed in the T cell derived JSL1 cell line (Lynch and Weiss, 2000). Consistent with the pattern of splicing of the endogenous *CD45* gene, RNA derived from a minigene in which CD45 variable exon 4 is flanked by constitutive exons 3 and 7 (Figure 4-1A, WT) shows only partial inclusion of exon 4 when expressed in resting JSL1 cells (basal exon repression), and a further 3-5 fold increase in exon 4 skipping when expressed in activated JSL1 cells (activation-induced exon repression)(Rothrock et al., 2003). The ESS1 splicing silencer sequence is necessary and sufficient for both the basal and

activation-induced repression of exon 4 *in vivo*, as evidenced in part by the fact that mutation or deletion of the ESS1 results in significantly higher levels of exon 4 inclusion in both resting and stimulated cells (Rothrock et al., 2003).

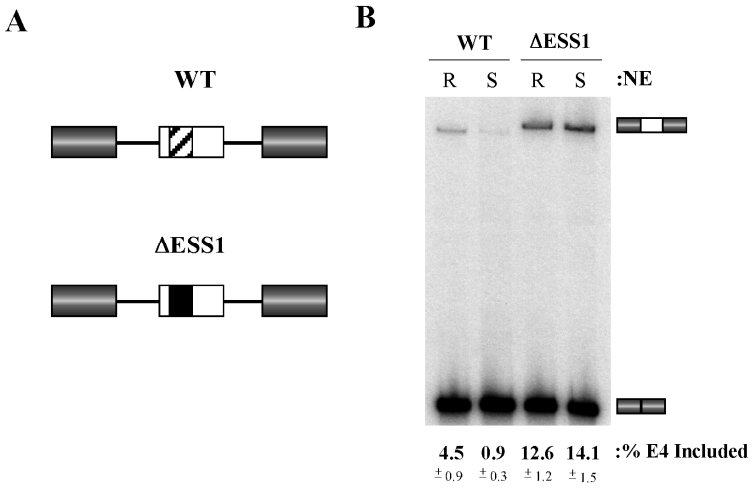

Figure 4-1: Endogenous CD45 Splicing Regulation is Reproduced *In Vitro*. (A) Schematic representation of the wildtype (WT) and ΔESS1 exon 4 minigenes. Variable exon 4 is flanked by constitutive exons 3 and 7 of CD45. The ESS1 sequence is represented by the crosshatched region and has been replaced in the ΔESS1 minigene. (B) *In vitro* splicing of the WT minigene showing basal exon repression with incubation in resting (R) nuclear extract (NE) and signal-induced exon repression in stimulated (S) nuclear extract. *In vitro* splicing of the ΔESS1 minigene shows loss of both basal and signal-induced exon repression.

As a first step toward characterizing the changes in splicing regulation that occur upon cellular activation, I sought to determine if nuclear extracts from resting and stimulated JSL1 cells contain the minimal factors necessary to confer the differences in isoform expression observed *in vivo*. Importantly, the signal-induced alternative splicing of CD45 exon 4 can be fully recapitulated *in vitro* using extracts derived from resting or stimulated JSL1 cells (see Chapter 7). As shown in Figure 4-1B, exon 4 is included in ~4% of products derived from *in vitro* splicing of the WT minigene in extract from resting cells (R-NE), while incubation of the same construct in extract from PMA-stimulated cells (S-NE) results in only ~1% inclusion of exon 4. This difference in exon 4 inclusion between resting and stimulated

extracts correlates with the same 3-5 fold increase in exon repression that is observed *in vivo* in both minigenes and the endogenous CD45 pre-mRNA. Importantly, this differential processing of exon 4 in extract from resting and activated cells is dependent on the ESS1 regulatory motif, since substitution of the ESS1 element with unrelated sequence of similar length (Figure 4-1A, ΔESS1) leads to increased inclusion of exon 4 in extract from resting cells, and no change in the level of exon inclusion between resting and stimulated extract (Figure 4-1B). Thus, nuclear extracts derived from resting and stimulated JSL1 cells support ESS1-dependent signal-induced changes in splicing to the same extent as observed *in vivo*.

Significantly, these results demonstrate that the change in isoform expression of CD45 observed upon T cell activation *in vivo* can be entirely accounted for by direct regulation of splicing and rules out alternative mechanisms of regulation by coordinated events such as transcription or mRNA export. In addition, these extracts provide a valuable tool for dissecting the changes in the ESS1-bound splicing regulatory complex that are induced upon activation to cause the signal-dependent silencing of exon 4.

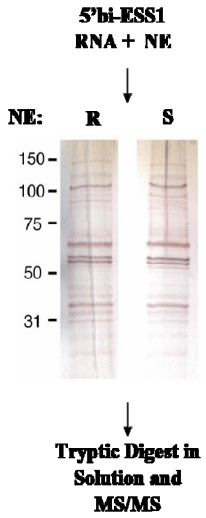

Figure 4-2: ESS1 RNA Affinity Purification. Biotinylated ESS1 RNA was incubated with nuclear extract and subjected to mass spectrometry analysis. Silver stain shows representative sample complexity and lack of detectable differences in banding pattern between resting and stimulated purifications.

PSF Mediates ESS1-Dependent Exon Silencing Specifically Under Stimulated Conditions

The Lynch lab has previously been unable to detect differences by silver stain in the protein

composition of the ESS1-bound complex purified from resting and stimulated extracts (Rothrock et al., 2005). However, since differences in protein composition could be below the detection limits of silver stain, I repeated the purification and subjected the entire sample to in-solution digestion with trypsin and analysis by mass spectrometry (Figure 4-2). The ESS1-associated proteins identified by multiple high-confidence tryptic peptides in the mass spectrometry analysis are listed in Figure 4-3. As anticipated from previous data, peptides derived from hnRNP L, PTB and hnRNP E2 were among the most frequently identified in both resting and stimulated purifications. In addition, several other members of the hnRNP family (hnRNP K, hnRNP D, hnRNP A1, and hnRNP A2) were also

Protein Name	Resting	Stimulated
hnRNP L	+	+
PTB	+	+
hnRNP E2	+	+
hnRNP K	+	+
hnRNP D	+	+
hnRNP A1	+	+
hnRNP A2	+	+
p54	-	+
PSF	-	+

Figure 4-3: Mass Spectrometry Protein Identification of ESS1 Purifications. HnRNP L, PTB, and hnRNP E2 associated with ESS1as reported previously in Rothrock et. al. (2005). These as well as the additionally listed hnRNP proteins were identified from resting and stimulated samples. HnRNP K, hnRNP D, hnRNP A1, and hnRNP A2 were not previously identified. PSF and $p54^{nrb}$ were identified after ESS1 purification in stimulated nuclear extracts and absent in purifications from resting nuclear extracts.

identified in both resting and stimulated samples (Figure 4-3). The most surprising result

from mass spectrometry, however, was the unique identification of PSF, and the highly-related protein p54^{nrb}, in the ESS1-purifications from stimulated nuclear extract (Figure 4-3).

To confirm and evaluate the specificity of the mass spectrometry results, RNA affinity purification experiments were repeated using a mutant version of ESS1 (Mut1) which abolishes both basal and activation-induced silencing activities of the sequence (Figure 5-1)(House and Lynch, 2006; Rothrock et al., 2003; Rothrock et al., 2005) and an unrelated RNA sequence that we have previously used as a non-specific control (House and Lynch, 2006; Rothrock et al., 2003; Rothrock et al., 2005). As reported before, hnRNP L, PTB, and hnRNP E2 each bind with higher affinity to ESS1 than to Mut1 or NS RNAs (Figure 3-3)(Rothrock et al., 2005). HnRNPs K and D exhibit similar specificity for ESS1 as observed for hnRNP L, PTB, and hnRNP E2 while hnRNP A1 and hnRNP A2 show a greater affinity for the control RNA sequence (Figure 4-4), suggesting that their presence in the ESS1 purification is due to weak background association. Importantly, however, the nuclear expression level, ESS1-association, and ESS1-specificity for each of these 7 hnRNP proteins remains unchanged between resting and stimulated conditions (Figure 4-3; Figure 4-4). Further evaluation of

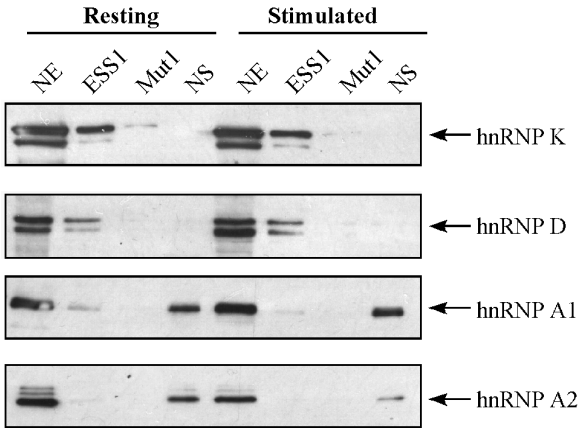

Figure 4-4: HnRNP K, HnRNP D, HnRNP A1, and HnRNP A2 Show Varied Affinities for ESS1 RNA. Western blotting of ESS1 purifications using either wildtype, mutant (Mut1), or nonspecific (NS) RNA show hnRNP K and hnRNP D bind to the wildtype ESS1 sequence with higher affinity than to mutant or NS RNA. Equal levels of these proteins are seen in resting and stimulated samples. hnRNP A1 and hnRNP A2 show increased levels of binding to the NS RNA over the ESS1 element.

functional roles for these additional hnRNPs has not demonstrated a requirement for these proteins in ESS1 activity (LMM, JJ, and KWL unpublished; data not shown).

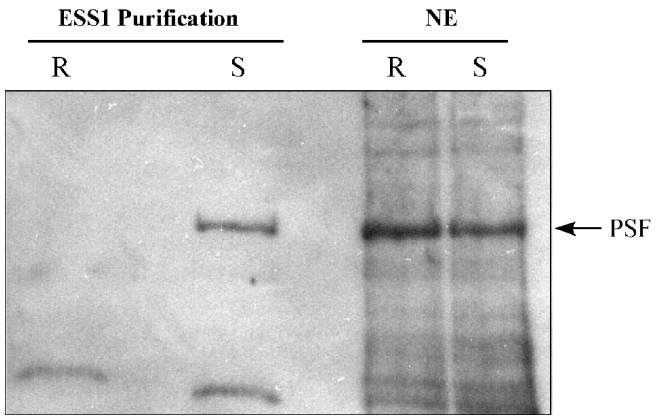

Figure 4-5: PSF Associates with the ESS1 Complex Only Under Stimulated Conditions. Western blotting of wildtype ESS1 RNA purifications show PSF association with incubation in stimulated nuclear extract but not in resting nuclear extract. Parallel analysis of total nuclear extract demonstrates equal levels of PSF expression in resting and stimulated nuclear extract.

In striking contrast to the results with the hnRNP proteins, western blot analysis of PSF binding to the wild-type ESS1 probe confirms robust PSF association with ESS1 only in purifications from stimulated extract (Figure 4-5, ESS1 purification). We note that this

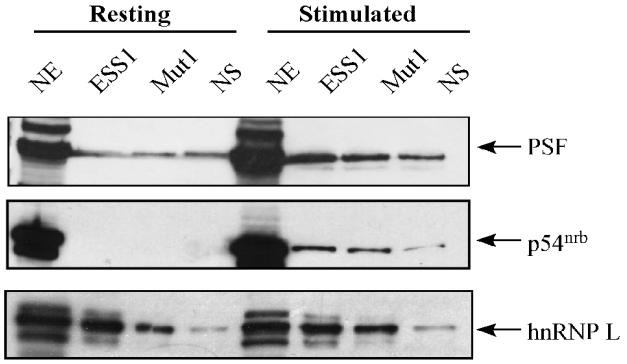

Figure 4-6: PSF and p54^{nrb} Show Increased Association with ESS1 Under Stimulated Conditions. Western blotting of RNA affinity purifications using wildtype, mutant (Mut1), and nonspecific (NS) RNA shows increased levels of PSF and p54^{nrb} binding to wildtype ESS1 over mutant or NS RNAs and increased association in stimulated purifications. HnRNP L is shown as a control.

change in ESS1-PSF association is independent of the level of nuclear PSF protein since western blot of total nuclear extract revealed no reproducible change in the expression of PSF protein between resting and stimulated conditions (Figure 4-5, NE). Further evaluation demonstrated that a weak non-specific interaction of PSF with ESS1 can be observed in resting nuclear extract upon overexposure; however, this level of bound PSF is similar to that associated with the NS RNA (Figure 4-6). In contrast, in activated extracts PSF exhibits enhanced binding to the ESS1 RNA relative to both the Mut1 and NS RNAs as well as relative to binding observed to any of the RNAs in resting cells (Figure 4-6). A similar specificity of ESS1-binding was obtained for $p54^{nrb}$, a protein highly homologous to PSF and often found together with PSF in cellular complexes. However, since PSF has been more clearly implicated in splicing than $p54^{nrb}$, and preliminary data from the knock-down of $p54^{nrb}$ suggest that it does not function to repress exon 4 (Figure 4-7), subsequent experiments focused solely on PSF.

