MODULATION OF PSMA SPLICE VARIANTS USING SPLICE SWITCHING OLIGONUCLEOTIDES IN PROSTATE CANCER CELLS

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ABSTRACT

Tiffany L. Williams: Modulation of PSMA Splice Variants using Splice Switching Oligonucleotides in Prostate Cancer Cells
(Under the Direction of Ryszard Kole, Ph. D.)

The Prostate Specific Membrane Antigen (PSMA), a product of the GCPII gene, is highly expressed as a largely extracellular membrane-anchored protein in malignant prostate tissues and in non-prostatic tumor neovasculature. Alternatively spliced variants of PSMA include the PSM', PSMAΔ6, and PSMAΔ18 transcripts. PSM' is produced by use of an alternate upstream 5' splice site in exon 1 and produces a cytoplasmic localized protein missing PSMA's transmembrane domain. The PSMA/PSM' splice ratio changes from predominately PSM' in normal and benign prostate tissues to predominately PSMA in prostate cancer. Variants PSMAΔ6 and PSMAΔ18 are produced by exon exclusion of exons 6 and 18, respectively, by the splicing machinery. If properly translated, PSMAΔ6 protein retains its membrane targeting, but lacks PSMA's enzymatic and dimerization domains; PSMAΔ18 lacks only the dimerization domain. Both exon exclusion products should not produce functional proteins.

Extensive studies have shown the ability of antisense oligonucleotides to target specific splicing elements in pre-mRNA to alter alternative splicing patterns by interfering with splicing machinery sequence recognition. Therefore,

antisense based splice switching oligonucleotides (SSOs) were targeted at or near the 5' splice sites of exons 1, 6, and 18 to alter PSMA splicing patterns by decreasing full length PSMA transcript levels and increasing PSM', PSMAΔ6, and PSMAΔ18 variant levels, respectively in LNCaP prostate cancer cells. We hypothesized that altering the PSMA/PSM' splicing ratio would affect specific properties of LNCaP malignancy including cellular proliferation and apoptosis levels. Also, splice switching to PSMAΔ6 and PSMAΔ18 variants while causing a decline in PSMA would aid identifying effects produced by a decline in PSMA alone.

Studies in this dissertation demonstrate that treatment of LNCaP cells with SSOs did modulate splicing of PSMA pre-mRNA from the full length PSMA splice variant to three splice variants: PSM', PSMAΔ6, and PSMAΔ18 variants. Application of SSOs decreased membrane PSMA mRNA and protein levels and increased PSM', PSMAΔ6 and PSMAΔ18 transcript levels. As a result, PSM' protein was translocated to the cytoplasm while switching to PSMAΔ6 and PSMAΔ18 downregulated PSMA expression. NAALDase assays confirmed splice switching at the protein functional level and showed that PSM' retained enzymatic activity. However, we were not able to determine any specific effects of splice switching on LNCaP malignancy as detected by cellular proliferation and apoptosis.

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ABBREVIATIONS

BBP	Branchpoint Binding Protein
Bcl-X	B-cell lymphoma/leukemia X
Bcl-2	B-cell lymphoma/leukemia 2
BMD	Becker Muscular Dystrophy
BPH	Benign Prostate Hyperplasia
BRCA1	Breast Cancer Type 1
CaPSURE	Cancer of the Prostate Strategic Urologic Research Endeavor
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CPDR	Center for Prostate Disease Research
DMD	Duchenne Muscular Dystrophy
EGFP	Enhanced Green Fluorescent Protein
ESE	Exon Splicing Enhancer
ESS	Exon Splicing Silencer
FDH	10-formyltetrahydro-folate Dehydronase
FGCP	Folylpoly-γ-glutamate Carboxypeptidase
FOLH	Folate Hydrolase
FTDP	Frontotemporal Dementia and Parkinsonism
GCPII	Glutamate Carboxypeptidase II
hnRNP	Heterogenous Nuclear Ribonucleoproteins
ICAM-1	Intercellular Adhesion Molecule 1

ISE	Intron Splicing Enhancer
ISS	Intron Splicing Silencer
PIN	Prostatic Intraepithelial Neoplasia
pkCα	Protein Kinase C alpha
PSMA	Prostate Specific Membrane Antigen
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MOE	2'-*O*-methoxyethoxy
NAA	*N*-acetyl Aspartate
NAAG	*N*-acetyl *l*-aspartal Glutamate
NAALADase	*N*-acetyl α-linked Acidic Dipeptidase
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMD	Nonsense Mediated Decay
OMe	2'-*O*-methyl
PAP	Prostatic Acid Phosphatase
PARP	Poly-(ADP ribose) Polymerase
PES	Phenazine Ethosulfate
PKC	Protein Kinase C
PSA	Prostate Specific Antigen
PNA	Peptide Nucleic Acid
PSMA	Prostate Specific Membrane Antigen
SMaRT	Spliceosome-mediated RNA *Trans*-splicing

SMA	Spinal Muscular Atrophy
SMN	Survival of Motor Neuron
snRNA	Small Nuclear RNA
snRNP	Small Nuclear Ribonucleoprotein
SSO	Splice Switching Oligonucleotide
TfrI	Transferrin Receptor I
U2AF	U2 Associated Factor

CHAPTER 1

Introduction

I. Pre-mRNA splicing

Almost all eukaryotic mRNA transcripts contain intronic sequences with noted exceptions being histone and heat shock transcripts (Kedes 1979; Hentschel and Birnstiel 1981; Hunt and Calderwood 1990). Moreover, the presence of introns can positively influence gene expression (Gruss, Lai et al. 1979; Brinster, Allen et al. 1988; Palmiter, Sandgren et al. 1991). The introns must be removed and exons precisely and efficiently connected to ensure gene sequence accuracy. It is estimated that more than 90% of pre-mRNA sequence is intronic and therefore removed from mature mRNA (Lander, Linton et al. 2001). Pre-mRNA splicing is the process by which nuclear factors act on pre-mRNA to remove introns and connect exons aiding in the transition to mRNA. *Cis*- and *trans*- splicing are the two types of pre-mRNA splicing that occur within the eukaryotic cell: *cis*-splicing is the more common type wherein a single pre-mRNA molecule is involved (Moore and Query 1993; Burge, Tuschl et al. 1999); the rarer *trans*-splicing requires the interaction of separate pre-mRNA sequences joined at specific exons (Konarska, Padgett et al. 1985; Solnick 1985; Mayer and Floeter-Winter 2005).

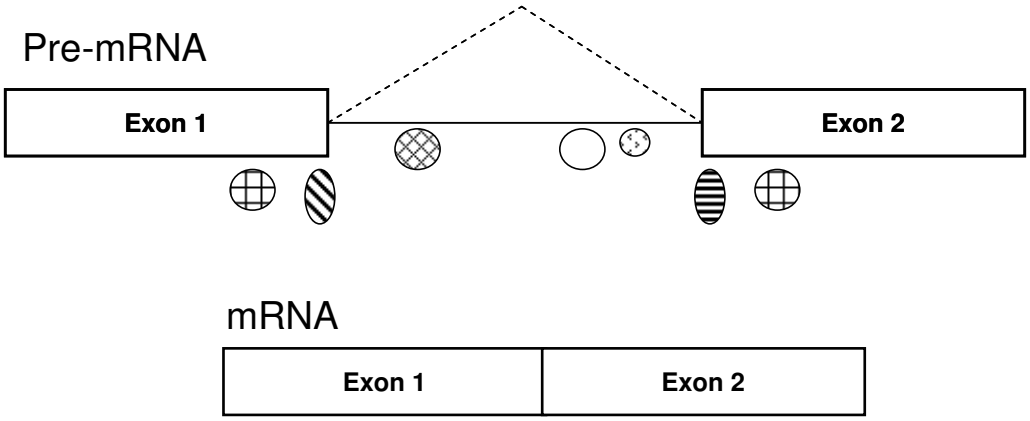
A. Splicing elements & factors

Splicing elements

Pre-mRNA splicing, i.e., the removal of the non-coding introns, and the subsequent joining of the coding regions, or exons, occurs at the exon-intron junctions termed splice sites, via two trans esterification steps (Moore and Query 1993). The four pre-mRNA sequences necessary for splicing are the 5' splice

donor site, 3' splice acceptor site, the intronic branch point, and a polypyrimidine sequence upstream of the branch point. These splicing elements have general consensus sequences utilized by the splicing machinery for effective identification of introns and exons (Fig. 1.1). Most of the nucleotides in each consensus sequence are degenerate, that is they can be replaced by other nucleotides and the element can still be recognized for splicing. Element strength is partially determined by adherence to consensus, but the inherent degeneracy is key in the process of alternative splicing (described later in more detail). Of the 5' and 3' splice site consensus sequences (C_{37} A_{58} G_{78} /**G_{100}** **U_{100}** A_{57} A_{71} G_{84} U_{47} and 12Y_{70} NC_{74} **A_{100}** **G_{100}** /G_{49}, respectively), only two nucleotides, GU in the donor 5' splice sites and AG in the acceptor 3' splices sites, are conserved in essentially all splice sites examined (Y=pyrimidine, N= any nucleotide, subscript number= percent presence of the nucleotide at that position). The remaining nucleotides of the consensus splice site sequences are represented at lower frequencies, resulting in functional splice sites with divergent sequences. Given the short and poorly conserved nature of the splice site sequences, the mechanism of selection of authentic splice sites among the many similar sequences that constitute cryptic and pseudo splice sites is still not completely understood. It is the degenerate branch point that determines which 3' splice site is utilized (Smith, Porro et al. 1989). The branch point is seven nucleotides long with an YNYUR**A**Y consensus sequence (Y=pyrimidine, R= purine, N= any nucleotide, **A**= invariant adenine nucleotide) and is located 18 to 40 nucleotides upstream of the 3' splice site (Zhuang, Goldstein et al. 1989); this

A.



B.

5' splice (donor) site = $C_{37}A_{58}G_{78}/\mathbf{G_{100}U_{100}}A_{57}A_{71}G_{84}U_{47}$

3' splice (acceptor) site = $12Y_{70}NC_{74}\mathbf{A_{100}G_{100}}/G_{49}$

Branch Point = YNYUR$\mathbf{A_{100}}$Y

Polypyrimidine tract

Exonic Splicing Enhancers/Silencers

Intronic Splicing Enhancers/Silencers

Figure 1.1. Pre-mRNA splicing elements.

A) Pre-mRNA sequences contain several intronic and exonic *cis* splicing elements that are recognized by the splicing machinery to produce mature intronless mRNA. B) Major splicing elements are listed. The 5' splice site, 3' splice site, and branch point are shown with their corresponding consensus sequences; subscript number is the percentage that nucleotide is found at that position in the consensus sequence; bold letters indicate invariant nucleotides.

range of location and sequence variability can lead to the identification of different branch points by the splicing machinery (Burge, Tuschl et al. 1999). Efficient recognition of suboptimal elements may be improved by additional sequences such as exonic or intronic splicing enhancers (ESEs, ISEs) (Hertel, Nomikos et al. 1997; Maniatis and Tasic 2002). There are also counteracting, regulatory sequence elements, known as exonic and intronic splicing silencers (ESSs, ISSs), which promote exon exclusion (Caputi, Mayeda et al. 1999; Chen, Kobayashi et al. 1999; Del Gatto-Konczak, Olive et al. 1999; Sironi, Menozzi et al. 2004) (Fig. 1.1).

snRNPs and SR proteins

The sequence elements involved in splicing are recognized by *trans*-factors including small nuclear ribonuclear proteins (snRNPs) and a number of other splicing factors such as heterogeneous nuclear ribonucleoproteins (hnRNPs) and SR proteins (see Blencowe 2000; Cartegni, Chew et al. 2002; Black 2003 for recent reviews), which contain arginine-serine-rich (RS) motifs (Blencowe, Bowman et al. 1999; Hastings and Krainer 2001).

Small nuclear RNAs are active within larger ribonucleoprotein complexes called snRNPs. Five snRNPs: U1, U2, U4, U5, and U6, have been identified as important factors in spliceosome formation, composition and function (Lerner, Boyle et al. 1980 and reviewed in Guthrie and Patterson 1988; Kramer 1996). Both the RNA and protein components of these snRNPs make key contacts to RNA and other splicing proteins, including: SR proteins, hnRNPs and many other miscellaneous proteins. By studying evolutionarily early type II splicing reactions

like those seen in the organelles of bacteria and the more recent production of a protein-less spliceosome, it is assumed that snRNPs evolved from an earlier RNA based splicing apparatus (Valadkhan 2005). The U1, U2, and U5 snRNPs exist as single particles in which individual snRNAs are complexed with Sm and other proteins unique to that snRNP. The U4 and U6 snRNAs are bound together in the nucleus as one snRNP (Bringmann, Appel et al. 1984; Hashimoto and Steitz 1984). Specifics of snRNP involvement in the splicing process are detailed later.

SR family proteins have N-terminal RNA-recognition motifs (RRM) and a serine/arginine (RS) rich C-terminal domain. The RRMs directly bind RNA and the RS region is involved in protein-protein interactions, such as recruiting other proteins to the splicing complex, and splice site pairing (Wu and Maniatis 1993; Min, Turck et al. 1997). SR proteins may also directly contact pre-mRNA at the branch point and 5' splice site (Shen, Kan et al. 2004).

The concentrations and the ratios between these splicing factors in different cell types and tissues clearly control splicing. For example, hnRNPA1 acts as an antagonist of the SR protein SF2/ASF, which functions in defining the 5' splice site proximal to the downstream 3' splice site by interacting with U1 snRNP. Higher A1/SF2 ratios shift splicing towards an alternative, distal 5' splice. Notably, this ratio can vary 100-fold in different tissues (Caceres, Stamm et al. 1994). Overall the combinatorial interactions of the factors with the sequence elements in pre-mRNA determine the tissue-specific expression of most pre-mRNAs (Smith and Valcarcel 2000; Roberts and Smith 2002). As a result of

these complex interactions there are a number of different alternative splicing events including exon skipping, inclusion of alternative exons; and use of alternative splice donor and acceptor sites and intron retention.

B. Nuclear cis-splicing: mechanism and the spliceosome

The understanding of pre-mRNA splicing has evolved with the use of *in vitro* splicing systems. New information continues to become available and the understanding of the complete mechanism of nuclear splicing is still in flux. In this section, I will summarize the current knowledge of spliceosome formation and the splicing reaction during *cis*-splicing in nuclear introns as described in *Genes VII* (Lewin 2000) and several primary and review articles.

Lariat structure

The splicing reaction occurs through a lariat structure (Fig. 1.2) (reviewed in Padgett, Grabowski et al. 1986). First, the 2'OH of the invariant 'A' nucleotide of the branch point performs nucleophilic attack on the phosphate bond upstream of the invariant 'G' nucleotide of the 5' splice site separating the first exon/intron junction. The upstream exon continues to be linear while the downstream intron forms a lariat structure where the newly formed terminus at the 5' end of the intron is linked by a 5'-2' bond between its invariant 'G' nucleotide of the initial 5' splice donor site and the 2' position of an invariant 'A' nucleotide in the branch point sequence. Next, the 3' splice site undergoes nucleophilic attack by the free OH of the upstream exon separating the 3' intron/exon junction. The intronic lariat is debranched at the branch point and readily degraded. The chemical reactions that occur at the splice sites and branch point are trans-esterification

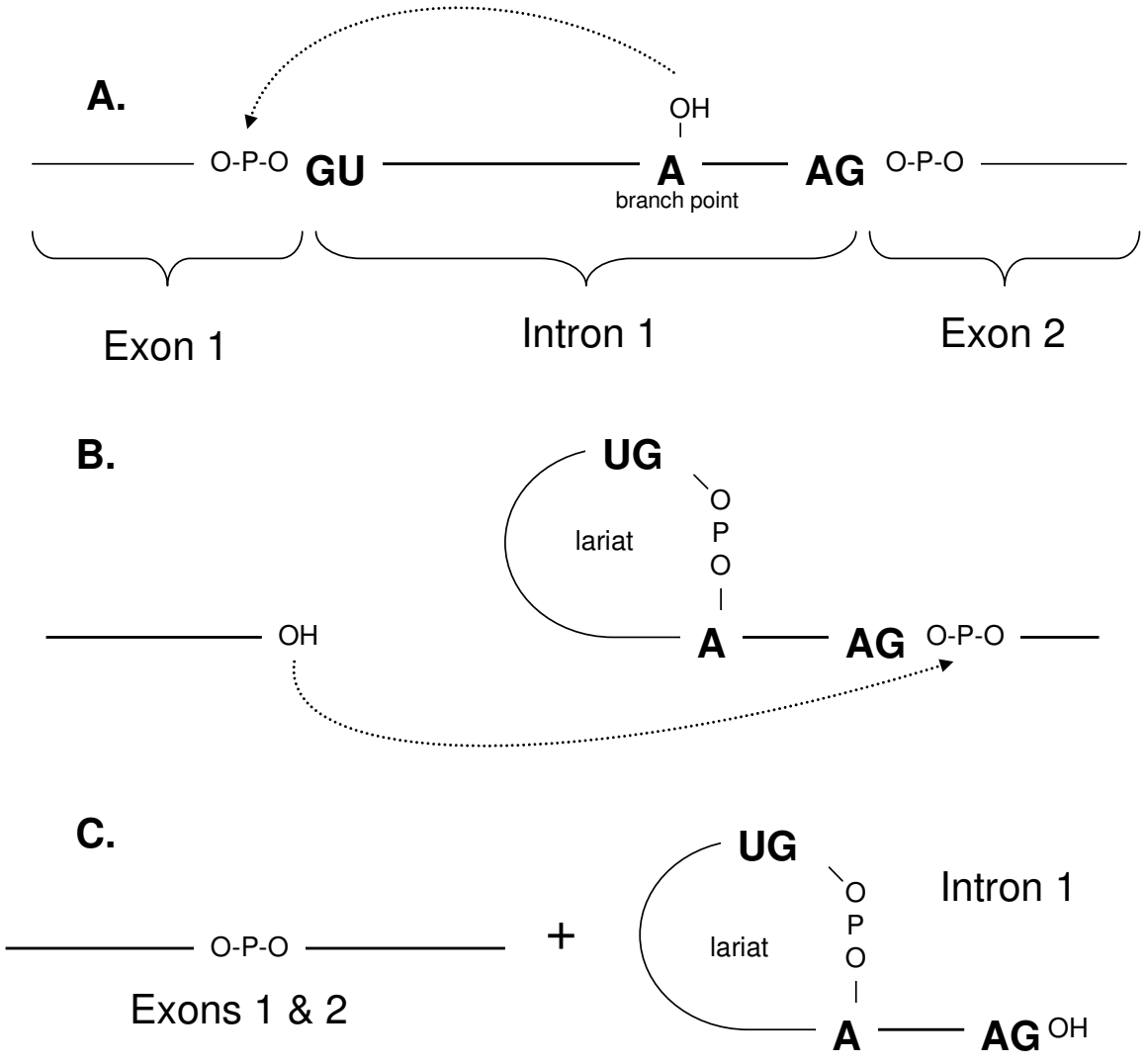


Figure 1.2. Transesterification steps and the intronic lariat intermediate in pre-mRNA splicing.

Intron removal and exon joining in *cis* splicing occurs via two transesterification steps and an intronic lariat structure. A) Representative pre-mRNA sequence containing 2 exons and 1 intron. The 2'OH group of the invariant adenine nucleotide in the intronic branch point attacks the phosphate group at the exon/intron junction of the 5' splice site. B) The lariat intermediate is formed. The resulting free 2'OH group of exon 1 performs nucleophilic attack on the phosphate group at the intron/exon junction of the 3' splice site. C) Intron sequence including lariat intermediate is released and degraded; exons 1 and 2 are joined.

reactions (Fig. 1.2). In the original pre-mRNA structure there were two 5'-3' bonds at the intron-exon junctions; these are exchanged for a 5'-2' bond between the 5' end of the intron and the branch point and a 5'-3' bond linking the two exons.

Stages of spliceosome formation

The process of splicing is accomplished by a large macromolecular complex in the nucleus called the spliceosome. The spliceosome is a large 50-60S structure that rivals a ribosomal subunit in size; it is composed of several hundreds of proteins and five snRNPs that come together and interact in stages in every splicing reaction (Rappsilber, Ryder et al. 2002). The major splicing components and steps are illustrated in Figure 1.3 and described here briefly, however significantly more can complex as more than 300 protein components have been identified (reviewed in Jurica and Moore 2003). The first stage called the E, or early presplicing, complex is sometimes referred to as the commitment complex since the pre-mRNA is first selected for splicing. Here, the U1 snRNP along with the ASF/SF2 SR protein base pairs with the 5' splice donor site (Jamison, Pasman et al. 1995) and the branch point binding protein (BBP) binds at the branch point sequence (Berglund, Chua et al. 1997). U1 recognizes the 5' splice site by simple base pairing between the 5' end of the U1 snRNA and the sequence at the intron/exon boundary and this recognition does not require ATP (Lerner, Boyle et al. 1980; Rogers and Wall 1980; Zhuang and Weiner 1986). A specific pyrimidine tract downstream of the branch point is then bound by U2 associated factor (U2AF) (Moore 2000). The polypyrimidine tract is highly

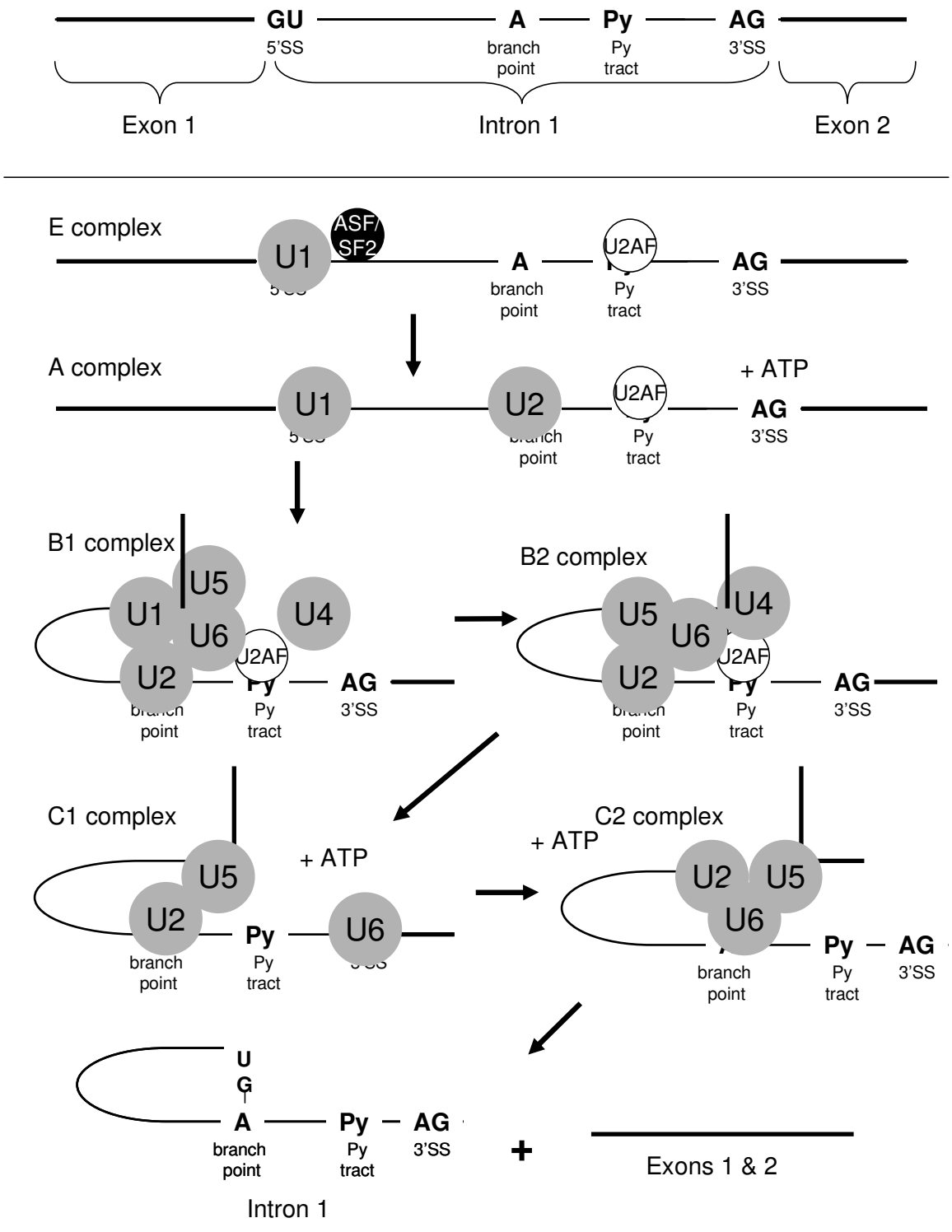


Figure 1.3. Major steps of spliceosome formation.

Figure 1.3. Major steps of spliceosome formation.

A representative pre-mRNA sequence including two exons and one intron goes through the major stages of spliceosome formation. Stages include the E complex (also termed the commitment complex), A, B1, B2, C1, and C2 complexes. Addition, positioning, and removal of splicing factors including snRNPs and SR proteins guide spliceosome assembly through each stage and facilitate the joining of exons and removal of intron sequence (see Chapter 1 for a detailed explanation).

conserved and is necessary for spliceosome assembly (Ruskin and Green 1985; Green 1986; Barabino, Blencowe et al. 1990). These two previous steps are required for the recognition of the branch point by U2 snRNP (Ruskin, Zamore et al. 1988); U2 snRNA binds complementary sequence in the branch point while other proteins in the U2 snRNP recognize RNA upstream of the branch point (Black, Chabot et al. 1985; Chabot and Steitz 1987). When U2 snRNP joins the action, ATP is required, and the E complex becomes the A presplicing complex. The B1 splicing complex is where the complete spliceosome containing the U5 and U4/U6 snRNPs is first identified. The U5 snRNP joins the spliceosome with the aid of an intron binding protein after U1 and U2 bind to the pre-mRNA (Grabowski and Sharp 1986; Bindereif and Green 1987; Konarska and Sharp 1987). The U5/U4/U6 pre-formed triple snRNP binds both the 5' splice site and the U2 snRNP with the aid of SR proteins (Roscigno and Garcia-Blanco 1995). Here is where the previously described lariat structure starts to take shape. In the B2 complex, U1 is released from the 5' splice site in order to position the 5' splice site and the U6 snRNP. In the C1 complex, ATP is required to release U4 in order for to sequester U6 for binding to U2 (Lamond, Konarska et al. 1988). The U6/U2 complex binds the RNA hairpin structure to perform the trans-esterification reactions where the 5' splice site is cleaved and the lariat intermediate is fully formed. The final C2 complex also requires ATP; the U5 snRNP of the U2/U6/U5 complex binds the 3' splice site which is then cleaved (Chiara, Palandjian et al. 1997). The two exons are then joined and the final mRNA sequence is defined.

II. Alternative Splicing

Since the discovery of DNA and gene structure in the middle of the 20th century, it was assumed widely that to achieve the phenotypic diversity seen in mammals and specifically humans, hundreds of thousands of genes would be required. Only recently with the sequencing of the human genome have researchers determined that only about 30,000 genes or even less make up our entire genome (Lander, Linton et al. 2001; Venter, Adams et al. 2001). Discovery of the number of different protein products that can be obtained from a single gene by alternative pre-mRNA splicing and other processes helps to resolve the issue of small gene number and large protein diversity. Several post-transcriptional processes, including alternative splicing, increase protein diversity without the need for increasing gene number; alternative splicing is the best example of a mechanism that exposes the fallacy of the 'one gene-one polypeptide' theory in eukaryotes.

