ACKNOWLEDGMENTS

I would like to thank Dr. Alfred Lewin for giving me the opportunity to join in his laboratory and for his advice, support, and trust during these projects. I would like to thank Dr. William Hauswirth for serving on my supervisory committee and for all of his guidance during completion of this thesis. I would like to thank Dr. Paul Oh for serving on my supervisory committee and for being an excellent mentor and confidant. I would especially like to thank my family in Korea, my mom and younger sister, as well as my mentors, Dr. Chang-Won Kang, Dr. Hoon-Taek Lee, Dr. Tae-Young Jung, Dr. Kil-Saeng Jung, and Dr. Hong-Yang Park, and friends here and Korea for their continuous support, cheer, and love over the years as I have pursued my dreams. I would especially like to thank my boyfriend, Koo Yung Jung, for his encouragement and love during my study. I would also like to thank all of my fellow laboratory colleagues for the advice, support, knowledge, and wisdom that they have bestowed upon me throughout the time that I have been in the master’s program, especially Dr. Marina Gorbatyuk and James Thomas, who supervised most of the work presented in this thesis.
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Ribozyme-mediated gene therapy is an anti-sense approach to inactivate gene expression in order to reduce the accumulation of mutated proteins that cause diseases. Rhodopsin-linked autosomal dominant retinitis pigmentosa (ADRP) has been an optimal objective for ribozyme gene therapy for several reasons: 1) it is a slowly progressive disease, 2) it is easy to access the target tissue, and 3) the eye is an immunologically privileged site. Retinitis pigmentosa (RP) is a term that refers to a group of inherited disorders in which abnormalities of the photoreceptor cells (rods and cones) of the retina lead to blindness in approximately 1 in 3500 people in the U.S. In order for ribozyme therapy to be effective, it is critical that the ribozyme is expressed at high levels.

Since efficient export of mRNAs from the nucleus into the cytoplasm plays an important role in their expression, the nuclear export element (NEE) can be beneficial to the field of ribozyme-mediated gene therapy. Transgene (i.e., ribozyme) expression can be regulated by both the transcriptional regulatory elements including the promoter,
enhancer, intron, and poly(A) sequence and the post-transcriptional regulatory elements such as splice signal (SS), constitutive RNA transport element (CTE), and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE).

The results of one of the studies being performed in our laboratory show that recombinant adeno-associated virus (rAAV)-delivered dog rod opsin-specific hammerhead ribozyme 448 (DogRhoRz448), which is an allele-independently designed ribozyme, can effectively reduce the levels of dog rhodopsin mRNA. The plasmid encoding DogRhoRz448 used in this previous study contains two self-cleaving hairpin ribozymes (new hairpin ribozyme [NHP] and old hairpin ribozyme [OHP]) as processing sequences. The function of the hairpin ribozyme is to free the hammerhead ribozyme 448 from the long primary transcript, thereby making Rz448 available to cleave its target.

The matrix (M) protein of vesicular stomatitis virus (VSV) has been proposed to effectively inhibit Ran-dependent nucleocytoplasmic bidirectional transport of both RNA and proteins in *Xenopus laevis oocytes*. By using this inhibitor, the newly selected ET-RNAs (Exceptional Transport RNAs) were discovered. In this current study, we have replaced a self-cleaving hairpin ribozyme (NHP) in our Rz448 construct with ET-208, which is one of the ET-RNAs, to examine whether ET-208 could promote better expression of Rz448, resulting in greater knock down of dog rhodopsin expression. We found that ET-208 was able to enhance the ability of the Rz448 plasmid to reduce the levels of rhodopsin mRNA within co-transfected cells.
CHAPTER 1
INTRODUCTION

Many advances have been made in understanding the steps in the pathway of gene activation to synthesis of functional proteins. The contemporary view of gene expression proposes that several processes occurring in the nucleus and the cytoplasm are connected (1). According to this model, this pathway can be divided into 5 steps: transcription initiation (the beginning of RNA synthesis), transcription elongation (the extension of transcript), transcription termination, polyadenylation (the cleavage of RNA and the addition of a polyadenosine tail to the 3’ end of the transcript) and translation (the synthesis of a protein from RNA). Growing evidence has revealed that these distinct stages of gene expression are functionally coupled (2-4) (Figure 1-1).

The export of mRNA is coupled to other steps in gene expression involving splicing (5), transcription, and 3’-end formation (2). For example, both splicing-dependent recruitment of the mRNA export machinery and exon-junction complex (EJC) formation are conserved in eukaryotic cell (2) (Figure 1-2). Spliced mRNAs are assembled into an individual spliced mRNP (ribonucleoprotein) complex that directs the mRNA for export (5,6). In addition to these export proteins, EJC contains numerous proteins involved in the cytoplasmic localization of mRNAs (2). EJC is made from that conserved mRNA export machinery and other components of the spliced mRNP are specifically recruited to a position 20 nucleotides upstream of exon-exon junctions (7). Once formed, the entire EJC is exported and then dissociates from the mRNA in the cytoplasm (8). Splicing is not essential for export but promotes the efficiency and/or
fidelity of the process by increasing the more efficient recruitment of export factors (2) and generating a specific nucleoprotein complex (5). The mRNA export machinery and some components are also co-transcriptionally recruited (2). They interact with nucleoporins of the nuclear pore complex that are required for mRNA export and/or several mRNA export factors (2). 3’-end formation is thought to be critical in co-transcriptional loading of the mRNA export machinery onto mRNAs (2).

The nucleus and the cytoplasm are physically and functionally separated by the nuclear envelope in the eukaryotic cell. Genetic information is stored and transcribed from DNA to RNA in the nucleus, whereas the transfer of information from RNA to protein occurs in the cytoplasm (9). Therefore, the export of RNA to the cytoplasm is an important process. Additionally, the intracellular localizations of RNAs are essential for their correct processing as well as function (10).

All major classes of RNA (mRNA, 5S rRNA, tRNA, snRNA, and rRNA) exit from the nucleus to the cytoplasm as RNA-protein complexes (9,11-13) via the nuclear pore complexes (NPCs) perforating the double-membraned nuclear envelope (14-16) using energy-dependent (17), carrier-mediated transport systems (18,19). The nuclear pore complex (125,000 kDa) is composed of 50-100 nucleoporin subunits. The subunits are arranged to produce a large central channel of 28nm and eight smaller peripheral channels of 9nm. The larger channel is used for active nucleocytoplasmic transport of large particles such as RNA-containing proteins and the smaller channels provide routes for passive diffusion of small molecules such as ions and metabolites (20,21).

The distribution of RNA between the nucleus and the cytoplasm results from the interactions between RNAs and nuclear export elements (NEEs) or nuclear retention
elements (NREs) as well as specific nuclear transport or localization factors (9,10,18,22-25). The export of RNA occurs in 3 steps. First, the RNA is transported to the nuclear envelope along the nuclear matrix and docked to specific nucleoporins at the nuclear entrance of the nuclear pore complex (15,18,26). Second, it is translocated through the central channel of the nuclear pore complex (27), and third it is transported along the cytoskeletal matrix (14). In addition, the Ran system is required for export of many RNAs dependent on Ran-GTPase and its associated binding, exchange, and activation factors (9,13,24,28,29) (Figure 1-3). The major guanine-nucleotide-exchange factor of Ran is the nuclear protein RCC1, whereas its main GTPase-activating protein, RanGAP1, is almost exclusively found in the cytoplasm (9) (Figure 1-4). This asymmetric distribution offers an attractive model to explain how direction could be achieved in nuclear transport (9). Binding of exportins to mRNAs is dependent on the presence of Ran-GTP. Export cargo release is accomplished by RanGTP hydrolysis, which is triggered in the cytoplasmic compartment by the RanGAP1 protein and additional factors (9). The Ran system is also needed for import of RNA export factors that must shuttle between the nucleus and the cytoplasm (18,30,31). Thus, mutation or inhibition of this system leads to obstruction of the export of most RNAs.

Previous experiments have suggested that the matrix (M) protein of vesicular stomatitis virus (VSV) effectively inhibits Ran-dependent nucleocytoplasmic bidirectional transport of both RNA and proteins in Xenopus laevis oocytes (32) when it is in the nucleus and associated with the nuclear pore complexes (33). James E. Dahlberg’s group used this inhibitor to select novel RNA sequences that overcome this block. Unlike typical transport of most RNAs, the newly selected ET-RNAs (exceptional
transport RNAs) are exported efficiently even when the Ran system and related processes are disrupted (10). It means that export of these RNAs does not require protein factors and export pathways thought to be essential for export of most RNAs. The ET-RNAs were selected from a collection of RNAs containing 20 random nucleotides. Those that served as nuclear export elements fall into one of three sequences (10). In this study, we used ET-208, the strongest exporter of the selected ET-RNAs, in an attempt to enhance the expression of a ribozyme previously tested for gene therapy.