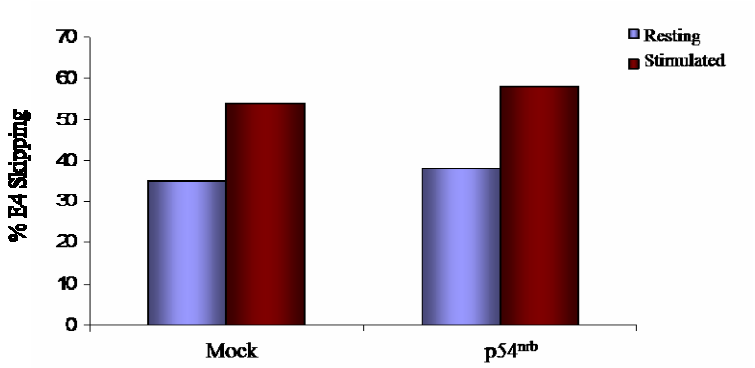

Figure 4-7: *In Vivo* Depletion of $p54^{nrb}$ Shows No Change in CD45 Exon Skipping After Stimulation. Graph represent the results from RT-PCR analysis of the wildtype exon 4 minigene following either mock transfection or $p54^{nrb}$ transfection of morpholino oligos. Following knock-down of $p54^{nrb}$, there was no change in the level of variable exon skipping under either resting or stimulated conditions when compared to mock treated JSL1 cells.

To determine if the association of PSF with the ESS1 sequence contributes to the increased repression of CD45 exon 4 in extract from activated cells, I evaluated the effect of inhibiting PSF in *in vitro* splicing assays. When monoclonal antibody directed against PSF is titrated into splicing assays using the wild-type minigene, a significant reduction in the level

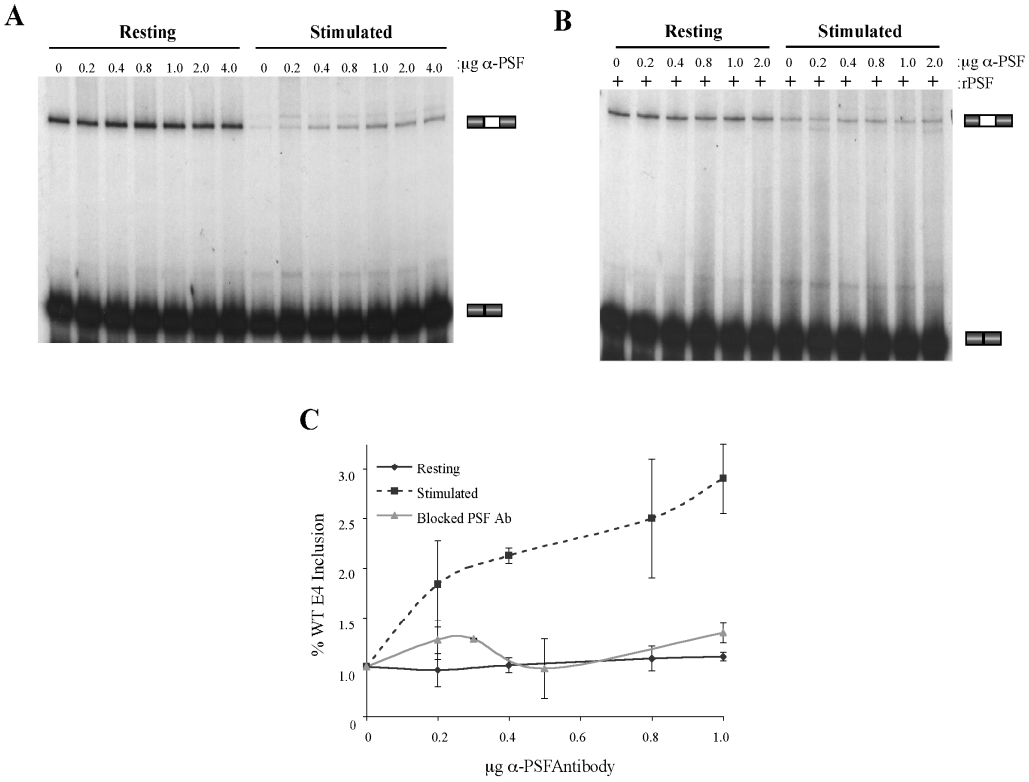

Figure 4-8: Functional Depletion of PSF Causes Increased Exon Inclusion in *In Vitro* Splicing Assays Only under Stimulated Conditions. (A) Titration of anti-PSF antibody to *in vitro* splicing assays with the wildtype exon 4 minigene causes an increase in exon inclusion specifically in stimulated samples with no change in resting samples. (B) Pre-incubation of anti-PSF antibody with inactive, recombinant PSF (rPSF) blocks the previously observed increase in exon inclusion in *in vitro* splicing asssys using the wildtype exon 4 minigene. (C) Graphical representation of quantitation of the above splicing assays.

of exon 4 skipping is observed in stimulated samples, leading to an increase in exon 4-containing product (Figure 4-8A, stimulated). Importantly, however, no change in the level of exon 4 inclusion is observed when the PSF antibody is titrated into resting extract (Figure 4-8A, resting). This effect is consistent with the fact that PSF is bound to ESS1 only under

stimulated conditions, and by extension, can only affect the splicing under these conditions. Quantitation of the PSF antibody effect on splicing is represented in the graph, demonstrating the dose-dependent and statistically significant ($p<0.05$) increase in exon inclusion under stimulated conditions (Figure 4-8C). It is of note, however, that the maximal effect of PSF

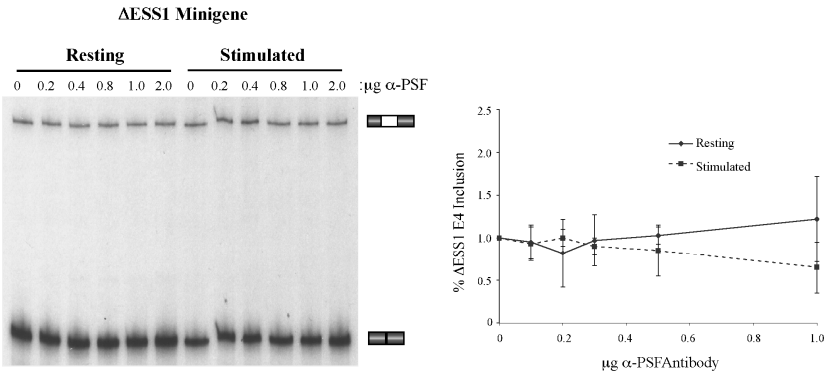

Figure 4-9: ESS1 Is Required for the Stimulation Specific Effects of PSF Depletion. Anti-PSF antibody titrated into *in vitro* splicing reactions containing the ΔESS1 minigene has no effect on the level of exon inclusion under resting or stimulated conditions. Numerical quantitation is shown in the graph.

antibody addition does not return the level of variable exon inclusion to the levels of inclusion seen under resting conditions (Figure 4-8A, compare first and last lanes).

The specificity of the PSF antibody effect was confirmed by pre-blocking the antibody with inactive recombinant PSF protein, which abrogates any effect of the antibody on splicing in stimulated extract (Figure 4-8B and C). Furthermore, the PSF antibody has no effect on the splicing of the ΔESS1 construct in either resting or stimulated extracts, demonstrating that the antibody only relieves ESS1-dependent exon silencing (Figure 4-9). In contrast to the addition of PSF antibody, the addition of hnRNP L antibody into *in vitro* splicing assays causes a decrease in the level of exon 4 skipping under both resting and

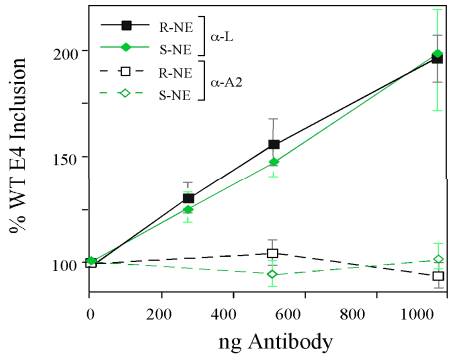

Figure 4-10: Addition of hnRNP L or hnRNP A2 Antibodies Does Not Show Stimulation Specific Effects *In Vitro*. *In vitro* splicing reactions using the wildtype exon 4 minigene show a similar increase in exon inclusion with the addition of anti-hnRNP L antibody under both resting and stimulated conditions. The addition of anti-hnRNP A2 antibody has no effect on exon inclusion under resting or stimulated conditions.

stimulated conditions, whereas the addition of hnRNP A2 antibody causes no change in the level of variable exon inclusion in either extract (Figure 4-10).

As additional functional confirmation, JSL1 cells expressing flag-tagged PSF at near endogenous levels were used to make nuclear extract under both resting and stimulated conditions (see Chapter 7). Flag-tagged PSF was purified from resting and stimulated nuclear extracts in parallel with a negative control purification using stimulated nuclear extract lacking flag-tagged PSF (see Chapter 7). *In vitro* splicing analysis was performed in the presence or absence of each of these purifications. The analysis of the wild-type exon 4 minigene shows no change in the level of variable exon 4 inclusion with the addition of either control or resting PSF purifications when compared to the addition of buffer alone (Figure 4-11A and D); however, the addition of flag-PSF purified under stimulated conditions causes a dramatic decrease in the level of variable exon inclusion as compared to either buffer alone or control purifications (Figure 4-11A and D). Similar analysis using a minigene containing CD45 variable exon 5 flanked by constitutive CD45 exons 3 and 7 provides the same pattern with only PSF purified under simulated conditions producing decreased levels of exon inclusion (Figure 4-11B and D). In parallel to the

previously described results, the addition of purified proteins to *in vitro* splicing reactions containing the ΔESS1 minigene shows no changes in the level of exon inclusion with the

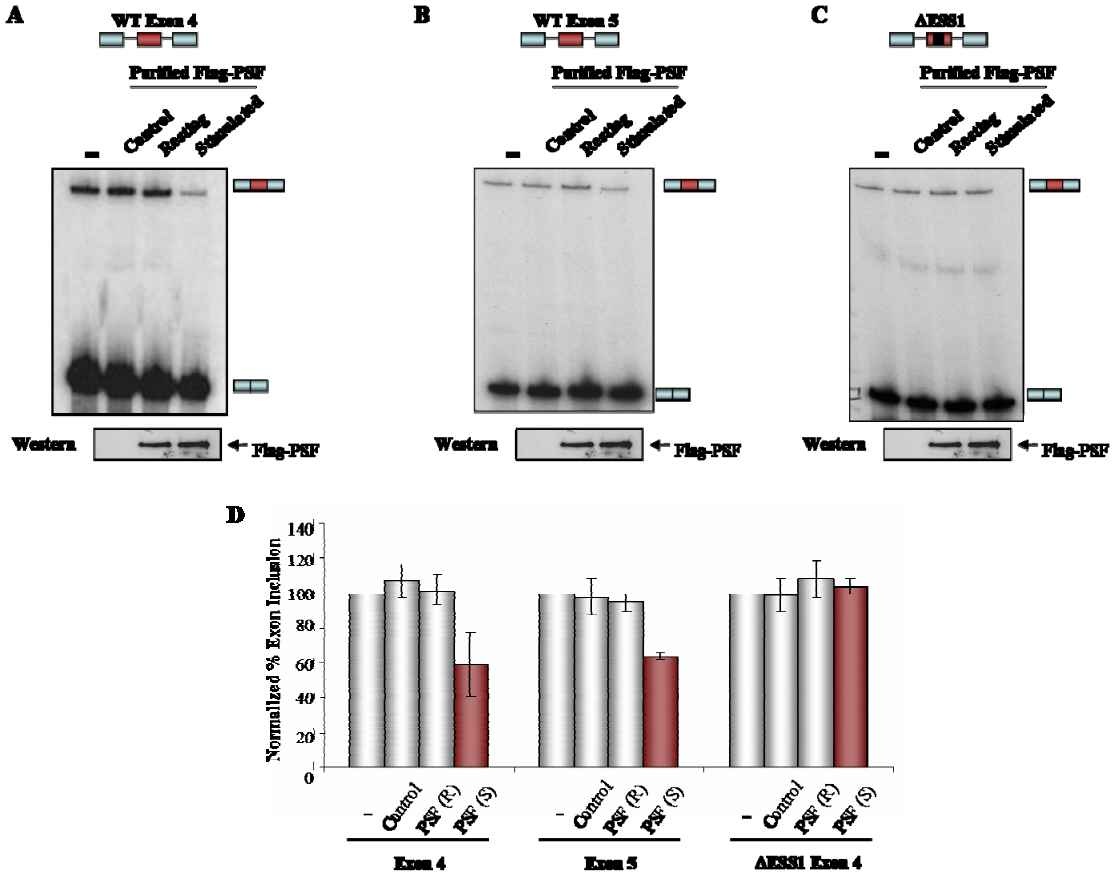

Figure 4-11: PSF Purified from Stimulated Cells but Not Resting Cells Causes ESS1-Dependent Exon Repression. Buffer (-), control, resting, or stimulated FLAG-PSF purifications were added to *in vitro* splicing assays containing the wildtype exon 4 (A), wildtype exon 5 (B), or ΔESS1 (C) minigenes. PSF purified under stimulated conditions causes decreased exon inclusion in the wildtype exon 4 and wildtype exon 5 minigenes while PSF purified under resting conditions has no effect. No effect on exon inclusion is observed in the analysis of the ΔESS1 minigene. Graphical representation of these results are shown (D).

addition of control, resting PSF, or stimulated PSF purifications (Figure 4-11C and D), demonstrating the activation-induced silencing activity of PSF is dependent upon the ESS1 element.