Alternative splicing is an essential, biological process that can regulate gene expression in higher eukaryotes and contributes greatly to the complexity of the proteome by generating multiple protein isoforms. Alternative splicing of eukaryotic pre-mRNA occurs when exonic or intronic sequences are selected to be either removed or retained, respectively, in mature mRNA. Our understanding of splice site selection in alternative splicing is limited. There is evidence that alternatively spliced introns have suboptimal splicing elements (Roca, Sachidanandam et al. 2003) that are poorly recognized by snRNPs and other splicing factors.

Previously, alternative splicing was thought to be a minor process occurring in only about 5% of human genes (Sharp 1994). By recent estimates using bioinformatics analysis, DNA microarrays and protein sequence data, the primary transcripts of approximately 59% to 74% of human genes are subject to alternative splicing, generating from two to several thousand different mRNA isoforms for each gene (Hanke, Brett et al. 1999; Croft, Schandorff et al. 2000; Lander, Linton et al. 2001; Johnson, Castle et al. 2003). Using a subset of genes on different chromosomes, the average number of alternatively spliced products per gene is estimated to be between 1.3 and 3.2 (Lander, Linton et al. 2001), but some genes including the human neurexin 3, giant muscle protein titin, calcium-activated potassium channels, and the *Drosophila* Down syndrome cell adhesion molecule (DSCAM) gene can achieve potentially thousands to tens of thousands of different mRNAs (Ushkaryov, Petrenko et al. 1992; Schmucker, Clemens et al. 2000; Bang, Centner et al. 2001; Strehler and Zacharias 2001). This makes it easy to understand how the alternative splicing process is one of the most important mechanisms that lead to protein diversity since the number of different protein products from a single gene can be larger than the total number of genes in an entire genome.

Regarding mRNA sequence, consequences of alternative splicing include complete or incomplete exon removal, partial or total intron retention and mutual exclusion of two different exons (Fig. 1.4). Since protein domains tend to migrate with exon sequence throughout chromosomal rearrangements, the complete removal of an exon by alternative splicing will usually delete a specific motif or

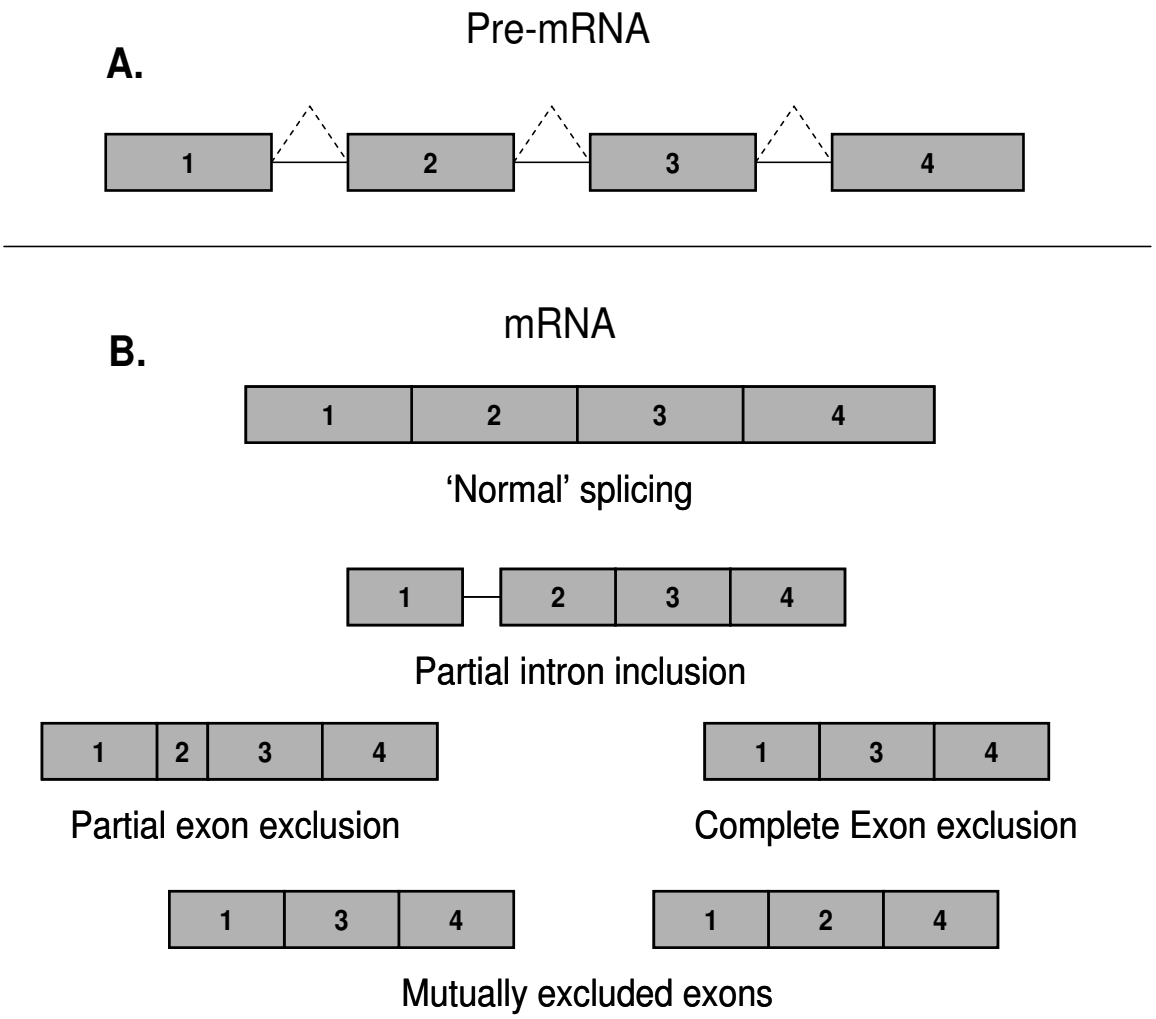


Figure 1.4. Multiple outcomes of alternative splicing.

A) A representative pre-mRNA sequence containing 4 exons and 3 introns. B) Alternative splicing of the pre-mRNA is able to produce several different types of mRNA transcripts.

domain from the final protein product. Commonly, protein domains that control membrane insertion are added or excluded by alternative splicing. This is a powerful method of protein modification since protein localization is such an important part of determining enzymatic, signaling and structural function. Examples of this type of regulation by alternative splicing include cyclo-oxygenase, Fas, Growth hormone receptor, Interleukin-2 and -4 receptors, Prostate specific membrane antigen and Vascular endothelial growth factor-I (Rosenfeld 1994; Cascino, Fiucci et al. 1995; Su, Huang et al. 1995; Blum, Wolf et al. 1996; Horiuchi, Koyanagi et al. 1997; Shibuya 2001; Chandrasekharan, Dai et al. 2002). Mutual exclusion of exons can lead to tissue specific protein types or simply exchange of one protein domain type for another.

By pure probability, almost two-thirds of alternative splicing events will change the reading frame of the mRNA sequence. This is by far the potentially most devastating type of alteration since it usually produces an early stop codon and thus a truncated protein. In many cases, early stop codons can initiate mRNA degradation by nonsense mediated decay (NMD); it has been estimated that about 18% to 25% of mRNA transcripts will be degraded by NMD due to the use of alternate sequence after alternative splicing (Lewis, Green et al. 2003). However, a change in reading frame also may cause the most significant alteration of protein sequence achieved by alternative splicing and produce a truly novel protein sequence. Alternative splicing also is able to increase or decrease the level of protein expression by altering poly-adenylation signals, producing unstable mRNAs or altering tissue specific spliced product expression.

A. Alternative Splicing in Disease

Mutations in either alternatively or constitutively spliced genes, or in genes coding or controlling the components of the splicing machinery can alter splicing, frequently leading to human disease. Annotated database surveys show that up to 50% of point mutations contributing to genetic diseases either damage or modify splice sites, branch points, polypyrimidine tracts and/or enhancer elements involved in splicing and, therefore, result in aberrant splicing of pre-mRNA and about 15% of all genetic disease mutations affect pre-mRNA splicing (Krawczak, Reiss et al. 1992; Cooper and Mattox 1997; Cartegni, Chew et al. 2002). Mutations in binding sites for SR proteins including SF2/ASF, SRp40 and SRp55 have also been associated with disease when found in genes such as BRCA1 (Liu, Cartegni et al. 2001) and SMN (Cartegni and Krainer 2002).

Disease causing alteration of splicing pathways is frequently due not to mutations present in pre-mRNA, but to a change in the expression of factors that influence alternative splicing. One example involves the CD44 family of membrane glycoproteins, which function as cell adhesion molecules. The CD44 gene is complex, and is comprised of 20 exons. Alternative splicing of 10 variable exons gives rise to a diverse range of CD44 protein isoforms. Some CD44 splice variants are associated with tumor metastasis and may be used in cancer diagnosis. A mutational analysis of one particular variable exon, v5, revealed a composite structure of enhancer and silencer elements regulated by signal transduction pathways (Woerfel and Bindereif 2001).

A clear cause and effect relationship between the modulation of splicing and cancer is exemplified by bcl-x, a gene involved in the apoptotic pathway where over-expression of a longer splice variant, bcl-xL, of this gene is associated with advanced, aggressive cancers (Reed 1998). Other alternatively spliced genes associated with human cancer have been reviewed elsewhere (Mercatante, Sazani et al. 2001) and examples of alternatively spliced genes implicated in other diseases are described later.

B. Antisense Modulation of Alternative Splicing

Antisense modification of gene expression exists in nature and also can be accomplished by synthetic small molecules and oligonucleotides. A comprehensive volume (Crooke 2001) and more recent reviews cover antisense technology (Dean and Bennett 2003; Kurreck 2003). It is noteworthy that even the newly discovered phenomenon of RNA interference (RNAi) ultimately is dependent on small interfering RNAs (siRNA) that interact with the target RNA via an antisense mechanism. In most cases the antisense effects lead to down-regulation of gene expression. As will be discussed below, using antisense molecules for modification of the complex mechanisms of pre-mRNA splicing allows not only down-regulation but also up-regulation and modulation of expression of targeted genes. This unique ability makes the application of antisense oligonucleotides particularly attractive, in view of the fact that a majority of genes generate alternatively spliced pre-mRNA. The next section focuses on antisense oligonucleotide targeting of previously discussed splicing elements to significantly alter the splicing patterns. This concept has clinical

relevance, since many diseases result from misguided, alternative or aberrant splicing (reviewed in Sazani and Kole 2003; Vacek, Sazani et al. 2003).

III. Splice switching oligonucleotides

Oligonucleotide based mechanisms have long been used for manipulation of intracellular RNAs and even DNAs *in vitro* and *in vivo* (reviewed in Crooke 2004; Aboul-Fadl 2005). Sequence-specific base pairing of engineered molecules with native cellular sequences has been able to sterically block gene expression at different stages including: transcription initiation through a triplex mechanism, spliceosome recognition, translation, and by recruiting proofreading machinery; all of these approaches have been used for the purpose of therapeutics and/or disease research. In most cases, short DNA and DNA-based nucleic acid sequences, or oligonucleotides, are the molecules of choice for performing antisense research, although (as will be described later) others including snRNAs and small organic molecules also have been useful. Moreover, antisense oligonucleotides are used most widely to target mRNAs for degradation by the RNAse H enzyme, presumably in the cytoplasm (reviewed in Nesterova and Cho-Chung 2004). RNase H has the ability to degrade any RNA sequence included in a DNA/RNA duplex, an advantageous behavior when trying to diminish RNA levels. This downregulation of gene expression has been almost universally effective in tested transcripts and particularly useful in bcl-2, protein kinase C alpha (pkCα), and intercellular adhesion molecule 1 (ICAM-1) research (Dean and McKay 1994; Gutierrez-Puente, Zapata-Benavides et al.

2002), even producing Vitravene®, a successfully approved therapeutic for AIDS related cytomegalovirus retinitis (de Smet, Meenken et al. 1999).

For some research applications using antisense technology, including alternative pre-mRNA splicing manipulation, degradation of transcripts by RNase H is not desired; the array of modified antisense oligonucleotides with specialized properties (including RNase H resistance) has grown and become increasingly more complex in chemistry to adapt to specific needs. These structural modifications have produced second generation antisense oligonucleotides with improved sequence specificity, binding capability, cellular and nuclear targeting and decreased toxicity (reviewed in Altmann, Fabbro et al. 1996; Monia 1997). For the purpose of targeting pre-mRNA sequences to interfere with splicing processes, it is desirable for antisense oligonucleotides to (1) reach the nuclear space where splicing takes place, (2) not recruit RNase H degradation, (3) have high binding affinity to targeted sequence, and (4) target known splicing elements in order to produce a predictable splicing pattern.

This laboratory has pioneered the use of antisense oligonucleotides targeted to pre-mRNA sequence in order to influence alternative splicing patterns. This specific use of short antisense sequences, termed splice-switching oligonucleotides (SSOs), has been successful in the modulation of mRNA level of several different transcripts including cftr, β-globin, bcl-x, and recently prostate specific membrane antigen (PSMA) (Sierakowska, Sambade et al. 1996; Friedman, Kole et al. 1999; Mercatante, Bortner et al. 2001), Williams and Kole, in press).

A. Chemistry

To be effective in modification of splicing, the oligonucleotides must bind to pre-mRNA without inducing degradation of the RNA duplexed with the oligonucleotide by RNase H, a ubiquitous enzyme. Thus, splice switching oligonucleotides include modifications such as 2'-*O*-methyl- and 2'-*O*-methoxyethyl- (MOE) substitutions of ribose, methylphosphonate oligodeoxynucleotides, morpholino, and peptide nucleic acid (PNA) (reviewed in (Sazani and Kole 2003).

2' modifications replacing the 2'OH group in RNA based oligonucleotides alone can grant RNase H resistance. 2'-*O*-methyl- and 2'-*O*-methoxyethyl-chemical modifications were shown to also have greater target affinity and nuclease resistance (Manoharan 1999). A 2'-*O*-aminopropyl sugar conformation involves an extended amine terminated – three carbon group bound to the 2' oxygen. This modification neutralizes the phosphate derived overall negative charge of the oligonucleotide molecule by introducing one positive charge from the amine group per sugar. 2'-*O*- aminopropyl substitutions improve cellular uptake properties compared to unmodified oligonucleotides because of its zwitterionic properties (Teplova, Wallace et al. 1999). Another available 2' modification includes a locked or bridged ribose conformation where the 4' ribose position also is involved; an oxygen molecule bound at the 2' carbon is linked to the 4' carbon by a methylene group. This ribose conformation grants RNase H resistance and greater binding affinity (Orum and Wengel 2001).

Morpholino oligonucleotides further deviate from standard nucleic acid structure by changing both the sugar and phosphate chemistries. Here, the five member ribose ring is substituted with a six member morpholino ring containing four carbons, one nitrogen and one oxygen. The ribose monomers are linked by a phosphordiamidate group instead of a phosphate group. These backbone alterations remove all positive and negative charges making morpholinos neutral molecules that more easily cross cellular membranes without the aid of cellular delivery agents often used by charged oligonucleotides. Morpholino oligonucleotides benefit from the same advantages granted other modified oligonucleotides, including RNase H and nuclease resistance and high binding affinity (Stein, Foster et al. 1997; Summerton 1999).

The peptide nucleic acid (PNA) chemically modified oligomers differ greatly from what is normally recognized as nucleic acid. There is no sugar ring or phosphate linkage; in their place, bases are attached and appropriately spaced by oligoglycine – like molecules. PNA oligomers also have no backbone charge and, due to their engineered chemical positioning of bases, have a high sequence binding affinity (Nielsen, Egholm et al. 1991; Hanvey, Peffer et al. 1992). It is also obvious as to why PNAs are not recognized by cellular nucleases or RNase H.

Oligonucleotide modifications that involve base chemistry are not widely used, but have been shown to be beneficial in some antisense studies. The C-5 propynyl cytosine modification amplified antisense activity in concert with phosphorothioate backbone linkages, where the oxygen with a double bond to

the phosphorous atom in a normal phosphate group is replaced by a sulfur atom. Note that phosphorothioate linkages can replace phosphates in combination with other backbone and base modifications to reduce nuclease recognition (Eckstein 2000). Two other cytosine base chemical modifications include heterocyclic phenoxazine and G-clamp (Moulds, Lewis et al. 1995; Raviprakash, Liu et al. 1995; Flanagan, Kothavale et al. 1996) that both give higher target binding affinities.

B. Delivery

Since splicing is a nuclear process, the oligonucleotides must have the ability to enter or be delivered efficiently to the cell nucleus. In cell culture, several different cationic carriers enhanced uptake of negatively charged oligonucleotides with sugar-phosphate backbones to the nuclei and led to a shift in splicing of several targets (reviewed in (Kole and Sazani 2001). Uncharged splice switching oligonucleotides such as morpholinos or PNAs cannot be delivered to cells by cationic carriers. Nevertheless, splice switching was observed in cultured HeLa and in erythroid cells from thalassemic patients when the oligonucleotides entered the cells subsequent to mechanical disruption of the cell membrane using a scrape-loading or syringe-loading technique (Lacerra, Sierakowska et al. 2000; Sazani, Kang et al. 2001; Suwanmanee, Sierakowska et al. 2002). Splice switching by morpholino and PNA oligonucleotides, but not by their negatively charged counterparts, also was observed, albeit at high concentrations, upon free uptake into these cells (Sazani, Kang et al. 2001; Suwanmanee, Sierakowska et al. 2002). Splice switching has also been

achieved in cell culture using viral vectors for stable and prolonged delivery of antisense RNA (Vacek, Ma et al. 2003) or by oligonucleotide conjugation with Tat or antennaepedia cell-penetrating peptides (Astriab-Fisher, Sergueev et al. 2002; Moulton, Hase et al. 2003).

C. Readout

Although antisense oligonucleotides are beginning to be accepted as therapeutics and are undergoing numerous clinical trials (http://www.clinicaltrials.gov/), their delivery and cellular uptake still need improvement. An assay that provides a clear, sequence specific read-out would be very useful in further investigations of the improved oligonucleotides. Modification of splicing by SSOs, which results in generation of a new splice variant on a null or low background, provides such an assay. This laboratory has developed reporter splicing assays in cell culture and in a transgenic mouse model, which allow determination of the levels of splice switching by any antisense molecule (Gemignani, Landi et al. 2001; Sazani, Vacek et al. 2002). One assay was devised and exploited to analyze in cell culture and *in vivo* the antisense effects of several novel oligonucleotide chemistries. The assay utilizes an enhanced green fluorescent protein (EGFP), the coding sequence of which is interrupted by a defective intron from human β-globin gene. The intron carries a thalassemic C- to T- mutation at position 654 that creates an aberrant 5' splice site and activates an additional 3' splice site upstream. The resultant aberrant splicing prevents production of correct EGFP mRNA and protein (Sazani, Kang et al. 2001). Antisense molecules targeted to the aberrant splice site restore

correct splicing, leading to uninterrupted EGFP mRNA properly translating full length EGFP with detectable fluorescence. For *in vitro* and *in vivo* testing, this construct was either stably transfected into HeLa cells or expressed in a transgenic mouse model. In the absence of oligonucleotide treatment, virtually no EGFP mRNA was present, and very little or no background fluorescence was detected in cultured cells or mouse tissues. This system tests sequence specific antisense activity; the generated fluorescence is directly proportional to the effectiveness of the antisense molecules in splice shifting; the level of fluorescence in analyzed cells and tissues directly correlates with the oligonucleotides' nuclear uptake and splice-shifting potential (Sazani, Kang et al. 2001; Sazani, Gemignani et al. 2002). The oligonucleotide-induced positive readout of EGFP fluorescence indicates that the active, *in vivo* injected oligonucleotides, distributed to different tissues, crossed the cell membranes, translocated to the nuclei where they blocked the aberrant splice site, interfered with the normal functioning of the spliceosome, and forced the splicing machinery to select an alternative splice site. The results can be quantified at the mRNA or protein level and by fluorescence-activated cell sorting (FACS) analysis of cultured cells or microscopy of sectioned mouse tissue. The assay allows comparison of effectiveness of oligonucleotide analog chemistries, delivery agents and different delivery approaches.

Using this assay, antisense properties of peptide nucleic acid (PNA), morpholino, 2'-*O*-methoxyethoxy (MOE) and 2'-*O*-methyl (OMe) were investigated. Interestingly, with 'free uptake' of the oligonucleotides into cultured

HeLa cells, with no special delivery methods or agents, antisense efficiency seemed to be related to a net backbone charge. The neutral PNA and morpholino analogs performed better than their negative counterparts (MOE and OMe). The addition of four lysine residues to PNA analogs increased their efficacy presumably due to a positive charge conferred by the basic amino acids (Sazani et al., 2001). Consistent with *in vitro* cell line data, the PNA-4-lysine chemistry out-performed all other oligonucleotides tested in vivo, exhibiting significant fluorescence in the liver, kidney, heart and lung tissues of the EGFP mouse. Interestingly, only the PNAs induced significant EGFP fluorescence both in HeLa cells when delivered without adjuvants from the media (10 µM) or *in vivo* by I.P injection (50 mg/kg) in the EGFP mouse. Unexpectedly, MOE treated animals also showed intense response to treatment with detectable fluorescence in several tissues; in contrast a neutral morpholino oligonucleotide analog induced significant fluorescence in HeLa cells but was only marginally effective *in vivo* showing little response in all tissues tested (Sazani et al., 2002b). Lack of morpholino effects are likely a consequence of rapid renal clearance of such molecules (Agrawal et al., 1995). 2'-*O*-methyl-oligonucleotides were not tested *in vivo* in the transgenic model. The tissues that showed the highest fluorescence were liver, kidney, lung, heart and small intestine (Sazani, Gemignani et al. 2002). Recently the EGFP-based splicing assay also was used for cell culture testing of oligonucleotide based modifications that increased binding of these compounds to their target. No effect on splice shifting was observed (Sazani et al., 2003). An earlier version of the assay, based on luciferase read-out (Kang et

al., 1998), also was used to study morpholino oligonucleotides conjugated to peptides, some of which were found to improve cellular and nuclear uptake of the conjugates (Moulton et al., 2003).

This stringent test of antisense activity can be used in investigations of different delivery agents and/or oligonucleotides with diverse chemistries. For example, the EGFP based splicing assay was used to test in cell culture the antisense effect of increased binding of the oligonucleotides to the target RNA. Surprisingly, base substitutions such as G-clamp, C-propynyl or phenaxazine in oligonucleotides with a MOE backbone had no effect when the oligonucleotides were freely taken up by the cells or delivered by the scrape-loading technique (Sazani et al., 2003c). Note that a similar lack of increased effect was observed *in vivo* when chimeric MOE oligonucleotides were derivatized with C-propynyl groups and used in RNase H dependent antisense downregulation assay (Shen et al., 2003).

D. Other splice-switching techniques

snRNAs

Small nuclear RNAs that exist in the cell as RNA processing factors have been exploited for modification of splicing by replacing their authentic antisense sequences with those directed toward a specific splicing element in a targeted gene (reviewed in Vacek, Sazani et al. 2003). RNA recognition sequences of the U1 and U7 snRNAs were modified to identify and bind to 3' cryptic splice sites in the β-globin pre-mRNA. These altered snRNAs used for splice switching were able to reduce aberrant β-globin splicing and induce correct splicing *in vitro*

(Gorman, Suter et al. 1998; Suter, Tomasini et al. 1999; Gorman, Mercatante et al. 2000). Similarly, U7 snRNA targeted to splice sites around exon 23 of the dystrophin transcript was able to cause exon exclusion in wild type and immortalized mdx muscle tissue culture cells, and to rescue the production of functional dystrophin protein (Brun, Suter et al. 2003). Delivery of splice switching snRNAs into the cell can be improved by placing them into viral vectors where long term, stable expression can be achieved (Vacek, Ma et al. 2003; Liu, Asparuhova et al. 2004). U1, U2, and U7 snRNAs encoded in viral vectors also have been used to induce exon skipping in dystrophin pre-mRNA as described in more detail later.

Trans-splicing

Although most pre-mRNAs undergo *cis*-splicing to form mature mRNA, *trans*-splicing, whereby sequences from two independently transcribed pre-mRNAs are spliced to form a composite mRNA, also exists. In two recent papers the latter process was exploited for therapeutic purposes. In the spliceosome-mediated RNA *trans*-splicing (SMaRT) technique a fragment of pre-mRNA, which carries exon sequences with correct reading frame of the targeted gene, is designed to base pair with the intron of a targeted pre-mRNA upstream of the exon with a disease causing mutation, such as 508 in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene. The construct enhances *trans*-splicing between the two molecules and suppresses *cis*-splicing of the target thereby replacing the defective exon with a correct one. Recently, Liu *et al.* demonstrated that SMaRT corrected endogenous mutant CFTR pre-mRNA *ex-*

vivo in airway epithelia from patients with cystic fibrosis and partially restored CFTR-mediated chloride transport. The same construct was inserted in the adenoviral vector and shown to be effective *in vivo* in xenografts of human CF bronchial cells (Liu, Jiang et al. 2002). SMaRT also has been used to repair pre-mRNA of Factor VIII in a mouse model of hemophilia A. The levels of functional Factor VIII reached 12% of normal (Chao, Mansfield et al. 2003). Note that in the cystic fibrosis model the *trans*-splicing molecules were injected directly into the xenograft while in the hemophilia model, large volumes were delivered by tail vein injections, exploiting hydrodynamic pressure to improve uptake of the construct (Budker, Zhang et al. 1996). Thus, although the progress is very encouraging, this and other related technologies need to overcome the challenges of delivery of relatively large molecules.

IV. Gene targets of splice switching

A. β-globin

β-Thalassemia is a blood disease resulting from reduced or absent production of functional β-globin, a subunit of hemoglobin. Severe cases of thalassemia, if untreated, result in early death. Current treatment consists of frequent transfusions combined with iron chelation therapy. Inability to produce hemoglobin is caused by mutations in the β-globin gene, affecting various stages of β-globin biosynthesis. While over 200 mutations lead to this disease, several common forms of thalassemia are due to mutations in β-globin gene introns that create alternative splice sites and lead to aberrant splicing of pre-mRNA. The result is inclusion of the intron fragment, which creates a nonsense codon in the

β-globin coding sequence, preventing translation of the protein (Olivieri 1999). This laboratory has used splice switching oligonucleotides to target aberrant splice sites and induce a shift from aberrant to correct splicing, restoring expression of hemoglobin. Sequence specific correction of splicing was achieved in model cell lines and subsequently by *ex vivo* treatment of erythroid progenitors cells from thalassemia patients (Vacek, Ma et al. 2003). In these studies, the SSOs were delivered by cationic lipid delivery agents or by temporary, mechanical damage to cell membranes (Lacerra, Sierakowska et al. 2000). Free uptake of SSOs by the cultured erythroid cells also was accomplished using oligonucleotides with a neutral, morpholino backbone. High levels of correct RNA and hemoglobin expression were achieved after several days of treatment (Suwanmanee, Sierakowska et al. 2002; Suwanmanee, Sierakowska et al. 2002). Like most other pharmacological agents, SSOs effects are relatively short term due to their degradation and excretion. Thus, the possibility of long-term repair of β-globin pre-mRNA also has been investigated. The sequence antisense to the aberrant splice sites in β-globin pre-mRNA was cloned into a U7 snRNA gene, and the modified anti-β-globin U7 snRNA expressed from its own promoter served as a means to deliver antisense sequences to erythroid cell nuclei. Previous work relied on transfection of appropriate plasmids, but later the modified U7 gene was incorporated into lentiviral vectors. Treatment of cells, both model cell lines and thalassemic patients' erythroid progenitors, resulted in significant upregulation of β-globin mRNA and functional protein expression. Although the level of expression was not as high as in the SSO treated cells, the

effects were permanent in the cell lines and lasted as long as the primary erythroid cells could be maintained in culture (Vacek, Ma et al. 2003). Antisense RNA delivery via lentiviral vectors is especially appealing in chronic diseases such as β-thalassemia that require lifelong correction of splicing. One advantage of RNA repair, especially for a gene that is as tightly developmentally controlled as β-globin is that the target gene is expressed in its natural environment and therefore cannot be over- or inappropriately expressed. As mentioned previously, RNA repair allows up-regulation or re-expression of a defective gene, a result that cannot be achieved by RNAi or standard antisense approaches.

