Characterization and Development of Strategies for Altering Protein Expression in JSL1 cells

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Characterization and Development of Strategies for Altering Protein Expression in JSL1 cells

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Alternative splicing is a common mechanism for regulating gene expression in eukaryotic cells. This process of differentially including or excluding variable exons provides a means for increasing proteome complexity. Alternative gene splicing occurs in a cell specific manner and may be influenced by changes in the extracellular environment. Despite the importance of this method for regulating gene expression, little is known about the factors involved in regulating its function.

The T cell tyrosine phosphatase CD45 provides a valuable model for investigating the factors involved in regulating alternative splicing. The CD45 gene contains three variable exons whose splicing is regulated in response to T cell activation. Studies of this gene have revealed the presence of an exonic silencer sequence within variable exon 4

that is capable of influencing exon skipping under both resting and stimulated conditions. Biochemical assays have shown that the regulatory protein hnRNP L binds to this silencer sequence and results in basal exon repression during resting conditions and undergoes modifications which further influence exon skipping upon stimulation. Furthermore, *in vitro* assays indicate that upon stimulation, an additional regulatory protein, PSF, binds to the regulatory complex associated with the silencer sequence.

Although these studies have provided novel information regarding the regulation of splicing, biochemical assays are unable to fully mimic the signaling pathways inside a cell, thus creating a need for a cell culture system. A Jurkat derived cell line, JSL1 cells, has been identified as being able to recapitulate the signal induced alternative splicing of the CD45 gene as seen in primary human T cells. This cell line presents a cell based system for evaluating the factors involved in splicing. However, in order to conduct *in vivo* experiments one must be able to modify protein expression. JSL1 cells present limitations due to difficulties in being able to alter protein expression.

A strong promoter, EF1-α, has been employed to drive the expression of candidate proteins in JSL1 cells. Transient transfections and stable cell lines expressing cDNAs driven by this promoter have shown little if any overexpression of candidate proteins normally expressed at high levels within the cell; however, significant overexpression has been achieved with the transfection of at least one protein that exists at a lower concentration. Initial experiments indicate that stably expressed flag-tagged proteins, driven by the EF1-α promoter, may be easily purified from JSL1 cells during resting and stimulated conditions and analyzed. Such data suggests that this promoter may afford more flexibility in altering and analyzing protein expression in JSL1 cells, thereby facilitating the investigation of signaling pathways involved in regulating

alternative splicing. Furthermore, strategies for regulating protein expression, through the use of a Tet-suppressor system, are in initial stages of being developed and hold the potential for providing an additional tool for evaluating the factors involved in regulating alternative splicing.

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List of Abbreviations

snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SF1	splicing factor 1
hnRNP	heterogeneous nuclear ribonucleoparticle
ESS	exonic splicing silencer
ESE	exonic splicing enhancer
ISS	intronic splicing silencer
ISE	intronic splicing enhancer
SR protein	serine/arginine-rich protein
U1 70K	70K protein of U1 snRNP
HIV-1	human immunodeficiency virus type 1
PSF	PTB-associated splicing factor
TCR	T cell receptor
PLCγ1	phospholipase C-gamma 1
PIP2	phosphatidylinositol 4, 5-bisphosphate
IP3	inositol triphosphate
DAG	diacylglycerol
PKC	protein kinase C
JSL1	Jurkat splicing line 1
PTB	polypyrimidine tract binding protein
TR	tetracycline repressor
$TetO_2$	tetracycline operator sequences

Chapter One

Introduction

Chapter Overview

The expression of eukaryotic genes involves numerous processing steps. One common step, referred to as pre-mRNA splicing, involves the removal of non-coding intron sequences and the ligation of exonic coding sequences to form mature mRNA (Alberts et al.; Sharp, 2005). The importance of this process is evidenced by the observation that approximately 15% to 50% of human genetic diseases may be a result of aberrant splicing events (Matlin et al., 2005).

Pre-mRNA splicing is facilitated by the activity of a macromolecular complex, referred to as the spliceosome. While the basic assembly and catalytic activity of the spliceosome is directed by conserved sequences at the exon-intron boundaries, additional regulatory proteins and sequences can influence these actions leading to a process known as alternative splicing. Alternative splicing is defined as the inclusion or exclusion of variable exons from pre-mRNA and is a widespread mechanism for regulating protein expression in eukaryotes.

Here I review the basic concepts of pre-mRNA splicing, spliceosome assembly and alternative splicing. Additionally, I introduce the CD45 gene which has proven to be a powerful model for the study of alternative splicing particularly in terms of how this process is influenced by cellular environment and signaling pathways.

Pre-mRNA Splicing and Spliceosome Formation

Pre-mRNA splicing is directed by 5' and 3' splice site sequences that reside at the intron/ exon boundaries. The 5' splice site sequence marks the 5' end of the intron and contains a GU dinucleotide that is surrounded by a more loosely conserved consensus sequence. The 3' splice site sequence is located at the 3' end of the intron and is characterized by the presence of a

branch point sequence, a polypyrimidine tract, and an AG at the 3'terminal end of the intron (Figure 1-1). Recognition of these splice site sequences by components of the spliceosome results in removal of the intron (Black, 2003).

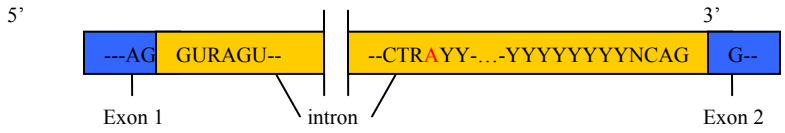

Figure 1-1. 5' and 3' splice site sequences
The 5' splice site contains a conserved GU dinucleotide. The 3' splice site contains a branch point sequence (CTRAYY), polypyrimidine tract (YY…), and an AG at its 3' terminus. Figure adapted from Alberts et al.

The spliceosome is a complex structure composed of five small nuclear RNAs (snRNAs), U1, U2, U4, U5, and U6, which are associated with accessory proteins and form small ribonucleoproteins (snRNPs) (Kramer, 1996). Assembly of the spliceosomal components onto the pre-mRNA occurs in a coordinated manner and facilitates the two transesterification steps of splicing (Figure 1-2). In the first step, the 2' hydroxyl of the branch point A attacks the phosphate at the 5' splice site causing cleavage of the 5' exon and ligation of the 5' end of the intron to the branch point A, resulting in the formation of a lariat intermediate (Figure 1-2A). The second step proceeds by the 3' hydroxyl group of the free exon attacking the 3' end of the intron, thus leading to the ligation of the two exons and removal of the intron (Figure 1-2B)(Black, 2003).

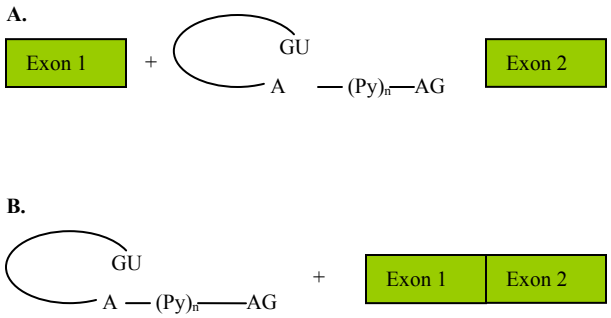

Figure 1-2. The Steps of a Splicing Reaction. (A) Attack of the phosphate at the 5' splicesite by the 2' hydroxyl of the branch point results in formation of lariat and free exon intermediates. (B) Attack of the 3' end of the intron by the 3' hydroxyl group of the free exon results in intron removal and exon ligation. Figure adapted from Black, 2003.

The coordinated process of spliceosome assembly on pre-mRNA has been defined as occurring through the step-wise formation of four complexes: E, A, B, and C. The E complex (Early complex) is the first well characterized step in the spliceosome assembly process and is considered a commitment to splicing. In this step, the exon is recognized by the binding of U1 snRNP to the 5' splice site, SF1 (splicing factor 1) binding to the branch point A sequence, U2AF65 (the large auxiliary factor of U2) binding to the poylpyrimidine tract, and the binding of U2AF35 (the small auxiliary factor of U2) to the 3' splice site (Figure 1-3A). Progression to the A complex proceeds by the binding of U2 snRNP to the branch point sequence, thereby positioning the branch point A residue for nucleophilic attack on the 3' splice site (Figure 1-3B). Assembly of tri-snRNP, a complex of U4/U5/U6 snRNPs, on residues upstream of the 5' splice site marks progression to the B complex. Extensive rearrangement of B complex consisting of the replacement of U1 snRNP, bound at the 5' splice site, with U6 snRNP and the dissociation of U4 snRNP results in the formation of the catalytic complex, or C complex (Figure 1-3C) (Black, 2003). A fifth complex, the H complex, is not as well defined as the other complexes, but has been characterized as being composed of mostly heterogeneous nuclear ribonucleoparticle (hnRNP) proteins (Matlin et al., 2005).

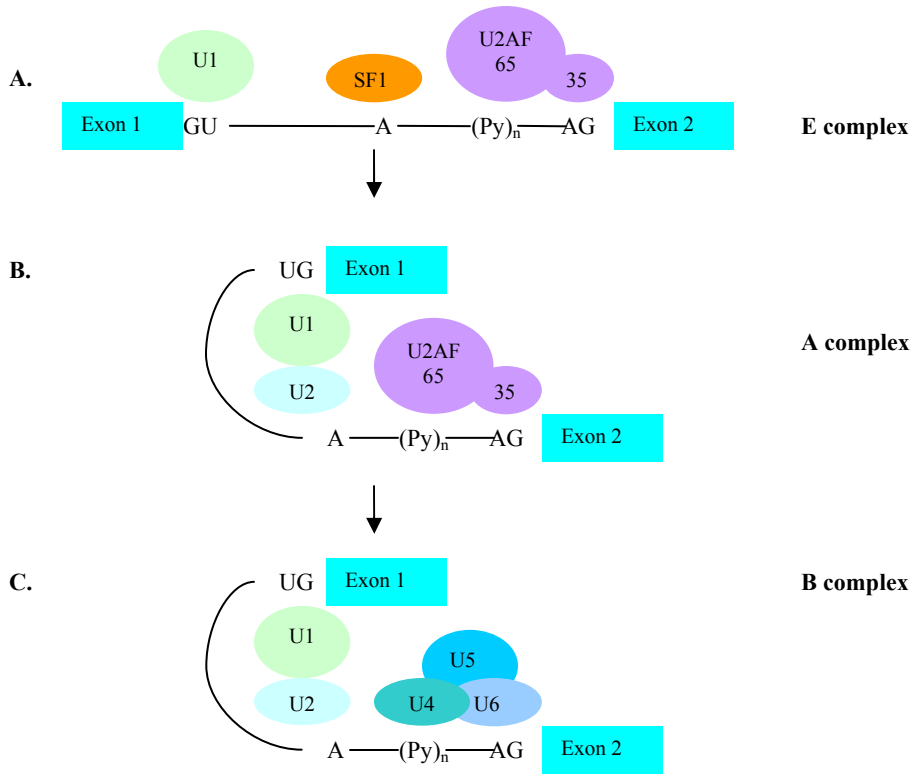

Figure 1-3. Spliceosome Assembly. Spliceosome assembly occurs through the step-wise formation of four complexes: E, A, B, and C. (A) E complex is characterized by the binding of U1 snRNP to the 5' splice site, SF1 binding to the branch point A sequence, U2AF65 binding to the poylpyrimidine tract, and U2AF35 binding to the 3' splice site. (B) A complex is formed by the binding of U2 snRNP to the branch point sequence and release of SF1. (C) B complex involves the binding of tri-snRNP (U4/U5/U6) and the release of U2AF 65 and U2AF 35. B complex undergoes extensive rearrangement to form C complex. Figure adapted from Black, 2003.

Splice site recognition by the spliceosome is determined not only by the splice site sequences, but also by the activity of auxiliary proteins. Evidence for this is seen by the fact that although the 5' and 3' splice site sequences are necessary, they are not sufficient for recruitment of the spliceosome due to their poorly conserved sequences. The additional binding of auxiliary proteins to regulatory sequences within and/or flanking the exons play an additional role in spliceosomal recruitment (Black, 2003). This combined need for splice site sequences and binding of auxiliary proteins sets up the potential for the regulation of splicing events. This process of regulated splicing and the influence of auxiliary factors on this process will be addressed in more detail in the following section.

Alternative Pre-mRNA Splicing

Alternative splicing is defined as the differential inclusion or exclusion of variable exons in a mature mRNA transcript. It is estimated that 60-75% of the human genome is subject to alternative splicing, with approximately 70-90% of these alternative splicing events resulting in the production of alternate protein products (Black, 2003; Shin and Manley, 2004). The existence of such a process allows for increased proteome complexity in eukaryotes.

In contrast to constitutive exons, which are always included in mature mRNA, variable exons exist in multiple forms and may be included or excluded from the final transcript in a variety of combinations (Figure 1-4). Variable cassette exons exist as full exons that are differentially included or excluded (Figure 1-4A). Mutually exclusive cassette exons are variable exons that can only be used in a mutually exclusive manner (Figure 1-4B). The presence of alternative 3' or 5' splice sites within exons allows for the differential inclusion of portions of an exon (Figure 1-4C and D). Determination of the inclusion or exclusion of cassette exons from mature mRNA depends on factors such as cell type and extracellular environment (Black, 2003).