Ribozymes are RNA molecules with enzymatic activities such as sequence-specific cleavage, ligation, and polymerization of nucleotides (34). Naturally occurring ribozymes have been grouped into several classes (34). Two of the more characterized ribozymes, which are from the group of self-cleaving viral agents, are the hairpin and hammerhead that naturally function as self-cleaving agents in viral replication (34,35). Their substrate specificity and small size provide therapeutic benefits for ribozyme-mediated gene therapies (34,36). Both ribozymes catalyze sequence-specific cleavage of RNA that results in products with 2’, 3’-cyclic phosphate and 5’-hydroxyl termini (34,37). The hairpin ribozyme consists of a 34 nucleotide catalytic core, comprised of stems A and B that include all the nucleotides essential for hairpin ribozyme function (37), and four helices, of which helices I and II are responsible for target recognition (34) (Figure 1-5). The hammerhead ribozyme has a catalytic core of 11 nucleotides (37,38) that is stabilized by a hairpin structure and is flanked by two helices that are responsible for substrate recognition (34) (Figure 1-5). Both hairpin and hammerhead ribozymes can be designed to recognize substrates having a disease-causing point mutation (34,35). The hairpin ribozymes target gene transcripts containing a quintuple sequence 5’YNGUC3’,
where N is any nucleotide and Y is a pyrimidine, while hammerhead ribozymes recognize less restricted sequences of substrate RNA containing a 5’NUX3’ triplet, where X is any nucleotide except guanosine (34-36).

Ribozyme-mediated therapy is an anti-sense approach to inactivate gene expression in order to reduce the accumulation of mutated proteins that cause diseases such as rhodopsin-linked autosomal dominant retinitis pigmentosa (ADRP). Ribozymes have also been tested to block replication of RNA viruses or retroviruses and to inactivate dormant oncogenes such as ras or bcr-abl (36). There have been two kinds of therapeutic ribozyme design. One is that a ribozyme specifically targets and cleaves an mRNA substrate containing the nucleotide sequence of a point mutation, but fails to cleave an mRNA substrate containing the wild-type nucleotide sequence (allele-specific ribozymes) (35). The other is that a ribozyme targets the defective mRNA at site that is not altered by mutation (allele-independent ribozymes) (35). Such ribozymes could reduce the expression of both toxic (mutant) proteins and normal homologues encoded by the partner chromosome. This allele-independent approach is used as part of an RNA replacement strategy in which the ribozyme is accompanied by a ribozyme resistant mRNA encoding the wild-type protein.

Retinitis pigmentosa (RP) is a term that refers to a group of inherited disorders in which abnormalities of the photoreceptor cells (rods and cones) of the retina (39) lead to blindness in approximately 1 in 3500 people in the U.S. (36). The disease is typically detected in the second decade of life as night blindness and loss of peripheral vision. The retinal degeneration is usually slowly progressive and may take decades before leading to legal blindness. Mutations affect the rod cells specifically, but later in the course of the
disease, cone cells also undergo apoptosis and central vision is lost. Retinitis pigmentosa is caused by one of three types of a genetic defect: autosomal dominant inheritance, autosomal recessive inheritance, and X-linked inheritance. About 25% of RP is dominantly inherited (36). One of the causes of ADRP is a dominant negative mutation in rhodopsin where a histidine is substituted for a proline at codon 23 (P23H) in rhodopsin gene (39,40). This leads to the accumulation of abnormal rhodopsin proteins that ultimately results in the apoptotic death of photoreceptor cells (39) causing loss of vision (34,36). While P23H rhodopsin is most prevalent in North America, over 100 disease-causing mutations in rhodopsin lead to ADRP. In addition, mutations in over 25 other genes can lead to retinitis pigmentosa (40).

One of the studies being conducted in our laboratory is to examine the therapeutic potential of recombinant adeno-associated virus (rAAV)-delivered hammerhead ribozymes targeting retinal mRNAs associated with ADRP. It is our hypothesis that ribozymes can diminish the production of mutated rhodopsin protein by selectively cleaving mRNA molecules encoding the aberrant forms of the proteins (34,36). The effectiveness of ribozyme-mediated reduction of mutant mRNAs that cause ADRP has been demonstrated in vivo in a P23H transgenic rat model. Eyes of rats that were injected with P23H specific ribozymes showed significant reduced rate of photoreceptor degeneration and vision loss (36,41,42).

In order for ribozyme therapy to be effective, it is critical that the ribozyme is expressed at high levels. Since efficient export of mRNAs from the nucleus into the cytoplasm plays an important role in their expression, the nuclear export element (NEE) can be beneficial to the field of ribozyme-mediated gene therapy.
The results of another study being preformed in our laboratory have shown that dog rod opsin-specific hammerhead ribozyme 448 (DogRhoRz448) can effectively reduce the levels of dog rhodopsin mRNA. This is a part of an RNA replacement strategy in which endogenous rhodopsin mRNA would be digested by ribozymes and replaced with a ribozyme resistant form of wild type mRNA. The ability of DogRhoRz448 to knock-down dog rod opsin was assessed in tissue cultured cells by co-transfecting HEK 293 cells with target (dog rhodopsin) cDNA and ribozyme 448 inserted in plasmids under the control of the chicken beta-actin (CBA) promoter coupled with the cytomegalovirus (CMV) enhancer. Ribozyme activity was assayed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) 48 hours post transfection. At a molar ratio of 1:4 and 1:6 of target to ribozyme, the level of dog rhodopsin mRNA was reduced by approximately 83% and 94% respectively compared to control.

The significance of the study about the effectiveness of DogRhoRz448 is that dogs with a point mutation at T4R exhibit a retinal phenotype that closely imitates that in humans with rhodopsin mutations (43). Naturally occurring hereditary retinal degeneration in dog, which is caused by this mutation, is referred to progressive retinal atrophies (PRAs) (43). Therefore, using this nonhuman rhodopsin mutant large animal provides an inestimable tool to evaluate ribozyme-mediated therapies for ADRP before beginning of human clinical trials.

Many examples of recombinant proteins whose expression in mammalian cells requires the presence of an intron (5,44,45). Especially, mRNAs that are transcribed from cDNAs are expressed poorly compared to the same mRNAs transcribed from a gene containing introns (5). For this reason, many available vectors contain an intron (5). The
supporting evidence for a link between splicing and efficient mRNA export explains this intron requirement (5). Insertion of an intron into CMV expression cassettes greatly promotes gene expression in the cases that have been examined (5,46-48). In the absence of an intron within expression vectors, polyadenylation might target the mRNA lacking an intron for efficient export (5).

The plasmid encoding dog rod opsin-specific hammerhead ribozyme 448 previously used in our research contains the ubiquitous chicken beta-actin promoter coupled with the CMV-enhancer driving high-level and stable expression of the ribozyme from AAV vector in vivo (49). Immediately downstream of the promoter is an intron, which should help in the accumulation and localization of ribozyme to cytoplasm (5). Two self-cleaving hairpin ribozymes are found immediately downstream of the ribozyme 448 which henceforth will be referred to as new hairpin ribozyme (NHP) and old hairpin ribozyme (OHP). The function of the hairpin ribozyme is to free the hammerhead ribozyme 448 from the long primary transcript, thereby making ribozyme 448 available to cleave its target. In addition, the plasmid contains two 145bp inverted terminal repeats (ITRs) of AAV2 that are absolutely required for packaging DNA into rAAV.

Adeno-associated viruses (AAVs) contain a single-stranded, relatively small (~4.7kb) genome (50) which enables its manipulation in a plasmid to produce recombinant AAV in high titers (51). AAV is a good candidate for the ribozyme delivery with several features: 1) it has been shown to be able to infect a wide range of tissues including both dividing and non-dividing cells such as photoreceptors, 2) it does not induce an inflammatory immune response and has not been associated with any diseases in humans or animals, 3) it has a broad cell tropism, and 4) it can be integrated stably into
the host cell genome or remain stable as an episome in non-dividing cells, leading to long-term expression of the delivered gene (52,53,54). It has been demonstrated that recombinant AAV injected subretinally was able to transduce all layers of the neuroretina as well as cells of the retinal pigment epithelium of adult nude mice (55). rAAV also has been shown to have no toxicity when injected subretinally into the eyes of rodents (54). rAAV vectors are deleted of both rep that encodes four regulatory proteins and cap that encodes three structural proteins genes (56), thus limiting its natural spread because both wild-type AAV and adenovirus would be required for its propagation (57).