B. Dystrophin

Exons, i.e. coding sequence mutations that disrupt the dystrophin reading frame lead to premature termination of protein synthesis, resulting in Duchenne muscular dystrophy (DMD). In contrast, deletions that maintain the reading frame lead to the synthesis of a shorter, semi-functional dystrophin protein, resulting in the less severe Becker muscular dystrophy (BMD). Therefore, skipping of the mutated exon may remove the offending mutation and restore the reading frame, converting DMD to a milder BMD phenotype. Indeed, a series of recent papers showed restoration of dystrophin expression in the mdx mouse model of the disorder. In this model, exon 23 carries a mutation that introduces a stop codon. Intramuscular injection of the 2'-*O*-methyl oligonucleotides complexed with Lipofectin targeted to exon 23 splice sites restored detectable dystrophin protein near the site of the injection (Mann, Honeyman et al. 2001). More recently, 2'-*O*-methyl oligonucleotides injected into the tibialis muscle in the

presence of the nonionic block polymer, F127, widely used in drug formulations, restored dystrophin expression to at least approximately 20% of normal. Importantly, the treatment measurably increased the strength of the injected muscle (Lu, Mann et al. 2003; Lu, Rabinowitz et al. 2005). One notes that the volume of the oligonucleotide formulation (30 µl) was close to the volume of the injected muscle. Thus, it seems possible that in addition to F127 a hydrodynamic pressure created in the muscle by the large amount of the injected liquid might have induced intracellular uptake of the oligonucleotides. Similar results were obtained by intramuscular injection with morpholino oligonucleotides alone (Alter, Lou et al. 2006) or complexed with Lipofectine (Gebski, Mann et al. 2003). Since morpholino oligonucleotides are uncharged Gebski et al. hybridized these molecules to negatively charged “leash” oligonucleotides. The leash concept was previously applied to morpholino oligonucleotides (Morcos 2001).

Antisense RNAs encoded in viral vectors have also been used to induce exon skipping in human dystrophin pre-mRNA by replacing sequences in U1, U2 and U7 snRNAs with sequences antisense to the 5’ and 3’ splice sites of dystrophin exon 51 (De Angelis, Sthandier et al. 2002). These modified snRNA genes were then cloned into the 3’ LTR of the pBabe puro retroviral vector. Viral particles were used to transduce muscle cells from a DMD patient having a deletion encompassing exons 48-50. Since this deletion created a premature termination codon in exon 51, skipping of this exon partially restored the reading frame of the gene and rescued dystrophin synthesis. The most efficient skipping was obtained when both the 5’ and 3’ splice sites of exon 51 were targeted with

antisense molecules. Similarly to the work in mdx mouse these results indicate that restored expression of dystrophin, even carrying substantial deletions, may alleviate the symptoms in patients with DMD.

C. Sma

Spinal muscular atrophy (SMA), an inherited neurodegenerative disorder is caused by mutations in one of the survival of motor neuron genes (SMN1) even though a duplicated copy of the gene (SMN2) also exists. SMN2 retains a correct open reading frame for SMN protein but its exon 7 is skipped, leading to expression of a nonfunctional gene product. To reverse exon skipping 2'-*O*-methyl oligonucleotides were targeted to the 3' splice of the downstream exon 8, based on the assumption that blocking of that splice site will tilt the balance of splicing to favor inclusion of exon 7. Indeed, this was accomplished in model cell lines that stably express minigene constructs of SMN2 (Lim and Hertel 2001). Thus, this therapeutic strategy may be useful in counteracting the progression of SMA. Modulation of inclusion of SMN exon 7 also was accomplished in cell culture by 2'-O-methyl-oligonucleotides targeted to the newly discovered intronic splicing silencers and enhancers located, respectively upstream and downstream from exon 7 (Miyajima, Miyaso et al. 2002; Miyaso, Okumura et al. 2003).

D. Tau

The 5' splice site of exon 10 of the *tau* gene is sequestered in a stem-loop structure and, therefore, this exon is to a large extent excluded from the mature Tau mRNA. A mutation implicated in the frontotemporal dementia and Parkinsonism associated with chromosome 17 (FTDP-17) destabilizes the stem

and exposes the splice site, leading to inclusion of the exon. 2'-*O*-methyl phosphorothioate oligonucleotides, targeted either to the 3' or the 5' splice sites of tau exon 10, forced exon skipping and restored a proper ratio of the splice variants. This effect was achieved by electroporation of the oligonucleotide into rat neuronal pheochromocytoma PC12 cells or AR42J pancreatic acinar cells. In the latter cells, decreasing the levels of exon 10 containing protein, which includes a microtubule-binding domain, caused rearrangement of cytoskeletal-structure, a potentially therapeutically favorable outcome (Kalbfuss, Mabon et al. 2001).

E. Bcl-x

Bcl-x, like other members of the bcl-2 family of genes is involved in apoptosis. Its two primary splice forms, bcl-xL and bcl-xS, are generated by alternative splicing of bcl-x intron 2 (Cory and Adams 2002 and references quoted therein), via the use of a common 3' splice site and two alternative 5' splice sites. Bcl-xL, the longer splice variant, is anti-apoptotic. Moreover, high levels of bcl-xL have been correlated with resistance to chemotherapy (Liu, Page et al. 1999). The shorter variant, bcl-xS, which uses a 5' splice site farther upstream from the 3' splice site than bcl-xL, has been shown to have pro-apoptotic properties (Minn, Boise et al. 1996). SSOs targeted against the bcl-xL 5' splice site were shown to shift splicing towards production of bcl-xS in the lung epithelial cancer cell line A549 (Taylor, Zhang et al. 1999). Although the splice shift alone was insufficient to induce apoptosis, it enhanced the apoptotic effects of chemotherapeutics administered with the oligonucleotide. In contrast, prostate

cancer PC3 cells were sensitive to SSO treatment in the absence of anti-cancer drugs (Mercatante, Bortner et al. 2001). The cell sensitivity was correlated with the level of bcl-x expression or more accurately with the absolute amounts of bcl-xS generated by the anti-bcl-xL SSO. In addition, the SSO treatment sensitized the cells to chemotherapeutics and radiation and interestingly, led to cell death in a cell line that was resistant to doxorubicin, a common anti-cancer drug (Mercatante, Mohler et al. 2002). Bcl-x is a particularly useful target for SSOs because the two splice variants have opposing functions. Thus, a shift in splicing simultaneously reduces the level of anti apoptotic bcl-xL and increases the level of pro-apoptotic bcl-xL, enhancing the effects of SSOs. The results also suggest that expression of possibly thousands of alternatively spliced genes can be manipulated by SSOs either as research tools in investigations of gene function, or possibly as drugs when targeted to disease associated genes, following the example of bcl-x (Mercatante and Kole 2000).

V. Prostate Cancer

Prostate cancer is the primary malignancy and the second leading cause of cancer deaths in men behind lung cancer (Jemal, Murray et al. 2005). Fortunately, most men who get prostate cancer during their lifetime will die with the disease instead of from it. However, although prostate cancers are normally slow growing and progressing, if not completely eradicated, they will eventually become androgen independent, causing a high mortality rate in men with uncontrolled prostate cancer. Management of those diagnosed with prostate cancer can be a challenging balance between treatment and quality of life (Wei,

Dunn et al. 2002). To add to the dilemma of how to treat prostate cancer, new diagnostic, screening and imaging techniques are now able to detect cancers at a much earlier stage than previous technologies; it is not yet known if these advances actually decrease the morbidity and/or mortality rates of the disease. Several groups have been involved in the research on this topic (i.e. risk assessment and decision making in treatment of prostate cancer); two of the largest research groups are the Cancer of the Prostate Strategic Urologic Research Endeavor (CaPSURE) and the Department of Defense Center for Prostate Disease Research (CPDR) (reviewed in Cooperberg, Moul et al. 2005). Their studies along with others have been crucial in mapping the progression of prostate cancer. It has been determined that for the first 10 to 15 years many prostate cancers are usually idle and slow to progress, but beyond 15 years, the mortality rate triples (Johansson, Andren et al. 2004). Since the overdiagnosis and very early diagnosis of prostate cancer may not necessarily benefit patients, current protocol is to identify men that are at a higher risk for aggressive cancers; noted risk factors include a family history of prostate cancer and ethnic background, primarily having an African American ethnicity (Reddy, Shapiro et al. 2003; Rebbeck 2005). There is conflicting evidence as to whether obesity is an important risk factor (Giovannucci, Rimm et al. 2003; Amling 2004; Amling, Riffenburgh et al. 2004; Freedland, Aronson et al. 2004).

A. Treatment of Prostate Cancer

Due to the above problems with effective treatment of prostate cancer, current treatments are moving toward the approach of ‘active surveillance’ after

prostate cancer diagnosis instead of 'watchful waiting'. The difference in these approaches is the level of monitoring and the expectation of a more aggressive treatment when the disease begins to progress. Localized prostate tumors are most often treated with radiation therapy or surgery while refractory or recurrent disease is treated with hormonal therapy. The Prostate Cancer Outcomes Study between 1994 and 1995 determined that 47% of patients received a prostatectomy, 23% received radiotherapy, 11% received androgen deprivation therapy and 19% received observation only (Harlan, Potosky et al. 2001). A more recent estimate by the CaPSURE study found only 5.5% of patients electing to use observation only (Harlan, Cooperberg et al. 2003).

B. Protein markers of prostate cancer

Biomarkers are important for the study, diagnosis, detection, prediction and possibly treatment of cancer, and prostate cancer is no exception. Useful molecular biomarkers of prostate cancer must be able to accurately measure cancer stage, progression and prognosis. The search for the perfect prostate cancer predictor is still going strong because there is still no one biomarker that can alone differentiate between benign and malignant disease. Efficacy of markers is measured by their ability to predict accepted measures of prostate cancer, including: Gleason score and clinical/pathological disease stage (Bostwick, Grignon et al. 2000; Quinn, Henshall et al. 2001). To be a useful prostate cancer marker for potential immunotherapy, Sbulke et al. suggest a few prerequisites: the biomarker must be found predominantly in the prostate, be highly expressed in all prostate disease states, have a cell surface presentation,

	Date of Discovery	*Localization for Detection*	*Biological Function*	*Diagnostic Usage*	*Associated Therapeutics*
Prostatic Acid Phosphatase (PAP)	1935	Secreted: Prostate gland, Serum, Blood, Semen	Catalyzes the conversion of orthophosphoric monoester to alcohol and orthophosphate	Blood test detects metastatic prostate cancer (no longer routinely used)	none
Prostate Specific Antigen (PSA)	1971	Secreted: Prostate gland, Serum, Blood, Semen	Hydrolyzes seminal vesicle proteins seminogelin I and II, resulting in liquefaction of the seminal coagulum; hydrolyzes insulinlike growth factor–binding protein-3 (IGFBP3)	Blood test approved by the FDA detects benign prostate hyperplasia, prostatitis, prostate cancer and its metastases. PSA levels generally mirror clinical progression of prostate cancer, but false positives and negatives are common.	none
Prostate Specific Membrane Antigen (PSMA)	1987	Plasma Membrane Bound/ Cytoplasmic: Prostate, Small Intestine, Kidney, Brain, Tumor Neovasculature	Hydrolyzes *N*-acetyl-aspartyl-glutamate to *N*-acetyl aspartate and glutmate; hydrolyzes the terminal glutamates of poly-glutamyl folates	Radiolabeled monoclonal antibody body scan detects localized and metastatic prostate cancer	Radiolabeled or dendrimer-linked PSMA antibodies can target PSMA expressing cells

Table 1.1 Biomarkers of prostate cancer: PAP, PSA, PSMA

but not be secreted, and have some enzymatic or signaling activity. One review by Tricoli et al. lists and describes the cellular localization, biochemical function, and cellular function of several potential prostate cancer markers including the three most studied prostate cancer markers, prostatic acid phosphatase (PAP), prostate specific antigen (PSA), and prostate specific membrane antigen (PSMA) (Tricoli, Schoenfeldt et al. 2004). Here, I will describe PAP and PSA, two historically popular prostate cancer markers the latter of which has become almost synonymous with assessing prostate cancer risk. The PSMA biomarker will be discussed later in great detail.

PAP

PAP levels were first identified and linked to the prostate in 1935 (Kutscher and Wolbergs 1935). High levels of PAP in serum then became associated with prostate cancer and its metastases, making it the first verified prostate cancer marker (Gutman, Sproul et al. 1936; Gutman and Gutman 1938). Serum levels of PAP declined after antiandrogen therapy, but then rebounded after disease resurgence (Huggins and Hodges 1941; Schacht, Garnett et al. 1984). Analysis of PAP levels was successful in identifying men with advanced metastatic disease, but failed to detect cancer in men with primary tumors and early stage disease (Sullivan, Gutman et al. 1942; Nesbit and Baum 1951). PAP did not become widely used as a diagnostic measure of cancer because its relatively high limits of detection made it undesirable; there was a need to identify malignancy earlier when the cancer is more likely to be cured.

PSA

PSA was first found in serum from patients with prostate cancer in 1980 (Papsidero, Wang et al. 1980), but had been previously identified in 1971 as a kallikrein-like serine protease (Hara, Koyanagi et al. 1971). Its function was determined to be to proteolytically liquefy semen in the prostate (Dawson and Vogelzang 1994). Originally, it was thought to be a prostate specific protein, but later studies confirmed its presence in other tissues including some female tissues (Papsidero, Wang et al. 1980; Myrtle, Klimley et al. 1986). After a link was made between PSA and prostate cancer, serum limits of PSA in patients were established; a serum level of 4 ng/mL or less was defined as normal, whereas a higher serum level indicated the need for further evaluation and possible treatment (Myrtle, Klimley et al. 1986). Over time, PSA became touted as the golden marker for prostate cancer diagnostics with large public campaigns to get all men over 50 years old to receive PSA blood tests on a regular basis. PSA was indeed effective in detection especially when combined with the use of digital rectal exams (Cooner, Mosley et al. 1990; Catalona, Smith et al. 1991; Brawer, Chetner et al. 1992; Labrie, Dupont et al. 1992), however, many became concerned with the sensitivity and specificity of the test (Wang and Kawaguchi 1986; Guinan, Bhatti et al. 1987; Stamey, Yang et al. 1987; Stamey, Kabalin et al. 1989). One problem involved the increase of PSA levels in the absence of cancer with conditions including benign prostate hyperplasia (BPH) and prostatitis. False positives became the great blemish of PSA tests leading to unnecessary follow up techniques including biopsies. Moreover, it was determined that 20 to 30% of men with test readings in the ‘normal’ range did

indeed have prostate cancer (Cooner, Mosley et al. 1990; Catalona, Smith et al. 1991; Labrie, Dupont et al. 1992). This level of inaccuracy in PSA screening due to false positives and false negatives make PSA highly criticized as a useful biomarker, although PSA remains the best and only United States Food and Drug Administration approved clinically available prostate cancer marker to date (FDA 1986; FDA 1994).

VI. PSMA

The PSMA gene has become increasingly relevant to the varied fields of prostate and general cancer research, neuroscience, and folate/one-carbon metabolism due to its unique enzymatic functions and localization in different tissue types (reviewed in Heston 1997; Chang, Gaudin et al. 2000; Ghosh and Heston 2004). PSMA may prove to be a more useful prostate cancer biomarker for imaging, detection and drug targeting than PSA or PAP since it is a membrane-bound protein on prostate cancer cells while the other two proteins are secreted.

A. Discovery and identification of PSMA

The PSMA protein was first identified in 1987 by Horoszewicz et al. during the production of monoclonal antibodies of spleen cells from Balb/c mice immunized with membranes taken from the LNCaP cell line they established in 1978 (Horoszewicz, Kawinski et al. 1987). That 7E11C-5.3 monoclonal antibody stained strongly LNCaP membranes, and also was highly specific for human primary prostate tissues, including benign prostate hyperplasia, prostate cancers and prostate metastases. Israeli et al. later sequenced the PSMA polypeptide

after isolation of PSMA using the CYT-356 monoclonal antibody and peptide microsequencing by proteolytic cleavage and HPLC fractionation (Israeli, Powell et al. 1993). Using degenerate primers followed by a cDNA hybridization probe, they were able to sequence and clone the entire PSMA cDNA. The sequencing predicted a 2653 base pair cDNA and a 750 amino acid protein. Rinker-Schaeffer et al. determined PSMA was located on human chromosome 11 using southern blotting analysis of mouse or hamster/human somatic cell hybrids (Rinker-Schaeffer, Hawkins et al. 1995). Two signals were detected, one localized to 11p and the other to 11q. It was later realized that the 11p11-p12 location encoded the PSMA gene and the 11q14 produced a PSMA-like gene presumably due to a duplication event (O'Keefe, Su et al. 1998).

PSMA is highly expressed in LNCaP cells and to a lesser extent in CWR-R1 cells, but not in the much used PC3 prostate cell line or DU-145 cells (Ghosh, Wang et al. 2005). In normal prostate, PSMA is expressed in secretory epithelial cells and is upregulated with higher levels of expression in prostatic intraepithelial neoplasia (PIN) and prostate adenocarcinoma (Horoszewicz, Kawinski et al. 1987; Bostwick, Pacelli et al. 1998). Prostate cancers express PSMA at a higher level than benign prostate tissues and its expression has a positive correlation to cancer grade and stage (Lapidus, Tiffany et al. 2000; Ross, Sheehan et al. 2003).

Monoclonal antibodies specific to PSMA are currently being used in clinical settings. The 7E11 mAb that was used to originally isolate PSMA has been improved by the linkage of ^{111}Indium, ^{90}Yttrium, and ^{131}Iodine radiolabel to

detect tissues expressing PSMA *in vivo* (Kahn, Williams et al. 1994; Deb, Goris et al. 1996). The ^{111}Indium 7E11 modified mAb has been marketed by the Cytogen company (Princeton, NJ) as ProstaScint and was approved for detection of primary prostate cancer and metastasis by the Food and Drug Administration in 1997.

Perhaps the most surprising and potentially important discovery in PSMA research is its localization and abundance in tumor neovasculature (Liu, Moy et al. 1997; Chang, Reuter et al. 1999; Chang, Reuter et al. 2001). Therapeutic targeting of PSMA may be able to treat not just prostate cancers, but all types of tumors by destroying the route by which they receive nutrients. Expression of PSMA also has been detected to a much lower extent in other tissues, including: brain, small intestine, kidney and muscle (Slusher, Robinson et al. 1990; Murphy, Ragde et al. 1995; Troyer, Beckett et al. 1995).

B. Function of PSMA: NAALADase and FGCP

NAALADase

Just prior to the isolation and identification of PSMA, other groups were studying an enzyme that hydrolyzes the *N*-acetyl-aspartyl-glutamate (NAAG) dipeptide to *N*-acetyl aspartate (NAA) and glutamate in the brain (Robinson, Blakely et al. 1987). This protein was isolated using polyclonal antisera and monoclonal antibodies to the NAAG carbodiimide linkage and was named *N*-acetyl α-linked acidic dipeptidase (NAALADase). NAA and glutamate are both important neurotransmitters in the brain; the latter being the principal excitatory neurotransmitter in the central nervous system (Hollmann and Heinemann 1994).

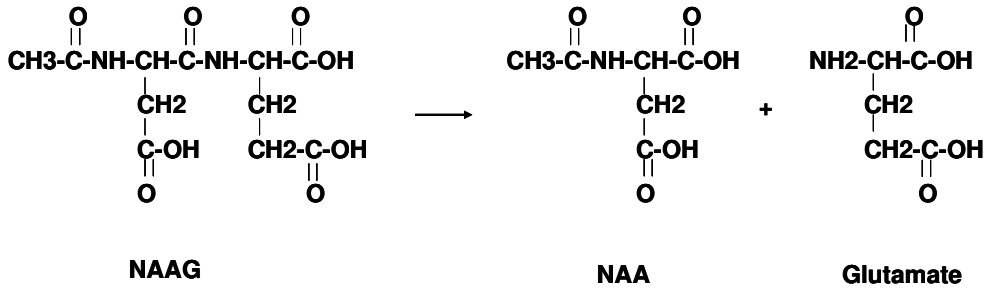
NAAG may act as a storage form of these two molecules and/or may also play its own role in signal transmission. There is a linking between PSMA and neuronal signaling since both NAAG and NAALADase have been associated with several CNS disorders related to the dysfunction of glutamate neurotransmission, including schizophrenia (Tsai, Passani et al. 1995) and epilepsy (Meyerhoff, Carter et al. 1992; Meyerhoff, Carter et al. 1992). It was hypothesized, and later confirmed by chromosome mapping, that NAALADase and PSMA were the same protein since PSMA also was able to hydrolyze NAAG (Fig. 1.5) and was sensitive to quisqualic acid, a potent inhibitor of NAALADase activity (Carter, Feldman et al. 1996).

FGCP

Another intersection of two seemingly unrelated fields, folate nutrition and prostate cancer, attributed another function to the PSMA protein. In the late 1990s, genetic mapping determined that PSMA, NAALADase, and a third gene, folylpoly-γ-glutamate carboxypeptidase (FGCP) were all expressed from a single gene (Pinto, Suffoletto et al. 1996; Luthi-Carter, Barczak et al. 1998). Note that the nomenclature committee of the International Union of Biochemistry and Molecular Biology has officially named the gene Glutamate Carboxypeptidase II (GCPII) (Barrett 1997), but in this work it is generally referred to as PSMA for simplicity.

FGCP was first identified by Chandler et al. in 1986 (Chandler, Wang et al. 1986). Its function is to cleave terminal glutamate residues at the gamma linkage from poly-glutamated folates (Fig. 1.5). The family of folates are B-vitamins

A. *N*-acetyl α-linked acidic dipeptidase (NAALADase)



B. Folylpoly-γ-glutamate carboxypeptidase (FGCP)

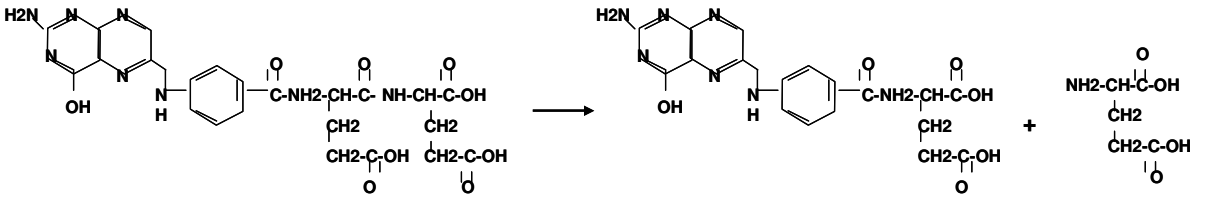


Figure 1.5. Enzymatic functions of PSMA.

A) *N*-acetylated-a-linked-acidic-dipeptidase (NAALADase) function: the *N*-acetyl-aspartyl-glutamate (NAAG) molecule is hydrolyzed to *N*-acetyl aspartate (NAA) and glutamate. B) Folylpoly-γ-glutamate carboxypeptidase (FGCP) function (also termed Folate Hydrolase (FOLH1)) : terminal glutamate residues of poly-glutamyl folates (in this case di-glutamyl folate) are hydrolyzed.

essential in the mammalian diet, but not natively synthesized by mammalian cells. Folate is required for several essential cellular processes including nucleic acid synthesis and methylation, the methionine cycle, and many others affected by the transfer of single carbon methyl groups (Eto and Krumdieck 1986; Jennings 1995).

A low folate level causes hyperhomocystenemia due to deficiencies in metabolism of methionine (Selhub, Jacques et al. 1993; Clarke, Woodhouse et al. 1998). Hyperhomocystenemia in turn leads to cardiovascular disease (Kang, Wong et al. 1987; Clarke, Daly et al. 1991; Boushey, Beresford et al. 1995; Danesh and Lewington 1998; Schnyder, Roffi et al. 2001; Klerk, Verhoef et al. 2002), neural tube defects (Smithells, Sheppard et al. 1976; Czeizel and Dudas 1992; Mills, McPartlin et al. 1995), anemia (Herbert 1967), and cognitive disease (Joosten, Lesaffre et al. 1997; Clarke, Smith et al. 1998; McCaddon, Davies et al. 1998; Selhub, Bagley et al. 2000; Seshadri, Beiser et al. 2002). Sauberlich et al. proposed that the folate bioavailability, or percentage of nutrient absorbed versus intake amount, of folate may be about 50% (Sauberlich, Kretsch et al. 1987). This is due mostly to the inability of cells to effectively transport polyglutamated folates across plasma membranes (Godwin and Rosenberg 1975; Halsted, Baugh et al. 1975; Halsted, Reisenauer et al. 1978; Bailey, Cerda et al. 1984; Keagy, Shane et al. 1988).

The poly-glutamyl storage form of folate may account for upwards of two-thirds of all ingested dietary folate (Melse-Boonstra, de Bree et al. 2002). Effective uptake of polyglutamyl folate requires hydrolysis of the glutamyl chain to

produce the monoglutamyl form of folate (Chandler, Harrison et al. 1991). The extracellular portion of the FGCP protein performs these hydrolysis steps in the brush border of the small intestine and possibly the proximal tubule cells of the kidney (Liu, Moy et al. 1997) and allows for folate uptake by the reduced folate carrier). A 1561 C>T polymorphism in exon 13 of FGCP that causes a H475Y amino acid substitution was associated with low serum folate and high plasma homocysteine levels in a population of Caucasian males (Devlin, Ling et al. 2000). *In vitro*, COS-7 cells expressing this polymorphism gave a 50% lower FGCP activity level than wild type FGCP expressing cells. Although PSMA's specific role as FGCP in the small intestine is well defined and understood, the reasons for its localization and function in prostate tissues remains unknown.

It has not been determined whether PSMA's folate hydrolase activity is functionally unique in humans or absolutely necessary for proper folate nutrition although other reports acknowledge that another protein must be able to hydrolyze NAAG (Bacich, Ramadan et al. 2002; Bzdega, Crowe et al. 2004). Bzdega et al. characterized an NAALADase expressed in cerebellar astrocytes of PSMA knockout mice which they called GCPIII (Bzdega, Crowe et al. 2004).

There are a few hypotheses as to why PSMA is so highly expressed in normal and malignant prostate tissues compared to other tissues including brain and small intestine. One idea is that PSMA helps maintain extracellular glutamate levels in the prostatic fluid compartment since glutamate is found in seminal fluid. The prostate may be subject to an inherent folate deficiency that makes it prone to genetic instability and therefore cancer; expression of PSMA

may be one mechanism prostate cells use to increase intracellular and/or local folate levels. It is also possible that PSMA localization on the prostate cell surface may itself play a role in malignant transformation by acting as a signaling or receptor molecule. Finally, high expression of membrane bound PSMA in prostate tumors may ensure adequate folate levels in rapidly dividing cells by recycling stored folates that were sequestered in necrotic cells.

PSMA knockouts

To add to the complexity of the PSMA story, two different groups produced PSMA knockout mice with differing, almost opposite results. Tsai et al. used a targeting vector that disrupted a 2.5 kb region containing exons 9 and 10 with a 1.8 kb PGK-neomycin cassette. PSMA null mutants were obtained in mice with a C57BL/6 background, but no live offspring were obtained. They demonstrated the need for PSMA in normal development and that the absence of PSMA results in early embryonic death in mice (Tsai, Dunham et al. 2003). Conversely, Bacich et al. produced a PSMA knockout in mice with a chimeric 129/SVJ on C57Bl/6 background also by insertion of a neomycin resistance cassette. The 2.5 kb region of the insert was targeted to the Sac II site in exon I and the Hind III site in intron I. Most of intron I was replaced by the PGK-neomycin cassette and multiple stop codons were introduced in exons I and II. This group found evidence of an alternate route of NAAG hydrolysis. They not only found no embryonic lethality, but describe “little detectable effect on the behavior and neurochemistry” of PSMA null mutant mice (Bacich, Ramadan et al. 2002).