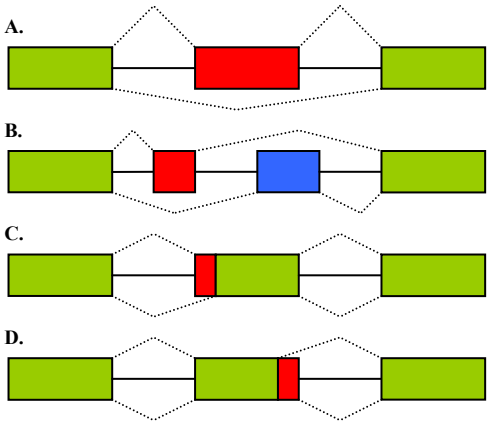

Figure 1-4. Patterns of Alternative Splicing. Boxes and solid lines represent exons and introns, respectively. Dotted lines show patterns of splicing. Constitutive exons are shown in green, while variable exons are represented by red and blue. (A) A cassette exon that is either included or excluded from mature mRNA. (B) Mutually exclusive cassette exons, with alternating inclusion of one over the other. (C) Alternative 5' splice site changes length of exon. (D) Alternative 3' splice site changes length of exon. Figure adapted from Lynch, 2004.

The regulation of splicing is critical as is evidenced by the estimate that 15% to 50% of human genetic diseases may be a result of mutations which disrupt splicing (Matlin et al., 2005). Multiple points of regulation can be identified throughout the splicing process; from the presence of the aforementioned 5' and 3'splice site sequences to the existence of positive and negative regulatory sequence elements. These regulatory sequence elements are characterized by their function and location and are as follows: exonic splicing silencers (ESSs), exonic splicing enhancers (ESEs), intronic splicing silencers (ISSs) and intronic splicing enhancers (ISEs). The splicing enhancers, ESE and ISE, are associated with exon inclusion; while the splicing silencers, ESS and ISS, are associated with exon exclusion from mature mRNA. Changes in the binding pattern of auxiliary proteins, such as serine/arginine-rich (SR proteins) and hnRNP proteins, to the regulatory sequences can alter splice site choice, thus providing an additional method for regulating splicing (Black, 2003).

RNA-binding Proteins

The SR proteins are a family of proteins that play crucial roles in the splicing of both constitutive and variable exons. There are at least ten identified canonical SR proteins, which are characterized by the presence of N-terminal RNA binding domains (RRM domains) and C-terminal arginine/serine rich domains (RS domains). The RRM domains recognize a variety of distinct RNA sequences, while the RS domains participate in protein-protein interactions and may be phosphorylated at the serine residues resulting in the regulation of functional activity (Figure 1-5) (Graveley, 2000).

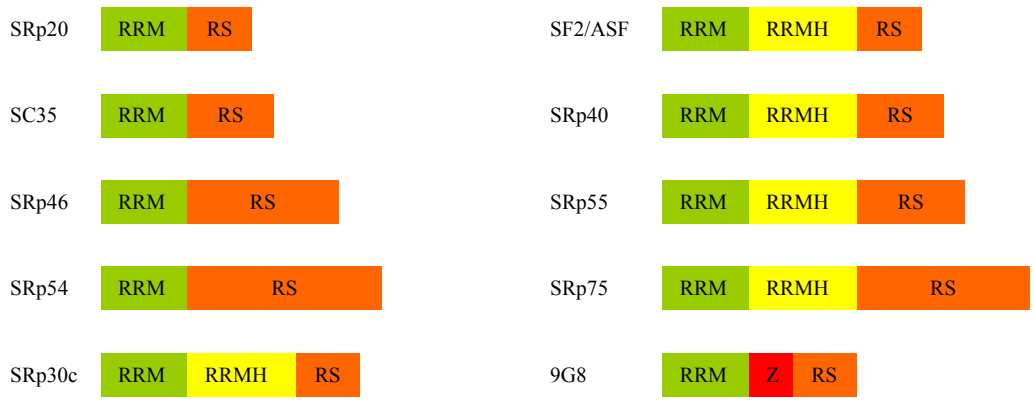

Figure 1-5. The SR Protein Family. There are ten identified members of the SR protein family. RRM: RNA recognition motif, RS: arginine/serine rich domain, RRMH: RRM homology, Z: zinc knuckle. Figure adapted from Graveley, 2000.

In addition to the canonical SR protein family, there exists a related group of proteins referred to as SR-related proteins. Although these proteins contain RS domains, similar to the SR proteins, not all of them possess RNA-binding motifs. SR-related proteins have been shown to interact with SR proteins and to participate in pre-mRNA splicing (Graveley, 2000).

The role of SR proteins in regulating constitutive splicing is demonstrated through studies showing that S100 cytoplasmic extracts, which are depleted of SR proteins, are unable to perform splicing; however, when exogenous SR proteins are added back to the extracts, splicing activity is restored (Krainer et al., 1990). At least one aspect of the role of SR proteins in constitutive splicing is their binding to the SR binding sites, referred to as constitutive splicing enhancers, of constitutive exons. This interaction between the constitutive splicing enhancers and SR proteins facilitates splice site recognition and formation of the E complex (Zuo and Maniatis, 1996).

The function of SR proteins in alternative splicing involves the regulation of variable exon splicing by the binding of SR and/or SR-related proteins to ESE regions (Graveley, 2000). As seen in SR protein binding with constitutive splicing enhancers, SR protein binding with alternative splicing enhancer regions also facilitates splice site recognition. A well-known example of SR protein regulation of alternative splicing is seen in the doublesex (dsx) gene of *D.*

melanogaster, where gender based expression of the splicing regulatory protein Tra, which interacts with SR and SR-related proteins, recruits SR proteins downstream of a weak 3' splice site resulting in alternative splicing of exon 4 of the dsx gene (Lynch and Maniatis, 1996).

The mechanistic function of SR proteins in mediating splice site recognition and thus E complex formation is reflected in their ability to recruit $U2AF^{65}$ and U1 snRNP to pre-mRNA. SR proteins function at the 3'exon by simultaneously binding to the exon and $U2AF^{35}$, thereby facilitating the recruitment of $U2AF^{65}$ to the 3' splice site (Figure 1-6A) (Wu and Maniatis, 1993; Zuo and Maniatis, 1996). The function of SR proteins at the 5' exon involves their binding with both upstream splicing enhancer complexes and the 70K protein of U1 snRNP (U1 70K). This interaction between the SR proteins and U1 70K leads to the binding of U1 snRNP to the 5' splice site (Figure 1-6B) (Kohtz et al., 1994; Zahler and Roth, 1995). Although the function of SR proteins in both constitutive and alternative splicing is similar in that they facilitate splice site recognition and E complex formation, a process known as exon definition; their roles differ in the sense that in constitutive splicing the proteins bind to the exon constitutively, whereas in alternative splicing SR protein binding is regulated (Graveley, 2000).

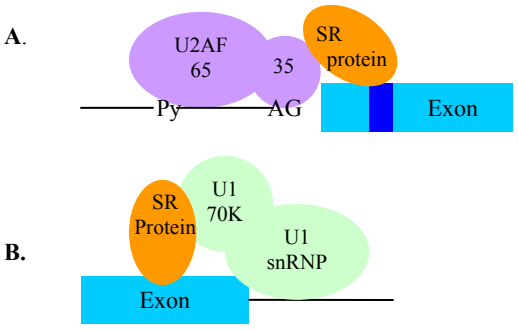

Figure 1-6. SR proteins facilitate splice site recognition. (A) An SR protein interacts with $U2AF^{35}$, thereby recruiting $U2AF^{65}$ to the poylpyrimidine tract at the 3' splice site. (B) An SR protein bound to the upstream exon interacts with U1 70K, thereby recruiting U1 snRNP to the 5' splice site. Figure adapted from Graveley, 2000.

In addition to their function in initiating E complex formation, SR proteins may also be involved in other steps of spliceosome assembly. For example, there has been evidence suggesting that SR proteins participate in the pairing of 5'and 3' splice sites. Additionally, SR protein involvement has been implicated in the recruitment of tri-snRNP into the spliceosome. Furthermore, antibodies against various SR proteins have indicated their association during multiple steps of spliceosome assembly (reviewed in Graveley, 2000). Such data suggests that SR proteins are present throughout the splicing process.

Another group of splicing regulatory proteins shown to influence pre-mRNA processing is the hnRNP proteins. Although characterized by their association with pre-mRNA transcripts, hnRNP proteins do not comprise a single family of related proteins (Black, 2003). These proteins are modular in structure with one common feature being the presence of one or more RNA binding domains, represented as RRMs (RNA Recognition Motif), KH motifs (K homology RNA binding domain), and RGG (Arg-Gly-Gly) boxes. Additionally, each hnRNP protein contains at least one domain that facilitates protein-protein interactions and determines protein localization (Table 1-1) (Dreyfuss et al., 2002).

Proteins	Domains	Size (kDa)	Functions
A1	2 RBDs, 1 RGG	34	mRNA splicing, export, telomere biogenesis
A2/B1	2 RBDs, 1 RGG	36/38	mRNA splicing, localization
C1/C2	1 RBD	41/43	mRNA splicing, stability
D (AUF1)	2 RBDs, 1 RGG	44-48	Telomere biogenesis, mRNA stability, recombination
E1/E2 (αCP1,2 or PCBP1,2)	3 KHs	38,39	mRNA stability, splicing, Translational control
F	3 RBDs	53	mRNA splicing
H/H' (DSEF-1)	3 RBDs	56	mRNA splicing, Polyadenylation
I (PTB)	4 RBDs	59	mRNA splicing, localization, Polyadenylation
K	3 KHs, 1 RGG	62	Transcription, Translational regulation
L	4 RBDs	68	mRNA export, stability, splicing
Q	3 RBDs, 1 RGG	55-70	mRNA splicing
U	1 RGG	120	Nuclear retention

Table 1-1. The structure and functions of hnRNP proteins. The hnRNPs are a group of related proteins characterized by their association with pre-mRNA. They contain one or more RNA binding domains, indicated as: RRM (RNA Recognition Motif), KH (K homology RNA binding domain), and RGG (Arg-Gly-Gly boxes). Figure adapted from Dreyfuss et al., 2002.

While hnRNP proteins show preferences for binding to distinct RNA sequences, these proteins also have a tendency towards overlapping binding specificity. The binding of hnRNPs with various RNA sequences influences their interaction with pre-mRNA, thereby altering processing to form mRNA (Dreyfuss, 2002). HnRNP proteins have also been implicated in a variety of other cellular events, such as mRNA localization, translation and stability, telomere biogenesis, and translational and transcriptional regulation (Table 1-1) (Hoek et al., 1998; Ostareck et al., 2001; Xu, 2001; Fiset and Chabot, 2001; Du et al., 1998).

Although poorly characterized, the formation of the H complex prior to spliceosome assembly may be one example of a regulatory function of hnRNP proteins in constitutive splicing. The sequence-specific binding of hnRNPs to the pre-mRNA, resulting in the formation of the H complex, may conceal alternative splice sites, thus allowing for appropriate binding of the spliceosome (Matlin et al., 2005). The role of hnRNP proteins in alternative splicing has been demonstrated through the study of HIV-1 (human immunodeficiency virus type 1) tat exon

2. In this model, hnRNP-A1, has been shown to bind with the ESS sequence of tat exon 2, causing exclusion of the exon from mature mRNA (reviewed in Black, 2003 and Dreyfuss, 2002). In general, the binding of hnRNP proteins to silencer sequences is thought to inhibit splice site recognition and thus prevent spliceosome assembly (Figure 1-7).

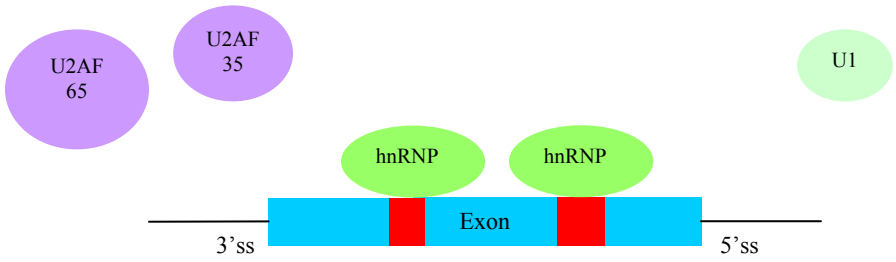

Figure 1-7. HnRNP proteins inhibit splice site recognition. The binding of hnRNP proteins to silencer sequences prevents E complex formation, thereby resulting in exon exclusion from mature mRNA.

