INITIAL DEVELOPMENT OF A RIBOZYME GENE THERAPY AGAINST HERPES SIMPLEX VIRUS TYPE I (HSV-1) INFECTION

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Herpes simplex virus keratitis is the most common infectious cause of corneal blindness in the western world. Although primary ocular or oral infection of herpes simplex virus type 1 (HSV-1) usually resolves within weeks, it leads to a latent infection of the trigeminal ganglia. The recurrent infection causes immunoinflammatory effects in the cornea which leads to blindness. Currently antiviral drugs (oral or topical) can effectively reduce acute infection, but they cannot inhibit the recurrent infection. The toxicity of current drugs as well as the emergence of drug resistant viruses leads to the need for an alternative therapy that can prevent corneal blindness caused by recurrent HSV-1 infection.

Ribozymes have been extensively studied and broadly applied for gene therapy. Several hammerhead ribozymes were designed to target messenger RNAs (mRNAs) of essential HSV-1 genes, and they were tested *in vitro* and *in vivo* for their therapeutic

effect against HSV-1 infection. A ribozyme targeting a late essential gene, U_L20, showed a significant inhibitory effect to HSV-1 viral replication *in vitro* and *in vivo*. U_L20 ribozyme was packaged in an adenoviral vector and the treatment significantly reduced the viral replication by sequence-specific cleavage of target mRNA in the cell culture. Even at a very high dose, no morphological difference was observed between cells with or without adenoviral infection. By knocking down U_L20 mRNA, this ribozyme greatly reduced the progeny viral DNA level consistent with the reduction of viral yield. The adenovirus packaged U_L20 ribozyme-154 inhibited HSV-1 infections caused by drug resistant strains, while no effect was detected in acyclovir treatment of these strains. *In vivo* testing of U_L20 ribozyme-154 was conducted in two animal models of HSV-1 infection: a rabbit ocular model and a mouse footpad model. By using iontophoresis to deliver chemically modified ribozyme RNAs to rabbit corneas, a significant reduction in the severity of lesions was observed. In the mouse footpad model, adenovirus packaged U_L20 ribozyme-154 protected mice from death due to spread of the HSV-1 infection to the central nervous system (CNS).

Overall, our studies showed promise for the application of a ribozyme based gene therapy approach to prevent HSV infection. By exploring different delivery methods, this therapeutic reagent targeting HSV-1 late gene mRNA can potentially be applied against recurrent infection at different tissues to achieve therapeutic effects.

CHAPTER 1
INTRODUCTION

Herpes Simplex Virus

Herpes simplex viruses (HSVs) belong to the Herpesviridae family, subfamily Alphaherpesvirinae according to the International Committee on Taxonomy of Viruses descriptions (ICTVD). These viruses were the first among the human herpesviruses to be discovered and have been extensively studied. The word "herpes" comes from the ancient Greek word "herpein", meaning “to creep or crawl” in the writings of Hippocrates some 25 centuries ago.[281] This reflects the ability of this virus to spread from initial infection sites (skin or mucosal surfaces), become latent in various human tissues, and reactivate themselves later. HSVs are evolutionary successful DNA viruses with a high level of host specificity. There are two serotypes of HSV, HSV-1 and HSV-2 (formal designations under ICTV description are human herpesviruses 1 and 2).[297] HSV-1 and 2 infect the human body in a very similar way; however, they have evolved not only anatomic tropism[115,142,367,368], but site-dependent incidences of reactivations.[203,286] HSV-1 causes orofacial and ocular infections in most cases and establishes latency in trigeminal ganglia, while HSV-2 prefers sacral ganglia and causes genital infections.[203,286] The seroprevelence of HSV-1 increases with age and reaches around 88% of the population at 40 years of age, while HSV-2 has an average seropervelence of 12-15%.[396] HSV transmits by direct contact with infected secretions and enters the human body through lesions or mucous membranes. Epithelial cells represent the primary targets of HSV infection.

Herpes Simplex Virus Biology

The herpesvirus virion comprises an envelop, an amorphorus protein layer called tegument, the icosahedral capsid, and an inner core containing viral genomic DNA. The genome of herpes simplex virus type 1 (HSV-1) is 152kb linear double-stranded DNA duplex with a G+C (guanosine+cytosine) base composition of 67%. HSV-1 encodes more than 80 open translational reading frames (ORFs) and most ORFs are transcribed into single transcripts (shown in Figure 1-1.). Reiterated HSV DNA sequences divide the genome into two unique sequences: designated unique long (U_L) and unique short (U_S) sequences. During viral DNA replication, two or four different isomers can be generated by inverting reiterated sequences and/or inverting the orientations of U_L and U_S. Furthermore, intragenomic and intergenomic recombination events create polymorphisms.