Reasons for the great divergence in results from these two groups are not readily apparent, but could be explained by a couple of differences in methodology. The slight difference in mouse strain may have had an effect on viability; this cannot be ruled out since the total function of PSMA is not well defined. Also, the method of producing the knockout mice is important; introducing stop codons in all three frames in exon I and the beginning of exon II may not be effective since splicing differences allow for the use of start codons in the second exon. Considering the number of alternative splicing events that involve exon I and intron I, it is more desirable to use a gene knockout approach that targets another portion of the gene, as done by Tsai et al. Nevertheless, both groups did verify their knockout by PCR genotyping, southern, and western blot analysis; explanation of their differing results remains elusive.

C. Structure of PSMA

Protein

Several reports describe PSMA as a 100-110 kD protein composed of 750 amino acids (Israeli, Powell et al. 1993). Computational analysis followed by protein crystallization of its extracellular region verified the domain composition of PSMA (Davis, Bennett et al. 2005). A short 19 amino acid intracellular region is located at the amino terminus and contains the first five amino acid residues, an MXXXL motif for protein internalization (Rajasekaran, Anilkumar et al. 2003). PSMA does internalize when bound to a ligand or specific antibodies which may implicate it as a receptor molecule (Liu, Moy et al. 1997; Anilkumar, Rajasekaran et al. 2003). The cytoplasmic domain is followed by another small 24 amino acid

transmembrane domain. This makes PSMA an unusual type II membrane protein in which the N-terminus is cytoplasmic while the C-terminus is extracellular. PSMA's protease domain is located at amino acids 56-116 and 352-591 and is similar to the transferrin receptor and bacterial binuclear zinc exopeptidases' protease domains (Rees, Lewis et al. 1983; Davis, Bennett et al. 2005). PSMA is also a metalloproteinase as it binds two zinc atoms in the enzymatic domain for functional activity (Rawlings and Barrett 1997). The C-terminal domain, amino acids 592 – 747, is called the helical domain and contains a dimerization motif. Deletion of the final 15 PSMA residues was shown to abolish PSMA enzymatic activity (Barinka, Mlcochova et al. 2004), which is consistent with the observation that protein dimerization is necessary for PSMA function (Schulke, Varlamova et al. 2003). PSMA also contains an apical protease associated domain (amino acids 117-351) that aids in making the substrate binding cavity and forming the dimer interface. The overall structure of PSMA, i.e. one that includes a short cytoplasmic domain, one transmembrane domain, and a large extracellular enzymatic region is frequent among known membrane bound hydrolases (Kenny and Maroux 1982).

PSMA shows significant sequence homology to the transferrin receptor (TfrI). Structurally, TfrI is also a type II protein and contains an N-terminal internalization domain made up of a YXRF amino acid sequence that allows internalization of its extracellular region when bound to iron-containing transferrin (Trowbridge and Collawn 1992). TfrI also exists as a functional dimer and has an aminopeptidase domain using two zinc atoms at the active site (Schneider,

Sutherland et al. 1982; Mahadevan and Saldanha 1999), although TfrI's peptidase function is thought to be vestigial.

The PSMA protein contains 10 potential glycosylation sites that are important if not vital to its extracellular function; removal of asparagine binding sites partially or completely blocked PSMA's NAALADase activity (Ghosh and Heston 2003).

mRNA

In normal prostate epithelium PSMA was determined to be not as highly expressed as the PSM' splice variant, in which 266 nucleotides of PSMA exon 1 is spliced out via an alternative 5' splice site, is a predominant splice variant (Su, Huang et al. 1995). PSM' protein lacks the first 60 amino acids that wholly encompasses the transmembrane domain and, as a result, localizes to the cytoplasm (Grauer, Lawler et al. 1998). Although PSM' retains the folate hydrolase active site, similar to the domain isolated by Davis et. al (Davis, Bennett et al. 2005), its cytoplasmic enzymatic activity has been questioned (Ghosh, Wang et al. 2005) and the function of PSM' is not known. In addition to PSMA and PSM' other splice variants, PSMA-C, -D (Schmittgen, Teske et al. 2003), PSMAΔ6 (direct submission to NCBI #AF254357) and PSMAΔ18 (Devlin, Ling et al. 2000) have been noted, but their expression level and function are largely unknown.

PSMA C pre-mRNA is spliced from the 3' splice site of intron 1 to an alternative 5' splice site located between those of PSMA and PSM' in exon 1. As in PSM' splicing, this pathway deletes the PSMA start codon; use of the AUG in

exon 2, predicts a protein identical to PSM'. In PSMA-D, the PSM' 5' splice site is spliced to a cryptic 3' splice site in intron 1, retaining a portion of intron 1 sequence. The retained sequence includes a start codon, presumably adding 24 extra amino acids to PSM-D protein. The rest of its sequence is in frame and identical to corresponding amino acids in PSMA and PSM'.

PSMAΔ6 and PSMAΔ18 variants have had little if any prior molecular characterization and analysis; this is the first study of these variants. Sequence analysis of PSMAΔ6 mRNA shows complete exclusion of exon 6 by the splicing machinery and predicts that removal of this exon would produce a frameshift in mRNA sequence. If translated and stable, the first 243 amino acids of the putative PSMAΔ6 protein would coincide with those of PSMA followed by 30 different C-terminal amino acids. The resulting PSMAΔ6 protein could be membrane-associated, but should be functionally inactive due to the lack of the enzymatic and dimerization domains. In PSMAΔ18, exon 18 is excluded leading to a putative 695 amino acid protein PSMA-like protein where the terminal 39 amino acids, coded downstream of exon 17 are from the alternate reading frame. Although PSMAΔ18 should contain the folate hydrolase active site it would lack the dimerization domain, which was found to be essential for enzymatic activity of PSMA or its extracellular domain (Schulke, Varlamova et al. 2003; Davis, Bennett et al. 2005). Thus, PSMAΔ18 is expected to be inactive. PSMA splice variants and their protein products are illustrated in Figure 1.6.

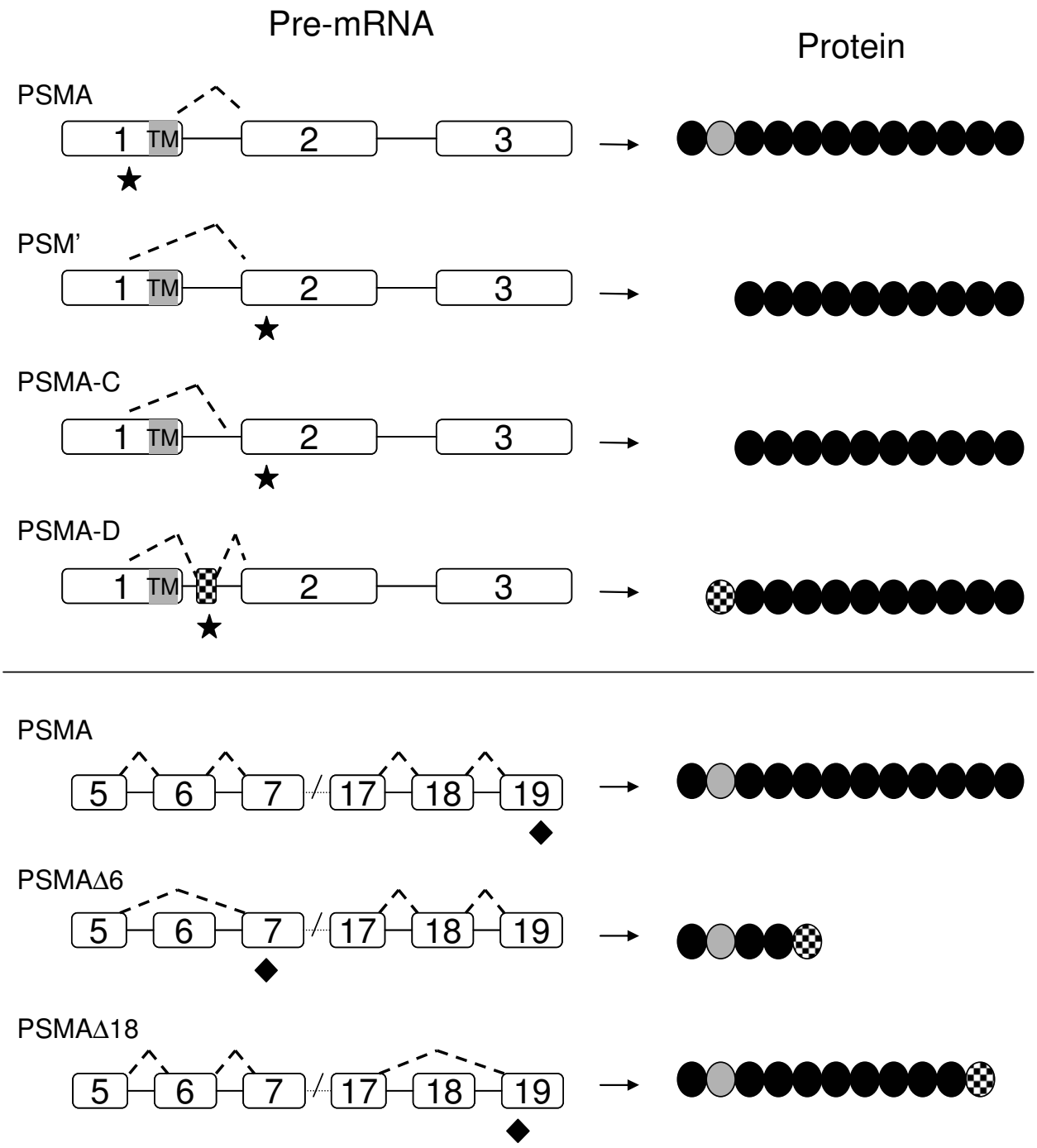


Figure 1.6. Alternative splice variants of PSMA pre-mRNA.

Figure 1.6. Alternative splice variants of PSMA pre-mRNA.

A) Splice variants from alternative splicing involving PSMA's first intron: full length PSMA contains all exon and no intron sequence; PSM' utilizes an upstream 5' splice site in exon 1 resulting in a protein that lacks the transmembrane domain; PSMA-C utilizes the same 5' splice site as PSM' and an alternate upstream 3' splice site resulting in a protein identical to PSM'; PSMA-D utilizes the same 5' splice site as PSM' and PSMA-C, but includes a novel exon located in PSMA's intron 1. The first amino acids of the PSMA-D protein are not present in the other variants, but the rest of the protein is in frame. B) Splice variants from alternative splicing involving exon exclusion: PSMAΔ6 results from exon exclusion of PSMA's exon 6, the protein is severerly truncated compared to PSMA with its final amino acids out of frame; PSMAΔ18 results from exon exclusion of PSMA's exon 18, its protein product is slightly truncated with the final amino acids out of frame.
Grey boxes and circles indicate PSMA's transmembrane domain, dashed lines show alternate splicing patterns, stars indicate location of the start codon, diamonds indicate location of the stop codon, checkered circles indicate an alternate reading frame.

CHAPTER 2

Materials and Methods

Cell Culture and Tissue Samples

Human prostate cancer cell lines, LNCaP-FGC and PC3, were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Facility (Chapel Hill, NC). They were maintained in a humidified cell culture incubator with 5% CO_2 at 37°C. LNCaP cells were cultured in RPMI 1640 (Gibco), supplemented with 1 mM sodium pyruvate and 5% penicillin/ streptomycin solution (Gibco), and 10% fetal bovine serum (FBS) (Gemini). PC3 cells were cultured in DMEM/F12 (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 1mM sodium pyruvate, 1X non essential amino acids, 5% penicillin/streptomycin solution (Gibco), and 10% FBS. For oligonucleotide transfections, 48 hours pre-transfection, cells were plated to approximately 50% confluence in an appropriate dish size for the assay (approximately $2.5X10^6$ cells per 10 cm dish, $5x10^5$ cells/ well - 6 well dish, $2x10^5$ cells/well - 24 well dish, and $5x10^4$ cells/well - 96 well dish). Snap frozen human prostate cancer tissue samples were obtained from UNC's Tissue Procurement Facility (Chapel Hill, NC). Samples were thawed and small sections homogenized in TRI-Reagent (Sigma) for RNA isolation.

SSO Treatment

18-mer 2'-*O*-methyl-oligoribonucleoside-phosphorothioates (OMe SSOs; TriLink BioTechnologies) and 18-mer 2'-*O*-methoxyethoxy- oligoribonucleotide-phosphorothioates (MOE SSOs; ISIS Pharmaceuticals/IDTDNA) were targeted to the 5' splice sites of exon 1 (SSO1OMe & SSO1MOE:

UACCGAAGAGGAAGCCGA), exon 6 (SSO6OMe & SSO6MOE: UAACCUGGUGUGAGAGGG), or exon 18 (SSO18OMe & SSO18MOE: AGGCCUGUCUGGUAACCC). 18-mer SSOs targeted to β-globin (705OMe & 705MOE: CCACUUACCUCAGUUACA), a randomized sequence (RANDOM-OMe: N_{18}), and a 4 nucleotide mismatch to SSO1 (SSOMM-OMe & SSOMM-MOE: UAACGAACAGGCAGGCGA) were used as negative controls. SSOs were delivered to cells (see figures for dosage and length of treatment) with Lipofectamine 2000 (Invitrogen) in the presence of 5% FBS according to manufacturer directions; SSO/Lipofectamine complex was not removed from cells throughout the duration of the transfection procedure.

RNA Isolation

Total RNA from treated and untreated cells was isolated with TRI-reagent (Molecular Research Center, Inc.) or the Gentra Systems Versagene™ RNA cell kit following the supplier protocols. Briefly, for the TRI-reagent protocol, media was removed from cells. Depending on well size, 250-500 μL TRI-reagent was added to cells and incubated at room temperature for 5-10 minutes. 1/10th volume 1-Bromo-3-chloropropane (BCP) was added to each volume of TRI-reagent, vortexed for 30 seconds, incubated on ice for 2 minutes, and centrifuged at 14000 RPM for 15 minutes at 4° Celsius. The top aqueous phase was transferred into 125-250 μL isopropanol with 1-2 μL Glycoblue (Invitrogen), inverted to mix and centrifuged at 14000 RPM for 10 minutes at 4° Celsius.

Supernate was removed and pellets washed with 125-250 μL ethanol. RNA pellets were air dried and resuspended in 25-50 μL autoclaved distilled water.

Reverse Transcription PCR (RT-PCR)

500ng total RNA was used in the RT reaction with MuLV reverse transcriptase (Applied Biosystems) at 42°C for 15 minutes and 95°C for 5 minutes to inactivate MuLV. PCR, in the presence of approximately 0.2 μCi [α-^{32}P] dATP, or 1-2 pM Cy5-dCTP (Amersham Biosciences) and Platinum Taq polymerase (Invitrogen), was: 95°C, 2 minutes followed by 21 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute. The final PCR step was 72°C, 7 minutes. For detection of PSMA/PSM' RT-PCR products, the forward and reverse primers were: AAACACTGCTGTGGTGG, and TAGCTCAACAGAATCCAGGC, respectively, yielding 511 bp PSMA and 245 bp PSM' products. These PCR reactions contained 6X PCR Enhancer with betaine (Epicentre) (Henke, Herdel et al. 1997). For the PSMA/PSMAΔ6 RT-PCR the forward primer was CTATGCACGAACTGAAGA, reverse, GAGCTTCTGTGCATCATAG, yielding 383 bp PSMA and 201 bp PSMAΔ6 product. For the PSMA/PSMAΔ18, the forward (TTCAGTGAGAGACTCCAGGACTTTG) and reverse, (CATTACGATTCTTTCTGAGTGAC) primers produced 358 bp and 261 bp products, respectively. Under the above conditions the change in product levels is linear in response to RNA input and extent of splicing. The PCR products were electrophoresed on a 10% TBE polyacrylamide precast gel in the Novex Mini-

Cell apparatus (Invitrogen). Dried gels were autoradiographed with Kodak BioMax film or scanned using Typhoon 9600 imager (GE Healthcare).

Protein Isolation and Immunoblotting

Treated and control cells were grown in 3 cm (6 well) or 10 cm plates. Media was removed and cells lysed on the plate with 0.5 and 1 ml water, respectively. The suspension was collected, sonicated for 10-30 sec using a Branson sonicator at power setting #2. Volume was increased to 2 ml and protein separated into membrane and cytosolic cell fractions by ultracentrifugation at 100,000g for 30 min using a Beckman Optima™ TL benchtop ultracentrifuge. Pelleted membrane (F2) fraction was resuspended in 250µL 1% SDS buffer. Protein concentration in the fractions was determined using the Bicinchonic Acid Solution protein assay kit with Bovine Serum Albumin as standard (Sigma).

For immunoblot analysis, 2-40µg protein was diluted to 40µl with 1X reducing reagent and 1X LDS sample buffer, incubated at 95℃ for 5 min and electrophoresed on a 4-12% gradient NuPage Bis-Tris precast polyacrylamide gel. The separated proteins were transferred onto Invitrolon PVDF membranes using the XCell II Blot Module. All reagents and instruments for immunoblot analysis were from Invitrogen (Carlsbad, CA).

Proteins were detected with: YPSMA1 mAb, 1:1000 dilution (Yes Pharmaceuticals, Anogen); β-tubulin mAb, 1:3000 (Sigma); anti-P, E, N-Cadherin pAb, 1:1000 (Calbiochem). Horseradish peroxidase conjugated anti-mouse

(Sigma) and anti-rabbit (Calbiochem) secondary antibodies were used where appropriate. The membranes were developed with the ECL Western Blotting Detection reagents (Amersham Biosciences) and exposed to Kodak BioMax film. Immunoblot and RT-PCR images were processed and quantified with the NIH Image J software.

PSMA NAALADase Activity Assay

Membrane and cytoplasmic fractions were obtained as described above except that all procedures were carried out in 1X NAALADase reaction buffer: 50mM Tris pH 7.4, 1mM CoCl2. 25µg protein was incubated with 2 mM NAAG in a volume of 100 µl for 24 h. The enzymatic reaction was stopped with 250 mM sodium phosphate and samples frozen until assayed (Slusher, Robinson et al. 1990; Schulke, Varlamova et al. 2003). Free glutamate was assayed as described using a commercial L-glutamic acid quantitation kit (r-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.

Cellular Malignancy Assays (MTS, ATP, Caspase 3/7)

The CellTiter 96® AQueous One Solution Cell Proliferation assay (MTS), CellTiter-Glo® Luminescent Cell Viability assay (ATP), and Apo-ONE® Homogeneous Caspase-3/7 assay (Caspase 3/7) (all Promega) were performed in 96 well dishes according to manufacturer's directions. Black-well dishes were used for the luminescent and fluorescent assays. Cells were plated 48 hours pre transfection and assayed 48 hours post transfection. Percent readings were

based on the equation [(sample reading – blank reading)/(mock transcription reading – blank reading)] X 100%.

Statistical Analysis

For analysis of splice switching oligonucleotide dose curves, EC_{50} values were calculated using non-linear regression analysis by the GraphPad Prism version 4 software. All error bars indicate standard error of the mean (SEM).

CHAPTER 3

Successful Modification of PSMA Splice Variants Using Splice Switching Oligonucleotides

I. Introduction

Antisense oligonucleotides are now well established tools for gene regulation at the mRNA level. Their sequence specificity, size, and chemical properties allow for efficient targeting of individual gene transcripts. Antisense technology has mostly been used for downregulation of mRNA by recruiting RNase H. When bound to target RNA, antisense oligonucleotides and RNA create an RNA/DNA duplex; RNase H recognizes this duplex as a potential cellular hazard and degrades the duplexed RNA. mRNA transcripts targeted by antisense oligonucleotides are rendered useless since they are cleaved and truncated prior to the translation process. Lesser utilized antisense oligonucleotide applications include blocking the maturation and transport of RNAs from the nucleus to the cytoplasm by targeting 5' cap and 3' polyadenylation sites and inhibiting translation by targeting the mRNA start codon (Crooke 2001).

The primary research in this laboratory and a few others focuses on a novel and potentially therapeutic application of antisense oligonucleotide technology. By targeting specific pre-mRNA splicing elements in the nucleus, antisense oligonucleotides are able to alter splicing patterns. Use of this process promotes desired alternative splicing patterns and can not only downregulate specific targets, but can simultaneously increase levels of desired transcripts. This specific application of antisense oligonucleotides was first applied to the β-globin gene by Dominski and Kole in 1993 (Dominski and Kole 1993). Since then, the splice altering capacity of antisense oligonucleotides has been tested

and confirmed for several targets including bcl-x, CFTR, tau, β-globin, and others (Sierakowska, Sambade et al. 1996; Friedman, Kole et al. 1999; Kalbfuss, Mabon et al. 2001; Mercatante, Sazani et al. 2001). Studies have shown the efficacy of splice-switching oligonucleotides not only in cultured cells, but also *in vivo* (Sazani, Gemignani et al. 2002; McClorey, Fletcher et al. 2005).

The properties of Prostate Specific Membrane Antigen splice variants and its alternative splicing patterns make PSMA an intriguing target for splicing modulation by SSOs. Even though PSMA is a relatively newly characterized gene and protein, several of its spliced products have been identified. Most studies involving PSMA address the full length membrane bound transcript where all 'normal' exons are included and introns excluded. This is the case even though one report maintains that this variant is not the primary transcript of the PSMA gene in some prostate tissues (Su, Huang et al. 1995).

Figure 1.6 illustrates all of the identified alternative splice variants of PSMA. Of these, PSMA is the best understood. PSM' has been at least partially characterized, while the properties of the other alternative splice products at the protein level have not been determined.

In this study, the PSMA, PSM', PSMΔ6, and PSMΔ18 variants are analyzed. PSM' is derived from a splicing pattern that utilizes the 3' splice site of PSMA's exon 2 and an alternate 5' splice site in exon 1. The start codon normally used for PSMA translation is 'spliced out' of PSM' as part of the first intron; an alternate start codon located in exon 2 encodes the initial amino acid for the PSM' protein. PSM' is missing the first 60 amino acids present in PSMA.

Consequently, PSM' does not have a transmembrane sequence and is localized to the cytoplasm instead of the plasma membrane. Su et al. first demonstrated that PSM' splicing predominates over PSMA in normal and benign prostate tissues tested. However, the ratio of PSM'/PSMA splicing reverses in prostate cancer (Su, Huang et al. 1995). A correlation also was drawn between a decrease in PSM'/PSMA ratio and increasing Gleason score and cancer metastasis (Schmittgen, Teske et al. 2003). From these observations, it is clear that upregulation of PSMA and/or downregulation of PSM' is related to malignancy of prostate tissues, but whether it is a causative factor or a consequence of malignancy remains to be determined. Applying splice switching technology to alter the PSM'/PSMA splicing pattern will help to characterize this relationship.

From PSMA sequence data we determined that removal of the sixth or eighteenth exons from the PSMA transcript causes a change in reading frame for the PSMAΔ6 and PSMAΔ18 splice variants compared to PSMA. With PSMAΔ6, the mRNA transcript encodes an early stop codon located upstream of the enzymatic and dimerization domains, while the usage of a novel stop codon in PSMAΔ18 leaves the enzymatic domain intact while eliminating the dimerization domain. Since it has been demonstrated that PSMA protein dimerization is required for its hydrolase and dipeptidase function (Schulke, Varlamova et al. 2003), we hypothesize that splice switching from PSMA to either PSMAΔ6 or PSMAΔ18 will disrupt PSMA function. However, both splice variants should maintain their membrane targeting potential if properly translated.

To induce splice switching from PSMA to the other three variants we utilize oligonucleotides with 2'-*O*-methyl (OMe) or 2'-*O*-methoxyethoxy (MOE) ribose conformations with phosphorothioate linkages (Figure 3.1 illustrates oligonucleotide chemistries). These SSOs were targeted to the 5' splice site of PSMA exons 1, 6, and 18.

Initially, three different OMe oligonucleotide sequences per target were tested in order to increase the chances of finding efficient, effective sequences. For the PSMA to PSM' shift, SSOs were targeted directly to the 5' splice junction of exon 1; the three tested SSOs each included the splice junction with intron and exon sequence, but differed in exact position at the splice junction (Fig 3.2). A more involved approach was taken to find SSO sequences for the PSMA to PSMAΔ6 and PSMAΔ18 shifts. Here, SSOs were targeted to exon splicing enhancer (ESE) sequences at or adjacent to the 5' splice sites. Previous reports had demonstrated that the blocking of ESE sequences with oligonucleotides was capable of inducing splice switching (Errington, Mann et al. 2003; Takeshima, Yagi et al. 2005). ESEfinder, a useful internet-based software, is able to analyze nucleic acid sequences to not only find ESEs, but quantify the strength of ESE sequences based on the recognition sequences of the SF2/ASF, SC35, SRp40, and SRp55 ESEs (Cartegni, Wang et al. 2003). Approximately 500 nucleotide sequences surrounding the 5' splice sites of PSMA's exons 6 and 18 were analyzed by ESEfinder. The graphic output of these analyses is shown in Figures 3.3 and 3.4. Also detailed in the figures are the locations and sequences of SSOs selected for testing. Of the nine original SSO sequences, the three that

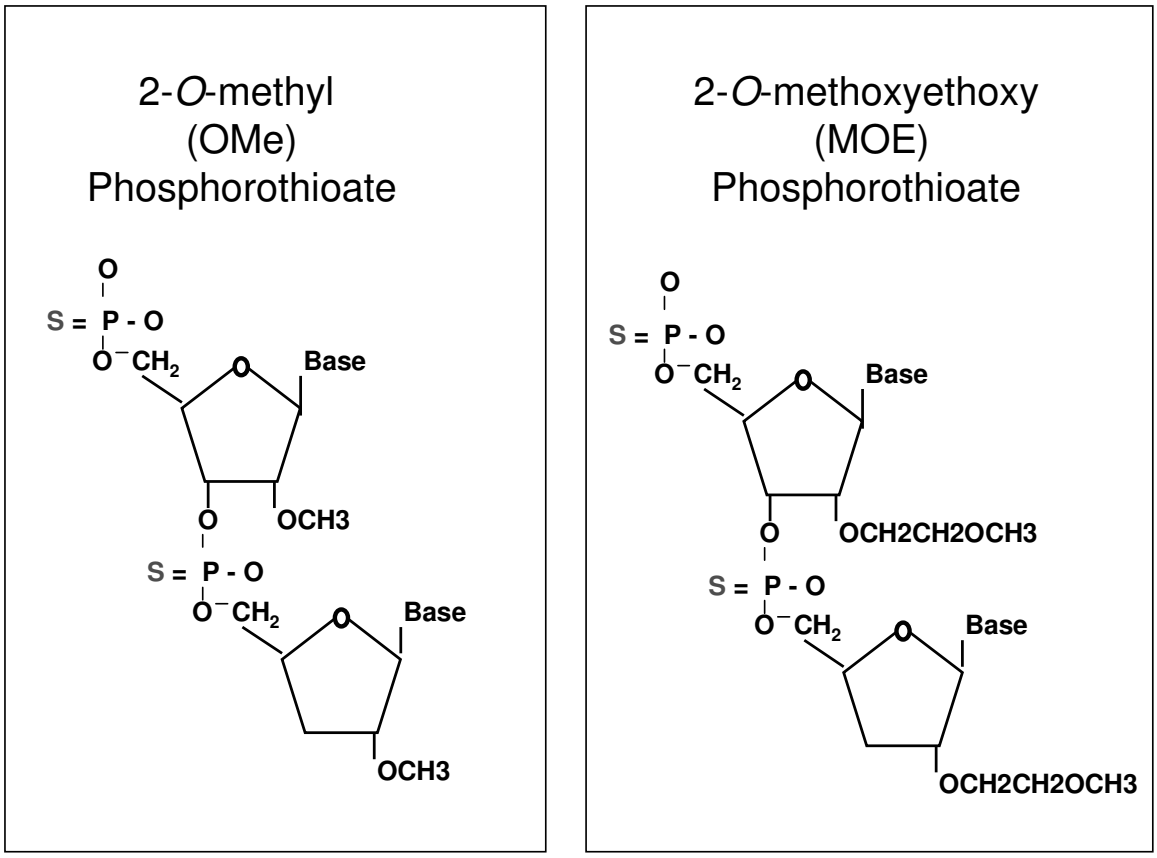
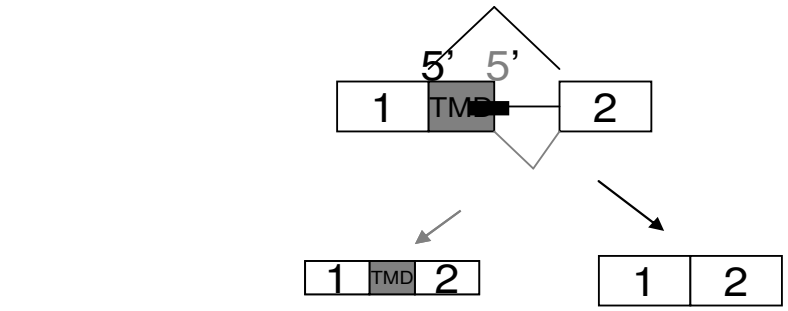
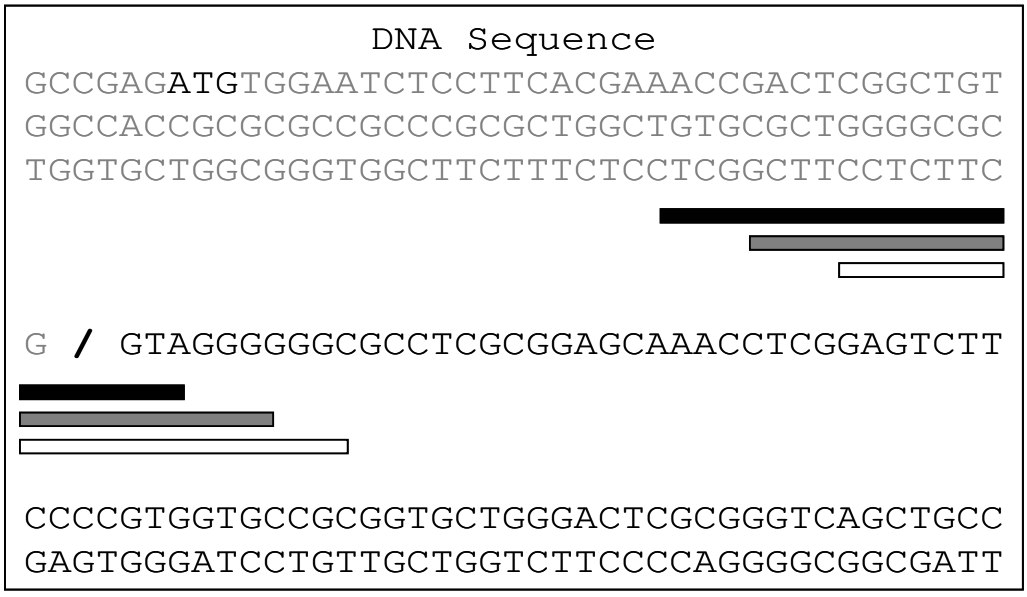


Figure 3.1. Chemical structures of 2'-*O*-methyl and 2'-*O*-methoxyethoxy phosphorothioate oligonucleotides.





