SILENCING TRANSCRIPTION: PROMOTER-TARGETED OLIGONUCLEOTIDES BIND CHROMOSOMAL DNA INSIDE CELLS

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DEDICATION

To my wife, Ashley R. Beane, and to my parents, Dennis and Sherrie Beane.

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by

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Aberrant gene expression can lead to multiple disease-states that can be difficult or impossible to treat using traditional small-molecule medications. An alternative approach to treating such diseases is oligonucleotide-based therapeutics, which are theoretically capable of treating or curing genetic diseases, infections, and abnormalities.

Oligonucleotide-based molecules targeted to DNA are referred to as antigene agents. These molecules can silence or activate gene transcription of alleles and have many potential medical applications. However, the growth of antigene technologies has been slow despite broad therapeutic potential and unique molecular applications.

Through the development of chemical modifications, oligonucleotide-based molecules are actively being improved and refined. Chemical modifications can alter the cellular uptake, toxicity, biodistribution, and plasma retention of oligonucleotides.

My research goal was to further the field of synthetic antigene oligonucleotides. To do this, I targeted endogenous genes in human cancer cell lines with chemically-

modified oligonucleotides, including MOEs, PNAs, ENAs, and LNAs. I established that LNAs were robust antigene agents capable of inhibiting transcription under multiple conditions.

Specifically, I established that mixed-base antigene agents physically associate with the hPR-B promoter and decrease the occupancy of RNA polymerase II on the hAR and hPR genes inside human cells. Furthermore, my research indicates that antigene LNAs function in an orientation-dependent manner and that functional LNAs must target the template strand of DNA to have appreciable potency.

This body of work comprised the first extensive study of a mixed-base antigene oligonucleotide in multiple human cell lines and provides the first evidence that mixed-base antigene agents can physically associate with chromosomal DNA and inhibit transcription of endogenous mammalian genes inside human cells. Collectively, my data suggest that antigene LNAs are a potent and general strategy for silencing gene expression, and that antigene LNAs also have potential therapeutic applications and possible utility in modern functional genomics.

TABLE OF CONTENTS

Chapter 1: Antigene LNAs and the Oligonucleotide World

Chapter 2: Initial Characterization of Promoter-Targeted Locked Nucleic Acids

Chapter 3: Establishing the Genomic Target and Mechanism of Antigene LNAs

Chapter 4: Comparison and Characterization of Additional Antigene Agents

Chapter 5: Materials and Methods

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	Adenine
ACN	Acetonitrile
agRNA	Antigene RNA
Bhoc	Benzhydryloxycarbonyl
BLAST	Basic Local Alignment Search Tool
BLAT	BLAST-Like Alignment Tool
Boc	Tert-Butyloxycarbonyl
C	Cytosine
cDNA	Complementary Deoxyribonucleic Acid
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E. coli	Escherichia Coli
EDTA	Ethylene Diamine Tetraacetic Acid
ENA	2'-O,4'-C-Ethylene Nucleic Acid
Fmoc	9-Fluorenylmethoxycarbonyl
G	Guanine

HATU	*O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-Tetramethyluronium Hexafluorophosphate
HCV	Hepatitis C Virus
hAR	Human Androgen Receptor
hPR	Human Progesterone Receptor
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
IC_{50}	Median Inhibition Concentration
LNA	2'-O,4'-C-Methylene Nucleic Acid (Locked Nucleic Acid)
MALDI-TOF MS	Matrix-Assisted Laser Desorption / Ionization Time of Flight Mass Spectroscopy
MOE	2'-O-(2-Methoxyethyl) Nucleic Acid
mRNA	Messenger RNA
nM	Nanomolar
NMP	1-Methyl-2-Pyrrolidinone
OMe	2'-O-Methyl Nucleic Acid
PNA	Peptide (Polyamide) Nucleic Acid
RNA	Ribonucleic Acid
RNAi	RNA Interference
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
siRNA	Short Interfering (Double-Stranded) RNA
SNP	Single Nucleotide Polymorphism
SP1	Specificity Protein 1

T	Thymine
TFA	Trifluoroacetic Acid
TFMSA	Trifluoromethanesulfonic Acid
TFO	Triplex-Forming Oligonucleotide
XAL-PEG-PS	Xanthenyloxy Alkanoic Acid-Polyethylene Glycol-Polystyrene

Chapter 1: Antigene LNAs and the Oligonucleotide World

This chapter is intended to be an introduction to the applications of oligonucleotide chemistries. In addition, relevant historical background has been included to provide the context necessary to appreciate advances and challenges in this field. Finally, this chapter introduces the concept of antigene technologies and emphasizes the properties and applications of locked nucleic acids.

1.1 Synthetic Oligonucleotides

The development of synthetic oligonucleotides and novel nucleic acid analogs has been a boon in many academic endeavors, ranging from fluorescent markers [1-3] and microarrays [4-7], to tools for transcriptional [8, 9] and translational [10-14] gene silencing. Since oligonucleotide-based molecules are being actively improved and refined through the development of novel chemical modifications [15-20], nascent applications of these versatile molecules are still emerging [21]. Most importantly, oligonucleotide-based therapeutics have the potential to address a currently unmet need for simple, therapeutic molecules capable of treating a large number of genetic diseases, infections, and abnormalities [10].

Modified oligonucleotides have many desirable characteristics compared with traditional small-molecule therapeutics [10]. For example, synthetic oligonucleotides can theoretically be used to target any disease-causing RNA or DNA sequences [10, 22-28]. This is an advantage that is becoming more obvious as we accumulate additional genomic data and are able to better understand the molecular basis of diseases that have been difficult or impossible to treat using conventional drugs [10].

An additional advantage of synthetic oligonucleotide-based therapeutics is the ability to capitalize on the genomic data currently available to alleviate the costs and difficulties associated with drug development. For an oligonucleotide-based therapeutic, knowledge of a genomic target sequence is all that is required to begin designing potential drug candidates [10, 24, 29, 30]. This is because oligonucleotides bind their targets by Watson-Crick or Hoogsteen pairing rules, which are well-characterized and predictable [10, 31, 32]. Currently, small-molecule therapeutics require extensive testing for each new target, and prior knowledge of functional small molecules is unlikely to aid in the design of small molecules to a novel drug target [33]. However, the opposite is true with oligonucleotide-based drugs.

Oligonucleotide-based drugs are unique among therapeutics, as the delivery methods, distribution, cellular uptake, toxicity, and pharmacokinetics are largely sequence-independent [10, 24, 29, 30]. This means that oligonucleotides can be considered as a ‘family’ of therapeutics, whose members would be expected to have similar biochemical and biophysical characteristics. Although this is an incentive for pharmaceutical companies, since there would be a diminishing risk of failure for each additional synthetic oligonucleotide-based drug developed, the field of oligonucleotide-based therapeutics has been stigmatized by clinical failure [22] and many poorly controlled research publications [32, 34-37].

Oligonucleotide-based technologies were first appreciated in 1978, when Zamecnik and Stephenson demonstrated that a complementary oligonucleotide had antiviral activity [38, 39]. Since this early demonstration, there has been substantial progress in our understanding of oligonucleotide-based technologies, including

mechanistic insights, an appreciation of chemical modifications, and a diverse range of novel applications [40]. For example, the predictable and reliable binding of an oligonucleotide to its complementary strand is the basis for multiple innovations in functional genomics, including microarrays [4, 6], antisense technologies [14, 23], and molecular probes [1-3, 41].

The advances made in chemical modifications of oligonucleotides are generally divided chronologically as ‘generations’ [23, 26, 40, 42, 43]. The first generation of oligonucleotide-based drugs was phosphothioate-modified [10]. Phosphothioate modifications were made to increase nuclease resistance and serum stability of oligonucleotides [10, 40]. Phosphothioate-modified oligonucleotides have a substituted sulfur atom in place of one of the non-bridging oxygen atoms in the phosphate group [40]. This modification creates a stable oligonucleotide-based drug with an ‘attractive’ pharmacokinetic profile, and has led to the commercial drug Vitravene [40]. However, phosphothioate-modified oligonucleotides bind to some proteins, including heparin-binding proteins, and can cause acute toxicities in animals [10, 24, 26]. The limitations of phosphothioate-modifications were the catalyst for researching additional chemical modifications, which led to the second generation of antisense technologies [26].

Second generation oligonucleotides, such as 2’-O-methyl RNAs (OMe) or 2’-O-methoxy-ethyl RNA (MOEs), contain modifications at the 2’ position of the ribose [10, 26, 29, 30]. Many of these modifications were developed through a research collaboration between Novartis and Isis Pharmaceuticals, which consisted of screening multiple chemical modifications for nuclease resistance, binding affinity, and cell culture performance [29]. Relative to other oligonucleotide chemistries, the properties of MOEs

have been studied intensely and currently there are multiple MOE-based drugs in clinical trials (Isis Pharmaceuticals) [10, 29, 30]. MOEs are considered a benchmark for antisense activity and are often analyzed in comparative studies with other chemical modifications [8, 44-48].

The third generation of chemically modified-oligonucleotides is large and consists of a variety of modifications [10, 26]. Examples include Peptide Nucleic Acids (PNAs) and Morpholino phosphoroamidates (MFs), which have radically altered backbone chemistries, while 2'fluro-arabino nucleic acids (FANA) contain relatively simple chemical substitutions [26]. Many of these third-generation chemistries have promising and unique properties. One such modification is the 2'-O-4'-methylene bridge ribose, which is also called a locked base. Oligonucleotides containing locked bases are generally referred to as Locked Nucleic Acids (LNAs) or Bridged Nucleic Acids (BNAs) [24].

LNAs were independently discovered by the Imanishi and Wengel laboratories in 1997 [24, 49]. Since 1997, LNAs have become popular antisense molecules and are commonly used in a number of applications ranging from aptamers [50-56], DNAzymes [57-62], and molecular probes [63-65] to therapeutic agents [66] and antagomirs [63, 67-69]. Currently, LNA-based therapeutics are being developed by Santaris Pharma, which has multiple ongoing phase I/II clinical trials [24, 49]. In addition to therapeutic applications, LNAs also have been recently used in microarray platforms to improve specificity and reproducibility of functional genomics screens [4-7, 70]. The success and utility of the LNA chemistry is largely due to the chemical and physical properties that it conveys to oligonucleotides [23, 49].

1.2 Desirable Characteristics of Synthetic Oligonucleotides

Chemical modifications can drastically alter the biochemical and biophysical properties of oligonucleotides [13, 44, 55, 71, 72]. As mentioned above, phosphothioate-modified oligonucleotides were originally developed to increase nuclease resistance and improve serum stability in oligonucleotides [10, 40]. In addition to nuclease resistance, high target affinity is a desirable characteristic for a synthetic oligonucleotide. For example, a single locked-base substitution can increase the melting temperature of a duplex by over 9°C [24, 73]. Another desirable property is specificity, which is the ability of an oligonucleotide to tolerate mismatches. For example, LNAs are commonly used as antagomirs [66-69, 74] and as probes to identify single nucleotide polymorphisms (SNPs) [15, 75-78] because they have high target specificity. In fact, a single mismatch between an LNA and its target can substantially reduce or even abolish binding [24].

For oligonucleotide modifications to become widely used, they must be cost-effective. A substantial factor determining the cost of a synthetic molecule is the difficulty or ease of synthesis [33]. Basic oligonucleotide synthesis is an automated process, which has been optimized over the course of several decades [79-88]. Many oligonucleotide modifications, such as LNA bases, are compatible with these existing technologies and can be manufactured on a scale large enough to accommodate commercial availability (Exiqon, Vedbaek, Denmark) [24]. However, some oligonucleotide chemistries are not yet commercially available, which limits the comparative studies that can be conducted by laboratories lacking the resources to synthesize the molecules. Some unique nucleic acid mimics, notably PNAs, are not

compatible with oligonucleotide synthesis, but can still be manufactured on a large scale. In fact, PNAs are synthesized using conventional peptide synthesis techniques [89], and can be modified with peptide conjugates [69, 90-93]. However, chemistries such as LNAs are conducive to chemical conjugates normally used with oligonucleotides, which can be advantageous in many applications [63-65]. Recently, it was shown that LNAs could be incorporated into oligonucleotides enzymatically, which may lead to even more cost-effective methods of producing modified-oligonucleotides [94].

Pharmacological properties of oligonucleotides are also drastically altered by chemical modifications [10, 24, 29, 30, 95]. For oligonucleotides to be effective drugs, they should not be rapidly excreted or degraded [10, 29]. Phosphothioate-modified oligonucleotides have favorable pharmacological profiles because they are not rapidly degraded or excreted. The pharmacological profile results from the nonspecific binding of phosphothioate-modified oligonucleotides to proteins, which can also lead to toxicity [40]. Therefore, phosphothioates are often used in conjunction with additional modifications to reach a balance of toxicity and pharmacological properties [10, 24, 44].

In general, chemical modifications can alter the cellular uptake, toxicity, biodistribution, and plasma retention of oligonucleotide-based therapeutics. Improving these properties is a constant and continuing area of research [10]. Many of the modifications used to improve pharmacological properties can also be used to enhance other oligonucleotide applications, such as microarrays [4, 6], probes [96, 97], or other functional genomic assays.

1.3 Functional Genomics: the Impact of Synthetic Oligonucleotides

Functional genomics is an area of molecular biology that focuses on the dynamic interactions between proteins and nucleic acids, such as transcription, translation, and signaling pathways [98-105]. Modified oligonucleotides have been critical in advancing our knowledge of the human transcriptome [106], noncoding RNAs [6, 69], alternative splicing [12, 23, 107-09], and protein functions [9, 10, 95, 110, 111]. Some of the common uses of synthetic oligonucleotides include: antisense [10], antigene [8, 9, 13, 40, 90, 112-115], probes for specific nucleic acids [4, 41, 96, 116, 117] , DNAzymes [57-62], aptamers [50-56], modified-siRNAs [118, 119], and PCR reagents [21, 75-77, 120-27].

Sequence-specific control of gene expression is a powerful tool in modern medicine and functional genomics [10]. For more than a decade, people have been trying to modulate cellular functions by targeting DNA and RNA using oligonucleotide-based molecules [40]. These approaches can be separated into two basic categories depending on the nucleic acid being targeted. Oligonucleotide-based molecules targeted to mRNA are referred to as antisense agents [26]. Oligonucleotide-based molecules targeted to chromosomal DNA are referred to as antigene agents [113].

Antisense agents have assisted functional genomics research for many years. The ability to silence a gene and then evaluate the resulting phenotype has greatly increased our collective understanding of protein functions. Likewise, antigene agents have been recently used to study isoform linkages [8, 9], transcription factor binding sites [8], putative open complexes [8, 9], gene activation [92, 128], and multiple other important features of chromosomal DNA.

Our knowledge and understanding of the human genome has increased dramatically over the last few years [129-137]. In fact, the rapid accumulation of genomic data has generated many questions concerning transcription, non-coding transcripts, alternative promoters, alternative polyadenylation, intronic antisense transcription, and locus control regions, among many other topics [129-136, 138-142]. These questions have been a stimulus for newer, more potent, sequence-specific oligonucleotides, which are capable of targeting specific regions of RNA or chromosomal DNA in a reproducible, reliable fashion. Specifically, there is a substantial push for oligonucleotide technologies compatible with high-throughput screens and arrays [4, 6, 123, 143, 144].

Modified oligonucleotides have also been used as probes to detect microRNAs [4], RNA transcripts [96], DNA sequences [5, 145], transcript dynamics [1-3, 41, 146], and levels of gene expression [121, 122]. A specific example is LNA-tiling arrays, which are used to probe for the presence or absence of microRNAs in high-throughput assays [4, 6]. Also, LNAs have been used to detect RNA transcripts in northern blots [96, 97], and have been used to isolate poly(A) RNAs from cell extracts [147]. Another specific example is molecular beacons, which fluoresce when bound to a complementary nucleic acid, and have been used inside cells to measure the dynamics of mRNA transcripts [1-3, 41]. Modified oligonucleotides have also been used to detect the presence of specific gene alleles, and to evaluate their level of expression [75-77, 123, 124, 143, 148]. There are a number of examples available in the primary literature that illustrate the utility of synthetic oligonucleotides. Many of these applications have been thoroughly reviewed [10, 13, 21, 149-51].

Functional genomics has greatly benefited from the development of siRNAs and the RNAi pathway. The RNAi pathway is endogenous to mammalian cells, and can be manipulated to inhibit specific gene products by introducing complementary duplex RNAs into cells [152-155]. siRNAs are now routinely used to study protein function in laboratories around the world [10, 152-156]. Chemical modifications, including LNAs, have been used to improve the specificity and stability of siRNAs [118, 157, 158]. Since either strand of an siRNA can theoretically be loaded into the RISC complex [159], chemical modifications have been used to bias strand loading, as well [157]. In addition, chemical modifications have also been used to improve the therapeutic properties of siRNAs and other oligonucleotide-based drugs [10].

1.4 Therapeutics: the Impact of Synthetic Oligonucleotides

The therapeutic applications of oligonucleotides have been extensively reviewed [10, 31]. Theoretically, antigene molecules could be used to treat any disease caused by the unwanted expression of a gene. This would include viral infections, gene amplification events, cancer, inflammatory diseases, etc. [13, 40, 112, 113, 150]. As previously mentioned in § 1.1, oligonucleotide-based drugs have many unique advantages compared to traditional small-molecule based drugs. Since oligonucleotide-based strategies utilize similar chemistries, entire families of drugs can be synthesized and expected to have similar cell permeability, cellular distributions, biodistributions, pharmacokinetics, etc. This is a very significant advantage over other classes of drugs, such as small molecules, which must be screened and tested individually to characterize pharmacological properties. In addition, many potential off-target effects of

oligonucleotide-based drugs can be computationally predicted relative to small molecules. However, despite ease of synthesis and design, oligonucleotide-based therapeutics was stigmatized early on by clinical failure and poorly controlled research publications [32, 34-36]. These setbacks have substantially slowed the growth of oligonucleotide therapeutics.

Despite these setbacks, academic and commercial laboratories have made many advances in oligonucleotide-based technologies in the past decade. As discussed in §§ 1.2 and 1.3, these advances have significantly improved the biodistribution, cellular uptake, nuclease-resistance, target-specificity, and toxicological aspects of oligonucleotide-based therapeutics [10, 24, 31, 157]. For example, an oligonucleotide-based therapy was recently used to treat West-Nile infected penguins at the Milwaukee County Zoo. AVI BioPharma, a company specialized in antisense technologies, designed and synthesized a drug targeting the virus in a matter of hours and reportedly saved the animals' lives [22]. This incident illustrates how quickly functional oligonucleotide-based drugs can be designed and synthesized. Additionally, with bioterrorism becoming a global concern, oligonucleotide-based drugs offer a relatively rapid treatment for many diseases and bioterrorism threats. To this end, the US Defense Threat Reduction Agency has recently contracted for the construction of antisense drugs against possible biological warfare agents [22].

There are many examples of diseases that have been difficult or impossible to treat using traditional medications. With the advent of high-throughput sequencing and genomic profiling, the molecular basis of many of these diseases is becoming clear. This understanding has led to an increasing enthusiasm for oligonucleotide-based

therapeutics, because they may be the only realistic therapy for targeting noncoding regulatory RNAs [24], such as microRNAs, which have been associated with cancer and other diseases [14, 160-162].

Since oligonucleotides can be extensively altered using various chemical modifications, it is theoretically possible to fine-tune these molecules to target specific sequences, inside specific organelles, inside specific cells, inside specific tissues. In fact, chemical modifications have been shown to change an oligonucleotide's cellular uptake [47], biodistribution (target organs) [163, 164], toxicity [118, 119, 157], and cellular distribution [163]. In addition, new delivery methods are being developed that will allow antisense oligonucleotides to be administered to patients orally [11, 165]. Also, lipid-based delivery systems are also being developed that may help target oligonucleotides to specific organs [166]. Another promising approach to improving the properties of oligonucleotide-based therapeutics is the combination of multiple chemistries. For example, the combination of amino-LNAs, thio-LNAs, and MOEs may yield potent, specific oligonucleotides with a targeted biodistribution, and very low toxicity.

Today, over eighty oligonucleotide-based drugs are at some stage of clinical development (www.clinicaltrials.gov). These have diverse therapeutic applications. For example, AVR118 (Advanced Viral Research Corporation, U.S. Patent 6,921,542) is a solution containing a peptide-(nucleic acid)-peptide conjugate. AVR118 has wound-healing properties and is currently being used as a treatment for clinical cachexia. ISIS Pharmaceuticals is currently testing multiple oligonucleotide-based compounds designed to treat a range of diseases, including: genital warts, CMV retinitis, HIV,

Crohn's disease, multiple sclerosis, diabetes, hypercholesterolemia, diabetic macular edema, and cancer [167]. Most of these drugs are currently in clinical trials and are either phosphothioate-modified oligonucleotides or MOEs.

Another pharmaceutical company, Santaris Pharma, is currently testing multiple LNA-based therapeutics in clinical trials [24]. In 2005, the first LNA-based drug, SPC2996, an anti-cancer agent, entered phase I/II clinical trials. This drug was designed to potently and specifically inhibit Bcl-2, a gene that is commonly overexpressed in cancer, and prevents cells from entering apoptosis. In addition, Santaris Pharma is testing two other LNA-based drugs, SPC2968 and SPC3042 [24]. SPC2968 is designed to inhibit Hif-1α, a gene commonly upregulated in tumors. Finally, SPC3042 is an antisense LNA-based drug designed to inhibit the gene survivin, which prevent cells from entering apoptosis and is generally associated with tumors that are resistant to treatment [24]. Although the majority of oligonucleotide-based drugs in clinical trials are designed to be antisense agents, some are aptamers (such as ARC1779, Archemix Corporation) or immunostimulatory oligonucleotides (such as HEPLISAV or TOLAMBA, Dynavax Technologies Corporation). To date, there are no antigene molecules being tested in clinical trials.

1.5 Antigene Technologies

When DNA is targeted by an oligonucleotide instead of RNA, it is referred to as an antigene approach. Generally speaking, antigene agents are intended to inhibit the process of transcription [8, 43, 90, 112, 113, 115, 168]. Relative to antigene technologies, antisense technologies have flourished, especially since the advent of

RNA interference [26, 40]. For multiple reasons, the growth of antigene technologies has been slow, despite broad therapeutic potential and unique molecular applications [113]. However, there are multiple reasons for pursuing antigene technologies.

Compared to traditional antisense approaches, antigene agents offer several unique advantages [113]. First, antigene agents have, on average, two targets per diploid cell, whereas antisense agents may have in excess of a thousand targets per cell [40, 113]. This is an intrinsic advantage of targeting chromosomal DNA and suggests that antigene agents may be able to inhibit gene expression at reduced concentrations relative to antisense agents [113]. Second, antisense agents can block translation and may deplete mRNA levels via RNase H, but they do not prevent additional mRNA from repopulating the cell. However, antigene agents inhibit gene expression at the level of transcription, so this is not a problem. Furthermore, antigene agents can silence and activate gene expression [92, 128]. Most importantly, antigene agents have a broader range of applications. For example, antigene agents have the potential for “targeted mutagenesis, targeted recombination, and sequence-selective manipulation of genomic DNA” [113]. When comparing antigene and antisense technologies, it is important to realize that these are actually complementary technologies, and that each one affords unique therapeutic opportunities.

Despite the multiple therapeutic opportunities afforded by antigene agents, development has been slow for multiple reasons. Antigene technology has traditionally suffered from an inability to target most DNA sequences [40, 112, 113], poor activity in physiologically-relevant conditions [40, 112, 113, 169], limited knowledge of DNA accessibility [113, 134], and from problems common to all oligonucleotide-based

approaches: cellular delivery, biodistribution, non-specific binding, and biostability [13, 26, 34, 37, 40, 170]. However, recent advances in antigene technology have alleviated many of these problems [9, 43, 112, 113, 171].

The rapid accumulation of genomic data has created questions concerning transcription, non-coding transcripts, alternative promoters, alternative polyadenylation, intronic antisense transcription, and locus control regions, among other topics [129-36, 138-42]. Dr. Thomas Gingeras has recently shown that the human genome contains many overlapping transcription start sites and previously unobserved sense-antisense pairs [132, 133, 140]. Specifically, transcription start sites have been found in exons and 3' untranslated regions (3'UTR) of protein-coding genes [140]. Furthermore, Dr. Gingeras has reported that many previously unobserved overlapping transcripts are also alternatively spliced [132, 140]. The regulatory roles of these transcripts are completely unknown—they are even referred to as "transcripts of unknown function" or TUFs [139]. These discoveries have created an increasing need for sequence-specific agents capable of targeting chromosomal DNA and probing putative transcriptional elements in a physiologically relevant context. Current methods for addressing these questions are lengthy and time-consuming. To address these questions efficiently and succinctly, we need to complement existing antisense technologies with sequence-specific antigene agents. However, the majority of existing antigene agents have severe limitations that reduce their utility for studying the human transcriptome [113].

In 1957, it was first observed that nucleic acids could form triplex structures; the binding mode of nucleobase triads was later described by Karst Hoogsteen [112, 113], and are commonly referred to as Hoogsteen base pairs. Triplexes are formed by a third

oligonucleotide-strand binding in the major groove of duplex DNA in either a parallel or anti-parallel orientation [13, 40, 112, 113, 172, 173]. For a parallel orientation to a purine-rich strand of DNA, the triplex-forming oligonucleotide (TFO) must be pyrimidine-rich and consist of cytosine and thymine. Sequence specificity is achieved because the thymine binds to A:T base pairs and cytosine binds to G:C base pairs. For an anti-parallel orientation to a purine-rich strand of DNA, the TFO must be purine-rich and consist of adenine and guanine. In the anti-parallel orientation, sequence specificity is achieved because adenine binds to A:T base pairs and guanine binds to G:C pairs [112, 113, 172, 174]. The therapeutic potential of TFOs to modulate gene expression by binding to chromosomal DNA was obvious to many, and today TFO-based molecules form the majority of existing data on antigene research [40, 112, 113, 169].

TFOs have been used for multiple applications [113] including site-directed mutagenesis [175, 176], site-specific recombination [177], site-specific inhibition of replication [178], site-specific delivery of DNA intercalators [169, 174], and gene-specific inhibition of expression [40, 179, 180]. TFOs have been successfully used in adult mice [176] and can be delivered on vectors with tissue-specific promoters [172]. However, TFOs are intrinsically limited to a small subset of target sequences, a fact that has hindered clinical applications, but has inspired multiple synthetic nucleotide modifications [13, 40, 43, 112, 113, 150].

Limitations of TFOs include relatively poor activity under physiological conditions, a small target-space restricted to homopurine/homopyrimidine sequences, and self-association [40, 113]. Current research has addressed the majority of these issues [113]. Self-association has been addressed by replacing canonical guanine bases with

6-thioguanine, 7-deazaguanine, or other modified bases [40]. Functionality under physiological conditions can be dramatically improved by conjugating TFOs to DNA-binding small molecules like daunomycin [169], or modifying TFOs with synthetic bases such as LNAs [13, 113, 173, 174]. However, triplex-formation is still intrinsically limited to homopurine/homopyrimidine sequences. This limitation created a need for antigene molecules capable of binding DNA via Watson-Crick base pairing.

Peptide Nucleic Acids (PNAs) were first synthesized by the Buchardt laboratory, which demonstrated that these nucleic acid mimics were capable of displacing a single strand of DNA by binding the complementary strand [181]. By displacing one strand of DNA and binding to the other via Watson-Crick base pairing, or “strand-invasion,” PNAs can theoretically be targeted to any sequence in the genome and are not limited to homopurine/homopyrimidine sites [171]. However, strand-displacement is not favorable under physiological conditions because the repulsive forces of the negatively-charged DNA strands are neutralized by sodium, magnesium, and other ions [182]. To increase the chance of successful strand invasion, it is possible to target regions of DNA expected to be less stable than surrounding areas. In 2002, the Richelson laboratory reported that a mixed-base PNA had antigene effects when administered to mice but the exact mechanism was unclear [114]. Recently, it was demonstrated that multiple mixed-base antigene PNAs were capable of inhibiting gene expression when targeted to a putative open complex, which is expected to form at transcription start sites [9].

The observation that a mixed-base oligonucleotide mimic could inhibit gene expression by targeting chromosomal DNA led directly to the work presented within this dissertation. Specifically, it led to the testing and characterization of mixed-base

antigene LNAs [8]. LNA was an ideal candidate for an antigene molecule as it had high target-affinity, specificity, and nuclease resistance [24]. Furthermore, LNAs were reported to localize to the nucleus [163], and LNA-modified TFOs were shown to have increased activity at the level of chromosomal DNA [173, 174, 183]. Also, several laboratories reported that LNAs could ‘strand-invade’ duplex plasmid DNA [184-186], and LNA-FISH probes were demonstrated to work under nondenaturing conditions [64]. Collectively, these data suggested that mixed-base LNAs could bind chromosomal DNA inside cells and inhibit gene transcription.

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Chapter 2: Initial Characterization of Promoter-Targeted Locked Nucleic Acids

2.1 Introduction

Aberrant gene expression can lead to multiple disease-states [1, 2]. One of the major applications of antigene technologies is to inhibit gene expression at the level of chromosomal DNA and to prevent disease-causing mRNA or proteins from being produced [3, 4]. Antigene agents have many potential uses, ranging from therapeutic applications to probes for functional genomics studies [5]. Although other molecules can be used as antigene agents, oligonucleotide-based agents are well-suited for these applications, since they can be easily designed to target a specific sequence via Watson-Crick base pairing [2] or Hoogsteen base pairing [3].

Over the past two decades, multiple laboratories have tested oligonucleotide-based antigene agents and have made progess toward clinical applications [2, 3, 6]. As discussed in Chapter 1, triplex-forming oligonucleotides are among the most commonly used and referenced of all available antigene agents. These molecules can bind double-stranded DNA in vitro [5] and have recently been tested in cells [1]. But triplex-forming oligonucleotides have a fundamental flaw; they are largely restricted to polypyrimidine-polypurine tracts [7] and cannot be used to target the much of the human genome. The ideal antigene agent should be able to target the entire human genome.

For years, many laboratories have experimented with mixed-base antigene agents, which can theoretically target any DNA sequence [7]. The majority of previous research on antigene agents was conducted *in vitro*, and most studies conducted in intact mammalian cells do not target endogenous chromosomal genes, but artificial

vectors [8-10]. Many of the cell culture studies have relied on pre-hybridizing the antigene molecule to a vector and then simultaneously transfecting both into cells [11]. Such studies test the ability of a molecule to function inside a cell, but do not address the challenges of introducing a molecule into the nucleus and specifically binding to its target. Any clinically useful antigene agent must overcome multiple cellular obstacles to be potent and specific. Compounds must be able to survive serum exposure without degrading, enter cellular membranes, potentially escape endosomes, pass into the nucleus, enter the appropriate nuclear organelle, and hybridize to the targeted chromosomal DNA with sufficient specificity and affinity to potentiate physiological effects [2, 7, 12].

The Corey laboratory has contributed to the field of synthetic antigene agents by demonstrating that they can be introduced into mammalian cells using standard transfection techniques and be used to inhibit expression of an endogenous chromosomal target [12-14]. In 2005, the Corey laboratory inhibited the gene expression of progesterone receptor using PNAs targeted to the transcriptional start site of the gene [12]. This suggests that synthetic, mixed-base oligonucleotides are capable of inhibiting gene expression at the level of chromosomal DNA. These initial studies warranted further investigation with additional synthetic oligonucleotide-based chemistries.

LNAs are ideal candidates for antigene applications. They have a high affinity for RNA and DNA [15-18], and they are potent antisense agents [19]. They are also nuclease-resistant [5] and show high target specificity [15]. However, the outcome of an LNA-based approach to silencing gene expression was not obvious because the

negatively charged backbone of LNAs would be expected to hinder interactions with double-stranded DNA relative to neutral or positively charged PNAs.

My hypothesis was that LNAs would recognize chromosomal DNA and inhibit transcription by physically blocking RNA polymerase II from moving along the DNA (i.e., a steric block mechanism, similar to the mechanism used by antisense LNAs to inhibit translation [20]). My initial project was to characterize the antigene potential of LNA-modified oligonucleotides. Specifically, my goal was to determine if promoter-targeted LNAs could inhibit the expression of human progesterone receptor (hPR), to evaluate the physical and chemical characteristics of functional antigene LNAs, and to demonstrate the generality of antigene LNAs.

2.2 General Experimental Design

Our initial experiments were designed to screen multiple target sites within the promoter of hPR. Specifically, we targeted the open complex of the B isoform of hPR (hPR-B). An open complex is the region from -9 to +2 surrounding a transcriptional start site [21], and is expected to be partially single-stranded with a higher frequency than surrounding regions of the promoter [22]. This makes it a logical target for synthetic antigene agents. The prior results with antigene peptide nucleic acids supported this early hypothesis [12, 14, 23].

hPR was selected as our initial model system for several reasons. First, it was a well-established model system in the Corey laboratory and had previously been used to characterize antigene PNAs [12]. Second, this allowed us to directly compare the efficacy of LNAs with PNAs. Finally, the transcriptional start sites for hPR-A and hPR-B,

the two major isoforms of hPR, were previously characterized by Kastner, et al. and Misrahi, et al. [24, 25] and later verified by the Corey laboratory [26]. Each isoform of the hPR gene has an independent minimal promoter, but some regulatory elements that control precise cellular expression patterns may be shared between the isoforms. However, like all endogenous genes, the precise regulation of hPR is not completely understood. For example, the extent of transcriptional and translational coupling between hPR-A and hPR-B is currently unknown.

Single-stranded LNAs were designed to target the four promoter sequences that were previously shown to be susceptible to antigene PNAs [12]. In addition to these four genomic targets, LNAs were also designed to target other regions of the hPR promoter, including a potential cruciform-forming region [7]. Targeting LNAs to the same model gene and to the same regions previously targeted using peptide nucleic acids allowed for a direct comparison of the antigene potential of these two distinct chemistries. The sequences of the LNAs tested are listed in **Table 2.1**.

LNAs were designed to both the sense and antisense DNA strands of the hPR promoter. Initially, all LNAs were synthesized to be 19 base pairs in length so that the antigene LNAs could be compared with antigene RNAs [26] and PNAs [12]. The 19-base LNAs were synthesized and provided by Sylvie Gabillet and Khalil Arar of Sigma-Proligo. These LNAs were designed to target sequences believed to be physiologically significant within the hPR promoter. The first series of LNAs tested each contained 8 modified LNA bases, evenly distributed throughout the sequence. Synthesis was simplified by minimizing the variety of modified bases (i.e., LNAs did not contain all four modified LNA bases).

Human T47D cells were used as a cancer cell model to test the efficacy of antigene LNAs. This cell line was originally selected because it expresses hPR-B at high levels, making inhibition of gene expression easier to verify [12]. T47D cells were derived from human breast cancer and have been used as a model cell line since 1979 [27]. This cell line was previously used to characterize antigene PNAs [12]. Additional information about T47D cells is available in Chapter 5.

LNAs were introduced into T47D cells using standard cationic lipid-mediated transfections. Antigene PNAs were previously observed to require two separate transfections to achieve high levels of potency [12]. Since we wanted to directly compare the antigene potential of these two chemistries, we minimized the number of variables by using a protocol for LNAs identical to that used for the PNAs. This protocol was not altered during any stage of initial antigene LNA characterization. Specifically, cells were plated 48 hours prior to the initial transfection and then transfected again 96 hours later. Cells were harvested 8 days following the initial transfection and split only once, 72 hours after the initial transfection. The protocol used and the exact timeline of transfections, splitting, and harvesting is described in detail in Chapter 5.

Analysis was based on the hPR-B isoform, which is located 751 bases upstream of the hPR-A isoform transcriptional start site. Cells were harvested using cell dissociation solution and pelleted by centrifugation. Cells were resuspended in proteolysis buffer and lysed using a freeze-thaw cycle at -80°C. Protein concentrations for each cell lysate were assayed using the BCA method. Experimental samples were analyzed using SDS-PAGE and standard immunoblotting. An HRP-conjugated secondary antibody was used to visualize hPR levels via chemiluminescence on film.

The levels of hPR-B were quantified by the intensity of each band using ImageJ, a freely available image analysis program. Detailed protocols are provided in Chapter 5.

2.3 Targeting the B Isoform of Human Progesterone Receptor with LNAs

LNA PR1, PR2, and PR3 were designed to target the putative open complex of the hPR-B isoform. LNA PR4 was designed to target an inverted repeat that could potentially form a cruciform structure in vivo. LNA PR5 and PR6 were both designed to target the putative open complex of the hPR-A isoform. Sequences information for each LNA is provided in **Table 2.1**.

LNAs were tested at two concentrations: 25 nM and 50nM. Preliminary results showed that only LNA PR3 inhibited hPR-B expression at both concentrations. LNA PR3 targeted from -9 to +10 relative to the transcription start site of hPR-B. Notably, this target sequence was the most potent silencing agRNA previously tested [26]. At 50 nM, LNA PR1, which was targeted from -2 to +17 relative to the hPR-B transcription start site, was also capable of inhibiting hPR-B. Both LNA PR1 and PR3 share no complementarity with the hPR-B mRNA and are targeted to the template strand of the chromosomal DNA. LNA PR2 was directed to the non-template strand and targeted -9 to +10 relative to the hPR-B transcription start site (the opposite orientation relative to LNA PR3). LNA PR2 did not inhibit hPR-B gene expression as assayed by immunoblotting.

Initial results suggested that some LNAs were inhibiting gene expression as indicated by reduced levels of hPR-B. Typical results are shown in **Figure 2.1**. These

data indicated that LNAs were capable of inhibiting gene expression in a similar fashion as antigene PNAs. But some LNAs, namely the LNAs targeting the transcription start site of hPR-A, did not inhibit gene expression at all. This suggested that the functional targets of antigene PNA may not correspond to functional antigene LNA targets. The most important implication of these experiments, however, was that LNAs were potent antigene agents and that further experiments were warranted.

2.4 Evaluation of the Potency of LNA PR3

Our preliminary experiments indicated that LNA PR3 was the most potent antigene LNA available. To characterize its potency, we performed dose response experiments and tested multiple concentrations ranging from 100 nM to 1.5 nM. Typical results are shown in **Figure 2.2**. Inhibition was observed as low as 3 nM with an IC_{50} value of ~8 nM. Scrambled LNA controls did not inhibit hPR-B expression at any dosage, suggesting that LNAs were potent and specific antigene agents. These data indicate that antigene LNAs had an IC_{50} value roughly 3-fold less than the IC_{50} value previously observed for antigene PNAs [12] and slightly better than the IC_{50} value found for agRNAs under the same experimental conditions [13]. The IC_{50} value for antigene LNAs could be lower under optimal transfection conditions.