In addition to what has been outlined thus far, it is important to note that there are likely many other proteins that perform crucial roles in regulating pre-mRNA splicing. For instance, the binding of PSF (PTB-associated splicing factor) to the ESS of variable exon 4 in the CD45 gene appears to result in decreased exon inclusion (Melton, unpublished data). Additionally, the RNA and DNA binding nuclear factor, p54, has significant homology to PSF and may have a role in regulating splicing (Kramer, 1996). As will be discussed below, the identification and regulation of such proteins is the target of current investigation.

Alternative Splicing in Response to Extracellular Stimuli

Over the past few years, it has become increasingly apparent that a number of genes throughout the human genome undergo alternative splicing in response to extracellular stimuli. In many cases, the signal-induced splicing of these genes leads to altered mature mRNA expression. Three mechanisms that have been shown to change alternative splice site usage in response to a stimulus are synthesis, phosphorylation, and a change in localization of splicing

regulatory proteins. One example of this regulatory process is demonstrated by the STREX (stress axis) exon of the large-conductance Ca^{2+} and voltage-activated potassium (BK) channel, where the differential inclusion and exclusion of the BK STREX exon is regulated in response to neuronal activity (reviewed in Stamm, 2002).

Another example of signal mediated alternative splicing is seen in the altered expression of PKCβ isoforms upon activation of the insulin receptor. In this example, insulin binding to its receptor induces a switch from the expression of the PKCβI isoform to the PKCβII isoform through the activation of 5' splice sites flanking the PKCβII exon (Chalfant et al., 1998). Additionally, activation of the insulin receptor results in the phosphorylation of the SR protein SRp40. The phosphorylation of this SR protein may be involved in the insulin induced switch in PKCβ isoform expression (reviewed in Stamm, 2002).

In addition, there has been substantial evidence for the regulation of alternative splicing by extracellular stimuli within the immune system. As will be discussed in more detail in the following section, proper T cell functioning relies on a number of signaling cascades. The expression of many of the genes involved in these cascades is regulated by the activation state of the T cell. Table 1-2, provides a list of some of the genes within T cells that are regulated through extracellular stimuli (reviewed in Lynch, 2004; Ip et al., 2007). From such data, it is evident that signal induced alternative splicing is an important process for many, if not most, human cellular functions. It is for this reason that developing a better understanding of the cellular processes involved in facilitating stimulus regulated alternative splicing is necessary.

Gene Family	Gene	Effect on protein
PTK	FYN	Change in SH2 and kinase domains
Adhesion molecule (Ig superfamily)	ICAM1	Altered number of immunoglobulin domains
Adhesion molecule (Ig superfamily)	PECAM1 (CD31)	Altered cytoplasmic domain
Adhesion molecule	CD44	Altered membrane-proximal extracellular domain
PTP	CD45	Altered extracellular domain
Cell-surface receptor	CTLA4	Change in inclusion of transmembrane domain
PTK	PYK2	Deletion of a portion of one of the two proline-rich regions

Table 1-2. Example of genes within T cells that undergo signal induced Alternative Splicing. PTK, protein tyrosine kinase; Ig, immunoglobulin; PTP, protein tyrosine phosphatase; SYK, spleen tyrosine kinase, ICAM1, intercellular adhesion molecule 1; PECAM1, platelet/endothelial cell-adhesion molecule 1; CTLA4, cytotoxic T-lympnocyte antigen 4; SH2, SRC homology; PYK2, protein tyrosine kinase 2; ERK1, extracellular signal-regulated kinase. Table adapted from Lynch, 2004.

T cell Activation

The immune system of an organism is responsible for recognizing and appropriately responding to antigenic stimuli. For an effective immune response a variety of physical and biochemical changes must occur within the cell types of the system. One group of cells that demonstrate this response to antigen challenge is the T cells. Through the altered expression of numerous proteins, T cells are able to become activated in the presence of an antigenic stimulus and then return to a resting state once the stimulus is removed (Miosge and Goodnow, 2005). T cell stimulation is conferred through the interaction of the T cell receptor (TCR) with an antigen stimulus. Upon activation, a complex cascade of signaling events occurs. Accessory molecules, molecules which aid the TCR in antigen recognition, regulate cellular protein kinases, such as Lck, Fyn, and ZAP-70. The transmembrane accessory protein, CD45, activates Fyn and Lck by removing inhibitory phosphates. Once activated, Lck activates ZAP-70 via

phosphorylation, thereby resulting in the phosphorylation and activation of PLCγ1 (phospholipase C-gamma 1). Activated PLCγ1 hydrolyzes PIP2 (phosphatidylinositol 4, 5-bis-phosphate) into IP3 (inositol triphosphate) and DAG (diacylglycerol). IP3 causes Ca2+ release and the translocation of the transcription factor NF-AT into the nucleus. DAG activates the protein kinase C (PKC) and Ras pathways (Figure 1-8) (reviewed in Mustelin and Tasken, 2003).

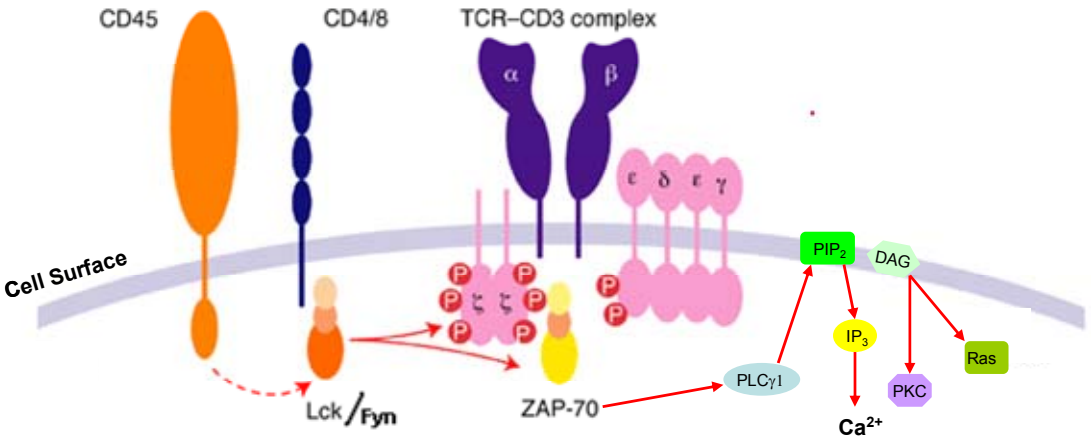

Figure 1-8. The T cell signaling pathway. Antigen stimulation of TCR results in a signaling cascade involving the accessory protein CD45. CD45 activates Lck and Fyn via dephosphorylation. Lck activates ZAP-70 through phosphorylation allowing for the phosphorylation and activation of $PLC_{\gamma}1$. Activated $PLC_{\gamma}1$ hydrolyzes PIP_2 into IP_3 and DAG. IP_3 casues Ca^{2+} release. DAG activates PKC and Ras pathways. Figure adapted from Wilkinson et al., 2005.

CD45 as a Model for Signal Induced Alternative Splicing

The CD45 gene is an excellent model for the study of signal induced alternative splicing, as both the splicing pattern and biological consequences of this process upon the gene have been well studied (Lynch, 2004). CD45 is a transmembrane protein and a member of the protein tyrosine phosphatase family (reviewed in Trowbridge and Thomas, 1994). The gene consists of thirty-three exons, with three, exons 4, 5, and 6, being variably expressed. The varied expression

of these exons results in the existence of five different CD45 isoforms (Figure 1-9) (Hermiston et al., 2002).

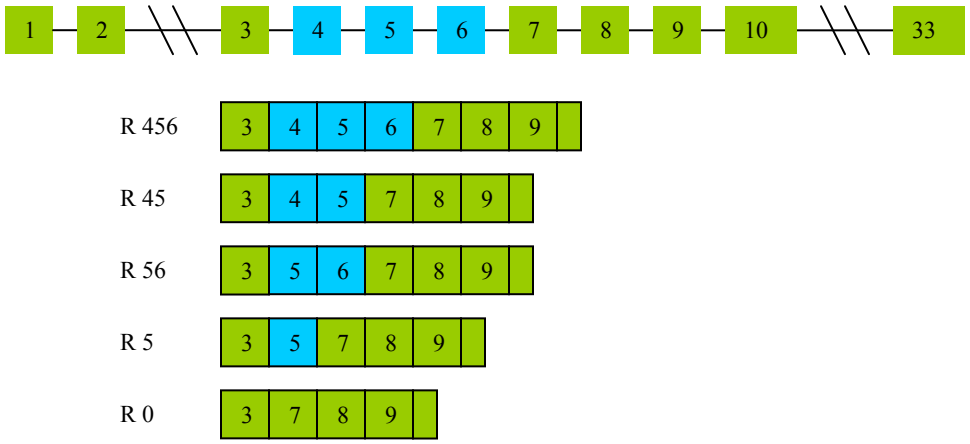

Figure 1-9. The five isoforms of CD45. Green boxes represent constitutive exons. Blue boxes represent variable exons. Each of the five different mature mRNA isoforms is shown.

Expression of the different CD45 isoforms relies on the activation state of the T cell. A naïve T cell, a T cell that has not been exposed to an antigen, displays the larger (R 456) CD45 protein. This isoform appears to have greater phosphatase activity, allowing for T cell activation (Figure 1-10A). Following antigen challenge, the smaller isoforms of CD45 are expressed, resulting in protein dimerization. The smaller, dimerized isoform has inhibited phosphatase activity which leads to inhibition of T cell activation (Figure 1-10B) (Majeti et al., 2000; Xu and Weiss, 2002). Through such a process, T cell activity can be regulated in response to antigen stimulation.

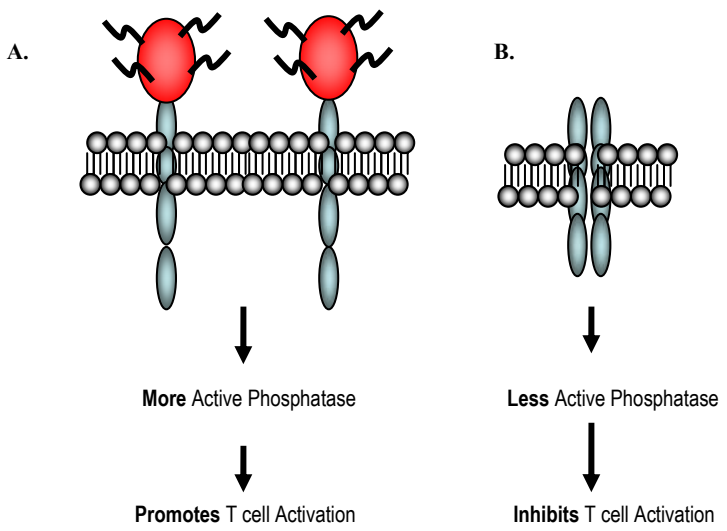

Figure 1-10. Expression of different CD45 isoforms regulates T cell activity. (A) A naïve T cell with the larger CD45 isoform being expressed. (B) A stimulated T cell with the smaller CD45 isoform being expressed.

The large isoform of CD45 is known to be expressed in a small subset of T lymphoma derived from the Jurkat cell line. A single clone from this cell population has been selected for its high level of expression of the full-length CD45 mRNA, and was called JSL1 (Jurkat Splicing Line 1) (Lynch and Weiss, 2000). Stimulation of JSL1 cells with the DAG analog, PMA, recapitulates the signal induced alternative splicing patterns of the CD45 gene seen in primary human T cells. Furthermore, stable expression of minigene constructs containing the variable exons of CD45 in JSL1 cells mimics the regulated splicing of endogenous CD45, thus providing a useful model system for studying the regulation of signal induced alternative splicing (Lynch and Weiss, 2000).

Through the investigation of a minigene construct, regulatory elements within exon 4 have been identified (Figure 1-11) (Lynch and Weiss, 2001). Analogous to the endogenous CD45 gene, inclusion of exon 4, when expressed in a minigene context in resting JSL1 cells, is ineffieient (basal exon repression), and in activated JSL1 cells there is a further 3-5 fold increase in exon 4 skipping (activation-induced exon repression). Deletion of the ESS1 splicing silencer

sequence of exon 4 results in significantly higher inclusion of the exon in both resting and stimulated cells; identifying this regulatory sequence as sufficient for regulating both the basal and activation-induced repression of exon 4 and suggesting that the regulated expression of this variable exon is dependent on the interaction of ESS1 with a splicing inhibitory complex (Rothrock et al., 2003).

Figure 1-11. The regulatory splicing elements of CD45 exon 4. Shown are the relative arrangements of the splicing enhancer and silencer sequences within exon 4 of CD45. Figure adapted from Lynch and Weiss, 2001.

Analysis of proteins bound to the ESS1 sequence in resting cells reveals a complex consisting of hnRNP L, PTB (polypyrimidine tract binding protein), and hnRNP E2. Further investigation of these proteins revealed that basal exon repression of exon 4 is conferred via the binding of hnRNP L to ESS1 (Figure 1-12A) (Rothrock et al., 2005). Additionally, biochemical assays performed by the Lynch lab have revealed that hnRNP L is differentially modified in resting versus stimulated cells. This change in the modification state of hnRNP L correlates with a slight increase in exon repression. Furthermore, *in vitro* assays have shown the recruitment of an additional protein, PSF, to the ESS1-regulatory complex under stimulated conditions. The binding of this protein to ESS1 demonstrates a greater effect on exon skipping than is seen with hnRNP L (Figure 1-12B) (Melton, unpublished).