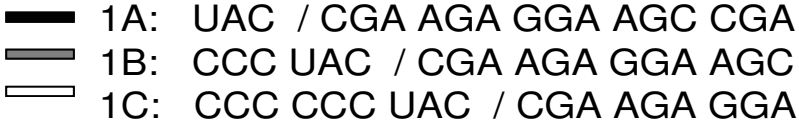


Figure 3.2. SSO1 candidate sequence selection.

18-mer oligonucleotide sequences were selected by their position at the 5' splice junction of PSMA's exon 1. Candidate SSO1 sequences (1A, !B, and 1C) included the 5' splice site junction with intronic and exonic sequence.

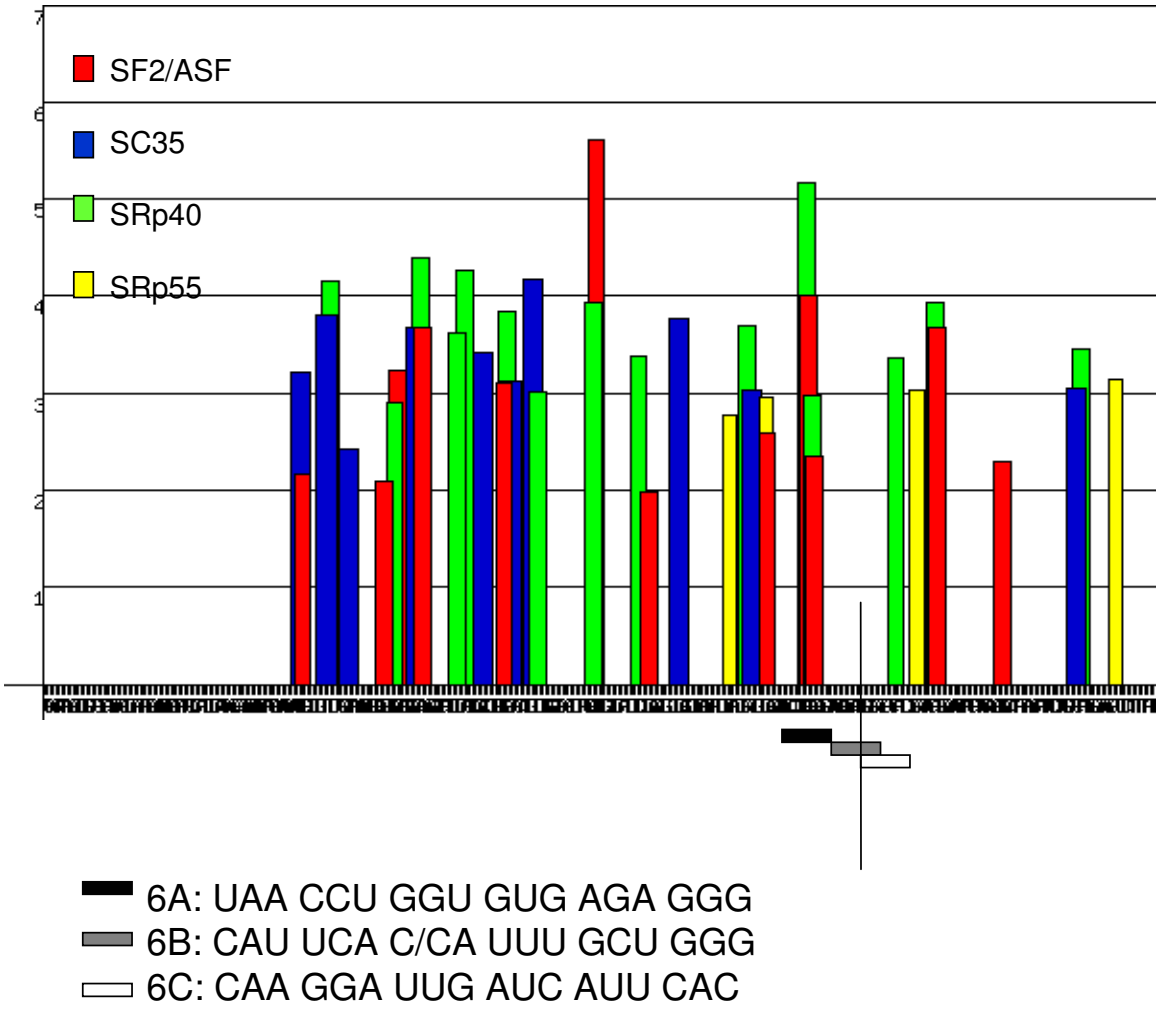


Figure 3.3. SSO6 candidate sequence selection.

Graphic ouput of ESEfinder software analysis of PSMA pre-mRNA sequence from exon 6 and intron 6. Three 18-mer oligonucleotide sequences were selected for their localization near the 5' splice junction and ESE recognition sequences. The X-axis shows the pre-mRNA nucleotide sequence. Colored bars indicate areas similar to the SF2/ASF, SC35, SRp40, and SRp55 ESE consensus sequences; height of bars is directly proportional to sequence similarity of ESE recognition sequences. The 6A oligonucleotide is positioned in the 6th exon at an ESE hotspot, 6B is positioned at the 5' splice junction, and 6C is positioned in the 6th intron.

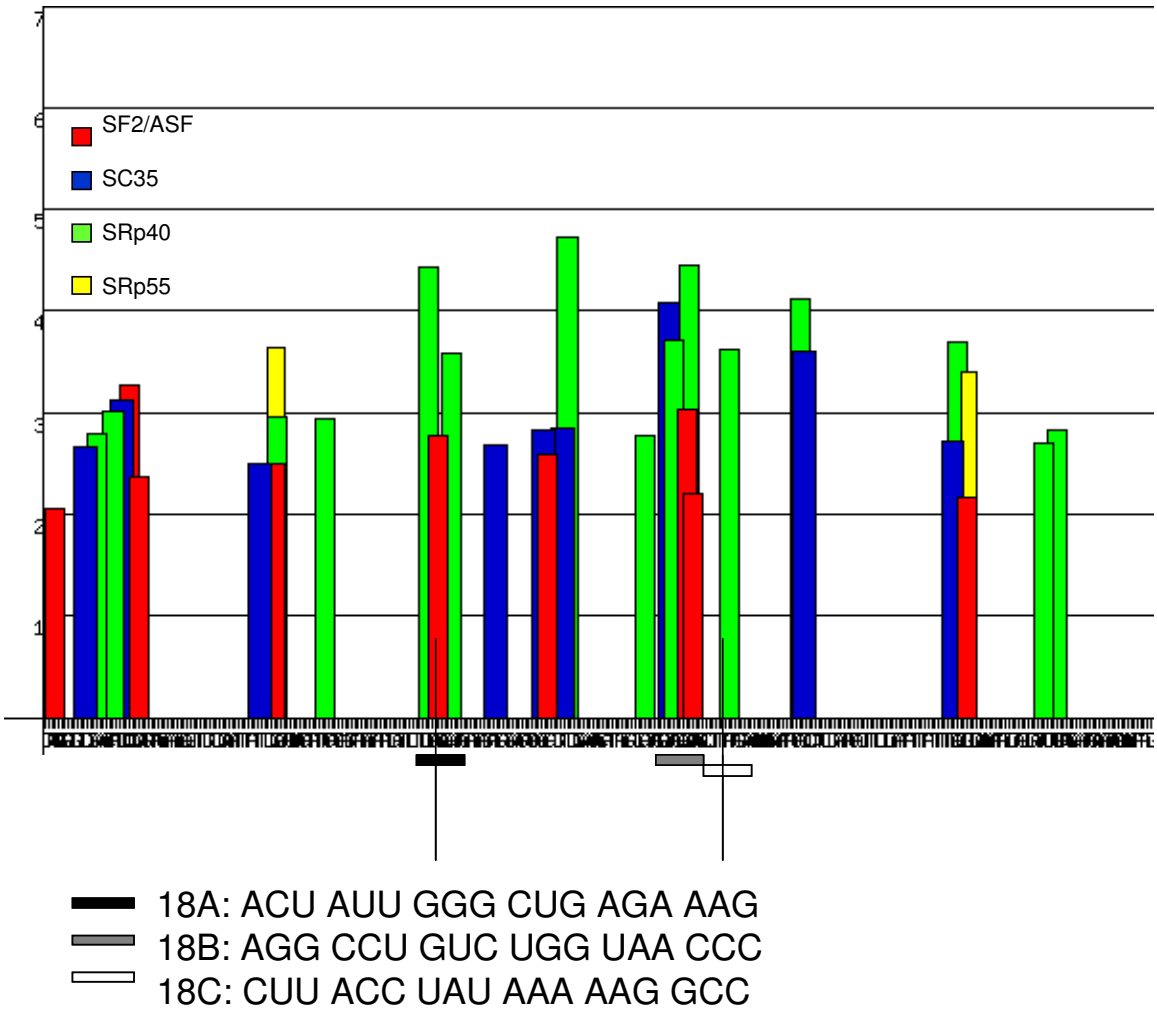


Figure 3.4. SSO18 candidate sequence selection.

Graphic ouput of ESEfinder software analysis of PSMA pre-mRNA sequence from exon 18 and introns 17 and 18. Three 18-mer oligonucleotide sequences were selected for their localization near exon 18's 3' and 5' splice junction and ESE recognition sequences. The X-axis shows the pre-mRNA nucleotide sequence. Colored bars indicate areas similar to the SF2/ASF, SC35, SRp40, and SRp55 ESE consensus sequences; height of bars is directly proportional to sequence similarity of ESE recognition sequences. The 18A oligonucleotide is positioned at exon 18's 3' splice site, 18B is positioned in the 18 exon at an ESE hotspot, and 1C is positioned at exon 18's 5' splice site.

induced the best splice switching (one per target) were selected for use in this study (Fig. 3.5).

II. Results

A. PSMA to PSM', PSMAΔ6, and PSMAΔ18 splice switching

Levels of PSMA splice variants were determined by RT-PCR amplification using target specific primers that flank alternatively spliced regions. Prostate tissue samples obtained from the University of North Carolina's Tissue Procurement Facility were tested to determine *in vivo* levels of the PSMA, PSM', PSMAΔ6, and PSMAΔ18 splice variants prior to SSO analysis (Fig. 3.6). This step was important since the PSMAΔ6 and PSMAΔ18 mRNA levels had not been previously established. Native levels of the PSMA and PSM' splice variants in cell lines including prostate cancer PC3, CWR-R1, LNCaP and cervical cancer HeLa were also analyzed (Fig. 3.7). Consistent with prior reports, LNCaP cells expressed a large amount of PSMA and PSM' mRNA and the PSMA/PSM' ratio was similar to that obtained from an RNase protection assay performed by Su et al. (Su, Huang et al. 1995). Antisense 2'-*O*-methyl-phosphorothioate (OMe) and 2'-*O*-methoxyethoxy-phosphorothioate (MOE) modified 18-mer SSOs were targeted to specific PSMA pre-mRNA sequences. These SSO chemistries are able to hybridize strongly to target RNA, are resistant to cellular nucleases and do not induce RNase H degradation of SSO-bound RNA (Manoharan 1999). To induce splice switching from the full length PSMA transcript to PSM', PSMAΔ6and PSMAΔ18 splice variants, LNCaP cells were treated with SSO1, SSO6, or SSO18, respectively.

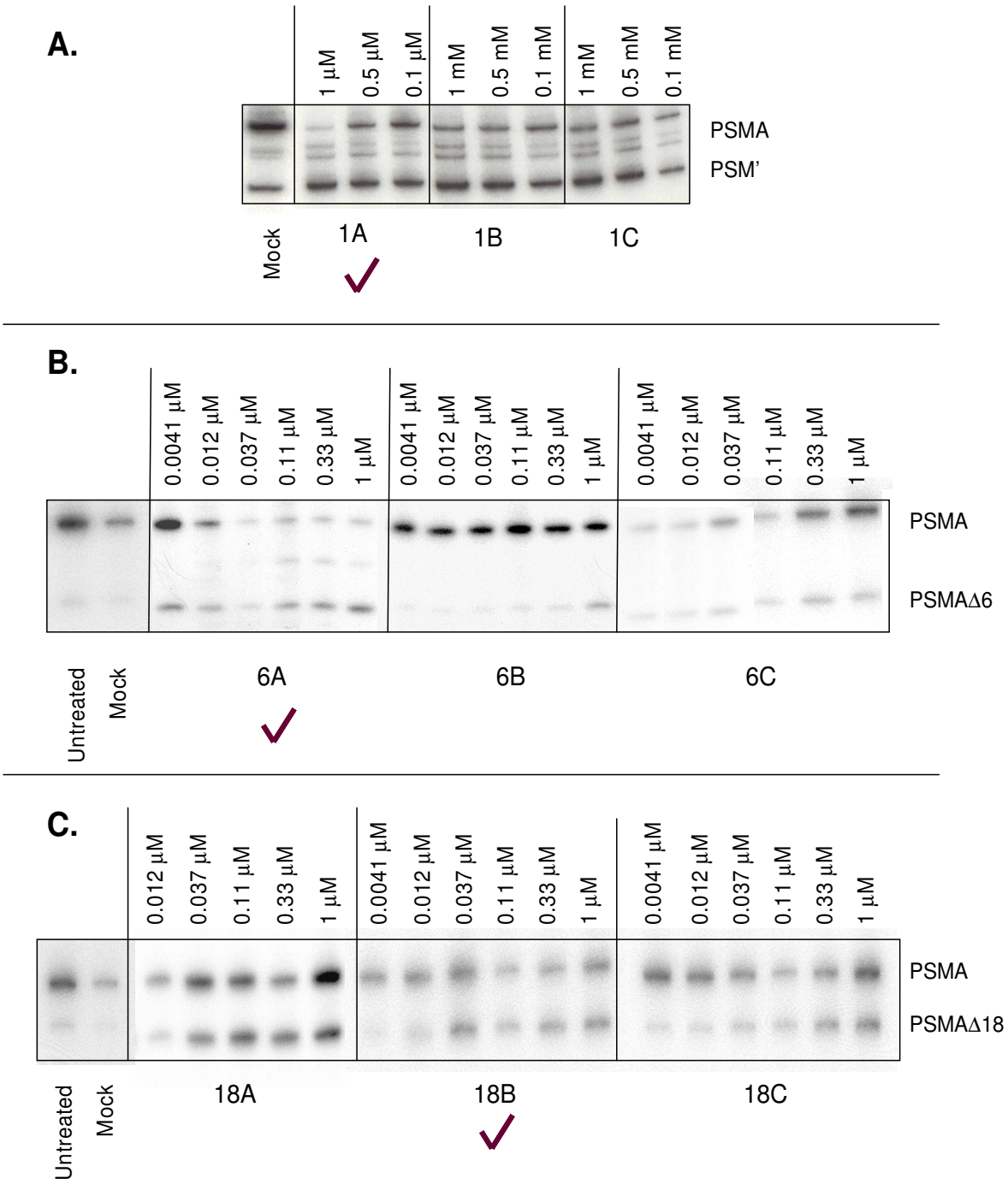


Figure 3.5. PSMA SSO 1, 6, and 18 selection.

Figure 3.5. PSMA SSO 1, 6, and 18 selection.

Candidate OMe SSOs were tested in LNCaP cells for their ability to switch PSMA splicing patterns after 48 hour transfections. RT-PCR analysis was of total cellular RNA using target specific primers. A) SSOs 1A, 1B, and 1C were tested at 1μM, 0.5 μM, and 0.1 μM concentrations. B) SSOs 6A, 6B, and 6C were tested using a 1:3 dose curve from 1 μM to 0.0041 μM concentrations. C) SSOs 18A, 18B, and 18C were tested using a 1:3 dose curve from 1 mM to 0.0041 mM concentrations. SSOs 1A, 6A, and 18B were determined to be the most efficient at splice switching and were chosen to be SSOs 1, 6, and 18.

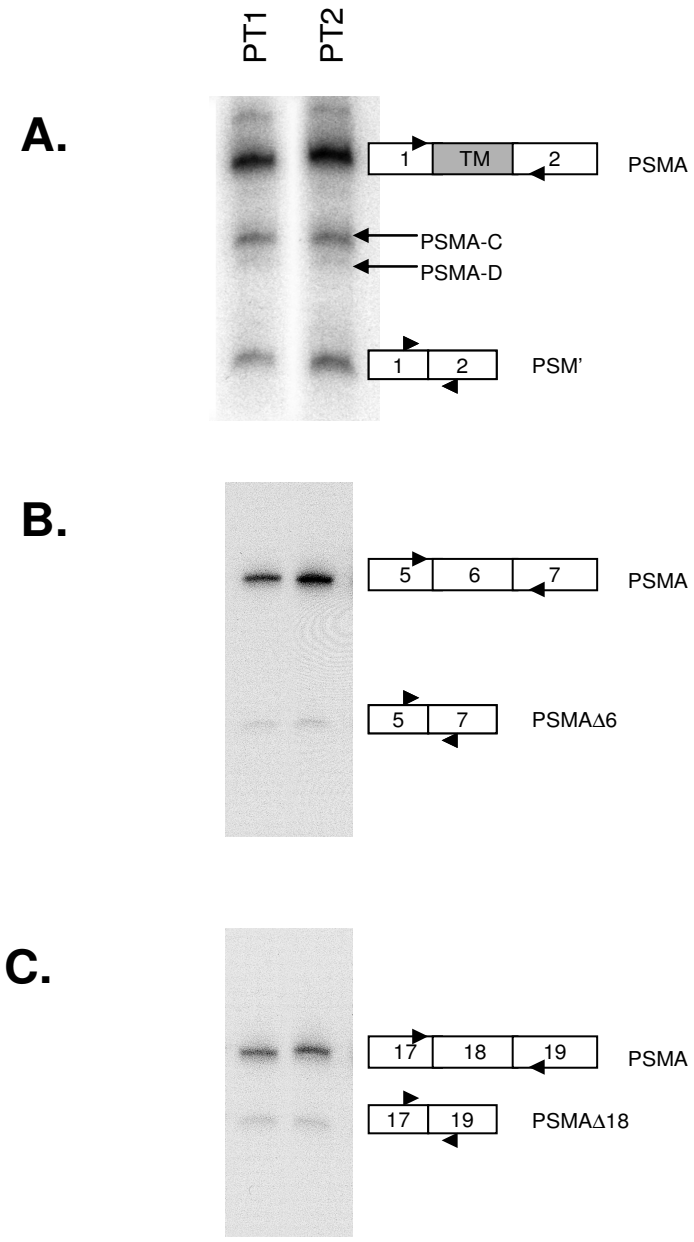


Figure 3.6. PSMA splice variant levels in prostate tumor samples.

RT-PCR analysis using target specific primers for PSMA splice variants. A) Splice variants produced from alternative splicing involving PSMA's intron 1 detected using an exon1 forward primer and exon 2 reverse primer: PSMA, PSM', PSMA-C, and PSMA-D. B) PSMA and PSMAΔ6 variants detected using primers in exons 5 and 7. C) PSMA and PSMAΔ18 variants detected using primers in exons 17 and 19. Two prostate tumor samples were obtained from the Tissue Procurement Facility of the University of North Carolina.

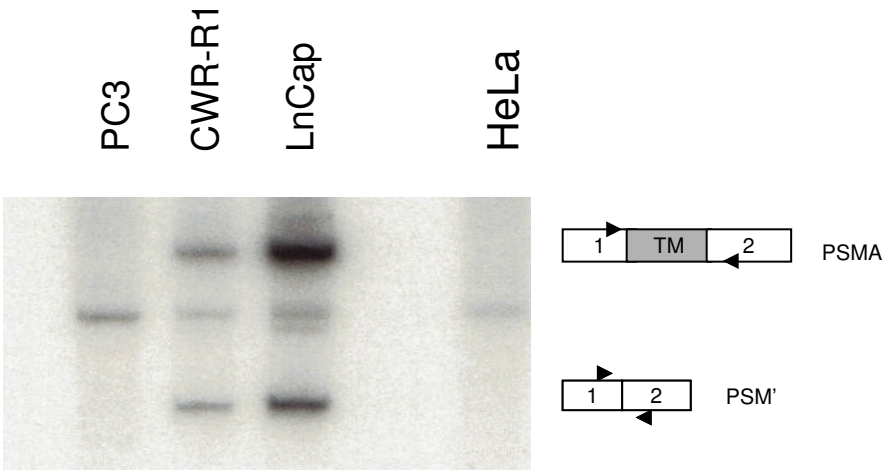


Figure 3.7. PSMA and PSM' splice variant levels in the PC3, CWR-R1, LNCaP, and HeLa cell lines.

RT-PCR analysis using target specific primers for PSMA and PSM'. PSMA is not detected in the PC3 prostate cancer cell line or the HeLa cervical cancer cell line. LNCaP cells express high levels of the two PSMA splice variants.

Without SSO treatment, the endogenous PSM' mRNA amount was approximately 22% to 28% of total, similar to a previous report (Su, Huang et al. 1995). The initial PSMAΔ6 percentage was 4% to 6%, indicating that in LNCaP cells PSMAΔ6 is a very minor PSMA splice variant. The PSMAΔ18 splice percentage of total was between 20% and 23%, i.e. at levels similar to the PSM'. RT-PCR analysis 48 hours post SSO transfection showed the desired, dose dependent decrease in PSMA mRNA and increase in PSM', PSMAΔ6, and PSMAΔ18 splice variants (Figs. 3.8, 3.9, 3.10, 3.12, 3.13, 3.14). The effects were sequence specific since there was no change in the levels of the splice variants in mock treated samples or in 1 μM 705 and negative control oligonucleotide (random/mismatch) treated samples. With SSO1, both the OMe and MOE chemistries induced similar amounts of splice switching in the dose curve analysis with the 0.3 μM dose being the only exception (Figs. 3.8 and 3.12). SSO1 was able to increase PSM' transcript levels to 80% and 73% of total at the best dose for OMe and MOE chemistries, respectively. The MOE SSOs 6 and 18 were not as efficient as their equivalent OMe chemistry; they were able to increase PSMAΔ6 and PSMAΔ18 transcript levels to only about 75% and 85% of that accomplished by OMe SSOs 6 and 18. However they were effective enough to increase PSMAΔ6 and PSMAΔ18 levels to 60% and 62% of total, respectively (Figs 3.13, 3.14). The fact that for SSOs 1 and 6 with OMe chemistry, the splice switching activity diminished between 0.3 and 1μM OMe SSO is most likely attributed to surpassing the optimum charge ratio between the negatively charged oligonucleotides and positively charged cationic lipid used for cellular

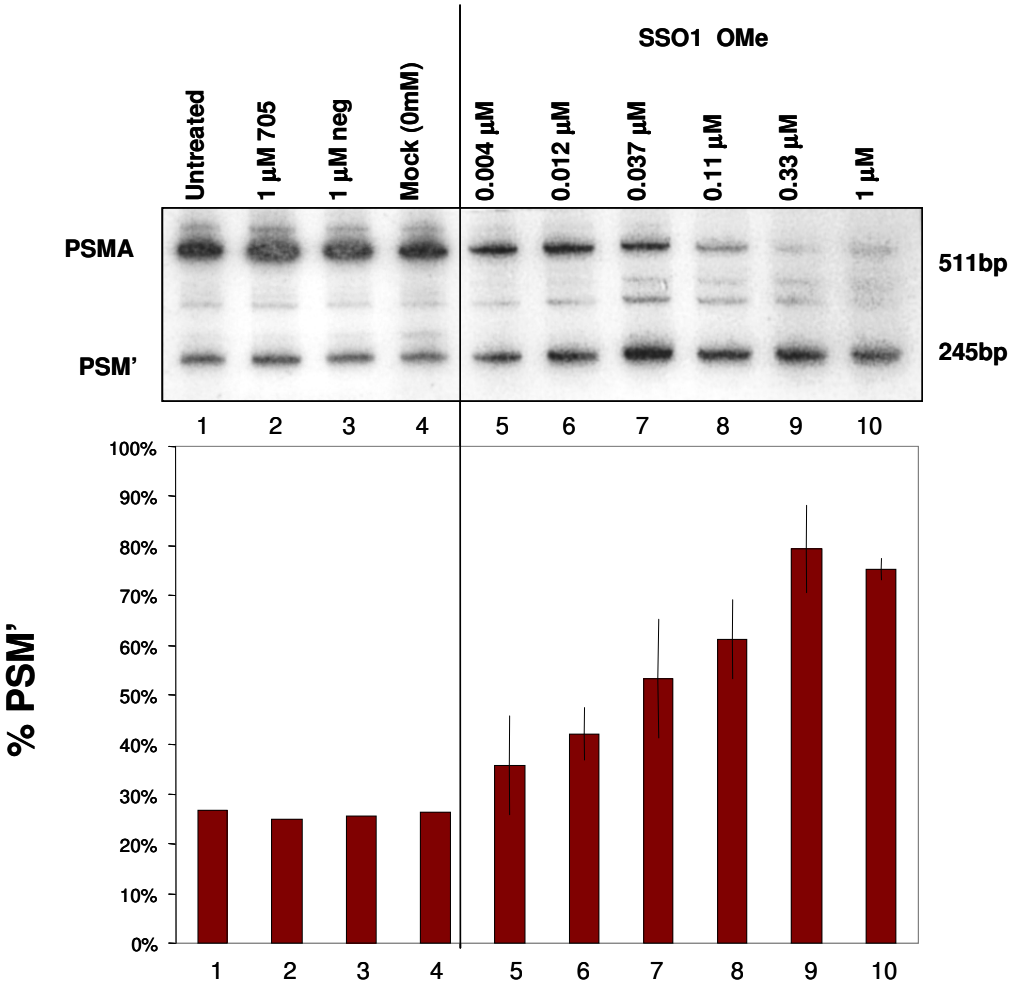


Figure 3.8. Dose curve of splice switching by PSMA SSO1 OMe.