Previously, it was observed that agRNAs could inhibit gene expression after a single transfection (~5 days) [26], but that antigene PNAs required two transfections to achieve potent inhibition of hPR expression (~8 days) [12, 14]. Without optimizing transfection conditions specifically for the LNA chemistry, I was not able to draw conclusions about the amount of time required for antigene LNAs to inhibit gene

expression. However, it was expected that a synthetic agent would not inhibit gene expression as rapidly as a molecule recognized and assisted by endogenous protein mechanisms such as antigene RNAs [23, 26, 28].

2.5 Linkage of Expression of hPR-A and hPR-B Isoforms

During the analysis of Western immunoblots, we observed that both the hPR-B and hPR-A isoforms appeared to be inhibited even though only the hPR-B isoform was being targeted. This observation had previously been made with both antigene RNAs and antigene PNAs [12, 26]. To characterize the linkage between hPR-A and hPR-B, we plotted the levels of hPR-A inhibition as a function of hPR-B gene expression. These data, shown in **Figure 2.3**, were gathered from multiple dose response curves and graphed using Microsoft Excel. These plots showed that the levels of hPR-B and hPR-A were strongly correlated and appeared to vary linearly with antigene agents and exponentially with antisense agents [12, 13, 29].

These data could be explained by two alternative hypotheses: (1) The two isoforms are coupled biologically and inhibition of hPR-A is occurring at the level of translation or transcription via an endogenous pathway. (2) Antigene LNAs are sterically blocking transcription from occurring in a large genomic region. I was unable to reject either hypothesis using Western blots. This is because Western blots show post-translational gene expression, and cannot be used to distinguish translational or transcriptional control in the absence of additional biochemical assays. However, this data is important because it suggests that antigene agents could complement existing

techniques for evaluating biological coupling and feedback mechanisms among protein isoforms.

2.6 Effect of LNA Length on Inhibition of hPR-B Expression

The cost of synthesizing any oligonucleotide is directly proportional to its length and the number of modifications required for its activity. Oligonucleotide length is also critical for potency, specificity, biodistribution, and toxicity. Minimizing the length of an oligonucleotide has obvious disadvantages, including reducing specificity. Since mathematically, shorter sequences can target a larger number of DNA sequences, the potential for toxicity is increased. Longer oligonucleotides also have disadvantages. For example, long oligonucleotides can be less specific than shorter oligonucleotides if their extra bases allow them to compensate for mismatches. Moreover, It has been demonstrated that longer triplex-forming oligonucleotides were often less potent than their shorter counterparts [30]. Therefore, it was important to survey the effect of LNA length gene silencing efficacy.

Specifically for antigene LNAs, it was possible that shorter oligonucleotides could potentially bind to the open complex more readily than bulkier and longer oligonucleotides. This was a reasonable hypothesis since promoters are bound by multiple proteins and certain sequences might not be accessible to longer LNAs.

To test the potency of shorter LNAs, a series of truncated LNAs were designed and synthesized. LNA PR3 was selected as a model LNA since it was the most potent LNA found in preliminary tests. LNAs were truncated form the 3’ end to maximize the coverage of the open complex. Truncated versions of LNA PR3 were synthesized by

Sigma-Proligo. The positions of modified bases were not altered (see **Table 2-1**). LNA PR7 (16 bases), LNA PR8 (13 bases), and LNA PR9 (10 bases) were compared with LNA PR3 using immunoblotting.

The results indicated that truncated antigene LNAs had limited functionality. At 25 nM, LNA PR3, PR7, and PR8 inhibited hPR-B expression. At 50 nM, PR3 and PR7 both inhibited hPR-B expression over 80%. These data indicate that longer LNAs are more potent under these conditions. This suggests that additional bases may increase potency, and that length is an important factor in determining the effectiveness of antigene LNAs. Based on this information, a 17- or 18-base LNA may be more cost-effective than a 19-base antigene LNA, while retaining similar levels of potency.

2.7 Effect of Locked-Base Placement Within an LNA upon Potency

Synthesis of LNA-substituted oligonucleotides is chemically straight-forward since LNA bases share the same chemical backbone as RNA and DNA bases [5, 15, 19, 20, 31, 32]. LNA bases may be introduced at any position of an oligonucleotide, making the number of different LNA designs per target sequence virtually unlimited. However, it is likely that LNA base placement can affect the potency of an antigene LNA and that only a small subset of LNA designs would yield optimal potency for any targeted sequence [33]. This is a reasonable hypothesis since the position and number of LNA base substitutions in an oligonucleotide would be expected to affect target affinity, melting temperature, and biological activity, among other variables.

To evaluate the importance of LNA base placement upon the optimization of antigene LNA design, we systematically varied the location and number of LNA

substitutions. LNA PR3 was selected as a model LNA since it was the most potent LNA found in preliminary tests. LNAs PR17, PR18, PR19, and PR20 were synthesized by Sigma-Proligo and were targeted to -9 to +10 relative to the hPR-B transcription start site. All of these sequences were 19 basepairs in length. By targeting the same DNA sequence, we were able to directly compare the efficiency of multiple LNA designs under identical experimental conditions.

This set of LNAs was designed based on existing antisense LNA motifs that were known to be potent. LNA PR17 was modeled after "gapmers," which were originally designed to recruit RNAse H to mRNA in order to cleave the sequence and reduce gene expression [15]. This design consists of modifying the 3' and 5' extremities of an oligonucleotide, while leaving at least 8 central DNA bases unmodified. Specifically, LNA PR17 has 3 modified LNA bases at both the 3' and 5' ends and 11 unmodified DNA bases in the central region of the oligonucleotide. LNA PR18 was synthesized with only 6 locked bases in the central region of the oligonucleotide. This design would be expected to facilitate binding if the central portion of the oligonucleotide nucleated target recognition. LNA PR19 was synthesized with pairs of locked bases evenly distributed throughout the oligonucleotide (with 9 locked bases in all) and was similar to antisense LNAs designed to sterically inhibit translation [15]. LNA PR20 was similar to LNA PR19, but had a total of 5 locked bases distributed throughout the oligonucleotide. LNA PR17, PR18, PR19, and PR20 were predicted to have similar melting temperatures (see **Table 2.1**).

Two additional LNAs were synthesized and tested in addition to LNAs PR17-20. Like the other LNA designs tested, these designs were also modeled after LNA PR3.

LNA PR10 and LNA PR11 were designed to cover a broad range of T_m values. This was accomplished primarily by increasing or decreasing the number of locked bases in the original LNA PR3 design. LNA PR10 contained only 5 modified bases, whereas LNA PR3 had 8 modified bases. LNA PR11 contained 11 locked bases, and had the highest predicted melting temperature of all of the LNAs tested (see **Table 2.1**).

Each LNA design tested, except for PR11, noticeably inhibited the expression of hPR-B. However, none was as efficient as the initial design, PR3 (**Figure 2.5**). We observed that LNA PR10 inhibited hPR-B expression almost to the extent that PR3 inhibited such expression (see **Figure 3-6**), yet LNA PR11 did not inhibit gene expression at any concentration assayed during this series of experiments.

The lack of efficacy observed with LNA PR11 could be explained by increased intra- and inter-molecular structures causing aggregation among the LNA molecules. This is a reasonable hypothesis based upon the high number of modified bases within LNA PR11. Non-specific Watson-Crick basepairing is a possible cause for the lack of LNA PR11 efficacy; however, no toxicity was observed upon LNA PR11 treatment. Moreover, other LNA studies have demonstrated an increased specificity with the addition of LNA bases, and even fully modified LNAs show decreased non-specific binding [34-38].

In addition to the LNA designs tested and previously reported [13], another design was also tested and found to be toxic to T47D cells at 25 nM and 50 nM concentrations even after additional purification using G-25 spin columns (described in detail in Chapter 5). The design was:

5’+T+G+TCTGGC+C+A+GTCCAC+A+G+C 3’

The locked bases are indicated by a preceding '+' sign. This indicates that LNA base placement can critically affect the toxicity of an oligonucleotide. However, multiple other designs to the same target did not show noticeable toxicity, suggesting that if an individual tests multiple LNA designs, they will find a design that is both non-toxic and highly potent.

These data indicate that a wide range of LNA designs are capable of inhibiting gene expression, but optimal functionality is dependent upon the exact locked-base placement within an oligonucleotide. Although potent LNA designs can be approximated by following known LNA guidelines, the optimal LNA design for any specific sequence would need to be determined empirically.

2.8 Targeting Transcription Factor Binding Sites Within the hPR-B Promoter

Our data suggest that LNAs could be targeted effectively to regions surrounding transcriptional start sites. But the utility of any antigene chemistry is directly proportional to the percent of the human genome it can effectively target. Therefore, following the initial experiments that validated the efficacy of promoter-targeted LNAs, we wanted to target additional sites within the hPR promoter. This included known and predicted transcription factor binding sites. This experiment was significant because the role of multiple transcription factors in gene regulation is not well understood and a tool to specifically inhibit transcription factor binding in a gene-specific manner would be beneficial for modern functional genomics.

Multiple transcription factor binding sites had previously been reported for hPR. In particular, upstream of the hPR-B transcription start site are two previously characterized SP1 sites [39, 40]. Two LNAs were designed to target these sites: LNA PR13 was designed to target the proximal SP1 site, and LNA PR14 was designed to target the distal SP1 site. Downstream of the hPR-B transcription start site was a highly conserved region that had high similarity with known AP-1 binding sites, as determined using available online tools for evaluating transcription factor binding sites. LNA PR15 and LNA PR16 were designed to target this potential transcription factor binding site. Finally, LNA PR12 was designed to target a region previously observed to elicit activation of the hPR gene [41]. Exact sequences and the position of LNA bases are given for these sequences in **Table 2.1**.

Designing oligonucleotides to specifically target a transcription factor binding site in a gene-specific manner is not a simple task, as the sequences surrounding a transcription factor binding site may be conserved in other genes as well. Many transcription factor binding sites contain DNA sequences that are known to cause potential artifacts and off-target effects in antisense molecules. For example, some transcription factors bind guanine-rich sequences, but oligonucleotides with multiple guanines in a row have been reported to cause multiple off-target effects [42-45]. This makes some sequences difficult to target, and prone to artifacts.

LNAs PR12, PR13, PR15, and PR16 were tested but showed no activity by Western blotting analysis. Typical results are shown in **Figure 2.7**. But LNA PR14, which was targeted to the distal SP1 site [39], showed low (~40%), but reproducible, inhibition of hPR-B gene expression. This result suggests that transcription factor

binding sites can be targeted by antigene LNAs. It is possible that an optimized sequence could achieve more potent inhibition of gene expression. These data also suggest that the open complex region near the transcriptional start site of genes may be particularly vulnerable to antigene LNAs compared to other conserved regions of the promoter.

2.9 Inhibition of Human Androgen Receptor using Antigene LNAs

The efficacy of antigene LNAs was validated in the hPR gene, but it was unknown if additional genes could be easily targeted by LNAs. We reasoned that if LNAs were general antigene agents, we would be able to target a second gene with high efficacy. To test this hypothesis, we selected the human androgen receptor (hAR) because it had previously been used as a model system for promoter-targeted RNAs [26] and we could use agRNAs as positive controls.

LNA AR1, AR2, and AR3 were designed to target the template strand of the hAR gene, and to overlap the putative open complex region of hAR. LNA AR1 showed slight inhibition during some initial experiments. Although not consistent, this inhibition did suggest that this sequence could be optimized for further testing. LNA AR2 did not work at any concentration tested. Upon testing, LNA AR3 showed potent and reproducible inhibition of AR expression as assayed by Western blotting (**Figure 2.8**). The IC_{50} value of AR3 was determined to be approximately 12 nM through multiple dose response curves. This result was intriguing since the analogous antigene RNA to this sequence was nonfunctional, and it exemplifies critical differences in targetable sequences and mechanisms for inhibiting gene expression between these two

chemistries. This series of experiments demonstrates that additional genes can be successfully inhibited using antigene LNAs in a straight-forward manner.

2.10 Antigene LNAs are Functional in MCF7 Cells

The expression of any particular gene in a cell will vary depending on environmental conditions and the tissue from which the sample originated. This generalization is exemplified with hPR, which is not significantly expressed in many cell-lines (NCBI GEO). For the convenience of high signal-to-noise ratios, T47D cells were originally selected to characterize gene-silencing agents. The inhibition of hPR is easy to qualify and quantify in T47D cells as hPR is expressed at very high levels in T47D cells. However, the level of endogenous gene expression may be a variable that determines the efficacy of an antigene LNA. This is a reasonable hypothesis as other antigene agents have shown expression-dependent activity [46]. To test this hypothesis, an additional cell line was needed that expressed hPR, but at much lower levels than that expressed in T47D cells. MCF7 cells, another human breast cancer cell line, were selected for this purpose.

The MCF7 cell-line was first established in 1973 [47] and expresses hPR at mRNA levels approximately ten-fold lower than that observed in T47D cells [41]. Previously, antigene RNAs were tested in MCF7 cells and were found to be incapable of silencing gene expression to any extent in that cell line [41]. However, antigene PNA-peptides were capable of inhibiting hPR expression in MCF7 cells, suggesting that gene silencing was possible [14]. To determine if antigene LNAs were capable of

inhibiting gene expression in a cell line that expressed hPR at much lower levels, we introduced LNA PR3 into MCF7 cells using cationic lipid.

Much like antigene PNA-peptides, antigene LNAs reproducibly inhibited hPR-B levels (**Figure 2.9**) in MCF7 cells and T47D cells. The level of inhibition achieved was significantly less than in T47D cells. Dose response curves for hPR-B in MCF7 cells suggests that LNA PR3 has an approximate IC_{50} value of 20 nM in MCF7 cells and a maximum efficacy of 50% inhibition (**Figure 2.9**).

These data suggest that the level of gene expression directly affects the potency of antigene LNAs. This conclusion is reasonable since highly expressed regions are single-stranded with a higher probability, which would be expected to facilitate initial LNA binding according to our proposed model. This interpretation is also consistent with previously reported “suicide inhibitors,” which are antigene agents that work best in highly transcribed genes [46]. This trend has also been observed for triplex-forming oligonucleotides [48].

2.11 Comparison of Antigene LNAs with other Antigene Approaches

The Corey laboratory has demonstrated that multiple synthetic antigene agents are capable of inhibiting gene expression. Excluding LNAs, these agents include antigene PNAs [12], antigene PNA-peptides [14], and antigene RNAs [26]. The data presented in this chapter suggest that LNAs are also potent antigene agents and that the open complex region surrounding the transcriptional start sites of hPR and hAR are especially accessible to antigene LNAs. These data also indicate that antigene LNAs

can be designed to additional genes and that transcription factor binding sites are viable targets for further antigene LNA development.

Although multiple synthetic antigene agents can be used to achieve gene inhibition, antigene LNA has several advantages over the other available chemistries. For example, PNAs and LNAs have substantially different backbone chemistries. LNAs have a negatively charged phosphodiester backbone, whereas PNAs have an uncharged amide backbone. The uncharged amide backbone of PNAs causes this molecule to aggregate and to be less soluble than LNAs [49]. Furthermore, LNA usage and handling is much simpler than PNAs (compare the methods as detailed in Chapter 5). Although PNA-peptides appear to be easier to transfect into cells, it is important to remember that LNAs can be introduced intravenously into animals without the need for lipid or further chemical modifications [50]. Also, peptide-mediated cellular delivery is poorly understood and may have unknown off-target effects [49].

Antigene RNAs are very potent molecules and can inhibit gene expression rapidly, reproducibly, and potently [26, 29]. However, the silencing capabilities of antigene RNAs are less predictable relative to LNAs since in some cells lines agRNAs activate gene expression and in other cell lines agRNAs silence gene expression [41]. Also, since agRNAs can enter numerous endogenous pathways including the RISC pathway, the potential for overwhelming cellular mechanisms is present [51]. In addition, antigene RNAs are double-stranded and can potentially target two different sets of sequences [52], each with different potential off-target effects. Small RNAs have also been reported to have off-target effects based on limited complementarity to non-targeted sequences [53] and to have different effects on gene expression depending on

cell line [41] and cell cycle [54]. Furthermore, dsRNAs have the potential to affect genes at the level of both transcription [26, 28, 41, 55] and translation [51]. These characteristics make small RNAs relatively unpredictable compared to synthetic molecules that do not specifically interact with endogenous biological pathways. For these reasons, antigene LNAs stand out among other antigene agents for their potency, efficiency, ease-of-use, specificity, and their lack of specific interference with known endogenous pathways.

2.12 Concluding Remarks

These data suggest that antigene LNAs are a potent and general strategy for silencing gene expression. These data were collected under conditions optimized for antigene PNAs, and not antigene LNAs. This decision was made to directly compare LNA and PNA efficiency. At the time, it was assumed that synthetic antigene agents would require two consecutive transfections to inhibit gene expression. As discussed in later chapters, under more optimal conditions, antigene LNAs can inhibit gene expression in only a single transfection and can rival antigene RNAs in speed and efficacy.

Although antigene LNAs were tested for potency, efficiency, and design, among other factors, the actual mechanism was not validated. The simplest possible mechanism to envision is a simple steric block mechanism as previously proposed for antigene PNAs [12]. In this mechanism, an antigene LNA would bind to single-stranded DNA during replication or transcription and would associated with a specific DNA sequence. This would prevent RNA polymerase II from binding and initiating

transcription, resulting in decreased gene expression. This mechanism is supported by *in vitro* data from other laboratories collected for TFOs [9], PNAs [56, 57], and LNAs [58].

Mechanistically, the data presented within this chapter support an LNA-mediated steric block model of gene inhibition. To confirm this mechanism, it would be necessary to show that LNAs inhibit gene expression at the level of transcription. The data presented within this chapter do not address if mRNA levels are changing, if transcription is stopped, or if LNAs are physically associating with chromosomal DNA. It is plausible that LNAs are interfering with the cellular function of one or more of the antisense transcripts present within the hPR promoter and only indirectly silencing the expression of hPR. Although these data collectively support an antigene model occurring through a steric block mechanism, we had not confirmed whether these promoter-targeted LNAs were actually working in this manner.

2.13 Conclusion

We have demonstrated that LNAs can be used to target regions within promoters and inhibit gene expression. Specifically, we showed that antigene LNAs can inhibit the expression of hPR and hAR. This body of work comprised the first extensive study of a mixed-base antigene agent in multiple human cell lines. No other published study had tested such a large number of sequences, designs, cell lines, genes, or functionally significant targets. This work established LNA as a potent antigene technology that could be used in modern functional genomics with potential therapeutic applications.

TABLE 2.1: Shows all of the antigene LNAs used during the experiments presented in this chapter. LNAs denoted by 'PR' and 'AR' target the specified sequences to the human progesterone receptor or the androgen receptor respectively. Each LNA used was single-stranded and capable of targeting one strand of chromosomal DNA. The targeted strand of DNA is denoted template or nontemplate.

LNA	Target	DNA Strand	Sequence
LNA PR1	-2/+17	(Template)	CCagtCCaCagCtgtCaCt
LNA PR2	-9/+10	(Nontemplate)	gCTgTggaCTggCCagaCa
LNA PR3	-9/+10	(Template)	tGtctGGccAGtccAcAGc
LNA PR4	-47/-28	(Template)	TTcccTccTcccTGGaGac
LNA PR5	+145/+126	(Template)	tGaGctGaaGGcaaaGGGt
LNA PR6	+137/+156	(Template)	TCaTgaCTgagCTgaaggC
LNA PR7	-9/+7	(Template)	tGtctGGccAGtccAc
LNA PR8	-9/+4	(Template)	tGtctGGccAGtc
LNA PR9	-9/+1	(Template)	tGtctGGccA
LNA PR10	-9/+10	(Template)	tGtctGGccaGtccacaGc
LNA PR11	-9/+10	(Template)	tGTcTGGccAGTccAcAGc
LNA PR12	-11/+8	(Template)	gCTgtCTggCcagTccACa
LNA PR13	-52/-33	(Template)	TtTGggCGggGccTccCTa
LNA PR14	-70/-51	(Template)	aTtGgGgTaGggAgGggCt
LNA PR15	+90/+109	(Template)	gCTttCActTgtCaTtTGa
LNA PR16	+106/+125	(Template)	TgAgtgAaaTCtaCaaCcC
LNA PR17	-9 /+10	(Template)	TGTctggccagtccacAGC
LNA PR18	-9/+10	(Template)	tgtctgGCCaGTCcacagc
LNA PR19	-9/+10	(Template)	tgTCtgGCcaGTccACagC
LNA PR20	-9/+10	(Template)	tGtcTggcCagtCcacAgc
LNA AR1	-8/ +9	(Template)	cACCtccCAgcgCcccCTc
LNA AR2	-13/ +6	(Template)	CTctcCaccTCccaGCgcC
LNA AR3	-24/ -6	(Template)	gTTgcATttGctctcCACc
LNAscr1	N/A	N/A	cCacaGCtgTCcagTtGGc
LNA HCV	N/A	N/A	CTAcgaGaCctCccGggGC
LNAscr2	N/A	N/A	cAgttGTcaGCtggCccac

Figure 2.1 shows our initial series of experiments designed to analyze the antigene potential of LNAs. (A) A model of the hPR gene with the relative positions of biologically important sequences and the relative target sites for the individual LNAs tested. Arrows denote the targeted DNA strand. Arrows to the right represent LNAs targeting the template strand of the DNA, and arrows to the left denote LNAs targeting the nontemplate strand. (B-C) Western analysis of multiple LNAs at 25 nM (B) and at 50 nM (C).

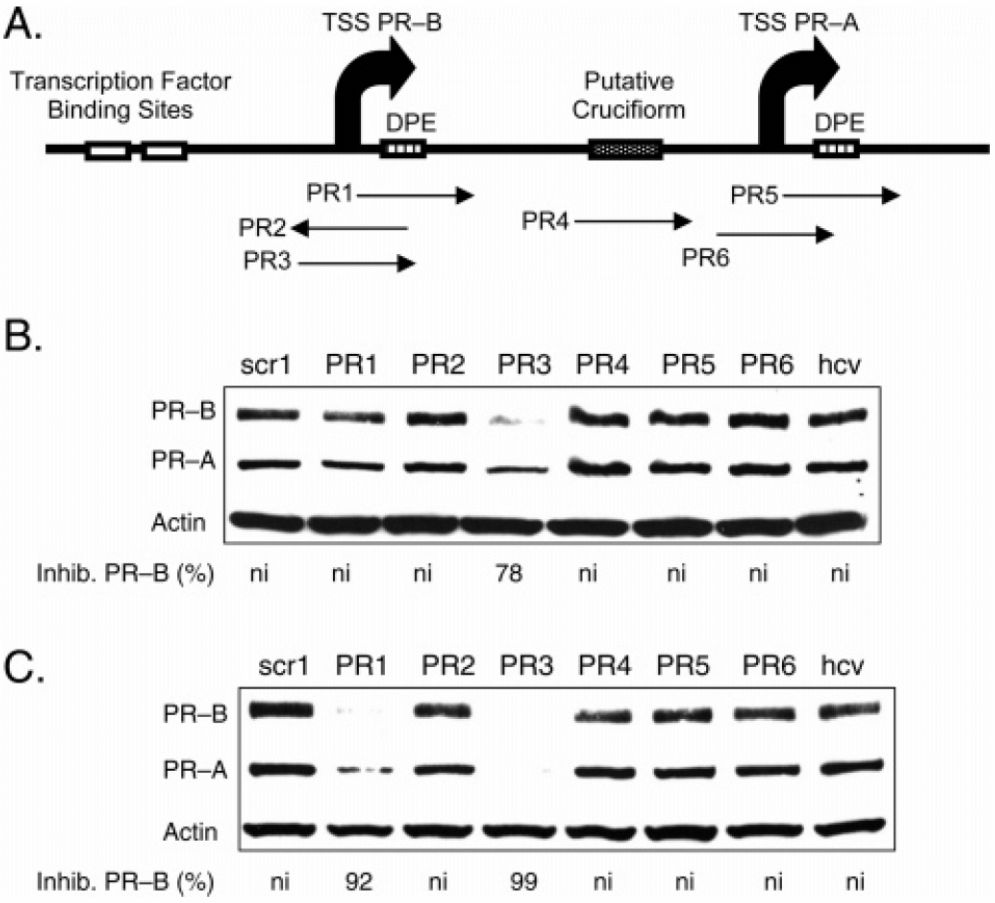


Figure 2.2 shows Western analysis of the indicated treatment at varying concentrations. (A) LNA PR3 inhibits gene expression with an IC_{50} value of approximately 8 nM. (B) A scrambled LNA control does not inhibit hPR expression at any concentration tested. (C) An agRNA targeting -9 to +10 relative to the hPR-B transcription start site potently inhibits hPR expression with an IC_{50} value of approximately 12 nM.

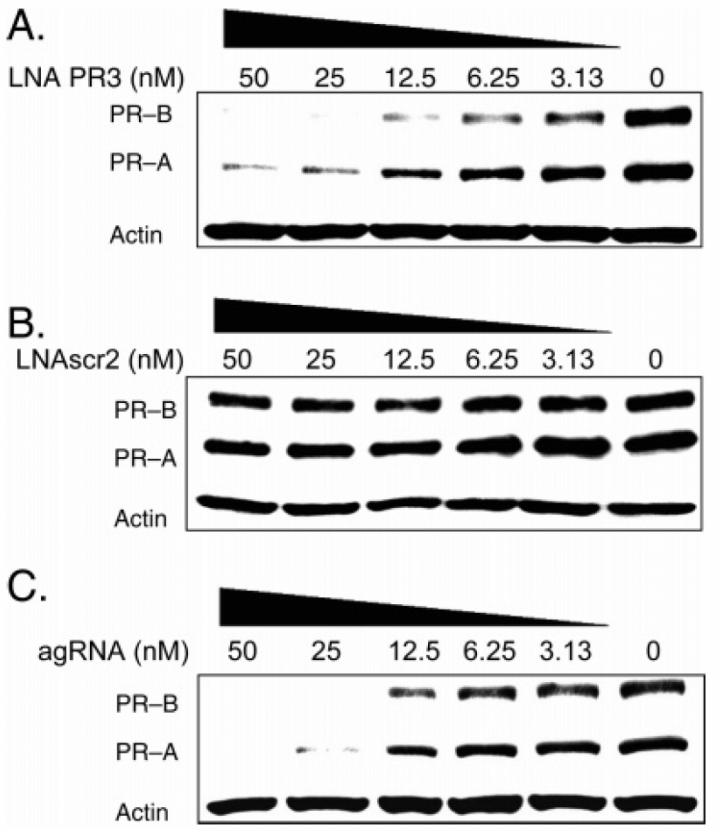


Figure 2.3 shows the relative levels of hPR-A as a function of hPR-B levels upon the indicated treatments. (A-C) Antigene agents consistently resulted in a linear correlation between the levels of hPR-B and hPR-A. (D) Antisense agents reproducibly yielded an exponential relationship between these two isoforms.

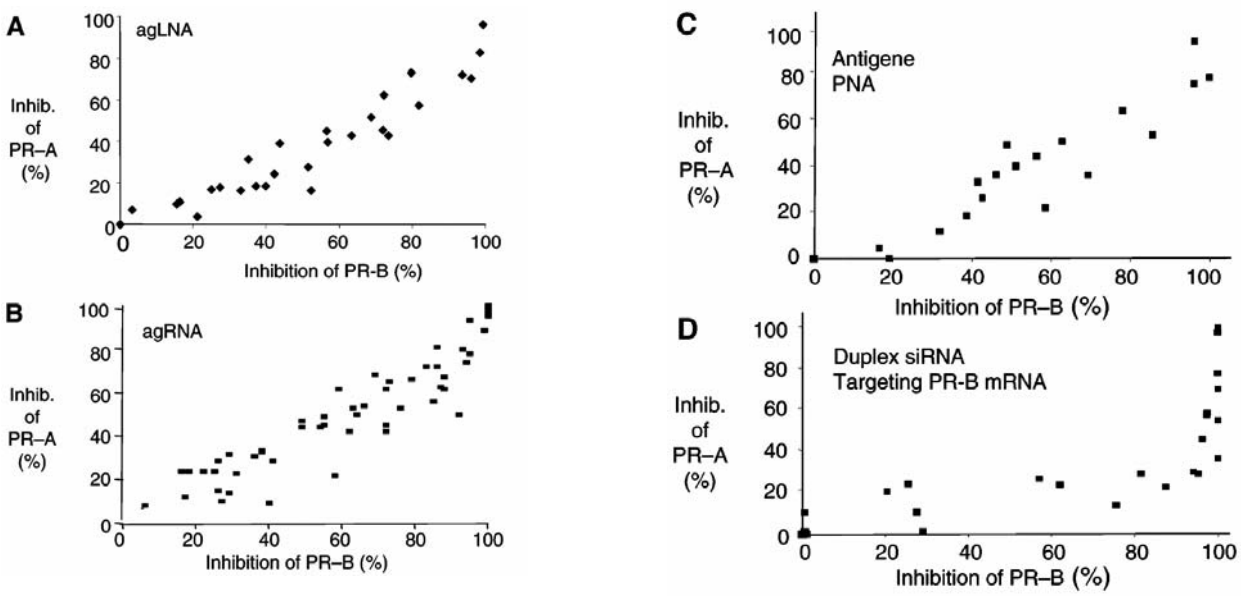


FIGURE 2.4: LNA Length Experiment. Figure 2.4 shows the effect of truncating LNA PR3 as described in the text. These LNAs were tested at 25 nM (A) and at 50 nM (B).

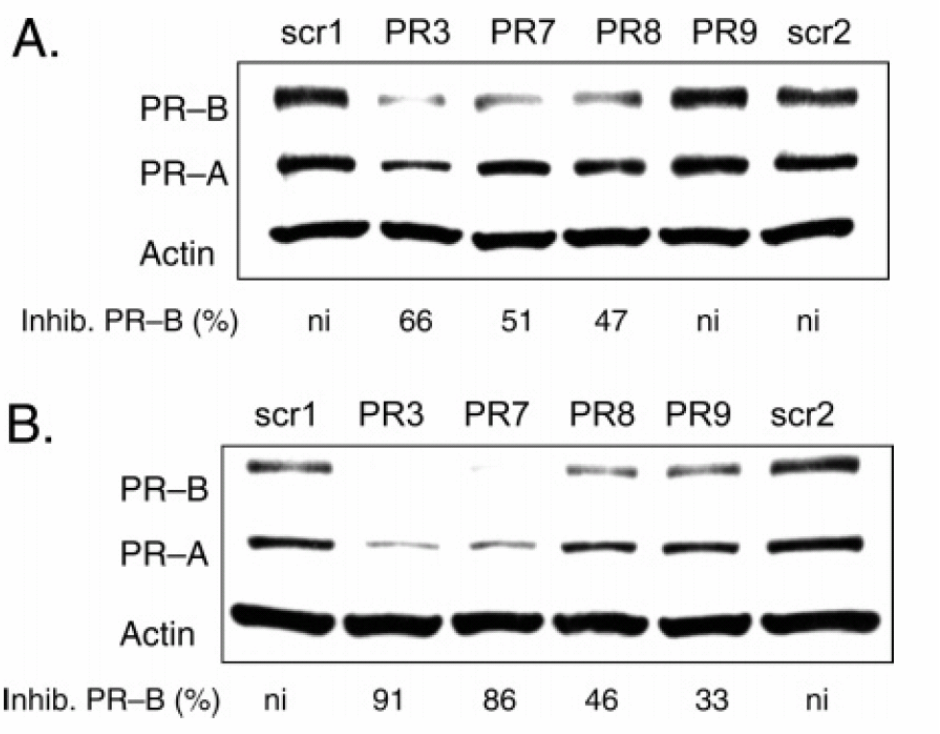


FIGURE 2.5: Effect of Locked-base Placement upon the Potency of antigene LNAs. Figure 2.5 shows Western analysis of hPR levels in T47D following treatment with LNAs of various designs targeted to the -9 to +10 region of the template strand surrounding the hPR-B transcription start site. This series of experiments were performed at 50nM and suggest that a wide range of LNA designs are functional and potent.

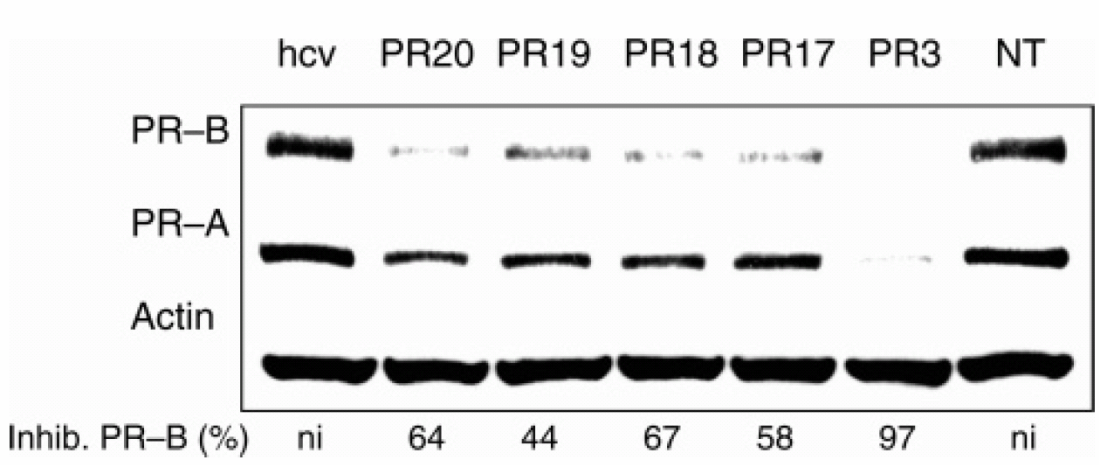


FIGURE 2.6: Evaluation of Tm Minimizing and Maximizing Designs upon Antigene LNA Potency. Figure 2.6 shows representative data from a series of experiments designed to ascertain the effect of melting temperature on the potency of antigene LNAs. LNAs were tested at 25 nM (A) and 50 nM (B) concentrations. LNA PR3 potently inhibited hPR-B at both concentrations. LNA PR10 inhibited hPR-B expression at higher concentrations. LNA PR11 did not work at any concentration tested.

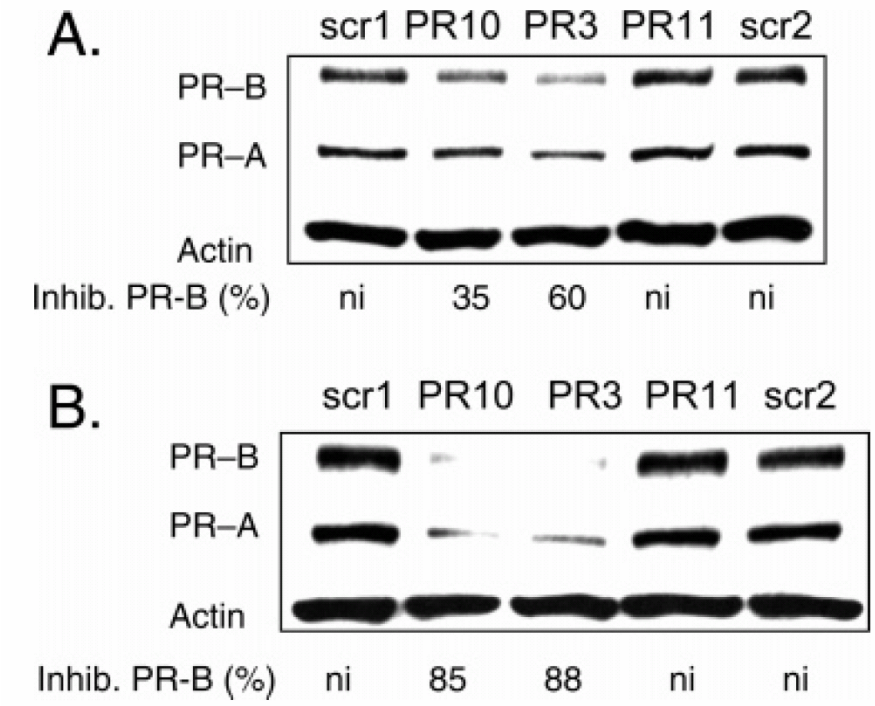


FIGURE 2.7: Evaluation of Antigene LNAs targeting Transcription Factor Binding Sites. Figure 2.7 shows representative data from a series of experiments designed to test whether or not antigene LNAs could potently inhibit expression when targeted to transcription factor binding sites. (A) A model of the relative location of the LNAs used in this series of experiment relative to the hPR-B transcription start site. (B) Western data showing the LNA PR14, which targeted the distal SP1 site, appeared to have reproducible, but limited functionality.

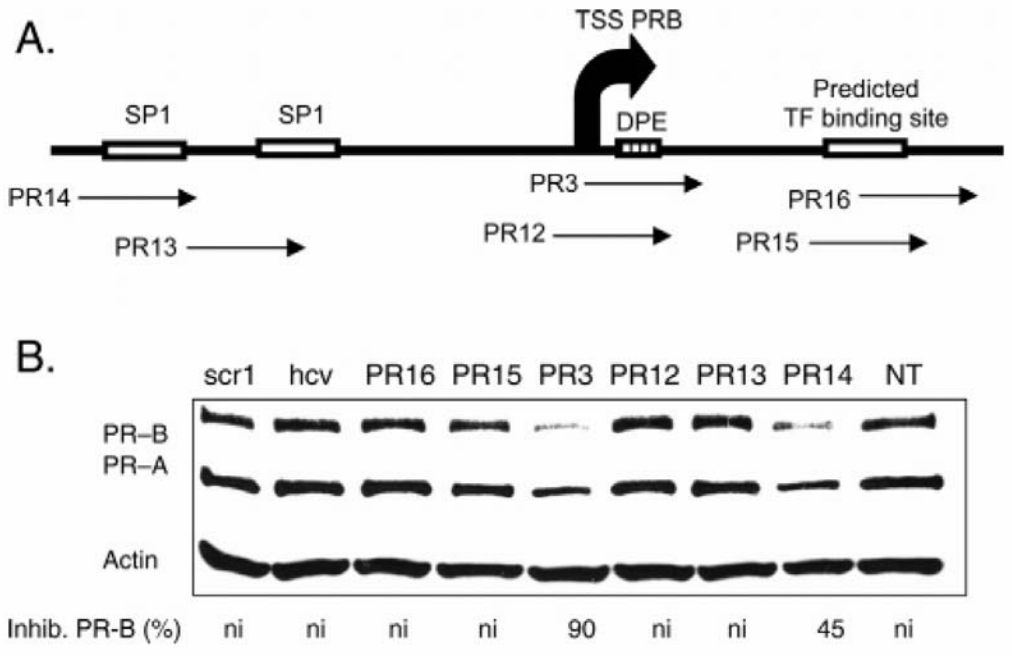


FIGURE 2.8: Antigene LNAs Targeting the Human Androgen Receptor. Figure 2.8 shows Western blots representative of a series of experiments designed to assay the ability of antigene LNAs to inhibit gene expression of androgen receptor. (A) Shows that LNA AR3 can inhibit AR expression potently. (B) Shows that LNA AR3 has an IC_{50} value under 25nM.