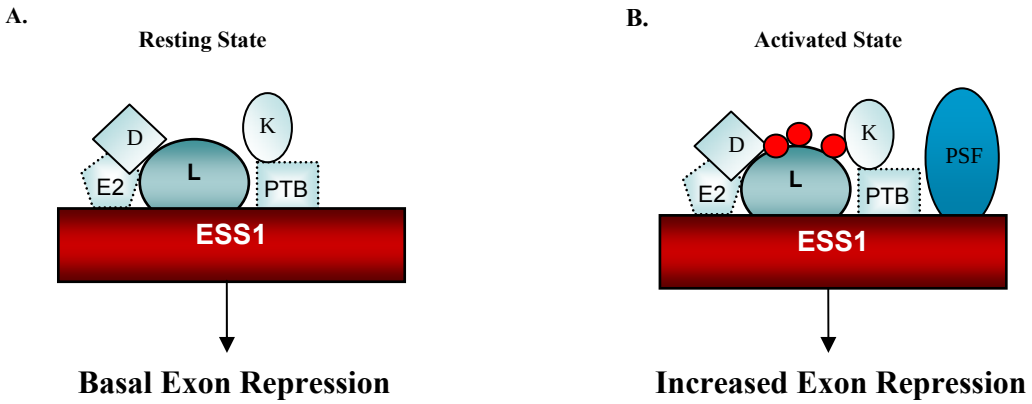

Figure 1-12. The binding and modification of regulatory proteins to ESS1 conferrers changes in the level of variable exon 4 inclusion. (A) Under resting conditions, the binding of hnRNP L to the ESS1 sequence of variable exon 4 in CD45 results in basal exon repression. (B) Protein modification to hnRNP L and binding of PSF to the ESS1 sequence under activated conditions results in increased exon repression.

Although *in vitro* studies have allowed for the identification of important signal induced regulatory proteins, these studies cannot fully recapitulate the signal induced cascades which occur within cells. It is for this reason that it is necessary for us to have the ability to reproduce these findings *in vivo*. In order to identify, *in vivo*, the proteins involved in regulating alternative splicing, one must be able to alter the levels of candidate gene expression. While there are many strategies for manipulating proteins within cells, most of these methods have proven to be difficult or unsuccessful in JSL1 cells. One common method for altering protein activity is the use of chemical inhibitors. Although this strategy is generally successful in JSL1 cells, inhibitors specific to all proteins of interest do not exist. A second approach for changing protein expression is RNAi (RNA inhibition). For reasons we have not fully characterized, JSL1 cells are unable to support this technique for knocking down protein expression. However, there has been recent success in achieving protein knockdown through the use of morpholinos (KWL, unpublished data). Another common strategy for manipulating protein expression is through transient transfection. In JSL1 cells, this technique only yields a 10-20% efficiency. The

analogous strategy of infection yields a higher, 70-90%, efficiency but relies on a promoter that is inducible by PMA.

As is evident by the obstacles discussed above, there is a significant need for the establishment of techniques that will allow for the altered expression of proteins within JSL1 cells. The next two chapters will focus on the development of strategies aimed towards facilitating the overexpression of target proteins, the stable expression of flag-tagged proteins, and the regulated expression of proteins of interest in JSL1 cells. It is anticipated that these methods will better enable the identification of protein associations and modifications involved in the regulation of alternative splicing in response to extracellular stimuli.

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Chapter Two

Development and Characterization of Methods for Overexpression of Proteins in JSL1 Cells

Introduction

To date, the study of CD45 has revealed novel information regarding the regulatory elements involved in signal induced alternative splicing. The enhancer and silencer regions within CD45 variable exon 4 that influence exon inclusion or exclusion have been characterized (Lynch and Weiss, 2001; Rothrock et al., 2003). Additionally, biochemical assays have identified hnRNP L and PSF as the primary regulatory proteins that bind to the signal-responsive silencer (ESS1) region of CD45 exon 4 (Rothrock et al., 2005; Melton, unpublished data). Although these findings have provided information that is critical to developing a better understanding of the mechanisms regulating CD45 splicing, the cell signaling pathways involved in inducing activation mediated alternative splicing are still elusive.

Cell signaling pathways are often analyzed through the use of cell culture systems, where protein expression may be altered and changes in cellular processes can be observed. As described in chapter 1, the Jurkat derived JSL1 cell line faithfully recapitulates the signal induced alternative splicing of the CD45 gene as seen in primary human T cells (Lynch and Weiss, 2000). Therefore, this cell line provides a cell culture system for observing the signaling pathways involved in regulating the alternative splicing of CD45 in response to stimulus.

Due to limitations inherent to JSL1 cells, altering protein expression has proven to be a challenge. One of the limitations imposed by JSL1 cells is their inability to support RNAi. For reasons we have been unable to identify, these cells fail to use this common method for protein knockdown. Another limitation to altering protein expression in JSL1 cells is the inefficient transfection of plasmid DNA via traditional transfection techniques. Low transfection efficiency

causes only a small fraction of the cells to express the desired protein product; therefore, any changes in splicing which may occur are masked by the effects of the larger, unaltered portion of cells. In efforts to address the challenge associated with transfection, a retroviral infection system was established in JSL1 cells and although this system provided higher levels of plasmid incorporation, it demonstrated complications due to variable protein expression and the PMA induction of its promoter.

Given the difficulties associated with manipulating protein expression in JSL1 cells, there is a significant need for the establishment of a technique that will allow for the altering of protein expression within this cell line. In this chapter, I describe the development and characterization of a strategy aimed towards circumventing these limitations so that it may serve as a tool for facilitating the investigation of the pathways involved in signal induced alternative splicing.

Results

The expression of cDNA driven by the EF1-α promoter may compensate for low transfection efficiency in JSL1 cells.

Previously, in an effort to increase the efficiency of plasmid transduction in JSL1 cells, a retroviral infection system was established. The MaRX II retroviral infection system was chosen because both transient expression as well as stable integration of plasmid DNA can be achieved through the use of this system. The MaRX II retroviral vector relies on two mammalian promoters for driving expression in cells: the LTR and the PGK promoters (Hannon, et al., 1999). When transfected into a viral packaging cell line, this plasmid is packaged into viral particles and secreted into the cell culture medium. ϕNX cells, a 293T derived cell line that stably expresses the gag, pol, and env retroviral proteins (Nolan, webpage), was used as the viral packaging cell line. In order to accommodate this viral packaging cell line, which produces a

murine specific virus, a JSL1 cell line stably expressing the murine specific viral receptor was developed and named JVR cells.

Viral infection of JVR cells is achieved through a two-step process. First, the MaRX II plasmid is transfected into ϕNX cells where it is packaged into viral particles and secreted into the cell media. Second, the virus containing media is added to the JVR cells resulting in their infection with the virus and subsequent expression of the cDNAs encoded within the MaRX II vector (Figure 2-1). Once infected, the JVR cells are treated as desired and protein and RNA is harvested for analysis.

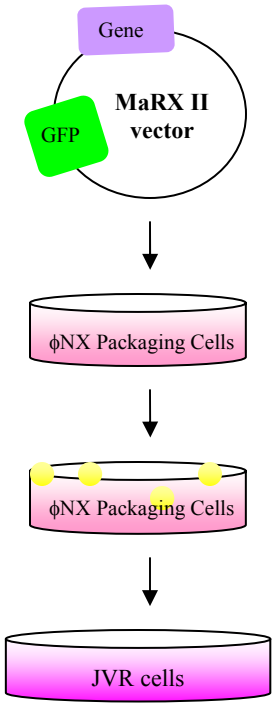

Figure 2-1. Schematic of retroviral infection of JVR cells. MaRX II plasmid is transfected into the ϕNX viral packaging cell line. Media containing virus (yellow circles) is added to JVR cells to allow for infection.

To evaluate the transduction efficiency of the retroviral infection system FACS analysis of GFP production, driven by the PGK promoter of the MaRX II vector, was conducted. This analysis showed significantly higher GFP expression (70-90%) in cells that were infected with the MaRX II packaged retrovirus when compared to JVR cells electroporated with the same MaRX II plasmid (30%) (Figure 2-2) (Experiment by Alexis Melton). Such data suggests that the retroviral infection system provides a technique that would allow for the sufficient delivery of plasmid DNA to the modified JSL1 cell line, thereby enabling the evaluation of candidate proteins. However, when candidate protein expression from the MaRX II plasmid was attempted two technical issues with the system were detected.

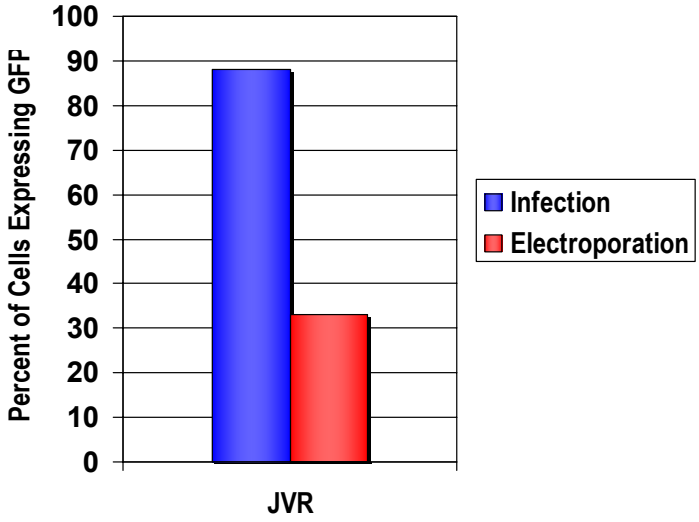

Figure 2-2. Infection results in increased plasmid transduction efficiency as compared with electroporation in JVR cells. Following either infection or electroporation of JVR cells with the MaRX II plasmid, the percentage of GFP expression, driven off of the PGK promoter of the MaRX II vector, was determined via FACS analysis. Experiment by Alexis Melton.

One issue associated with the MaRX II retroviral infection system became apparent upon the evaluation of candidate protein expression in resting JVR cells following infection. Previously, Lynch and Weiss demonstrated that transfection of a constitutively active form of the small G protein Ras (Ras R12, T59) resulted in at least a partial decrease in variable exon inclusion in resting cells (Lynch and Weiss, 2000). However, when Ras R12 was cloned into the MaRX II vector downstream of the LTR promoter and virally transfected into JVR cells, RT-PCR analysis detected no change in the level of exon inclusion. Furthermore, little to no protein overexpression was detected by Western blot analysis despite adequate transduction efficiency as determined by FACS analysis of GFP production driven by the PGK promoter (experiment by Alexis Melton). Similar experiments using different candidate protein cDNAs with a flag-tag sequence demonstrated the same low level of protein expression in resting JVR cells despite acceptable transduction efficiency (Figure 2-3A). Since the primary purpose for establishing the retroviral infection technique was to achieve the ability to overexpress candidate proteins, the low levels of expression afforded by the MaRX II system show that this goal was not achieved.

A second issue with the retroviral infection system was detected upon stimulation of JVR cells. In order to induce a change in CD45 splicing, the JSL1 cell line is treated with PMA. When JVR cells infected with retrovirus containing MaRX II plasmid encoding candidate protein cDNAs were exposed to PMA, Western blotting for flag-tagged proteins detected an increase in candidate protein expression (Figure 2-3B). This finding suggests that the LTR promoter driving candidate protein cDNA expression is PMA inducible. Since it is necessary to evaluate splicing during resting and stimulated conditions, PMA induction of the LTR promoter makes it difficult to draw necessary correlations, thereby presenting another limitation of the retroviral infection system.

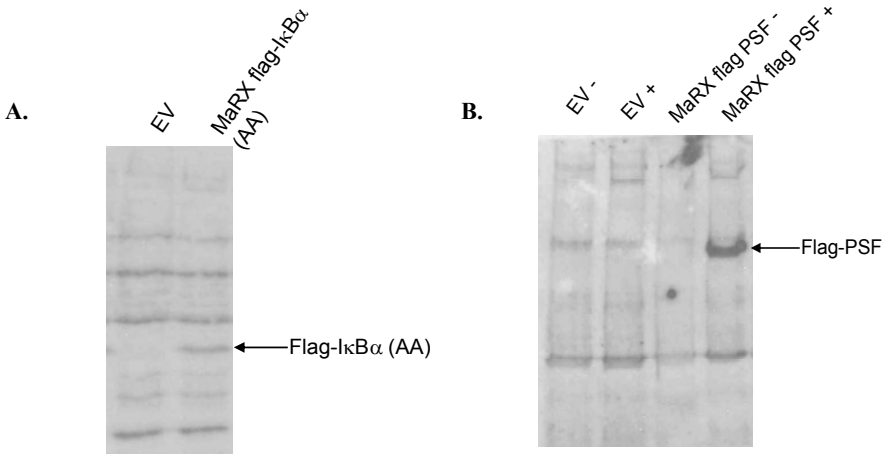

Figure 2-3. The MaRX retroviral infection system causes low and variable protein expression. (A) Western blotting for flag-tagged protein detects low expression from the LTR promoter. IκBα (AA) is a dominant negative mutant of IκBα. (B) Western blot analysis for flag-tagged protein reveals that the LTR promoter is PMA inducible. EV represents empty vector. For each Western blot, 10μL of samples at a concentration of $7.5x10^4$ cells/mL were loaded.