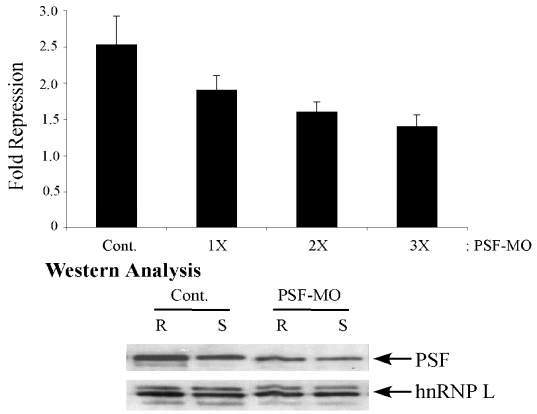

Figure 4-12: *In Vivo* Depletion of PSF Causes a Decrease in Response to Stimulation. Morpholino knock-down of PSF causes a decrease in signal-induced exon repression with increasing doses (1, 2, or 3 transfections) of PSF morpholino as compared to mock treated JSL1 cells. Western blot analysis shows a modest decrease in PSF protein levels with hnRNP L shown as a loading control.

In vivo studies of PSF have been complicated by the essential role of PSF in numerous nuclear processes. A morpholino that causes partial reduction of PSF protein reduces the change in CD45 splicing observed upon activation (Figure 4-12), but interpretation of these results is complicated by a number of pleotropic effects including a marked decrease in cell viability and general alterations in splicing. A requirement for normal expression of PSF to maintain viability is consistent with studies implicating PSF as an essential factor in numerous nuclear processes, including splicing, transcription, DNA unwinding and nuclear retention of mRNA (Shav-Tal and Zipori, 2002), and emphasizes the importance of the *in vitro* system which uncouples PSF-dependent regulation of splicing from other functional roles of this protein. We have also utilized a second morpholino that does not reduce PSF protein significantly and has no global effects on cellular splicing or viability. Not surprisingly, this second morpholino does not influence activation-induced splicing of CD45 on its own, but importantly, when CD45 splicing is sensitized by the reduction of hnRNP L, treatment of cells with the second morpholino does specifically reduce activation-induced silencing of CD45 exon 4 (Figure 5-4). Together the studies in

this section confirm a significant role for PSF in associating with ESS1 under activated conditions and regulating the signal-induced increase in ESS1-dependent silencing.

Differential PSF Protein Associations May Regulate Activation-Dependent PSF Binding and Function

The regulation of PSF addition to the ESS1-binding complex is critical to the understanding of the signal-induced exon repression of CD45 since PSF is the primary contributor to this increase in activity. Data presented previously in this chapter demonstrated that PSF is present in the nucleus at equal levels under resting and stimulated conditions, but is associated with ESS1 only after cellular activation (Figure 4-5). Additionally, PSF purified under resting conditions is not able to cause an increase in exon repression *in vitro*; however, PSF purified under stimulated conditions causes a striking decrease in exon inclusion (Figure 4-11). Further, two-dimensional gel analysis of PSF shows no significant change in migration between resting and stimulated samples (Figure 4-13), suggesting PSF is not differentially modified under these two conditions. At this time it is not understood what controls the binding and function of PSF in this system. PSF has been shown to play a role in many nuclear processes (Shav-

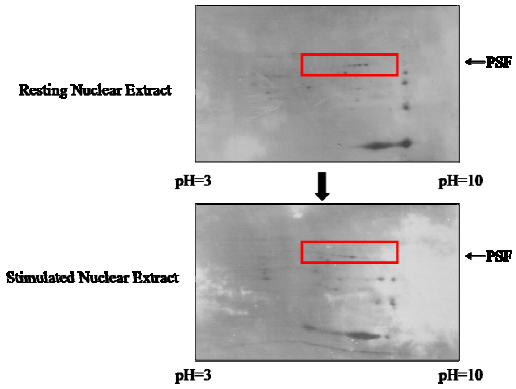

Figure 4-13: Two-Dimensional Gel Analysis of PSF Shows No Change in Isoelectric Focusing. Following two-dimensional gel electrophoresis and Western blotting, PSF shows no significant shift in migration when comparing resting to stimulated conditions (red boxes).

Tal and Zipori, 2002), and consistent with this diversity, PSF interacts with numerous nuclear and cytoplasmic factors, including PKCα and MEKK1(Peng et al., 2006; Rosenberger et al., 2002)(Melanie Cobb—personal communication). Therefore, it is possible that differential protein associations under resting and stimulated conditions regulate the activation-induced PSF addition and function.

To begin to identify potential differential protein interactions, flag-tagged PSF was purified using salt concentrations similar to those in *in vitro* splicing where PSF shows function (see Chapter 7). Silver stain analysis of these purifications shows differential banding patterns under resting and stimulated conditions (Figure 4-14). The most striking of these differences include an increase in intensity of a band running at approximately 55 kDa and a decrease in intensity of a doublet at approximately 50 kDa after stimulation (Figure 4-14). As a first step toward identifying functionally relevant interactions, I performed Western blot analysis for known PSF-interacting proteins (Figure 4-15). The majority of the proteins evaluated were present at approximately equal levels in both resting and stimulated purifications. These proteins include hnRNP L, hnRNP K, p54nrb,

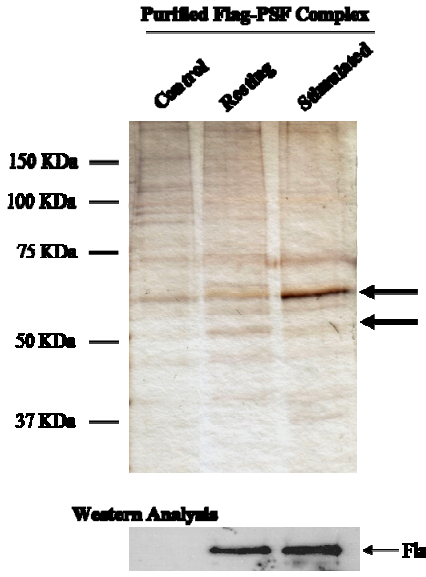

Figure 4-14: Silver Staining of Flag-PSF Purifications Show Different Banding Patterns from Resting and Stimulated Samples. Immunopurification of flag-tagged PSF and its associated proteins shows differential banding patterns from resting and stimulated nuclear extracts. Arrows indicate two of the most striking changes.

U1A, PKCα, and MEKK1; however, PTB associates more highly with the PSF complex under resting conditions as compared to stimulated conditions (Figure 4-15).

Further evaluation of these PSF-associated proteins was performed by subjecting these complexes to high salt washes and RNase A treatment (Figure 4-16). Comparing a high salt wash to a low salt wash, some of the interactions were reduced (hnRNP L, U1A), some proteins were lost completely (PTB, hnRNP K, PKCα), and others were unaffected (p54, MEKK1) (Figure 4-16). Treatment with RNase showed similar results to those obtained following a high salt wash with some interactions being decreased, some lost, and some unaffected when compared to purifications performed under low salt conditions (Figure 4-16). Thus, the association of these interacting factors display differential binding affinities for the PSF complex and differential requirements for RNA in the stability of the complex.

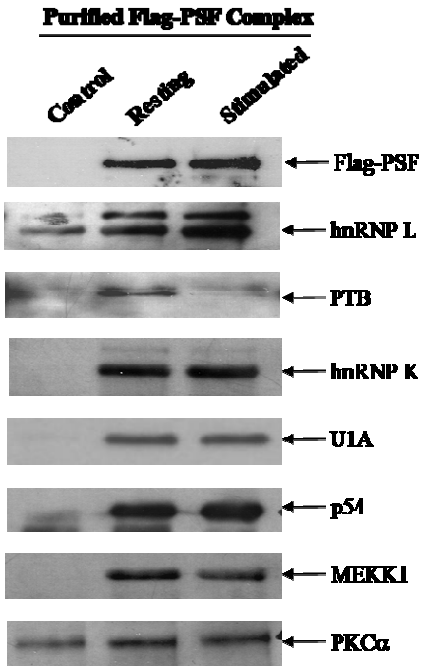

Figure 4-15: Western Blot Analysis of PSF Interacting Proteins from Resting and Stimulated Flag-PSF Purifications. Western blot analysis for the indicated proteins in control (no flag-tagged protein), resting flag-PSF, or stimulated flag-PSF purifications performed under low-stringency conditions (100 mM KCl binding and wash). Flag-PSF blot shows equal levels of tagged-PSF in each purification.

Protein	Low Salt		High Salt		+RNase	
	R	S	R	S	R	S
hnRNP L	++	++	+	+	-	-
PTB	++	+	-	-	+/-	-
hnRNP K	++	++	+	+	+/-	+/-
U1A	++	++	+	+	+	+
$P54^{nrb}$	++	++	++	++	++	++
MEKK1	++	++	++	++	++	++
PKCα	+	+	-	-	-	-

++ Strong Interaction
+ Weak Interaction
+/- Very Weak Interaction
- No Interaction

Figure 4-16: PSF Interacting Proteins Show Differential Sensitivity to High-Salt and RNase Treatment. Western blot analysis was performed as in Figure 4-15 following washing in high-salt (500 mM KCl) or pretreatment with RNase A. Low-salt conditions represent the results shown in Figure 4-15. Each of the PSF interacting proteins shows individual changes in binding following washing in high-salt or RNase treatment.

Discussion

In this chapter, I describe the identification and functional evaluation of the signal-induced activity of PSF on the ESS1 element. PSF and $p54^{nrb}$ were identified as activation-specific ESS1 binding factors by mass spectrometry analysis of total ESS1 purifications from resting and stimulated cells. The total purification was subject to tryptic digestion and mass spectrometry analysis due to lack of observed differences in banding pattern by silver stain. This analysis identified several additional hnRNP family members bound to the ESS1 element under both resting and stimulated conditions. Follow-up analysis of these additional

hnRNP proteins has not demonstrated a functional requirement; however, it is possible these proteins provide increased stability to the ESS1 complex.

Functional evaluation of PSF demonstrated a critical role for this protein in mediating the increase in exon silencing after stimulation. The signal-dependent function of PSF was demonstrated by both the functional depletion of PSF using anti-PSF antibody and the addition of PSF purified from stimulated cells. Both the depletion and addition of PSF show the function of PSF is dependent upon the presence of the ESS1 sequence and is of much larger magnitude than the increase in silencing mediated by increased hnRNP L function.

While each of the functional PSF experiments were performed using extensive controls, neither technique provides direct evidence that the PSF protein is itself responsible for the increase in silencing function because the depletion of PSF may also remove associated proteins and the PSF added into these *in vitro* reactions was not purified to homogeneity. However, if PSF is not the functional protein causing this increase in silencing activity upon stimulation, the data proves that it is required and, at minimum, functions as a necessary adapter protein.

PSF function was also evaluated *in vivo* using a knock-down approach. The *in vivo* depletion of PSF resulted in the expected decrease in exon skipping in response to cellular activation; however, the visual evaluation of these cells shows decreased cell viability as well as changes in size and morphology. Given the involvement of PSF in a variety of nuclear functions, it is not surprising that even mild reduction in the level of PSF protein within the cell causes decreased viability, leading to various cellular changes and complicating the interpretation of the CD45 splicing analysis. Thus, while the data does show a decrease in

the fold repression in response to stimulation, the splicing pattern of CD45 shows some unexpected abnormalities that are most likely due to global changes in cellular function in response to decreased levels of PSF protein.

Preliminary experiments purifying PSF with its associated proteins show differential banding patterns by silver stain as well as changes in protein associations with increased stringency and RNase treatment. PTB was identified with increased association to the purified PSF complex under resting conditions and is likely to correspond to the silver stained band at approximently 50 kDa that decreases upon stimulation. The investigation of PSF protein interactions is still in its initial stages and further work should be performed to further identify and evaluate the PSF-associated proteins under resting and stimulated conditions. Mass spectrometry analysis of both the differential bands observed by silver stain and the total reaction should provide the needed identifications. Eventually, functional evaluation of the PSF-associated proteins may provide important information regarding the mechanisms of signal-dependent PSF function.