In this current study, we examined whether ET-208 could promote better expression of ribozyme 448, resulting in greater knock down of dog rod opsin expression. We have replaced a self-cleaving hairpin ribozyme (NHP) in our dog rod opsin-specific ribozyme 448 construct with ET-208. We discovered that this element enhanced the ability of the ribozyme plasmids to reduce accumulation of rhodopsin mRNA.
Figure 1-1. A contemporary view of gene expression. Recent findings suggest that each step regulating gene expression is a subdivision of a continuous process. In this contemporary view of gene expression, each stage is physically and functionally connected to the next, ensuring that there is efficient transfer between manipulations and that no individual step is omitted (Source: Reference 1).
Figure 1-2. Model for splicing coupled mRNA export in Metazoans. (A) UAP56 and Aly associate with the splicesome. Simplified pre-mRNA with a 5’ cap, 2 exons and an intron, and a poly(A) tail, hnRNP proteins package the pre-mRNA, and SR proteins associate with exons. (B) The Tap-p15 heterodimer targets the mRNP to the nuclear pores. Aly acts as bridging protein between the exon-junction complex (EJC) and Tap-p15. (C) mRNA export factors dissociate from the mRNP after export to the cytoplasm. Factors involved in NMD (e.g. Upf3, Y14, and RNPS1) remain bound to the mRNP (Source: Reference 4).
Figure 1-3. A model for the regulation of cargo binding and release by Ran. The asymmetric distribution of the two different Ran forms can be used to regulate the binding and release reactions between different carriers and their cargos. Binding of exportin to their export substrates is dependent on the presence of Ran-GTP. Cargo release is achieved by RanGTP hydrolysis, which is triggered in the cytoplasmic compartment by the RanGAP1 protein and additional factors (Source: Reference 5).
Figure 1-4. The Ran cycle. The small nuclear GTPase Ran switches between a GDP- and a GTP-bound state. Ran GTP/GDP cycle is regulated by Ran-guanine-Nucleotide-exchange factors (RanGEFs) and by Ran-GTPase-activating proteins (RanGAPs). The major RanGEF is RCC1, whereas the major RanGAP is RanGAP1 (Source: Reference 5).
Figure 1-5. Hairpin and hammerhead ribozymes. Schematic diagram of hairpin and hammerhead ribozymes hybridized to their target sequences. Short bars represent conventional base pairs, and dots represent non-standard base pairings. Target sequences are shown in gray shading, and arrows indicate cleavage sites. Helices are numbered with roman numerals (Source: Reference 32).
CHAPTER 2
MATERIALS AND METHODS

ET-208 Design

Sense and antisense DNA oligonucleotides coding for ET-208 and including a region of 20 nucleotides (5’ TTGAGGGCCCTCATTGCCGC 3’) that served as NEE were designed with flanking restriction sites, SpeI and NsiI and ordered from Invitrogen. The length of the sense and antisense strands are 45 and 37 nucleotides respectively, and the 5’ ends of both oligonucleotides were phosphorylated.

Rz448-NHP-OHP, Rz448-ET208-OHP, and Rz448-OHP Constructs

The construct, Rz448-NHP-OHP (Figure 2-2) containing dog rod opsin-specific hammerhead ribozyme 448, was designed by inserting the sequence for Rz448 between the HindIII and SpeI restriction sites in the P21NHP plasmid (Figure 2-1). This plasmid contains the CMV enhancer coupled with the CBA promoter driving the expression of the ribozyme, an intron, two self-cleaving hairpin ribozymes (NHP and OHP) that are found immediately downstream of the RZ448, and two ITRs. The Rz448-ET208-OHP construct (Figure 2-3) was designed by removing the NHP ribozyme with an SpeI and NsiI digest and inserting the ET-208 sequence between those sites. The Rz448-OHP construct was made by removing the ET-208 sequence from the Rz448-ET208-OHP plasmid with an SpeI and NsiI digestion. The SpeI and NsiI cohesive ends were then treated with T4 DNA polymerase to blunt the ends and ligated to seal the plasmid.
Gel Purification of Oligonucleotides

The cloning efficiency of Rz448 and ET-208 can be improved by polyacrylamide gel purification of the oligonucleotides before using them in a ligation reaction. 900 picomoles of each sense and antisense oligonucleotide were mixed with 4ul of formamide loading dye [90% (w/v) formamide, 50mM EDTA, 0.4% (w/v) xylene cyanol, and 0.4% (w/v) bromphenol blue] and incubated at 90°C for 1 min, and then loaded on the acrylamide gel. The oligos were separated on a 10% (w/v) acrylamide gel run in 1X TBE buffer [89mM Tris borate, pH 8.3, and 20mM EDTA]. The gel was run at 500-600V and no more than 40 mA. After the bromphenol blue tracking dye had run about two-thirds the length of a 20cm gel, the gel was transferred to a 20×20 cm Fluor-coated TLC plate. In a dark room, a short-wavelength UV hand lamp was used to visualize the DNA band. The bands were then excised with a scalpel, crushed in 200ul of elution solution [1M ammonium acetate, 50mM Tris HCl (pH 7.5), 20mM EDTA, 0.5% (w/v) SDS] in a sterile 1.5ml microcentrifuge tube, and eluted overnight at 37°C. The elution solution was then removed from the gel slices, and the DNA was ethanol (EtOH) precipitated, resuspended in 10ul of dH2O. Its concentration was determined by the absorbance at 260nm and stored at -20°C.

Annealing Phosphorylated Oligonucleotides

In a sterile 1.5ml microcentrifuge tube, the following contents were combined 20ul of each oligonucleotide (sense and antisense), 10ul of 10X buffer (for restriction enzyme HindIII from Promega), and dH2O to 100ul. The final volume of reaction solution was 100ul. A reaction tube was then heated to 90°C for 5 min and slow-cooled at 37°C for another 10 min. The 10ul of the annealed DNA oligonucleotides, which was diluted in
990ul of dH2O (diluted at 1:100). This dilution was then used for the subsequent cloning step.

**Digestion of the Circular AAV Packaging Vector with Proper Restriction Enzymes**

The reaction solution was composed of 10ul of resuspended circular plasmid, 1ul of a desired restriction enzyme (here, HindIII, SpeI, and NsiI), 2ul of 10X enzyme buffer, 2ul of 10X BSA, and dH2O to 20ul in a sterile 1.5ml microcentrifuge tube. The reaction tube was then incubated at 37°C for 1 hr. The linearized AAV packaging plasmid DNA was ethanol precipitated, and then the second digestion was performed. Finally, the vector plasmid was ready for gel purification.

**Gel Purification of Vector DNA**

Before ligation, agarose gel purification of the linearized AAV packaging vector was performed by running the digest on a 1% agarose gel and staining with ethidium bromide (EtBr) [10mg/ml]. The proper band was then excised after visualization with low-intensity UV light. The excised band was then crushed in a sterile 1.5 ml microcentrifuge tube and mixed with an equal volume of phenol (~pH 8.0) to elute the DNA. The contents were mixed well by vortexing and then incubated at -70°C for 1 hour and centrifuged for 5 minutes at 13,200 rpm. Next, the aqueous phase was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (50:50:1, v/v/v) to eliminate all protein contamination. The linearized DNA was precipitated by the addition of two volumes of 100% EtOH. Finally, the sample is resuspended in 10ul of dH2O.

**Ligating Annealed DNA Oligonucleotide Insert and Vector DNA**

For different sized plasmid DNAs, the amount of oligonucleotide and linearized plasmid vector DNA should be adjusted to maintain an appropriate ratio of oligonucleotide: vector ends. Here, we used the ratio of annealed oligonucleotide and
linearized vector plasmid DNA that is 3:1. The following components were combined in a sterile 1.5ml microcentrifuge tube: 3ul of DNA oligonucleotide inserts that had been phosphorylated and annealed, 1ul of vector DNA that has been linearized with the proper enzymes, 1ul (10units) of T4 DNA ligase, 2ul of 10 ligase buffer [500mM Tris-HCl (pH 7.5), 100mM MgCl$_2$] (from BioLabs), and dH$_2$O up to 20ul. The reaction is incubated at room temperature for 2 hr or 16°C overnight. After incubation, the ligated plasmid DNAs (Rz448-NHP-OHP and Rz448-ET208-OHP) were ethanol precipitated, resuspended in 10ul of dH$_2$O.