Given the problem of low and variable expression seen with the LTR promoter, we screened multiple common promoters for their activity in JSL1 cells. Available in the lab were minigene constructs that used the EF1-α, CMV, or Act promoter to drive expression of the same mRNA. Following transfection of each construct into JSL1 cells, RNA was harvested and mRNA expression levels were analyzed. RT-PCR analysis of mRNA expression in resting cells

revealed no detectable function from the CMV promoter and only moderate function was detected with the Act promoter. However, expression from the EF1-α promoter was robust; making it a strong candidate for driving desired protein expression within JSL1 cells (Figure 2-4).

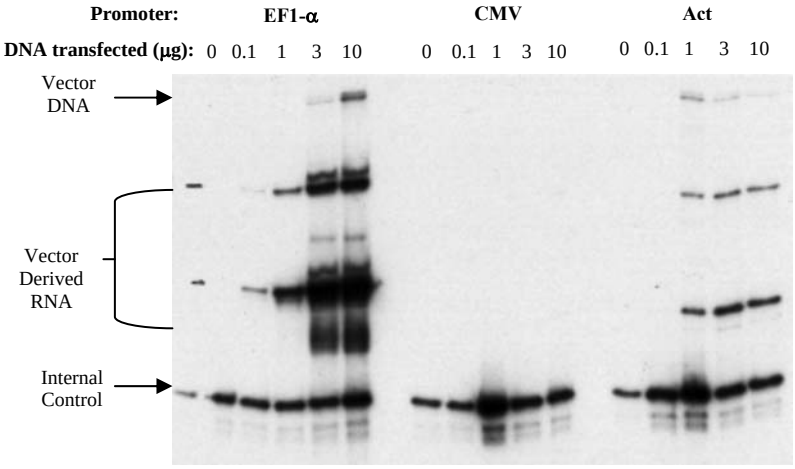

Figure 2-4. The EF1-α promoter allows for robust expression in JSL1 cells. RT-PCR analysis of mRNA expression driven by three different promoters shows no mRNA production from the CMV promoter, moderate production via the Act promoter, and robust production through the EF1-α promoter. RT-PCR by K. Lynch.

Despite the poor transfection efficiency in JSL1 cells, significant protein expression from a robust promoter may compensate for the low number of cells containing the plasmid of interest. Thus, a parental vector containing the EF1-α promoter and a multiple cloning site (MCS) was developed and named, pEFneo. An additional flag-tagged parental vector, pEFnFlag, was developed

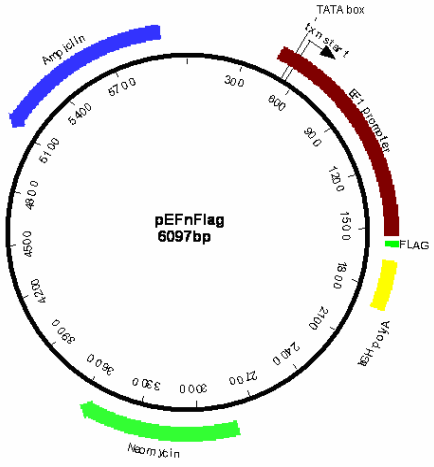

Figure 2-5. Plasmid map of pEFnFlag. A parental vector containing the EF1-α promoter, an MCS, and a flag-tag sequence was developed

from pEFneo (Figure 2-5). cDNAs encoding proteins of interest may be easily cloned into these two vectors and highly expressed when transfected into JSL1 cells. Examples of this technique are demonstrated by the cloning and transient transfection of regulatory proteins, whose expression and function in JSL1 cells have been previously characterized, into the parental EF1-α vectors.

To determine the effectiveness of the EF1-α parental vectors in expressing flag-tagged proteins and to monitor the levels of protein overexpression the regulatory proteins hnRNP L, PSF, PTB, and p54 were cloned into pEFnflag. Each plasmid was transfected into JSL1 cells via electroporation. Following 48 hours of recovery, cells were lysed and efficiency of protein expression was determined by Western blotting for the flag-tagged protein. When compared to the low flag-tagged protein expression observed in resting cells using the MaRX retroviral infection method; this technique provided a 3-5 fold increase in detectable flag-tagged protein expression for each plasmid transfected (Figure 2-6A, compare EFflag PSF to MaRX flag PSF – PMA in Figure 2-3B). Moreover, in contrast to the LTR promoter, the EF1-α promoter is not PMA inducible (Figure 2-6B). This data suggests that proteins inserted into the pEFflag vector may be successfully expressed at consistent and detectable levels within JSL1 cells.

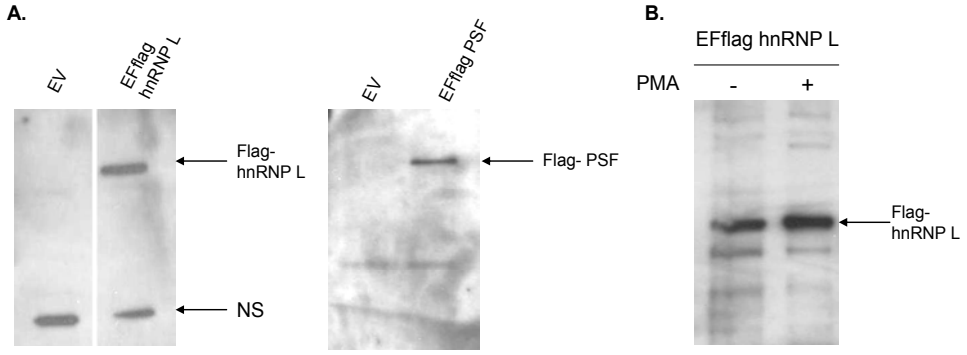

Figure 2-6. Western blots for flag-tagged proteins transiently transfected into JSL1 cells. (A) Western blot analysis for flag-tagged proteins transfected into JSL1 cells reveals that each construct was able to produce the protein of interest. EV represents empty vector. (B) Western blotting for flag-tagged protein expression in resting and stimulated cells shows no induction of the EF1-α promoter with PMA. NS represents non-specific band. For each blot, 10μL of samples at a concentration of $7.5x10^4$ cells/mL were loaded.

In order to determine whether the amount of protein expressed from the EF1-α promoter was changed, Western blot analysis was done with antibody that detects both endogenous and flag-tagged protein. Since the flag-tag is small compared to overall protein size, both forms co-migrate. For each transfection showing the expression of the desired flag-tagged protein, little to no overexpression was detected (Figure 2-7). It should be noted, however, that another member of the Lynch lab has been able to achieve significant overexpression of flag-tagged Ras when the protein's expression was driven by the EF1-α promoter and transiently transfected into JSL1 cells. Therefore, the inability to detect high levels of

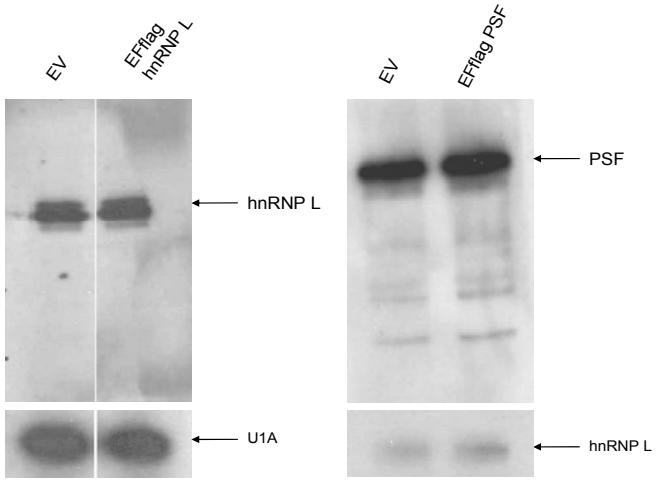

Figure 2-7. Western blots for endogenous proteins following transient transfections. Cells expressing flag-tagged proteins show little to no overexpression of the transfected proteins. EV represents empty vector. U1A was blotted for as loading control for hnRNP L blot and hnRNP L is shown as loading control for PSF blot. For each blot, 5μL of samples at a concentration of $7.5x10^4$ cells/mL were loaded.

expression for these initial transfections may be a result of their already high expression levels within the cell line, and not a direct reflection of the strength of the EF1-α promoter for driving protein expression. Another explanation for these findings may be that as regulatory proteins, their increased level of expression from the transfected plasmid causes a decrease in their own endogenous expression. In support of this idea, results from RT-PCR analysis of high and low expressors of pEFflag PSF stably transfected into JSL1 cells showed a direct correlation between increased flag-tagged PSF mRNA production and decreased endogenous PSF mRNA production (Figure 2-8).

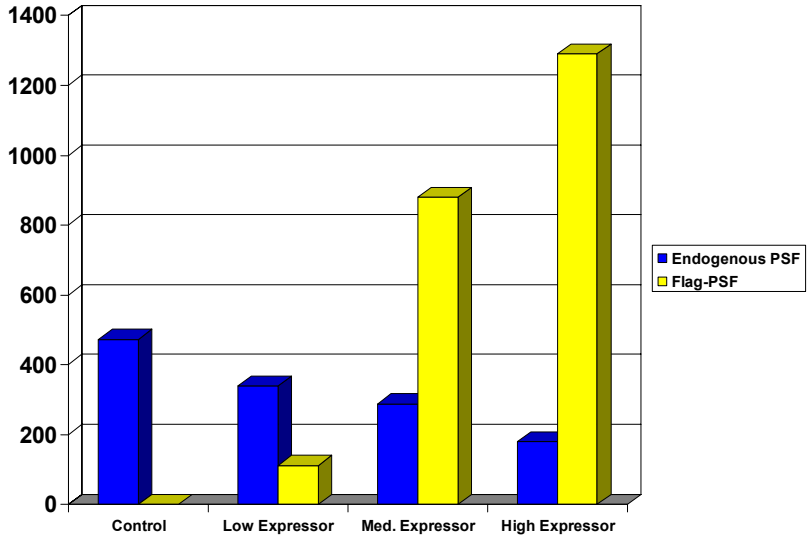

Figure 2-8. Overexpression of regulatory proteins causes a decrease in their endogenous expression. RT-PCR analysis, using primers specific for either endogenous PSF or flag, was conducted on RNA from cells stably expressing flag-tagged PSF driven by the EF1-α promoter. When compared to cells not expressing the flag-tagged RNA, one sees a direct correlation between increased flag-tagged RNA expression and decreased endogenous RNA expression (Experiment conducted with help of Alan Tong)

Previously, Rothrock et al. demonstrated that a 50% reduction in hnRNP L expression in 293 cells transfected with a CD45 exon 4 minigene, was sufficient to induce a significant increase in exon 4 inclusion (Rothrock, et. al., 2005). This data suggests that the splicing process is tightly regulated and slight changes in protein expression can significantly alter the splicing process. To observe whether the modest change in protein expression achieved by the EF1-α

promoter would alter splicing of CD45, RNA was harvested from JSL1 cells transfected with pEFnflag expressing various protein cDNAs of interest and analyzed by RT-PCR. Figure 2-9, shows RT-PCR of mRNA harvested from JSL1 cells stably expressing a minigene that recapitulates the splicing pattern of the endogenous CD45 gene (see chapter 1). Primers specific to the minigene enable the detection of variable exon 4 inclusion or exclusion. In cells expressing detectable levels of flag-tagged protein, no change in exon 4 splicing was observed under resting conditions (Figure 2-9). In addition, RT-PCR analysis of PMA stimulated cells detected no deviations from normal splicing.