Therefore, PSF was found to bind to the ESS1 sequence and function in an activation-dependent manner, accounting for a significant amount of the increase in exon silencing after cellular activation. Given the numerous protein-protein interactions of PSF, the signal-induced binding and activity of this protein may be regulated by changes in protein interactions, and the initial evaluation of PSF-associated proteins under resting and stimulated conditions demonstrates apparent differences in these interactions.

CHAPTER FIVE

Interplay of hnRNP L and PSF in Mediating Activation-Induced Exon Repression

Introduction

The CD45 gene is known to be subject to signal-regulated changes in alternative splicing. The basal regulation of CD45 variable exon repression has been characterized previously leading to the identification of the ESS1 silencing sequence present within each of the three alternatively spliced exons (Rothrock et al., 2003; Tong et al., 2005). The ESS1 sequence was shown to be both necessary and sufficient to induce the endogenous pattern of CD45 splicing regulation under both basal and stimulated conditions (Rothrock et al., 2003). HnRNP L, PTB, and hnRNP E2 were identified as members of a basal ESS1 binding complex, and further investigation identified hnRNP L as the main functional regulator of basal exon inclusion (Rothrock et al., 2005).

In the previous chapters, differential hnRNP L modification was shown to correlate with a modest increase in the repressive activity of the protein after stimulation. Additionally, PSF was identified as binding to the ESS1 complex in a signal-dependent manner, leading to a more sizable increase in exon repression. Given that both of these mechanisms serve to increase the exon repression within the cell and neither effect individually is of sufficient magnitude to account for the total increase in silencing after activation, this chapter focuses on the combined effects of hnRNP L and PSF following cellular stimulation. Binding and functional studies suggest that the effects hnRNP L and

PSF are largely independent, and the combined effects of these two mechanisms produce a total effect equivalent to total activation-induced change in splicing.

Results

HnRNP L and PSF Demonstrate Combinatorial Regulation of Stimulated Exon Repression

Given that signal-induced exon 4 repression mediated by both an increase in hnRNP L activity as well as the addition of PSF to the ESS1-binding complex is observed, evaluation of the interplay between these two factors using various ESS1 mutants was undertaken. The full 60 nucleotide ESS1 sequence contains a short and long copy of the activation responsive sequence (ARS) motif (Figure 5-1, ARS motif underlined), so defined because mutation of the conserved C's in each ARS repeat abolishes both basal and activation-induced skipping of exon 4 *in vivo* and *in vitro* (House and Lynch, 2006; Rothrock et al., 2003; Figure 5-1, Mut1). However, the ARS motif alone is not sufficient to confer the complete activity of the ESS1 element, even when present in multiple copies (A. Tong and K.W.L. unpublished). Further mutational analysis of the ESS1 element reveals an important role for the sequence that acts as a spacer between the two copies of the ARS motif. Remarkably, substitution of two nucleotides between the short and long copy of the ARS motif has little effect on basal repression, but significantly reduces the fold increase in exon repression in stimulated extract (Fig. 5-1, Mut2, Fold Repression=1.6 vs. 3.5) as well as in activated cells (data not shown). Thus, basal silencing activity of the ESS1 sequence can be at least largely separated from the signal-induced silencing activity of this regulatory element both *in vivo* and *in vitro*.

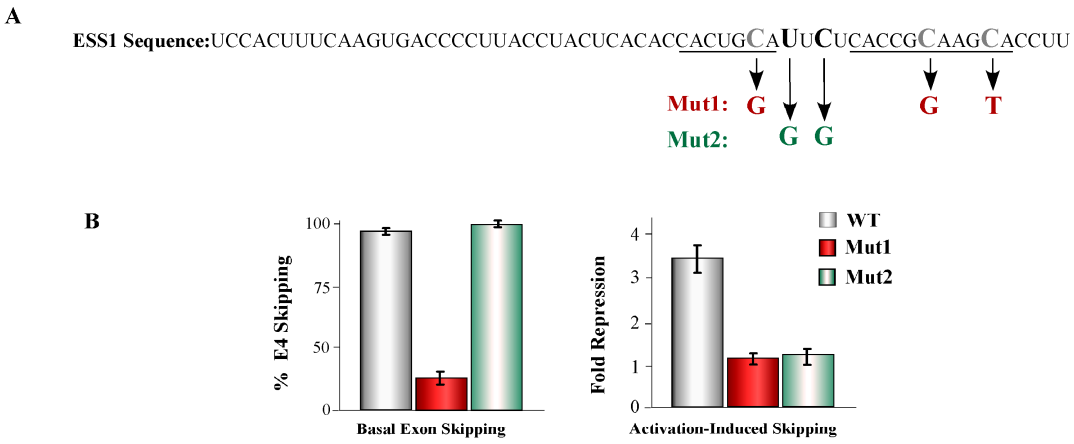

Figure 5-1: ESS1 Point Mutations Separate Basal and Activation-Induced Silencing. (A) The 60 nucleotide ESS1 element with the ARS motif underlined is shown. Nucleotide changes for Mut1 and Mut2 are as indicated. (B) *In vitro* splicing analysis demonstrates the Mut1 sequnce has lost both basal and activation induced silencing activity while the Mut2 sequence is only defective in mediating signal-regulated exon silencing when compared to the wildtype minigene. Quantitation of was performed as described in previous chapters. (Data contributed by Jiarong Wang)

The Mut1 mutation of the ESS1 element greatly reduces the affinity of hnRNP L for this sequence in resting cells, consistent with the loss of basal silencing of this mutation (Rothrock et al., 2005). The affinity purifications shown in chapters 3 and 4 strongly suggest that hnRNP L, as well as PSF, also have reduced binding to the Mut1 RNA under stimulated conditions. However, to more accurately assess the relative affinity of these proteins for the mutant versus wildtype ESS1 RNAs in stimulated extracts, competition assays were conducted to determine the ability of hnRNP L and PSF to discriminate between wildtype and mutant RNAs. As anticipated, addition of wildtype competitor to a binding reaction results in a dose-dependent decrease in the levels of hnRNP L and PSF associated with the biotinylated ESS1 probe (Figure 5-2, WT), while titration of the non-specific control RNA has little or no effect on the binding of these protein even at a 30-fold molar excess of competitor (Fig. 5-2, NS). In agreement with the direct affinity experiments in described in

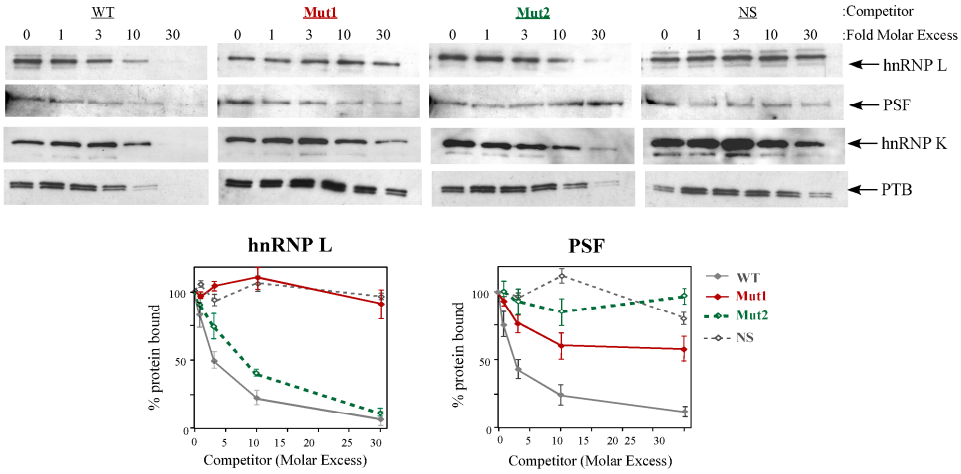

Figure 5-2: hnRNP L and PSF Show Differential Affinity for Mutant Sequences. Competition RNA affinity purification (see Chapter 7) was performed using biotinylated WT ESS1 probe and the indicated competitor sequence. Binding was evaluated by Western blotting. HnRNP L demonstrates minimal competition when the Mut1 sequence is added, whereas the addition of Mut2 competitor causes a decrease in hnRNP L binding. PSF binding is decreased following addition of Mut1 and unaffected following the addition of Mut2. Significant differential competition is not observed for the binding of hnRNP K and PTB to the mutant sequences. The addition of a nonspecific (NS) sequence to the reaction does not affect binding of any of the indicated proteins in a dose dependent manner. Graphs represent quantification of protein binding as determined by densitometry with values being normalized to protein binding in the absence of competitor RNA.

chapter 4, the Mut1 RNA also has little ability to compete for the binding of hnRNP L to the biotinylated ESS1, and has reduced affinity for PSF (Figure 5-2, Mut1), consistent with the inability of this mutant sequence to support either basal or activation-induced exon repression. In strikingly contrast to Mut1, Mut2 efficiently recruits hnRNP L, but not PSF, away from the biotinylated WT ESS1 RNA (Figure 5-2, Mut2). Therefore, Mut2 maintains the ability to bind to hnRNP L, consistent with the basal activity of this sequence; however, PSF is unable to associate with the Mut2 sequence and confer ESS1-mediated activation-induced exon skipping.

In addition to the experiments described above, similar competition experiments were performed for PTB and hnRNP K. Figure 5-2 also shows that both Mut 1 and Mut 2 compete equally for PTB and hnRNP K binding. The Mut1 and Mut2 sequences are less effective competitors than wild-type ESS1 as would be predicted from previous results (see

chapter 3 and chapter 4). Significantly however, the differential mutant binding observed for hnRNP L and PSF is absent.

The experiments with Mut1 and Mut2 suggest that the binding of hnRNP L and PSF to ESS1 is largely independent of each other. The autonomy of PSF and hnRNP L function is further implied by the residual activation-induced repression observed under conditions in which PSF activity is reduced by antibody or the Mut2 mutation (Figure 5-1 and Chapter 4), consistent with the remaining activation-induced increase mediated by an increase in hnRNP L function (See Chapter 3). Moreover, knock-down of hnRNP L does not significantly weaken the activation-induced repression of ESS1-dependent splicing (See Chapter 3), presumably due to the continued activity of PSF.

If hnRNP L and PSF do indeed function separately to repress the ESS1-containing exon 4, then blocking both hnRNP L and PSF in extract from activated cells should function in a combinatorial manner to return the level of exon inclusion to that of resting extract. As predicted, while addition of either hnRNP L or PSF antibody alone to stimulated extract causes a partial increase in the level of variable exon 4 inclusion, the addition of both antibodies has a markedly greater effect on exon inclusion, returning the level of exon inclusion in stimulated extract to nearly that observed under

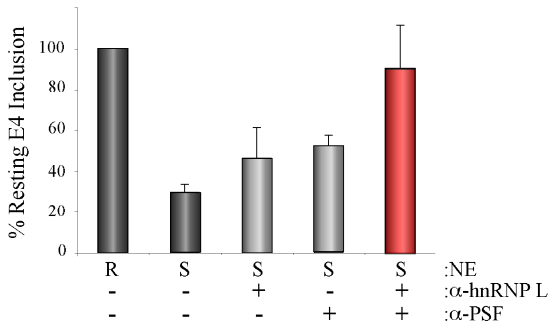

Figure 5-3: Inhibition of hnRNP L and PSF under Stimulated Conditions Return Levels of Exon Inclusion to Resting Conditions. *In vitro* splicing reactions were carried out in the presence of the indicated antibodies. Percent exon inclusion was quantitaed and normalized to the level of exon inclusion in resting nuclear extract in the absence of antibody addition. While the addition of hnRNP L antibody or PSF antibody individually cause an increase in exon inclusion, only when both antibodies are added in combination to levels do levels of exon inclusion in stimulated nuclear extracts equal those observed in resting nuclear extracts.

resting conditions (Figure 5-3). In contrast, addition of an equal mass of hnRNP A2 antibody to the reaction does not alter splicing nor is there a similar combinatorial increase in exon inclusion upon addition of hnRNP L and PSF antibodies to resting extract (data not shown).

Further evidence for the combinatorial regulation of CD45 exon 4 by hnRNP L and PSF comes from treatment of JSL1 cells with morpholino oligos targeting both proteins. As described in chapter 4, a morpholino targeting PSF that does not cause global changes in splicing and cell viability (PSF2 MO, see Chapter 7) also has no effect on CD45 splicing on its own (Figure 5-4, PSF). However, treatment of cells with this PSF morpholino in combination with the morpholino targeting hnRNP L results in a specific decrease in silencing of exon 4 in activated cells, with little alteration of splicing in resting cells (Figure 5-4, L vs. PSF+L). The combined effect of inhibition of PSF and hnRNP L under activated conditions both *in vivo* and *in vitro* strongly argues that the activation-induced skipping of CD45 exon 4 can be entirely accounted for by the combinatorial effects of hnRNP L and PSF within the ESS1-silencing complex.