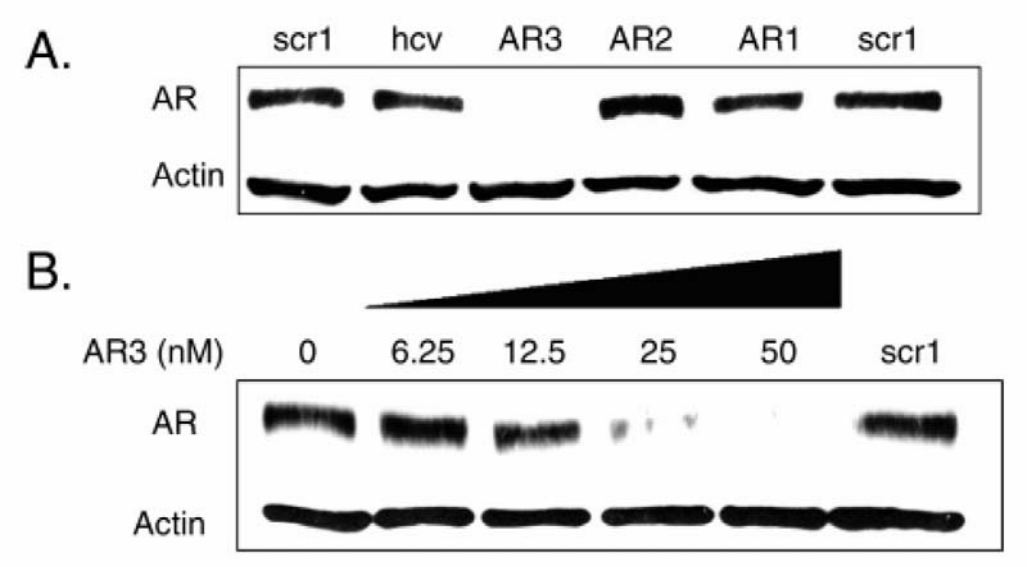
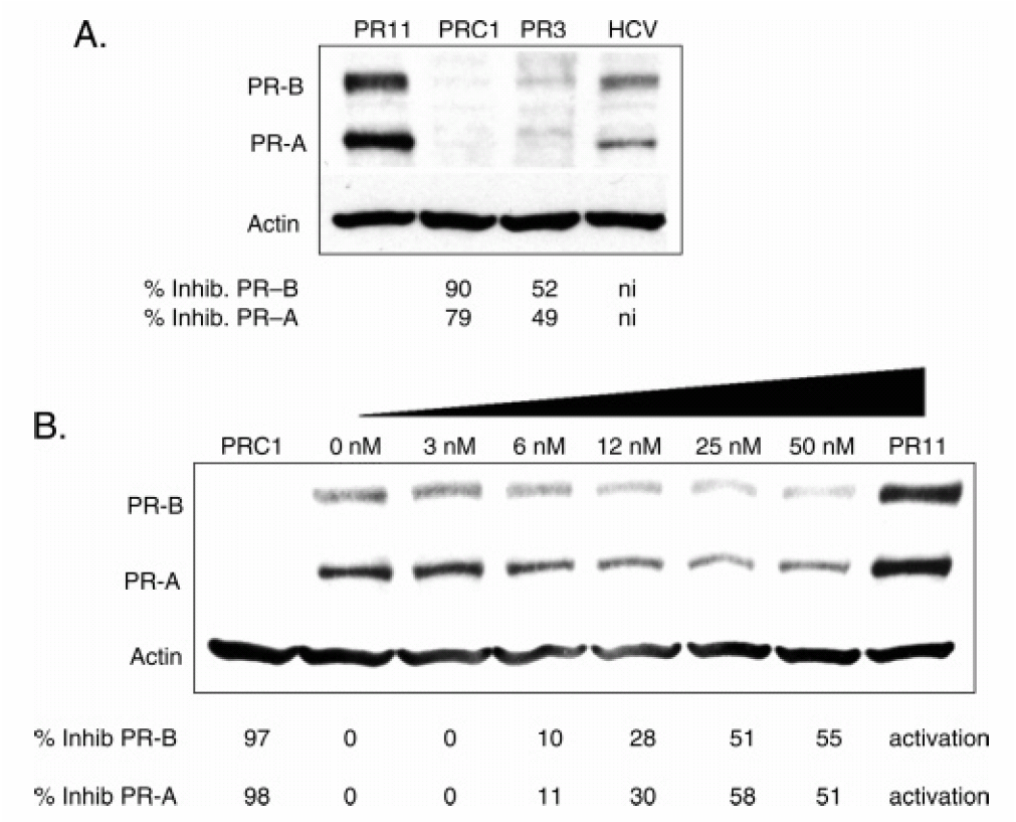


FIGURE 2.9: Antigene LNAs are Functional in MCF7 Cells. Figure 2.9 shows Western analysis of MCF7 cells after treatment with antigene LNAs targeting the human progesterone receptor. (A) Shows that LNA PR3 can actively inhibit gene expression in T47D cells. (B) Dose response curve with LNA PR3 in MCF7 cells. LNA PR3 was found to have an approximate IC_{50} value of 25nM and a maximum efficiency of approximately 50% in MCF7 cells. PR11 is an activating agRNA that was used as a control in this series of experiments.



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Chapter 3: Establishing the Genomic Target and Mechanism of Antigene LNAs

3.1 Probing the Antigene LNA Mechanism

Collectively, the data presented in Chapter 2 supports a mechanism of antigene LNA activity where LNAs bind directly to chromosomal DNA and sterically block RNA polymerase II from associating with the occluded DNA sequence. This mechanism is commonly referred to as a 'steric-block' and is depicted in **Figure 3.1**. A steric block model of gene inhibition is supported by *in vitro* data for TFOs [1], PNAs [2, 3], and LNAs [4].

Recently, the Corey laboratory has shown that multiple antisense transcripts are expressed in the hPR promoter [5]. In fact, recent studies indicate that antisense transcription is common in most human genes [6-8]. This is important because antisense transcripts provide an alternate target for synthetic antigene agents. Since the existence of these transcripts was previously unknown, they were not considered in earlier hypothetical models of antigene agent activity [9]. Although the steric-block model provides the most direct explanation for antigene LNA activity (**Figure 3.1**), it is possible that antisense transcripts play a role in the mechanism of synthetic antigene agents, such as LNAs.

To further investigate the mechanism of antigene LNAs, I addressed three critical questions that were not tested in our initial characterization of antigene LNAs [10]. First, the effect of antigene LNAs on mRNA levels was not previously evaluated [10]. The level of mRNA would be expected to decrease if LNAs inhibit transcription. Second, it was not confirmed whether or not LNAs were binding to chromosomal DNA inside living

cells, as expected in our model (**Figure 3.1**). The third and most important question was whether or not LNAs were actually inhibiting transcription. The work presented in this chapter is intended to address these critical questions, and to further our understanding of the antigene LNA mechanism.

3.2 Non-Coding RNAs and Mammalian Promoter Architecture

Recent evidence indicates that the mammalian transcriptome is highly complex. Many human genes have multiple transcription start sites within exons and introns that can be oriented in either the sense or antisense direction [6-8]. Although non-coding RNAs have been studied for decades [11, 12], it was not until the advent of high-throughput microarray technologies that the sheer number of non-coding RNAs was appreciated [13, 14]. Microarray technologies have also allowed for genome-wide surveys of mammalian promoter architecture [15]. These studies have revealed that transcriptional start sites are common throughout a gene, including within the exons and introns. CAGE analysis was used to demonstrate that distal promoters, up to >100k bases upstream of a gene, rather than proximal promoters, were not uncommon for most protein-coding and non-coding genes [8]. By conducting similar studies in multiple tissues and cell types, it was shown that alternative promoters are differentially expressed in a tissue-dependent manner [7, 8, 16]. Although scientists are beginning to appreciate the vast complexity of the mammalian transcriptome, the functions and endogenous regulation of these transcripts are poorly understood and have even been referred to as transcripts of unknown function, or “TUFs” [17].

Non-coding RNAs are versatile in function and are involved in a wide assortment of cellular pathways including translation, transcription, mRNA stability, mRNA degradation, cellular stress, imprinting, dosage compensation, modulation of protein function, cell fate, development, and binding to regulatory metabolites [12, 17]. Yet there is evidence that many non-coding RNAs may simply be the result of transcriptional noise. Specifically, Nobrega, et al., using mice as a model system, deleted over one megabase of non-coding sequences conserved between rodents and primates and reported that the mice were viable and had no obvious phenotypes [17, 18]. But similar experiments have been performed on microRNAs without yielding phenotypes due to the redundant layers of control within genomes [17, 19]. These genome-wide and whole-organism experiments have not shed light on the universal function of non-coding RNAs. However, studies of individual systems have led to a deeper appreciation for the roles that non-coding RNAs can play.

Although there are many examples of non-coding RNAs playing substantial roles in multiple cellular pathways [12], the role they play in transcription is the most relevant for this context. In 2004, Espinoza, et al. and Allen, et al. reported that a small non-coding RNA called B2 could repress transcription in response to heat shock by binding to RNA polymerase II [20, 21]. These studies show that non-coding RNAs can regulate mRNA levels in response to environmental stress. Another study conducted by the Junghas laboratory demonstrated that RNAs can bind transcription factors and influence transcriptional levels [22]. In 2007, Martianov, et al. reported that a small non-coding transcript in the dihydrofolate reductase gene repressed transcription by forming a stable triple-helix with the major promoter [23]. This report suggests that stretches of

pyrimidine and purine bases could be susceptible to repression via a similar mechanism involving non-coding RNAs. Notably, the Stutz laboratory has shown that the stabilization of antisense RNAs can induce transcriptional gene silencing in S.cerevisiae [24]. This particular study demonstrated that PHO84 antisense transcript became increasing stable as the cell aged due to a decrease in the activity of a regulatory protein. In 2008, Mariner, et al. reported that certain SINE-derived RNAs could bind to RNA polymerase II and that one such RNA could repress transcription during heat shock [25]. The prevalence of SINEs in the human genome has been a long-standing curiosity; this report suggests that certain non-coding RNAs play substantial roles in transcriptional regulation and that many can directly interact with RNA polymerase II. Alternative mechanisms for the prevalence of non-coding transcription have been proposed. In particular, the Orlando laboratory has proposed that non-coding transcripts play a critical role in maintaining active transcription via histone modifications [26]. The purpose of non-coding transcription has also been associated with cellular organization within the nucleus [27].

The mammalian transcriptome is complex, and the limitations of CAGE, RACE, and microarrays are becoming increasingly obvious. Yet the scientific community has learned a great deal about both the prevalence of transcription and the architecture of promoters in the past few years. Despite these gains, our understanding of the functional significance of distal transcriptional start sites, antisense transcripts, non-coding RNAs, transcriptional start sites within exons and introns, and aberrant transcription is still in its infancy. However, it is clear that non-coding RNAs can play a

substantial role in transcription and could be a potential substrate for antigene oligonucleotides.

3.3 General Experimental Design

To address how antigene LNAs affect gene expression, we used our previously established model system, hPR [9, 10, 28-32]. The transcriptional start sites for hPR-A and hPR-B, the two major isoforms of hPR, were previously characterized by Kastner, et al.[33], Misrahi, et al. [34], and were also verified by the Corey laboratory [31]. Furthermore, the Corey laboratory has analyzed the hPR gene for alternative transcriptional start sites and antisense transcripts in T47D cells [5].

Specifically, we targeted the transcription start site of hPR-B. Previously, we observed that LNA B2, which targets the template strand from -9 to +10, was the most potent antigene LNA tested [10]. Therefore, we used LNA B2 to probe the mechanism of antigene LNAs. LNA B2 overlaps the open complex (the region from -9 to +2 surrounding a transcriptional start site [35]), which is expected to be partially single-stranded with a higher frequency than surrounding regions of the promoter [36]. The sequences of the LNAs used for these experiments are given in **Table 3.1**. LNAs were synthesized and provided by Sigma-Proligo and were designed to both the template and non-template strands of the hPR promoter.

LNAs were introduced into T47D cells using standard cationic lipid-mediated transfections. Previously, we used a protocol optimized for antigene PNAs that required two consecutive transfections to achieve potent inhibition [9]. The work presented within this chapter was performed using a protocol optimized for antigene LNAs. This

protocol requires a single cationic lipid-mediated transfection to achieve potent inhibition. Cells were plated 48 hours prior to the initial transfection and harvested for mRNA 72 to 96 hours later. Protein samples were harvested 120 to 144 hours post-transfection. Following treatment, transcription was analyzed using the nuclear run-on assay and chromatin immunoprecipitation of RNA polymerase II. RNA levels were examined using QPCR. A detailed description of these protocols is provided in Chapter 5.

3.4 Antigene LNAs Decrease mRNA Levels of the hPR and hAR

Our initial experiments were designed to evaluate the effect of antigene LNAs on the mRNA levels of hPR and hAR. Although antigene LNAs can be designed as gapmers [37], the LNA designs we used for these experiments are not capable of recruiting RNase H and would therefore be unable to decrease mRNA through known antisense mechanisms. If single-stranded, template-directed LNAs worked through a steric block mechanism (**Figure 3.1**), we would expect mRNA levels to decrease.

To test this hypothesis, LNA AR24, B2, B1, and HCV were synthesized by Sigma-Proligo. LNA B2 and LNA AR24 were previously shown to potently decrease protein levels of hPR and hAR, respectively [10]. LNA AR24 is designed to target the template strand of the hAR gene 24 bases upstream from the transcriptional start site. LNA B2 is designed to target hPR from -9 to +10 across the hPR-B transcriptional start site and is also targeted to the template strand of the gene. LNA B1 is designed to target hPR-B from -9 to +10, but it is directed to the nontemplate strand of the hPR

gene. Finally, LNA HCV is a negative control that is directed to a sequence in the HCV genome and is not specific for any human targets.

LNAs were introduced into T47D cells at 50 nM concentrations using RNAiMAX according to the manufacturer's instructions. All samples were harvested 72 to 96 hours post-transfection. Levels of mRNA were assayed using QPCR as described in Chapter 5. Error bars represent the standard deviation of at least 3 biological replicates, which were technically replicated at least twice. All mRNA levels were normalized to a loading control, GAPDH, and LNA HCV, our negative LNA control.

As shown in **Figure 3.3**, these data indicate that antigene LNA B2 can potently reduce hPR-B mRNA levels. Antigene LNA B1, however, does not appear to inhibit hPR-B levels, which is consistent with previously reported Western analysis [10]. As shown in **Figure 3.3**, we also observed reproducible and statistically significant inhibition of hAR mRNA using LNA AR24. Since the inhibition was not as striking as the inhibition observed for hPR, we performed dosage-compensation experiments to confirm that the inhibition was real. Compiled data is shown in **Figure 3.4**. These data indicate that LNA AR24 is working in a dose-dependent manner. LNA AR24 may not be the optimal LNA for this hAR, and it is possible that increased inhibition of hAR expression may be achievable by testing additional LNAs.

The most important conclusion from these experiments is that antigene LNAs decrease mRNA levels. These observations are consistent with a steric block mechanism and suggest that LNAs affect genes at the level of transcription. However, these data are not sufficient to discount alternative mechanisms involving antisense

RNAs. To address this possibility, we sought to determine whether antigene LNAs were capable of physically associating with chromosomal DNA inside cells.

3.5 Antigene LNAs Physically Associate with Chromosomal DNA

LNA binding to double-stranded DNA is not without precedent. In 2003 and 2004, Silahtaroglu reported that LNA-FISH probes could hybridize to specific repeats under non-denaturing conditions, suggesting that LNA can strand-invade DNA [38, 39]. In 2005, the Pedersen laboratory published that short LNAs strand-invade plasmid DNA and inhibit transcription [4]. There are no studies demonstrating that mixed-base antigene LNAs can physically associate with chromosomal DNA in living cells. It was therefore critical to evaluate LNA binding in a whole-cell system.

The steric block model implies that an antigene LNA physically associates with chromosomal DNA to inhibit gene transcription (**Figure 3.1**). We directly tested this hypothesis by isolating and analyzing LNA-bound substrates from treated cells. To accomplish this, we used 3' biotin-modified LNAs provided by Sigma-Proligo. Biotinylated LNAs were introduced into T47D cells using a standard lipid-mediated transfection. All modified LNAs were first tested in cell culture. The compiled data is shown in **Figure 3.5**. To ensure that the substrate was located in the nucleus, we isolated nuclear fractions. The nuclear lysates were incubated with avidin-coated beads to isolate LNA substrates. The bound substrates were then eluted and precipitated. To ensure that only DNA substrates were amplified, these protocols did not include reverse transcription. Isolated DNA was then PCR-amplified and analyzed on a 3% agarose gel. Data interpretation was based strictly on the presence or absence of the

corresponding band on the agarose gel. Buffer conditions were designed to stabilize double-stranded DNA to prevent LNAs from binding after cells were lysed. These protocols are described in Chapter 5.

As a positive control, input samples for each biological sample were taken before incubation with the avidin-coated beads. This control validates that the original sample contained the nucleic acid substrate of interest. As LNA HCV does not target hPR and does not contain a modified biotin, it served as a negative control and demonstrated that the targeted DNA did not non-specifically bind to the avidin-coated beads. These results are shown in **Figure 3.5**.

Each input sample amplified DNA, which confirmed that each sample contained the desired chromosomal DNA sequence before affinity purification. These data indicate that the unmodified negative control did not associate with the targeted sequence following the pull-down. However, LNA B2 and LNA B1 both appear to pull-down a DNA substrate, which corresponds to the expected molecular weight of the amplicon.

The bands corresponding to LNA B2 and LNA B1 were excised from the agarose gel, and the amplified DNA material was eluted using a spin column. The amplified DNA sequences were cloned into TOPO vectors and sequenced. The sequences were then aligned with the hPR-B gene promoter using NCBI BLAST and BLAT, which confirmed that LNAs were associating with the specific portion of chromosomal DNA that contains hPR.

These data indicate that antigene LNAs can physically bind to chromosomal DNA inside cells. Both LNA B1 and LNA B2 were found to associate with chromosomal DNA

(**Figure 3.5**). This is a surprising result, as it suggests that the inactivity of LNA B1 is not caused by a lack of DNA accessibility. It is reasonable, however, that if the template strand is accessible, then the corresponding non-template strand could also be accessible.

Although these data are consistent with a steric block model of antigene action, they suggest that binding DNA is not sufficient to inhibit gene expression at the level of chromosomal DNA. Collectively, these data suggest that strand-orientation is a critical factor for antigene activity. However, this phenomenon has been previously observed in *in vitro* systems.

In *in vitro* systems, it was reported that antigene agents targeting the template strand of DNA could inhibit transcription, but an antigene agent targeting the non-template strand could not. Hanvey, et al. previously observed that PNAs targeting the template strand of DNA resulted in almost 100% inhibition of transcription, but targeting the non-template strand was ineffective even at much higher concentrations [2]. Nielson, et al. observed an identical trend and reported that targeting an antigene PNA to the non-template strand caused virtually no inhibition, concluding that "[i]t is quite clear that the RNA polymerase is quite insensitive to the structure of the non-template strand" [3]. Other laboratories have reported similar findings suggesting that oligonucleotide orientation is critical for the inhibition of transcription [36, 40]. Our observations of whole-cell experiments are consistent with these early observations based on *in vitro* systems. Our results suggest that targeting the non-template strand does not interfere with the ability of RNA polymerase to initiate transcription of hPR mRNA inside living cells.

In summary, these results indicate that LNAs are physically associating with chromosomal DNA, but that binding does not always lead to transcriptional silencing. These data also suggest that for an oligonucleotide to be a functional antigene agent, it must be targeted in the correct orientation to bind to the template strand of a gene. These conclusions are consistent with previously observed *in vitro* results [2, 3] for antigene PNAs. Our results may be applicable to other synthetic antigene agents.

3.6 Antigene LNAs Inhibit the Transcription of hPR-B

Following the confirmation that antigene LNAs were capable of binding to chromosomal DNA inside cells, we sought to verify if transcription was inhibited by antigene LNAs. To assay the level of transcription within the hPR gene, we utilized the nuclear run-on assay, a classic biochemical technique that is used to determine RNA polymerase density on genes.

Nuclear run-on experiments were designed to assay the expression of the hPR gene and a previously reported hPR antisense transcript [5]. To test for non-specific interactions with our probes, we used an empty vector as a negative control. A probe specific for GAPDH served as a positive control for transcriptional activity in T47D cells. The exact experimental protocol was modified from existing protocols [41-45], and is detailed in Chapter 5.

Before performing the *in vitro* transcription steps, a small aliquot of cells were taken from each plate and analyzed using QPCR. This was precautionary step to ensure that the treatment was successful. LNA B2, LNA B1, and LNA HVC were tested for their ability to inhibit the transcription of hPR. Typical results are shown in **Figure**

3.6. QPCR results indicate that LNA B2 inhibited hPR-B expression approximately 70% as observed earlier (see **Figure 3.3**).

Transcriptional levels for the hPR gene were assayed using a double-stranded probe corresponding to the coding portion of the hPR cDNA (See **Figure 3.6**) for the relative position of the probe). The first dot corresponds to an empty-vector control, which shows that non-specific binding is not occurring. The second row of dots indicates that GAPDH is being actively transcribed, representing a positive control for transcription. Each blot was individually normalized to GAPDH and to background. The third row of dots represents transcriptional activity in the coding region of the hPR gene. These results suggest that this region of hPR is being actively transcribed.

Although these data indicate that transcription is still occurring in the coding region of the hPR gene, QPCR and Western blotting have established that hPR-B levels (both mRNA and protein) are decreasing (see **Figures 2.2, 3.3**). Since these antigene LNAs cannot hybridize to the hPR-B mRNA, they cannot directly decrease the levels of mRNA. Furthermore, we had established that antigene LNAs were physically associating with chromosomal DNA (**Figure 3.5**). Therefore, I hypothesized that antigene LNAs were inhibiting transcription proximal to the targeted location, and not throughout the entire gene.

This hypothetical model would suggest that antigene LNAs silence the transcription of hPR-B, but do not prevent RNA polymerase II from initiating transcription from downstream transcription start sites. This hypothesis is reasonable because there are many transcription start sites located thousands of bases away in UTRs, exons, and introns of hPR ([5], Yue, unpublished). It is reasonable that these

remain transcriptionally competent when DNA is bound by an antigene LNA located thousands of bases away. If theses putative downstream transcripts did not express gene products detectable using our current antibodies and primer sets, then they could only be detected using hybridization assays such as northern blots or run-ons assays. Furthermore, Dr. Xuan Yue has routinely observed multiple RNA species in Northern blot assays.

To test the hypothesis that antigene LNAs were inhibiting transcription of hPR proximal to the targeted site, I designed a run-on probe to assay a much smaller region of the hPR-B promoter. Unfortunately, I was unable to achieve a sufficient signal-to-noise ratio for such a small probe using the nuclear run-on assay. This led me to perform an alternative method for determining RNA polymerase density: RNA polymerase II chromatin immunoprecipitation (RNAPol-ChIP).

RNAPol-ChIP can be used to assay the relative occupancy of RNA polymerase II on a segment of chromosomal DNA, which is directly correlated to the level of transcription [46-48]. RNAPol-ChIP has several distinct advantages over traditional run-on assays. It is faster, requires less material, does not involve radioactivity, and, since cells are cross-linked, the nuclei are not subject to rapid decay. Most importantly, however, is that QPCR-based analysis can be used to analyze much smaller regions with a higher sensitivity. The ability to assay small (~100 nucleotide) regions for significant changes in RNA polymerase occupancy made RNAPol-ChIP ideal to validate my hypothetical model of antigene action.

RNAPol-ChIP was modified from existing protocols [46]. I seeded 2,000,000 cells in 150 cm^2 dishes 48 hours before treatment. To obtain sufficient nuclei for a

RNAPol-ChIP, I required 2 150 cm^2 dishes per experimental treatment. Experimental samples were analyzed using QPCR, and PCR primers that bracketed the hPR-B transcription start site. Each sample was normalized to the initial loading (input), to the overall transcriptional level of the cells (GAPDH), to non-specific binding (IgG), and finally to the negative control, LNA HCV. Input and IgG controls were performed individually for each sample. Detailed protocols are provided in Chapter 5.

Results indicate that RNAPII occupancy across the hPR-B transcriptional start site was significantly decreased upon treatment with LNA B2 relative to our negative control, LNA HCV (**Figure 3.6**). All ChiP experiments were biologically replicated at least 3 times, and error bars represent the standard deviation among these replicates. These data demonstrate that LNA B2 is affecting the density of of RNA polymerase II associated with chromosomal DNA proximal to the hPR-B transcription start site.

Collectively, the nuclear run-on data and the RNAPol-ChIP data have led me to conclude that antigene LNAs can inhibit transcription, but do not prevent RNAPII from associating with downstream transcriptional start sites. This mechanism of transcriptional inhibition is distinct from promoter-targeted RNAs, which appear to silence the transcription across the entire gene [29, 49]. This distinction is not unexpected, since RNA-mediated transcriptional gene silencing has been shown to require endogenous proteins [32, 49-51] that are unlikely to specifically associate with synthetic molecules such as antigene LNAs.

3.7 Promoter-Targeted LNAs Inhibit the Transcription of hAR

To confirm whether the results obtained for hPR were indicative of a general mechanism of antigene action, we repeated the RNAPol-ChIP series of experiments with an additional gene. To this end, we assayed the effect of LNA AR24 on RNA polymerase II occupancy proximal to the transcriptional start site for hAR. LNA AR24 and LNA HCV were introduced into T47D cells using standard cationic lipid-mediated transfections. Samples were handled, harvested, and analyzed as described for hPR.

We observed that LNA AR24 leads to a reduction in RNAPII occupancy near the hAR TSS relative to the negative control LNA HCV (**Figure 3.7**). The reduction of RNA polymerase II occupancy is not as drastic as seen for hPR (**Figure 3.6**), but it does appear correlated to the observed mRNA levels for LNA AR24 (**Figure 3.4**). As mentioned above, if additional LNAs were tested, it may be possible to achieve more potent gene silencing. This result is significant, however, because it supports a general mechanism for antigene action, which involves the inhibition of transcription. These data are consistent with a steric block mechanism.

3.8 LNAs Associate with RNA

Although my data is consistent with a steric block model of gene inhibition, I was unable to completely reject an alternative mechanism involving antisense transcripts. Because antisense transcription is so prevalent in the mammalian genome [6, 7, 11, 52], it is unlikely that an antigene agent can be targeted specifically to DNA without the potential for binding an RNA species. It is likely that non-coding RNAs will become an increasingly important variable to address for site-specific mixed-base antigene agents.

Although I had established that antigene LNAs were capable of binding to chromosomal DNA, it was expected that they were also associating with complementary RNAs as well. This expectation was based on existing antisense LNA data [53]. To verify this hypothesis, I reasoned that I could isolate LNA-bound RNAs using a similar approach as previously outlined in § 4.5, but with minor modifications. Following the elution step, the isolated RNA was treated with DNase to prevent any contaminating DNA from being amplified. The RNA was then reverse-transcribed, and the cDNA was PCR-amplified and analyzed on a 3% agarose gel. Input controls for each sample were taken before incubation with avidin-coated beads to validate that each original sample contained the RNA of interest. Data interpretation was based strictly on the presence or absence of the corresponding band on the agarose gel.

I assayed the isolated RNA for the antisense RNA shown to be spliced and required for RNA-mediated gene activation [5]. **Figure 3.8** shows a 3% agarose gel with amplified DNA bands corresponding to a primer set that amplifies across the 5/6 exon boundary of the antisense transcript [5]. The bands correspond to the expected molecular weight. Each input sample contained the desired RNA and amplified the expected band. LNA HCV was a negative control and did not associate with the RNA of interest. As expected, LNA B1-3' did not associate with the antisense transcript because it did not share any sequence complementarity with the target. As expected, LNA B2-3' did associate with the antisense transcript.

This result is not surprising since LNAs are well-established antisense agents and are expected to bind complementary RNA species [53]. Furthermore, multiple antigene LNAs capable of hybridizing with each characterized antisense transcript were

previously tested, and only a few of these LNAs potently inhibited gene expression [10]. Since at least some of these additional, non-functional antigene LNAs would be expected to hybridize to the antisense transcript, it is reasonable to assume that merely binding a nucleic acid substrate is an insufficient predictor of antigene LNA activity. However, these data do support the validity of the biotin-pulldown assay and give credence to its accuracy and reliability.

3.9 Conclusion

These data demonstrate that antigene LNAs physically associate with chromosomal DNA and inhibit transcription. Available data suggests that antigene LNAs inhibit transcription in regions proximal to their targets. Furthermore, these data indicate that antigene LNAs are functioning in an orientation-dependent manner, and that functional LNAs must target the template strand of DNA to be potent.

This work represents a substantial advance in the field of synthetic antigene oligonucleotides. This is the first evidence that mixed-base antigene agents can physically associate with chromosomal DNA inside living cells, and inhibit the transcription of endogenous mammalian genes inside human cells. Our system demonstrates that LNAs can be introduced into cells, enter the nucleus, and bind the correct target site to cause transcriptional silencing. Future work will likely focus on the effects of antigene LNAs after they have bound their chromosomal target, such as the effects on additional transcription factors near the target site, whether DNA repair proteins are recruited to LNA-bound sites, and whether antigene LNAs can activate gene expression.

TABLE 3.1 LNAs used in the experiments presented within Chapter 3.

LNA	Target	DNA Strand	Sequence
LNA B1	-9/+10	(Nontemplate)	gCTgTggaCTggCCagaCa
LNA B2	-9/+10	(Template)	tGtctGGccAGtccAcAGc
LNA PR17	-9 /+10	(Template)	TGTctggccagtccacAGC
LNA AR3	-24/ -6	(Template)	gTTgcATttGctctcCACc
LNA HCV	N/A	N/A	CTAcgaGaCctCccGggGC

Figure 3.1 depicts two potential models for the molecular mechanism for antigene action. (A) Antigene LNAs could potential bind to antisense transcripts in a gene promoter and disrupt the transcripts normal cellular functions, which could indirectly lead to the inhibition of hPR. (B) Antigene LNAs could be functioning via a steric block mechanism. In this model, an antigene LNA would directly interact with chromosomal DNA and prevent RNA polymerase II from initiating transcription.

FIGURE 3.1A: Models of Antigene LNA Activity

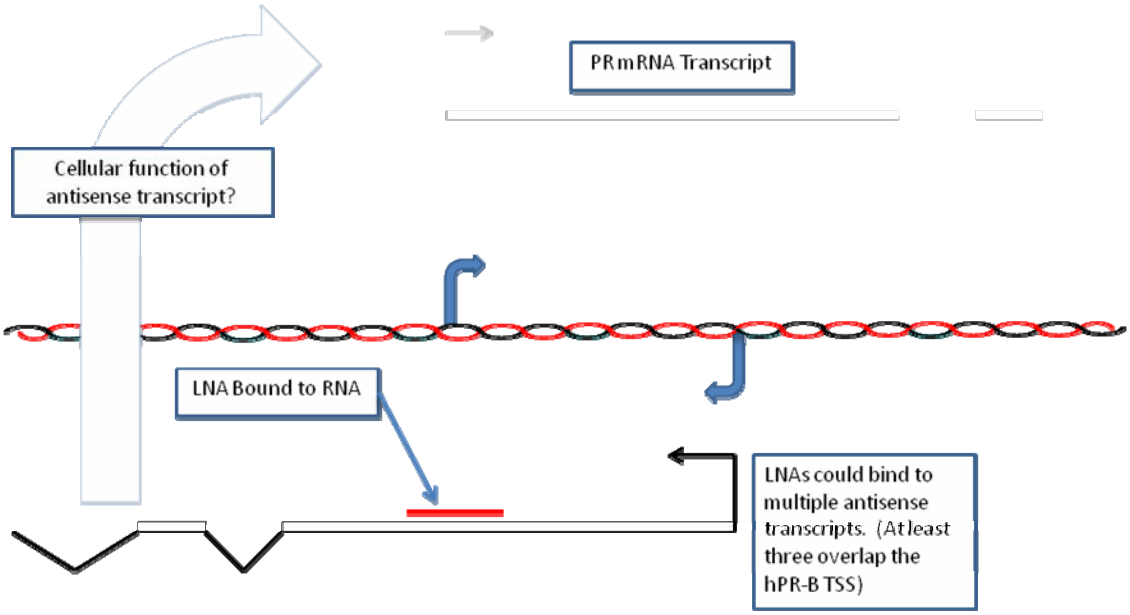


FIGURE 3.1B: Models of Antigene LNA Activity

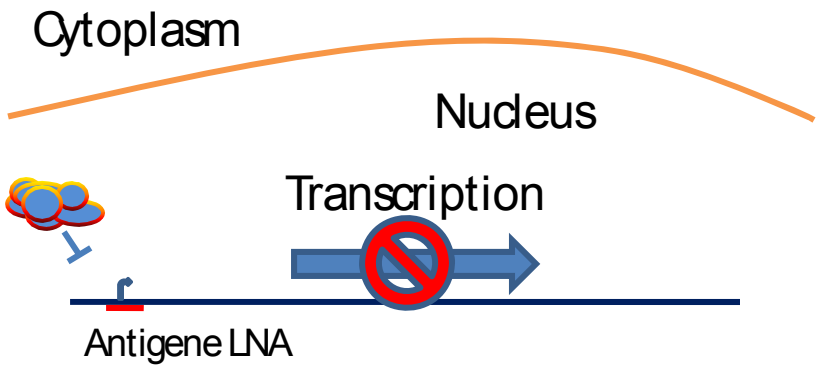


FIGURE 3.2: Non-Coding RNAs and Aberrant Transcripts in the hPR Gene. Figure 3.2 depicts a model showing the hPR gene with recently discovered alternative transcription start sites (symbolized here using small arrows). These transcripts have unknown cellular functions and are likely to be noncoding RNAs. The majority of these transcripts are not characterized.

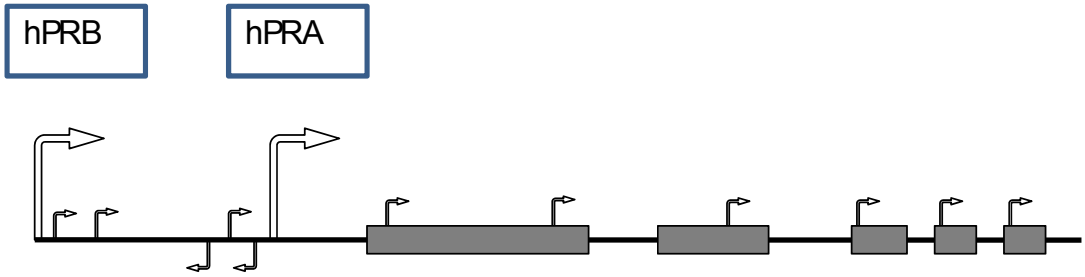


FIGURE 3.3: QPCR Data Showing Antigene LNAs Affect hPR mRNA Levels. Figure 3.3 is QPCR data showing the levels of hPR-B mRNA following treatment with antigene LNAs. These data support that LNA PR B2 is inhibiting hPR-B mRNA levels. LNA PR B1 showed very limited activity. All data was normalized to negative control LNA HCV. Error bars represent standard deviations of at least 3 biological replicates.

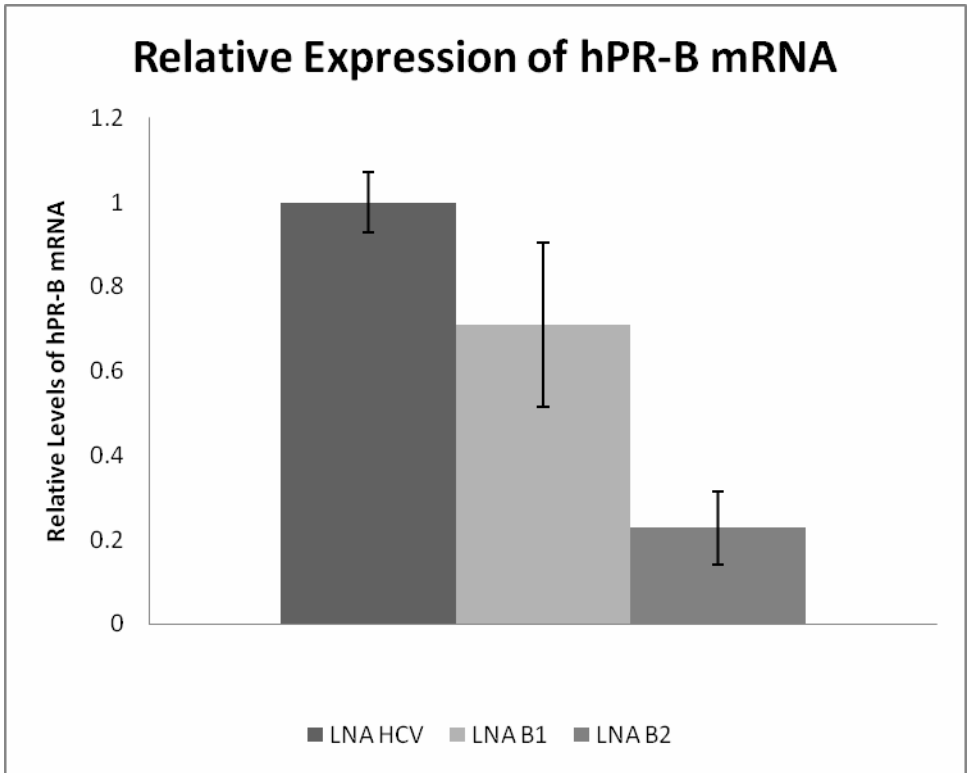


FIGURE 3.4: QPCR Data Showing Antigene LNAs Affect hAR mRNA Levels. Figure 3.4 shows QPCR data for the human androgen receptor following treatment with antigene LNAs. LNA AR24 was tested at multiple concentrations, as indicated, to verify that gene inhibition was reproducible and specific. These data demonstrate that LNA AR24 can inhibit the expression of human androgen receptor. Error bars represent the standard deviation among at least 4 biological replicates at each concentration.