Upper panel: RT-PCR analysis of total cellular RNA using target specific primers after 48 hour SSO1 OMe transfection.
Lower panel: Quantitated results from RT-PCR analysis: Percent PSM' mRNA of total. The results are from three independent SSO treatments, error bars indicate SEM.
Lanes 1: Untreated control LNCaP mRNA, *Lanes 2 & 3:* Negative control SSO1 OMe at 1µM concentration, *Lanes 4-10:* LNCaP cells transfected with SSO1 OMe at 0µM (Mock), 0.004µM, 0.01µM, 0.04µM, 0.1µM, 0.3µM and 1µM.

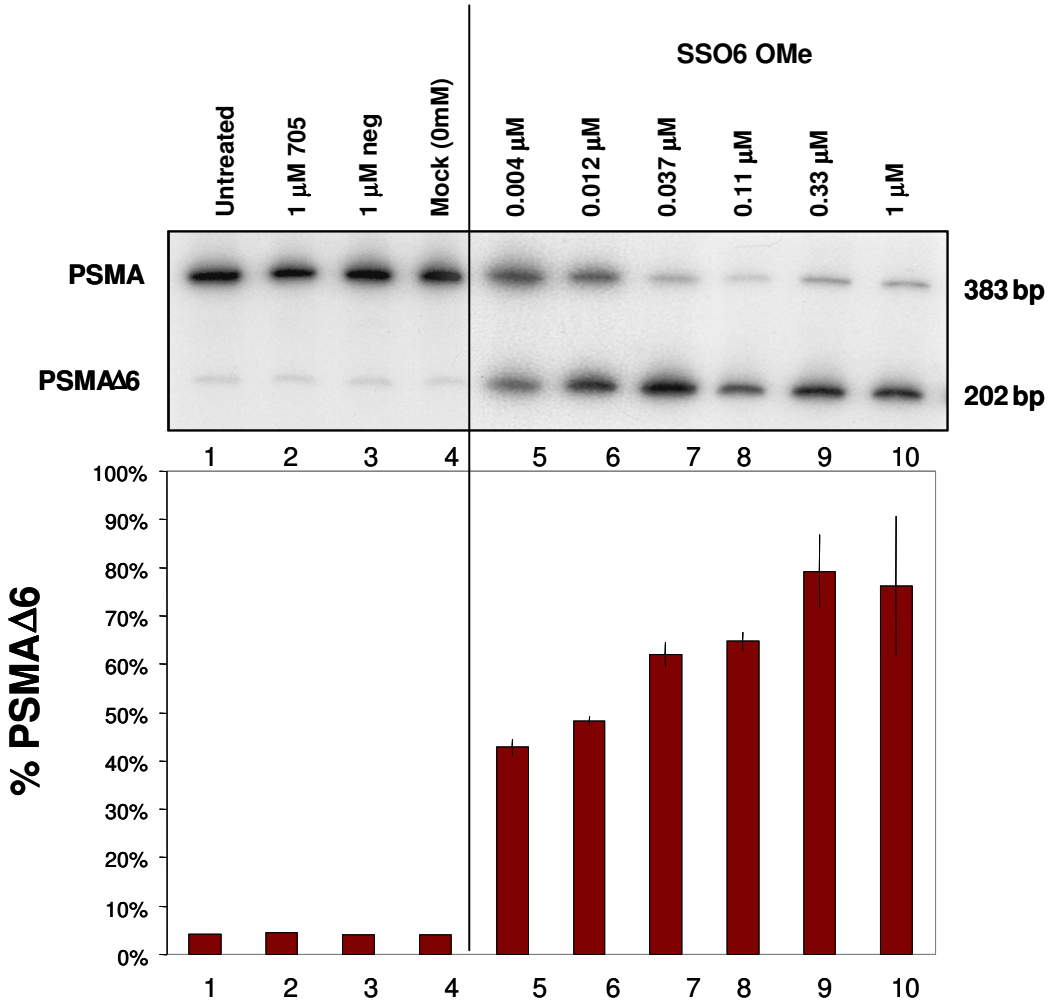


Figure 3.9. Dose curve of splice switching by PSMA SSO6 OMe.

Upper panel: RT-PCR analysis of total cellular RNA using target specific primers after 48 hour SSO6 OMe transfection.
Lower panel: Quantitated results from RT-PCR analysis: Percent PSMAΔ6 mRNA of total. The results are from three independent SSO treatments, error bars indicate SEM.
Lanes 1: Untreated control LNCaP mRNA, *Lanes 2 & 3:* Negative control SSO6 OMe at 1µM concentration, *Lanes 4-10:* LNCaP cells transfected with SSO6 OMe at 0µM (Mock), 0.004µM, 0.01µM, 0.04µM, 0.1µM, 0.3µM and 1µM.

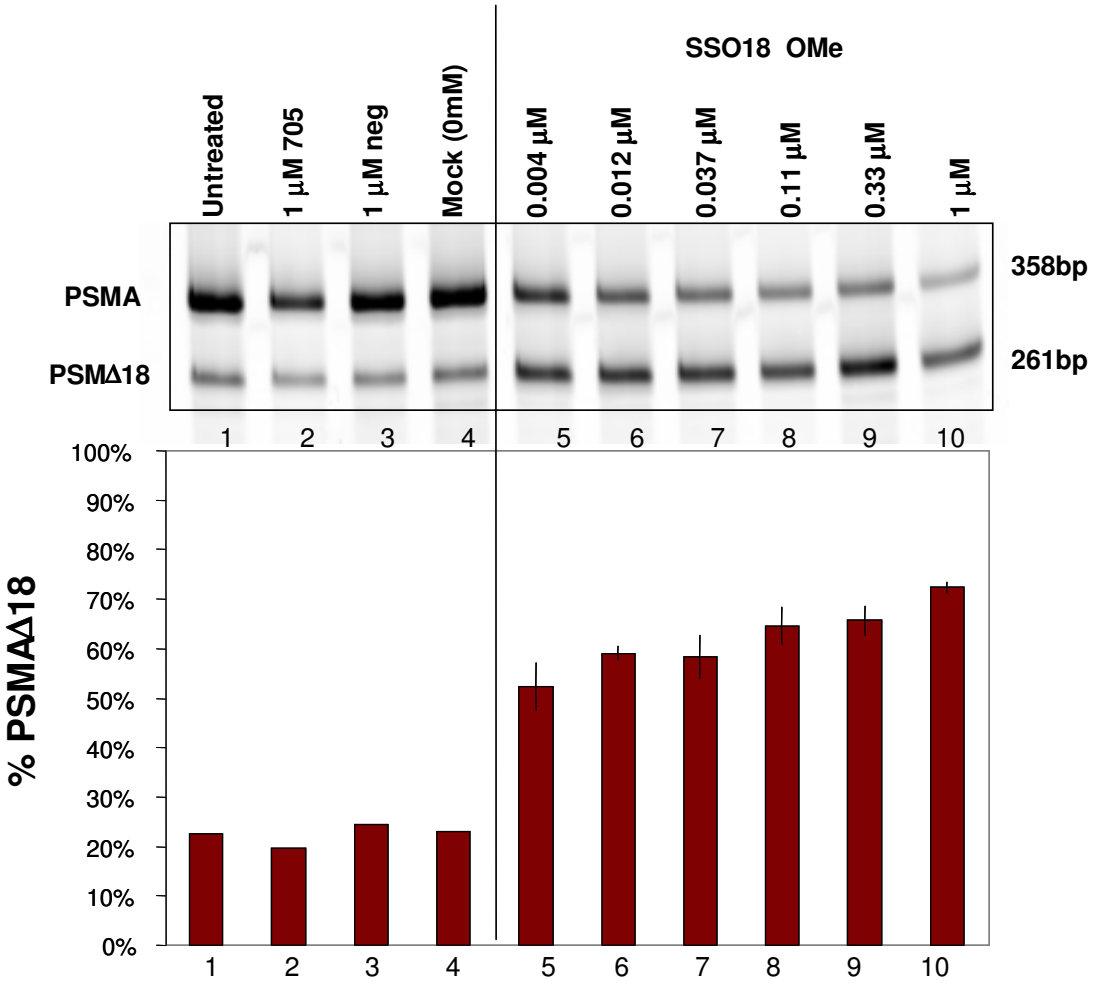


Figure 3.10. Dose curve of splice switching by PSMA SSO18 OMe.

Upper panel: RT-PCR analysis of total cellular RNA using target specific primers after 48 hour SSO18 OMe transfection.
Lower panel: Quantitated results from RT-PCR analysis: Percent PSMAΔ18 mRNA of total. The results are from three independent SSO treatments, error bars indicate SEM.
Lanes 1: Untreated control LNCaP mRNA, *Lanes 2 & 3:* Negative control SSO18 OMe at 1µM concentration, *Lanes 4-10:* LNCaP cells transfected with SSO18 OMe at 0µM (Mock), 0.004µM, 0.01µM, 0.04µM, 0.1µM, 0.3µM and 1µM.

delivery; this decrease in SSO activity at high concentration was not detected for the MOE chemistry.

The data from several independent RT-PCRs used to calculate the EC_{50} values for each SSOs indicate that for the OMe chemistry, SSO18 was the most effective at splice shifting with an EC_{50} of 1.9 nM; SSOs 1 and 6 gave EC_{50} values of 5.6 nM, and 3.5 nM, respectively. Time course experiments using 0.3 μM of each OMe SSO confirmed a persistent modulation of PSMA pre-mRNA splicing up to 96 hours (Fig. 3.11). The effects increased until 48 hours, and then subsequently decreased, presumably due to SSO decay or its dilution in dividing LNCaP cells. With the MOE chemistry, SSO1 had the lowest EC_{50} value of 2.1 nM while the EC_{50} for SSO6 was 18.8 and SSO18 was 6.25.

B. PSMA and PSM' protein expression in OMe SSO treated cells

The consequences of PSMA pre-mRNA splicing modulation were analyzed at the protein level by immunoblot analysis of cytoplasmic and membrane fractions of OMe SSO treated LNCaP cells. The YPSMA1 mAb used in these experiments detects both PSMA and PSM' because its epitope, near the C-terminus, at amino acids 716-723 of PSMA (Zhu et al. 1999), is retained in both proteins. However, YPSMA1 cannot detect the PSMAΔ6 or PSMAΔ18 proteins since both their mRNA transcripts contain stop codons upstream of the terminal amino acid sequence.

PSMA is a type II transmembrane protein and therefore the PSM' splice variant, lacking a transmembrane domain, is expected to be retained in the cytoplasm. Indeed, a shift in PSMA pre-mRNA splicing induced by OMe SSO1

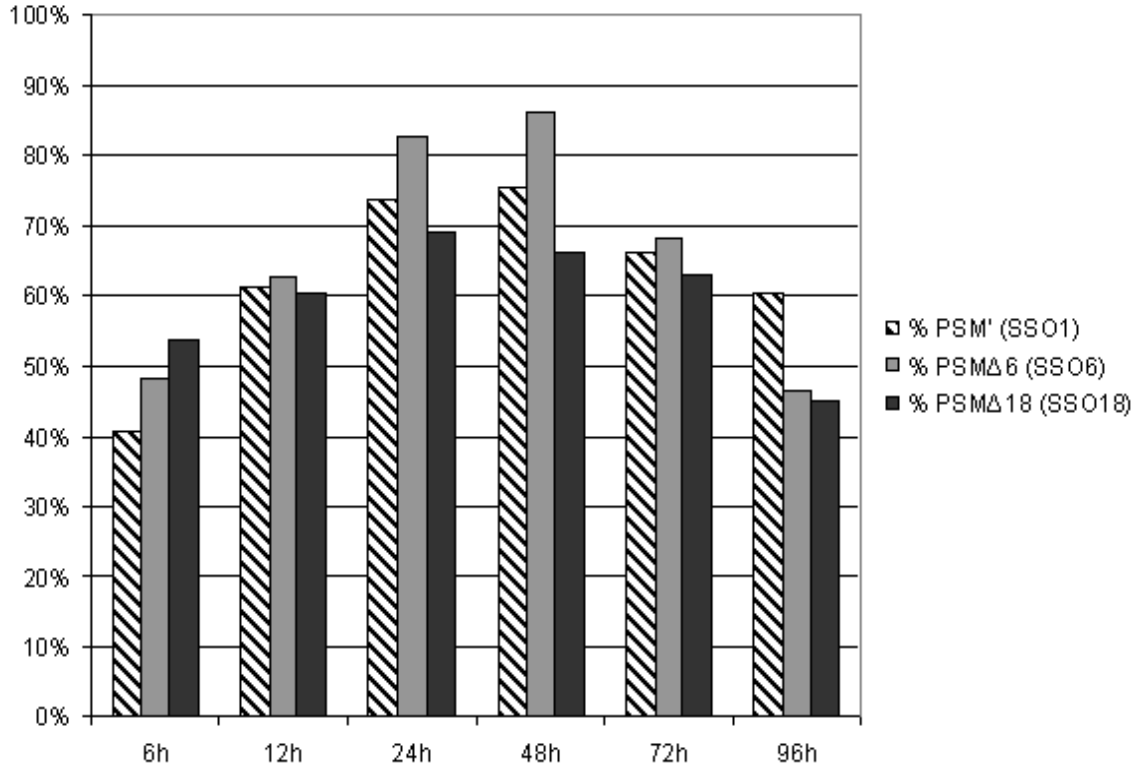


Figure 3.11. Time course of splice switching by PSMA OMe SSOs.

LNCaP cells were transfected with either SSO1, SSO6, or SSO18 at a concentration of 0.3µM. RNA was harvested at 6, 12, 24, 48, 72 and 96 hours post transfection. RT-PCR was performed using target specific primers and quantitated to determine splice variant percentage.

increased the level of PSM' in the cytoplasmic fraction by 133% over mock (Fig. 3.15, lane 3). Although, at the mRNA level, there was a clear decrease of PSMA mRNA post SSO1 treatment, that effect was less evident with PSMA protein in the membrane fraction (Fig 3.15, lane 7), presumably reflecting the long half-life of membrane PSMA protein. The effect of LNCaP cell treatment with OMe SSO6 as detected by immunoblot, was a 68% to 79% decrease of PSM' and PSMA proteins in the cytoplasm and membrane fractions (Fig. 3.15, lanes 4, 8). The decrease of PSMA reflects, as noted above, a more effective shift in PSMA pre-mRNA splicing by OMe SSO6 than OMe SSO1. The PSMAΔ6 splice variant protein was not detected because the truncated PSMAΔ6 protein lacks the YPSMA1 mAb epitope. Note that PSMAΔ6 protein is not likely to be functional and therefore the effect of SSO6 is presumably limited to elimination of PSMA and PSM' (see the discussion section of this chapter). Similar results were seen after OMe SSO18 treatment though the decline in PSMA product in the membrane fraction was less pronounced compared to OMe SSO6 (Fig 3.15, lanes 5, 9). Again, the YPSMA1 mAb did not detect the generated PSMAΔ18 variant due to alteration of the C-terminal amino acid sequence. Nevertheless, the downregulation of PSM' and PSMA after OMe SSO6 and SSO18 treatment verify splice switching activity of both oligonucleotides at the protein level.

C. Function of PSMA and PSM' in OMe SSO treated cells

Cytoplasmic and membrane fractions of LNCaP cells, treated with OMe SSO1, SSO6 or SSO18 at a concentration of 0.3 µM for 48 hours, were assayed for PSMA protein dipeptidase activity. *N*-acetyl-aspartyl-glutamate (NAAG) was

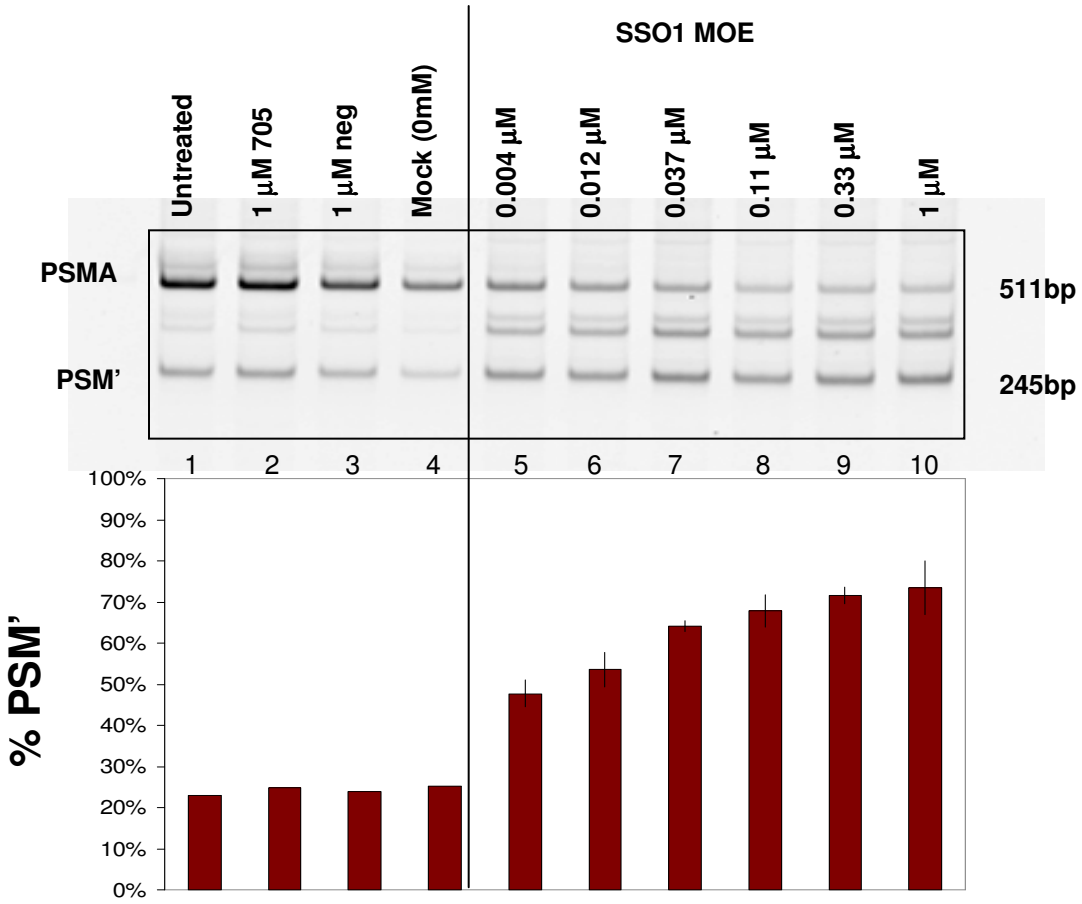


Figure 3.12. Dose curve of splice switching by PSMA SSO1 MOE.

Upper panel: RT-PCR analysis of total cellular RNA using target specific primers after 48 hour SSO1 MOE transfection.
Lower panel: Quantitated results from RT-PCR analysis: Percent PSM' mRNA of total. The results are from three independent SSO treatments, error bars indicate SEM.
Lanes 1: Untreated control LNCaP mRNA, *Lanes 2 & 3:* Negative control SSO1 MOE at 1µM concentration, *Lanes 4-10:* LNCaP cells transfected with SSO1 MOE at 0µM (Mock), 0.004µM, 0.01µM, 0.04µM, 0.1µM, 0.3µM and 1µM.

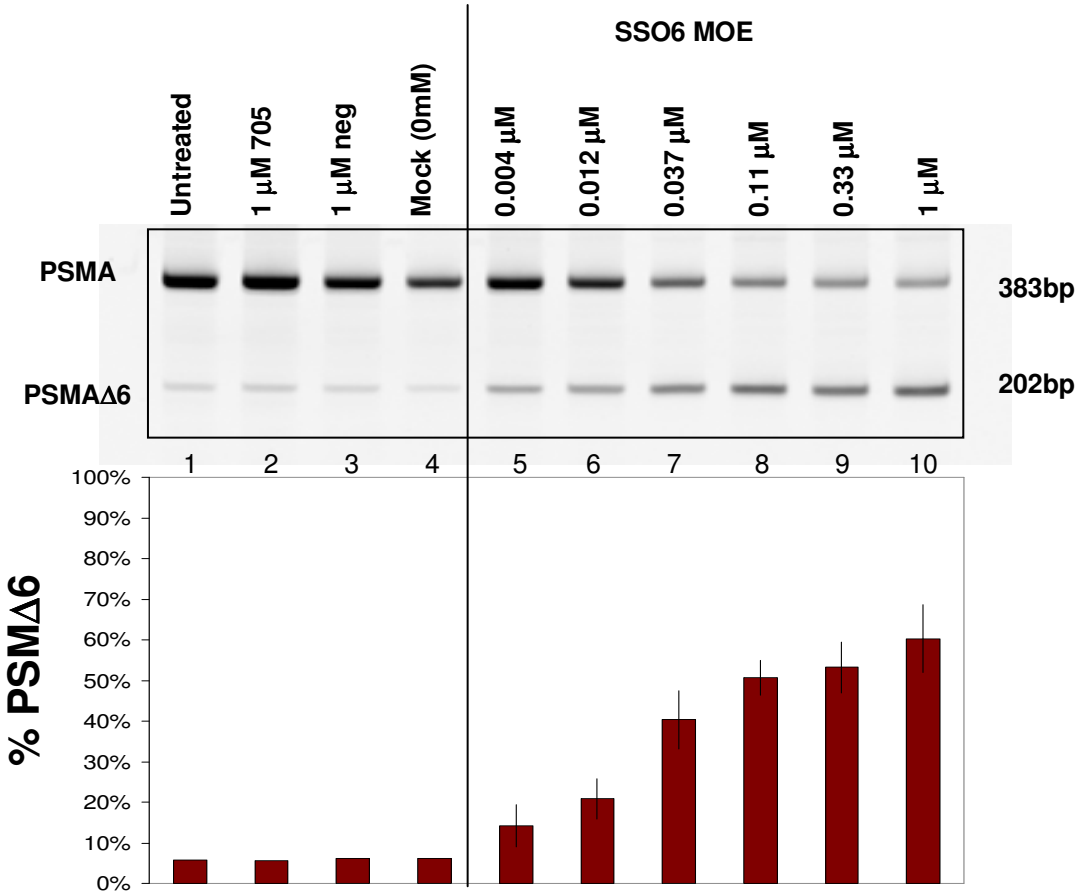


Figure 3.13. Dose curve of splice switching by PSMA SSO6 MOE.

Upper panel: RT-PCR analysis of total cellular RNA using target specific primers after 48 hour SSO6 MOE transfection.
Lower panel: Quantitated results from RT-PCR analysis: Percent PSMAΔ6 mRNA of total. The results are from three independent SSO treatments, error bars indicate SEM.
Lanes 1: Untreated control LNCaP mRNA, *Lanes 2 & 3:* Negative control SSO6 MOE at 1µM concentration, *Lanes 4-10:* LNCaP cells transfected with SSO6 MOE at 0µM (Mock), 0.004µM, 0.01µM, 0.04µM, 0.1µM, 0.3µM and 1µM.

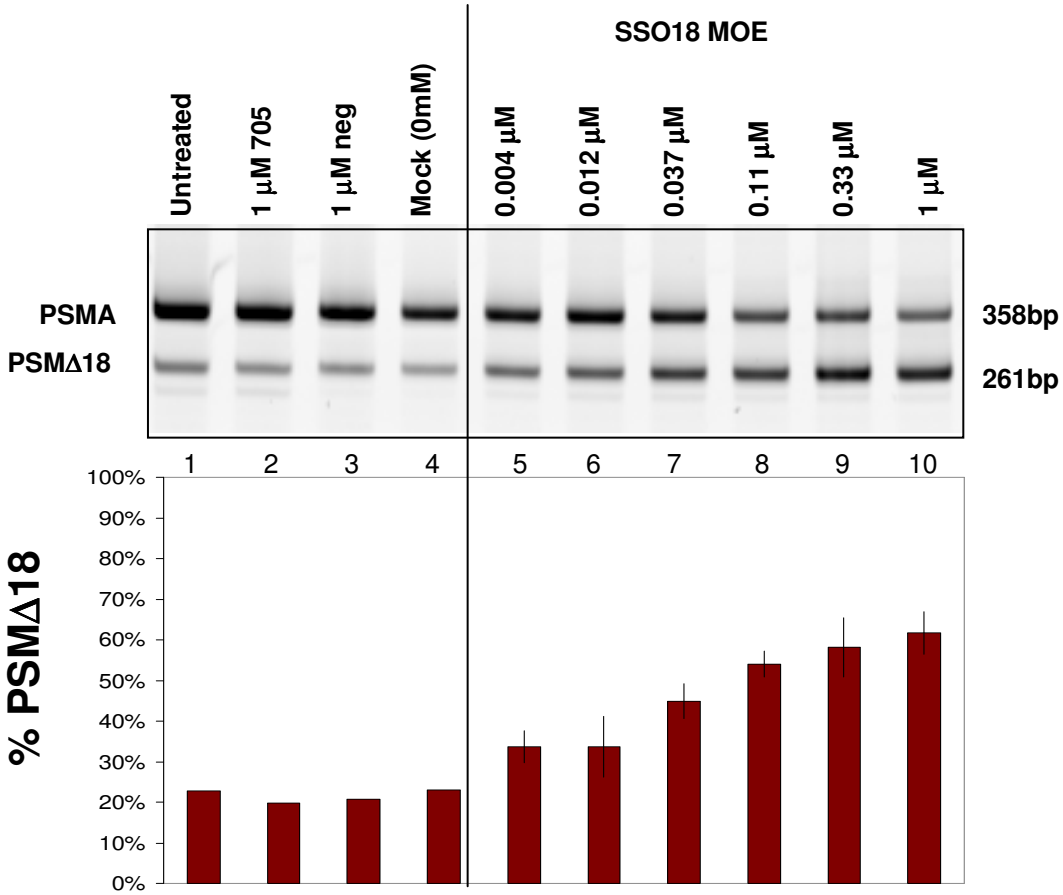


Figure 3.14. Dose curve of splice switching by PSMA SSO18 MOE.