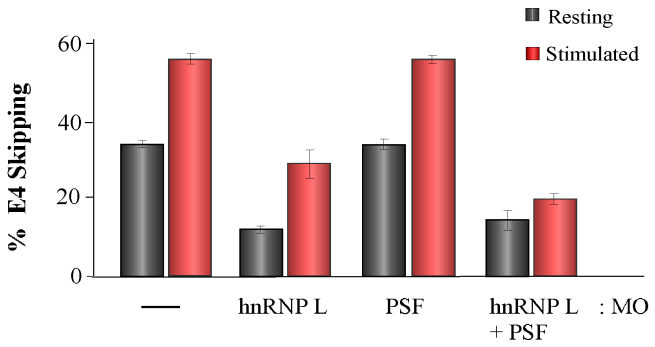

Figure 5-4: Knock-down of hnRNP L Sensitizes Cells to Knock-down of PSF and Causes a Decrease in Activation-Induced Silencing. Morpholino oligo knock-down of PSF causes no change in exon skipping and no change detectable change in protein level (data not shown) when added in isolation. However, PSF knock-down in combination with morpholio knock-down of hnRNP L causes a profound decrease in the signal-induced exon skipping observed when hnRNP L levels are decreased in isolation. (Data contributed by Jason Jackson)

Discussion

While the previous chapters characterize the individual contributions of hnRNP L and PSF to the activation-dependent change in CD45 splicing, the above experiments characterize the interactions between these two proteins in the context of ESS1 RNA and begin to develop a more complete biological picture of CD45 splicing regulation. While the functional contribution of PSF to exon silencing is significant, it does not account for the total increase in fold repression observed (Figure 5-3 and Chapter 4). Additionally, the functional change in repression caused by differential modification of hnRNP L is admittedly a modest effect (see Chapter 3). However, the experiments in this chapter demonstrate that when combined these two mechanisms produce an effect that approximates the total change in exon silencing both *in vitro* and *in vivo*.

The binding affinity of hnRNP L and PSF to the Mut1 and Mut2 sequences, correlated with the function of those mutant sequences, again supports PSF as the major functional contributor to signal-induced exon silencing with hnRNP L functioning mainly as the regulator of basal exon silencing. The binding data also suggest the binding of PSF is largely independent of hnRNP L, making it unlikely that the changes in hnRNP L modification are responsible for the activation-dependent recruitment of PSF to the ESS1 sequence. However, the sensitization of the system to PSF knock-down following a reduction in hnRNP L levels suggests that there may be some crosstalk between these effects. It is possible that while binding of hnRNP L and PSF is largely independent the overlap occurs at the functional level. In this case, depleting hnRNP L would cause small perturbations in the level of PSF to have a larger functional effect on exon repression as

observed *in vivo* when cells are treated with morpholio oligos to both hnRNP L and PSF. Thus, while individual effects of hnRNP L and PSF are not dependent upon one another, the *in vitro* splicing as well as the knock-down experiments presented in this chapter demonstrate increased functional efficiency when both mechanisms are in operation, and both hnRNP L and PSF are required to obtain the total increase in signal-dependent exon silencing following cellular activation.

CHAPTER SIX

Conclusion

While signal-induced alternative splicing of CD45 exon 4 via the ESS1 sequence is well documented, there has been no report of the mechanisms by which the activity of this silencer is induced upon cellular activation. In chapters 3, 4, and 5, I demonstrate that the signal-induced change in isoform expression observed *in vivo* is fully accounted for by a direct change in splicing and can be recapitulated in extracts from resting and activated cells. I have further identified the two major contributors to the activation-induced increase in exon repression mediated by the ESS1 element (Figure 6-1). A change in the modification state of hnRNP L upon stimulation correlates with an increase in the repressive activity of this protein, producing a modest increase in the skipping of CD45 exon 4. Thus, hnRNP L plays a major role in the basal level of exon repression under resting conditions as well as contributing to the signal-induced exon skipping following stimulation. A second, and more sizable, effect on activation-induced repression is produced by the addition of PSF to the ESS1-binding complex following activation. Importantly, the combined contributions of hnRNP L and PSF to ESS1-dependent silencing produce an effect that is of similar magnitude to the total observed increase in exon repression both *in vitro* and *in vivo*, suggesting that they confer most, if not all, of the mechanisms of signal-induced regulation in this system.

While I did identify four other proteins that bind with some specificity to the ESS1 element (Figure 6-1), at least two of these proteins (PTB and hnRNP E2) have little

contribution to the activity of ESS1 (Rothrock et al., 2005), and preliminary experiments have provided similar results for the remaining two (hnRNP K and hnRNP D). Moreover, we find no evidence for altered ESS1-association of any of these four hnRNP proteins between resting and activated conditions (Chapter 4).

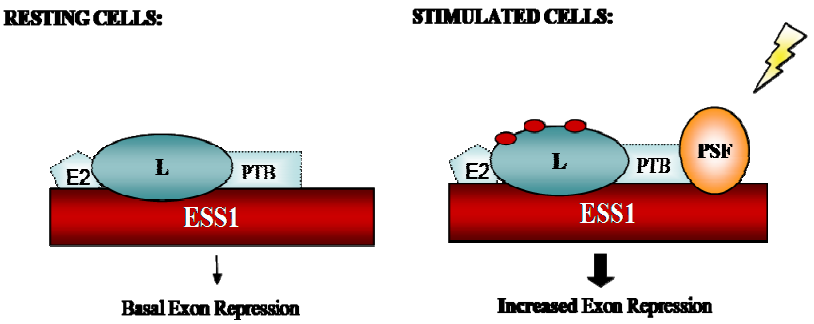

Figure 6-1: Model of ESS1-Binding Complex Under Resting and Stimulated Conditions. Under resting conditions, hnRNP L mediates basal exon silencing. After stimulation, the silencing function of hnRNP L is slightly increased over basal activity and PSF is added to the complex.

Mechanisms of Signal-Regulated Alternative Splicing via Post-Translational Modifications

Signal-induced changes in splicing patterns have been described to alter the expression of numerous genes and proteins (Shin and Manley, 2004; Tarn, 2007). However, the mechanisms by which specific cellular signaling events influence splicing regulatory proteins, and the subsequent effects of these changes on alternative splicing, are only beginning to be addressed. Several recent reports have characterized the nucleocytoplasmic regulation of hnRNP A1 in response to osmotic shock, in which induced phosphorylation of hnRNP A1 leads to its cytoplasmic retention (Allemand et al., 2005; Guil et al., 2006; van der Houven van Oordt et al., 2000). The corresponding decrease in nuclear hnRNP A1 results in alternative splicing of an E1A minigene reporter upon osmotic shock (Blaustein et

al., 2005; Patel et al., 2005; van der Houven van Oordt et al., 2000). Similar to hnRNP A1, the phosphorylation state of the SR proteins SF2/ASF, 9G8 and SRp40 have been shown to be regulated by the kinase Akt, in response to stimulation with growth factors or insulin (Blaustein et al., 2005; Patel et al., 2005). However, in contrast to hnRNP A1, phosphorylation of SF2/ASF by Akt does not change the localization pattern of SF2/ASF (Blaustein et al., 2005), suggesting that the phosphorylation state of this SR protein results in direct changes in its activity.

Signal-induced repression of CD45 exon 4 is due in part to the regulation of hnRNP L by post-translational modification as described for hnRNP A1. However, in this instance, modification of hnRNP L appears to function more similarly to Akt modification of SF2/ASF, in that we observe no change in the nuclear concentration of this protein, nor any change in its affinity for ESS1, despite the fact that we do observe an increase in hnRNP L activity. While the signal-induced changes in the modification state of hnRNP L have yet to be identified, an acidic shift in pI is often observed following an increase in the phosphorylation state of a protein. Thus, it is a reasonable assumption that the change in migration of hnRNP L in stimulated extract is due to an increase in phosphorylation. Regulation of hnRNP L by phosphorylation would also be consistent with known mechanisms of hnRNP regulation and thus provides a reasonable candidate for further investigation.

Regulated Recruitment of PSF to a Pre-mRNA Substrate is a Unique Means of Controlling Splicing

In contrast to the above mechanisms of altered localization or activity, the activation-dependent association of PSF to the ESS1-binding complex provides a unique example of the specific recruitment of a splicing factor to a regulatory sequence in response to signaling events. The mechanisms controlling the binding of PSF to the ESS1-regulatory complex remain unknown; however, the equal levels of nuclear PSF protein in resting and stimulated extract imply the binding of PSF is not regulated by changes in either protein expression or nuclear-cytoplasmic localization. Additionally, 2D gel analysis of PSF shows no evidence for changes in the modification state of PSF itself. Finally, mutational data as well as hnRNP L knock-down experiments suggest that under conditions of limited hnRNP L binding, PSF is still recruited to the ESS1 complex following stimulation, suggesting the change in the modification state of hnRNP L is not responsible for PSF binding. Therefore, the signal-induced addition of PSF to the ESS1-binding complex must be regulated by additional mechanisms.

One appealing mechanism of PSF regulation involves signal-induced changes in PSF association with other nuclear proteins. While PSF was initially identified as the binding partner of PTB (Patton et al., 1993), it has been shown to interact with many other nuclear proteins as well as both DNA and RNA (Peng et al., 2006; Shav-Tal and Zipori, 2002). Many of these interactions have been shown to be dynamic and regulated by various cellular events, including altered interaction with PKC in response to phorbol ester treatment and altered subnuclear localization and interactions with other splicing proteins upon apoptosis (Rosenberger et al., 2002; Shav-Tal et al., 2001). Thus, PSF may exist in a protein complex in nuclei of resting T cells that precludes its binding to the ESS1 element. In such a scenario,

cellular activation would be predicted to release PSF from sequestration, thereby allowing PSF recruitment to the ESS1-binding complex and an increase in exon skipping. Further study is still needed to determine if PSF association with CD45 exon 4 is indeed controlled by such a mechanism; however, preliminary PSF purifications from JSL1 cells demonstrate differential banding patterns following silver stain analysis, suggesting a promising outcome.

Combinatorial Control of Signal-Regulated Alternative Splicing

Of particular interest in this system is the fact that the overall signal-induced regulation of exon 4 is not controlled by a single mechanism, but by the separate activities of hnRNP L and PSF, which combine to yield the total increase in exon skipping. Combinatorial control of signal-induced alternative splicing of CD45 and other genes has been previously suggested by studies demonstrating cross-talk and/or redundancy in signaling pathways necessary for a particular splicing phenotype (Lynch and Weiss, 2000; Pelisch et al., 2005; Weg-Remers et al., 2001), but these studies have not investigated combinatorial changes in the RNA-bound splicing regulatory machinery. The use of multiple small effects to achieve a functionally significant change in splicing, as shown here for CD45, would be predicted to increase the specificity of signal-induced regulation, as only genes controlled by the specific combination of activated factors would be responsive to a particular stimulus. In addition, the requirement for the integration of multiple "hits" to alter splicing prevents accidental changes in splicing due to spurious activation of individual signaling cascades. Therefore, the combinatorial control of signal-induced splicing, such as

that identified here for CD45, may prove to be a more global mechanism of achieving specific regulation of alternative splicing in response to precise extracellular cues.

Future Experiments

While I have identified two contributions mediating the increase in CD45 exon 4 repression in response to cellular activation, many questions remain regarding the mechanisms of regulation in this system. First, further evaluation of hnRNP L should be performed to identify the specific modifications present under resting and stimulated conditions. This information may provide important information for further understanding the details of hnRNP L function in both resting and stimulated cells. The previous analysis was most likely hindered by the large amounts of unmodified hnRNP L within the sample; therefore, additional purification steps could be performed to enrich for the modified population. Enrichment could be accomplished by 2D gel separation and staining prior to mass spectrometry analysis; although, separation using an ion exchange column could also provide the needed separation based upon the difference in charge. Once specific differences in modifications have been identified, mutation of these residues to either inhibit or mimic the modification can be made and functional analysis of the mutants evaluated. These types of experiments will provide direct evidence for the involvement of these modifications in mediating the increase in exon silencing after stimulation which is currently only a correlative observation.

In addition to functional evaluation, the nature and location of the modifications of hnRNP L may provide insight into the enzymes responsible for the modification addition or

removal. These are also important remaining questions in this system as the upstream pathways remain unknown, and the identity of the modifying enzymes will aid in characterizing the required signaling pathways.