**T4 Polymerase Treatment**

The function of T4 DNA polymerase is to blunt cohesive ends on double-stranded DNA molecules with 5’- or 3’- protruding termini by 3’ overhang removal and 3’ recessed end fill-in. In a 1.5ml microcentrifuge tube, the following contents were combined: 10ul of digested DNA, 1ul of 2mM dNTPs, 2ul of T4 DNA polymerase, 2.5ul of 10X T4 DNA polymerase buffer, and dH$_2$O to 25ul. A tube was then incubated at 22°C for 30min and heated to 70°C for 5 min to inactivate T4 DNA polymerase. Rz448-OHP was then ethanol precipitated and resuspended in 10ul of dH$_2$O.

**Bacterial Transformations**

We use one of several recombination deficient strains of *Escherichia coli*, such as Sure cells (Stratagene), in order to prevent loss of the AAV inverted terminal repeat (ITR) sequences present in the AAV packaging vector. ITRs are critical for packaging plasmid inserts as recombinant AAV vectors (34). 2ul of the ligation reaction were used to transform electro-competent *E.coli* cells according to Sambrook and Russel (58). 20ul of Sure cells were mixed with 2ul of the ligated plasmids and electronically charged at
1.5V. Transformed cells were suspended in 1ml of autoclaved LB media are incubated in a thermomixer at 37°C shaking at 750 rpm for 1 hr to allow cell growth. Growing cells were then plated on LB/ampicillin (Amp) plates for selection of colonies containing the ligated plasmid DNAs, and grown at 30°C overnight to a low density to help preserve ITRs. After growing overnight, several colonies were picked out and inoculated in 5ml of LB media with ampicilin followed by another growing at 30°C overnight with shaking. After overnight incubation, increased amounts of three constructs were extracted by using Perfectprep Plasmid Mini kit from Eppendorf. The Eppendorf mini prep kit was performed according to the manufacturer’s recommendations. All clones are screened for ITR retention by an SmaI digest followed by running 0.7% or 0.6% agarose gels. The plasmids were then sequenced for proper Rz448 and ET208 orientation.

**Large-Scale Cesium Chloride (CsCl) DNA Prep**

This procedure allows us to increase the amount of the three constructs and to accomplish a highly purified preparation of DNA for transfection. After sequencing the plasmids, Sure cells were transformed with these plasmids and spread on and LB/Amp plate. A colony was inoculated in 5ml of LB/Amp media, and then 2 ml of this preculture was inoculated and grown in 1L of LB/Amp media at 30°C. After incubation for 14 hours, the culture was transferred to a bottle and centrifuged at 4000 rpm for 10 min to pellet the bacterial cells. The supernatant was decanted, making sure not to disturb the cell pellet. 30 ml of Tris-EDTA buffer was added to the cell pellet and completely resuspended. Cell lysis solution [0.2M NaOH, 1% SDS] is then added to the resuspension and mixed well. This resuspended mixture was incubated for 5 min at room temperature until the lysate is relatively clear with no visible clumps of cell material.
After the addition of 30 ml of neutralizing solution [3M sodium acetate (NaAc) pH 5.2] to the lysate, it was mixed well and placed on ice for 10 min. The neutralized lysate was centrifuged at 10,000 rpm for 15 min and the aqueous phase was poured off through 3 layers cheesecloth. The volume was then determined, and 0.6 volumes of 2-isopropanol was added. The mixture was incubated on ice for 1 hour. After centrifugation at 10,000 rpm for 30 min, the supernatant was removed. The pellet was gently washed 1 time with 70% EtOH and air dried for 30 min. The DNA pellet was then resuspended in 8 ml of Tris-EDTA buffer. 8.4 g of CsCl was dissolved in the DNA, and 150ul of ethidium bromide (EtBr) [10mg/ml] was added to the CsCl-DNA mixture. The DNA was then transferred to an ultracentrifuge tube, and spun overnight (at least 16 hours) in NVT 65 at 55,000 rpm, 20°C, and under vacuum. On the next day, the plasmid DNA band (lower band) was extracted using a short-wavelength UV hand light to visualize the DNA band stained with EtBr. Plasmid DNAs were transferred to a 15ml conical tube and the EtBr was removed from the DNA with a series of isoamyl alcohol extractions. 2.5 volumes of dH₂O were added to DNA, followed by an addition of 2 volumes of 100% ethanol in 20ml Corex tubes. Tubes were incubated on ice for 1 hour. After incubation, plasmid DNAs were pelleted at 12,000 rpm for 15 minutes at 4°C. The supernatant was removed, the DNA pellet was washed with 70% EtOH, and was dried in a speed vac. The DNA pellet was resuspended in 400ul of Tris-EDTA buffer, and then transferred to a sterile microcentrifuge tube. 40ul of neutralizing buffer [3M NaAc pH 5.2] and 1ml of 100% EtOH were added to the DNA and mixed well. The DNA is then incubated at room temperature for 10-15 minutes. The plasmid DNA was pelleted for 5 min at 13,200 rpm. The supernatant was decanted and washed with 70% EtOH followed by drying it in a
speed vac. The DNA was resuspended in 400ul of dH\textsubscript{2}O and its concentration is determined by absorbance at 260nm. Plasmid DNAs were stored at -20°C.

**Plasmid Labeling Reaction with Cy\textsuperscript{TM}3 and Cy\textsuperscript{TM}5 Dyes**

The use of the Cy\textsuperscript{TM}3 and Cy\textsuperscript{TM}5 dyes allows us to monitor the intracellular localization of plasmid DNA in cells following the transfection. According to manufacture’s description, the *Label* IT Reagents covalently attach marker molecules to nucleic acids in a chemical reaction. Prepare at least 25% more plasmid DNA in weight than needed to account for pipetting/transfer errors. In this study, three different constructs (Rz448-NHP-OHP, Rz448-ET208-OHP, and Rz448-OHP) were labeled with Cy\textsuperscript{TM}3, which gives you red color, and target plasmid DNA encoding dog rhodopsin are labeled with Cy\textsuperscript{TM}5, blue color. In a sterile 1.5ml microcentrifuge tube, the following components were combined for a 50ul plasmid labeling reaction: an appropriate volume of plasmid DNA in weight is diluted in dH\textsubscript{2}O to bring the DNA to the proper volume of labeling reaction, 2.5ul of *Label* IT Tracker\textsuperscript{TM} Reagent (Cy\textsuperscript{TM}3 or Cy\textsuperscript{TM}5 dye), and 5ul of 10X labeling buffer A. The non-enzymatic *Label* IT Tracker\textsuperscript{TM} Intracellular Nucleic Acid Localization Kit from Mirus was performed according to the manufacturer’s recommendations. After this, the concentration of labeled plasmid DNA is re-determined by absorbance at 260nm.

**Co-transfection of HEK 293 Cells with Plasmids Expressing Target and Ribozyme**

**Cell Preparation**

We chose to use human embryonic kidney (HEK) 293 cells, because they do not express our target (dog rhodopsin) which should eliminate target background. For our experiments, we used that under passage 41. Cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich Co.) supplemented with
10% fetal bovine serum (FBS, Sigma-Aldrich Co.), 100U/ml of penicillin, 100mg/ml of streptomycin (10X PenStrep, Mediatech, Cellgrow, VA). The day before transfections, the cells were trypsinized and counted on an hemacytometer so that they will be 90-95% confluent on the day of transfection.

Transfection

Transfections were preformed in triplicate or quadruplicate. To account for pipetting/transfer errors, all were prepared at least 25% more reaction volume than needed. DMEM media were removed from cells plated on day before transfection and replaced with fresh media lacking antibiotics (9ml for a 10cm dish or 1.5ml for 6-well [35mm] plate). Opti-Mem I Reduced Serum Medium (Sigma-Aldrich Co.) without serum is used to dilute plasmids carrying target and ribozymes and transfection Lipofectamine™ 2000 Reagent (Invitrogen). For multiple dishes, make a bulk mix of plasmids. For each 10cm tissue-culture dish, 10ug of total plasmid DNA (target + ribozyme) were diluted in 1ml of Opti-Mem. 4ug of total plasmid DNA were dissolved in 250ul of Opti-Mem for each well of a 6-well plate. The diluted target and ribozyme plasmid DNA were incubated separately for 5min at room temperature, and then mixed together and incubated for an additional 10 minutes. The diluted Lipofectamine™ 2000 Reagent was incubated at room temperature for 10 min. 3ul of Lipofectamine™ 2000 Reagent was used per 1ug of DNA. Once Lipofectamine™ 2000 Reagent was diluted, it was combined with the diluted plasmid DNA and incubated for 20 min at room temperature. The complexes were added directly to each dish or well containing cells and medium [DMEM with 10% FBS (without antibiotics)] and gently rocked. The plates
were incubated with transfected cells in a 5% CO2 incubator at 37°C to maintain growing
cells for 48 hours.