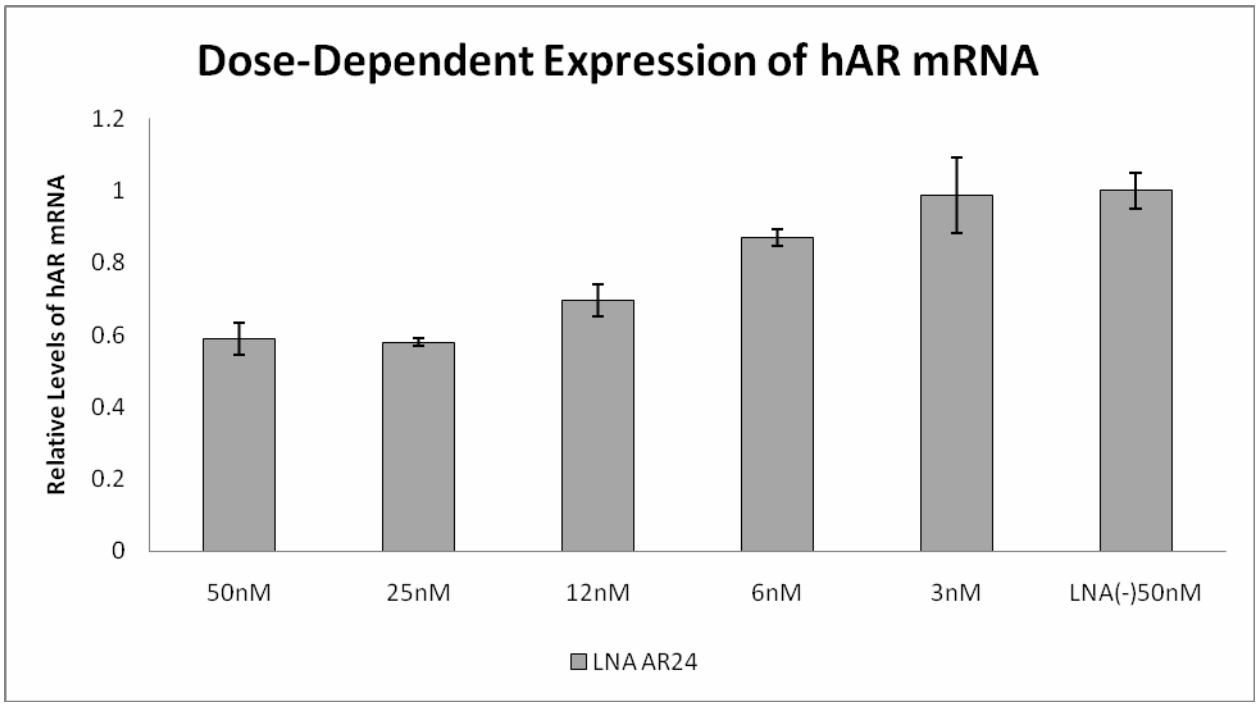
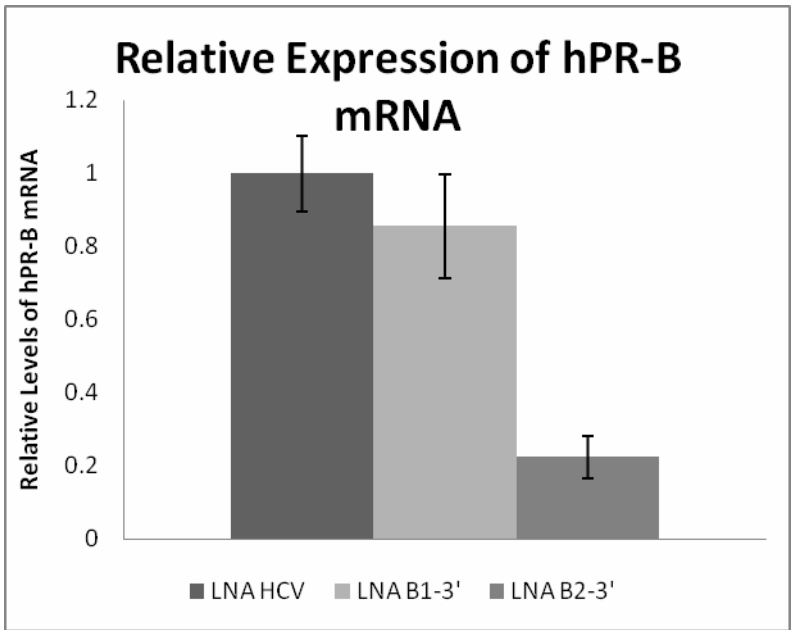


FIGURE 3.5: (A) QPCR data showing the relative levels of hPR-B mRNA following treatment with the indicated Biotin-modified antigene LNAs. These data show that the biotin-modified LNAs were functional and capable of inhibiting hPR-B gene expression. Error bars represent the standard deviation of at least 4 biological replicates. All data was normalized to our negative control, LNA HCV. Each sample was transfected at 50 nM. (B) A representative 3% agarose gel showing that hPR DNA was pulled down using the biotin-modified LNAs. Inputs were taken prior to incubation with avidin beads and indicate that the desired target nucleic acid is present. Both LNA B1 and B2 were capable of binding to chromosomal DNA. Products were excised from the gel, cloned, and sequenced.

A



B

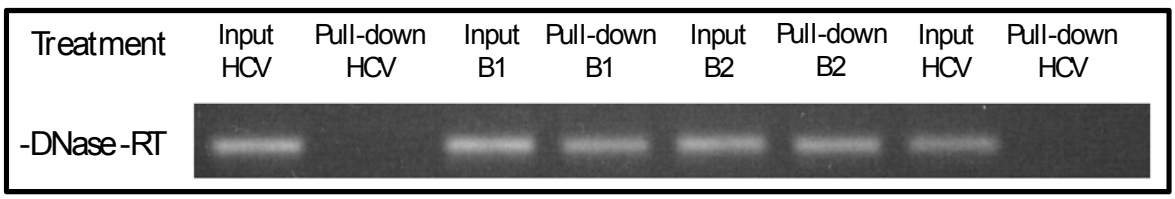
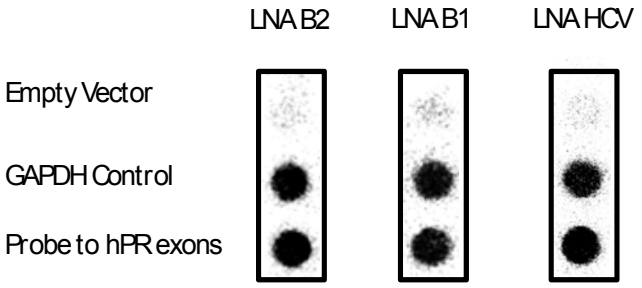


FIGURE 3.6: Antigene LNAs are capable of silencing hPR expression at the level of transcription. (A) Nuclear run-on experiment showing that transcription is still on in the coding region of hPR, which is shared by hPR-A and hPR-B. (B) RNAPol-ChIP showing that transcription is off at the hPR-B TSS where LNA B2 is being targeted. Error bars represent the standard deviation of three biological replicates.

A



B

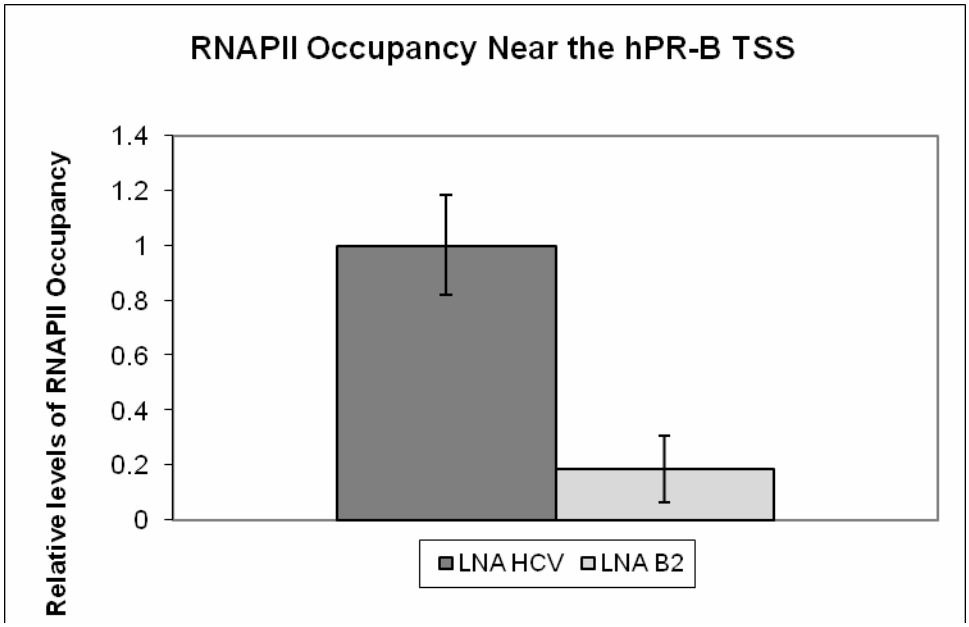


FIGURE 3.7: RNAPol-ChIP Data of the hAR Gene. Primers were located downstream of the hAR TSS. These data indicate that LNA AR24 silences the transcription of hAR. This suggests that transcriptional silencing is a general characteristic of functional antigene agents.

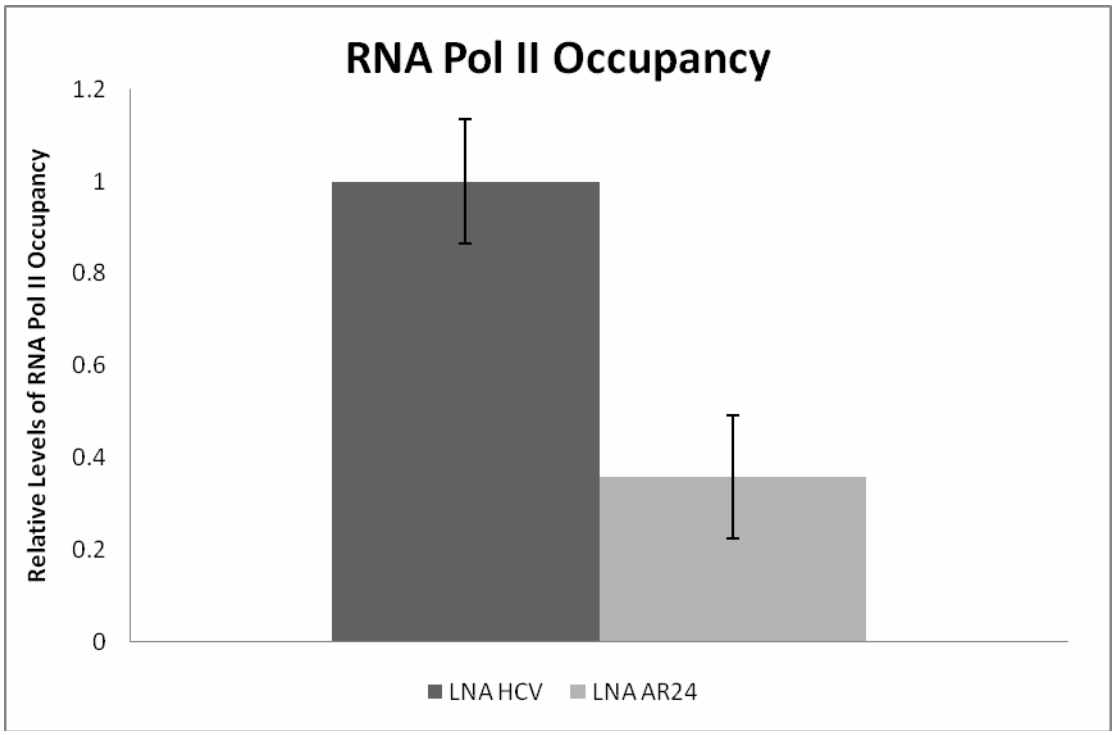
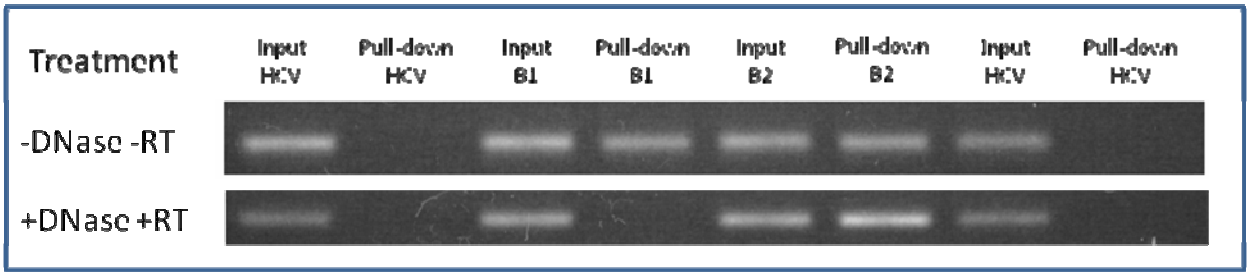


FIGURE 3.8: Biotin-Pulldown Data for Antisense Transcripts. Figure 3.8 addresses the ability of antigene LNAs to bind to antisense transcripts expressed within the hPR promoter. 3% agarose gel showing that antigene LNAs associate with DNA and with the antisense transcript.



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Chapter 4: Comparison and Characterization of Additional Antigene Agents

4.1 Introduction

In 2005, the Corey laboratory demonstrated that PNAs targeting the transcriptional start site of hPR could inhibit gene expression at the level of mRNA and protein [3]. In 2007, the Corey laboratory demonstrated that mixed-base LNAs [1] and PNA-peptides [2] could also be used to potently inhibit gene expression when targeted to gene promoters. Collectively, these data suggest that synthetic, mixed-base oligonucleotides are capable of inhibiting gene expression at the level of chromosomal DNA. Since some chemical modifications may confer substantial advantages to antigene oligonucleotides, it is prudent to evaluate multiple chemical modifications.

2'-O-methoxyethyl-modified oligonucleotides (MOEs) and 2'-O-4' ethylene nucleic acids (ENAs), were selected to test this hypothesis. This research was intended to evaluate the antigene potential of mixed-base MOEs and ENAs using our previously established model system of human progesterone receptor (hPR) and to further characterize antigene PNAs and antigene RNAs [3, 5, 6]. The chemical structure of each of these chemistries is presented in **Figure 4-1**.

4.2 2’-O-Methoxyethyl-Modified Oligonucleotides or MOEs

MOEs were originally identified by a research collaboration between Ciba-Geigy Ltd. (now Novartis) and Isis Pharmaceuticals. The 2’MOE modification was the product of screening multiple 2’-alkoxy derivatives and observing that the MOE modification conferred an oligonucleotide with nuclease resistance and increased hybridization affinity for RNA [11]. Upon further testing, it was discovered that MOEs had pharmacological properties similar to phosphothioate-modified oligonucleotides but with decreased toxicities [11, 12]. Although MOEs are often referred to as “second generation” antisense oligonucleotides [12], they are still frequently used in multiple applications.

MOEs have been used in a number of diverse applications. For example, MOE-modified oligonucleotides referred to as ‘gapmers’ can be designed to activate RNase H in order to effectively cleave targeted mRNA sequences [11, 12]. Completely MOE-modified oligonucleotides can be used to sterically block translation [12], or effectively alter splicing [13]. MOE-modified oligonucleotides have also been reported to be potent antagomirs, capable of completely inhibiting miR-122 expression [14, 15]. Moreover, MOE-based drugs are currently in clinical trials and a substantial amount of data is available about the pharmacokinetic properties of MOE-modified oligonucleotides [11, 13, 16]. MOE-based antisense drugs and their applications have been extensively reviewed, and are the subject of multiple book chapters [4].

As these examples imply, MOEs are a useful benchmark for oligonucleotide-based applications [1]. Therefore, the Corey Laboratory wanted to evaluate the

antigene potential of mixed-base MOEs. I am grateful to ISIS Pharmaceuticals for generously providing the MOEs necessary for these experiments.

These experiments were performed during 2006 and 2007 and were included in the original antigene LNA publication [1]. Therefore, the methods used were identical to those presented for LNAs in Chapter 2. These experiments were intended to compare MOEs with isosequential antigene LNAs. The sequences of all of the MOEs tested are given in **Table 4.1**.

Rosalyn Ram performed the original series of experiments, which were subsequently repeated and verified. A Western blot showing the typical observed phenotype is shown in **Figure 4.2**. This Western blot shows MOE PR2 and MOE PR3. These MOEs were single-stranded and targeted from -9 to +10 relative to the transcription start site for hPR-B. MOE PR2 is designed to target the non-template strand of DNA. MOE PR3 is designed to target the template strand of DNA and is analogous to LNA PR3, which was the most potent LNA tested [1]. LNA HCV was a negative LNA control, and agRNA PR9C was used as an additional positive control. Each MOE was transfected into T47D cells, twice, at a concentration of 50 nM using Oligofectamine. This procedure is discussed in Chapter 2 and Chapter 5. The results shown in **Figure 4.2** indicate that the fully-modified MOE oligonucleotides do not inhibit the hPR expression under these conditions.

MOEs may function as potent antigene agents under different conditions, at higher concentrations, or utilizing different target sequences. However, these data demonstrate that under identical conditions antigene RNAs and antigene LNAs silence gene expression and antigene MOEs do not.

4.3 Peptide Nucleic Acids or PNAs

4.3.1 PNA Background

A PNA is a nucleic acid mimic with a neutral polyamide backbone comprised of N-(2-aminoethyl) glycine units [17, 18]. Since PNAs have an unnatural polyamide backbone, they are not substrates for nucleases and therefore show high stability in serum, cell culture, and in vivo [12, 20]. Initial reports indicated that PNAs exhibited increased hybridization properties *in vitro*, and were promising antisense and antigene agents [17, 21, 22]. PNAs have been used successfully *in vitro* [17, 22-24], in cell culture [3, 20, 25], and *in vivo* [13, 20, 26]. The properties and applications of PNAs have been reviewed extensively and are the subject of multiple books [20, 27] and reviews [19, 28-30].

Like MOEs, PNAs have been utilized in many varied applications ranging from PCR-clamps [30], telomerase inhibitors [31], and antisense agents [17] to artificial transcription factors [32], FISH probes [29, 30], and antigene agents [2, 3, 17, 26]. Although PNAs have many unique advantages over other oligonucleotide mimics, they have several disadvantages as well, which include poor solubility, limited cellular uptake, and poor biodistribution [20]. However, many recent advances with additional chemical modifications, ligand conjugations, and delivery methodologies are being developed to address these obstacles [13, 20].

The Corey laboratory has demonstrated that antigene PNAs [3, 8] and PNA-peptides [2] can potently silence gene expression. Although antigene applications were reported in 1991 [21] and extensively studied by multiple laboratories [20], it was not until 2005 when the antigene properties of PNAs were tested and validated in

mammalian cells under well-controlled conditions [3, 20]. Specifically, this work established that PNA-DNA complexes, targeting the hPR-B promoter, could efficiently silence hPR gene expression at the level of protein and mRNA [3].

However, several key questions were left unresolved. First, only four PNAs were tested, which all targeted the template strand of DNA [3]. Therefore, the question remained whether PNAs targeting the non-template strand could silence gene expression. Second, only two regions of the hPR promoter (the regions immediately adjacent to the transcription start sites of hPR-B and hPR-A) were probed. Therefore, it was unknown whether or not PNAs could target sequences further away from the putative 'open complex.' Third, only PNAs 19 bases in length were tested. Therefore, it was unknown whether or not PNA-length was an important factor in antigene activity. Fourth, PNA activity was only monitored at the level of mRNA and protein. Therefore, direct physical interaction with chromosomal DNA was inferred and had not been directly tested. Fifth, transcriptional silencing was inferred from the observed decrease in protein and mRNA levels. Therefore, transcriptional silencing had not been verified directly. Finally, antigene PNAs were only tested in a single cell line and only on a single gene. Therefore, the robustness and generality of antigene PNA functionality had not been addressed.

4.3.2 PNA Experimental Design

The synthesis, purification, MALDI-TOF analysis, and general design of PNA is described in detail in Chapter 5. For each PNA, I designed and ordered three DNA carriers and analyzed melting curves to validate the presence of only one species of

DNA:PNA complex. For the previously validated antigene PNAs, I ordered the DNA carriers used in the original publication [3].

The PNA sequences tested are listed in **Table 4.1**. Transfections were initially performed as described in the 2005 antigene PNA paper [3], and the 2007 antigene LNA paper [1]. This transfection protocol consisted of seeding the cells 48 hours prior to transiently transfecting the cells using a cationic lipid, splitting the cells 72 hours post-transfection and then performing a second transient transfection 120 hours post-transfection. Cells were harvested for protein 7 to 8 days following the initial transfection.

It is important to note that, in early 2007, our laboratory noticed a decline in the activity of antigene oligonucleotides. Our standard lipid at the time, Oligofectamine, appeared to stop working in T47D cells (**Figure 4.3**). Our lab switched to RNAiMAX and noticed improved transfection efficiency. However, even with new cells from ATCC, Oligofectamine remained ineffective in T47D cells with agRNAs, siRNAs, and PNAs (**Figure 4.3**). Therefore, my initial experiments were performed with the cationic lipid RNAiMAX.

4.3.3 PNA Experimental Results

These initial experiments were intended to validate the PNA positive control and to determine optimal conditions for PNA transfections. Consecutive transfections were performed as previously described [1, 3, 8]. In addition, several variations of this protocol were attempted to omit the need for splitting the cells 72 hours after the initial transfection. Typical results from these experiments are shown in **Figure 4.4**. These experiments were performed utilizing either agRNA PR9, siRNA PRC1, or LNA PR9 as

a positive control. In each experiment, all positive controls tested worked under all of the conditions tested. However, PNA PR9 was not functional under these conditions. All PNA carriers are listed in **Table 4.2**, and are indicated by “c1,” “c2,” and “c3,” respectively, in each figure.

These data suggested that antigene PNA activity could be dependent upon the lipid used to transfect the cells. This was a logical assumption, since according to the accompanying product detail for Oligofectamine (available from Invitrogen’s website), it was originally designed for antisense agents, specifically phosphothioate-modified oligonucleotides. However, RNAiMAX was specifically formulated for duplex RNAs according to its accompanying product details (also available from Invitrogen’s website). We postulated that the observed lack of PNA functionality was due to the fact that the RNAiMAX lipid formulation was not optimized for antisense agents and our previous lipid, Oligofectamine, was optimized for antisense agents. Since Oligofectamine was no longer functional in T47D cells, we immediately proceeded to test other lipid formulations.

The first set of lipids tested were Dharmacon’s DharmaFECT reagents D1, D2, D3, and D4. Although these lipids were designed for duplex RNAs according to the manufacturer, they provided a straight-forward way to test a broad range of lipid formulations. Typical results observed using these lipids are shown in **Figure 4.5**, and the sequence of each oligonucleotide is given in **Table 4.1**. The results indicate that our positive controls, LNA PR9 and siRNA PRC1, exhibit potent inhibition of hPR under all condtions tested. However, PNA PR9 was not observed to inhibit hPR expression

compared to a scrambled PNA control. Three unique carriers were tested and are listed in **Table 4.2**.

These data suggest that the range of lipid formulations conducive to the efficient delivery of PNA:DNA complexes is not as wide as LNAs and duplex RNAs. However, at this time I began to consider that our double transfection protocol [3, 8], which was originally utilized for Oligofectamine, could be suboptimal for other lipid formulations. Representatives from Dharmacon, New England Biolabs (NEB), and Ambion stated that they had not tested the potency of their lipids under consecutive transfections, and each recommended following the product optimization guides provided by their corporation. I began testing antigene PNAs using the protocols provided by each manufacturer, in addition to performing double-transfections for each lipid.

Multiple lipids were tested under single-transfection conditions. **Figure 4.6** shows typical results obtained from single transient transfection experiments. Positive controls included at least two of the following: agRNA PR9, siRNA PRC1, or LNA PR9. Positive controls worked under all conditions tested except Oligofectamine or Roch X-tremeGENE transfection reagent. These two lipids did not work under any conditions tested in T47D cells.

These results suggest that antigene LNAs and duplex RNAs are potent and robust under single-transfection conditions. These were some of the first experiments that clearly indicated that antigene LNAs were capable of inhibiting gene expression in only a single transfection. Previously, it was assumed that a synthetic antigene molecule would require two consecutive transfections to potently inhibit gene expression [1, 3]. This result was important because it clearly demonstrated that

antigene LNAs had an activity similar to that observed for antigene RNAs rather than antigene PNAs. Antigene PNAs did not show appreciably activity under these conditions.

In addition to cationic lipids designed for nucleic acids, NEB Transpass P protein transfection reagent was also tested. Since PNAs have a polyamide backbone, it was reasonable to test a transfection reagent designed to interact with proteins. Using this reagent, DNA carriers would not be required, which would greatly simplify our antigene PNA protocols [8].

This reagent had not been validated in T47D cells, and we could not use our standard LNA and duplex RNA controls to verify efficient delivery. Therefore, in collaboration with a post-doctoral fellow, Dr. Kenneth Huffman, we confirmed that NEB Transpass P could successfully be used to introduce a control protein, IgG labeled with Dylight 549, into T47D cells. Optimal transfection conditions were obtained using the control protein as well. The lipid was noticeably toxic at higher concentrations. Antigene PNAs targeting multiple sequences within the hPR promoter were transfected into T47D cells and tested for activity. Typical results are shown in **Figure 4.7**. Results indicated that the NEB Transpass P transfection reagent was not an effective means of delivering antigene or antisense PNAs into mammalian cells.

After testing antigene PNAs under multiple conditions, we decided it would be simpler to test additional lipid and cell culture conditions using antisense PNAs, which could inhibit gene expression with a single transfection [3]. Typical results are shown in **Figure 4.8**. Antisense PNA PRC1 was tested using three carriers, including a previously validated DNA carrier. PNA AS75, which targeted the 3'UTR of hPR, was

also included. A scrambled PNA was used as a negative control. These DNA carriers are listed in **Table 4.2**. As a positive control, siRNA PRC1 was included. siRNA PRC1 potently inhibited hPR expression under all condtions tested. The antisense PNAs did not appreciably inhibit hPR gene expression under the tested conditions.

Collectively, these results indicated that PNA activity could be sensitive to specific lipid formulations or cell culture conditions. An alternative hypothesis was that efficient PNA:DNA:Lipid complex formation could require a chemical additive that had been removed by the increased stringency of PNA purification. This hypothesis was supported by two main observations. First, Dr. Jiaxin Hu had recently observed by NMR that trifluoroacetic acid (TFA) was present at an approximate ratio of 10 molecules of TFA to each molecule of PNA, following PNA synthesis. Second, PNAs synthesized by Panagene, a commercial PNA provider, were devoid of contaminants and truncated products (as verified by MALDI-TOF) but had no appreciable activity in cell culture.

Older PNAs, synthesized in 2005 or earlier, were used as benchmarks for comparison with the recently synthesized PNAs. PNA stocks were annealed with previously validated DNA carriers in the presence or absence of TFA. TFA was used at two ratios, 1:5 and 1:30. These ratios were selected to bracket the ratio observed by Dr. Jiaxin Hu. To control for potential lipid dependencies, experiments were performed using RNAiMAX, DharmaFECT 4, and Oligofectamine. Typical results are shown in **Figure 4.9**. In addition to this series of experiments, dose response curves using RNAiMAX and DharmaFECT 4 (shown in **Figure 4.10**) were also performed. As a positive control, RNA PRC1 was included. No activity was observed using PNAs under these conditions.

These data suggested that PNAs were not active under a wide range of lipid and cell culture conditions and were indicative of a systemic problem. Notable differences between T47D sublines have previously been reported [34, 35]. In fact, T47D cells are considered to be a cell line of “notable genetic instability” [36], and have even been referred to as “a highly unstable” cell line [37]. Therefore, it was a reasonable precaution to verify that the cells used for our current and previous experiments had not undergone subline differentiation. Samples of T47D stocks used in 2004, 2005, and 2006 were revived from cryostorage and cultured for two complete passages. These cells were tested for mycoplasma and genotyped (**Figure 4.11**). All cells tested had the expected molecular markers for T47D cells. Details of this assay are provided in Chapter 5. In addition, other members of the lab, including Dr. Jiaxin Hu, tested various cell culture conditions (e.g. serum formulations).

4.3.4 Conclusions of PNA Work

Collectively, these data suggest that PNAs are not as robust as siRNAs, agRNAs, and antigene LNAs in T47D cells. **Figure 4.12** summarizes the conditions tested during this course of experiments. Additional tests were performed by other members of the Corey laboratory. After exhausting multiple possibilities, I was unable to resolve the issues affecting the performance of the antigene PNA controls.

Overall, it is puzzling what factors are preventing PNA:DNA complexes to be active under the conditions tested. Oligofectamine was reformulated, according to the manufacturer, prior to July 9, 2004. It is likely that the original 2005 antigene PNA experiments were performed using the old formulation of Oligofectamine. However, the original formulation is no longer available, so this hypothesis cannot be tested.

However, other experiments with agRNAs and antigene LNAs using Oligofectamine in 2006 and 2007 showed potent inhibition [1, 38, 39]. These experiments would have been performed using the new formulation, as any remaining stocks of the old formulation would have been expired (lipids have a typical shelf-life on the order of months). In the first quarter of 2008, however, Oligofectamine ceased to work with any reagent tested (see **Figure 4.3**). This suggests that the observed lack of activity with agRNAs, antigene LNAs, and siRNAs was not due to Oligofectamine's reformulation in 2004.

From a practical standpoint, it appears that antigene LNAs have superior antigene qualities under the tested conditions. These experiments demonstrated that antigene LNAs were robust and worked under a wide range of cellular conditions, and could potently silence gene expression after only a single standard transfection. It is unfortunate that I was unable to resolve the issue with antigene PNA activity. However, these experiments elucidated the qualities of antigene LNAs and led directly to the work presented in Chapter 3.

4.4 Antigene RNA (agRNA or Promoter-targeted RNA)

The impact of small duplex RNAs upon modern molecular biology cannot be overstated, and has been the subject of multiple reviews [45-51]. Although RNAi was previously observed in plants, Andrew Fire and Craig Mello were the first to show that duplex RNA was capable of inhibiting gene expression in an animal model [52]. After their publication in 1998, multiple papers have followed, elucidating RNAi pathways [47, 50, 53].

Small duplex RNAs, chemically identical to the ones routinely used to cleave mRNAs and silence post-transcriptional gene expression, can also be used to mediate changes in chromosomal DNA in yeast [54, 55] and mammals [56]. Although some of the initial publications reporting the existence of transcriptional gene silencing in mammals were retracted [57, 58], multiple laboratories have reported similar findings [59, 60] that validate the existence of RNA-mediated transcriptional gene silencing. Recently, the Dahiya laboratory discovered that microRNA-373 could induce the expression of genes with complementary promoters, which confirmed that RNA-mediated transcriptional gene modulation occurred naturally [61].

The Corey laboratory has contributed significantly to the advancement of RNA-mediated transcriptional gene modulation. In 2005, the Baylin laboratory and the Corey laboratory reported that transcriptional gene silencing could be induced by duplex RNAs in the absence of DNA methylation [6, 62]. In 2006, the Rossi laboratory, the Morris laboratory, and the Corey laboratory reported that argonaute proteins were involved with mammalian transcriptional silencing [39, 63]. And in late 2006 and early 2007, the Dahiya laboratory and the Corey laboratory reported that duplex RNAs could also activate gene transcription in mammalian cells [38, 64].

Antigene RNAs are very potent and serve as an important benchmark for testing additional antigene agents. In general, cell culture conditions that are conducive to potent agRNA activity have also been conducive to potent antigene LNA activity. Therefore, agRNAs can often be used to to determine optimal transfection conditions for antigene LNA experiments.

Although multiple variables are involved with cell culture conditions, there are three major variables that strongly affect the potency and variability of transient transfection experiments. These variables are cell number, the ratio of lipid and oligonucleotide, and the timing of cell harvest. Among these important factors, cell number and lipid ratio appear to be similar between antigene LNAs and agRNAs. However, the optimal harvest time for agRNA activity and antigene LNA activity are sometimes different. This is probably indicative of their distinct mechanisms of antigene action. However, I have still utilized agRNAs to optimize lipid ratios and cell number conditions for use with LNAs. The sequences of the antigene RNAs tested are listed in **Table 4.1**. The specific methods used for antigene RNAs are detailed in Chapter 5.

Figure 4.13 is an example of a reverse transfection and a typical lipid ratio test. This experiment was performed by maintaining cell culture conditions among samples, with the exception of the amount of lipid used in conjunction with 50 nM of an agRNA or a negative control mismatch RNA. The numbers given in **Figure 4.13** are ratios of the volume of lipid to the volume of 20 μM agRNA stock used. Therefore, a “1:2” ratio indicates that twice the volume of lipid was used compared to the volume of agRNA used.

High lipid concentrations can cause off-target effects and cytotoxicity, therefore lipid ratio tests are critical for minimizing these factors in cell culture experiments. In addition, lipid ratio tests are important because lipid is often the most expensive reagent used in an experiment, and performing these tests allows the cost-effectiveness of multiple lipids to be directly compared. The data in **Figure 4.13** suggest that RNAiMAX is potent over a wide range of lipid concentrations.

Figure 4.14 is an example of a typical cell number optimization experiment. **Figure 4.14** is an example of a reverse transfection using the cationic lipid RNAiMAX. This experiment was performed using 1.8 µl of RNAiMAX for a 50 nM transfection. All experimental conditions were maintained except for the number of cells used in each sample. The cell number ranged from 40,000 to 140,000. As a negative control, a duplex RNA with no known targets in the human genome, MM4, was used. The agRNA, PR9, was used as a positive control for these experiments. At this concentration, 100,000 to 120,000 cells appeared to yield high potency with very little noticeable cell toxicity. Lower cell numbers exhibited noticeable toxicity and slowed growth.

Figure 4.15 depicts a dose response curve performed using the optimal conditions determined by experiments similar to the ones shown in **Figure 4.13** and **Figure 4.14**. This is a typical result showing that agRNA PR9 can potently inhibit hPR expression with an IC_{50} below 3.12 nM. This is notable, because this is a reverse transfection, and was harvested 72 hours post-transfection. Since reverse transfections do not require a 48-hour incubation prior to transfection (refer to Chapter 5), this protocol is a full 4 days faster than the previously used forward transfection protocol [6], and is therefore more high-throughput and more cost-effective.

Although optimization experiments generally produced conditions yielding potent inhibition, some lipids were not effective in T47D cells under any tested condition. For example, **Figure 4.16** shows a cell number and lipid ratio test for the Roche X-tremeGENE transfection reagent. Lipids that did not perform well in initial tests were not tested with additional antigene agents. One explanation for these results is simply

that lipids can affect the cellular distribution of oligonucleotides [65], and that this particular product may not be conducive to nuclear delivery.

Figure 4.17 and **Figure 4.18** are examples of typical cell number and lipid ratio tests using additional lipids. These figures illustrate the differences among available lipids. For example, although some lipid reagents are cheaper than others, a much higher lipid ratio may be required for activity, which can negate the cost-effectiveness of the lipid (see **Figure 4.18**). In general, agRNAs appear to be robust and capable of inhibiting gene expression using a wide range of lipid conditions.

In addition to Western blots, agRNAs have been used as standard controls in a range of other experiments. **Figure 4.19** shows typical QPCR results obtained utilizing agRNAs in tissue culture. These data show that agRNAs can typically inhibit hPR mRNA levels over 80 percent. It is intriguing that an 80 percent reduction of mRNA can correspond to complete inhibition of protein expression. This observation suggests that the remaining RNA may not code for protein.

In addition to standard QPCR, the level of RNA polymerase II density on the hPR promoter has been analyzed following agRNA PR9 treatment. Chromatin immunoprecipitation experiments were performed in collaboration with Dr. Xuan Yue. These data, shown in **Figure 4.19**, show that RNA polymerase II is reduced at the hPR promoter following agRNA treatment. These data suggest that agRNA PR9 silences gene transcription and is in agreement with previous studies [33, 39].

In conclusion, agRNAs appear to be robust antigene agents that function via an endogenous and conserved pathway. Collectively, these data suggest that agRNAs are powerful benchmarks for measuring the potency and robustness of additional antigene

agents. However, antigene LNAs have several potential advantages when compared with antigene RNAs. These advantages are discussed in § 2.11.

4.5 Ethylene-Bridged Nucleic Acids or ENAs

ENAs were first synthesized in 2001 [66] by the Koizumi laboratory, and are similar in design to LNAs except for the addition of a carbon group in the 2', 4' bridge. This additional carbon has been reported to substantially increase nuclease resistance and to improve other properties relative to LNAs [67]. ENAs have been (i) used in antisense applications [67-74], (ii) designed to activate RNase H [69, 71], (iii) used to form triplexes with DNA [67, 75], (iv) designed to detect SNPs [76], and (v) alter gene splicing [77-79], among other applications. These reports suggest that ENAs may have superior properties compared to existing antisense and antigene agents [67], and may have therapeutic applications [80]. Because of these qualities, mixed-base ENAs are very promising antigene molecules.

The antigene potential of ENA was evaluated during the summer of 2008. I am grateful to Sigma-Proligo for providing the reagents necessary to test and evaluate this chemistry. The handling and transfection protocols used for ENAs are given in detail in Chapter 5, and are identical to those used for the LNA experiments discussed in Chapter 3. The ENA sequences tested are given in **Table 4.1**.

ENAs targeting the hPR-B promoter were evaluated for antigene potential in T47D cells using QPCR. These data are shown in **Figure 4.20**, and represent the compilation of 5 biological replicates. The position of LNA bases within the oligonucleotides previously tested [1] were used as templates for designing the ENAs.