Although the regulation of hnRNP L in response to T cell activation is important in this system, the larger increase in exon repression is mediated by the signal-induced addition of PSF to the ESS1-binding complex. Thus, the regulation of PSF under resting and stimulated conditions is of great importance and currently remains a mystery. As mentioned previously, the protein-protein interactions of PSF are numerous, and alterations in these interactions following stimulation provide a logical mechanism for regulation of PSF binding and function. Initial experiments have already shown PSF purifications under resting and stimulated conditions show differential banding patterns when subjected to silver staining. Additionally, PSF purified under the same conditions is able to induce exon silencing only when purified from stimulated cells and not from resting cells. Taking advantage of these techniques, mass spectrometry analysis of these purifications should provide the identity of the PSF-interacting proteins. With this information, differentially associated proteins can be evaluated for their role in the regulation of PSF binding and function.

Functional evaluation of the PSF complexes can also be performed to determine the required protein associations. PSF purification could be performed under increasingly stringent conditions, continually decreasing the number of associated proteins. Functional analysis of these complexes using *in vitro* splicing assays will distinguish the minimal functional complex under stimulated conditions or reveal an inhibitory interaction under resting conditions.

While most of my work has focused on the characterization of variable exon 4, PSF also demonstrates functional exon repression under stimulated conditions in the analysis of variable exon 5 *in vitro*. Since exons 4, 5, and 6 show coordinated regulation, the mechanisms of activation-induced exon repression described for variable exon 4 may also function to regulate variable exons 5 and 6, and this possibility should be experimentally studied.

Importantly, the studies described here have focused on regulation at the level of RNA-protein interactions, but the intracellular signaling pathways required in this system remain unknown. Thus, investigation of intracellular signaling pathways which culminate in alterations in the function of hnRNP L and PSF in response to activation is of high priority.

Conclusion

In conclusion, I show that the signal-induced splicing regulation of CD45 exon 4 can be recapitulated using resting and stimulated nuclear extracts derived from JSL1 cells. These extracts provided a platform for the identification of a combinatorial mechanism of activation-induced repression of exon 4 via hnRNP L and PSF. Future characterization of the changes in hnRNP L and PSF induced upon T cell activation will provide further insight as to how integration of signaling pathways leads to the unique remodeling of a silencer complex to promote signal-induced changes in alternative splicing.

CHAPTER SEVEN

Materials and Methods

Minigenes and RNA

The WT, ΔESS1 and Mut1 minigenes have been described previously as WT, alt-ESS and mESS1 (House and Lynch, 2006). The Mut2 minigene was derived from the WT version by PCR-based mutagenesis as described previously (Rothrock et al., 2003). For *in vitro* splicing experiments minigene derived RNAs were transcribed *in vitro* with T7 polymerase (Promega). 5' biotinylated ESS1, Mut1, NS Control, and $(CA)_{20}$ RNAs as well as non-biotinylated ESS1, Mut1, Mut2, and NS Control RNAs were chemically synthesized by Dharmacon. The NS Control RNA sequence has been previously published as E14 (Rothrock et al., 2005).

Cell Culture, Morpholino Oligo Transfections, and RNA Isolation

JSL1 cells have been described previously (Lynch and Weiss, 2000). Stable cell lines expressing minigenes were prepared and analyzed as described in Rothrock et. al. (2003). Antisense knock-down was performed by electroporation of 20million JSL1 cells with the indicated amount of morpholino oligonucleotide (Gene-Tools Inc.) in RPMI without supplementation. Cells were allowed to recover overnight in RPMI +10% fetal calf serum before stimulation with PMA. Cells were harvested for RNA and protein 48 hours after treatment with PMA. PSF knock-down experiments where multiple dose of mopholino oligonuceotide was transfected were performed as described above with cells being allowed

to recover for 48 hours between transfections in RPMI +10% fetal calf serum. Morpholino oligonucleotide sequences are indicated below.

Oligo Name	Target Gene	Sequence
hnRNP L	hnRNP L	CGCCCGCCGCCGCCATCTTCACCAT
PSF 1	PSF	GTCGAGGCAAAAGCGAAGAAGACGC
PSF 2	PSF	CTCCGGAACCGATCCCGAGACATGT
PTB	PTB	CTATATCTGGGACAATGCCGTCCAT
$p54^{nrb}$	$p54^{nrb}$	TATTACTCTGCATTTTTGCACCCTC

MaRX II Plasmids and Viral Infections

MaRX II plasmids were derived by insertion of either GFP, Ras V12, Ras R12, DN-IκBα, myc-hnRNP L, myc-PTB, or myc-hnRNP A1 downstream of the LTR promoter. In each of the above constructs with the exception of the GFP cDNA construct, GFP cDNA was inserted downstream of the PGK promoter. The MaRX II construct was transfected into ϕNX cells using calcium phosphate. ϕNX cells were allowed to incubate and virus containing media was used to infect JVR cells. JVR cells were then treated as indicated and analyzed by FACS, RT-PCR, and Western blot (for detailed protocol see Appendix D).

Specific Post-Translational Modification Analysis and Immunoprecipitation of hnRNP L

Immunoprecipitation of hnRNP L was performed by lysing JSL1 cells at a density of 1×10^6 cells/100 μL of lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 20% Glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1X complete protease inhibitors (Roche), 1% TX100). Cells

were incubated on ice for 10 min. and membrane fractions were removed by centrifugation. Immunoprecipitation was performed by incubation of 300 μL cell lysate with 30 μL of 50:50 PGS bead slurry and 3 μL anti-hnRNP L antibody. Samples were washed 3X with lysis buffer containing a final concentration of 500 mM KCl and no TX100 and analyzed by SDS-PAGE and Western Blot. In the analysis of hnRNP L sumoylation, JSL1 cells were lysed as described above with the addition of NEM (Sigma) to a final concentration of 1 mM. In the analysis of O-GlcNAc modification, cells were stimulated as described previously with the addition of PUGNAc (Carbogen) to a final concentration of either 60 or 100 μM. Cells were lysed as described above with the addition of PUGNAc to a final concentration of 50 μM.

Nuclear Extract and Purified Proteins

JSL1 cells were grown in roller bottles either untreated or treated with 50ng/mL PMA for approximately 60 hours and nuclear extract was purified as described previously (Lynch and Weiss, 2000). Recombinant GST-hnRNP L was purified as described in Rothrock et. al. (2005). Recombinant His-tagged PSF was purified using the previously described pET-PSF construct (kind gift of J. Patton) and purification protocol (Patton et al., 1993). Flag-tagged PSF was purified out of nuclear extract from JSL1 cells stably expressing the protein from the pEF-FLAG-PSF vector. Nuclear extracts were prepared as described above. Purifications were preformed by incubation with FLAG-conjugated EZ-View Resin (Sigma) and washed (for detailed protocol see Appendix E). Low salt washes were performed in GFP100 (100mM KCl). High salt washes were performed in GFB500 (500mM KCl), and RNase A treatment was performed by adding 20 μg of RNase A to the Flag-PSF nuclear

extract and incubating at 30 degrees C for 10 minutes prior to purification. For Western blot analysis, 100 μL of 2X SDS sample buffer was added directly to the resin and samples. For functional analysis in *in vitro* splicing reactions, FLAG-PSF was eluted from the beads by incubating the resin with 3X-FLAG peptide (sigma) at a concentration of 500 ng/μL for 1 hour (see Appendix E).

RT-PCR

RT-PCR and analysis was performed as described previously (Lynch and Weiss, 2000).

In Vitro Splicing

In vitro splicing reactions were carried out as described in Rothrock et. al. (2005). Antibodies or proteins were added to the reaction prior to the addition of substrate RNA.

RNA Affinity Purification

Direct RNA affinity purifications were performed as described in Rothrock et. al. (2005) (for detailed protocol see Appendix B). Competition RNA affinity purifications were performed as describe above with the addition of competitor RNA to final concentrations of 0.1 μM, 0.3 μM, 1.0 μM, and 3.0 μM (for detailed protocol see Appendix C). Purification of hnRNP L from either resting or stimulated JSL1 nuclear extract was performed as described by Hui and Bindereif (2003); however, JSL1 nuclear extract was used in place of Hela nuclear extract and 5' biotinylated-$(CA)_{20}$ RNA (Dharmacon) was used in place of 5'

biotinylated-$(CA)_{32}$. Following elution with 6 M urea, samples were dialyzed overnight against 20mM Tris-HCl pH 7.5, 100 mM KCl, 20% Glycerol, 1mM EDTA, and 1mM DTT.

Mass Spectrometry

Mass spectrometry was performed by the Protein Technology Core at UT Southwestern Medical Center by digesting proteins in solution with procine trypsin. Tryptic peptides were dissolved and injected into reverse-phase HPLC/ion trap with a nanospray source, using a ThermoFinnigan LCQ Deca XP MS instrument and Xcalibur 1.3 software. MS/MS files were searched against NCBI-nr protein sequence data bases, using the Sonar database software (GenomicSolutions, Inc.).

Two-Dimensional Eletrophoresis

7 cm Immobiline DryStrip pH 3-10 NL (GE Biosciences) was rehydrated for 15 hrs. in 8 M urea, 2% CHAPS, 0.5% IPG Buffer (IPG Buffer 3-10 NL, GE Biosciences), and 20 mM DTT. Approximately 30 μg of JSL1 nuclear extract was loaded onto the strip during rehydration. Isoelectric focusing was performed using an Ettan IPGphor II Isoelectric Focusing Unit (Amersham Biosciences) according to the manufacturer's recommendations with the first step being extended to 60 min. at 300V. DryStrips were then incubated for 15 min. at room temp. in 0.5 M Tris-HCl pH 6.8, 8 M urea, 40% Glycerol, 2.5% SDS, and 65 mM DTT and sealed to the top of a 10% SDS-PAGE gel using 0.5% agarose. SDS-PAGE gel was run at 200V and transferred to nitrocellulose. Membranes were then subjected to Western blot analysis (for detailed protocol see appendix A).

Western Blotting

Western blotting was performed as described in Lynch and Weiss (2000) with the antibodies described below.

Antibodies

Antibodies used for the indicated applications were as follows:

Antigen	Antibody	Source
hnRNP L	4D11	Abcam
hnRNP E2	Rabbit Polyclonal	Gift from R. Andino
PTB N-term	Rabbit Polyclonal	Gift from D. Black
hnRNP A1	4B10	ImmunoQuest
hnRNP A2/B1	DP3B3	ImmunoQuest
hnRNP K/J	3C2	ImmunoQuest
hnRNP D	Rabbit Polyclonal	BioLegend
U1A	Rabbit Polyclonal	Gift from I. Mattaj
FLAG	M2	Sigma and Cell Signaling
Nmt55/p54^{nrb}	78-1C	Affinity BioReagents
Acetylated-Lysine	Rabbit Polyclonal	Cell Signaling
Phospho-Threonine	42H4	Cell Signaling
Phospho-Tyrosine	P-Tyr-100	Cell Signaling
O-GlcNAc	CTD 110.6	Pierce

PSF	B92	Sigma
PSF	6D7	Abnova

APPENDIX A

Two-Dimension Gel Protocol

A. Solutions:

Rehydration Buffer (stock stored at -20 without DTT)

Urea	12g	(8M final conc.)
Chaps	0.5g	(2% final conc.)
IPG Buffer	125 µL	(0.5% final conc.) (from Amersham for pH of strip)
Water	16 mL	

SDS Equilibration Buffer (stock stored at -20 without DTT)

0.5M Tris-HCl pH 6.8	20 mL
Urea	72 g
Glycerol	60 mL
10% SDS	40 mL
Water	27 mL

B. Sample Prep for 1st Dimension

1. Add fresh 1M DTT to Rehydration Buffer to final concentration of 20 mM(need 125 µL/sample)
2. Mix 120 µL Rehydration Buffer with 5 µL of NE (approx. 20-30 µg)
3. Carefully pipette sample into 7cm ceramic strip holder and spread evenly
4. Remove film cover from dry strip starting at the (+) end
5. Carefully place strip gel side down into the strip holder starting with the (+) end (try not to get bubbles)
6. Gently pipette cover fluid (mineral oil) over strip and add cover (notches toward the gel)

C. Running Conditions for 1st Dimension

Rehydration for 15 hours

	Voltage	Time
Step 1 and Hold	300 V	60-240 min.
Step 2 Gradient	1000V	30 min.
Step 3 Gradient	5000V	90 min.
Step 4 and Hold	5000V	20 min.