**Harvest of Co-Transfected Cells**

48 hours post-transfection, cells were trypsinized to remove them from culture
plates and pelleted by centrifugation at 4000 rpm for 5 min at 4°C. The trypsin
supernatant was removed and the cell pellet was then washed with 500ul of 1X PBS
twice.

**RNA Extraction from Co-Transfected HEK 293 Cells**

RNA was extracted by using GenElute™ Mammalian Total RNA kit from Sigma.
The GenElute™ Mammalian Total RNA kit was performed according to the
manufacturer’s recommendations. After RNA extraction, the eluted RNA is treated with
DNase (DNA- free™ from Ambion) to remove contaminating DNA from RNA
preparation. According to the product description, contaminating DNA is digested to
levels below the limit of detection by routine PCR by using DNA- free. The
concentration of DNase treated RNA was determined by absorbance at 260nm. PCR was
carried out to verify that no DNA contaminants have been eliminated from the RNA
preparation. In a sterile PCR tube, the following contents were combined: an appropriate
volume of 1ug of RNA, 1ul of primer I [15 pmol/ul] (sense strand of dog rhodopsin
oligonucleotide), 1ug of primer II [15pmol/ul] (antisense strand of dog rhodopsin
oligonucleotide), 2.5ul of 10X Mg free buffer, 2ul of MgCl2, 1ul of dNTPs [10mM],
0.5ul of Taq polymerase, and dH2O to bring the PCR solution to 25ul. After 20 or 30
cycles, PCR products are run on a 1.5% agarose gel, and then compared to the negative
control. If nothing is amplified, we consider our RNA preparation to be free of DNA.
Analysis of Ribozyme Activity by Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

This allows for generating first-strand cDNA from an RNA template by using proper primers. We used First-Strand cDNA Synthesis kit from Amersham Bioscience to reverse transcribe RNA to cDNA. The reverse transcriptase reaction was set up as described the protocol of the kit to synthesize first-strand cDNAs of dog rhodopsin and beta-actin (2 ug of RNA used for 15ul reaction). To avoid pipetting variation, the reverse transcription reaction for both genes was done in the same tube using sequence-specific antisense primers [40 pmol/ul]. PCR reaction for dog rhodopsin and beta-actin control was set up separately, since their optimized amplification conditions (annealing temperature and extension time) are different. For detection of PCR products, 5ul or 10ul of each reaction was loaded on an 1.5% agarose gel or an polyacrylamide gel respectively. 5% and 8% polyacrylamide gels were used for beta-actin and dog rhodopsin respectively. For quantitating PCR products run on the 1.5% agarose gel, the Gel Quantification program from BioRad was used to quantitate. After the bromphenol blue tracking dye has run about two-thirds the length of a 30cm gel at 400V/ no more than 40mA, the gels were stained with a nucleic acid attain, SYBR Green I (Molecular Probes), which is diluted 1:10,000, for 15-20 min. The stained gels are analyzed to determine the volume of each PCR product using the PhosphoImager and Image Quant program. Numerical values were obtained and ratios of rhodopsin to beta-actin were calculated.
Figure 2-1. Map of p21 NewHP packaging plasmid.

Figure 2-2. Map of Rz448 cloned into p21 NewHP packaging plasmid.
Figure 2-3. Map of ET-208 cloned into p21 NewHP Rz448 packaging plasmid.
CHAPTER 3
RESULTS

As discussed in the introduction, Dr. Marina Gorbatyuk had already tested a highly active ribozyme against dog rhodopsin mRNA. Ribozyme activity was assayed by quantitative RT-PCR 48 hours post transfection with HEK 293 cells. At a molar ratio of 1:4 and 1:6 of target to ribozyme, the level of dog rhodopsin mRNA was reduced by approximately 83% and 94% respectively compared to control (Figure 3-1). Therefore, we used this ribozyme (Rz448) to test the effects of the targeting sequences.

Construction of Rz448-ET208-OHP Packaging Plasmid

After inserting the ET-208 and dog rhodopsin Rz448 sequences into the p21NewHP plasmid, the presence of the ET-208 and Rz448 sequences in the cloned vector was examined by performing a 25 cycle PCR amplification of the sequences between SpeI and NsiI (Figure 3-2). PCR products were run on 1.5% agarose gel and stained with EtBr (Figure 3-3). In addition, the clones were screened for ITRs that are absolutely necessary for packaging of AAV. ITR retention was determined by performing a SmaI digest, and running the digested plasmid on a 0.6% agarose gel (Figure 3-4). Each AAV ITR contains two SmaI restriction sites within its sequence. If both ITRs are present, a SmaI digest yields two bands that are 3608bp and 3013bp in size (Figure 3-4). To verify proper orientation and correct sequence of ET208 and RZ448, all clones were examined by sequencing analysis.
Construct of Rz448-OHP Packaging Plasmid

The Rz448-OHP plasmid, which lacks both the NHP ribozyme and ET-208, was constructed to serve as a control plasmid in our comparison experiments. Since this construct is based on the Rz448-ET208-OHP plasmid, the absence of ET-208 was confirmed by performing a 30 cycle PCR with a sequence-specific sense primer for p21 NHP and the antisense strand of ET-208. Plasmids containing the ET-208 were also amplified to serve as positive controls (PC). Amplified PCR products were run on a 1.5% agarose gel and stained with EtBr (Figure 3-5). Rz448-OHP clones 1 and 2 do not have bands corresponding to the ET-208 band in the PC lanes (Figure 3-5). The Rz448-OHP clones were also screened for ITR and sequenced to confirm the absence of ET208 (Data not shown).

The Co-Localization of Plasmids Expressing Target and Ribozyme within a Cell after Co-Transfection of HEK 293 Cells

To visualize the co-localization of plasmids expressing target and ribozyme in co-transfected HEK 293 cells, plasmids were labeled with fluorescent dyes before transfection. Dyes were visualized with a confocal microscope (BioRad) at 48 hours post-transfection. In Figure 3-6 (X20), cells bearing plasmids encoding ribozyme are seen as a red dot and cells including a plasmid containing target are shown as a blue spot. In the case of cells holding both plasmids expressing target and ribozyme are indicated as a spot of violet color.

Analysis of Ribozyme Activity by Quantitative RT-PCR

The activity of ribozyme 448 targeting dog rod opsin RNA was assayed by quantitative RT-PCR 48 hours post transfection. For this, we extracted total RNA and compared the levels of target rhodopsin mRNA to an unchanged cellular mRNA, beta-
actin. The mRNAs for dog rhodopsin and beta-actin were reverse transcribed and their cDNAs were amplified. The PCR products from both templates were run on a 1.5% agarose and 5-8% polyacrylamide gel to quantitate the amount of beta-actin and dog rhodopsin mRNA levels.

**Results from 1.5% Agarose Gels**

**At a molar ratio of 1:4 of target to ribozyme**

Cotransfection of HEK 293 cells was conducted in triplicate. On a 1.5% agarose gel, rhodopsin ran as a single band corresponding to ~350bp (Figure 3-7a), whereas a band corresponding to ~750bp was obtained from beta-actin (Figure 3-7b). A brighter band indicates that the lane has more rhodopsin cDNA than a faint band. In a faint band, the level of rhodopsin cDNA was decreased by the ribozyme activity. The control cells did not have a plasmid expressing a dog rhodopsin-specific ribozyme. As shown in Figure 3-7a, the control samples loaded in last three lanes are much brighter than the other variants. This suggests that the other three variants (Rz448-NHP-OHP, Rz448-ET208-OHP and Rz448-OHP from left to right) expressing ribozyme caused the significant reduction in rhodopsin mRNA. On the other hand, in the case of beta-actin, the intensity of each band is similar between experimental and control variants (Figure 3-7b). Since beta-actin is a should not be affected by the ribozyme in HEK 293 cells, its expression level should be unchanged and can therefore be used as an internal control for these experiments. Using Excel (Microsoft), the ratio of rhodopsin to beta-actin was averaged and indicated in a bar graph (Figure 3-8). According to these calculations, the level of dog rhodopsin mRNA from all experimental variants was equally reduced by approximately 90% as compared to the control cells lacking ribozyme. This finding confirms the previous results that the levels of dog rhodopsin mRNA in transfected cells
can be decreased by the activity of dog opsin-specific ribozyme 448. However, since we could not see a difference among the three plasmids expressing the ribozyme, we could not ascertain whether ET-208 could promote better expression of ribozyme 448, and cause a resulting in greater knock-down of dog rod opsin mRNA expression at this ratio (1:4, target: ribozyme).