Upper panel: RT-PCR analysis of total cellular RNA using target specific primers after 48 hour SSO18 MOE transfection.
Lower panel: Quantitated results from RT-PCR analysis: Percent PSMAΔ18 mRNA of total. The results are from three independent SSO treatments, error bars indicate SEM.
Lanes 1: Untreated control LNCaP mRNA, *Lanes 2 & 3:* Negative control SSO18 MOE at 1µM concentration, *Lanes 4-10:* LNCaP cells transfected with SSO18 MOE at 0µM (Mock), 0.004µM, 0.01µM, 0.04µM, 0.1µM, 0.3µM and 1µM.

used as a substrate and the released free glutamate was measured colorimetrically as described in the Materials and Methods chapter. OMe SSO1 treatment, consistent with the generation of functional PSM' splice variant, resulted in a 30% increase of cytoplasmic activity and a 10% decrease in membrane PSMA activity (the latter result is within statistical error, Fig. 3.16). After OMe SSO6 and OMe SSO18 treatment, both cytoplasmic and membrane fractions showed significantly decreased levels of PSMA function (Fig. 3.16). Also in line with the results seen at the protein level, the decline in PSMA activity after OMe SSO18 treatment was not as pronounced as that seen with OMe SSO6.

III. Discussion

We have found that in LNCaP cells, SSO-induced shifts in splicing drove expression of PSMA mRNA and protein from the malignancy-associated membrane bound variant to other lesser expressed variants, including cytoplasmic PSM' and truncated, presumably non-functional PSMAΔ6 and PSMAΔ18. This change was also reflected in the shift in NAALADase activity of PSMA. The EC50 of SSOs 1, 6 and 18 are listed in table 3.1; the splice switching efficiency of SSOs may be improved if oligonucleotides with higher Tm values, for example, with PNA and LNA backbones (Sazani, Gemignani et al. 2002; Sazani, Dillman and Kole, unpublished) are used.

The increase in PSM' NAALADase activity in the cytoplasmic fraction of OMe SSO1 treated cells indicates that SSO induced PSM' retained its enzymatic function. In view of the recent reports that dimerization of PSMA or of its

	Sequence	GC Content	Selection Method	Chemistry	EC50 value	Highest Shift Percentage	Best Shift Concentration
SSO 1	UACCGAAGA GGAAGCCGA	55.6%	5'Splice Site	OMe, P=S	5.6nM	80% PSM'	0.3 µM
				MOE, P=S	2.1nM	73% PSM'	1 µM
SSO6	UAACCUGGU GUGAGAGGG	55.6%	ESEfinder	OMe, P=S	3.5nM	79% PSMΔ6	0.3 µM
				MOE, P=S	18.5nM	60% PSMΔ6	1 µM
SSO18	AGGCCUGUC UGGUAACCC	61.1%	ESEfinder	OMe, P=S	1.9nM	73% PSMΔ18	1 µM
				MOE, P=S	6.25nM	62% PSMΔ18	1 µM

Table 3.1 Properties of PSMA SSOs

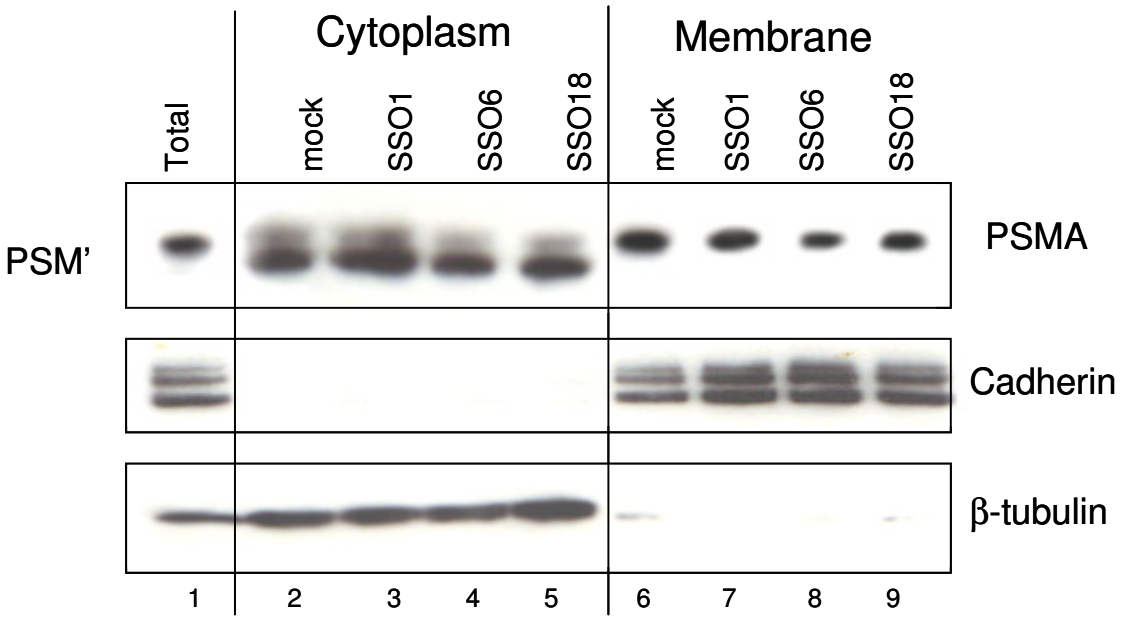


Figure 3.15. Western blot analysis of PSMA protein from OMe SSO transfected cells.

Total cellular protein was isolated from LNCaP cells 48 hours post transfection with 0.3 µM OMe SSOs 1, 6, or 18. Cytoplasmic and membrane fractions were separated by ultracentrifugation (see Materials and Methods).
Upper panel: PSMA protein (40 µg for cytoplasmic fraction, 2 µg for membrane fraction and total protein),
Middle panel: 10 µg Cadherin protein for membrane fraction identification,
Lower panel: 2 µg β-tubulin protein for cytoplasmic fraction identification.
Lane 1, total untreated PSMA protein, *Lanes 2 & 6*: mock treated cells, *Lanes 3 & 7* SSO1, *Lanes 4 & 8* SSO6, *Lanes 5 & 9*, SSO18 treatment. *Lanes 2-5* cytoplasmic section, *Lanes 6-9* membrane section.

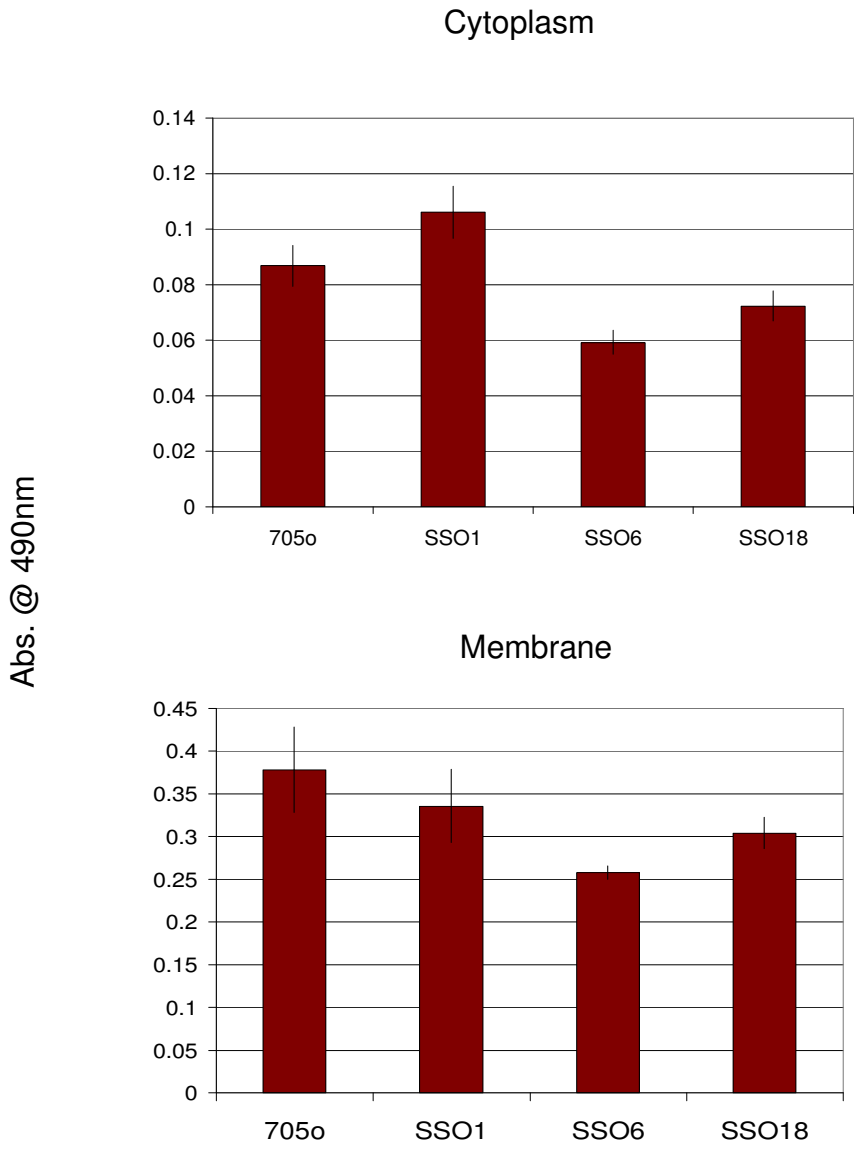


Figure. 3.16. L-glutamate cleavage in cytoplasmic and membrane fractions after OMe SSO treatment.

LNCaP cells were transfected with 0.3 µM of either SSO1, SSO6, SSO18, negative control 705 SSO, or mock treated for 48 hours. Protein was separated into cytoplasmic and membrane fractions. Fractions were assayed colorimetrically for NAALADase activity as measured by the amount of l-glutamate cleaved from the NAAG molecule after a 24 hour incubation.

extracellular domain is essential for activity (Schulke, Varlamova et al. 2003; Davis, Bennett et al. 2005) it follows that PSM' forms a homodimer in the cytoplasm. Enzymatic activity of PSM' was recently questioned because of its incompatible glycosylation pattern (Ghosh, Wang et al. 2005). The reason for this discrepancy with our results is not clear.

Since cytoplasmic PSM' retains activity in OMe SSO1 treated LNCaP cells, it is conceivable that PSM' may have a suppressor-like role in normal prostate cells. Its expression should lead to hydrolysis of cytoplasmic polyglutamed folate stores, freeing cellular folates for efficient export (Osborne, Lowe et al. 1993). This would affect cellular one carbon metabolism including DNA and RNA synthesis (Wagner 1995) possibly altering the cell cycle and apoptosis. The possible pro-apoptotic effects of expression of folate metabolism enzymes are not limited to PSM'. 10-formyltetrahydro-folate dehydrogenase (FDH) is highly expressed in a number of tissues, including prostate, and virtually eliminated in most tumor tissues and cell lines. Transient transfection of FDH inhibited growth of cancer cells (Krupenko and Oleinik 2002).

In LNCaP cells, the level of PSMA expression is very high as transcription of the gene is enhanced 140 fold in cancer versus normal prostate cells (Ghosh and Heston 2004). In consequence, SSO1-induced PSM' levels and its potential effect on folate metabolism will be much higher than in non-malignant prostate tissue. This observation points out the potential strength of the SSO approach in this system. If PSMA plays a role in the malignancy of prostate cells, they should be more sensitive to the SSO1 treatment; the cells that do not express PSMA will

be resistant. Thus, if SSO1 were to be used clinically, the side effects should be low and the therapeutic index high.

This report is the first to examine the PSMAΔ6 and PSMAΔ18 splice variants. Both variants are produced in LNCaP cells and are readily detectable by RT-PCR. Since the initial level PSMAΔ6 is very low, its role in LNCaP cells is not clear. However, PSMAΔ18 and PSM' transcripts are expressed natively at about 33% of PSMA, suggesting a function in relation to PSMA. RT-PCR analysis of the RNA from cells treated with SSO6 shows that PSMA mRNA was strongly decreased and PSMAΔ6 mRNA strongly increased. There was a concomitant, and expected, decrease of PSMA protein but we were unable to detect the PSMAΔ6 protein splice variant using a different polyclonal PSMA antibody (Williams and Kole, unpublished), suggesting that that protein was not properly translated or was unstable. Even if expressed, the truncated PSMAΔ6 would lack the catalytic site while both PSMAΔ6 and PSMAΔ18 lack the dimerization domain. Therefore, neither variant should have enzymatic activity.

PSMA is potentially a membrane signaling or a transport protein because of its cytoplasmic MXXXL internalization motif and its ability to internalize in response to ligand binding (Rajasekaran, Anilkumar et al. 2003). This is supported by the observation that aptamers bound to PSMA's extracellular region are transported internally and dispersed in the cytoplasm of LNCaP cells (Lupold, Hicke et al. 2002). Both the PSMAΔ6 and PSMAΔ18 splice variants would retain this MXXXL motif and could interfere with normal membrane PSMA signaling, giving a dominant negative effect. Until this hypothesis is further

examined, one concludes that any effects of SSO6 and SSO18 treatment on LNCaP cells are due to a decrease in PSMA alone.

CHAPTER 4

PSMA Splice Switching Effects on Malignancy of LNCaP Cells

I. Introduction

Cancer originates and progresses in response to a series of genetic alterations that are responsible for oncogene activation and/or inactivation of tumor suppressors and DNA repair regulators. This process though does not account for several other factors including epigenetic changes, post-transcriptional and post-translational regulation of protein expression, and the influence of tissue microenvironment (Clemens 2004; Feinberg, Ohlsson et al. 2006). Cellular proteins that influence or are influenced by these types of changes are not as readily or directly associated with malignancy since a cause/effect relationship is not always well established. Cancer biomarkers, or proteins whose expression levels are linked to an increased risk of cancer or progression of disease, may be useful for more than cancer diagnosis, screening, and staging; with increased investigation into their biological function and the basis of their modulation of expression with cancer, biomarkers may be implicated as oncogenes, tumor suppressors, or as accessory elements capable of influencing other aforementioned factors related to the initiation and/or advancement of malignancy. For example, expression levels of human chorionic gonadotrophin-β (hCG-β) are used to diagnose and monitor testicular and other types of cancer. hCG-β is considered a very sensitive cancer marker and specific assays that monitor hCG-β in serum, urine, and tumor tissue are currently in use (reviewed in Stenman, Alfthan et al. 2004). While most studies into its expression focus only on its use as a biomarker, hCG-β has been shown

to increase tumor cell proliferation by preventing apoptosis in tissue culture cells (Butler, Ikram et al. 2000). hCG-β's role may surpass biomarker to oncogene status and it could eventually be targeted for molecular therapy instead of simply being used for cancer diagnosis and staging.

PSMA is considered a biomarker of prostate cancer because its increased expression is correlated to prostate cancer grade, stage, and hormone insensitivity (Wright, Grob et al. 1996; Kawakami and Nakayama 1997; Bostwick, Pacelli et al. 1998; Schmittgen, Teske et al. 2003). Also, its presence in tumor neovasculature and absence in normal vasculature point to a potential link between PSMA and tumor maintenance (Liu, Moy et al. 1997; Silver, Pellicer et al. 1997; Chang, Reuter et al. 1999). Prostate malignancy may be directly influenced by PSMA expression; regulation of PSMA expression, particularly by alternative splicing, could be a key determinant of cancer initiation and/or progression.

Malignancy can be described as the sum of the acquired molecular and cellular changes that cancer cells gain to overcome regulation in growth, differentiation, and cell death. Although malignancy is relatively simple to define, the specific processes involved in malignant transformation and perpetuation are still being discovered and described. Hanahan and Weinberg suggested six categories of cellular changes that must be realized to achieve malignancy: "self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis" (Hanahan

and Weinberg 2000). Clinically, malignancy is correlated to tumor size, lymph node involvement, and presence of metastases in the TNM staging system (Sobin 2003; Gospodarowicz, Miller et al. 2004). In this study LNCaP cells are used for their high expression of PSMA and fortunately, they readily exhibit the major hallmarks of malignancy. LNCaP cells are derived from a human lymph node metastasis of prostatic adenocarcenoma and can form tumors when injected into nude mice (Horoszewicz, Leong et al. 1983).

We have successfully targeted PSMA pre-mRNA with three different SSO sequences of two different backbone chemistries to induce splice switching from the full length PSMA transcript to the PSM', PSMΔ6, and PSMΔ18 variants in LNCaP cells. Treatment with SSO1 was able to alter PSMA splicing and increase cytoplasmic PSM' protein level while simultaneously decreasing membrane-associated PSMA expression. Both SSOs 6 and 18 treatment increased PSMAΔ6 and PSMAΔ18 transcript levels, respectively, and induced downregulation of PSMA and PSM' protein. To determine if modulation of PSMA splicing by SSOs has an effect on LNCaP malignancy, cells were tested for alterations in proliferation and apoptosis post SSO transfection. PC3 cells, which do not express PSMA, were used as a control to verify specificity of SSO effects.

II. Results

A. Cellular proliferation/viability after SSO Treatment

LNCaP and PC3 cells were transfected with 2'-O-methyl (OMe) and 2'-O'methoxyethoxy (MOE) phosphorothioate SSOs at varying concentrations using the Lipofectamine 2000 (Invitrogen) cationic lipid delivery agent for 48 hours.

Splice switching of PSMA from the full length transcript to PSM', PSMAΔ6, and PSMAΔ18 by SSO1, SSO6 and SSO18, respectively, were previously verified at the mRNA, protein, and PSMA functional level (see Chapter 3). Post transfection, levels of cellular proliferation and viability were determined using a colorimetric metabolism assay and a luminescent ATP quantitation method. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) directly measures cellular metabolism by utilizing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) and an electron coupling reagent phenazine ethosulfate (PES). The MTS compound is reduced into a formazan molecule by NADPH or NADH in viable, active cells. Formazan is a soluble and colored compound in tissue culture medium; levels of formazan produced after the addition and conversion of MTS are determined by measuring absorbance at wavelength 490 nm. Cell number/metabolism is directly proportional to absorbance readings.

Figures 4.1 and 4.2 show the results of the MTS proliferation assay after PSMA SSO treatment of LNCaP and PC3 cells. Percent proliferation is based on readings at 490 nm compared to mock (Lipofectamine 2000 only) treatment. In LNCaP cells, OMe SSOs 1, 6, and 18 all induced a decline in cellular proliferation at 48 hours (Fig. 4.1). SSO1 gives the most prominent effect with a 60% decrease in MTS metabolism at the highest concentration (1 μM); OMe SSOs 6 and 18 also had substantial effects on LNCaP proliferation. The greatest effects observed were a 53% decline at 0.3 μM for SSO6 and a 50% decline at 1 μM for SSO18. However, the negative control OMe oligonucleotides, 705

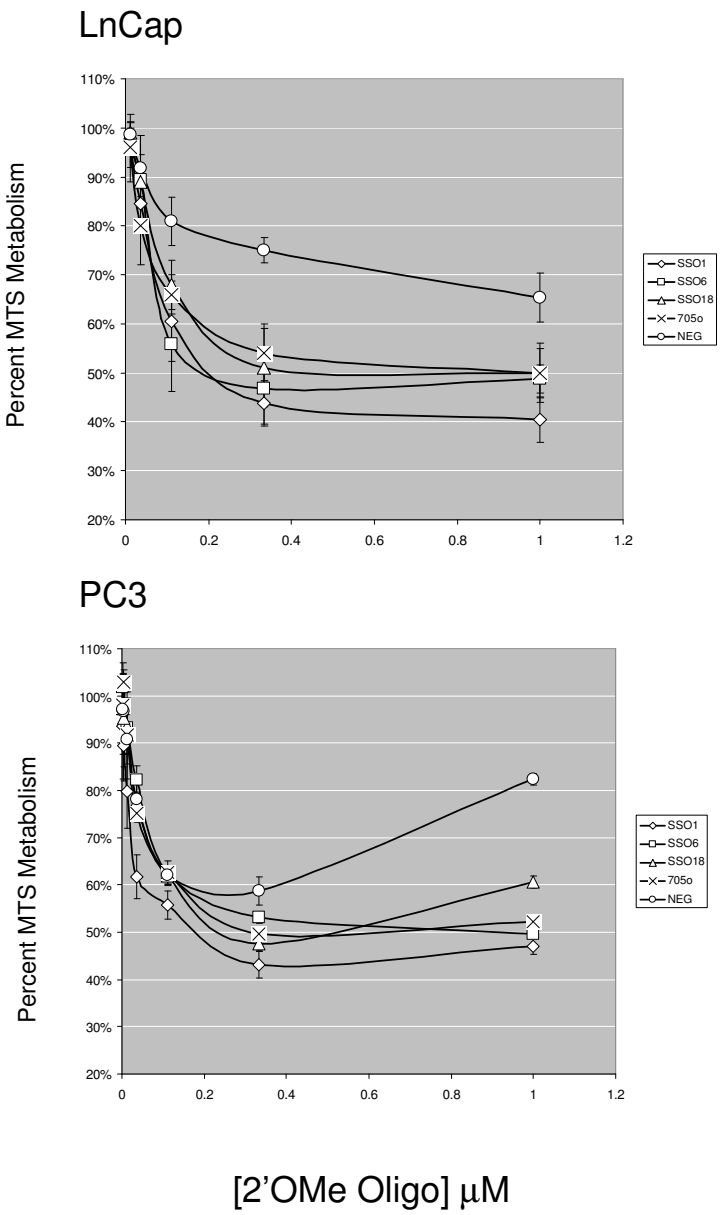


Figure. 4.1. MTS analysis after OME SSO treatment in LNCaP and PC3 cells.

LNCaP and PC3 cells were treated with OMe SSOs 1, 6, and 18, and control oligonucleotides using a 1:3 dose curve from 1 µM to 0.041 µM. Post transfection cells were assayed for proliferation/ total cellular metabolism using the colorimetric CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Absorbance readings detected at 490 nm is directly proportional to total metabolism; percent metabolism is based on oligonucleotide treatment absorbance readings compared to mock treatment absorbance.

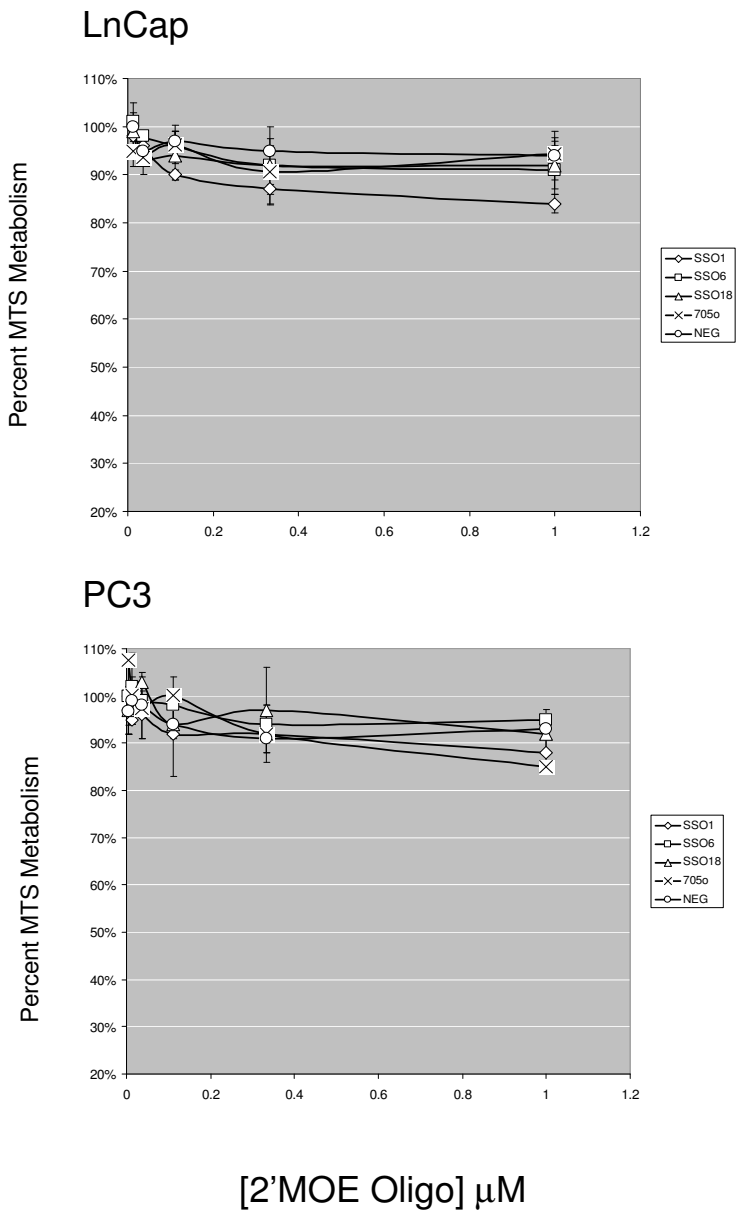


Figure. 4.2. MTS analysis after MOE SSO treatment in LNCaP and PC3 cells.

LNCaP and PC3 cells were treated with MOE SSOs 1, 6, and 18, and control oligonucleotides using a 1:3 dose curve from 1 μM to 0.041 μM. Post transfection cells were assayed for proliferation/ total cellular metabolism using the colorimetric CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Absorbance readings detected at 490 nm is directly proportional to total metabolism; percent metabolism is based on oligonucleotide treatment absorbance readings compared to mock treatment absorbance.

(targeted to the β-globin gene) and Random (N_{18}) /MM (SSO1 four nucleotide mismatch) also caused a decrease in cellular MTS metabolism. At most concentrations, negative control SSO effects were not as pronounced as targeted SSOs, but they were large enough to question the specificity of the relationship between PSMA splice switching and observed cellular effects. To further examine the validity of this relationship, MOE SSOs of the same sequence were tested in LNCaP cells (the decision to use the MOE chemistry is outlined in this chapter's discussion section); also both OMe and MOE SSOs were tested in PSMA negative PC3 cells (Figs. 4.1 and 4.2). The results of these studies confirm effects not related to PSMA splice switching of OMe oligonucleotides on LNCaP cells. First, OMe SSOs produced a similar decrease in MTS metabolism post transfection in PC3 cells, with SSO1 again giving the greatest effect. Moreover, the MOE SSOs of the same sequence that induced similar levels of splice switching at the mRNA level as OMe SSOs had very little if any effect on cellular proliferation as measured by MTS metabolism in both LNCaP and PC3 cells (Fig. 4.2).

The level of ATP, the predominant energy storage molecule of the cell, is one of the most direct measures of cellular metabolism. Here, ATP levels are correlated to cellular proliferation since abundance of ATP is directly related to the number of cells in culture (Crouch, Kozlowski et al. 1993). The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) provides an accurate readout of ATP levels by taking advantage of the light-emitting potential of the luciferin molecule. A recombinant firefly luciferase enzyme catalyzes the oxygenation of

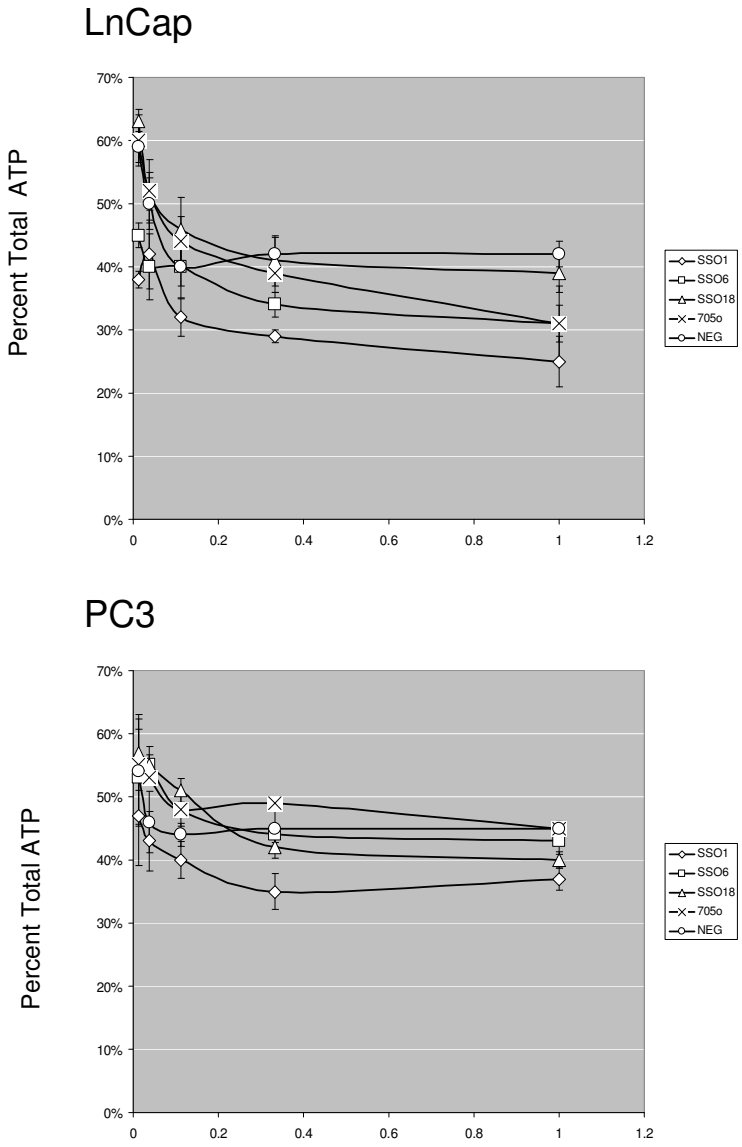


Figure. 4.3. ATP content analysis after OME SSO treatment in LNCaP and PC3 cells.