Temp. at 20 degrees C and Current at 50 µA/strip

D. Sample Prep for 2nd Dimension

1. Pour normal SDS-PAGE gels of desired percentage with no comb in the stacker (gel up to top)
2. Add 1 mL of 1M DTT to 14 mL of SDS Equilibration Buffer and place in small containers (one/sample…like sterile basins for tissue culture) and mix a 1% agarose/1X SDS Running Buffer Solution
3. Remove gels from holders and place into SDS Equilibration Buffer
4. Incubate with rocking at room temp. for 15 min.
5. Remove gels from SDS Buffer and place onto 3M paper soaked in 1X SDS Running Buffer
6. Rinse gels with 1X SDS Running Buffer
7. Microwave 1% Agarose Solution
8. Place strips with plastic side against the back of the plate on SDS-PAGE gel and push down with forceps (remove bubbles)
9. Seal to gel with 1% agarose solution and allow to solidify 5 min.
10. Run SDS-PAGE gels, transfer, and Western Blot as per normal lab protocol

APPENDIX B

Direct RNA Affinity Purification

A. Prep Streptavidin Beads (Ultralink Immobilized Streptavidin—Pierce)

1. Need 30 μL 50:50 bead slurry/sample
2. Wash beads 3X in 1 mL GFB100
3. Block 30 min at 4 degrees C in
   10 μL Heparin (50 μg/μL stock)
   1 mL GFB100
4. Wash 1X in 1 mL GFB100

B. Binding Reaction (500 μL total)

50 μL	BC400
132.5 μL	BC100
50 μL	13% PVA
20 μL	80mM $MgCl_2$
20 μL	25mM ATP
20 μL	0.5M Creatine Phosphate
50 μL	0.25 μg/μL tRNA in BC100
5 μL	10pmol/μL Bi-RNA (add to mixture last)
132.5 μL	Water
20 μL	Nuclear Extract

Incubate binding reaction at 30 degrees C for 30 min.

C. Couple RNA to Beads

500 μL	Binding Reaction
10 μL	50 μg/μL Heparin
20 μL	80mM $MgCl_2$
97 μL	BC400
394 μL	Water
15 μL	Streptavidin Beads

1. Make master mix of above without the binding reaction and use to resuspend the beads after last wash
2. Add 500 μL of master mix from above to each binding reaction
3. Incubate 1-2 hours with rotation at 4 degrees C
4. Wash 4X in 500 μL of GFB100 with 4mM $MgCl_2$

5. Add 50 μL 2X SDS Sample Buffer and run 10-50 μL on an SDS-PAGE gel as per normal lab protocol

APPENDIX C

Competition RNA Affinity Purification

A. Prep Streptavidin Beads (Ultralink Immobilized Streptavidin—Pierce)
   i. Need 20 μL 50:50 bead slurry/sample
   ii. Wash beads 3X in 1 mL GFB100
   iii. Block 30 min at 4 degrees C in
        10 μL Heparin (50 μg/μL stock)
        1 mL GFB100
   4. Wash 1X in 1 mL GFB100

B. Binding Reaction (125 μL total)*

12.5 μL	BC400
35 μL	BC100
12.5 μL	13% PVA
5 μL	80mM $MgCl_2$
5 μL	25mM ATP
5 μL	0.5M Creatine Phosphate
12.5 μL	0.25 μg/μL tRNA in BC100
1.25 μL	10pmol/μL Bi-RNA (add to mixture last)
12.5 μL	10X Conc. Competitor RNA** (see bottom)
21 μL	Water
3 μL	Nuclear Extract

Preincubate reaction without Bi-RNA probe for 10 min. at 30 degrees C
Add Bi-RNA probe and incubate reaction at 30 degrees C for 30 min.

C. Couple RNA to Beads

125 μL	Binding Reaction
2.5 μL	50 mg/mL Heparin
5 μL	80mM $MgCl_2$
24 μL	BC400
78.5 μL	Water
10 μL	Streptavidin Beads

1. Make master mix of above without the binding reaction and use to resuspend the beads after last wash
2. Add 125 μL of master mix from above to each binding reaction
3. Incubate 1-2 hours with rotation at 4 degrees C
4. Wash 4X in 250 μL of GFB100 with 4mM $MgCl_2$

5. Add 20-50 μL 2X SDS Sample Buffer and run 10-50 μL on an SDS-PAGE gel as per normal lab protocol

*Reactions can be scaled according to levels required for detection of desired protein
**I use final concentrations of 0.1μM, 0.3μM, 1μM, and 3μM competitor RNA (1X, 3X, 10X, and 30X fold excess)

APPENDIX D

Retroviral Infection Protocol

Day 1 Morning: Plate ϕNX cells

Plate ϕNX cells at approximately 30-40% confluency in DMEM, 5% FBS, P/S/G, 50 μM BME (split an 80-90% confluent plate approximately 1:5)

Day 1 Evening: Transfect ϕNX cells

Tube 1: 1 mL 2X HBSS pH 7.05

Tube 2: 10 μg MaRX DNA
125 μL 2M $CaCl_2$
865 μL Water

Add the contents of tube 2 dropwise to tube 1 while vortexing

Incubate at room temperature for 30 min.

Add mixture dropwise to ϕNX cells and gently rock plate from side to side to mix

Incubate cells overnight at 37 degrees C

Day 2 Morning: Wash ϕNX cells

Aspirate media from ϕNX cells

Gently wash 2X with 5 mL warm PBS (PBS must be warm)

Replace media with 10 mL RPMI, 5% FBS, P/S/G

Incubate 24-48 hrs. at 37 degrees C

Day 2 Anytime: Split JVR Cells

Count JVR cells to be infected and plate at a density of 0.5×10^6 cells/mL

Day 3: Infect JVR Cells

Transfer media from ϕNX cells (save cells for FACS analysis) to a conical tube and spin for 5 min. at 1000 rpm

Transfer media to a fresh conical tube and add polybrene to a final concentration of 10 μg/mL

Spin 0.5 mL JVR cells/1 mL of infection media to infect cells at a density of 0.5×10^6 cells/mL (each sample should be in a separate tube)

Aspirate media from JVR cells

Resuspend and plate JVR cells in viral media + polybrene

Incubate JVR cells overnight at 37 degrees C

Trypsinize ϕNX cells and analyze tranfection efficiency by FACS

Day 4: Dilute JVR Cells

Count JVR cells and split to desired density using fresh RPMI, 5% FBS, P/S/G and analyze after 24-72 hrs. (treat with PMA if desired)

Evaluate infection efficiency by FACS, harvest RNA and protein as desired

APPENDIX E

FLAG-PSF Purification from Nuclear Extract

A. EZ-View FLAG-resin (sigma)
   1. Remove 20 μL (40 μL 50:50 slurry) EZ-View FLAG-resin per sample
   2. Pre wash resin 3X in 500 μL GFB100
   3. Resuspend resin after washing to obtain a 50:50 slurry with GFB100

B. Conjugation of Washing of FLAG-PSF
   1. Add 100 μL of NE per sample to eppendorf tubes on ice
   2. Add 100 μL of GFB100 to each sample to aid in mixing
   3. Add 40 μL of 50:50 resin slurry to each sample
   4. Incubate 2-3 hours at 4 degrees C with rotation
   5. Wash samples 3X in 500 μL of desired salt GFB (Low-salt = GFB100, High-salt = GFB500)
   5. Wash 1X in 500 μL GFB100 and remove supernatant (if not eluting add 100 μL 2X sample buffer and analyze by SDS-PAGE)

C. Elution of FLAG-PSF Complexes with 3X FLAG peptide (sigma)
   1. Dilute 3X FLAG peptide stock to 500 ng/μL
   2. Add 30 μL 3X FLAG peptide to each sample
   3. Incubate resin on ice for 1 hour with mixing every 5-10 minutes
   4. Transfer eluted FLAG-PSF to fresh tubes
   5. Analyze 5-10 μL of eluted sample by anti-FLAG Western blot to evaluate elution efficiency

BIBLIOGRAPHY

Allemand, E., Guil, S., Myers, M., Moscat, J., Caceres, J. F., and Krainer, A. R. (2005). Regulation of heterogenous nuclear ribonucleoprotein A1 transport by phosphorylation in cells stressed by osmotic shock. Proc Natl Acad Sci U S A *102*, 3605-3610.

Batsche, E., Yaniv, M., and Muchardt, C. (2006). The human SWI/SNF subunit Brm is a regulator of alternative splicing. Nat Struct Mol Biol *13*, 22-29.

Bedford, M. T., and Richard, S. (2005). Arginine methylation an emerging regulator of protein function. Mol Cell *18*, 263-272.

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.

Blanchette, M., and Chabot, B. (1999). Modulation of exon skipping by high-affinity hnRNP A1-binding sites and by intron elements that repress splice site utilization. Embo J *18*, 1939-1952.

Blaustein, M., Pelisch, F., Tanos, T., Munoz, M. J., Wengier, D., Quadrana, L., Sanford, J. R., Muschietti, J. P., Kornblihtt, A. R., Caceres, J. F.*, et al.* (2005). Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nat Struct Mol Biol *12*, 1037-1044.

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

Burd, C. G., and Dreyfuss, G. (1994). RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. Embo J *13*, 1197-1204.

Byth, K. F., Conroy, L. A., Howlett, S., Smith, A. J., May, J., Alexander, D. R., and Holmes, N. (1996). CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4+CD8+ thymocytes, and B cell maturation. J Exp Med *183*, 1707-1718.

Caceres, J. F., Misteli, T., Screaton, G. R., Spector, D. L., and Krainer, A. R. (1997). Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J Cell Biol *138*, 225-238.

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.

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.

Chalfant, C. E., Mischak, H., Watson, J. E., Winkler, B. C., Goodnight, J., Farese, R. V., and Cooper, D. R. (1995). Regulation of alternative splicing of protein kinase C beta by insulin. J Biol Chem *270*, 13326-13332.

Cheng, C., and Sharp, P. A. (2006). Regulation of CD44 alternative splicing by SRm160 and its potential role in tumor cell invasion. Mol Cell Biol *26*, 362-370.

Comer, F. I., Vosseller, K., Wells, L., Accavitti, M. A., and Hart, G. W. (2001). Characterization of a mouse monoclonal antibody specific for O-linked N-acetylglucosamine. Anal Biochem *293*, 169-177.

Coulter, L. R., Landree, M. A., and Cooper, T. A. (1997). Identification of a new class of exonic splicing enhancers by in vivo selection. Mol Cell Biol *17*, 2143-2150.

Dadke, S., Cotteret, S., Yip, S. C., Jaffer, Z. M., Haj, F., Ivanov, A., Rauscher, F., 3rd, Shuai, K., Ng, T., Neel, B. G., and Chernoff, J. (2007). Regulation of protein tyrosine phosphatase 1B by sumoylation. Nat Cell Biol *9*, 80-85.

Das, R., Zhou, Z., and Reed, R. (2000). Functional association of U2 snRNP with the ATP-independent spliceosomal complex E. Mol Cell *5*, 779-787.

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.

Ding, J. H., Zhong, X. Y., Hagopian, J. C., Cruz, M. M., Ghosh, G., Feramisco, J., Adams, J. A., and Fu, X. D. (2006). Regulated cellular partitioning of SR protein-specific kinases in mammalian cells. Mol Biol Cell *17*, 876-885.

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.

Fairbrother, W. G., and Chasin, L. A. (2000). Human genomic sequences that inhibit splicing. Mol Cell Biol *20*, 6816-6825.

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

Grignani, F., Kinsella, T., Mencarelli, A., Valtieri, M., Riganelli, D., Grignani, F., Lanfrancone, L., Peschle, C., Nolan, G. P., and Pelicci, P. G. (1998). High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res *58*, 14-19.

Guil, S., Long, J. C., and Caceres, J. F. (2006). hnRNP A1 relocalization to the stress granules reflects a role in the stress response. Mol Cell Biol *26*, 5744-5758.

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.

Hannon, G. J., Sun, P., Carnero, A., Xie, L. Y., Maestro, R., Conklin, D. S., and Beach, D. (1999). MaRX: an approach to genetics in mammalian cells. Science *283*, 1129-1130.

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

Hoffman, B. E., and Grabowski, P. J. (1992). U1 snRNP targets an essential splicing factor, U2AF65, to the 3' splice site by a network of interactions spanning the exon. Genes Dev *6*, 2554-2568.

Honig, A., Auboeuf, D., Parker, M. M., O'Malley, B. W., and Berget, S. M. (2002). Regulation of alternative splicing by the ATP-dependent DEAD-box RNA helicase p72. Mol Cell Biol *22*, 5698-5707.

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.

Hui, J., and Bindereif, A. (2005). Alternative pre-mRNA splicing in the human system: unexpected role of repetitive sequences as regulatory elements. Biol Chem *386*, 1265-1271.

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.

Jacobsen, M., Schweer, D., Ziegler, A., Gaber, R., Schock, S., Schwinzer, R., Wonigeit, K., Lindert, R. B., Kantarci, O., Schaefer-Klein, J.*, et al.* (2000). A point mutation in PTPRC is associated with the development of multiple sclerosis. Nat Genet *26*, 495-499.

Johnson, J. M., Castle, J., Garrett-Engele, P., Kan, Z., Loerch, P. M., Armour, C. D., Santos, R., Schadt, E. E., Stoughton, R., and Shoemaker, D. D. (2003). Genome-

wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science *302*, 2141-2144.

Kanopka, A., Muhlemann, O., Petersen-Mahrt, S., Estmer, C., Ohrmalm, C., and Akusjarvi, G. (1998). Regulation of adenovirus alternative RNA splicing by dephosphorylation of SR proteins. Nature *393*, 185-187.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol *18*, 621-663.