**At a molar ratio of 1:1 of target to ribozyme**

As stated above, since a significant decrease was observed in all experimental variants tested (NHP-OHP, ET-208-OHP, or OHP) (Figure 3-7a), we could not judge which one was best in reducing the levels of dog rhodopsin mRNA. For this reason, we decided that decreasing the molar ratios of target to ribozyme was necessary to see differences among the three experimental variants. To accomplish this, we designed a second transfection experiment done in quadruplicate, at a molar ratio of 1:1 (target to ribozyme). These cells were processed using the same procedures for post-transfection incubation times and RNA isolation. RT-PCR was performed and equal volumes of DNA products were loaded. Representative results for rhodopsin and beta-actin are shown in Figures 3-9a and 3-9b, respectively. Based on the intensity of the rhodopsin bands relative to the beta-actin bands, we can assess a greater or lesser efficacy in ribozyme function. Of the three plasmids tested, Rz448-ET208-OHP resulted in the highest reduction of rhodopsin mRNA. In contrast, with the Rz448-OHP plasmid, virtually the same level of rhodopsin mRNA was observed compared to untreated control (Figure 3-9a). As seen in lanes 1-4 (Figure 3-9a), the plasmid having Rz448-NHP-OHP showed higher levels of rhodopsin as compared to lanes 9-12, Rz448-OHP used as a control for NHP and ET-208 (Figure 3-9a). Levels of beta-actin as seen of the agarose gel, mRNAs were determined to be equal from all variants and controls (Figure 3-9b). These findings
demonstrated that ET-208 elevated the expression of the ribozyme 448 and resulted in the best knock-down of dog rhodopsin mRNA among the experimental variants. As illustrated in Figure 3-10, the averaged mRNA ratio of rhodopsin to beta-actin for Rz448-ET208-OHP was reduced by approximately 73% compared to control. Using Rz448 with just the old hairpin ribozyme (Rz448-OHP) the level of dog rhodopsin mRNA was decreased by approximately 36%. However, in the case of Rz448-NHP-OHP, almost no difference (reduction of ~9%) was observed. As a result of these findings, we propose that the ET-208 sequence can significantly reduce the amount of dog rhodopsin mRNA by the expression of ribozyme 448 at a high level.

**At a molar ratio of 1:2.2 of target to ribozyme**

An intermediate ratio of 1:2.2 was also examined to confirm our findings in the 1:1 ratio experiment. Cotransfections were performed in quadruplicate. The results are shown in Figure 3-11. Approximately 55% reduction in mRNA levels was obtained using Rz448-ET208-OHP, whereas an approximately 32% decrease was observed using Rz448-OHP. Although the reduction difference was not as high as in the 1:1 ratio experiment, ET-208 was still the most efficient in reducing levels of dog rhodopsin mRNA among the three experimental variants. This finding confirms the previous results that the most decreased level of dog rhodopsin mRNA was shown in cells transfected with plasmids containing the ET-208 element.

**Results from 5% and 8% Polyacrylamide Gels Containing Urea**

A 10ul portion of the same cDNA created for the agarose gel experiment was used for polyacrylamide gel electrophoresis. An 8% polyacrylamide gel was used to resolve the ~350bp rhodopsin PCR product, while an 5% gel was used for the ~750bp beta-actin product. These gels were stained with a nucleic acid stain, SYBR Green I (Molecular
Probes). The fluorescence of the DNA bands was measured on the Storm Phosphorimager (Molecular Dynamics) using the program ImageQuant. Numerical values were obtained and ratios of rhodopsin to beta-actin were calculated (Figure 3-12).

A graphical representation of the averaged ratios of rhodopsin to beta-actin using the three molar ratios of target to ribozyme (1:4, 1:1, and 1:2.2) is illustrated in Figure 3-13, 3-14, and 3-15, respectively. In the case of molar ratio 1:4, the pattern of reduction of all three experimental variants was similar to the results obtained from agarose gel. However the rate of reduction was slightly decreased from 90% to 75% (Figure 3-13). At a molar ratio of 1:1 (Figure 3-14), the amount of dog rhodopsin mRNA that was reduced is the same as that observed in agarose gel. The levels of rhodopsin mRNA were reduced by approximately 58% for both NHP and ET-208, while a reduction rate of about 31% was observed using OHP alone. Strikingly, there was no significant difference in the reduction between NHP and ET208 (Figure 3-14). The results based on the agarose gel demonstrated approximately 73% and 9% reduction rate for ET-208 and NHP respectively compared to control.

Unexpected results were also obtained at the molar ratio of target: ribozyme, 1:2.2 and are shown in Figure 3-15. NHP showed a greater reduction of rhodopsin mRNA levels than ET-208. There was a decrease in the detected cleavage for the 1:2.2 ratio as compared to the 1:1. While the 1:1 ratio showed a decrease of 58% for both ET-208 and NHP, the 1:2.2 ratio showed a decrease of 20% and 43% respectively. From these observations, we concluded that ET-208 promotes the expression of Rz448 more efficiently when the target and ribozyme are expressed at equal molar ratios. This
combination of 1:1 (target: ribozyme) results in the greatest reduction of dog rhodopsin mRNA.
Figure 3-1. The Rz448 activity on the reduction of dog rhodopsin mRNA levels in transfected cells (Courtesy of Dr. M. Gorbatyuk).

Figure 3-2. Map of Rz448 and ET208 cloned into p21 NewHP packaging plasmid.
Figure 3-3. ET-208 PCR product from Rz448-ET208-OHP clones 1-6. NC= negative control.

Figure 3-4. SmaI digest of Rz448-ET208-OHP clones 1-5 demonstrating presence of AAV ITRs.
Figure 3-5. PCR amplification to confirm absence of ET-208 in Rz4448-OHP clones. PC= positive control.

Figure 3-6. Plasmids containing ribozyme (Rz448-NHP-OHP, Rz448-ET208-OHP, and Rz448-OHP) were labeled with Cy™3 (Red), while plasmids coding for target were treated with Cy™5 (Blue) (A confocal microscope by BioRad X20).
Figure 3-7. 1:4 ratio of target to Rz separated on agarose gels. A) Rhodopsin cDNA (~350 bp). Rz448-NHP-OHP clones 1-3, Rz448-ET208-OHP clones 4-6, Rz448-OHP clones 7-9, and control 10-12. C= control containing dog rhodopsin DNA for PCR. B) Beta-actin cDNA (~750 bp). Rz448-NHP-OHP clones 1-3, Rz448-ET208-OHP clones 4-6, Rz448-OHP clones 7-9, and control 10-12. C= control containing beta-actin DNA for PCR.
At a Molar Ratio of 1:4
(Target : Ribozyme)

Figure 3-8. Ratio of rhodopsin to beta-actin at a molar ratio of 1:4 (target: ribozyme)
Figure 3-9. 1:1 ratio of target to Rz separated on agarose gels. A) Rhodopsin cDNA (~350 bp). Rz448-NHP-OHP clones 1-4, Rz448-ET208-OHP clones 5-8, Rz448-OHP clones 9-12, and control 13-16. C= control containing dog rhodopsin DNA for PCR. B) Beta-actin cDNA (~750 bp). Rz448-NHP-OHP clones 1-4, Rz448-ET208-OHP clones 5-8, Rz448-OHP clones 9-12, and control 13-16. C= control containing beta-actin DNA for PCR.
Figure 3-10. Ratio of rhodopsin to beta-actin at a molar ratio of 1:1 (target: ribozyme)

Figure 3-11. Ratio of rhodopsin to beta-actin at a molar ratio of 1:2.2 (target: ribozyme)
Figure 3-12. Picture of polyacrylamide gel containing urea stained with SYBR Green I and scanned by ImageQuant

Figure 3-13. Ratio of rhodopsin to beta-actin at a molar ratio of 1:4 (target: ribozyme)
At a Molar Ratio of 1:1
(Target to Ribozyme)

Figure 3-14. Ratio of rhodopsin to beta-actin at a molar ratio of 1:1 (target: ribozyme)

At a Molar Ratio of 1:2.2
(Target to Ribozyme)