LNCaP and PC3 cells were treated with OMe SSOs 1, 6, and 18, and control oligonucleotides using a 1:3 dose curve from 1 μM to 0.041 μM. Post transfection cells were assayed for cellular proliferation using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Luminescence produced is directly proportional to cellular proliferation; percent proliferation is based on oligonucleotide treatment luminescence readings compared to mock treatment luminescence.

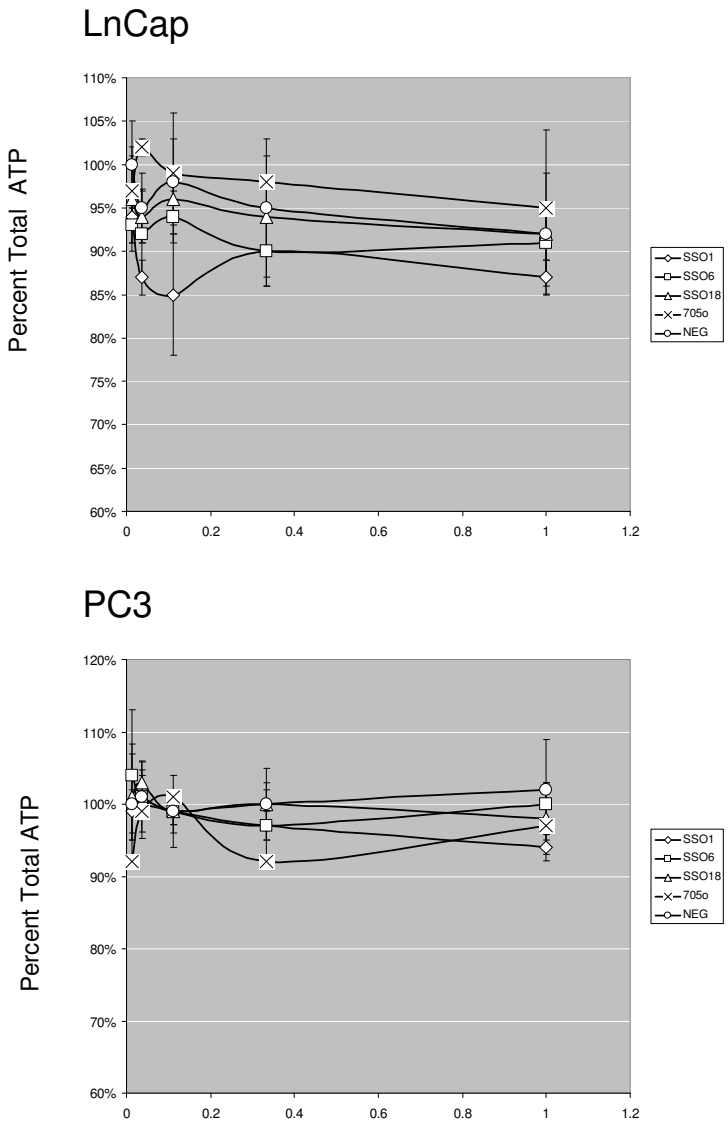


Figure. 4.4. ATP content analysis after MOE SSO treatment in LNCaP and PC3 cells.

LNCaP and PC3 cells were treated with MOE SSOs 1, 6, and 18, and control oligonucleotides using a 1:3 dose curve from 1 μM to 0.041 μM. Post transfection cells were assayed for cellular proliferation using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Luminescence produced is directly proportional to cellular proliferation; percent proliferation is based on oligonucleotide treatment luminescence readings compared to mock treatment luminescence.

beetle luciferin in the presence of ATP, magnesium, and molecular oxygen; oxyluciferin, AMP, diphosphate, carbon dioxide and light are the products of the reaction. The luminescence or amount of light produced is directly related to ATP present when luciferin is in excess. After oligonucleotide transfections, LNCaP and PC3 cells were lysed and total ATP levels were accessed. The results of this luminescent ATP assay after OMe and MOE SSO1, 6, and 18 treatments mostly echoed data from the MTS proliferation assay. The total amount of ATP detected did decline significantly after 48 hour of transfection with all OMe SSOs and controls in both LNCaP and PC3 cells, although the effect was more prominent in LNCaP cells (Fig 4.3). Using the same sequences with MOE chemistry, changes in ATP content were not impressive in both cell lines with none of the treatments giving more than a 15% decrease in LNCaP cells or an 8% decrease in PC3 cells (Fig. 4.4). Again, these results indicate that the SSO oligonucleotide effects induced are not specific to the manipulation of PSMA splice variants.

B. Apoptosis induction after SSO treatment

LNCaP and PC3 cells were treated with SSO and control oligonucleotides as previously described. Levels of apoptosis, or programmed cell death, were measured 48 hours post treatment using a caspase 3/7 detection assay.

The ability of cancer cells to avoid apoptotic death by apoptosis is a key measure of malignancy that must be acquired in addition to proliferation to buffer against cellular attrition rates. Apoptosis is marked by several biochemical and physical changes, including activation of cellular proteases called cysteine

aspartic acid-specific proteases (caspases), disruption of mitochondrial and plasma membranes, chromosome degradation, cell surface phosphatidylserine exposure, and cell shrinkage (recently reviewed in Gerl and Vaux 2005; Vermeulen, Van Bockstaele et al. 2005). Accurate detection of apoptosis is best achieved by measuring several of these processes since not all are required and/or sufficient for apoptosis. Here, we utilize the Apo-ONE® Homogeneous Caspase-3/7 assay (Promega) to determine levels of active caspases 3 and 7 as a measure of apoptosis. Caspases are considered 'active' after they are cleaved from the inactive pro-caspase form and able to act on downstream effectors such as poly-(ADP ribose) polymerase (PARP), protein kinase C (PKC), topoisomerases, and other caspases (Vaux and Strasser 1996; Nicholson and Thornberry 1997). Caspase 3 is able to cleave and activate downstream caspases 2, 6, 7, 8, 9, and 10 (Slee, Harte et al. 1999). Janicke et al. demonstrated that caspase 3 activity are necessary for DNA fragmentation in the MCF-7 breast cancer cell line (Janicke, Sprengart et al. 1998) and detailed the many possible effects of caspase 3 in apoptosis signaling (Porter and Janicke 1999). Caspase 7 is considered a caspase 3-like protease with the same substrate recognition (Nicholson and Thornberry 1997; Talanian, Quinlan et al. 1997). In the caspase 3/7 assay, a pro-fluorescent substrate of caspase 3 and 7, Z-DEVD-R110 [rhodamine 110, bis-(N-CBZ-L- aspartyl-L- glutamyl- L- valyl-L-aspartic acid amide)], is cleaved after a cell lysis step. The rhodamine 110 group is released and fluoresces with excitation at 499 nm. Fluorescence emitted at 521 nm is directly proportional to caspase 3/7 activity. Interestingly, caspase 3/7

activity increased with all oligonucleotides tested in both LNCaP and PC3 cell lines. With OMe SSOs and controls, large increases in caspase 3/7 activity were detected with a 14 fold upsurge over mock treatment in LNCaP and PC3 cells, respectively (Fig. 4.5). Again, MOE oligonucleotides did not have an impact on cells to the extent of their OMe counterparts; they produced a more modest 3.25 and 2.4 fold increase in LNCaP and PC3 cells, respectively (Fig. 4.6). Also, as the graphs illustrate, there were no significant differences between the readings of SSOs and negative controls with both chemistries in both cell types.

The evidence for non-specific effects of OMe oligonucleotides was overwhelming; no verifiable link between manipulation of PSMA splice ratio by SSOs and cellular proliferation/apoptosis level could be established.

III. Discussion

Here, LNCaP cells were tested for changes in cellular proliferation and apoptosis rates post PSMA specific 2'-*O*-methyl and 2'-*O*-methoxyethoxy phosphorothioate SSO treatment using three different assays: 1) a MTS cell viability assay, 2) an ATP content assay, 3) and a caspase 3/7 assay. The PSMA-less PC3 prostate tumor cell line, and negative control oligonucleotides, were used under the same conditions to subtract non-PSMA specific effects of SSO treatment. We previously had demonstrated effective splice switching of PSMA at the mRNA level using SSOs 1, 6, and 18 over a range of oligonucleotide concentrations up to 1 μM after 48 hours. Modest changes in PSMA and PSM' protein level consistent with, but not as prominent as, the mRNA shift were observed using 0.3 μM of the 2'-*O*-methyl SSOs. The three

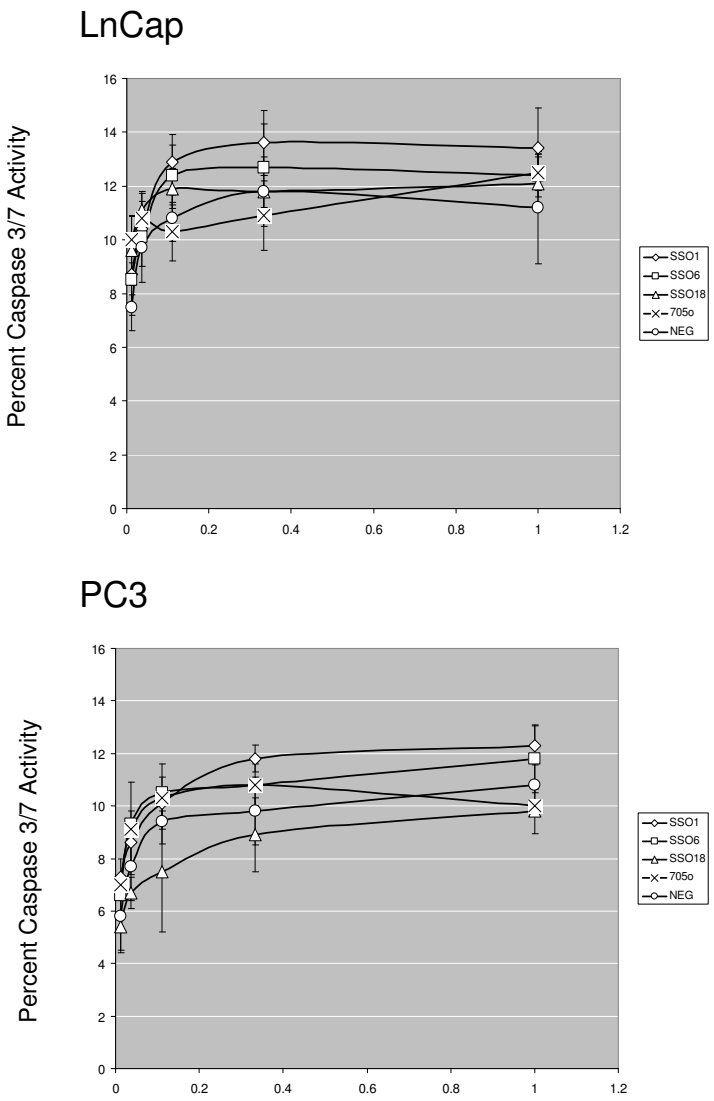


Figure. 4.5. Caspase 3/7 analysis after OME SSO treatment in LNCaP and PC3 cells.

LNCaP and PC3 cells were treated with OMe SSOs 1, 6, and 18, and control oligonucleotides using a 1:3 dose curve from 1 μM to 0.041 μM. Post transfection cells were assayed for apoptosis by detecting active caspase 3/7 levels using the fluorescent Apo-ONE® Homogeneous Caspase-3/7 assay (Promega). Fluorescence produced is directly proportional to active caspase 3/7 levels; percent apoptosis is based on oligonucleotide treatment fluorescence readings compared to mock treatment fluorescence.

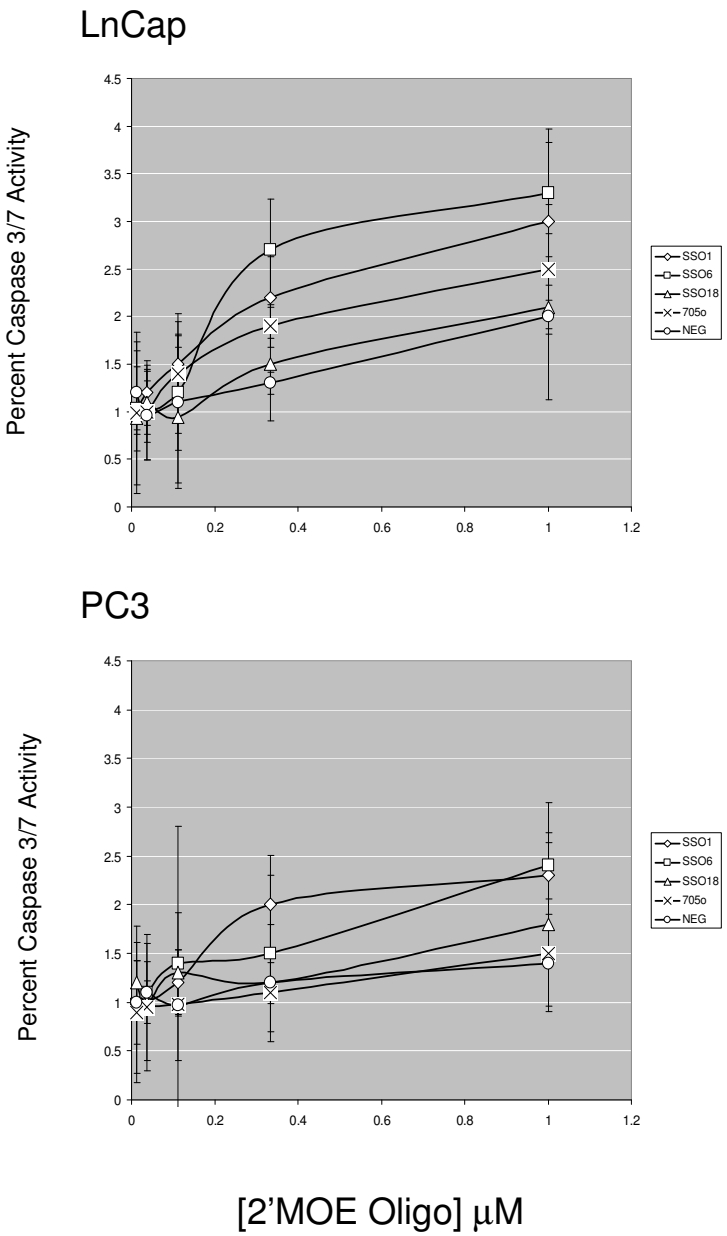


Figure. 4.6. Caspase 3/7 analysis after MOE SSO treatment in LNCaP and PC3 cells.

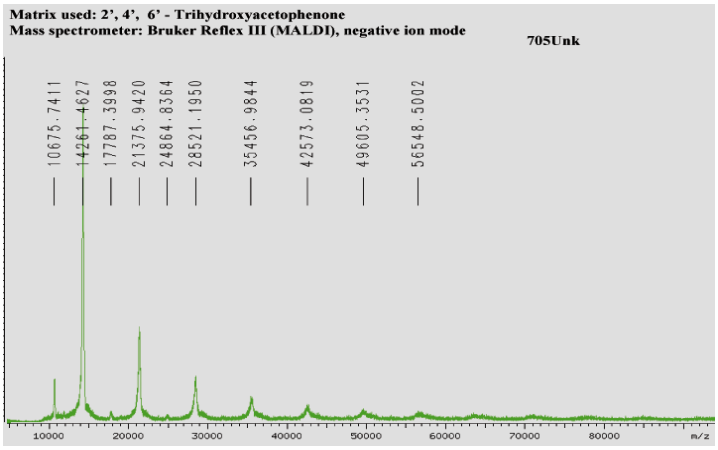
LNCaP and PC3 cells were treated with MOE SSOs 1, 6, and 18, and control oligonucleotides using a 1:3 dose curve from 1 μM to 0.041 μM. Post transfection cells were assayed for apoptosis by detecting active caspase 3/7 levels using the fluorescent Apo-ONE® Homogeneous Caspase-3/7 assay (Promega). Fluorescence produced is directly proportional to active caspase 3/7 levels; percent apoptosis is based on oligonucleotide treatment fluorescence readings compared to mock treatment fluorescence.

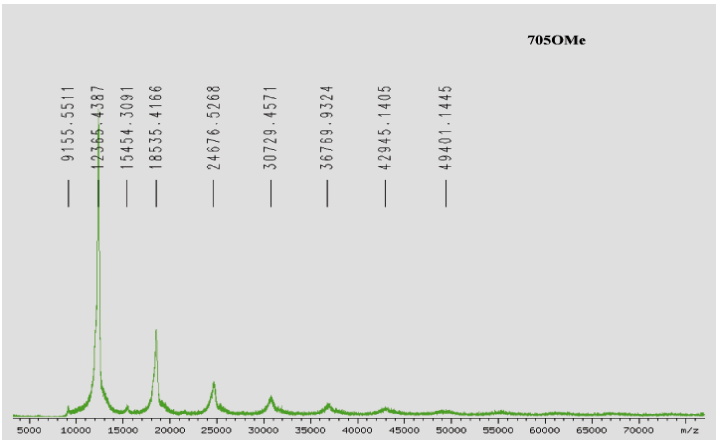
assays used to evaluate specific aspects of LNCaP malignancy were performed using the same dose range and 48 hour time point. In LNCaP cells the 2'-*O*-methyl SSOs induced a marked decline in cellular proliferation and increased in apoptosis, however these effects were not limited to PSMA specific oligonucleotides; both control 2'-*O*-methyl negative control oligonucleotides gave a similar effect. Additionally, 2'-*O*-methoxyethoxy oligonucleotides of the same SSO1, 6, and 18 sequences failed to produce any significant changes in LNCaP proliferation and apoptosis compared to mock (no oligonucleotide) or negative control oligonucleotide treated cells. One could argue that the effects of SSO1 could be PSMA specific since they consistently produced a more pronounced effect on proliferation and apoptosis compared to the other SSOs and control oligonucleotides. However, results of PC3 treated cells refute this conclusion and further detail the non-specific effects of PSMA targeted SSOs; measurements after PC3 transfections were similar to those from LNCaP treatment even exhibiting the enhanced effect of SSO1 even though PC3 cells lack PSMA.

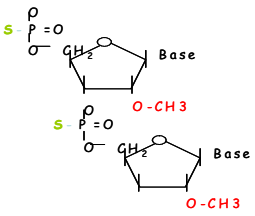
Explanation of the dissimilarity in results from the two different SSO chemistries continues to be speculative. The 18-mer sequences of SSOs 1, 6, and 18 were identical and their backbone chemistries contained all phosphorothioate linkages. What remains is the difference in ribose modification, chemical synthesis, and possible presence of contaminants. Current literature shows no evidence of any inherent toxicity with 2'-*O*-methyl oligonucleotides in cell culture at the concentrations used in this study. TriLink BioTechnologies

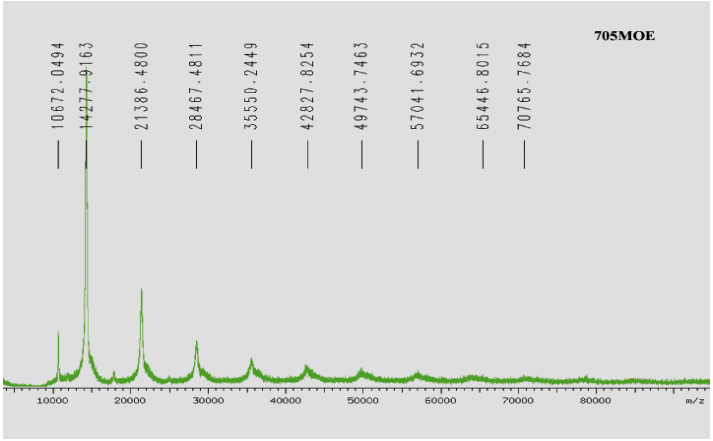
(San Diego, CA), the manufacturer of the 2'-*O*-methyl oligonucleotides used here, confirmed their confidence in a lack of toxicity. Note that while all oligonucleotides were purified by HPLC prior to our handling, two different companies were used to obtain the two sets of oligonucleotides. The 2'-*O*-methoxyethoxy oligonucleotides were obtained from Isis Pharmaceuticals (Carlsbad, CA), the sole provider of the chemistry, using IDTDNA (Coralville, IA) as their distributor. We cannot discount or verify any differences in oligonucleotide quality based on synthesis that may have an effect on cells in culture. An effort was made to identify contaminants in 2'-*O*-methyl oligonucleotide solutions using PAGE separation followed by visualization with a UV shadowing technique. Single bands consistent with oligonucleotide size were detected with no visual contaminants, although this process cannot rule out the presence of small molecule contaminants.

Initially, only 2'-*O*-methyl SSOs and controls were utilized in this study. The 705 negative control oligonucleotide consistently showed little effect on LNCaP cells after MTS analysis, a result not shared with other negative controls. After tracking the origin of the 705 sample, we determined that it was mislabeled as 2'-*O*-methyl, and was possibly of 2'-*O*-methoxyethoxy chemistry. A chemical comparison was made using mass spectrometry of oligonucleotides with the 705 sequence of both chemistries. The spectrometry profile of the 'unknown' 705 oligonucleotide was highly similar to that of the 705 2'-*O*-methoxyethoxy (Fig. 4.7). To increase the validity of our results, 2'-*O*-methoxyethoxy SSOs and controls were added to the study.









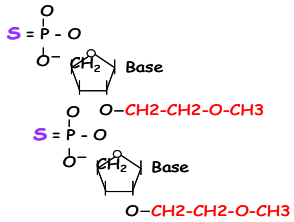


Figure. 4.7. Mass spectrometry of 705 negative control oligonucleotide samples.

Figure. 4.7. Mass spectrometry of 705 negative control oligonucleotide samples.

Mass spectrometry was performed to determine the oligonucleotide chemistry of a 705 negative control sample (705Unk). Known 705 oligonucleotide samples of 2'-*O*-methyl (705OMe) and 2'-*O*-methoxyethoxy (705MOE) chemistry were tested for comparison. Mass spectrometry profile of the 705Unk sample was identical to the 705MOE sample. Analysis was performed by the University of North Carolina's Michael Hooker Proteomics Core Facility.

We were unable to directly link splice switching of PSMA by these SSOs with changes in malignancy of LNCaP cells as measured by cellular proliferation and apoptosis. However, this does not remove PSMA from being considered a factor or contributor of prostate cancer initiation or progression. Although the SSOs used here were efficient in altering PSMA mRNA levels, the modest change in protein level induced may not have been sufficient to detect any real effects in culture. As mentioned previously, PSMA SSOs of other chemistries may be able to lower EC50 values and produce a more significant change in PSMA protein levels. Also, it would be informative to separately introduce PSMA splice variants into cell lines that do not natively express PSMA and test for specific cellular changes. This would remove background effects of due to the presence of other variants, but not be able to directly address the consequence of splice ratios.

To speculate into PSMA's possible contribution to prostate malignancy, its cell surface presentation puts it into the categories of extracellular signal transducer, surface membrane receptor, or extracellular matrix protein. Cell surface signaling for growth stimulation, angiogenesis, and tissue invasion can be exploited to sustain cancer cells. As detailed earlier, PSMA is capable of binding a ligand outside of the cell and signal intracellularly for the initiation of internalization. Although its intracellular domain is very small, its five C-terminal amino acids are required for this internalization; PSMA is capable of at least this level of signal transduction. Overexpression of the membrane bound form of PSMA in prostate cancer compared to the cytosolic variant is consistent with, but

not evidence of it being utilized by cancer cells for survival, growth, or invasion signaling. There is one published report that PSMA is able to act as a receptor molecule by transporting extracellular cargo into the cytoplasm (Lupold, Hicke et al. 2002), also a study in progress demonstrates that nucleic aptamer conjugates targeted for uptake by PSMA are internalized by LNCaP cells, enter the cytoplasm, and downregulate specific mRNA transcripts (Giangrande and Sullenger, unpublished data). More research is required to define PSMA's receptor capacity. PSMA may prove to be important in physical cellular linkages as part of the extracellular matrix superstructure. Surprisingly, Ghosh et al. determined that expression of PSMA in PC3 cells decreased invasiveness (Ghosh, Wang et al. 2005). Although this is opposite of the expectation, it does suggest that PSMA has some role in the physical links that cancer cells make for tissue invasion and metastasis.

Chapter 5

Conclusions

Summary of Dissertation Results

Splice switching oligonucleotides have been successfully used with several gene targets for the manipulation of alternatively spliced variants (see Chapter 1). Results presented here verify that we are now able to add PSMA to this growing list by targeting its pre-mRNA with SSOs of OMe and MOE chemistries. SSOs 1, 6, and 18 targeted to the 5' splice sites of exons 1, 6, and 18, respectively, were able to induce a decline in full length PSMA mRNA and increase mRNA levels of PSM', PSMAΔ6, and PSMAΔ18 (Chapter 3). In addition, splice switching to the endogenously less prominent variants in LNCaP cells caused a decline in membrane bound PSMA protein. Treatment with SSO1 for 48 hours also increased PSM' protein levels in the cytoplasm (Chapter 3). A functional assay measuring PSMA's NAALADase activity verified splice switching at the protein level and, importantly, indicated that the cytoplasmic PSM' retains functional activity despite its lack of glycosylation (Chapter 3). We attempted to link the change in PSMA splice variant levels from SSO treatment to a change in properties of LNCaP cellular malignancy by testing levels of cellular proliferation and apoptosis post SSO transfection (Chapter 4). Our study was based on the observation that PSMA splice ratios are naturally altered in prostate cancer compared with normal prostate tissue (see Chapter 1 for a detailed explanation), i.e. the PSM'/PSMA mRNA ratio decreases from normal and benign prostate tissue to prostate cancer and continues to decrease with an increase in cancer grade (Chapter 1). However, we were not able to correlate PSMA specific splice switching by our SSOs with cellular proliferation and apoptosis levels as

measured by three different assays (Chapter 4).

It is possible that the amount of splice switching we were able to induce at the protein level is not adequate enough to detect any PSMA specific changes in LNCaP cells; testing SSOs of different chemistries and concentrations may identify more efficient SSOs that are able to significantly alter PSMA protein levels. Moreover, future studies into PSMA's function in prostate tissue and tumor neovasculature will be invaluable in determining whether our splice switching technique targeted at PSMA pre-mRNA will be useful toward the treatment of prostate and other types of cancers. It is also a definite possibility that native changes in PSMA splicing based on prostate tissue malignancy are a consequence or effect of cellular changes rather than a causative factor. If that is the case, SSO technology used here would not be a useful therapeutic. However, PSMA splice ratio could still be used as a diagnostic marker in prostate cancer in conjunction with screening by PSA and other markers.

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