Kearse, K. P., and Hart, G. W. (1991). Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. Proc Natl Acad Sci U S A *88*, 1701-1705.

Kim, S. C., Sprung, R., Chen, Y., Xu, Y., Ball, H., Pei, J., Cheng, T., Kho, Y., Xiao, H., Xiao, L.*, et al.* (2006). Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol Cell *23*, 607-618.

Kishihara, K., Penninger, J., Wallace, V. A., Kundig, T. M., Kawai, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P. S., Thomas, M. L., and et al. (1993). Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. Cell *74*, 143-156.

Kohtz, J. D., Jamison, S. F., Will, C. L., Zuo, P., Luhrmann, R., Garcia-Blanco, M. A., and Manley, J. L. (1994). Protein-protein interactions and 5'-splice-site recognition in mammalian mRNA precursors. Nature *368*, 119-124.

Konig, H., Ponta, H., and Herrlich, P. (1998). Coupling of signal transduction to alternative pre-mRNA splicing by a composite splice regulator. Embo J *17*, 2904-2913.

Krecic, A. M., and Swanson, M. S. (1999). hnRNP complexes: composition, structure, and function. Curr Opin Cell Biol *11*, 363-371.

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

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.

Lim, L. P., and Burge, C. B. (2001). A computational analysis of sequence features involved in recognition of short introns. Proc Natl Acad Sci U S A *98*, 11193-11198.

Lin, C. H., and Patton, J. G. (1995). Regulation of alternative 3' splice site selection by constitutive splicing factors. Rna *1*, 234-245.

Liu, H. X., Chew, S. L., Cartegni, L., Zhang, M. Q., and Krainer, A. R. (2000). Exonic splicing enhancer motif recognized by human SC35 under splicing conditions. Mol Cell Biol *20*, 1063-1071.

Liu, H. X., Zhang, M., and Krainer, A. R. (1998). Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. Genes Dev *12*, 1998-2012.

Lukong, K. E., and Richard, S. (2003). Sam68, the KH domain-containing superSTAR. Biochim Biophys Acta *1653*, 73-86.

Lynch, K. W. (2004). Consequences of regulated pre-mRNA splicing in the immune system. Nat Rev Immunol *4*, 931-940.

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.

Magistrelli, G., Jeannin, P., Herbault, N., Benoit De Coignac, A., Gauchat, J. F., Bonnefoy, J. Y., and Delneste, Y. (1999). A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. Eur J Immunol *29*, 3596-3602.

Manley, J. L., and Tacke, R. (1996). SR proteins and splicing control. Genes Dev *10*, 1569-1579.

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.

Matter, N., Herrlich, P., and Konig, H. (2002). Signal-dependent regulation of splicing via phosphorylation of Sam68. Nature *420*, 691-695.

Mayeda, A., Munroe, S. H., Caceres, J. F., and Krainer, A. R. (1994). Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. Embo J *13*, 5483-5495.

Mayrand, S. H., Dwen, P., and Pederson, T. (1993). Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA. Proc Natl Acad Sci U S A *90*, 7764-7768.

Mee, P. J., Turner, M., Basson, M. A., Costello, P. S., Zamoyska, R., and Tybulewicz, V. L. (1999). Greatly reduced efficiency of both positive and negative selection of thymocytes in CD45 tyrosine phosphatase-deficient mice. Eur J Immunol *29*, 2923-2933.

Modafferi, E. F., and Black, D. L. (1997). A complex intronic splicing enhancer from the c-src pre-mRNA activates inclusion of a heterologous exon. Mol Cell Biol *17*, 6537-6545.

Modafferi, E. F., and Black, D. L. (1999). Combinatorial control of a neuron-specific exon. Rna *5*, 687-706.

Modrek, B., and Lee, C. (2002). A genomic view of alternative splicing. Nat Genet *30*, 13-19.

Najib, S., Martin-Romero, C., Gonzalez-Yanes, C., and Sanchez-Margalet, V. (2005). Role of Sam68 as an adaptor protein in signal transduction. Cell Mol Life Sci *62*, 36-43.

Nakielny, S., and Dreyfuss, G. (1999). Transport of proteins and RNAs in and out of the nucleus. Cell *99*, 677-690.

Navakauskiene, R., Treigyte, G., Gineitis, A., and Magnusson, K. E. (2004). Identification of apoptotic tyrosine-phosphorylated proteins after etoposide or retinoic acid treatment. Proteomics *4*, 1029-1041.

Patel, N. A., Chalfant, C. E., Watson, J. E., Wyatt, J. R., Dean, N. M., Eichler, D. C., and Cooper, D. R. (2001). Insulin regulates alternative splicing of protein kinase C beta II through a phosphatidylinositol 3-kinase-dependent pathway involving the nuclear serine/arginine-rich splicing factor, SRp40, in skeletal muscle cells. J Biol Chem *276*, 22648-22654.

Patel, N. A., Kaneko, S., Apostolatos, H. S., Bae, S. S., Watson, J. E., Davidowitz, K., Chappell, D. S., Birnbaum, M. J., Cheng, J. Q., and Cooper, D. R. (2005). Molecular and genetic studies imply Akt-mediated signaling promotes protein kinase CbetaII alternative splicing via phosphorylation of serine/arginine-rich splicing factor SRp40. J Biol Chem *280*, 14302-14309.

Patton, J. G., Porro, E. B., Galceran, J., Tempst, P., and Nadal-Ginard, B. (1993). Cloning and characterization of PSF, a novel pre-mRNA splicing factor. Genes Dev *7*, 393-406.

Pelisch, F., Blaustein, M., Kornblihtt, A. R., and Srebrow, A. (2005). Cross-talk between signaling pathways regulates alternative splicing: a novel role for JNK. J Biol Chem *280*, 25461-25469.

Peng, R., Hawkins, I., Link, A. J., and Patton, J. G. (2006). The splicing factor PSF is part of a large complex that assembles in the absence of pre-mRNA and contains all five snRNPs. RNA Biol *3*, 69-76.

Perez, I., Lin, C. H., McAfee, J. G., and Patton, J. G. (1997). Mutation of PTB binding sites causes misregulation of alternative 3' splice site selection in vivo. Rna *3*, 764-778.

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

Prasad, J., Colwill, K., Pawson, T., and Manley, J. L. (1999). The protein kinase Clk/Sty directly modulates SR protein activity: both hyper- and hypophosphorylation inhibit splicing. Mol Cell Biol *19*, 6991-7000.

Robberson, B. L., Cote, G. J., and Berget, S. M. (1990). Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol Cell Biol *10*, 84-94.

Rooke, N., Markovtsov, V., Cagavi, E., and Black, D. L. (2003). Roles for SR proteins and hnRNP A1 in the regulation of c-src exon N1. Mol Cell Biol *23*, 1874-1884.

Rosenberger, U., Lehmann, I., Weise, C., Franke, P., Hucho, F., and Buchner, K. (2002). Identification of PSF as a protein kinase Calpha-binding protein in the cell nucleus. J Cell Biochem *86*, 394-402.

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.

Sacco-Bubulya, P., and Spector, D. L. (2002). Disassembly of interchromatin granule clusters alters the coordination of transcription and pre-mRNA splicing. J Cell Biol *156*, 425-436.

Sanford, J. R., and Bruzik, J. P. (1999). SR proteins are required for nematode trans-splicing in vitro. Rna *5*, 918-928.

Sanford, J. R., and Bruzik, J. P. (2001). Regulation of SR protein localization during development. Proc Natl Acad Sci U S A *98*, 10184-10189.

Schaal, T. D., and Maniatis, T. (1999). Selection and characterization of pre-mRNA splicing enhancers: identification of novel SR protein-specific enhancer sequences. Mol Cell Biol *19*, 1705-1719.

Senapathy, P., Shapiro, M. B., and Harris, N. L. (1990). Splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. Methods Enzymol *183*, 252-278.

Shav-Tal, Y., Cohen, M., Lapter, S., Dye, B., Patton, J. G., Vandekerckhove, J., and Zipori, D. (2001). Nuclear relocalization of the pre-mRNA splicing factor PSF during apoptosis involves hyperphosphorylation, masking of antigenic epitopes, and changes in protein interactions. Mol Biol Cell *12*, 2328-2340.

Shav-Tal, Y., and Zipori, D. (2002). PSF and p54(nrb)/NonO--multi-functional nuclear proteins. FEBS Lett *531*, 109-114.

Shin, C., and Manley, J. L. (2004). Cell signalling and the control of pre-mRNA splicing. Nat Rev Mol Cell Biol *5*, 727-738.

Sironi, M., Menozzi, G., Riva, L., Cagliani, R., Comi, G. P., Bresolin, N., Giorda, R., and Pozzoli, U. (2004). Silencer elements as possible inhibitors of pseudoexon splicing. Nucleic Acids Res *32*, 1783-1791.

Stickeler, E., Fraser, S. D., Honig, A., Chen, A. L., Berget, S. M., and Cooper, T. A. (2001). The RNA binding protein YB-1 binds A/C-rich exon enhancers and stimulates splicing of the CD44 alternative exon v4. Embo J *20*, 3821-3830.

Subra, J. F., Cautain, B., Xystrakis, E., Mas, M., Lagrange, D., van der Heijden, H., van de Gaar, M. J., Druet, P., Fournie, G. J., Saoudi, A., and Damoiseaux, J. (2001). The balance between CD45RChigh and CD45RClow CD4 T cells in rats is intrinsic to bone marrow-derived cells and is genetically controlled. J Immunol *166*, 2944-2952.

Tacke, R., and Manley, J. L. (1999). Functions of SR and Tra2 proteins in pre-mRNA splicing regulation. Proc Soc Exp Biol Med *220*, 59-63.

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.

Tarn, W. Y. (2007). Cellular signals modulate alternative splicing. J Biomed Sci.

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.

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.

Trop, S., Charron, J., Arguin, C., Lesage, S., and Hugo, P. (2000). Thymic selection generates T cells expressing self-reactive TCRs in the absence of CD45. J Immunol *165*, 3073-3079.

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 Immunol *12*, 85-116.

Valcarcel, J., and Gebauer, F. (1997). Post-transcriptional regulation: the dawn of PTB. Curr Biol *7*, R705-708.

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*, 307-316.

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

Vosseller, K., Sakabe, K., Wells, L., and Hart, G. W. (2002). Diverse regulation of protein function by O-GlcNAc: a nuclear and cytoplasmic carbohydrate post-translational modification. Curr Opin Chem Biol *6*, 851-857.

Vosseller, K., Wells, L., and Hart, G. W. (2001). Nucleocytoplasmic O-glycosylation: O-GlcNAc and functional proteomics. Biochimie *83*, 575-581.

Wagner, E. J., and Garcia-Blanco, M. A. (2001). Polypyrimidine tract binding protein antagonizes exon definition. Mol Cell Biol *21*, 3281-3288.

Wang, Z., Rolish, M. E., Yeo, G., Tung, V., Mawson, M., and Burge, C. B. (2004). Systematic identification and analysis of exonic splicing silencers. Cell *119*, 831-845.

Weg-Remers, S., Ponta, H., Herrlich, P., and Konig, H. (2001). Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway. Embo J *20*, 4194-4203.

Wells, L., Vosseller, K., and Hart, G. W. (2001). Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. Science *291*, 2376-2378.

Woerfel, G., and Bindereif, A. (2001). In vitro selection of exonic splicing enhancer sequences: identification of novel CD44 enhancers. Nucleic Acids Res *29*, 3204-3211.

Wu, J. Y., and Maniatis, T. (1993). Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell *75*, 1061-1070.

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 Sci U S A *100*, 8776-8781.

Yeakley, J. M., Tronchere, H., Olesen, J., Dyck, J. A., Wang, H. Y., and Fu, X. D. (1999). Phosphorylation regulates in vivo interaction and molecular targeting of serine/arginine-rich pre-mRNA splicing factors. J Cell Biol *145*, 447-455.

Zhang, X. H., and Chasin, L. A. (2004). Computational definition of sequence motifs governing constitutive exon splicing. Genes Dev *18*, 1241-1250.

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*, 1351-1361.

Zuo, P., and Maniatis, T. (1996). The splicing factor U2AF35 mediates critical protein-protein interactions in constitutive and enhancer-dependent splicing. Genes Dev *10*, 1356-1368.

VITAE

Alexis Allyson Melton was born in Loveland, Colorado on September 28th, 1981, the daughter of Michael Neil Melton and Vicki Lynn Melton. After receiving her high school diploma from Cherry Creek High School, Englewood, Colorado in May 1999, she began studies at the University of Colorado at Boulder. In December 2001 she completed her Bachelor of Arts degree in Biochemistry with a minor in Chemistry. In June 2002 she entered the Medical Scientist Training Program at the University of Texas Southwestern Medial Center at Dallas. In June 2007 she received the degree of Doctor of Philosophy from the University of Texas Southwestern Medical Center at Dallas.

Permanent Address: 3987 Truman Dr.
Frisco, TX 75034