Figure 3-15. Ratio of rhodopsin to beta-actin at a molar ratio of 1:2.2 (target: ribozyme)
Figure 3-16. Picture of polyacrylamide gels containing urea stained with SYBR Green I and scanned by ImageQuant at a molar ratio of 1:2.2 (target: ribozyme). A) Beta-actin (5%, ~750 bp). Rz448-NHP-OHP clones 1-4, Rz448-ET208-OHP clones 5-8, Rz448-OHP clones 9-12, and control 13-16. B) Rhodopsin (8%, ~350 bp). Rz448-NHP-OHP clones 1-4, Rz448-ET208-OHP clones 5-8, Rz448-OHP clones 9-12, and control 13-16.
CHAPTER 4
DISCUSSION AND FUTURE STUDIES

Two of the best characterized ribozymes that have been used in gene therapy are the hairpin and the hammerhead that act naturally as viral self-cleaving agents (34,35). Rhodopsin linked autosomal dominant retinitis pigmentosa (ADRP) has been an ideal target of ribozyme-mediated therapy for a number of reasons: (1) it is a slowly progressive disease; (2) it is easy to access the target tissue, and (3) the eye is an immunologically privileged site (34,36). There are over 100 mutations in the rhodopsin gene, leading to ADRP with the P23H mutation of rhodopsin being the most widespread in the U.S. One of the studies being performed in our laboratory is rAAV-mediated hammerhead ribozyme gene therapy for the T4R point mutation in dog rhodopsin that is associated with progressive retinal atrophies (PRAs) (43). The importance of PRAs is that dogs with this mutation display a retinal phenotype that mimics that in humans with dominant rhodopsin mutations (43).

In order for gene therapy of genetic disorders to be effective, it is crucial that the transgene (i.e., ribozyme) is expressed at high levels. Transgene expression can be changed at both the transcriptional and post-transcriptional levels. In numerous previous studies, the transcriptional regulatory elements have been optimized for efficient transgene expression, including the promoter, enhancer, intron, and poly(A) sequence (59). To fulfill this, some researchers have begun to focus on the NEEs (nuclear export elements). Recent studies have investigated the post-transcriptional regulatory elements such as splice signals (SS), constitutive RNA transport elements (CTE), and the
woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). All of these signals are known to increase RNA export from the nucleus.

The best known example of stimulation at post-transcriptional level is the inclusion of an intron within the expression cassette \(5,46,47,60\). It has been reported that the addition of an intron can facilitate gene expression by causing the cytoplasmic accumulation of mRNA \textit{in vitro} and \textit{in vivo} \(60\). In this regard, splicing, which is biochemically linked to RNA export, promotes the efficiency of the process by formation of a specific nucleoprotein complex \(2,61\). However, both CTE and WPRE have the advantages of not requiring splicing events \(60\). These elements compensate for the lack of an intron in the transgene expression \(61\). Several studies have demonstrated the utility of these \textit{cis}-acting RNA elements for increasing transgene expression in context of either plasmid or viral gene vectors \(5,62,63\).

Retroviral replication requires the nuclear export unspliced forms of viral RNAs \(61,64-67\), because eukaryotic cells encode factors that inhibit the nuclear export of incompletely spliced mRNAs \(67\). To avoid the requirement for splicing prior to export, retroviruses have evolved mechanisms that allow their intron-containing RNAs to exit the nucleus \(66\). The constitutive transport element (CTE) of the simian type D retroviruses is \textit{cis}-acting element and stem-loop structure \(67\) that promotes the nuclear export and cytoplasmic accumulation of incompletely spliced mRNAs \(61\). The CTE-mediated nuclear export involves the interaction between CTE and the host-encoded RNA binding protein TAP, a host factor which is thought to be required for export for cellular RNAs \(63,66-69\). Some studies have tested the utility of CTE in the creation of an antisense system \(48,65,70\). The CTE has also been proposed as an RNA motif that has the ability
to interact with intracellular RNA helicase that unwinds target mRNAs and facilitates cleavage by ribozymes (48,65). Futami and coworkers connected the constitutive transport element (CTE) to an antisense sequence (65). According to their results, a hybrid antisense oligonucleotide (ODN) containing CTE and targeted to the bcl-2 gene suppressed the expression of this gene more effectively than did the antisense ODN alone (65). Warashina’s group attached the CTE to their ribozyme (70). They hypothesized that an RNA helicase coupled to a ribozyme might efficiently guide the ribozyme to its target site by resolving any inhibitory mRNA structures, thereby leading to efficient substrate cleavage (70). This modification significantly enhanced ribozyme activity in vivo and permitted cleavage of sites previously found to be inaccessible, resulting in suppression on the expression of genes (70). From the findings from Liu et al., the CTE addition did not alter in vitro ribozyme activity (48). However, in vivo, Rz-coupled-CTEs were more than 20% more active than non-CTE Rz (48). In this experiment, the CTE probably assists ribozymes through RNA transport and not through RNA unwinding (48).

Besides the SS and the CTE, the third RNA element that can significantly enhance transgene expression is the post-transcriptional regulatory element of hepadnaviruses, such as hepatitis B virus or woodchuck hepatitis virus (WPRE) (62), the latter being more efficient (48,71). Other post-transcriptional elements, such as SS and CTE, depend on the transgene of interest for their enhancing effect (48). On the other hand, the stimulatory ability of the cis-acting WPRE (63), when it is inserted into the 3’ untranslated region of coding sequences (71), is transgene-, promoter-, and vector-independent (48,61). The 600 bp size of the WPRE contains at least three distinct cis-
acting sub-elements required for maximal function (48,62). The WPRE seems to act independently on transcription and splicing and may improve gene expression by facilitating RNA export (48,62). Since woodchuck hepatitis virus (WHV) encodes intronless messages (62,63), WPRE evolved to stimulate the expression of intronless viral messages (71). This element has been shown to enhance the transgene expression, which requires no viral proteins to function (63), from vectors of adeno-associated virus (AAV), lentivirus, and retrovirus (48,61,71) both in vitro and in vivo (60). To investigate the efficacy of the WPRE in enhancing transgene expression from adeno-virus vectors, some studies have tested the level and distribution of the green fluorescent protein (GFP) (71) and the luciferase expression encoded by a cDNA (60,72). Increases in the efficiency of AAV-directed expression both in vivo and in vitro were demonstrated (60,71,72). In an experimental rat glaucoma model, incorporation of WPRE into AAV improved GFP expression in retinal ganglion cells (RGCs) (64). Work done by Paterna’s group proposed that the WPRE, which was inserted into rAAVs, improved the production of certain neuroprotective proteins for human neurodegenerative diseases (73).

As discussed above, efforts aimed at increasing the transgene expression have been developed the post-transcriptional enhancer elements associated with optimized transcriptional regulatory elements in several ways. Many researchers have used the CTE and WPRE to enhance the expression of mRNA and ribozymes. However, the CTE addition did not change in vitro ribozyme activity (48). Unpublished observations from Alan White, a graduate student from our laboratory, demonstrated that there was no significant improvement made by insertion of WPRE in a ribozyme expressing AAV
vector. Despite all of these efforts, there still room for improvement in nuclear transport of ribozymes for gene therapy.

Since efficient export of mRNAs from the nucleus into the cytoplasm plays an important role in their expression, the NEE can be beneficial. One of the AAV2 ribozyme vectors, p21NHP RZ448, previously used in our studies uses two self-cleaving hairpin ribozymes found immediately downstream of dog rhodopsin ribozyme 448 as a processing sequence to free ribozyme 448 from the polyA tail of the primary transcript. Additionally, expression of the ribozyme in this plasmid is under the control of CBA promoter paired with the CMV-enhancer and has an intron located immediately downstream of the promoter as well as two ITRs required for AAV packaging.

As discussed in the introduction, it has been found that the matrix (M) protein of VSV effectively hinders Ran-dependent nucleocytoplasmic bidirectional transport of RNA and proteins in *Xenopus laevis* oocytes (32). In one of the studies conducted by James E. Dahlberg, they selected new RNA sequences that overcome the M protein mediated RNA transport inhibition. (10). These new RNA sequences function as NEE and fall into one of three sequences in a part of a collection of RNAs containing 20 random nucleotides (10). Of these ET-RNAs, ET-208 was recommended by James E. Dahlberg for our experiment. Here, we investigated whether ET-208 could increase export of ribozyme 448 to the cytoplasm, leading to greater knock-down of dog rhodopsin expression.

We tested this by constructing three different plasmids (Rz448-NHP-OHP, Rz448-ET208-OHP, and Rz448-OHP) for our comparison experiments. The presence or absence and correct orientation of the ET-208 and the NHP ribozyme within these
constructs were verified with PCR and sequencing analysis. The plasmids were also screened for ITRs. Large-scale cesium chloride (CsCl) preps were made of the constructs to get highly purified and large amount of DNA for transfection. Plasmids containing target and ribozyme sequences were labeled with fluorescent dyes. After the co-transfection of the plasmids, the localization of the two within the transfected cells was visualized with a confocal microscope.