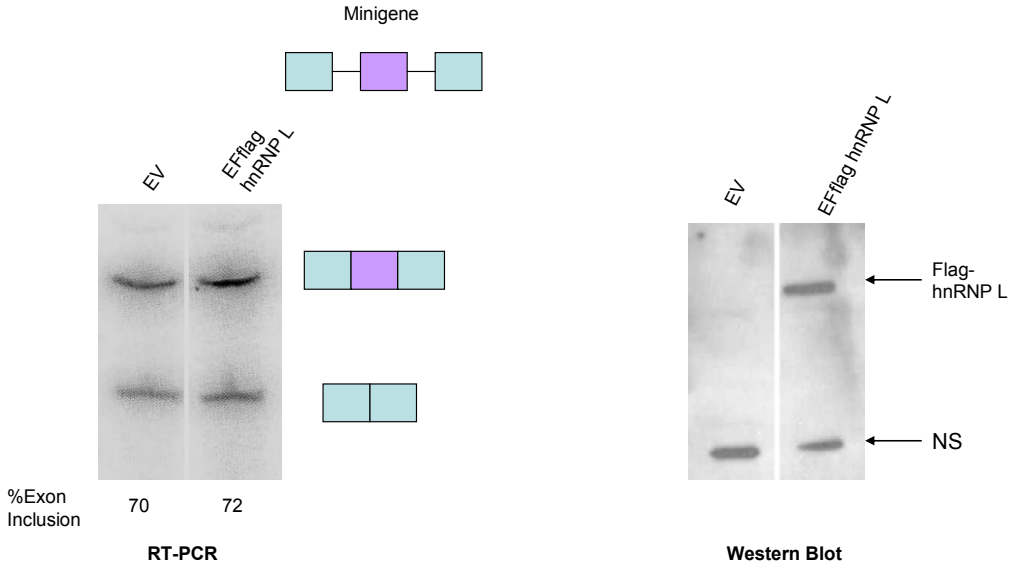

Figure 2-9. RT-PCR analysis shows no change in variable exon 4 splicing in resting JSL1 cells transfected with EFnflag constructs. RT-PCR analysis of RNA from JSL1 cells, stably expressing a minigene that recapitulates CD45 splicing, shows no change in variable exon 4 inclucsion in resting JSL1 cells expressing flag-tagged hnRNP L. Western blot shows detectable flag-tagged hnRNP L in the same cells. The blot was loaded as follows: 10μL of samples at a concentration of $7.5x10^4$ cells/mL. Figure labeled minigene depicts the organization of the minigene used in this experiment. The purple box represents variable exon 4. The boxes to the side of the RT-PCR show the isoforms represented in the gel. NS represents non-specific band.

Furthermore, to determine times of optimal protein expression following transfection, RNA and protein was harvested from JSL1 cells 24, 48, and 72 hours after electroporation. Flag-tagged protein expression was analyzed via Western blot with anti-flag antibody. In cells transfected with the pEFnflag vector expressing cDNA for either hnRNP L or PSF it appears that

the highest level of expression is 24 hours following electroporation (Figure 2-10). However, RT-PCR analysis of endogenous CD45 detected no change in splicing at any timepoint (data not shown). While protein and RNA has been analyzed 48 hours following electroporation in work described here, harvesting cells 24 hours after electroporation may show more dramatic changes in protein overexpression and subsequently CD45 splicing.

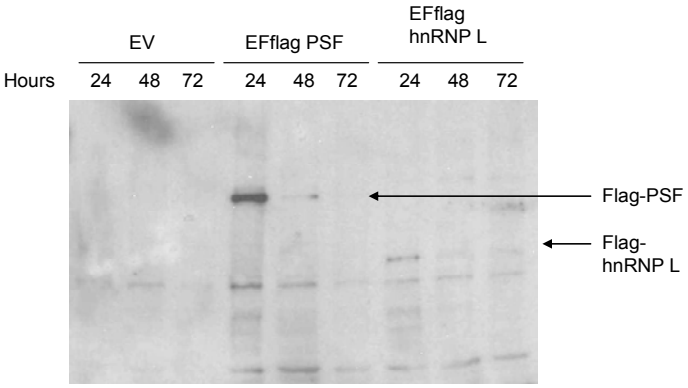

Figure 2-10. Analysis of flag-tagged protein expression in JSL1 cells at 24, 48, and 72 hours following electroporation. Western blotting for flag-tagged protein expression at 24, 48, and 72 hours following electroporation reveals that both PSF and hnRNP L expression driven by the EF1-α promoter are highest at 24 hours. EV represents empty vector. For each sample, 10μL at a concentration of $7.5x10^4$ cells/mL was loaded.

Stable cell lines expressing EF1-α Flag-tagged proteins

As a complementary approach to transient transfections, efforts towards establishing JSL1 cell lines stably expressing proteins driven by the EF1-α promoter have been made. The development of stable cell lines expressing flag-tagged proteins may provide at least two benefits that will aid in the study of the factors involved in regulating signal induced alternative splicing. First, by enriching for a population of cells stably expressing the EF1-α driven protein of interest, the problem associated with low transient transfection efficiency can be overcome. As previously mentioned, low transient transfection efficiency causes only a small portion of the cells to express the desired protein product. As a result, any changes in splicing which may occur are masked by the effects of the larger, unaltered population of cells. Since stable cell lines

represent a population of cells that posses the same altered protein of interest, the effects of these proteins would no longer be masked. Therefore, it may be possible to detect changes in alternative splicing in stable cell lines, where no change was detected from the corresponding transient transfections. Second, stably expressed flag-tagged proteins may be easily isolated. Since the activation of different signaling pathways in response to stimuli can cause a variety of changes in both protein modification and localization, having the ability to isolate specific proteins of interest and analyze their signal responsive changes will be a valuable tool.

To generate stable cell lines the transfected JSL1 cells were serially diluted into cell culture medium containing Neomycin, after 48 hours of recovery following electroporation. This drug selection process allowed for the isolation of only those cells containing the pEFflag + candidate protein cDNA vector. Three cell lines stably expressing the pEFflag constructs have been established. The vectors that have been stably integrated into the JSL1 cells are pEFflag PSF, pEFflag PTB, and pEFflag p54. Individual clones expressing each of these flag-tagged proteins were identified via drug selection and Western blotting for the flag-tagged protein of interest (Figure 2-11). Western blotting specifically for the protein of interest revealed no dramatic changes in expression level (data not shown); but as mentioned previously this may be due to the already high abundance of these proteins and/or the autoregulation of their expression.

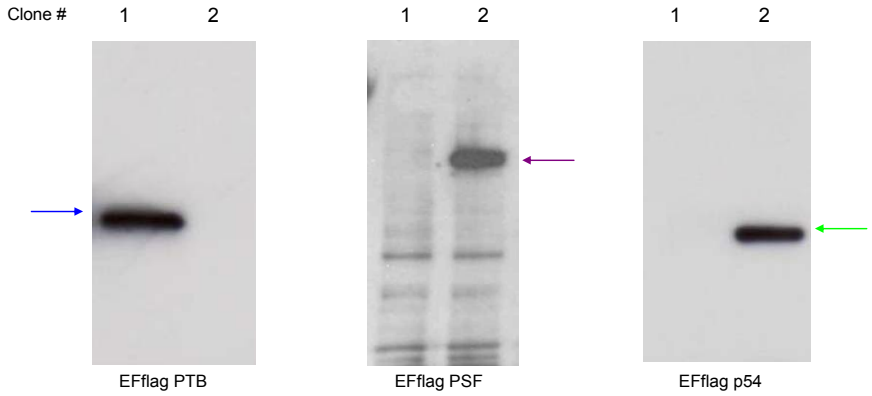

Figure 2-11. Western blots of stable cell line clones expressing flag-tagged proteins. Numbered lanes indicate individual clone samples. Western blot analysis using anti-flag antibody indicates cells expressing flag-tagged protein of interest. Blue arrow: flag-PTB. Purple arrow: flag-PSF. Green arrow: flag-p54. For each Western blot, 10μL of samples at a concentration of $7.5x10^4$ cells/mL were loaded.

As mentioned previously, one hurdle associated with low transient transfection efficiency is the masking of effects caused by altered protein expression in a small fraction of cells by the unaltered effects resulting from the larger unmodified cell population. The stable cell lines represent a homogenous cell population expressing the same EF1-α promoter driven protein expression. Therefore, the aforementioned hurdle should be overcome in this cell population. To evaluate any splicing changes in the stable cell lines, RT-PCR analysis of endogenous CD45 was performed. As seen with the transient transfections, there was no detectable change in CD45 splicing under resting conditions (Figure 2-12). However, it is necessary to evaluate any changes in splicing that may occur with PMA stimulation before conclusions can be drawn about the involvement of these stable cell lines in the regulation of CD45 splicing.

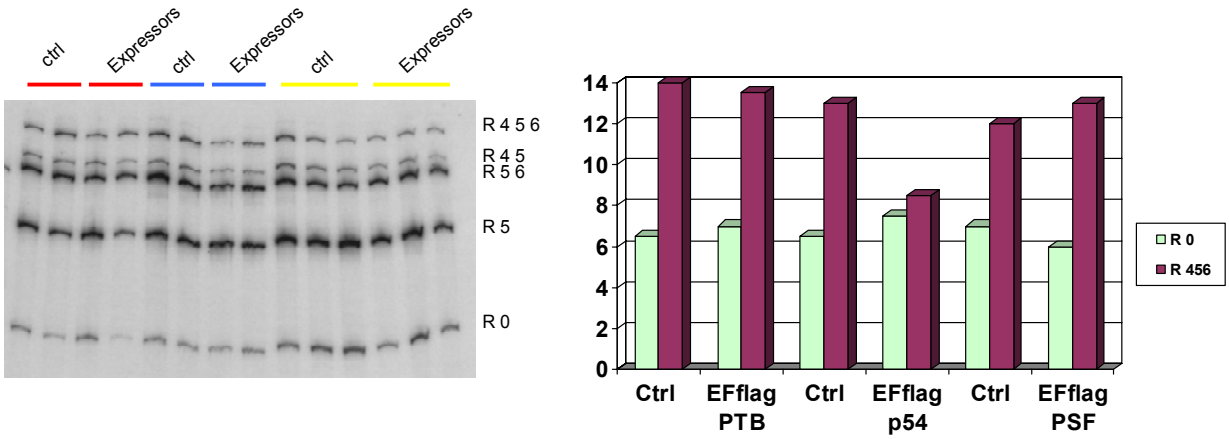

Figure 2-12. RT-PCR analysis of endogenous CD45 mRNA in cells stably expressing flag-tagged candidate proteins. RT-PCR analysis of RNA from cells stably expressing EFflag PTB (red lines), EFflag p54 (blue lines), or EFflag PSF (yellow lines) indicates no change in CD45 isoform expression when compared to control (ctrl). R456, R45, R56, R5, and R0 represent variable exons shown on autoradiogram (isoforms discussed in chapter 1). The chart shows amount of R 456 isoform versus R 0 isoform for each cell line. Percentages for each isoform were averaged for each cell line and its corresponding control.

Discussion

With the development of parental vectors that are able to be successfully transiently transfected into JSL1 cells, many opportunities for studying the factors involved in signal responsive alternative splicing become available. Evidence that proteins, such as Ras, can be overexpressed in JSL1 cells suggests that other genes of interest may be cloned into the parental EF1-α vectors and expressed at high levels. Success of this technique will allow for the overexpression of constitutively active or dominant negative mutant proteins. Such alterations in protein expression will play a key role in identifying the factors involved in the signal responsive cell signaling pathways.

The stable expression of flag-tagged proteins within the JSL1 cell line will provide a valuable tool for isolating and analyzing the changes incurred by various proteins in response to extracellular stimuli. As more pEFflag containing stable cell lines are established, overexpression of proteins that exist at lower concentrations and that are not autoregulated may be achieved. The impact of this sustained altered protein expression on signal responsive

alternative splicing may also provide insight into the signaling pathways involved in splicing. However, it is important to consider that the constant overexpression of certain proteins may have a detrimental effect on cell growth and survival. It is for this reason that a strategy that will allow for the controlled overexpression of target proteins is in the process of being developed and will be discussed in detail in the following chapter.

Materials and Methods

Cell Culture

JSL1 cells, described previously (Lynch and Weiss, 2000) were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum, 2mM glutamine, penicillin, and streptomycin. The cells were grown at 37°C in the presence of 5% CO_2.

ϕNX cells were maintained in DMEM supplemented with 5% heat-inactivated fetal calf serum, 2mM glutamine, penicillin, and streptomycin, and 50μM Beta Mercaptoethanol.

MaRX II retroviral infection

On the morning of day 1, ϕNX cells were plated at ~20% confluency in ϕNX cell media. That afternoon ϕNX cells were transfected as follows: Tube 1: 1mL 2XHBSS pH7.05, Tube 2: 10μg MaRX DNA, 125μL 2M $CaCl_2$, 865 μL water. The contents of tube #2 was added dropwise to tube #1 while vortexing. The mixture was incubated at room temperature for 30 min. Following incubation, the mixture was added dropwise to ϕNX cells and gently swirled. Cells were incubated onverniht at 37°C.

The morning of day 2 ϕNX cells were washed as follows: media was removed from the cells, they were washed twice with warm PBS, resuspended in JSL1 cell media (see cell culture), and

incubated at 37°C for 24-48 hours. That afternoon JVR cells were split to a density of 0.5 x 10^6 cells/mL.

On day 3 JVR cells were infected as follows: media from ϕNX cells was transferred to a conical tube and centrifuged for 5 min. at 1000rpm., media was then transferred to a fresh tube and polybrene was added to a final concentration of 10μg/mL, 0.5 mL of JVR cells per mL of viral media was spun at 1000rpm for 5 min. and media was aspirated off, JVR cells were then resuspended in viral media + polybrene and transferred to a 6 well plate, the infection plate was spun at 2500 rpm for 90 min. at room temp. then incubated overnight at 37°C.