Therefore, these ENAs are isosequential to previously tested LNAs. ENA HCV served as a negative control and was not expected to interact specifically with any sequence within the human genome. ENA sequences targeting -9 to +10 of both the template and non-template strands were tested.

As shown in **Figure 4.20**, ENA B1, which targeted the non-template strand of hPR from -9 to +10, was not functional. The analogous LNA was also nonfunctional in our previous tests [1]. ENA DPE was targeted -2 to +17 relative to the hPR-B transcription start site (**Table 4.1**). No significant inhibition was detected using ENA DPE. However, the analogous LNA was previously shown to inhibit hPR expression [1]. ENA B2 was targeted -9 to +10 and was capable of inhibiting hPR-B mRNA levels by approximately 50 percent. The analogous LNA, LNA B2, is capable of inhibiting hPR-B levels by over 70 percent (see Chapter 3).

Figure 4.21 shows data from two independent chromatin immunoprecipitation experiments. These experiments were designed to assay the level of RNA polymerase II at the hPR-B transcriptional start site following ENA treatment. These data suggest that ENAs only marginally decrease the density of RNA polymerase II at the hPR-B promoter.

These data suggest that ENAs can inhibit hPR-B expression using a single transient transfection. These data indicate that ENAs are not as potent as their isosequential LNA counterparts. However, this may be due to suboptimal placement of the ENA bases. It is possible that additional designs could improve the potency of these antigene molecules. In conclusion, these data demonstrate that ENAs do have antigene potential and warrant further characterization.

4.6 Concluding Remarks

The Corey laboratory has demonstrated that multiple synthetic antigene agents are capable of inhibiting gene expression. These include LNAs [1], antigene PNAs [3], antigene PNA-peptides [2], and antigene RNAs [5, 6]. Data presented within this chapter suggests that some oligonucleotide-modifications may be better suited for antisense applications rather than mixed-base antigene applications. For example, 2'MOE-modified oligonucleotides are potent antisense agents, but do not show appreciable antigene functionality under the conditions tested. Overall, these observations are likely due to the specific properties conveyed by each chemical modification upon an oligonucleotide (e.g. cellular distribution, uptake, hybridization properties, etc.).

Collectively, these data indicate that multiple oligonucleotide-modifications can be used to create potent antigene agents. Specifically, we have demonstrated that LNAs and agRNAs are robust antigene agents and can work under multiple conditions. In addition, we have demonstrated that ENAs have appreciable antigene potential, and may prove to be more potent under optimized conditions and using optimized sequences.

Chapters 2, 3, and 4 represent the most comprehensive study of mixed-base antigene agents conducted in whole cells. No other published study had tested such a large number of sequences, designs, cell lines, genes, functionally significant targets, or oligonucleotide-chemistries. These results suggest that mixed-base antigene agents could be useful tools for functional genomics and therapeutic potential.

Table 4.1: Sequences Used and Their Genomic Targets. "Target" refers to the promoter sequence targeted by the molecule, and the numbers are relative to the transcription start site for hPR-B. Antisense molecules and negative controls do not target promoter sequences, and are simply labeled as "Antisense" or "(-)control." "Orientation" identifies the strand of DNA targeted by antigene molecules and is relative to the hPR-B gene. MOE molecules were fully modified. LNA bases and ENA bases are indicated by lowercase letters within the given sequence. All RNA sequences are double-stranded, and the identity of only one complementary strand is given. PNA sequences are given form the N terminus to the C terminus. The "K" in PNA sequences represents lysine molecules.

Chemistry	Name	Target	Orientation	Sequence 5' to 3' (N to C)
MOE	PR2	-9 to 10	Nontemplate	GCUGUGGACUGGCCAGACACA
MOE	PR3	-9 to 10	Template	UGUCUGGCCAGUCCACAGCUG
LNA	PR3 (B2)	-9 to 10	Template	tGtctGGccAGtccAcAGc
PNA	PR9	-9 to 10	Template	KTGTCTGGCCAGTCCACAGCK
PNA	PR13	-13 to +6	Template	KAAGCTGTCTGGCCAGTCCAK
PNA	PR26	-26 to -7	Template	KAGGCGTTGTTAGAAAGCTGK
PNA	PRC1	Antisense	N/A	KAGTTGTGCTGCCCTTCCATK
PNA	AS75	Antisense	N/A	CATGACGACTGGACTCCCC
PNA	Scr	(-)control	N/A	KGTACCTCAAGCACCCGGTCK
ENA	DPE	-2 to +17	Template	ccAGTccAcAGcTGTcAcT
ENA	B1	-9 to 10	Nontemplate	GctGtGGActGGccAGAcA
ENA	B2	-9 to 10	Template	tGtctGGccAGtccAcAGc
ENA	HCV	(-)control	N/A	CTAcgaGaCctCccGggGC
RNA	PR9	-9 to 10	N/A	UGUCUGGCCAGUCCACAGCTT
RNA	PR9C	-9 to 10	N/A	UGUCUGGCCAGUCCACAGC
RNA	PRC1	Antisense	N/A	AGUUGUGCUGCCCUUCCAU
RNA	MM4	(-)control	N/A	UCUCUCGCCAGUGCACACCTT

Table 4.2: Sequences of PNA carriers used in the experiments discussed in Chapter 4.

Carrier for PNA:	Name of DNA Carrier	DNA Sequence 5' to 3'
PR9	c1	ACTGGCCAGACAGCTTTTCT
PR9	c2	AGTGACAGCTGTGGACTGG
PR9	c3	GACTGGCCAGACAGCTTTCT
PRC1	c1	CATGCATGGAAGGGCAGCA
PRC1	c2	AGGGCAGCACAACTCCGCA
PRC1	c3	GGCAGCACAACTAAGCTT
SCR	c1	TGACACATGGAGTTCGT
AS75	c1	CCAGTCGTCATGAGCTG

Figure 4.1: Structures of LNA, PNA, MOE, and ENA Molecules

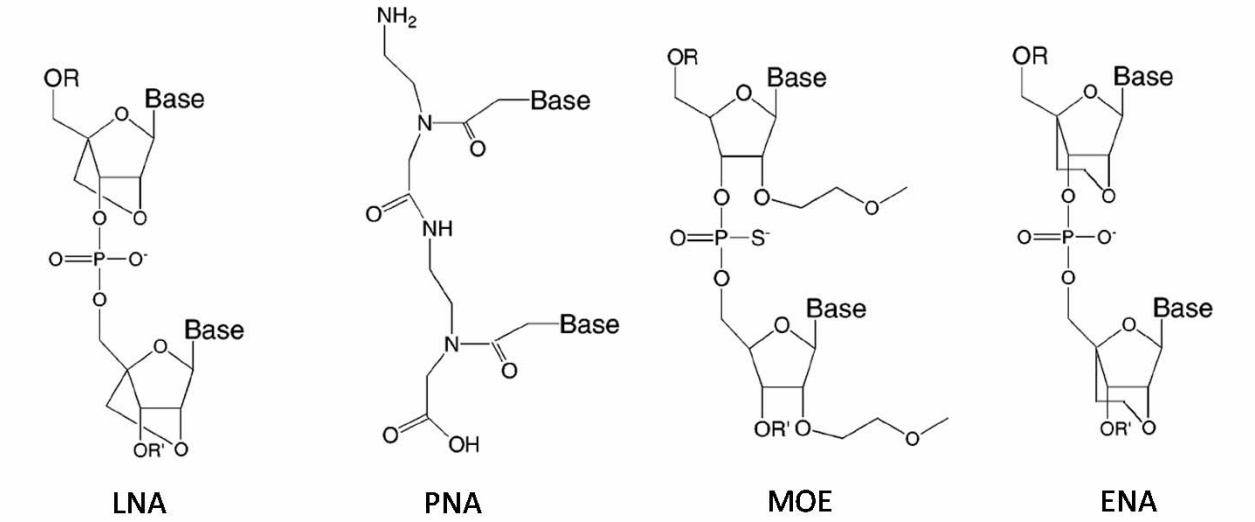


Figure 4.2: Western Blot of Typical Antigene MOE Results. LNA HCV (HCV) is a negative control. All samples are targeted to -9 to +10 across the transcriptional start site of hPR-B. All samples were tested at 50 nM. LNA PR3 and agRNA PR9C are positive controls. MOE PR3 is targeted to the nontemplate strand of DNA, and MOE PR2 target the template strand. No activity was observed for these sequences.

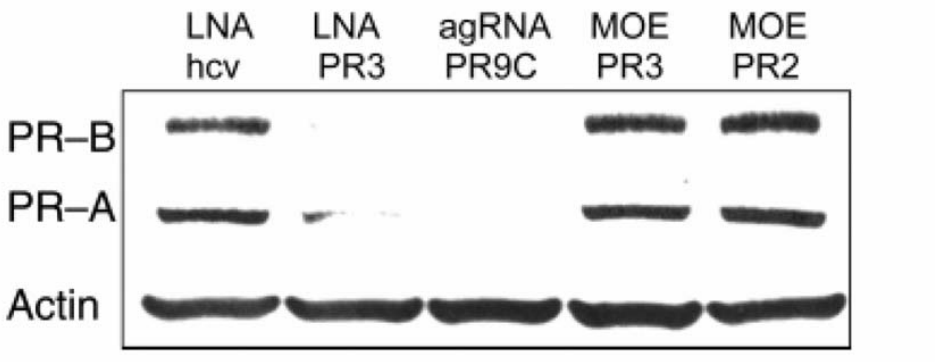


Figure 4.3: Western blot showing typical results obtained by using Oligofectamine transfection reagent in T47D cells. Corresponding target sites are listed in Table 5-1. “c1,” “c2,” and “c3” indicate the carrier used to anneal the PNA as listed in Table 5-2. Positive controls included agRNA PR9 and siRNA PRC1. PNAs were used at a concentration of 100 nM. Positive controls were used at 50 nM.

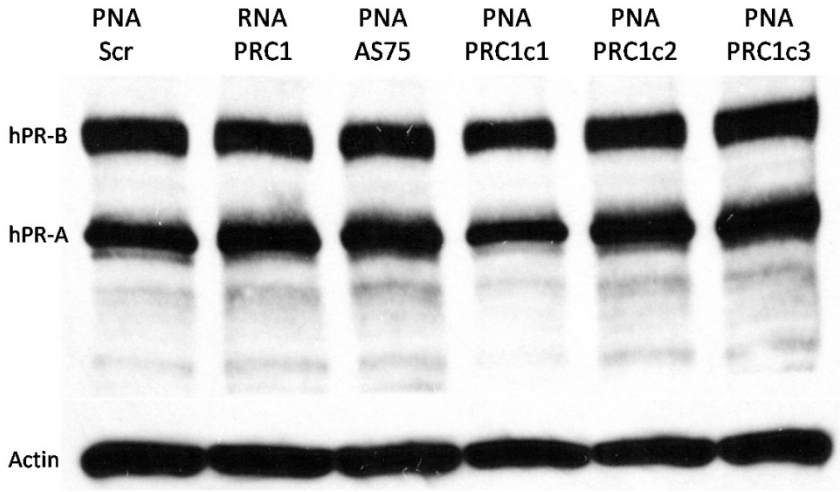


Figure 4.4: Western blot showing typical results of a double-transfection with antigene PNAs using RNAiMAX cationic lipid reagent. (A) Samples harvested on day 6. (B) Samples harvested on day 8. Corresponding target sites are listed in Table 4.1. "c1," "c2," and "c3" indicate the carrier used to anneal the PNA as listed in Table 4.2. Positive controls included agRNA PR9 and siRNA PRC1. PNAs were used at a concentration of 100 nM. Positive controls were used at 50 nM.

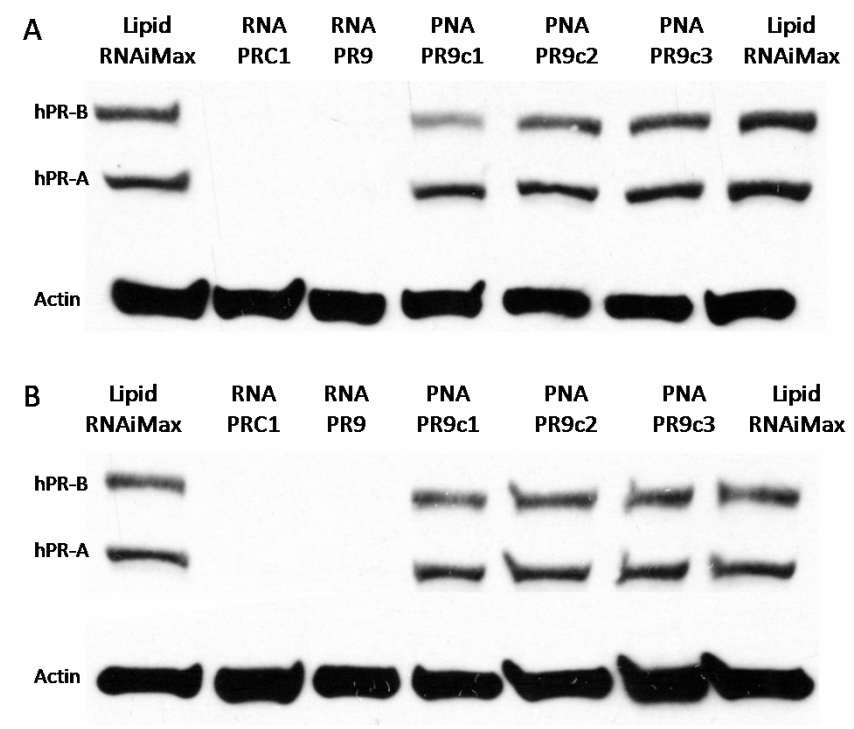


Figure 4.5: Western blot showing typical results of a double-transfection with antigene PNAs using (A) DharmaFECT 1, (B) DharmaFECT 2, (C) DharmaFECT 3, and (D) DharmaFECT 4 cationic lipid reagents. Corresponding target sites are listed in Table 4.1. "c1," "c2," and "c3" indicate the carrier used to anneal the PNA as listed in Table 4.2. Positive controls included LNAs B2 and siRNA PRC1. PNAs were used at a concentration of 100 nM. Positive controls were used at 50 nM.

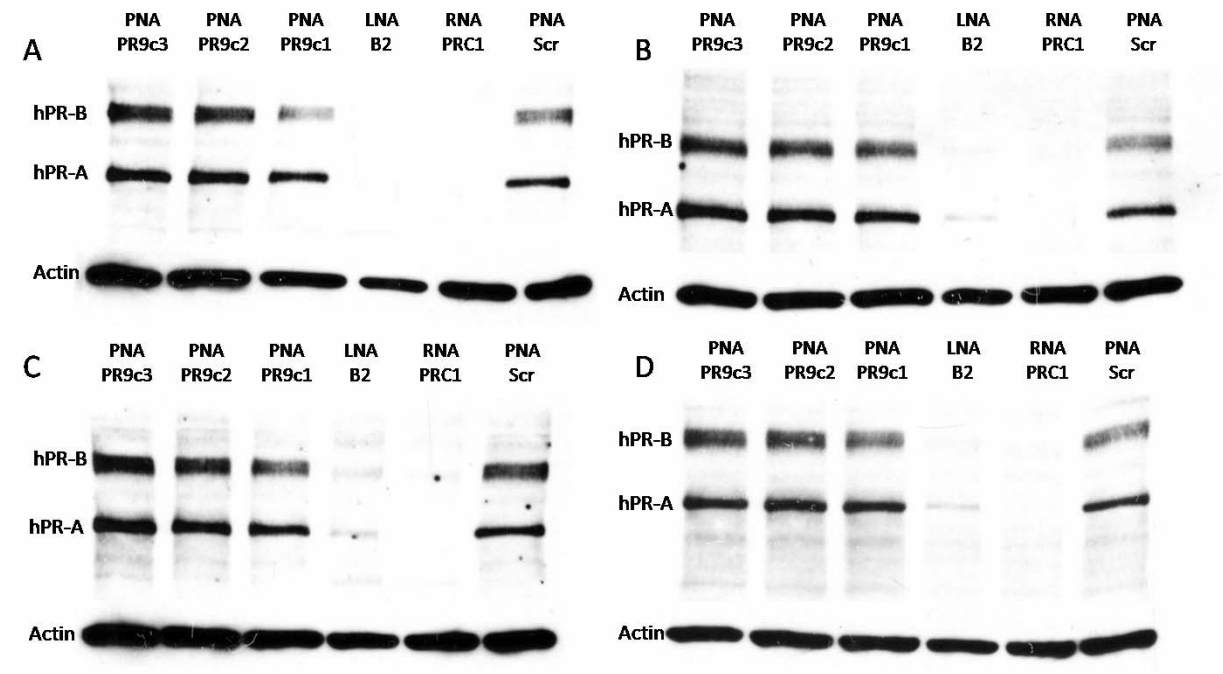


Figure 4.6: Western blots showing examples of the typical results of a single transfection experiments with antigene PNAs using various lipids. Specifically, results using (A) RNAiMAX, (B) DharmaFECT 2, and (C) DharmaFECT 3 cationic lipid reagents are shown below. Corresponding target sites are listed in Table 4.1. "c1," "c2," and "c3" indicate the carrier used to anneal the PNA as listed in Table 4.2. LNA B2, agRNA PR9, and siRNA PRC1 are positive controls. PNAs were used at a concentration of 100 nM. Positive controls were used at 50 nM.

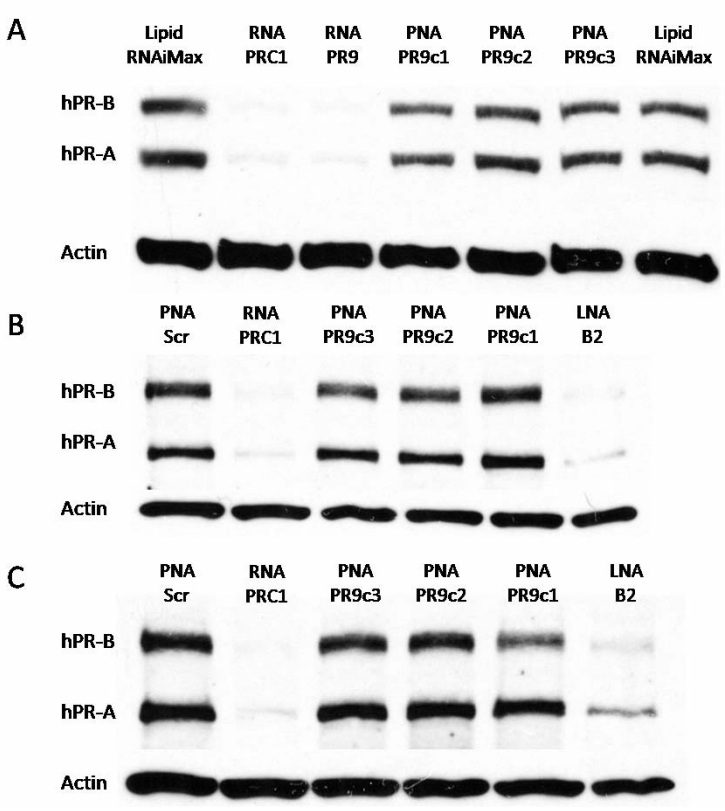


Figure 4.7: Western blot showing typical results of a single-transfection experiment with antigene PNAs using NEB Transpass P protein transfection reagent. NEB Transpass P was tested using multiple sequences and conditions and does not appear to facilitate PNA transfections.

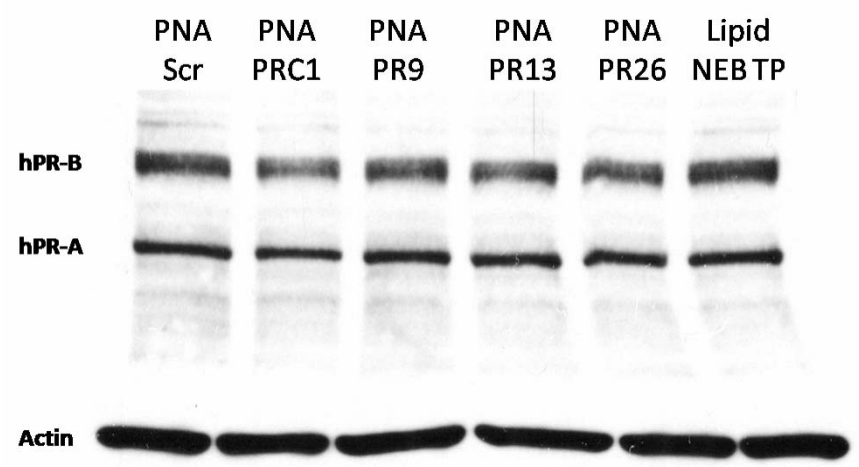


Figure 4.8: Western blot showing an example of the typical results of a single transfection experiments with antisense PNAs using various lipids. Specifically, this Western was performed using DharmaFECT 4 cationic transfection reagent. PNA scr represents a negative PNA control. RNA PRC1 is an siRNA positive control. PNA AS75 and PNA PRC1 are experimental PNA samples. "c1," "c2," and "c3" indicate the carrier used to anneal the PNA as listed in Table 4.2. PNAs were used at a concentration of 100 nM. Positive controls were used at 50 nM.

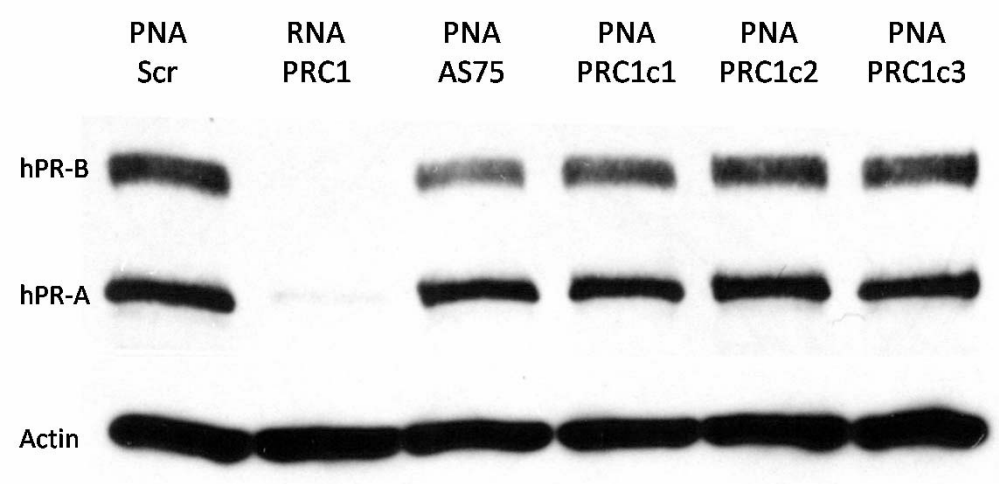


Figure 4.9: Western blots showing examples of the typical results of single transfection experiments with antisense PNAs testing the possibility that TFA affected PNA potency. This was tested with several lipids, specifically (A) DharmaFECT 4, (B) RNAiMAX, and (C) Oligofectamine cationic lipid transfection reagents. Ratios indicate the number of PNA molecules per molecules of TFA. RNA PRC1 is an siRNA positive control. PNA PRC1 is a positive PNA control sequence annealed to the previously validated sequence [3]. 'Old' indicates that the PNA was synthesized in 2006 or earlier.

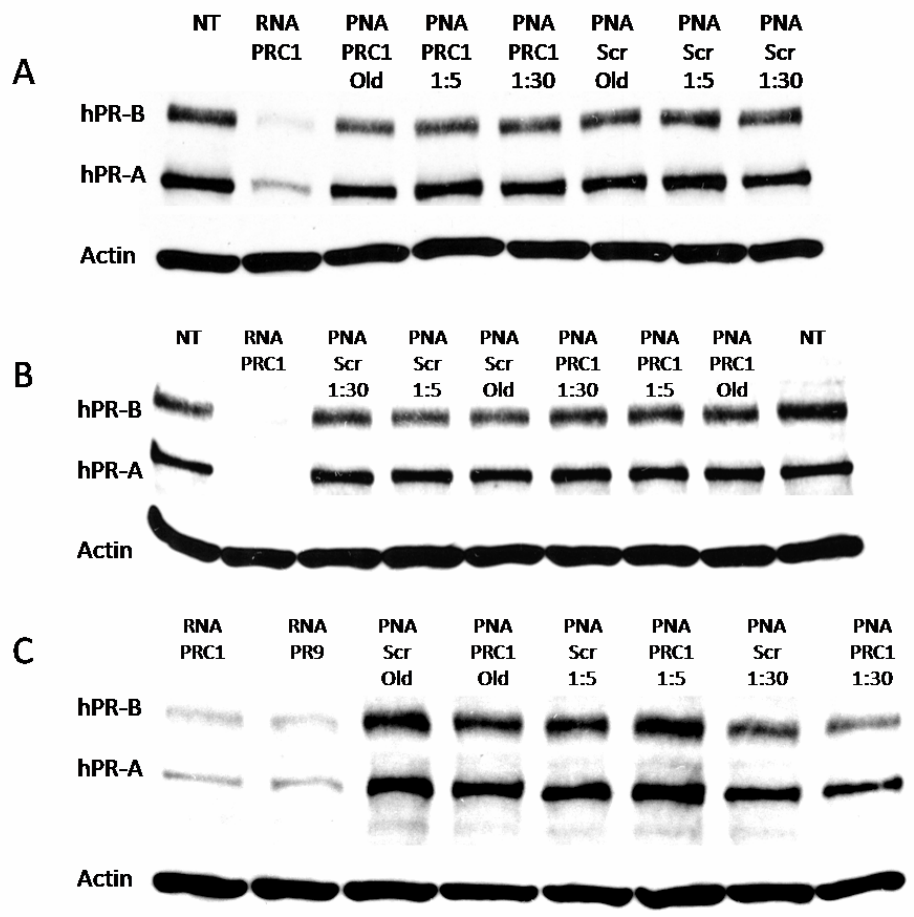


Figure 4.10: Western blots showing examples of the typical results of single-transfection dose response curves performed with antisense PNAs, which tested the possibility that TFA affected PNA potency. This was tested with several lipids, show here are (A) DharmaFECT 4 and (B) RNAiMAX cationic lipid transfection reagents. Ratios indicate the number of PNA molecules per molecules of TFA. RNA PRC1 is an siRNA positive control. PNA PRC1 is a positive PNA control sequence annealed to the previously validated sequence [3].

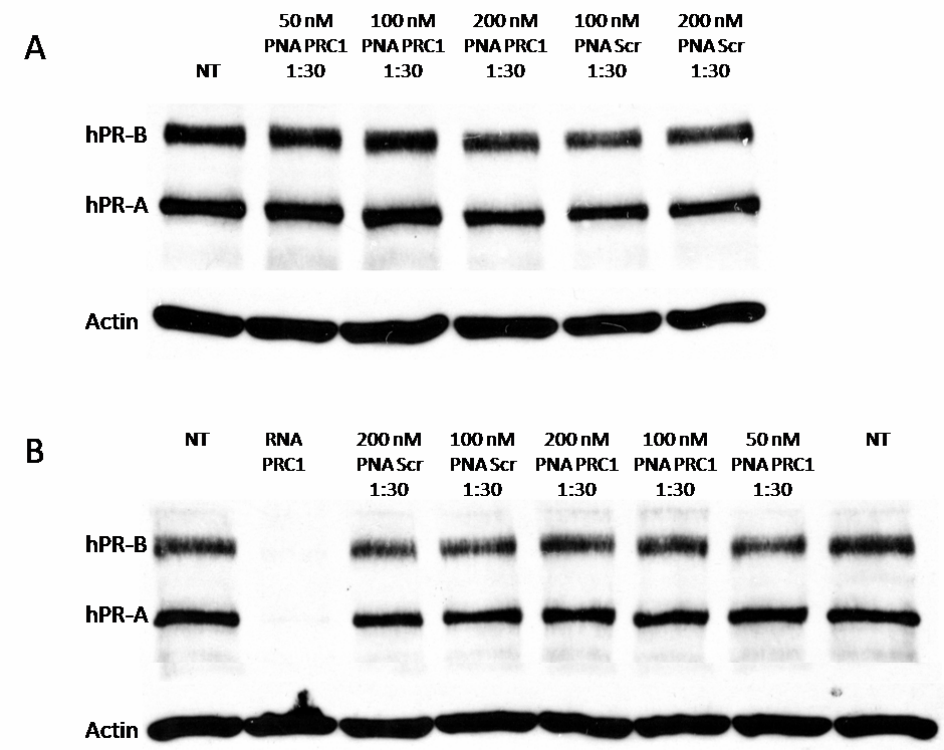


Figure 4.11: Example of Powerplex Genotyping Data from UTSWMC Sequencing Core

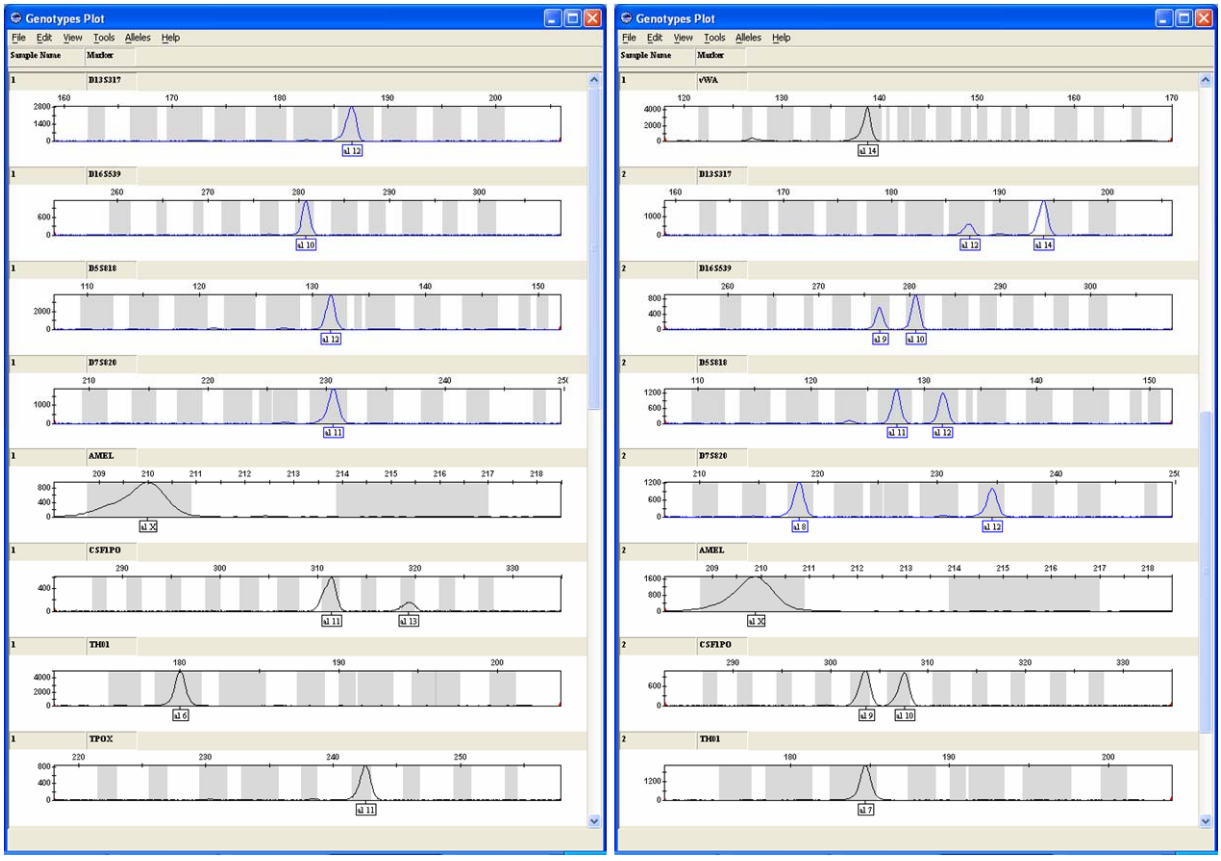


Figure 4.12: Summary of Conditions and Approaches Tested Using Antigene PNAs

- **Tested additional carriers**
- **Tested multiple lipids and transfection conditions**

Name	Single	Double	Reverse
RNAiMAX	•	•	•
DharmaFECT-1	•	•	
DharmaFECT-2	•	•	
DharmaFECT-3	•	•	
DharmaFECT-4	•	•	
NEB-D2	•	•	
NEB-TP	•	•	
Oligofectamine	•	•	

- **Tested PNAs synthesized in Korea**
- **Tested PNA Stocks used during 2003, 2004, and 2005**
- **Tested 2004 and 2006 T47D cells from cryostorage**
- **Tested all cells for mycoplasma contamination**
- **Genotyping verified that all cells were T47D**
- **Tested if TFA was required for potency**

Figure 4.13: Western blots showing examples of the typical results of single-transfection lipid ratio test. These experiments were performed with antigene RNAs using RNAiMAX cationic lipid transfection reagent, at the given ratio of RNA duplex to lipid. Duplex RNA was tested at 50 nM. MM4 is a negative control duplex RNA that does not have a specific target within the human genome. PR9 is an agRNA targeting the -9 to +10 region around the transcriptional start site of hPR-B.

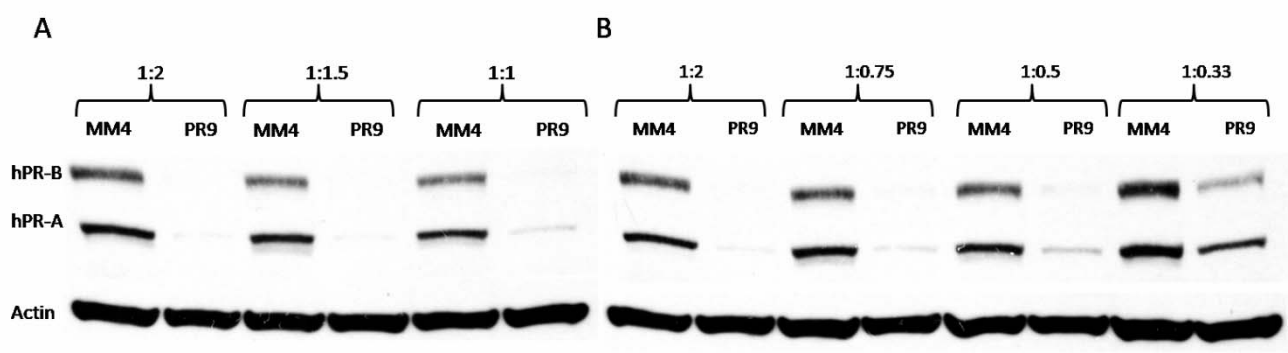


Figure 4.14: Western blots showing examples of the typical results of single-transfection cell number test. These experiments were performed with antigene RNAs using 1.8 µl of RNAiMAX cationic lipid transfection reagent. Duplex RNAs were tested at 50 nM. MM4 is a negative control duplex RNA that does not have a specific target within the human genome. PR9 is an agRNA targeting the -9 to +10 region around the transcriptional start site of hPR-B. Cell numbers varied as indicated from 40,000 cells to 140,000 cells.

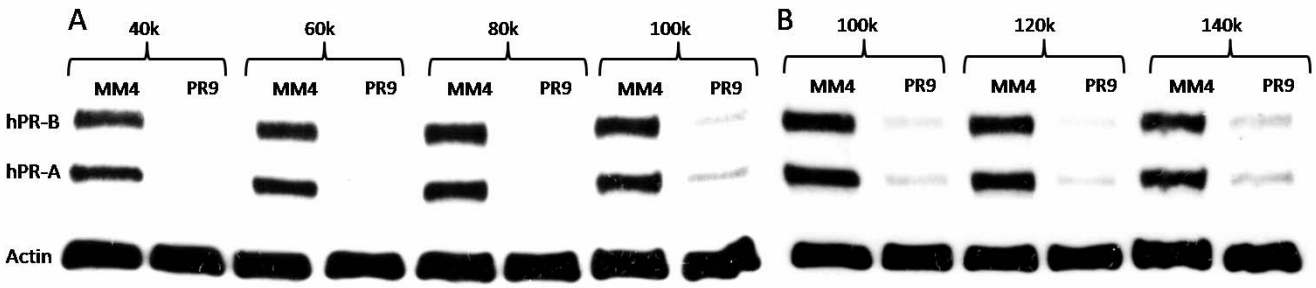


Figure 4.15: Western blot showing an example of the typical results of a reverse transfection experiment performed with antigene RNAs and using RNAiMAX cationic lipid transfection reagent. Duplex RNA was tested at 50 nM. MM4 is a negative control duplex RNA that does not have a specific target within the human genome. PR9 is an agRNA targeting the -9 to +10 region around the transcriptional start site of hPR-B. The estimated IC50 value is below 3.12 nM. This experiment was completed within 72 hours, which is 4 full days faster than the analogous forward transfection.

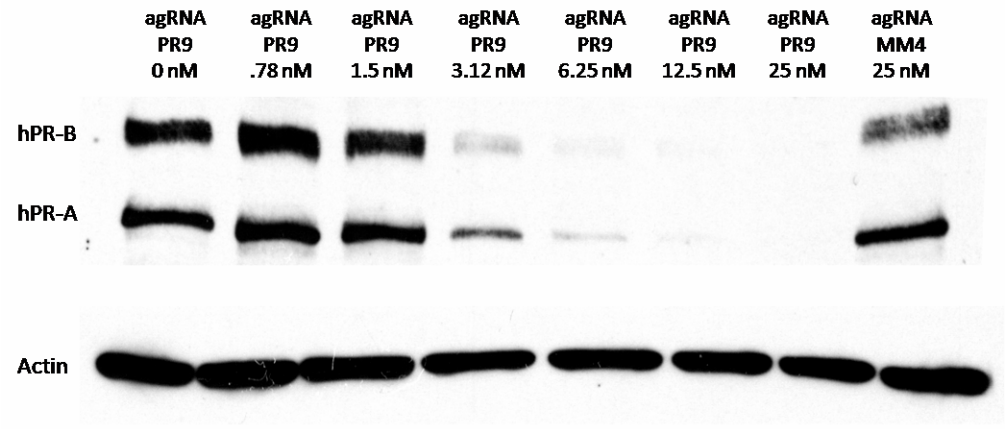


Figure 4.16: Western blot showing typical results of a (A) lipid ratio test and (B) cell number optimization test using antigene RNAs with Roche X-tremeGENE cationic lipid transfection reagent. Duplex RNA was tested at 50 nM. MM4 is a negative control duplex RNA that does not have a specific target within the human genome. PR9 is an agRNA targeting the -9 to +10 region around the transcriptional start site of hPR-B. The cell number optimization experiment was performed at a 5:1 lipid to RNA ratio as explained in the text.