Ribozyme activity leading to the reduction of dog rhodopsin mRNA was analyzed by quantitative RT-PCR. To assay reduced levels of dog rod opsin mRNA, two kinds of gel (agarose gel and polyacrylamide gel containing urea) were used and stained with EtBr and SYBR Green I, respectively.

First, rhodopsin and beta-actin RT-PCR products were run on 1.5% agarose gel. The ratio of rhodopsin to beta-actin was averaged. Transient co-transfection at a molar ratio of 1:4 of target to three different ribozyme constructs, all showed a greater than 90% reduction compared to a control plasmid lacking ribozyme (Figure 3-8). As described in the results, since we could not see a difference among the plasmids expressing ribozyme; we could not determine whether ET-208 could promote better expression of ribozyme 448. To overcome this obstacle, we tried decreasing target to ribozyme ratios during transfection to determine at which ratio a difference might appear among the variants. At a molar ratio of 1:1, a slightly decreased rate of reduction of the variants was observed (Rz448-ET208-OHP: ~73%, Rz448-OHP: ~36%) (Figure 3-10), but these results are consistent with findings from a ratio of 1:4 (Figure 3-8). Rz448-NHP-OHP variant showed a decreased amount of reduction (9%) (Figure 3-10). An interesting result was obtained at a ratio of 1:2.2. The level of target following transfection by Rz448-NHP-
OHP construct was not different than transfection with the control plasmid not carrying ribozyme (Figure 3-11). Approximately a 55% and 32% reduction was observed for Rz448-ET208-OHP and Rz448-OHP, respectively (Figure 3-11). In view of the results from the 1.5% agarose gel, which demonstrated that the levels of dog rhodopsin mRNA are markedly reduced by plasmid carrying ET-208 as its NEE, we can propose that the ET-208 can enhance the activity of ribozyme at a low ratio of target to ribozyme. However, at a high ratio, the extent of reduction is not significantly different between NHP and ET-208.

Polyacrylamide gels were also used to quantitate the levels of dog rhodopsin target after treatment our various Rz448 constructs. The results based on this analysis were consistent with those obtained previously by 1.5% agarose gel assay. As expected, as ratios of target to ribozyme declined, the amount of reduction was also decreased. About 75% reduction was observed from all experimental variants at a molar ratio of 1:4 (Figure 3-12). In the case of a ratio of 1:1, around 58% reduction rate was obtained by both NHP and ET-208, whereas that of Rz448-OHP was 31%. Interestingly, there was no significant difference in reduction between NHP and ET208 at this ratio. But the error bars which represent the differences on data for each sample for NHP is greater than that for ET-208 (Figure 3-13). For this reason, although there was a small difference between NHP and ET-208, this assay system does not allow us to conclude that ET-208 promotes greater activity than the NHP ribozyme. Two results above provided direct evidence supporting our hypothesis that the Rz448-ET208-OHP construct works as well or better than Rz448-NHP-OHP. On the other hand, it was surprising to find that NHP resulted in the greatest knock down of dog rod opsin expression at a molar ratio of 1:2.2 (Figure 3-
The opposite was observed from the agarose gel. The decreased reduction rate of each was 43% for NHP and 20% for ET-208, respectively.

There are several factors that could account for the observed differences. First, analyses by an agarose gel and a polyacrylamide gel containing urea are done by different methods. The agarose gels and polyacrylamide gels were stained with EtBr [10mg/ml] and SYBR Green I (diluted 1:10,000), respectively. The increased sensitivity of SYBR green increased the background fluorescence in the gels and influenced the detection of bands, possibly resulting in incorrect estimation of RNA levels. Second, as shown in Figure 3-16, the bands were not as tightly formed as expected at a ratio of 1:2.2. Especially in the case of rhodopsin, all bands were very faint. As a result of this, the levels of rhodopsin cDNA might not be correctly detected. Finally, at a ratio of 1:2.2 of target to ribozyme, the error bars for NHP were much greater than those for ET-208 (Figure 3-15). Therefore, there is not enough direct evidence for us to interpret our data to mean that the NHP ribozyme is better than the NEE ET-208.

In conclusion, we found that ET-208 was able to enhance the ability of ribozyme to reduce the levels of rhodopsin mRNA within co-transfected cells. In order to truly compare the NEE ET-208 to the hairpin ribozyme processing sequence, we need to remove the OHP ribozyme in all of our constructs used in this experiment. This experiment will enable us to obtain direct evidence on whether the ET208 acting as an NEE is better on promoting high expression of our hammerhead ribozymes than the hairpin ribozyme that functions as a processing sequence in the separation of the hammerhead ribozyme from the long transcript. Since splicing is known to enhance nuclear-cytoplasmic transport of mRNA (2,5,44-47), we should also test ribozyme
constructs that lack an intron sequence, to determine if RNA export is enhanced by ET208 in the absence of an intron.

In addition, some issues regarding methods used for this experiment will be examined to get more information from the results. First, here, we used quantitative RT-PCR to assay the activity of Rz448 targeting dog rhodopsin RNA determined by the reduction of dog rhodopsin mRNA levels. Since RT-PCR is highly reproducible only in the exponential phase of amplification, it is necessary to collect quantitative data at a point in which every sample is in this phase to obtain the accuracy and precision. After the reaction rate ceases to be exponential, it enters a linear phase of amplification. The number of cycles of PCR used for this experiment was determined by being performed for a greater or lesser number of cycles 15 -30 (M. Gorbatyuk, personal communication). Real-time PCR automates this process by quantitating reaction products for each sample in every cycle (74). The advantage of real-time PCR is that the result is a broad dynamic range, with no user intervention required. Data analysis, including standard curve generation and copy number of calculation, is performed automatically. Because of this advantage, in the future experiments we will use real-time PCR to determine rhodopsin RNA levels in transfected cells. Second, when we took pictures of co-transfected cells by using a confocal microscope, we did not count the number of cells visualized as different colors (blue or red) depending on that they contain which plasmids (target or ribozyme). For this reason, we could not determine the efficiency of transfection from these pictures. In the future experiment, we will sort cells depending on their colors labeled with different fluorescent dyes to see the efficiency of our transfection.
Lastly, in the field of antisense approaches to knock down the expression of mutated genes that cause diseases, small interfering RNA (siRNA) has also held promise for the development of therapeutic gene silencing. RNA interference (RNAi) is a post-transcriptional process triggered by the introduction of double-stranded RNA (dsRNA) which leads to gene silencing in a sequence-specific manner (75). siRNA typically consists of two 21-23 nucleotide single-stranded RNAs that form a 19 bp duplex with 2 nucleotide 3’ overhangs (76). siRNAs are an intermediate of RNA interference, the process by which double-stranded RNA inactivates homologous genes (76). Since siRNA target less specific sequences than ribozyme, it can be easily designed to recognize RNAs encoding aberrant proteins. Although ribozymes are also able to elicit strong and specific suppression of gene expression, they have limited target sequences, a quintuple sequence for hairpin ribozymes and a triplet sequence for hammerhead ribozymes (34-36). For this reason, siRNA has become popular in studying the biology of organisms ranging from unicellular protozoans to mammals (77). However, the effect of siRNA delivered as RNA is only transient and this fact severely limits the applications of siRNAs (78). To overcome this limitation, the construction of plasmids for better expression of siRNA within cells is necessary. With our findings from this experiment, we will examine whether ET-208 could promote better expression of siRNA, resulting in greater reduction of dog rhodopsin expression, by replacing Rz448 with siRNA sequence in our Rz448-ET208-OHP construct.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Eun-Jung Choi was born November 21, 1976, in Seoul, Korea. She was educated in Korea from elementary to undergraduate schools. She obtained her bachelor’s degree in animal and life sciences from KonKuk University in February 2001. She was awarded scholarships and a research assistantship during her time in the college. She worked at a nutrition lab during her sophomore and junior years. Then, she worked at a genetic engineering lab. She spent about 1 year in the states for English training at the University of Tennessee. Then, she worked at the National Livestock Research Institute.

She was admitted to the University of Florida, College of Medicine, for fall 2002 as a master’s student. She majored in molecular genetics and microbiology and worked in Dr. Alfred Lewin’s lab with a focus on ribozyme-mediated gene therapy for eye diseases. Upon graduation from the master’s program, she will attend the Interdisciplinary Program in Biomedical Sciences at the University of Florida to attain her degree of doctor of philosophy.