Subcloning

Insertion of sequences encoding the proteins hnRNP L and p54 into the Bam HI and Eco RI sites of pEFflag formed the plasmids pEFflag L and pEFflag p54, respectively. The hnRNP L sequence was digested from the plasmid pMaRX flag L; whereas the sequence encoding p54 was removed from pMaRX flag p54. The plasmid pEFflag PSF was cloned by blunt-end ligation of the PSF coding sequence, removed from the Xho sites of pMaRX flag PSF, into the Nde I region of pEFflag. Insertion of the sequence encoding PTB into the Eco RI site of pEFflag formed the construct pEFflag PTB.

Transient Transfections and Stable Cell Lines

Transfections were carried out via the electroporation of 10-20 million cells with 10μg of plasmid to be transfected. Cells were then plated in serum free RPMI 1640 medium and allowed to recover for 2 days. For transient transfections, protein was harvested after 2 days recovery and analyzed via Western blot as described. For stable cell lines, cells were recovered for 2 days in RPMI 1640–5% serum, then serially diluted into medium containing Neomycin 2 mg per ml, and

grown for an additional 2 to 3 weeks. Neomycin-resistant clones were then expanded further and analyzed for flag-tagged protein expression via Western blotting.

Western Blots

Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with Tris-buffered saline, 0.1% Tween 20 containing 5% nonfat dried milk or 5% BSA, washed, and incubated with indicated antibody. Detection was performed using enhanced chemiluminescence (ECL, Amersham).

Antibodies

Antibodies used for the indicated applications were as follows: anti-hnRNP L (4D11, Abcam), anti-PTB N-term (rabbit polycolonal, gift from D. Black), anti-PSF (B92, Sigma), anti-p54 (78-1C, Affinity BioReagents) and anti-flag (M2, Cell signaling).

RT-PCR

Total RNA was isolated by using RNAzol (Tel-Test, Friendswood, Tex.) according to the included protocol. For RT-PCR analysis of endogenous CD45, 1.5 mg of total RNA was heated to 90°C in the presence of 1 ng of RT primer, 300 mM NaCl, 10 mM Tris (pH 7.5), and 2 mM EDTA and allowed to cool to 43°C. This annealed reaction was diluted into an RT mix containing final concentrations of 10 mM Tris (pH 7.5), 6 mM MgCl2, 10mM dithiothreitol, 50 mM NaCl, and 1 mM deoxynucleoside triphosphates, and incubation was continued at 43°C for 30 min. For PCR, a third of the RT reaction mixture was diluted into a PCR mix containing final concentrations of 1.5 mM MgCl2, 10 mM Tris (pH 8), 50 mM KCl, 0.2 mM deoxynucleoside

triphosphates, 20 ng of RT primer, and 10 ng each of primers and overlaid with mineral oil. PCR was done by heating samples to 94°C for 2 min followed by 20 cycles of 1 min at 94°C, 1 min at 70°C, and 2 min at 72°C. RT-PCR analysis of minigene RNA was done similarly.

All of the above conditions were determined empirically to give a signal which was linear with respect to input RNA. Following completion of PCR, the reaction products were extracted with phenol-chloroform-isoamyl alcohol and ethanol precipitated in the presence of glycogen as carrier. The resulting pellets were resuspended in formamide loading buffer and resolved on a 5% denaturing polyacrylamide gel. Quantitation of all RT-PCR assays was done with a Typhoon Phosphoimager (Amersham Biosciences).

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Chapter Three

Development of EF1-α driven Tet-suppressor System

Introduction

Being able to control the expression of various genes extends the same benefits as achieved through the establishment of stable cell lines, but should relieve the complications associated with the potentially harmful constant overexpression of certain proteins. Regulating gene expression in mammalian cells is frequently made possible through the use of drug regulatory systems. Although there are a number of such systems commercially available, we are interested in adopting the T-REx™ System, marketed through Invitrogen, for facilitating the controlled expression of target proteins in JSL1 cells. We selected this system based on the fact that it has been characterized as successfully regulating gene expression in a variety of mammalian cell lines (Asp et al., 2000; Qi et al., 2004; Jones et al. 2005) and because it relies on regulatory elements from the native Tet operon, as opposed to other systems which use viral transactivators (Yao et al., 1998). This latter characteristic prevents secondary, non-specific activation of host genes. In order to implement a method for regulating gene expression, vectors containing promoters and drug resistance genes which are supported by the cell line must be developed. This chapter describes the mechanism of action of the Tet-suppressor system we propose to use, the modifications which must be made to the system so that it may function properly in JSL1 cells and initial progress towards this goal.

Results

The T-REx™ System is a tetracycline regulated mammalian expression system that utilizes a repressor mechanism to block transcription from the promoter in the absence of tetracycline. The system consists of two components: the regulatory vector (pcDNA™6/TR),

which provides expression of the tetracycline repressor (TR) protein and the expression vector (pcDNA™4/TO), which contains the gene of interest (Figure 3-1). Inserted between the TATA box of the promoter and the transcriptional start site of the gene of interest are two tetracycline operator sequences ($TetO_2$).When these two components are transfected into cells, expression of TR from the regulatory vector binds to the $TetO_2$ sequence and represses transcription of the gene of interest. Addition of tetracycline to the cell culture media binds to the TR protein, resulting in a conformational change and release from the $TetO_2$ sites, thereby allowing transcription of the gene of interest (Figure 3-1) (Scott, 2007).

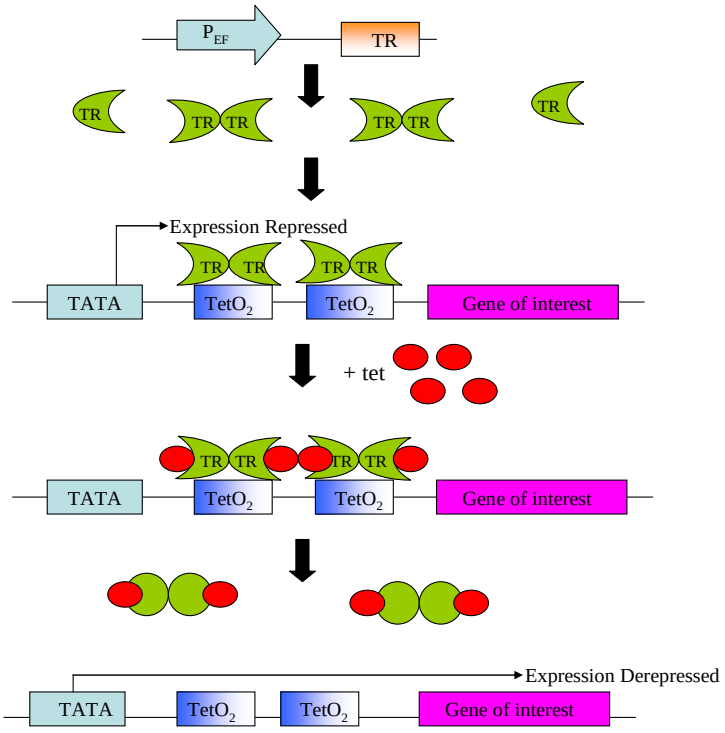

Figure 3-1. Functioning of Tet-Suppressor System. The Tet-repressor vector expresses the TR protein. In the absence of tetracycline, TR binds to the $TetO_2$ region of the promoter, inhibiting expression of the gene of interest. Addition of tetracycline causes a conformational change to the TR releasing it from the $TetO_2$ sequences, thereby allowing for gene expression. Figure adapted from Invitrogen.

The Tet-Repressor

While the commercial vectors for regulating gene expression are readily available, they are all based on the CMV promoter. As mentioned in chapter 2, this promoter fails to drive expression in JSL1 cells (Figure 2-1). Therefore, we have generated an EF1-α promoter based vector that drives expression of TR and renamed it pcDNA6/TR-EF. This construct has been transfected into JSL1 cells. Although Western blotting for TR protein production in cells 48 hours after electroporation shows little protein expression (Figure 3-2), we have proceeded with the process of establishing a stable cell line and are currently awaiting results.

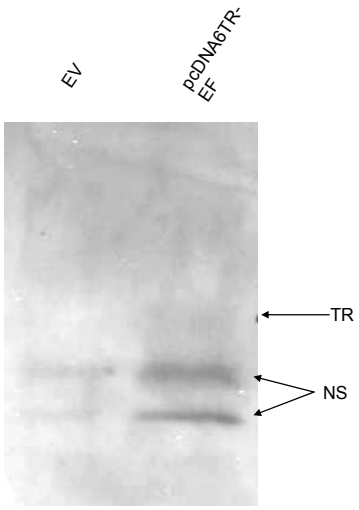

Figure 3-2. Western blot analysis of TR expression 48 hours after electroporation. Western blotting for TR protein expression 48 hours after electroporation detects small amounts of the protein. TR arrow is pointing to a faint band at the expected size for the tet repressor protein. EV represents empty vector. NS represents non-specific bands. For each sample, 10μL at a concentration of $7.5x10^4$ cells/mL was loaded.

The Tet-Operator

The expression vector also relies on the CMV promoter to drive gene expression. Therefore, we attempted to engineer the EF1-α promoter so that it would be Tet responsive. It has been previously determined that maximal gene suppression is

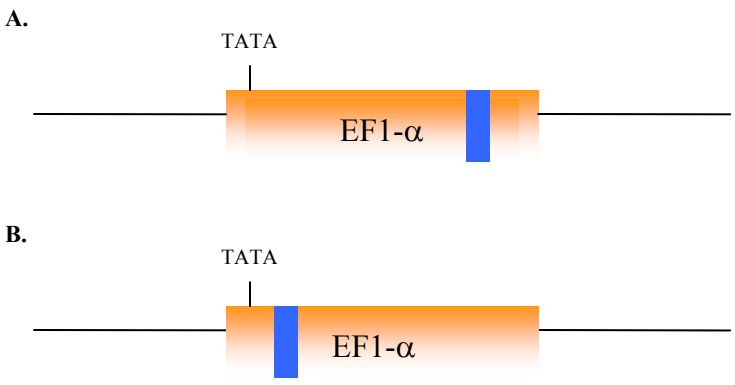

Figure 3-3. Schematic of the placement of the $TetO_2$ sequences within the EF1-α promoter. (A) The $TetO_2$ sequences were originally cloned into the far 3' end of the promoter. (B) The $TetO_2$ sequences have been shown to function optimally 10 nucleotides downstream of the TATA box. Blue box represents $TetO_2$ sequences.

achieved when the $TetO_2$ sequences are placed 10 nucleotides after the last nucleotide of the TATA box (Yao et al., 1998). Due to ease of cloning, we initially subcloned the $TetO_2$ sequences into a region at the far 3' end of the promoter (Figure 3-3A). However, the placement of these sequences into this region appears to inhibit protein expression (Figure 3-4). Possible reasons for this inhibitory effect may be due to the insertion of the $TetO_2$ sequences into transcription regulatory regions such as the Kozak sequence or due to the formation of hairpin structures within the inserted sequence. Therefore, we are currently introducing the $TetO_2$ sequences into the optimal region of the EF1-α promoter by PCR mutagenesis (Figure 3-3B). Future studies will be required before it can be determined how the location of these sequences within this promoter will function in regulating protein expression.

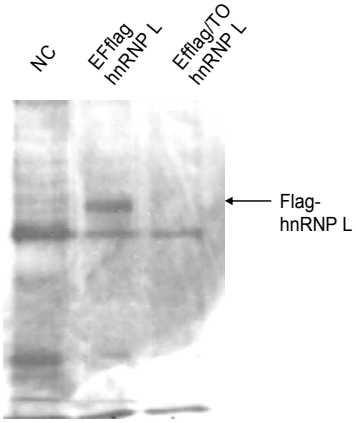

Figure 3-4. Insertion of $TetO_2$ sequences greater than 10 nucleotides downstream of the TATA box of the EF1-α promoter inhibits protein expression. Western blot of flag-tagged hnRNP L expression, driven by either the EF1-α or EF1-α/TO promoter, shows that flag-tagged protein is expressed by the unaltered promoter, but is undetectable with the modified promoter. NC indicates negative control. For each sample, 10μL at a concentration of $7.5x10^4$ cells/mL was loaded.

Once a functional expression vector is developed various proteins of interest may be cloned into the vector. These constructs can then be transfected into cells stably expressing pcDNA6/TR-EF. Cells co-expressing the regulatory and expression vectors can be identified by Neomycin resistance conferred via the expression vector. Proper functioning of the suppressor system can be ascertained by Western blot analysis of flag-tagged protein expression in the presence and absence of Tetracycline.

Discussion

Having the ability to regulate the expression of various proteins may prove to be one of the most valuable tools for identifying the factors involved in regulating signal induced alternative splicing. Being able to induce the expression of either a wild type or mutant protein at specific timepoints and observing their effects on signal mediated alternative splicing, can lead to key inferences about the signaling pathways involved in this protein expression regulatory process. Furthermore, the establishment of a Tet-inducible system should provide a means for achieving the expression of flag-tagged and/or mutant proteins while circumventing the complications associated with constant overexpression of proteins. Unfortunately, there is a risk that this modified regulated expression system will be unable to fully repress promoter function, negating the aforementioned benefit. Additionally, since a system that relies on the EF1-α promoter for protein expression has not been previously developed, we are uncertain whether any gene suppression will be achieved through the binding of the TR protein. As more advances are made on this system these questions may be addressed and strategies for optimization of a regulated expression system can be explored.