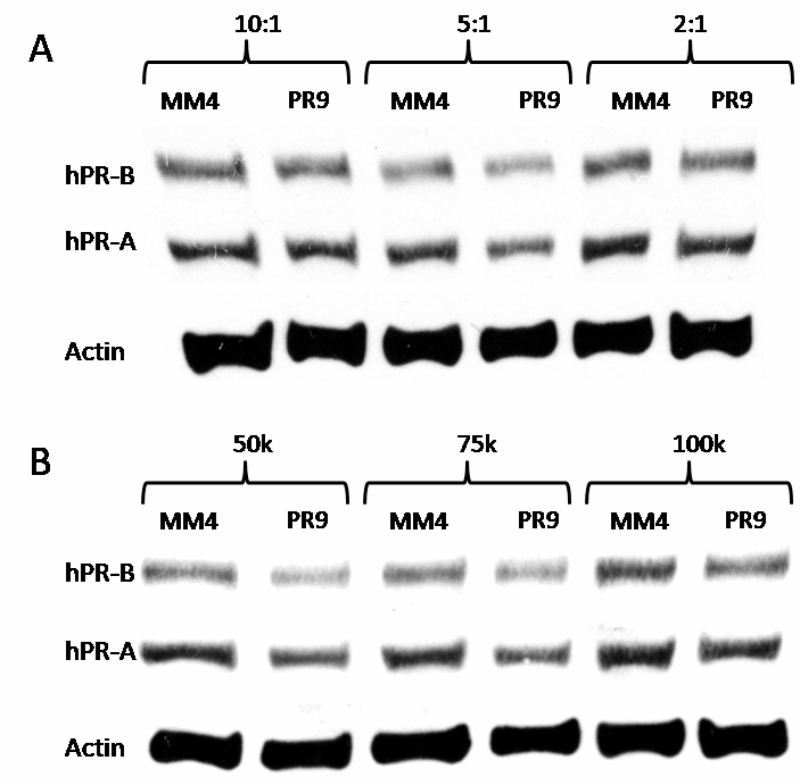


Figure 4.17: Western blot showing typical results of a (A) cell number and (B) lipid ratio optimization test using antigene RNAs with DharmaFECT-1 cationic lipid transfection reagent. Duplex RNAs were tested at 50 nM. MM4 is a negative control duplex RNA that does not have a specific target within the human genome. PR9 is an agRNA targeting the -9 to +10 region around the transcriptional start site of hPR-B. The cell number optimization experiment was performed at a 1:1 lipid to RNA ratio as explained in the text.

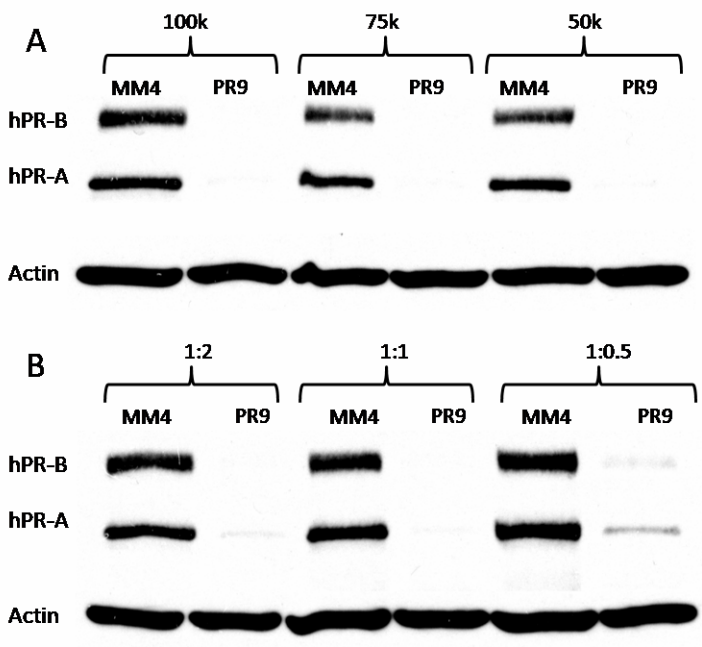


Figure 4.18: Western blots showing typical results of lipid ratio optimization tests using A) DharmaFECT-2, B) DharmaFECT-3, and C) NEB R1 lipid reagents. MM4 is a negative control duplex RNA, and PR9 is a positive control agRNA.

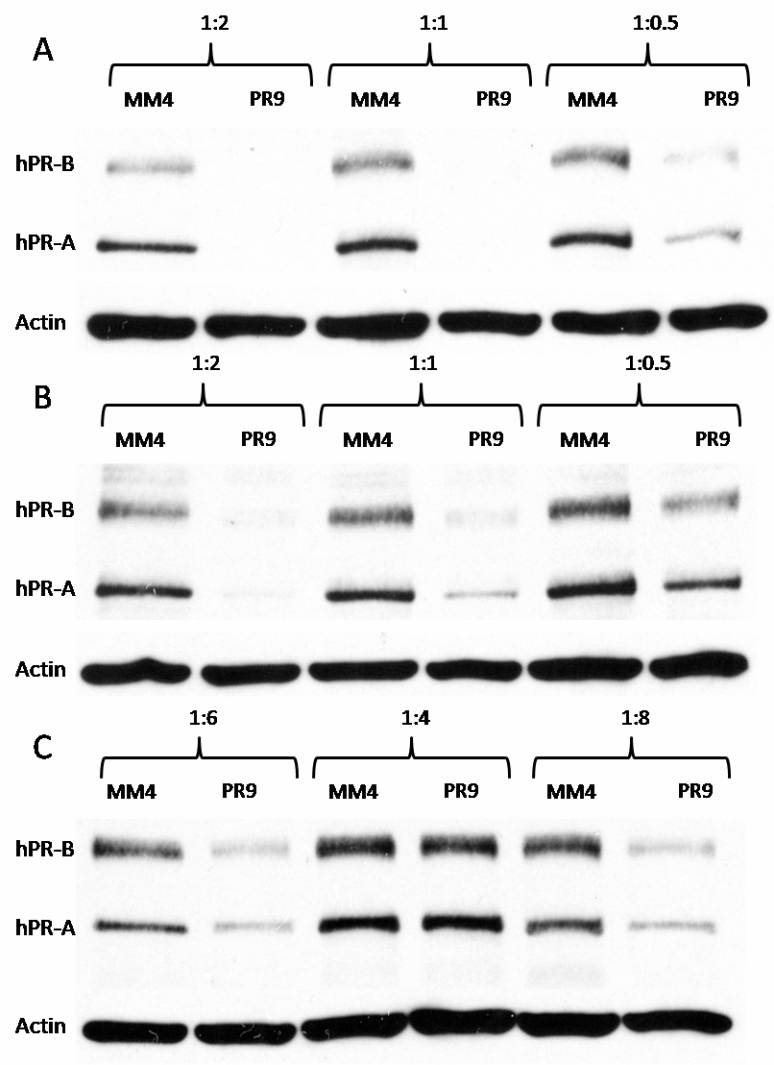


Figure 4.19: The QPCR data in figure (A) shows the relative mRNA levels following agRNA treatment in T47D cells. Data is typical of the normal observed inhibition of agRNA PR9. Data is normalized to GAPDH and MM4 as described in Chapter 6. (B) RNA polymerase II ChIP data indicating that RNA polymerase density is reduced following agRNA treatment relative to MM4 (signal above IgG background). This is indicative of transcriptional silencing.

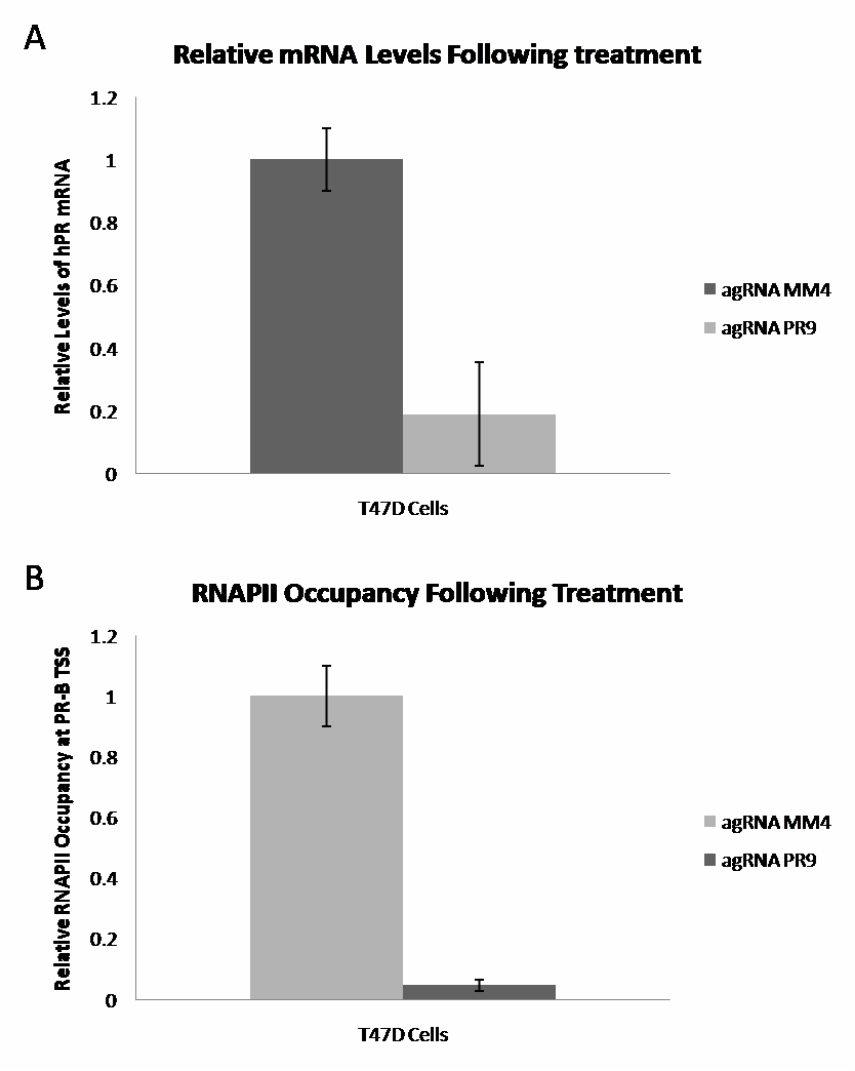


Figure 4.20: QPCR data showing the relative mRNA levels following ENA treatment in T47D cells. Data are normalized to GAPDH and ENA HCV (negative control) as described in Chapter 6. ENA targets are listed in Table 5-1. Data indicate that ENA B2 inhibits hPR-B levels approximately 50 percent. Data shown are the averaged results of 4 biological replicates. Error bars represent the standard deviation.

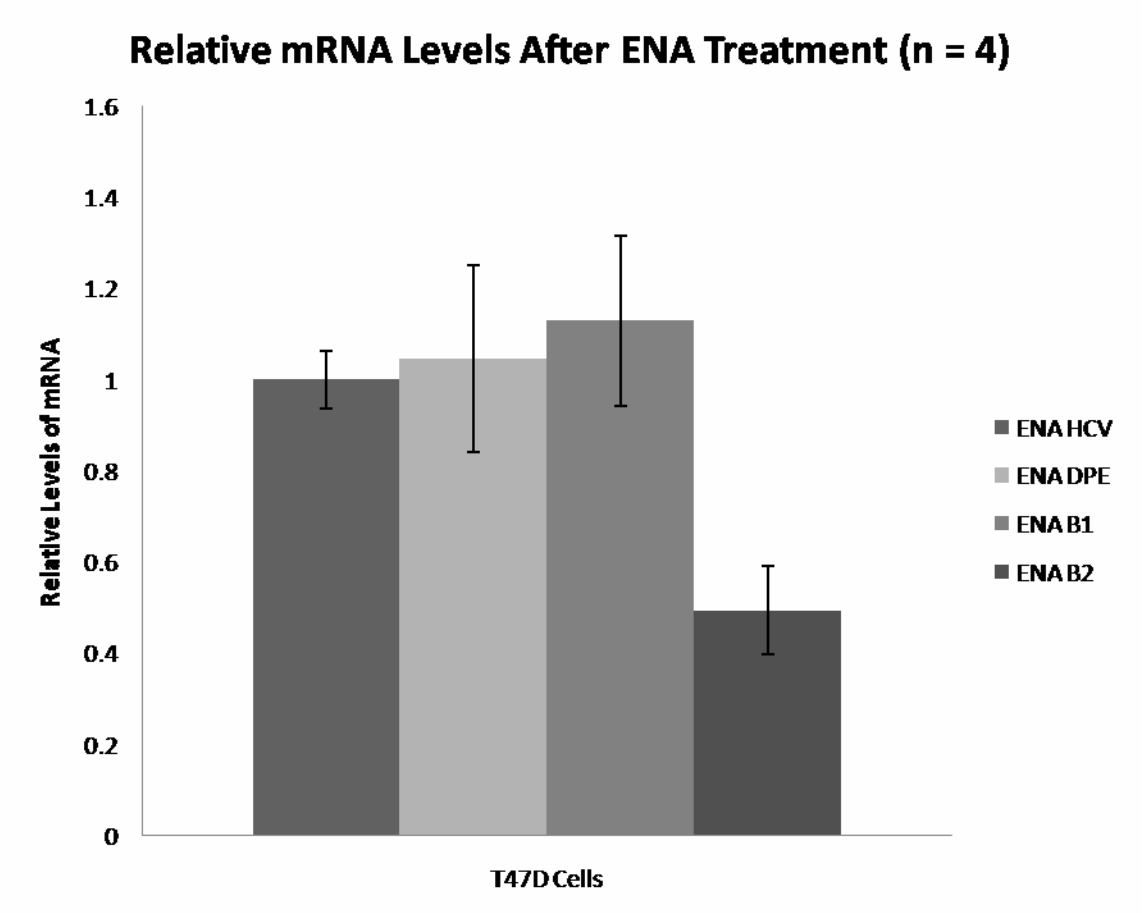
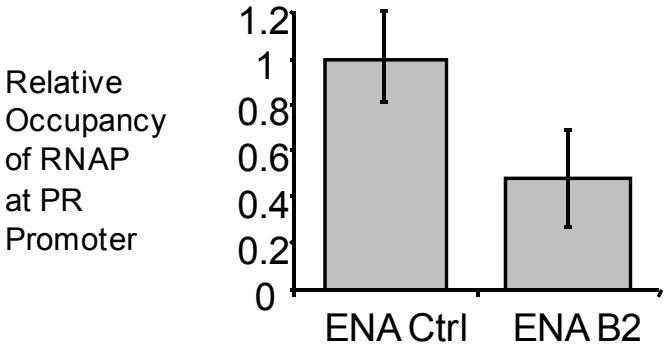


Figure 4.21: RNA polymerase II ChIP data indicating that RNA polymerase density is partially reduced following ENA treatment relative to ENA Ctrl (also ENA HCV) (Signal above IgG background). This may be indicative of transcriptional silencing.



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Chapter 5: Materials and Methods

5.1 Introduction

I would like to begin this chapter by thanking past lab members that I never met. Their carefully documented notes and protocols have been an invaluable resource. In turn, I hope that these protocols will help other researchers.

5.2 General Protocols

5.2.1 Absorbance Spectroscopy

The concentration of synthetic oligonucleotide, RNA, or DNA was measured using a Cary 100Bio UV-Visible spectrophotometer (Varian) at 260 nm and at ambient temperature. Concentrations of DNA, RNA, ENA, MOE, and LNA were determined by Beer's law using the extinction coefficients provided by the manufacturer. Concentrations of PNA were determined by summing the extinction coefficients of each individual monomer (A = 13.7, C = 6.06, G = 11.7, T = 8.6, mL/μmol*cm). Full spectrums ranging from 200 nm to 800 nm were obtained for each oligonucleotide to ensure quality. TE buffer was used in place of water for the samples and the blanks.

5.2.2 Melting Temperature (T_m) Analysis

For determining melting temperatures, the Cary 100Bio UV-Visible spectrophotometer (Varian) was used to measure the change of absorbance (hyperchromicity/hypochromicity) at 260 nM as the sample was repeatedly heated and cooled from 14°C to 95°C. The spectrophotometer was equipped with a 12-sample

Peltier temperature control accessory. PNA and complementary DNAs, DNA, LNA and complementary DNA, or duplex RNAs were suspended in a 0.1 M phosphate buffer (Na_2HPO_4, pH 7.5) at a concentration of 2 μM per strand. To prevent evaporation, mineral oil was placed over each sample.

Melting curves were generated only after an initial ramp to 98°C for a two-minute duration. This initial heating ensured that complementary oligonucleotides (or synthetic oligonucleotides) were dissociated prior to analysis. After the two-minute holding step at 98°C, the samples were gradually cooled to 14°C at a constant rate of 2°C/minute with a 6 second hold at each reading. At the same rate, the samples were gradually heated to 98°C. This process was repeated a finite number of times, which was at the user's discretion. Data were collected on each cycle following the initial cycle, and in both the heating (denaturation) and cooling (renaturation) parts of the cycle.

Theoretically, these two curves should be identical indicating a reversible process. However, it is not uncommon to observe hysteresis of the cooling path (annealing) relative to the heating path (dissociation). This observation is due to the entropic penalty of two complementary oligonucleotides finding incorrect binding partners and orientations prior to properly annealing to another oligonucleotide. This process does not occur during dissociation, hence the apparent hysteresis.

General analysis of the melting curves involves taking the first derivative of each curve plotted as absorbance observed versus the temperature. The shape of this curve can be very informative. A steep transition curve from double to single-stranded oligonucleotides indicates a very pure sample of annealed oligonucleotides. Multiple inflection points can be indicative of other stable complexes, including triplexes, higher

order structures, and also self-complementarity. An elongated sigmoidal curve is indicative of multiple semi-stable species, which can occur if the reagents contain truncated oligonucleotides, have substantial self-complementarity, or a combination of both.

For analysis, at least six complete runs should be examined, including denaturation and renaturation curves. The exact T_m and melting curve will depend on the concentration of the oligonucleotides present, the salt concentration, and the presence of additional chemicals or truncated products. Therefore, T_m values should be reported with these conditions stated.

5.3 General Cell Culture

5.3.1 MCF7

MCF7 (also MCF-7) cells were obtained from ATCC (American Type Culture Collection. Manassas, Virginia). MCF7 cells were originally described by Soule, et al. in 1973 [1]. The cell line was derived from a pleural effusion of a 69 year old Caucasian female. MCF7 cells were reported by Soule, et al. to have a mean of 88 chromosomes, most of which were metacentric.

MCF7 cells were maintained in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS), 0.5% nonessential amino acids (NEAA), 1 mM sodium pyruvate, 10 mM HEPES, and 0.4 units/ml bovine insulin. Cells were cultured at 37 °C and 5% CO_2. Cells were subcultured at 1:3 or 1:4 ratios.

Notably, Nugoli, et al. reported that MCF7 cells are subject to high genetic variability [2], which suggests that these cells can be very sensitive to culture conditions.

In fact, in 1987, MCF-7 cells from 4 laboratories were studied and found to be genetically distinct, and even responded differentially to hormone treatments [3]. Different cell conditions in long term culture were suggested to be one of the major contributing factors for this observation [3].

5.3.2 T47D

T47D (also T-47D) cells were obtained from ATCC (American Type Culture Collection. Manassas, Virginia). T47D cells were originally described by Keydar, et al. in 1979 [4]. The cell line was derived from a pleural effusion of a 54-year-old female with an infiltrating duct carcinoma of the right breast [4]. T47D cells were reported by Keydar, et al. to have a mode of 66 chromosomes, with considerable variation of chromosome number within the cell population. 2-3% of the originally observed cells were reported to have close to 100 chromosomes [4]. In addition, Keydar, et al. reported that acentric fragments and translocated chromosomes were frequently observed in this cell line [4].

T47D cells were maintained in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS), 0.5% nonessential amino acids (NEAA), 1 mM sodium pyruvate, 10 mM HEPES, and 0.4 units/ml bovine insulin. Cells were cultured at 37 °C and 5% CO_2. Cells were subcultured at 1:3 or 1:4 ratios.

It is important to note that T47D cell have been reported to be genetically unstable [5, 6], and are subject to high genetic variability. This suggests that these cells can be very sensitive to culture conditions. For the highest reproducibility, it is prudent to maintain exact culture conditions for the duration of experiments, and to use consistently use low passage numbers.

5.3.3 Cell Counting

In preparation for transient transfections, cells were counted using a hemocytometer. A hemocytometer is an optically flat chamber containing an aliquot of the cell suspension to be quantified, and is used in conjunction with a microscope. This method works because the hemocytometer has a defined area of known depth. The following protocol originated with R.I. Freshney's Culture of Animal Cells [7].

In preparation for counting cells using a hemocytometer, cells were trypsinized or chemically dissociated from their culture dish and suspended in media. From this cell suspension, a 10 µL aliquot was immediately transferred to the hemocytometer. (The aliquot should not be injected underneath the coverslip, but rather drawn under the coverslip by capillary action to ensure an even cellular dispersion.) Care was taken to avoid overfilling the hemocytometer, as this reduces accuracy. Once the cell suspension was loaded, the hemocytomer was placed onto the stage of a microscope and viewed under the lowest magnification to check even cell dispersion. (If the cells are not evenly dispersed, counts are not accurate.)

The microscope was adjusted for the highest magnification required to observe each 1 mm^2 square (delimited by triple parallel lines, nine total squares) individually. Usually, counting the cells in the four corner squares was sufficient to approximate cell numbers (the more cells counted, the more accurate the estimate will become, 300- 700 cells should be counted). Cells located along the left or upper boundaries of the 1 mm^2 area (i.e. the cells on the triple parallel lines) were not counted. However, the cells on the right and lower boundaries of the 1 mm^2 area were counted. The total number of

cells counted was then divided by the number of 1 mm^2 squares counted. The resulting number was then multiplied by 10^4. This calculation yielded the cells/mL of the original cell suspension.

This method is prone to several potential errors. Most errors are due to the improper loading of the hemocytometer chamber. A second cause of error is cell aggregation resulting from the improper mixing of the original cell. The final cell suspension should be a single-cell suspension to minimize error. Another source of error is using a dilute cell suspension. Although using a dilute cell suspension facilitates cell counting, concentrations under 10^6 cells/mL are prone to error.

5.3.4 Mycoplasma Testing

To test cells for mycoplasma contaminations, the Takara PCR Mycoplasma Detection Set (Catalog # 6601 from Takara Bio, Inc.) was used. The basic principle of this detection kit is that the mycoplasma contaminations can be detected in the media of contaminated cells after 72 hours. Therefore, an aliquot of old media is sufficient to detect multiple species of mycoplasm. This is possible because multiple species of mycoplasma have a conserved region of DNA, which can be amplified using the correct pair of PCR primers and the correct PCR conditions. The PCR conditions require low annealing temperatures to allow for potential mismatches due to the nonconserved bases present in some mycoplasma species, and a long extension time for this same reason.

Per sample, 0.1 µL of Hotstart Taq, 1.6 µL of 10 µM dNTPs, 2 µL Hotstart Buffer, 1 µL of primer R1, 1 µL of primer F1, and 9.3 µL of nuclease water should be added to 6 µL of cellular media. Samples should be fresh, as nucleases in the media can break

down the mycoplasma DNA and give false negatives. As a positive control, 1 µL of mycoplasma DNA was included. The mycoplasma DNA should be diluted in 5 µL of nuclease-free water and used in place of the cellular media listed above. As a negative control, 6 µL of water is used in place of cellular media. The negative control ensures that the pipettes being used have not been contaminated with PCR products.

The exact sequences and PCR conditions are described in the product manual. Specifically, the forward primer is 5' ACACCATGGGAGCTGGTAAT 3' and the reverse primer is 5' CTTCATCGACTTTCAGACCCAAGGCAT 3'. The first stage of PCR consists of one cycle of 94°C for 30 seconds. The second stage of PCR consists of 30-35 cycles of 94°C for 30 seconds, followed by 55° C for 2 minutes, and then 72°C for 1 minute. The total PCR reaction is approximately 3 hours.

PCR products were analyzed using agarose gel electrophoresis (described below). Expected product sizes ranged from 369 to 681 bases in length, depending on the mycoplasma species. A 2-3% agarose gel was used to separate these products. Approximately 6 µL of the PCR product was loaded with 2-4 µL of loading dye. The positive control, negative control, and the appropriate DNA ladder were viewed on the same agarose gel. Approximately 100 V was applied to the gel for 35 minutes in TBE buffer to achieve suitable separation.

Agarose gels were analyzed on the AlphaImager HP system (Alpha Innotech, San Leandro, CA). The following command was used to view and capture the image for analysis: "Acquire Image" (focus was adjusted to approximately 2.00, with an aperture of 1.20); auto exposure options were deselected. The UV transilluminator was turned

on, and the exposure time was adjusted as needed. Image properties were adjusted as required and the image was printed for documentation.

Positive results are indicated by the presence of any band or bands within a sample. There are two major errors associated with this protocol. First, prolonged storage of a sample allows mycoplasma DNA to degrade when stored in media containing nucleases. Second, false positives can be observed if PCR routine is repeated for enough cycles that primer-dimers begin forming.

5.3.5 Genotyping

Genotyping is a service provided by the UT Southwestern McDermott Center Sequencing Core and can be used to verify the identity of cell lines. This is useful because cells that are kept too long in culture may not be reliable models of the original source tissue. Also, cell lines may become mixed with other cell lines or mislabeled in cryostorage. To address these potential problems, PCR can be used to amplify molecular markers within cells. ATCC lists the expected genotype of each cell type that it provides on its website (www.atcc.org). The genotype is listed as “DNA Profile” and is given by the expected number of times each of 9 STRs (Short Tandem Repeats) are repeated. For example, MCF-7 cells have a “DNA Profile” given as the following:

Amelogenin: X

CSF1PO: 10

D13S317: 11

D16S539: 11,12

D5S818: 11,12

D7S820: 8,9

THO1: 6

TPOX: 9,12

vWA: 14,15

T47D cells are defined as having the following genotype:

Amelogenin: X

CSF1PO: 11,13

D13S317: 12

D16S539: 10

D5S818: 12

D7S820: 11

THO1: 6

TPOX: 11

vWA: 14

Since the probability of each marker being identical in two distinct cell lines is very low, this form of identification is a reliable way to validate cell lines.

DNA can be purified using Trizol per the manufacturer's instructions. As an alternative, other kits that directly purify DNA from mammalian cells are also commercially available. For genotyping analysis, the UT Southwestern Medical Center McDermott Center Sequencing Core requires a minimum of 3 µL of high-quality DNA at a concentration of 10 µg/mL. Data is returned as shown in **Figure 4.11**, and as a text file summarizing the results in a format similar to the DNA profiles listed above. These results should be interpreted with the caveat that observed gene expression may vary among cell lines even with identical genotypes depending upon cell culture conditions.

5.4 Transient Transfection Protocols

5.4.1 Forward Transfection

Transient transfections are used to temporarily transfect cells as opposed to stably transfected cells. Here, forward transfections specifically refer to transfections performed on monolayer cells adhered to the surface of a normal culture vessel. This procedure is accomplished by seeding the cells in advance (the exact amount of time depends on the cell line used).

For T47D experiments, cells were plated at 80,000-120,000 cells per well in six-well plates (Costar) two days prior to transfection. Optimal cell number was determined empirically, and adjusted as needed depending on the rate of cell growth (cells generally grow at different rates depending on the passage number). Transfection of oligonucleotides was performed with Oligofectamine or RNAiMax (Invitrogen) according to the manufacturer's instructions. Duplex RNA, MOE, LNA, PNA:DNA complexes, and ENAs were diluted to the desired concentration in a final volume of 1.25 mL using Optimem (Invitrogen) containing cationic lipid reagent. This mixture was added to cells. Media was changed 24 hours post-transfection. For Western blots, cells were harvested approximately 4-6 days post-transfection. For QPCR, mRNA was harvested approximately 3-4 days post-transfection.

For MCF7 experiments, cells were plated at 120,000-180,000 cells per well in six-well plates (Costar) two days prior to transfection. Optimal cell number was determined empirically, and adjusted as needed depending on the rate of cell growth. Transfection of oligonucleotides was performed with Oligofectamine or RNAiMax (Invitrogen) according to the manufacturer's instructions. Duplex RNA, LNA, PNA:DNA

complexes, and ENAs were diluted to the desired concentration in a final volume of 1.25 mL using Optimem (Invitrogen) containing cationic lipid reagent. This mixture was added to cells. Media was changed 24 hours post-transfection. As with T47D cells, cells were harvested approximately 4-6 days post-transfection for Western blots and approximately 3-4 days post-transfection for mRNA.

For high reproducibility, cells should only be used if they are evenly dispersed prior to transfection. Cell confluency (as measured by how much of a cell's edges are touching another cell) should be optimal for the transfection reagent being used (the manufacturer will specifically state the expected optimal confluency for their lipid reagent). Cell number optimization experiments can be used to estimate the optimal confluency of a lipid for a given cell line, assuming the cell growth is relatively stable.

Forward transfections allow the user the advantage of inspecting cells prior to the transfection. In addition, forward transfections allow the user to inspect for potential cell counting errors or unexpected cell growth rates. However, forward transfections have the disadvantage of taking additional time and media as compared to reverse transfections.

5.4.2 Reverse Transfection

Here, "reverse transfection" specifically refers to a transfection performed on monolayer cells, which have been suspended in media prior to the addition of a lipid/oligonucleotide complex. This type of transfection has become popular since the advent of RNAi screening procedures [8]. This procedure is accomplished by trypsinizing or chemically dissociating the cells and counting them just prior to the actual transfection. The counting of the cells generally occurs while the oligonucleotide and

lipid reagents are being allowed to complex in solution. Reverse transfections are distinct from forward transfections because they do not require the seeding of cells prior to the actual experiment.

Transfection of oligonucleotides was performed according to the manufacturer's instructions, when provided. Each unique cationic lipid will need to be individually optimized. Duplex RNA, LNA, PNA:DNA complexes, and ENAs were diluted to the desired concentration in a volume of 0.25 mL using Optimem (Invitrogen) containing a cationic lipid reagent at an optimized ratio. This mixture was evenly dispersed into six-well plates and allowed to incubate for 20 minutes. During the 20-minute incubation, T47D cells were chemically dissociated and suspended in RPMI-1640 for counting. T47D cells were diluted to yield a final concentration of 100,000-140,000 cells per mL. 1 mL of diluted cells was added to each well in six-well plates (Costar) following the 20 minute incubation, for a total final volume of 1.25 mL. An additional 1 mL of RPMI-1640 Media was added 24 hours post-transfection for a total volume of 2.25 mL. 48 hours post-transfection, media was replaced with RPMI-1640. For Western blots, cells were harvested approximately 3-4 days post-transfection. For QPCR, mRNA was harvested approximately 3 days post-transfection.

MCF7 cells were reverse transfected in the same manner and using the same final volumes. MCF7 cells were transfected at a final concentration of 120,000 to 160,000 cells per mL. As with T47D cells, protein samples were harvested 3-5 days post transfection, and mRNA samples were harvested 3-4 days post transfection.

For reproducibility, the 0.25 mL mixture of lipid/oligonucleotide/Optimem should be evenly dispersed along the bottom of each well. This process is facilitated by pre-

wetting the surface of the plate using Optimem, and aspirating the Optimem prior to adding the 0.25 mL mixture (This reduces surface tension). Cell number optimization experiments can be used to estimate the optimal confluency of a lipid for a given cell line, assuming the cell growth is relatively stable. Time trials may be necessary for determining the optimal incubation time for each lipid, each unique gene, and each unique oligonucleotide chemistry.

Reverse transfections are more conducive to high-throughput experiments and screens as compared to traditional forward transfections. As described in Chapter 4, agRNA PR9 was capable of potently inhibiting hPR-B protein within 72 hours post-transfection (see **Figure 4.15**). Since this experiment did not require cells to be plated 48 hours in advance, this data was obtained a full 4 days faster than the analogous forward transfection.

5.4.3 Double Transfection

The term ‘double transfection’ refers to a protocol that requires two consecutive transient transfections. In general, a double transfection requires that the protocol for a forward transient transfection be performed twice, and often requires that the cells are passaged prior to the second transfection to prevent the cells from becoming overconfluent. This protocol has been utilized by the Corey laboratory to successfully transfect antigene PNAs into T47D cells [9, 10], and has also been used to introduce antigene LNAs into T47D and MCF7 cells [11]. In addition, this protocol has been utilized to evaluate the mechanism of antigene RNAs [12].

For T47D experiments, cells were plated at 80,000-90,000 cells per well in six-well plates (Costar) 48 hours prior to the initial transfection. Optimal cell number was

determined empirically, and adjusted as needed depending on the rate of cell growth. Transfection of oligonucleotides was performed with cationic lipids according to the manufacturer's instructions (Oligofectamine was used exclusively for the original antigene PNA [10] and LNA [11] work). Duplex RNA, LNA, and PNA:DNA complexes were diluted to the desired concentration in a final volume of 1.25 mL using Optimem (Invitrogen) containing cationic lipid reagent. This mixture was added to the monolayer of adhered cells and allowed to incubate for 24 hours. Media was changed 24 hours post-transfection.

72 hours after the initial transfection, cells were inspected for health (toxicity, growth rate, and distribution) and confluency in preparation for passaging. In general, cells were passaged 1:3, however, if toxicity was observed (i.e. slow growth and floating cells relative to the controls) cells were passaged at higher ratios such as 1:2 or 3:4. This procedure was accomplished by washing the cells with warmed Dulbecco's PBS (Sigma) to remove residual media. The PBS was aspirated off the cells, and 200 µL of trysin was added for approximately 2 minutes to dissociate the cells. 800 µL of warm RPMI-1640 was added to stop the trypsin reaction. Cells were dissociated using a pipette. The required ratio of cells was added to a new well, and additional RPMI-1640 was added for a final volume of 2 mL. Splitting the cells was necessary to ensure exponential cell growth, and to prevent cells from becoming overconfluent.

5 days after the initial transfection, cells were transfected a second time. The protocol detailed above for a transient forward transfection was repeated. Media was changed 24 hours after the second transfection. Protein samples were harvested on days 7-9 depending on cell health and confluency (generally, day 8 was optimal).

Double transfections may be carried out without splitting by performing the second transfection 72 hours after the initial transfection and harvesting 5-6 days after the initial transfection. However, the confluency of the cells cannot be optimal for both transfections if the cells are growing at a stable rate (i.e., cells are initially transfected at optimal confluency, but continue to grow. Therefore, the second transfection must be performed under suboptimal conditions if the initial transfection was done correctly). This can affect reproducibility. Also, some genes are sensitive to the confluency level of the cells, which may lead to an apparent modulation of gene activity independent of treatment.

Double transfections have the advantage of allowing the user to split the cells into multiple plates. For example, a single plate can be used for the initial transfection. Cells are passaged at a 1:3 ratio into three separate six-well dishes. This ensures that the first transfection was performed under the same exact conditions, and allows the user to have three technical replicates of the second transfection or to perform time trials by harvesting each plate on a different day.

However, double transfections have many disadvantages including lower reproducibility, additional handling, additional media and reagents, and are more prone to user error. Since oligonucleotides can affect cell growth differentially, it is highly unlikely that cells will be uniformly distributed during the second transfection. This means that the transfection efficiency for each well and each sample will be different for the second transfection relative to the first transfection. This inherent error is difficult to estimate since the effect of an oligonucleotide may not become pronounced (toxic) until after the cells have already been split. This results in lower reproducibility. Double

transfections require additional handling relative to single transfections. As described above, double transfections require cells to be passaged during the experiment. Each well may need to be passaged at different ratios, and may be growing at different rates. Therefore this process requires either guesswork or extensive user experience with each particular cell line. Double transfections are more expensive to perform than single transfections relative to the amount of data obtained. This is due to the use of more oligonucleotide, more media, more plates, more trypsin during splitting, more lipid, and more time. Finally, double transfections are more prone to user error since it requires additional handling. If cells are passaged incorrectly, the wrong well is transfected, or wells are mislabeled during the splitting steps the experiment is lost.

5.5 Bacteria and Plasmid

General protocols for making LB plates, LB media, and growing bacterial cells were directly taken from Chapter 1 of Current Protocols in Molecular Biology.

5.5.1 Cloning

Cloning was accomplished using the *TOPO TA Cloning Kit for Sequencing* (Invitrogen). The kit was used according to the manufacturer's instructions with a few small modifications. Only one-half of the amount of each reagents specified by the instructions was used, which effectively doubled the number of reactions per kit without any noticeable decrease in efficiency. Immediately following the cloning reaction, the TOPO plasmid with the desired insert was transformed into competent bacterial cells.

5.5.2 Transformation of Bacteria

Competent E. coli cells were obtained from commercial sources. 1-3 µL of purified plasmid DNA was added to nuclease-free water for a total of 6 µL. The 6 µL was then added to 50 µL of competent bacterial cells and incubated on ice for 20 minutes. Cells were placed at 41°C for 30 seconds and then immediately returned to ice for2 minutes. 200 µL of S.O.C. media was added to the transformed bacteria, and the solution was allowed to grow at 37°C in a shaker at 240 rpm for 1 hour.

Following the incubation, 50 µL and 200 µL of bacterial cells are plated on two separate LB plates containing ampicillin. Having two concentrations helps the user to identify individual colonies. Plates are placed at 37°C and allowed to grow for approximately 16 hours.

5.5.3 Plasmid DNA Purification Using Alkaline Lysis

DNA was purified from bacterial minipreps by alkaline lysis using a protocol modified from *Current Protocols In Molecular Biology* 1.6.1. (1.7.2). A single bacterial colony was used to inoculate 5 mL of LB broth containing ampicillin and was grown to saturation (8-12 hours) at 37°C and 240 RPM. 2.0 mL of bacterial cells were placed into a 2.0 mL Ependorf microcentrifuge tube and spun at maximum speed using a tabletop microcentrifuge for approximately 15 seconds. Cleared media was aspirated from the pelleted cells and the process was repeated for a total of 4 mL of the original culture.

Cells were resuspended in 100 µL GTE (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA) solution and incubated at room temperature for 5 minutes (RNase can be added to any of these steps in an attempt to lower the amount of contaminating RNA).

200 µL of NaOH/SDS solution (0.2N NaOH, 1% SDS) was added to lyse the cells, and the reaction should be allowed to continue for no longer than 5 minutes on ice. The reaction is neutralized by adding 150 ul of potassium acetate solution (3M potassium acetate, pH 5.5) and then vortexing the solution at maximum speed for 2 seconds to mix. (Prolonged mixing can result in sheared bacterial DNA contaminating the plasmid DNA.) The reaction should be allowed to progress on ice or 5 minutes. The precipitate can be visually confirmed. The desired plasmid DNA is suspended in the clear portion of this mixture.

To remove the precipitate, spin cells at 12,000 rpm for 5 minutes. The clear supernatant is then transferred to a fresh tube. 800 µL of 95% ethanol should be added to the supernatant and allowed to incubate for 2-5 minutes at room temperature to precipitate nucleic acids. DNA and RNA was pelleted by spinning the samples at 12,000 rpm for 2 minutes (The pellet should be visible). The supernatant was aspirated and the pellet was washed with 1 mL of 70% ethanol. 70% ethanol was removed by centrifugation at 8,000 rpm for 3 minutes. The resulting pellet was resuspended in 50 µL of TE buffer. To remove RNA, DNA was purified using 1% agarose gel electrophoresis. The resulting DNA was sent to the UTSWMC McDermott Center Sequencing Core for verification.

5.5.4 Maxi-prep Plasmid DNA Purification

Plasmid DNA was purified from maxi-preps (200-1000 mL) using the GenElute HP Plasmid Maxiprep Kit (catalog # NA0300) from Sigma-Aldrich. Purification was performed using the “spin” format and resulted in high-quality DNA without detectable

RNA contamination. Although alkaline lysis can be used as an alternative to this kit, it is not recommended because the yield is generally lower due to the necessity of RNase treatments to remove RNA contamination.

5.5.5 Direct Colony PCR

Direct colony PCR is a relatively fast way to verify that a colony contains a plasmid DNA of interest. It is conducive to high-throughput screening since it circumvents the need to grow overnight cultures to prepare minipreps for sequencing. This procedure can also be used to determine the orientation of an insert by using additional primer sets. Required materials include general PCR equipment, PCR reagents, and primers flanking the insert are required to verify the presence or absence of a DNA insert. The following protocol was adapted from methods in Molecular Biology [13].

A master reaction mix can be prepared for all samples. The following was added per sample: 0.1 µL of Hotstart taq, 1.6 µL of 10 µM dNTPs, 2 µL Hotstart Buffer, 1 µL of forward primer, 1 µL of reverse primer, and 14.3 µL of nuclease water. A sterile pipette tip was used to obtain a small sample of each colony (or mini-prep solution) to mix with the PCR reaction mixture detailed above (‘small sample’ means that the PCR reagents should not look cloudy or significantly change color upon addition). A positive control should be included and can consist of a small aliquot of the original insert. Negative controls should include a sample without DNA template to ensure primer-dimers are not forming, and a second negative control consisting of an empty vector template should also be included.

Exact PCR conditions are dependent upon the primer pair selected and the insert to be examined. An example of a general protocol would include a ten-minute incubation at 95°C to lyse the bacterial cells and to activate the Hotstart taq. Then the PCR reaction should be heated to 95°C for 15 seconds for denaturation, cooled to 57°C for 30 seconds for annealing, heated to 72°C for 1 minute for extension, and then these three steps should be repeated 40 to 45 cycles. This should be followed by a 10 minute incubation at 72°C. After these steps the reaction should be held at 4°C.

PCR products should be examined by electrophoresis on a 3% agarose gel. If needed, the PCR product can be purified from the agarose gel. The presence of a band at the expected molecular weight indicates that the colony contains the insert of interest. The results can be used to screen dozens of colonies in order to decide what samples should be further studied or sent to sequencing.

5.6 Preparation of Short dsRNAs

5.6.1 Design and Target Selection

Duplex RNAs can have multiple off-target effects [14-16] and therefore require considerable attention when designing and selecting a target. Multiple companies now provide in-house software for determining the optimal siRNAs for a given target sequence, however, this software does not apply to the design of antigene RNAs. Since a promoter is located in a static region of DNA, antigene RNAs may not be considered optimal siRNAs. However, since agRNAs are chemically identical to siRNAs [17], it is prudent to avoid sequences that may elicit off-target effects. This task is accomplished using NCBI BLAST (Basic Local Alignment Search Tool) and ensuring

that additional sequences are not being targeted. A caveat: BLAST is limited by algorithm parameters and was not intended to search for short sequences that may constitute the source of off-target effects.

Since it is difficult to find unique RNA targets with no other potential off-target effects, it is prudent to select and test two or three sequences with a single target gene. If each sequence has a different set of potential off-target effects, but yields the same phenotype, it lends credence to a claim of specific effect. In addition, scrambled control sequences or mismatch containing sequences should be designed to verify the observed effect is specific.

5.6.2 Preparation of RNA Stocks

Lyophilized single-stranded RNAs were obtained from Integrated DNA Technologies (IDT). RNAs were re-suspended into 100 μM stocks using nuclease-free water (Ambion, Foster City, CA). Absorbance spectroscopy was used to confirm the concentration and purity of each RNA using the extinction coefficient provided by IDT. Complementary RNA strands were combined to a produce a final concentration of 20 μM RNA duplex in 2.5X PBS. RNAs were annealed using a thermocycler. The general protocol was as follows: 95 °C for 5 min, 85°C for 1 min, 75 °C for 1 min, 65 °C for 5 min, 55 °C for 1 min, 45 °C for 1 min, 35 °C for 5 min, 25 °C for 1 min, and 15 °C for 1 min, and held at 15 °C. Stocks were stored at -20°C or -80°C. This protocol was modified from a previously published protocol [9].

5.7 Preparation of LNA-modified Oligonucleotides

5.7.1 Design and Target Selection

LNA target sequences were selected primarily on the observed activity of antigene PNAs [10] and RNAs [17]. Additional targets were selected if they were conserved and appeared functionally significant [11]. Conservation was determined using UCSC Genome Browser. NCBI BLAST was used to ensure that obvious off-target effects were minimized. Unless otherwise specified, the placement of LNA-bases were intended to evenly distribute the modifications within the oligonucleotide, and to minimize the number of modified bases required to synthesize a specific oligonucleotide [11].

5.7.2 Preparation of LNA stocks

Single-stranded LNAs were provided in suspension from Sigma-Proligo. The LNA stocks were heated to 65°C for 5 minutes to ensure the absence of aggregate LNA clusters. LNA concentrations were determined using absorbance spectroscopy and the extinction coefficient provided by Sigma-Proligo. Single-stranded LNAs were diluted to 20 μM stocks. Prior to transfections, LNAs were heated at 65°C for 5 minutes. Stocks were stored at -20°C or -80°C.

If LNAs showed obvious toxicity in cell culture, the original stock was purified using a G-25 spin column (Illustra MicroSpin G-25 Columns, GE Healthcare). LNA stocks were remade as described above and tested in cell culture. G-25 purification should be avoided unless it is required due to the substantial loss of LNA reagent.

5.8 Preparation of PNA Oligonucleotides

The following description of PNA protocols were largely taken from a single source, the dissertation of Christopher Nulf [18]. Dr. Nulf was a previous graduate student in the Corey laboratory, and although we have never met, I have learned a great deal from his protocols and descriptions. These original protocols have been revised and expanded to include design parameters, cell culture experience, and antigene applications.

In general, PNAs were designed to target promoter sequences in the hPR and hAR genes, and then the sequences were checked for potential off-target effects computationally using NCBI BLAST. Following these steps, the PNA was synthesized using an Expedite 8909 PNA Synthesizer. The crude product was verified using MALD-TOF mass spectrometry and then purified using HPLC. HPLC samples were collected and MALDI-TOF mass spectrometry was used to identify aliquots containing pure PNA. These samples were collected, lyophilized, and then resuspended in water. For delivery to cells, DNA carriers were designed and ordered for each PNA. Carriers were annealed to the PNA using a thermocycler, and then the stability and purity of PNA:DNA complexes were verified using melting temperature analysis. Suitable carriers were further tested in cell culture and the optimal carrier was used for additional studies. These protocols are presented below in the general order that they were used.

5.8.1 PNA Design and Target Selection

PNA target sequences were selected primarily on the previously observed activity of antigene PNAs [10], RNAs [17], and LNAs [11]. Additional targets were selected if they were conserved and appeared functionally significant. Conservation

was determined using UCSC Genome Browser [19-27]. NCBI BLAST was used to ensure that obvious off-target effects were minimized [28-30]. For general information regarding sequence alignments and the limitations of local and global alignments please refer to the 1994 review by Waterman and Vingron [31].

5.8.2 PNA Synthesis Using the Expedite 8909 PNA Synthesizer

PNAs were prepared by automated Fmoc-based solid-phase synthesis using an Expedite 8909 synthesizer (PE Biosystems, Foster City CA) [32]. The Expedite synthesizer was modified with proprietary software and designed to control delivery time and amounts of reagents across a solid-support resin. Additional bottle positions are available, which can be used to introduce multiple amino acids into PNA oligonucleotides during synthesis, provided that the molecules are compatible with the Fmoc-based chemistry.

Fmoc-protected PNA monomers with Bhoc-protected bases (A, T, G, and C), HATU activator, Fmoc-XAL-PEG-PS resin (2 μmol scale) and other reagents for PNA synthesis were obtained from ABI (Applied Biosystems, Foster City, CA). Base solution (0.2 M DIPEA/0.3 M 2,6-lutidine) and capping solution (5% v/v acetic anhydride/6% v/v 2,6-lutidine in DMF) were also obtained from ABI. Fmoc-amino acids were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Deblocking solution (20% v/v piperidine in DMF) was prepared from anhydrous solutions of DMF and piperidine using a sterile and dry graduated cylinder.

Unwanted side reactions are caused by water and amines, therefore it is essential to keep all reagents as anhydrous and amine-free as possible. Powdered reagents were either lyophilized or placed in a DrieRite dessicant container for two

hours after being removed from refrigeration to minimize water absorption. Each PNA monomer was dissolved in 3.75 mL Diluent (anhydrous NMP) (216 mM final). Some monomers required 30 minutes of shaking or vortexing to fully dissolve. HATU activator is dissolved in 13.5 mL anhydrous DMF (182 mM final). It is critical to maintain anhydrous activator, therefore the DMF was maintained under N_2 when used. Fmoc-protected L-lysine was dissolved in anhydrous DMF at a final concentration of 200 mM using spare bottles compatible with the Expedite. Upon completion of synthesis, the column containing the resin-bound PNA was washed, using a syringe, with 50 mL of isopropyl alcohol, and then dried in either the lyophilizer or using the in-house air.

Dry, washed resin was then transferred into a fritted (0.2-um) PTFE (polytetrafluroethylene) spin column (Millipore). PNA was 'cleaved' from the resin using a 'cleavage solution' that was comprised of 20% (v/v) m-cresol (Sigma-Aldrich) in trifluoroacetic acid (TFA). This was accomplished by adding 250 µL of cleavage solution to the resin for 90 minutes. The cleavage solution was then spun down and the process repeated for 10 minutes. After the cleavage, the PNA is released from the resin. The PTFE column is then centrifuged at 6,000 rpm to force the PNA solution through the filter and into the bottom of the tube, separating it from the resin.

PNA is precipitated from the cleavage solution by adding 1 mL of ice cold diethyl ether. A substantial amount of white precipitate should immediately appear if the synthesis was successful. The mixture was then briefly vortexed, followed by 5 minutes of centrifugation at 8,000 rpm to pellet the crude PNA product. The supernatant was decanted and the pellet was washed with an additional 1 mL ice cold diethyl ether. This washing step is repeated for a total of 5 times. The remaining diethyl ether was allowed

to evaporate from the washed PNA pellet via air drying. Once dry, the PNA pellet appears yellowish white, and should appear cracked. PNAs are resuspended in 300 µL of nuclease-free water and stored at -20°C for further purification and MALDI-TOF verification.

5.8.3 MALDI-TOF Mass Spectrometry

The application of Matrix-Assisted Laser Desorption-Ionization Time-of-Flight (MALDI-TOF) Mass Spectroscopy to PNAs has been specifically researched and the advantages and disadvantages of particular matrices discussed at length [33]. For the work presented within Chapter 4 of this dissertation, PNA Mass spectrometry was performed by MALDI-TOF [34] on a Voyager-DE Workstation (Applied Biosystems, Foster City, CA) using a saturated solution (1:1 ratio of ACN and dH2O, with 0.1% TFA) of sinapinic acid (3,5-Dimethoxy-4-hydroxy-cinnamic acid) as a matrix (Sigma-Aldrich, St Louis, MO). The 'sandwich' method was used to plate samples. On a gold-plated target, 1 µL of the matrix was pipette and allowed to dry for at least 10 minutes. Then 1 µL of the PNA sample was pipetted on top of the dried matrix and allowed to dry for 5 minutes. Finally, an additional 1 µL of matrix was pipette onto the plate and was allowed to dry for at least 15 minutes until crystal formation was observed. MALDI-TOF analysis is sensitive to the concentration of the PNA solution; therefore multiple concentrations of PNA solution can be tested on the same plate. The following setting were used as general guidelines and starting points for MALDI-TOF analysis: laser intensity was set to 2250; accelerating voltage was set to 20000V, grid was set at 94.5, the guide wire setting was set to 0.5, and the delay time was set to 100 ns. Most of these settings are default on the Voyager-DE Workstation. MALDI-TOF mass

spectroscopy (see below) of truncated PNA products can also be used to assist in troubleshooting the Expedite 8909 synthesizer by determining positions and sequences that commonly fail.

5.8.4 Reverse-Phase High Performance Liquid Chromatography of PNAs

PNA oligomers, with lysine conjugates, have both hydrophobic and hydrophilic properties, and may be analyzed and purified by reverse phase-high performance liquid chromatography (RP-HPLC). PNAs will associate with the hydrophobic C_{18}-column in an aqueous environment (filtered water with 0.1% TFA), but can be competed off the column using a non-polar buffer (ACN, 0.1%TFA). Hydrophobicity is a function of the PNA length, therefore, longer PNAs, which are more hydrophobic, require a higher concentration of ACN to dissociate from the C_{18}-column. By applying an increasing gradient of ACN across the C_{18}-column, it is possible to elute shorter, truncated PNA products that contaminate the full length PNA product. This makes it relatively simple to separate trunctated, undesirable products, which result from failed reactions, from the desired PNA product.

A Delta-Pak C_{18}-column (Waters Life Sciences Solutions, Milford, MA; part #WAT011804) was used with a Rainin HPLC system (two Rainin HPLX Solvent Delivery units, Rainin Pressure Module, and a Rainin Dynamax UV-1 absorbance detector) to purify PNAs. The column was periodically equilibrated with buffer B (ACN, 0.1% TFA) for 1-3 hours as routine maintenance to evacuate bubbles and remove residual hydrophobic particles from the column. Prior to PNA purification, the C_{18}-column was equilibrated with buffer A (filter-sterilized dH_2O, 0.1% TFA) for a minimum of 30 minutes at a rate of 2 mL per minute.

The Dynamax DA Data Acquisition software package was used to program a purification scheme consisting of the following steps: buffer A was run for 5 minutes after the injection of the PNA sample; the concentration of buffer B was increased from 0% to 100% over the course of 49 minutes to form an increasing gradient; 100% Buffer B was allowed to run for 6 minutes; the system was then equilibrated with buffer A as a holding step. These steps were performed at a flow rate of 4 mL per minute for purification of PNAs. The elution of PNA products from the C_{18}-column was monitored at 260 nm using the Dynamax UV-1 absorbance module.

Fractions were collected between the desired absorbance range and the each fraction was analyzed using MALDI-TOF mass spectrometry. For the experiments presented in Chapter 4, RP-HPLC was only used for purification, and was not used as an analysis tool. Prior to injection, all reagents were centrifuged at 12,000 rpm to prevent any precipitates from entering the HPLC. Unlike previously published work, the HPLC column was not heated during the PNA analysis. This was a precautionary step taken after Dr. Jiaxin Hu observed a visible precipitate caused by the degradation of the column due to heating. MALDI-TOF analysis did not indicate any noticeable differences, which may be due to the exclusion of syntheses of longer bisPNAs, which were previously synthesized using a heated water jacket.

5.8.5 Lyophilization

Following RP-HPLC purification, PNAs are analyzed and verified using MALDI-TOF mass spectrometry. HPLC fractions containing the desired PNA product are pooled and lyophilized. This is important, because PNAs are dissolved in ACN, which is toxic to cells and must be removed prior to use in cell culture. The pooled fractions are

frozen and covered with Parafilm. The Parafilm was perforated using a syringe tip (18 gauge or smaller) to allow the ACN to evaporate. The frozen, pooled, Parafilm-covered, perforated PNA samples were then placed into the Labconco Freeze Dry System/ Freezone 4.5 lyophilizer (Kansas City, MS; catalog # 77500-03) for approximately 2-24 hours to remove the ACN. Lyophilized PNAs were resuspended in 300 µL of nuclease-free water. The concentration was determined by absorbance spectroscopy and the stocks were maintained at -20°C or -80°C.

5.8.6 Biotin-Labeling of PNAs

The addition of biotin to PNA was performed after PNA synthesis, but before the cleavage of PNA from the resin. Reagents are needed in excess as follows: 40X excess d-biotin; 36X excess HATU; 80X excess DIPEA. The exact amount of each reagent required depends on the theoretical yield of the resin used during synthesis and the weight of resin used for the synthesis. The mathematical product of these numbers will yield the theoretical yield in moles, and this number is used to determine the exact amount of each reagent required.

After the PNA was washed with isopropyl alcohol and dried, it was placed in a clean test tube with a stir bar and sealed using a rubber stopper. HATU was added to the d-biotin and dissolved in 1 mL of anhydrous DMF. To facilitate mixing, the sample was vortexed for 30-45 minutes at the highest setting (heat was not applied). DIPEA was added to the dissolved d-biotin mixture and the solution changed to a yellow-brown color. The solution was then added to the test tube containing the resin-bound PNA, and the reaction was allowed to proceed overnight (a minimum of two hours is required).

Resin was removed from the test tube using a dropper, and placed into a 15 mL fritted glass filter in the septa of a 100 mL flask with a vacuum to pull the reaction mixture into the flask. The resin was washed using DMF and then DCM. This washing cycle was repeated 5-6 times. To dry the resin, generous amounts of methanol (20-50 mL) was added. The resin was allowed to dry for 30 minutes. The dry resin was removed from the fritted glass filter and placed into into a fritted (0.2-um) PTFE (polytetrafluroethylene) spin column (Millipore). The biotin-labeled PNAs were then cleaved from the resin as described above in 6.8.2.

5.8.7 Design and selection of Carrier DNA for PNAs

DNA carrier design has been described in detail for PNAs [9] and for additional oligonucleotide mimics [35]. DNA carriers are generally18–20 nucleotides in length and are designed to have partial complementarity to a PNA. The carriers are designed to overlap the PNA from either the 5’ or 3’ end of the oligonucleotide. A portion of the carrier DNA, usually 4-10 bases, will overhangs the the end of the PNA. This portion of the DNA carrier should contain a randomly generated sequence. Previously it was demonstrated that the observed inhibition of a PNA varied from 45-80% by changing the base composition of the overhanging DNA [36].

DNA carriers were obtained from from Sigma-Genosys or Integrated DNA Technologies and resuspended in nuclease-free water at a concentration of 100 μM. The carriers were then analyzed using absorbance spectroscopy. If the spectrum appears abnormal, the samples were purified using G-25 spin columns. Stocks were stored at -20°C.

A minimum of three carrier DNAs were tested for each PNA as carrier choice can greatly affect the potency and activity of a PNA [36]. This observation has been previously explained: a carrier with low affinity for a PNA will dissociate prior to cellular uptake and a carrier with high affinity for a PNA will fail to dissociate with the PNA inside cells [9]. The affinity of a carrier correlates to observed melting temperatures [36], and it can be useful to determine the melting temperature (T_m) for a PNA:DNA duplex. Melting curve analysis of PNA:DNA duplexes can be used to evaluate the potential for higher-order structures to form, self-complementarity, etc. T_m values between 55°C and 75°C (0.1M) are active within cells [9, 36].

5.8.8 Annealing Carrier DNA to Antigene PNAs

PNA samples were heated at 80°C and concentrations were verified using absorption spectroscopy prior to annealing. Purified PNA was placed into a thin-walled PCR tube and diluted using nuclease-free water to a concentration of 40 μM. In a separate PCR tube, the DNA carrier was diluted using nuclease-free water to a concentration of 80 μM of DNA. An equal volume of 10X PBS was added to the 80 μM stock of carrier DNA to produce a 5XPBS, 40 μM DNA solution. An equal volume of the PNA and DNA solutions were added together and mixed by pipetting. This produced a final concentration of 20 μM PNA and DNA oligonucleotides in 2.5X PBS and nuclease-free water.

PNAs were annealed using a thermocycler. The following steps were used: 95°C for 5 minutes; 85°C for 1 minutes; 75°C for 1 minutes; 65°C for 1 minutes; 55°C for 1 minutes; 45°C for 1 minutes; 35°C for 1 minutes; 25°C for 1 minutes; 15°C for 1 minutes; hold at 15°C. This protocol is a general guideline for annealing. The most

critical point is to heat the solution and allow the oligonucleotides to dissociate, and then cool the solution slowly to ensure that the PNA:DNA duplexes form properly. Since PNAs can form aggregates and must be heated prior to use in cell culture, the annealing process must be repeated immediately before transfection into cultured cells.

5.8.9 PNA Transfection Protocols

Antigene PNAs were transfected using the double-transfection method detailed in section 6.4.3. PNAs require approximately 30 minutes of additional preparation time prior to transfections relative to siRNAs, LNAs, or agRNAs. This is because an critical procedure for working with PNAs is that all PNA solutions must be re-annealed immediately prior to use in tissue culture, and this procedure takes approximately 20-30 minutes (depends on the thermocycler). The heating and cooling process is intended to denature any aggregation of PNAs that may have occurred over time, and to ensure properly annealed duplexes.

In addition, DNA carriers can substantially alter the activity of a PNA [36], and multiple (minimum of three carriers) should be tested in cell culture and analyzed prior to characterization and optimization experiments. The observed lack of functionality of a PNA may not be due to accessibility or physiological importance of a target site, but instead due to problems with carrier DNAs (see 6.8.7). Therefore, multiple carriers should be tested for each individual PNA before concluding that the target sequence is not viable. Each carrier should be tested in duplicate, preferably in triplicate to ensure that the optimal carrier is selected for each PNA. This process will take a minimum of two weeks. Additionally, PNA:DNA duplexes may interact differently with different lipids, therefore carriers should be retested for each lipid used.

5.9 Examination of Protein levels

5.9.1 Harvesting Protein from Mammalian Cells

Media was aspirated from each well, and the cells were immediantely washed with 1X PBS (room temperature). PBS was aspirated and cells were dissociated from the well using 200 µL of trypsin (0.05% with EDTA•4Na, Invitrogen, catalog # 25300-112) per well and incubating for 2-4 minutes at 37°C. The reaction was stopped using 800 µL of RPMI-1640. Cells were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 15 minutes at 3500 rpm (4°C).

Cleared RPMI-1640 supernatant was aspirated from the pelleted cells, and the cells were washed with 1 mL of 1XPBS and centrifuged for 15 minutes at 3500 rpm (4°C). The PBS supernatant was aspirated, and the cell pellet was resuspended in 35-50 µL of cell lysis buffer (50 mM Tris-Cl, 120 mM NaCl, 0.5% nonidet P-40, 1 mM EDTA-Na, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 0.1 mM NaF, 0.1 mM sodium orthovanadate, pH 7.4) containing 1X Complete Protease Inhibitor cocktail (Roche Applied Science, Indiapolis, IN) [37]. Cell lysates were frozen at -80 °C (freeze/thaw cycle will help complete cell lysis) and stored for concentration determination and further analysis.

5.9.2 Bicinchoninic Acid Assay

Protein concentration was determined using a prepared kit version of the bicinchoninic acid (BCA) assay. Specifically, protein concentration was determined using the Micro BCA Protein Assay Kit (Peirce, Rockford; IL; product # 23235). BCA assays were performed as described by the manufacturer, using multiple concentrations of bovine serum albumin to create a standard curve. Each sample was performed in

duplicate to minimize pipetting error. Samples were detected using a PowerWave$_X$ Select microplate reader (Biotek Instruments, inc., Winooski, VT) and data analyzed using the KC4 microplate reader software package provided with the instrument.

5.9.3 Western Blotting

Western blots were performed essentially as described [38]. Protein inhibition was analyzed using Western blotting. Generally 20-40 µg of protein was loaded into a gel. This range of protein was selected because it yields a clear signal with low background, and is readily separated using SDS-PAGE. Total protein was prepared in 4X SDS buffer, and loaded into a 7.5% Tris-HCl Ready Gel (Biorad, Hercules, CA; catalog # 161-1100) polyacrylamide gel and separated using electrophoresis at a constant voltage, in 1XTGS (Tris/Glycine/SDS) (Biorad, catalog #161-0772). A voltage of 75 V was applied for 15 minutes, and then increased to 100 V for approximately 1.5 hours. Gels were removed from their cassettes and the stacking gel was removed using a razor blade. The remaining gel was placed onto Hybond-C Nitrocellulose (Amersham, Buckinghamshire, UK), and transferred at 100 V for 2 hours in transfer buffer (20% methanol, 0.3% w/v Tris, 1.44% w/v glycine).

The polyacrylamide gel was discarded and the membrane was stained using Ponceau S. The stained gel was visually inspected for indications of transfer failure (bubbles, loose cassette, etc.). The membrane was trimmed, and excess membrane was discarded. The remaining membrane was cut below the bovine serum albumin (green) prestained standard (Kaleidoscope Prestained Standards, Biorad, catalog # 161-0324) and separated into two pieces. These blots were placed at 4°C in 5% milk (in PBS-T) for blocking for 1-3 hours (longer blocking steps help decrease background).

Following the blocking steps, the blots were incubated with antibodies. The “top” blot (containing the green, magenta, and blue standards) contains both the hPR isforms and hAR. The “bottom” blot (violet, orange, red, and blute standards) contains β-Actin. Primary antibodies were diluted into 7 mL 5% milk (in PBS-T) and incubated with the appropriate blot. Progesterone receptor monoclonal antibody (6A1) (Cell Signaling, Danvers, MA) was diluted 1:1000 and incubated overnight. Androgen receptor (Cell Signaling, catalog #3302) was diluted to 1:1000 and incubated and incubated overnight. Monoclonal β-Actin antibody (Sigma-Aldrich, catalog #A5441) was diluted 1:10,000 and incubated for 1 hour.

Following the incubation with primary antibodies, blots were washed three times for 5 minutes in PBS-T. Peroxidase AffiniPure Donkey Anti-Mouse IgG secondary antibody (Jackson Immunoresearch, Westgrove,PA) for hPR, or β-Actin; or Peroxidase AffiniPure Donkey Anti- Rabbit IgG (Jackson Immunoresearch) for hAR was added to the blots and allowed to incubate. Secondary antibody was incubated with the appropriate blots for the following times and ratios: anti-mouse 1:10,000 for β-Actin for 30 minutes; anti-mouse 1:5000 for PR for 45-60 minutes; anti-rabbit 1:5000 for hAR for 45-60 minutes.

After the incubation with the secondary antibody, blots were washed three times for 15 minutes in PBS-T (longer times and higher volumes were used to reduce background when necessary). Blots were developed using the Supersignal West Pico Chemiluminescent Substrate kit (Pierce, catalog #34080) according to the manufacturer’s instructions. Blots were developed by exposing Blue X-Ray film (Phenix

Research Products, Candler, NC) at exposure times. Final exposures were scanned and analyzed using the ImageJ image analysis suite (NIH, freeware).

Analysis consisted of background subtraction, normalization to loading control (β-Actin band intensity), and then normalization to a negative control (a mismatched or scrambled sequence). The resulting number was the relative band intensity of each sample as compared to the mismatch. The percent inhibition can be determined by subtracting this number from 1. This calculation was used for the initial antigene LNA work [11].

5.10 Examination of RNA levels

5.10.1 RNA Purification from Mammalian Cells

For purification of RNA, cells were harvested using TRIzol (Invitrogen) according to the manufacturer's instructions. The concentration of RNA was determined by absorbance spectroscopy using an extinction coefficient of 22.629. 1-2 µg of total RNA was diluted to 8 µL using nuclease-free water. 1 µL of DNase I (2 units/ul) and 1 ul of 10X DNase I Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 20 mM MgCl2) were added to the RNA solution and incubated at 25 °C for 10 minutes. DNase I was heat-inactivated at 70°C for 15 minutes. Following the DNase I treatment, RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. Each sample was diluted to a final volume of 20 µL using RT mastermix (1X RT Buffer, 1X dNTPs, 1X Random Primers, 1 µL Reverse Transcriptase) and the following program was used for the reverse transcription: 25°C

for 10 minutes, 37°C for 2 hours, 75°C for 10 minutes, and held at 4°C. Single-stranded cDNA was stored at -80°C for less than 24 hours prior to QPCR analysis.

5.10.2 Primers and Computational Primer Design

For routine amplification of hPR and hAR mRNA, validated primer sets were ordered from Applied Biosystems (Taqman Gene Expression Assays) and used per the manufacterer's protocols. For use with iTaq SYBR Green Supermix w/ ROX (BIO-RAD), validated primer sets were taken from the RT primer database (RTprimerDB [39, 40]), primary literature sources, or were designed using Primer3 [41-43]. Primers designed using Primer3 were tested for nontarget amplification using *in silico* ePCR [21]. Primers designed to amplify cDNA were positioned across exon boundaries to decrease the possibility of DNA amplification [44]. Standard curves were performed using whole cell cDNA, essentially as described [45].

For chromatin immunoprecipitation, primers were generally designed to amplify genomic DNA across the transcription start site of the targeted gene, or in a neighboring region (within 60 bases). ChIP primers were designed using Primer3 [41-43], and the sequences were obtained using the database of transcriptional start sites [46-50]. The possibility of nontarget amplification was checked using *in silico* ePCR [21]. Standard curves were performed using purified genomic DNA. Only those primer sets that show linear amplification over several orders of magnitude were used.

5.10.3 RT-QPCR

QPCR was performed on an ABI7500 Real Time PCR System (Applied Biosystems) using iTaq SYBR Green Supermix w/ ROX (BIO-RAD, catalog # 172-5851) or Taqman Universal PCR Master Mix (Applied Biosystems, catalog # 4324018).

Primers were designed as described in 6.10.2 with the exception of primers for GAPDH which were supplied as a control (Applied Biosystems) and were used with both Biorad SYBR and ABI Taqman reactions. Reactions for QPCR analysis were performed in 20 µL reactions containing 10 µL of SYBR Green Supermix (or Taqman Universal PCR Master Mix) , 100 nM primers, and approximately 100 µg of cDNA using the following thermal profile: 50°C for 2 minutes, 95°C for 2 minutes (10 minutes for Taqman Universal PCR Master Mix), followed by 45 cycles of 95°C for 20 seconds, 57°C for 30 seconds, and 72°C for 45 seconds. Melting curves were performed as needed and performed according to the ABI7500 manual.

QPCR data was analyzed and reported as the relative fold change in expression relative to a negative control sample. The relative fold change in expression for each sample was calculated using the following steps: first, the C_t value of the control primer set (GAPDH, ABI) was subtracted from the C_t value of each experimental primer set. This yielded ΔC_t for each sample, which is the change in C_t normalized to the amount of cDNA loaded. Second, the normalized C_t value of the negative control (mismatched or scrambled oligonucleotide-treated cells) was then subtracted from the other experimental samples. This calculation yields the $\Delta\Delta C_t$, which is the change in a normalized sample relative to a normalized control. Finally, fold change was determined using $2^{-(\Delta\Delta Ct)}$. Error for QPCR experiments was shown as the standard deviation as determined by the fold change of least three biological replicates.

5.11 Biotin-Pull down of RNA or DNA by LNAs

T47D cells were plated in six-well dishes and transfected with single-stranded, biotinylated LNA (supplied by Sigma-Proligo) as previously detailed in 6.4.1. Each sample was harvested 72-96 hours post-transfection. Replicated samples were pooled during harvest. Cell nuclei were harvested using 2 consecutive 5 minute washes in 1 ml of hypotonic lysis buffer (10 mM Tris pH 8.0, 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40) and centrifugation at 3500 rpm for 5 minutes each time. The supernatant was discarded. Nuclei were re-suspended in 100 µL of nuclear lysis buffer (150 mM NaCl, 0.5% NP-40, 2 mM MgCl2, 2 mM CaCl2, 20 mM Tris pH 7.5, 1 mM DTT, and 1X Complete-Mini Protease Inhibitor (Roche)) and placed at -80°C as a freeze/thaw cycle helped complete nuclear lysis. Samples were stored at -80°C.

Approximately 30 µL of Avidin-coated beads (NeutrAvidin Agarose Resin, Thermo Scientific, Rockford, IL) were blocked using 100 ng of salmon sperm DNA (Ambion, Foster City, CA) for each sample prior to use. The resin was handled using bead-loading tips. The lysed nuclei were thawed on ice and then centrifuged at 12,000 rpm for 10 minutes to pellet cellular debris. The supernatant for each sample was transferred to a sterile tube. From each sample, a 10 µL aliquot was placed into another sterile tube and stored at -80°C for use as an input control. The remaining 90 µL of supernatant was combined with 870 µL of wash buffer (500 mM NaCl, 2 mM MgCl2, 2 mM CaCl2, 20 mM Tris pH 7.5) containing 3 µl of RNasein (Promega) and 30 µl of pre-blocked avidin-beads. The samples were rotated with the beads at 4°C for two hours.

Following the incubation, beads were centrifuged for 20 seconds using a C-1200 mini-centrifuge (Labnet, Edison, NJ). Supernatant was discarded and the beads were washed with 1 mL of wash buffer, mixed by flicking, and allowed to sit on ice for 2 minutes. Samples were centrifuged with the C-1200 mini-centrifuge, the supernatant was discarded, and the wash repeated for a total of 4 times. The final wash supernatant was discarded, and the beads were incubated with 500 µL of elution buffer (1.5 % Biotin, 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5 % sodium N-lauroyl sarcosinate) for 2 hours at 45°C with continuous vortexing using a Thermomixer 5436 (Eppendorf, Hamburg, Germany) at a setting of 10. The incubation with elution buffer releases the RNA from the beads. Samples were centrifuged and the supernatant was transferred to a sterile 1.5 mL tube.

Samples were combined with 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich (Fluka Analytical)) and allowed to incubate on ice for 10 minutes. After phase separations were visible, samples were centrifuged at 12,000 rpm at 4°C for 12 minutes. The aqueous phase (~450 µL), containing the nucleic acid, was removed and transferred to a sterile 1.5 mL tube. To assist precipitation, 3 µL of glycogen (Invitrogen) and 25 µL of Sodium Acetate (3M, pH 5.5; Ambion) were added to each sample. To precipitate RNA and DNA, 525 µL of isopropyl alcohol was added to each sample. Samples were incubated at -80°C for a minimum of 1 hour and a maximum of overnight. Although 2 volumes of 100% ethanol can be used to precipitate RNA, isopropyl alcohol was used as it avoids the need to split samples or use 2 mL tubes. The pellet was washed using 1 mL of 75% ethanol. Samples were centrifuged at 7,000 rpm at 4°C, and the supernatant was discarded. Residual isopropanol was

removed by pipette, but the pellet was not allowed to dry as drying reduces the final yield. The pellet was resuspended in 24 µL of nuclease-free water and heated to 65°C for 5 minutes.

For the analysis of LNA binding to DNA, PCR reactions were performed in a total volume of 20 µL containing 10 µL of SYBR Green Mastermix (Qiagen, Valencia, CA), 100 nM primers, and 2-4 µL of resuspended DNA. The sequence of the forward primer was 5' CCTAGAGGAGGAGGCGTTGT 3', and the sequence of the reverse primer was 5' ATTGAGAATGCCACCCACA 3'. These primers were designed to amplify a 103 basepair region corresponding to chr11:100505689-100505791. Each sample was amplified in quadruplicate using the following thermal profile: 50°C for 2 minutes, 95°C for 2 minutes, followed by 50 cycles of 95°C for 20 seconds, 57°C for 30 seconds, and 72°C for 45 seconds. PCR products were resolved on a 3% agarose gel and analyzed by the presence or absence of a detectable signal. Products were excised and cloned as described in 6.5.1 and sequenced by the UTSWMC McDermott Center Sequencing Core. Sequences were aligned to hPR using NCBI BLAST and BLAT.

For the analysis of RNA binding, RNA concentration was not determined due to low recovery. Instead 8 µL of the resuspended RNA was reverse transcribed. Samples were DNase-treated by adding 1 µL of DNase I (2 units/ul) and 1 ul of 10X DNase I Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 20 mM MgCl2) to the RNA solution, followed by an incubation at 25 °C for 10 minutes. DNase I was heat-inactivated at 70°C for 15 minutes. Following the DNase I treatment, RNA was reverse transcribed as described in 6.10.1. The resulting cDNA was used immediately. PCR reactions were performed in a total volume of 20 µL containing 10 µL of SYBR Green Mastermix

(Qiagen, Valencia, CA), 100 nM primers, and 2-4 µL of cDNA. The primer sequences used were designed to amplify across the 5/6 exon boundary of a ncRNA [51]. PCR products were resolved on a 3% agarose gel and analyzed by the presence or absence of a detectable signal.

5.12 Nuclear Run-On Assay

Dot blots made in advance using purified plasmid DNA, Nytran SPC .45 µm transfer membrane (Whatman, Sanford, ME), and the Schleicher & Schuell (S&S) Minifold-I Dot-Blot System (Whatman). For 5 blots, 30 µg of plasmid should be denatured using 250 mM NaOH and 500 mM NaCl diluted in nuclease-free water for a total volume of 400 µL. Plasmids were incubated for 30 minutes at room temperature, and then the reaction was neutralized on ice using 800 µL of neutralization buffer (0.1X SSC, 0.125 M NaOH) for 3 minutes. Membrane was soaked in 0.4 M Tris-HCl, pH 7.4 for 5 minutes prior to use.

The S&S minifold was assembled essentially according to the manufacturer's instructions. Spacers were placed under the rim to increase the seal, grease was applied to gaskets that did not contact the membrane, and all unused ports ('dots') were sealed using tape. Blotting paper was placed beneath the membrane. Filtered water was used to ensure a proper vacuum was present.