Materials and Methods

Cell Culture

JSL1 cells, described previously (Lynch and Weiss, 2000) were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum, 2mM glutamine, penicillin, and streptomycin. The cells were grown at 37°C in the presence of 5% CO_2.

Transient Transfections and Stable Cell Lines

Transfections were carried out via the electroporation of 10-20 million cells with 10μg of plasmid to be transfected. Cells were then plated in serum free RPMI 1640 medium and allowed to recover for 2 days. For transient transfections, protein was harvested after 2 days recovery and analyzed via Western blot as described. For pcDNA6/TR-EF transfections, cells were recovered for 2 days in RPMI 1640-5% serum, then serially diluted into medium containing Blasticidin 0.5 μg per ml and grown for an additional 2 to 3 weeks. Blasticidin-resistant clones were expanded and analyzed for TR protein expression via Western blotting.

Western Blots

Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with Tris-buffered saline, 0.1% Tween 20 containing 5% nonfat dried milk or 5% BSA, washed, and incubated with indicated antibody. Detection was performed using enhanced chemiluminescence (ECL, Amersham).

Antibodies

The anti-TetR (rabbit polyclonal, Abcam) antibody was used for detection of Tet repressor protein.

Subcloning

The pcDNA6/TR-EF vector was cloned by removal of the region between the SpeI and NheI sites of the pcDNA™6/TR vector. After removal of this section, the EF1-α promoter was inserted between the Hind III and Kpn I sites of the vector.

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Chapter Four

Conclusions

Alternative splicing has been identified as a common mechanism for regulating gene expression in eukaryotic cells. This process of differentially including or excluding variable exons provides a means for increasing proteome complexity. Its significance is reflected by the estimate that between 15 and 50% of human genetic diseases can be attributed to aberrant splicing events (Matlin et al., 2005). Despite the importance of alternative splicing little is known about the factors involved in regulating its function.

The alternative splicing of genes occurs in a cell specific manner and may be influenced by changes in the extracellular environment. One example of a gene which undergoes signal induced changes in alternative splicing is the CD45 gene. This gene has been characterized as having three variable exons (exons 4, 5 and 6), which are variably included or excluded in response to T cell activation (Hermiston et al., 2002). CD45 is a transmembrane protein and is abundantly expressed on the surface of T cells. In a naive T cell, the larger isoform is expressed and the protein exists as a monomer. However, once a T cell is exposed to antigen, the smaller CD45 isoform is expressed and assumes a dimeric state. It is believed that these signal induced changes in CD45 isoform expression function to regulate the activation state of the T cell, with the larger isoform allowing for T cell activation and the smaller isoform inhibiting activation (Majeti et al., 2000; Xu and Weiss, 2002).

The signal responsive changes in splicing characteristic of CD45 make this gene an ideal model for evaluating the factors involved in regulating alternative splicing. Previously, Lynch and Weiss described a Jurkat derived cell line, JSL1 cells, which recapitulate the signal responsive alternative splicing of the CD45 gene as seen in primary human T cells (Lynch and

Weiss, 2000). In addition, a CD45 minigene has been described as mimicking the signal induced exclusion of exon 4 as seen in the full-length gene (Lynch and Weiss, 2001). Together, these findings have established a useful model system for studying the factors involved in regulating CD45 alternative splicing.

To date, the use of this model system has provided novel information regarding the regulation of CD45 splicing. A 60 nucleotide regulatory sequence within variable exon 4, ESS1, has been characterized as influencing exon skipping under both resting and stimulated conditions (Lynch and Weiss, 2001; Rothrock et al., 2003). Biochemical studies have revealed that the regulatory protein hnRNP L binds to this sequence and results in basal exon repression (Rothrock et al., 2005), and that under stimulated conditions this regulatory protein undergoes modifications which further influence exon skipping (Melton, unpublished). Furthermore, biochemical assays have shown that upon stimulation, an additional regulatory protein, PSF, binds to the regulatory complex associated with ESS1 (Melton, unpublished). While these *in vitro* studies provide important and useful information about the regulation of CD45 alternative splicing, they are unable to mimic all of the signaling pathways that are present within the cell. Therefore, it is important that we study the splicing process not only in an *in vitro* system but also in an *in vivo* system.

The JSL1 cell line provides an *in vivo* system that may be used for the study of CD45 alternative splicing. However, in order to conduct experiments *in vivo* one must be able to alter protein expression. Unfortunately, common strategies for altering protein expression, such as RNAi and transient transfection with widely used vectors, have proven unsuccessful in JSL1 cells. Chapters 2 and 3 described techniques that have been attempted or are being developed in efforts towards overcoming the obstacle of manipulating protein expression in JSL1 cells. In this chapter, I will review those techniques and discuss the progress each technique has afforded us.

The MaRX retroviral infection system

Previously, a retroviral infection system was established in an effort to increase the transfection efficiency in JSL1 cells. This system used the MaRX II vector which contains two promoters, PGK and LTR. A MaRX vector with GFP expression driven by the PGK promoter was established and various cDNAs were cloned into the vector downstream of the LTR promoter. FACS analysis of GFP production revealed a significant increase in transfection efficiency with the use of the infection technique when compared to electroporation (70-90% vs. 30%). Despite this increase, Western blot analysis of candidate proteins revealed low expression from the LTR promoter. Furthermore, induction of the LTR promoter was detected upon PMA stimulation of JSL1 cells.

Since low transfection efficiency may result in the masking of changes induced by the expression of altered proteins, it is necessary to have a system that will allow for high transfection efficiency and/or overexpression of candidate proteins. Although the MaRX II retroviral infection system provides the necessary high transfection efficiency, it fails to produce significant overexpression of candidate proteins. In addition, PMA induction of the LTR promoter makes this system unusable, since it is necessary to draw correlations between any changes observed in resting and stimulated cells. It is for these reasons that we have sought to characterize and develop other strategies for manipulating protein expression in JSL1 cells.

Identification and characterization of increased protein production via the EF1-α promoter

Given the deficiencies associated with the MaRX II retroviral infection system, we took a different approach to achieving altered protein expression. From a screen of common promoters, the EF1-α promoter was identified as being the most robust in JSL1 cells. We established a parental vector containing the EF1-α promoter, a multiple cloning site, and a flag-tag sequence

(pEFnflag). Various candidate protein cDNAs were cloned into this vector and their expression levels were evaluated. Western blotting for flag-tagged protein expression in resting JSL1 cells revealed a 3-5 fold increase in expression when compared with resting JSL1 cells virally infected with the same flag-tagged protein. However, RT-PCR analysis detected no change in CD45 splicing relative to cells transfected with an empty vector. Although, Western blot analysis of PSF, PTB, hnRNP L and p54 reveal little increase in total protein expression when compared to endogenous alone, another member of the Lynch lab has been able to achieve overexpression of Ras when cloned into pEFnflag. There are a few possible reasons I have been unable to achieve overexpression of proteins driven by the EF1-α promoter. First, I have transfected plasmids containing cDNAs for the regulatory proteins hnRNP L, PSF, PTB, and p54. Since these proteins normally exist within the cell at high concentrations, it may be difficult to achieve higher levels of expression. Second, these proteins are responsible for regulating protein expression. It is possible that they autoregulate their own production, thereby negating the effects of the transfected DNA. Indeed, such autoregulation has been shown for many splicing proteins (Ares, 2007; Lareau et al., 2007) and directly shown for PTB (Wollerton et al., 2004). In support of this idea, RT-PCR analysis of JSL1 cells stably expressing EFflag PSF showed a direct correlation between increased flag-tagged mRNA production and decreased endogenous PSF mRNA production. Third, the transfected DNA may not be being expressed in a large enough population of cells; thereby causing any changes in protein expression level to be masked by the larger, unaltered population of cells. It is for this final reason that we established stable cell lines.

Establishment of stable cell lines expressing flag-tagged proteins driven by the EF1-α promoter

The establishment of stable cell lines expressing flag-tagged candidate proteins whose expression is driven by the EF1-α promoter are expected to provide two benefits. First, by

establishing a clonal cell line expressing the flag-tagged protein of interest, the risk of dilution from those cells not expressing the flag-tagged protein is removed. Thus, effects caused by the expression of cDNAs introduced into the cell can be clearly observed. Second, stably expressed flag-tagged proteins may be easily isolated. This is beneficial because flag-tagged proteins may be isolated from resting and stimulated cells and any changes in modification in response to extracellular signaling can be detected.

Currently, three stable cell lines containing EF1-α driven flag-tagged proteins have been developed and are as follows: EFflag PSF, EFflag PTB, and EFflag p54. Although Western blotting for endogenous proteins indicates little if any overexpression of these proteins, the same considerations mentioned previously must be made here. RT-PCR analysis also failed to detect any changes in CD45 splicing under resting conditions, but changes in response to stimulation have not been evaluated. It is possible that under stimulated conditions, these cell lines will demonstrate altered CD45 splicing as a result of protein modifications and/or newly activated signaling pathways.

Thus far, the full potential of stable cell lines containing EF1-α driven flag-tagged proteins has not been determined. It appears that this method will be extremely useful for the isolation and analysis of changes to candidate proteins during resting and stimulated conditions. Initial experiments with flag-tagged PSF purified from stable cell lines demonstrates differential protein association and activity. The establishment of stable cell lines may also be useful for achieving overexpression of less abundant candidate proteins, but this cannot be determined until other stable cell lines have been developed.

Establishment of a Tet-suppressor system

Although stable cell lines may provide valuable benefits, there is a potential problem associated with one of these benefits. This problem is related to the constant overexpression of proteins. While protein overexpression is a result we are trying to achieve, constant overexpression may prove detrimental to the cell. So that we may address this issue, efforts toward establishing a Tet-suppressor system are currently underway.

The system we have chosen to build from is the commercially available T-Rex system. The system contains two parts: the Tet-operator vector and the Tet-repressor vector. The Tet-operator vector contains a $TetO_2$ sequence within the promoter, while the Tet-repressor vector encodes the tet-repressor protein. In the absence of tetracycline, the tet-repressor protein binds to the $TetO_2$ sequence of the Tet-operator and blocks protein expression. When tetracycline is introduced, the drug binds with the tet-repressor protein causing it to release from the $TetO_2$ sequence, thus allowing protein expression. This system, as do all Tet-suppressor systems, relies on the CMV promoter, which does not express in JSL1 cells. Therefore, in the Tet-repressor vector, we have replaced this promoter with the EF1-α promoter. We are currently in the process of establishing cell lines stably expressing this plasmid. So far, transient transfection efficiency, as determined by Western blotting for the tet-repressor protein, appears to be low. If this transfection does not result in clones expressing the Tet-repressor vector, other strategies for introducing the plasmid will have to be evaluated. One possible approach may be to linearize the vector, as this may be more easily incorporated. Additionally, we are in the process of engineering the $TetO_2$ sequence into the EF1-α promoter. Due to ease, we initially subcloned this sequence at the far 3' end of the promoter; however, this appeared to inhibit promoter function. Since it has previously been determined that positioning of the $TetO_2$ sequence 10 nucleotides downstream of the TATA box allows for optimal functioning (Yao et al., 1998),

future studies will begin by inserting the Tet operator in this region of the EF1-α promoter via PCR mutagenesis.

Having the ability to regulate protein expression would be a valuable tool for evaluating the signaling pathways involved in signal induced alternative splicing. In addition to preventing constant overexpression of stably incorporated cDNAs, a Tet-suppressor system could offer other benefits like being able to alter protein expression at various timepoints following cell stimulation. Such a benefit would provide useful information about the sequence of events that occur upon stimulation. Since the establishment of this system is still in progress, it is difficult to comment on how it will perform in helping us achieve our goal of altering protein expression in JSL1 cells. There are a few concerns associated with this system that must be considered. One concern is that since all other Tet-suppressor systems rely on the CMV promoter, we do not know if the EF1-α promoter will be appropriately regulated by the Tet repressor. Another concern is regarding the leakiness of the system. If it is too leaky (unable to sufficiently repress promoter function), then it will not provide the benefit of restricting protein overexpression. As further advancements are made on this system, a better determination about its effectiveness can be ascertained.

The need for a method for manipulating protein expression in JSL1 cells

As discussed earlier in the chapter, alternative splicing is an important process in gene regulation. Many human genetic diseases have been linked to aberrant alternative splicing. The significance of this process makes it important that we identify and understand the factors involved in its regulation. Although biochemical studies have provided important information about some of the regulatory elements in alternative splicing there is still a great deal unknown. Establishing a successful *in vivo* model system will facilitate the identification of signaling

pathways involved in the regulation of splicing. JSL1 cells provide a cell culture in which *in vivo* studies may be carried out; however, the limitations they pose must first be overcome before they can be considered a successful *in vivo* model system. The strategies and techniques presented within this thesis have been attempts towards characterizing a method for altering protein expression in JSL1 cells. While they all appear to be promising, each method must be further developed before any final conclusions about their usefulness can be made.

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VITAE

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