For each "dot," 200 µL of denatured plasmid was applied in a drop-wise fashion over the course of 1-3 minutes under vacuum. After application of plasmid was completed, vacuum was applied for 10 minutes and then turned off. Prior to disassembly of the minifold, remaining liquid was aspirated from each seal to prevent

DNA “halos” from forming around the exterior of each dot. Orientation was marked using a pencil. Membranes were soaked for 2 minutes in pre-crosslink buffer (0.5 M NaCl, 0.5 M Tris-HCl pH 7.5), and then placed on top of Glad Clingwrap (not wrapped) and cross-linked while wet, using a Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation, Westbury, NY) at 1200 μJ/cm^2 for 100 seconds. Blots were wrapped in Glad Clingwrap and stored at 4°C for up to three weeks.

Just prior to use, blots were soaked in 2X-SSC. Dot blots should be placed at 43°C into ~10 mL of hybridization buffer (20 mM PIPES pH 6.7, 50% Formamide, 2 mM EDTA, 800 mM NaCl, 1X Denhardt’s solution, 0.5% SDS, 500μg/ml denatured salmon sperm (boil & ice before use) and nuclease-free water) for 4-24 hours in advance of the in vitro transcription portion of the nuclear run-on assay. This step is essential for preventing non-specific binding to the membrane. Dot blots included an empty vector control, GAPDH positive control, and two experimental vectors.

Large-scale forward transfections were used for run-on assays. Dishes (150 mm, Corning) are approximately ~18X larger than a 6-well dish, so the equivalent of 100k cells seeded in a 6-well plate is 1.8 million cells per dish. Approximately 2 million cells were plated per dish, and in order to obtain the required number of nuclei, 2 dishes were used per sample. Cells were incubated for 48 hours prior to transfection. Reagents were scaled linearly from the 6-well protocol detailed in § 6.4.1. All numbers for antigene agents are given assuming a 20 μM stock. 51.2 μl of LNA or agRNA was combined with with 1.34 mL of OptiMEM. 28.8 μL of Lipid was combined with 580 μl of OptiMEM. These two solutions were combined for a total of 2 mL and allowed to incubate for 20 minutes. During this incubation step, media was aspirated from each

dish and replaced with 18 mL of OptiMEM. Immediately following the 20 minute incubation, the 2 mL of Lipid/LNA solution was added to the cells. Cells were allowed to incubate for 24 hours. Media was changed to RPMI 24 hours post-transfection.

Samples were harvested 3-4 days post-transfection. Media was aspirated from each dish and the cells were washed with 1X PBS. Cells were removed from dishes using a rubber policeman. Replicates were combined and centrifuged at 500Xg for 5 minutes in a S4180 rotor at 4°C. PBS supernatant was discarded and the pelleted cells were resuspended in hypotonic lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, 0.5% NP-40) and allowed to incubate on ice for 5 minutes. The samples were centrifuged at 500Xg for 5 minutes in a S4180 rotor at 4°C, and the hypotonic lysis was repeated. Following the second hypotonic lysis, samples were centrifuged at 500Xg for 5 minutes in a S4180 rotor at 4°C, and samples were resuspended in storage buffer (50 mM Tris-HCl pH 8.5, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and flash-frozen using liquid nitrogen. Nuclei were stored at -80°C for in vitro transcription.

To prepare for the in vitro transcription assay, it is essential to prepare all buffers required for the assay. Before beginning the actual protocol, there are two buffers that have to be made. Unless otherwise noted, all buffers should be freshly prepared. If the NaCl and guanidinium solutions form crystals, simply heat the reagent at 42°C for ~1 hour. Each sample requires 110 µL of elongation buffer (200mM TRIS-HCL pH 8.0, 100 mM NaCl, 0.8 mM EDTA, 0.2 mM PMSF, 2.4 mM DTT, 4 mM MnCl2, 8 mM MgCl2, and nuclease-free water). Each sample will also require 27 µL of SET buffer (5% SDS, 50 mM EDTA, 100 mM TRIS-HCL pH 7.4, and nuclease-free water). In addition, each

sample will require 400 µL of extraction buffer (4 M guanidinium-isothocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.1M β-mercaptoethanol, and nuclease-free water).

The following steps were performed in an area approved for radiation. Per sample, 110 µL of elongation buffer, 2.5 µL of CTP, 2.5 µL GTP, and 20 µL of ATP were added together in 2 mL tubes along with approximately 60 units of RNase Inhibitor. 14 µL of α-P32 UTP was added to each sample. Bead-loading P200 tips were used to transfer 135 µL of nuclei into the reaction (thawed, in storage buffer). Bead-loading tips were used to gently mix the nuclei by pipetting up and down. The in vitro transcription assay was allowed to proceed for 25 minutes at 26°C.

The in vitro transcription assay was stopped through the addition of 50 µL of DNase I. The DNase was allowed to incubate for 10 minutes at 26°C and was added using bead-loading tips to facilitate mixing, as flicking radioactive solutions is not recommended. Following the ten-minute incubation, 3.5 µL of proteinase K and 27 µL of set buffer were added to each sample and mixed using bead-loading tips. Tubes were placed at ~37°C for 30 minutes and continuously mixed using the Thermomixer 5436 at the minimum setting.

After the thirty-minute incubation, 400 µL of extraction buffer and 80 µL of 3M Sodium Acetate pH 5.5 were added to each sample and allowed to incubate for 4 minutes. To extract nucleic acids, 750 µL of phenol:chloroform:isoamyl alcohol was added to each sample and vigorously mixed (inside a sealed Nalgene container). Samples were then placed on ice for 20 minutes, and then centrifuged at 14,000 rpm at 4°C for 15 minutes to separate phases. Following the spin, the aqueous phase was transferred to two new 1.7 mL tubes (approximately 400 µL to each tube). Yeast tRNA

was used as a carrier, and 3µL was added to each tube and briefly vortexed. To remove radioactive liquids from the seal, the samples were briefly centrifuged. RNA was precipitated by the addition of 500 µL of cold isopropyl alcohol (2-propanol) to each sample. Precipitation was allowed to proceed at -20°C for approximately 2 hours.

Following the two-hour incubation, the samples were centrifuged at 13,000 rpm at 4°C for 12 minutes to pellet the precipitated RNA. The supernatant was removed using a P1000 and discarded into radioactive waste. A critical point in this assay was not to allow the pellet to dry at any step. Therefore, immediately after the supernatant was removed, 1 mL of 75% ethanol was added to each sample. Samples were inverted several times and centrifuged at 8,000 rpm at 4°C for 10 minutes. The supernatant was removed using a pipette and was discarded into radioactive waste. Each pellet was immediately resuspended in 200 µL of hybridization buffer, and placed on the Thermomixer 5436 at 45-50°C and vortexed for 15 minutes at the minimum setting. Following this fifteen-minute incubation, a critical step was taken to reduce noise caused by radioactive particulates, the samples were centrifuged at 12,000 rpm for 20 minutes.

Cryogenic vials with internal threads (2 mL capacity, Corning Incorporated, New York, New York) were used to incubate the product of *in vitro* transcription with dot blots. Membranes were placed into the cryogenic vials with the plasmid-bound face of the membrane facing towards the center of the vial. 1 mL of hybridization buffer was placed into each vial, and then the 400 µL of sample was added (200 µL from two tubes) for a total volume of 1.4 mL. Cryogenic vials were placed into a glass roller tube

and placed into the hybridization oven (Thermo Hybaid, UK) at 42 °C for 36 hours at 10 rpm.

Prior to washing, blots were removed from the cryogenic vials and placed into washing buffer B (2XSSC), sealed, and inverted 4 times to remove radiation. Membranes were washed with 30 mL of washing buffer A (2XSSC, 0.1%SDS) at 40°C for 2 hours at 10 rpm. Next, membranes were washed for 20 minutes in 25 mL of wash buffer B and then placed into 2 mL vials with 1.6 mL of wash buffer B and 25 µL of RNase for 1 hour at 43°C without shaking. Following the RNase incubation, membranes were washed with 30 mL of wash buffer B for 2 hours at 48°C at 12 rpm in a vertical position (this is accomplished by taping a tube-rack onto the rotisserie bar inside the Hybaid oven). Membranes were dried using blotting paper, placed inside Glad Clingwrap, and taped onto a developing cassette (BAS 2025, Fujifilm, Japan) and exposed using an imaging plate (BAS-IP SR 2025, Fujifilm). The imaging plate was developed after being exposed for 20-72 hours at -80°C using a phosphoimager (FLA-5100, Fujifilm, Japan). Analysis of dot blots was conducted using ImageJ image analysis software (NIH) and Microsoft Excel. Images were normalized to background, and to the positive GAPDH controls.

5.13 Chromatin Immunoprecipitation Assay (ChIP)

Large-scale forward transfections were used for ChIP assays. Approximately 2 million cells were plated per dish (150 mm, Corning), and in order to obtain the required amount of material, 2 dishes were used per sample. Cells were incubated for 48 hours prior to transfection. Reagents were scaled linearly from the 6-well protocol detailed in

§ 6.4.1. All numbers for antigene agents are given assuming a 20 μM stock. 51.2 μl of LNA or agRNA was combined with with 1.34 mL of OptiMEM. 28.8 μl of Lipid was combined with 580 μl of OptiMEM. These two solutions were combined for a total of 2 mL and allowed to incubate for 20 minutes. During this incubation step, media was aspirated from each dish and replaced with 18 mL of OptiMEM. Immediately following the 20 minute incubation, the 2 mL of Lipid/LNA solution was added to the cells. Cells were allowed to incubate for 24 hours. Media was changed to RPMI 24 hours post-transfection.

Samples were harvested 3-4 days post-transfection. Media was aspirated from each dish and the cells were washed with 1X PBS. PBS was aspirated and 10 mL of 1% Formaldehyde (in PBS) was added to each dish for 10 minutes. Immediately following the ten-minute incubation, the cross-linking reaction was quenched by adding 1 mL of 1.25 M glycine (in PBS) followed by a five-minute incubation. The formaldehyde and glycine solution was aspirated and the cells were washed once with 1XPBS. PBS was then aspirated. A total of 12 mL of 1X PBS was added to each sample in order to harvest cells. Cells were removed from dishes using a rubber policeman.

Replicates were combined and centrifuged at 500Xg for 5 minutes in a S4180 rotor at 4°C. PBS supernatant was discarded and the pelleted cells were resuspended in hypotonic lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl pH 8.0, 0.5% NP-40) and allowed to incubate on ice for 5 minutes. The samples were centrifuged at 500Xg for 5 minutes in a S4180 rotor at 4°C, and the hypotonic lysis was repeated. Following the second hypotonic lysis, samples were centrifuged at 500Xg for 5 minutes

in a S4180 rotor at 4°C, and samples were resuspended in buffer B (1%SDS, 10 mM EDTA, 50mM Tris-HCl pH 8.1, 1X Roche Protease inhibitor cocktail, 50 units RNase inhibitor) and flash-frozen using liquid nitrogen.

Nuclear lysate was stored at -80°C for sonication. Lysed nuclei were sonicated, and DNA was sheared into base fragments approximately 500 bases long using a Model 150 V/T ultrasonic homogenizer (Biologics; five 20 second pulses at 40% power, in an ice bath, with 1 minute between each pulse). Aliquots of sheared DNA were analyzed on an 3% agarose gel to ensure proper fragment size.

Following sonication, samples were pre-cleared. Samples were centrifuged for 10 minutes at 12,000 rpm at 4°C to pellet cell debris. Prior to continuing, 25 µL of supernatant was transferred to a sterile 1.7 mL tube for use as an “input” control. The input control is critical because it is used to normalize the ChIP samples to the amount of chromatin originally present prior to immunoprecipitation. Approximately 40 µl of Protein G Plus/Protein A agarose suspension (Calbiochem, cat. No. IP05) was added per sample into 1 mL of IP buffer. Beads were washed 3 times with1 mL of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167mM NaCl, 1X Roche protease inhibitor, 50 units/mL of RNase inhibitor). Per sample, two tubes were prepared (IgG control and experimental). For each sample, 770 µL of IP Buffer, 30-35 µL of 3X washed beads and 200 µL of each sample were combined. Samples were allowed to incubate for over 1 hour at 4°C while continuously being inverted. This step is intended to eliminate anything that might nonspecifically stick to the beads. Once the samples were pre-cleared, the samples are centrifuged down using a C-1200 microcentrifuge and the supernatant was transferred to a new 1.7 mL

tube. Following this step, 4 µg of the antibody of interest was added to the appropriate samples. Samples were rotated at 4°C for over 12 hours.

Beads were blocked prior to immunoprecipitation to decrease nonspecific noise. Nonspecific noise is the observed signal caused by DNA binding to the beads or to the antibody in a nonspecific manner. Blocking was accomplished by adding approximately 50 µL of Protein G Plus/Protein A agarose suspension (Calbiochem) per sample into 1 mL of IP buffer containing 15 µL of sheared salmon sperm DNA. Samples were rotated at 4°C for a minimum of 1 hour. Following this incubation, samples were centrifuged (using a C-1200) for 10 seconds to pellet the beads and the supernatant was discarded. Beads were washed with 1 mL of IP buffer a total of three times.

Immunocomplexes were isolated by adding the immunoprecipitated samples to the beads blocked in the manner described above. The beads were resuspended and rotated at 4°C for 2 hours. After this incubation, the beads were pelleted using centrifugation (C-1200 microcentrifuge). Supernatants were removed and stored at -80°C for potential reprobing. Beads were resuspended and washed for a total of five times, which consisted of a five minute incubation period with each wash, centrifugation to pellet the beads, and the removal of the supernatant. The washes were performed in the following order: (i) Low-salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl); (ii) High-salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl); (iii) LiCl wash; (iv) TE pH 8.0; (v) TE pH 8. After the final wash, samples were prepared for elution.

Complexes were eluted from beads eluted by adding 250 µL of freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$, and 50 units/mL of RNAse inhibitor) to the

washed beads. Samples were placed on a thermomixer (setting 5-6) at 25-28°C for 15 minutes. Following this incubation, samples were centrifuged to pellet the beads, and the supernatant was transferred to an sterile tube. An additional 250 µL of elution buffer was added to each sample, and this process was repeated for a total of 500 µL. Input samples were diluted to 500 µL using elution buffer. 20 µL of 5M NaCl was added to each sample for a final concentration of 200 mM. Samples were placed at 65°C for a minimum of 2 hours (maximum of overnight) to reverse cross-links.

Following reversal of crosslinks, add 1 µL of RNase to each sample and heat at 37°C for 30 minutes. Following the thirty-minute incubation, 20 µL of 1M Tris-HCL pH 6.5, 10 µL of 0.5M EDTA, and 20 µg of proteinase K was added to each sample and allowed to incubate at 42°C for 45 minutes. DNA was isolated from each sample by adding 520 µL of phenol:chloroform:isoamyl alcohol extraction. Samples were briefly vortexed and allowed to incubate on ice for 15 minutes. Samples were centrifuged at 14,000 rpm at 4°C to separate phases. The aqueous phase was transferred to a 2 mL tube. To facilitate precipitation, 2.5 µL of glycogen and 25 µL of sodium acetate were added to each sample, mixed thoroughly, and allowed to incubate for 5 minutes. DNA was precipitated by adding 1.3 mL of 100% ethanol to each sample. Samples were placed at -80°C for a minimum of 2 hours (maximum of overnight) to allow DNA to precipitate.

DNA was pelleted by centrifugation at 14,000 rpm at 4°C. The supernatant was discarded and the pellet was washed with 2 mL of 75% ethanol. Samples were centrifuged at 8,000 rpm for 10 minutes at 4°C to pellet the DNA after the 75% ethanol wash. The supernatant was discarded and samples were resuspended in 30-40 µL of

nuclease-free water. Detection of specific DNA sequences was done using QPCR. Primers were designed to amplify regions of chromosomal DNA near the transcriptional start sites of genes. QPCR was performed in 20 µL reactions containing 10 µL of SYBR Green Mastermix, 100 nM primers, and 2 µL DNA using the following thermal profile: 50°C for 2 minutes, 95°C for 10 minutes, followed by 52 cycles of 95°C for 20 seconds, 57°C for 30 seconds, and 72°C for 45 seconds.

QPCR data was analyzed and as the relative fold change in RNA polymerase II occupancy relative to a negative control sample. This was calculated using the following steps: First, the C_t value of the input control sample was subtracted from the C_t value of each experimental primer set. This normalized each sample to the amount of DNA in the original samples. Second, the normalized C_t value of the IgG negative control was subtracted from each sample (each sample had its own IgG control). This normalized each sample to the amount of nonspecific binding present in each individual sample. Third, the normalized C_t value for GAPDH was subtraced from each experimental sample. This step normalized each sample to the relative amount of RNA polymerase II present on GAPDH, and was intended to normalize the results to the overall amount of transcription present in a control gene. Finally, the normalized C_t value for a negative control LNA (mismatched or scrambled) was then subtracted from the other experimental samples. Fold change was determined using the $2^{-(\Delta\Delta Ct)}$ method. Error for ChIP experiments is shown as the standard deviation as determined by the fold change of least three biological replicates.

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Chapter 6: Future Directions: Characterization of Bidirectional Promoters Using Antigene Locked Nucleic Acids

6.1 Introduction

Since the completion of the Human Genome Project, an abundance of bidirectional promoters have been analyzed and computationally determined [1-3]. Bidirectional promoters are promoters that produce transcripts in both the sense and the antisense orientations (**Figure 6.1**). Over 10% of known genes are actually organized "head-to-head," with less than 1 kb separating their transcriptional start sites [4]. 10% is a very conservative estimate, and does not include the noncoding RNAs that been recently observed [5]. Notably, if the human genome was organized in a similar fashion as simpler organisms, we would expect, on average, for genes to be spaced ~85 kb apart [2]. This unexpected organization allows genes to be regulated by the same promoter.

A bidirectional promoter allows the expression of two genes to be coregulated [6] (**Figure 6.1**). Microarray analysis has confirmed that the transcripts of many bidirectional genes are coexpressed. However, some transcripts in bidirectional promoters are actually inversely expressed or even expressed in a mutually exclusive manner [1, 3]. It is unknown how the intervening promoter sequence can be modulated to preferentially express one gene, while repressing the other [7]. In fact, Trinklein, et al. demonstrated that the deletion of a transcriptional start site of one gene often leads to the increased transcription of the opposing gene [3]. This result suggests that there is competition for shared resources such as transcription factors [3].

Although bidirectional promoters are very abundant, the physiological consequences of such a large number of bidirectional gene pairs are currently unclear [2]. However, bidirectional promoters are strongly conserved, which suggests that they are functionally significant [3]. It is intriguing that even some viruses have bidirectional promoters at gene loci of “outstanding regulatory importance” [8]. Usually, bidirectional promoters are associated with known housekeeping and cell-cycle genes. In fact, 30% of housekeeping genes [4], 40% of DNA repair genes [4], and 50% of DNA replication initiation genes [2] are regulated by bidirectional promoters. These classes of genes are implicated in multiple diseases, and recently, Yang, et al. were able to show that bidirectional promoters were significantly enriched in multiple cancer-related gene-sets [7]. This implies that a thorough characterization of a bidirectional promoter could have a large impact on our understanding of cancer mechanisms. However, the regulatory mechanisms of all but a few bidirectional genes are unknown [3].

Recently, it was demonstrated that bidirectional promoters can be regulated by a small subset of transcription factors [1], suggesting a general mechanism for how bidirectional promoters are regulated [7]. This observation is relevant to any transcripts that may share regulatory features; this would also include the growing field of noncoding RNAs. Many noncoding RNAs form sense-antisense pairs with coding transcripts [9], which originate within the same 1 kb region, and would, by definition, be considered a bidirectional gene. Whether a transcript is noncoding or coding, it is likely that all bidirectional promoters share a common, conserved regulatory mechanism, e.g. regulation by the same subset of transcription-factors. Therefore, it is reasonable to

expect that a deeper understanding of bidirectional promoters may have a substantial impact on our understanding of the transcription of both noncoding and coding genes.

Previous studies of bidirectional promoters have relied heavily upon plasmid constructs and luciferase assays to test the effect of various mutations. However, these types of assays cannot be used to test if elements located outside the construct—transcriptional elements, epigenetic markers, etc., might impart a different response. Genetic testing where the mutations are placed back into their proper cellular context are extremely difficult, time-consuming, and expensive since individual constructs have to be made for each mutation tested [10]. However, antigene LNAs could provide a powerful alternative for the initial characterization of transcriptional landscapes in their full cellular context. To this end, I propose to study dihydrofolate reductase (DHFR) and the MutS Homolog 3 (MSH3).

6.2 Background of DHFR and MSH3

DHFR and MSH3 share a bidirectional promoter, are positioned in a “head-to-head” orientation, and are divergently transcribed. The DHFR/MSH3 gene pair has been conserved in mice, hamsters, and other vertebrates, and has been studied for almost 20 years. DHFR (EC 1.5.1.3) converts dihydrofolate into tetrahydrofolate, which is required for the de novo synthesis of purines, thymidylic acid, and certain amino acids. DHFR has been linked to several medical conditions including spina bifida [11], and is also a common drug target for antitumor agents [12] and for parasitic chemotherapy [13].

The exact function of MSH3 is currently unknown, however it shares significant homology to the bacterial DNA mismatch repair protein MutS [14, 15]. Inokuchi, et al. have presented data supporting that inactivation of MSH3 is involved in the development of hematological malignancies, and have postulated that MSH3 may be involved in antirecombination events instead of a single-nucleotide repair pathways [16]. These data strongly suggest that further characterization and study of the DHFR/MSH3 system could have important therapeutic implications.

Despite years of research, by multiple laboratories, there are still multiple questions to address about the transcriptional regulation of DHFR/MSH3. Like many bidirectional promoters, DHFR/MSH3 is very GC-rich and has a putative G-quadruplex forming region [17]. These quadruplex-forming regions are believed to play essential regulatory roles in many genes; however, almost nothing is known about how these structures may regulate bidirectional transcription. It has been long established that genes with GC-rich promoters often have multiple transcription start sites [18], and, as expected, DHFR and MSH3 both have alternative transcription start sites that may affect tissue-specific expression.

One of the most controversial and intriguing characteristics of the DHFR/MSH3 promoter is the existence and function of a noncoding RNA transcript [19-21]. Masters, et al. found an upstream transcript in the DHFR promoter, which produced a transcript that was only found in the nucleus [20]. Blume, et al. reported that this minor transcript could affect the transcription of the major promoter and functioned by sequestering transcription factors [21]. However, in 2007, Martianov, et al. provided evidence that the noncoding DHFR transcript physically binds to the promoter of DHFR via a triple-helix

formation and inhibits transcription during cell quiescence [19]. Yet the authors do not address how MSH3 expression is affected by the noncoding RNA, and do not provide strong evidence that this interaction can truly occur in a relevant transcriptional context.

These controversial issues, combined with a rich base of literature, and therapeutic potential, make DHFR/MSH3 a very attractive model system. In addition, since these genes are expressed in almost all cell types, checking for cell-line specific effects would be straight-forward. Finally, the completion of this proposal will address important biological questions and may have broader implications for any sense/antisense transcripts produced from the same genomic loci.

6.3 Aim 1

Aim 1 is to inhibit the expression of DHFR using agLNAs and to monitor the expression of neighboring genes under normal and serum-starved conditions. The purpose of this aim is to address if: (1) LNAs can inhibit the expression of DHFR; (2) LNAs can activate expression of MSH3 by blocking divergent transcription; (3) LNAs can specifically inhibit DHFR without affecting MSH3 expression; (4) LNAs can be used to determine if a transcription factor is shared between the divergent gene or is unidirectional; (5) LNAs can target putative G-quadruplex forming regions; and (6) Neighboring genes are affected by targeting DHFR.

My hypothesis is that agLNAs can inhibit transcription of DHFR, and I expect to see an increase in levels of MSH3. This hypothesis is supported by previous observations that the deletion of a divergent transcriptional start site leads to an increase in the expression of the other gene product [3]. Based on this and previous

studies, it is reasonable to expect a similar effect if a transcriptional start site is sterically blocked using an agLNA. Previously, it was demonstrated in the mouse dhfr gene, that certain putative transcription factor binding sites were dispensable for the proper expression of the mouse MSH3 homolog but were required for dhfr expression [22]. This suggests that although many regulatory elements are shared, some are unidirectional and dedicated to a specific gene. Therefore, it is reasonable to expect that a thorough characterization of known transcription factor binding sites may yield targets capable of gene-specific inhibition, and dual-gene inhibition. This information would be valuable therapeutically, and would also advance our understanding of the underlying regulation of bidirectional promoters.

The experimental design of this aim is simple and straight-forward—I will design agLNAs targeting transcription state sites and known transcription factor binding sites. I will then transfect these LNAs into T-47D, MCF-7, and HeLa cells, and monitor the expression of neighboring genes using QPCR and immunoblotting. Serum-starvation is a simple addition to our existing protocol and is simply accomplished by leaving cells in serum-free media for 24-48 hours. Although I expect LNAs to perform adequately, they are a new antigene agent and are relatively untested in multiple genes and cell lines. It is possible that the LNAs may not be functional. If this appears to be the case, I will use chemically modified promoter-targeted RNAs (strand-biased) to test these hypotheses.

Potential therapeutic applications of a DHFR-directed agLNA: DHFR is inhibited by methotrexate (MTX), which is an important chemotherapeutic agent used to treat several types of malignancies [23]. A single nucleotide polymorphism in DHFR mRNA can lead to methotrexate resistance, which can cause some patients to be intrinsically

resistant to chemotherapy [23]. A DHFR-directed agLNA could potentially be used in combination with other chemotherapy drugs to treat MTX-resistant patients. Additional therapeutic applications might include sequence-specific parasitic chemotherapy. Also, if MSH3 expression can be increased by sterically blocking the transcription start site of DHFR, it may be a potential treatment for patients with hematological malignancies [16] (the resulting DHFR deficiency would be easily treatable with N(5)-formyltetra hydrofolate).

6.4 Aim 2

Aim 2 is to inhibit the expression of MSH3 using agLNAs and to monitor the expression of neighboring genes under normal and serum-starved conditions. The purpose of this aim is to address if: (1) LNAs can inhibit the expression of MSH3; (2) LNAs can activate expression of DHFR by blocking divergent transcription; (3) LNAs can specifically inhibit MSH3 without affecting DHFR expression; (4) Neighboring genes are affected by targeting MSH3. The experimental design and approach to addressing these questions will be the same as above, in Aim 1. The potential therapeutic applications for MSH3-targeted agLNAs are stated in Aim 1.

6.5 Aim 3

Aim 3 is to inhibit the noncoding DHFR transcript and to monitor the expression of neighboring genes under normal and serum-starved conditions. The purpose of this aim is to address if: (1) agLNAs can be used to inhibit noncoding RNA transcription; (2) the noncoding DHFR transcript can regulate the expression of MSH3; (3) the noncoding

RNA can affect the expression of neighboring genes; (4) agLNAs can block the putative physical interaction of the noncoding transcript with the DHFR promoter [19].

6.6 Discussion

My hypothesis is that agLNAs can inhibit transcription of the noncoding DHFR transcript, and that I will observe a significant increase in levels of DHFR transcription under serum-starved conditions. This hypothesis is supported by the recent work by Martianov, et al. [19]. However, Martianov, et al. used an siRNA to cleave the noncoding transcript, and the effect of inhibiting the noncoding transcript at the level of chromosomal DNA may be quite different. Some of the putative transcription factors in the DHFR/MSH3 promoter may actually be required for proper regulation and expression of the noncoding transcript and not for the protein coding transcripts. To test this hypothesis, I can monitor the noncoding transcript levels in Aim 1 and in Aim 2 using Northern blots. This would provide additional insights into the interrelationships between regulatory elements in the DHFR/MSH3 promoter.

The mechanism of this transcript is very controversial [19, 21], and previous laboratories have lacked the experimental tools and model systems to adequately address many fundamental questions. Existing techniques in the Corey laboratory, such as our proficiency with antigene agents, the ability to perform RIPS, ChIPs, etc. present a unique opportunity to probe these questions. For example, Blume, et al. postulated that the noncoding transcript in DHFR sequesters transcription factors (SP1) required for DHFR transcription [21]. A modified RIP assay, where we immunoprecipitate for SP1, could directly address this potential mechanism. To my

knowledge, a RIP has never been used to evaluate transcription-factor binding to noncoding RNAs.

Previously the noncoding transcript was mapped to positions -480, -470, --466, -460, -456, and -449 relative to the DHFR gene [20]. The exact start site of the noncoding transcript is ambiguous and 5'-RACE would be required to determine it. After 5'RACE, LNAs targeting the exact transcription start site(s) of the noncoding RNA could be designed.

Although agLNAs targeting noncoding RNAs may have therapeutic applications in the future, the primary purpose of this aim is to address very important biological questions concerning the regulation, mechanism, and expression of an established noncoding RNA. Results could have implications for the roles of other noncoding RNAs.

6.7 Future Directions

The final extension of this research would be to test additional bidirectional promoters and sense-antisense gene pairs to establish general relationships about how bidirectional genes are regulated. The burden of this task could be greatly alleviated by taking advantage of existing microarray databases such as NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) [25, 26], or Arrayexpress [27-29]. With these tools, it is possible to sort through over 100,000 microarray experiments, and examine differential expression of a set of genes in multiple cell lines, representing multiple tissue types, from multiple laboratories from around the world. These databases are freely available.

6.8 Concluding Statements

This project is feasible using existing techniques in the Corey Laboratory. The completion of each aim will address important biological questions, and functional agLNAs have clear therapeutic applications. Finally, this model system is very flexible and could be used to address additional questions in the future.

Table 6.1 agLNAs designed to target chromosomal DNA. These are not capable of targeting DHFR mRNA. Therefore, if DHFR expression is inhibited, it must be downregulated either by a steric block, or by targeting MSH3 mRNA. Relative Chromosomal positions are depicted in Figure 6.2, below.

	Gene	NM	Target notes	dbTSS	Sequence (5' to 3')
1	DHFR	NM_000791	Major TSS	79,986,556	TGCCCAGTCCCAGACAGAA
2	DHFR	NM_000791	Major TSS	79,986,556	TCCCAGACAGAACCTACTA
3	DHFR	NM_000791	dbTSS Alt Pr 2	79,986,136	GCGGCCACAATTTCGCG
4	DHFR	NM_000791	dbTSS Alt Pr 2	79,986,136	CACAATTTCGCGCCAAACT
5	DHFR	NM_000791	HIT000031619	79,986,539	ACAGAACCTACTATGTGCG
6	DHFR	NM_000791	HIT000031619	79,986,539	AGTCCCAGACAGAACCTAC
7	DHFR	NM_000791	ENT0000343331	79,986,857	AAGGATGGAGGTTAAAAGA
8	DHFR	NM_000791	ENT0000343331	79,986,857	ATGGAGGTTAAAAGACGCA
9	DHFR	NM_000791	dbTSS Alt Pr 1	79,987,199	ACCCTTGGGCTTTTCCTGT
10	DHFR	NM_000791	dbTSS Alt Pr 1	79,987,199	CTTTTCCTGTGGCAGTATC
11	DHFR	NM_000791	Quadruplex_1	79,986,489	GGCGCTGCGGCCGCT
12	DHFR	NM_000791	Quadruplex_2	79,986,489	TGGGGGCGCTGGGGGC

Table 6.2 : agLNAs designed to target chromosomal DNA. These are not capable of targeting MSH3 mRNA. Therefore, if MSH3 expression is inhibited, it must be downregulated either by a steric block, or by targeting DHFR mRNA. Relative Chromosomal positions are depicted in Figure 6.3, below.

	Gene	NM	Target notes	dbTSS	Sequence (5' to 3')
13	MSH3	NM_002439	dbTSS Alt Pr1	79,983,877	TAGGCTGATGTTAGGGCTC
14	MSH3	NM_002439	Main TSS	79,986,050	ATGATAGGGCTGGAGGA
15	MSH3	NM_002439	Main TSS	79,986,095	CTTGTGGGTAAGGCGGGCG
16	MSH3	NM_002439	dbTSS Alt Pr 2	79,986,727	CCCGGCAGATACCTGAG
17	MSH3	NM_002439	dbTSS Alt Pr 2	79,986,735	CGTTTGGGTCCCATCGCCC

Figure 6.1 Transcriptional landscape of a typical bidirectional promoter. The transcriptional start sites (TSS) of both genes are within 1 kb (The transcripts can overlap forming a sense-antisense pair, as well). Some regulatory elements will be shared between the two genes. However, some regulatory elements can be dedicated to a single gene. *These genes can be protein coding or noncoding*.

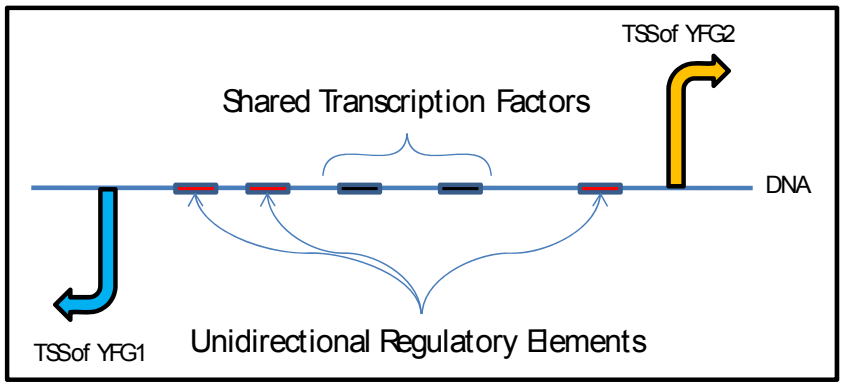


Figure 6.2: Model of the DHFR transcriptional landscape according to literature references and the dbTSS. Shown in red are the relative target sites for agLNAs. Two LNAs have been designed for each reported transcript. MSH3 promoters are shown in grey.

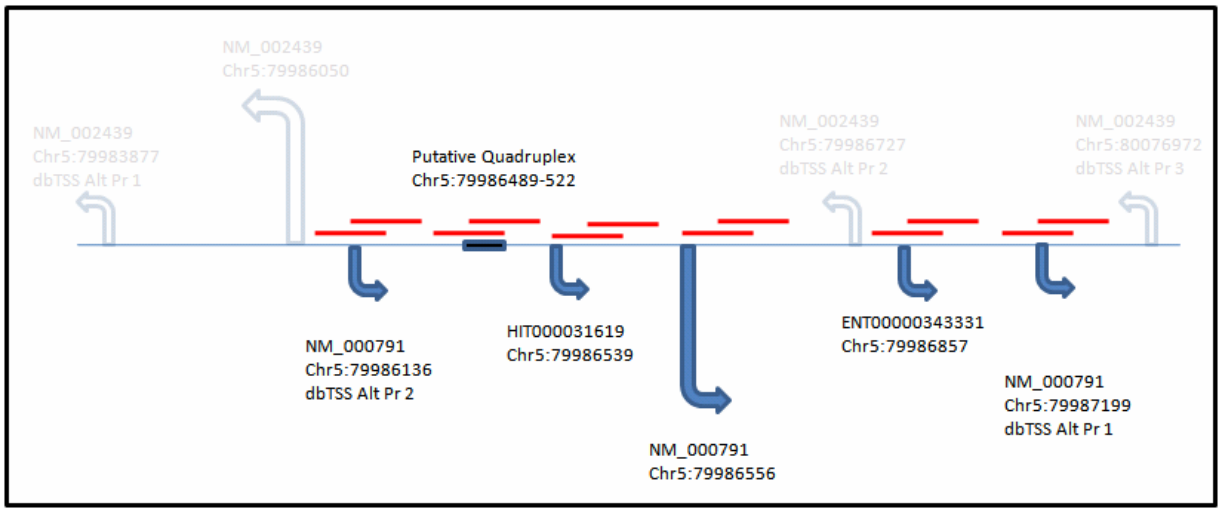
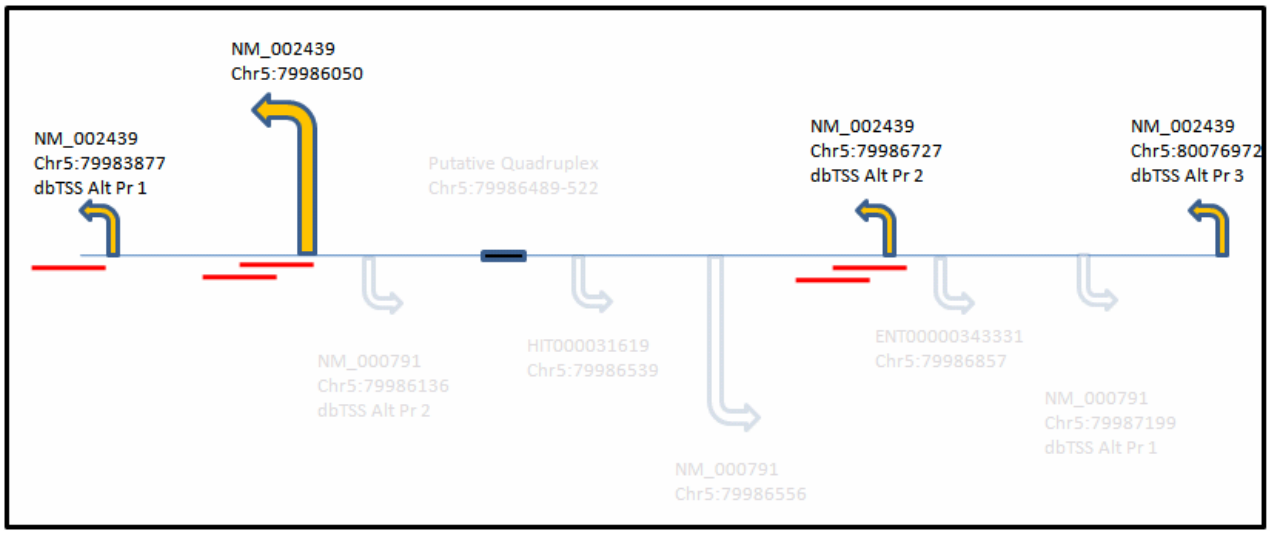


Figure 6.3 Model of the MSH3 transcriptional landscape according to literature references and the dbTSS. Shown in red are the relative target sites for agLNAs listed in Table 2. Alternative promoter 3 will not be targeted as it is significantly downstream (>89,000 bases) and does not form any known sense-antisense pairs. DHFR promoters are shown in grey.



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