HSV infection is initiated by interactions of viral membrane proteins with cell surface components, and five out of twelve HSV membrane proteins have defined roles in viral entry. They are glycoprotein B (gB), gC, gD, gH and gL, and entry events involve interactions including binding and fusion of viral envelope proteins with the cellular membrane. HSV recognizes glycosaminoglycan (GAG) chains of cell surface proteoglycans, preferentially heparin sulfate, which is considered as the binding receptor. Two viral glycoproteins, designated gB and gC, mediate the binding to heparin sulfate and substitute each other during the binding event.[143] Following binding of virions to cells, fusion event takes place essentially by gD to trigger cell entry. Other viral envelop glycoproteins, gB and a heterodimer of gH-gL, are required to facilitate successful fusion.[329,330] In addition to heparin sulfate, there are two other cellular surface receptors participating in the fusion event. One was originally called HVEM (herpesvirus entry

mediator)[254] and later designated as HveA (Herpesvirus entry protein A)[379], which is a human member of the tumor necrosis factor (TNF) receptor family. Second human entry receptors were identified as related members of immunoglobulin superfamily including CD155[239], which is poliovirus receptor, nectin-2 (originally HveB), and nectin-1 (HveC) which are homophilic cell adhesion molecules localizing to sites of cadherin-based cell junctions.[6,307] A newly discovered HSV-1 entry receptor is generated in heparin sulfate by specific glucosaminyl-3-O-sulfotransferases.[321] In summary, HSV entry of cells can be separated as two different events, binding and fusion. Viral membrane proteins can interact with each other and compensate in the absence of others to facilitate entry. The abundant existence of cellular surface receptors also contributes to HSV viral entry, which determines the broad host range of HSV infection. Taking these into consideration, it is difficult to inhibit HSV infection by only preventing viral entry, since the entry is such a complex event and multiple factors from virus and host have to be considered.

Herpes simplex virus can cause both lytic and latent infections, and persist in the host life-long. During lytic infection, HSV expression is tightly regulated. There are three kinetic classes of genes transcribed in strictly ordered sequence by the cellular RNA polymerase II: immediate early (IE or α), early (E or β), and late (L or γ) gene. Transcription of α genes (ICP0, ICP4, ICP22, ICP27, and ICP47) start once viral DNA enters the nucleus. These genes are regulated by promoters that are responsive to VP16, a tegument protein functioning as trans-activator by associating with cellular transcription factors. Immediate early gene products initiate later viral gene expression, and early gene products are mostly responsible for viral DNA replication, while late proteins are mainly structural proteins for virion assembly (shown in Figure 1-2.). After primary infection,

HSV is capable of establishing latency in host sensory ganglia but may periodically reactivate and cause outbreaks. During latency HSV genomic DNA exists as an episome in the nucleus and no viral protein is detected. However, certain stimuli to host immune surveillance, which might be triggered by trauma, stress, UV-light or any kind of immunosuppression, initiate a brief viral replication in sensory neurons and transport the virus back to the peripheral epithelium where HSV propagates causing the next episode of HSV infection.

Herpes simplex virus enters neuron endings during primary infection and undergoes retrograde transport through direct interaction of viral U_L34 protein with the intermediate chain of cytoplasmic dynein.[276,401] Once reaching the nucleus, the viral capsid docks at the nuclear pore complex (NPC) to inject viral DNA into the nucleoplasm.[238] During latency, expression of all viral genes except the latency-associated transcripts (LATs) is shut off, and HSV-1 persists as a stable episomal element in the neuronal cell nucleus.[238] During reactivation, it is presumed that the lytic replication cycle ensues within the nucleus, and viral genomes are packaged in capsids, which then bud through the inner and outer nuclear membranes. At this stage, the virus travels by anterograde transport along the axons[251,295] through the interaction of the viral RNA-binding protein U_S11[300] with the ubiquitous kinesin heavy chain. Upon reaching the axon terminal, the virus exits the terminal and infects neighboring cells. These special mechanisms of intraneuronal transport give HSV-1-based vectors an advantage for non-invasive inoculation targeting the peripheral nervous system (PNS)[119,232,273], for example, in chronic pain therapy[120,123,133] and preventing periphery neuropathy.[54,55,306]

HSV-1 vector can also be used for CNS delivery, e.g., for therapy of neurodegenerative diseases.[64,156,220]

Herpes Simplex Virus Pathogenesis

Herpes simplex virus type 1 infection affects 70-90% of people in most populations[1,218], and it has been recognized as a human pathogen with significant morbidity, commonly causing lesions on skin or mucosal surfaces. Primary infection of HSV-1 usually takes place early in life in humans and very often has subclinical indications which heal within weeks without scarring. Reactivations from latent HSV infection often cause asymptotic shedding of viral particles which promotes the transmission of the virus. Occasionally, HSV infection can cause severe diseases, including sporadic encephalitis, neonatal HSV-1, ocular infections, and even lethal infections. Individuals with inherited or acquired immune deficiencies (organ transplant recipients, patients under chemotherapy, or HIV patients) have a higher risk of developing serious conditions.

Humans are the only natural reservoir of HSV. During its evolution, HSV has developed multiple strategies to escape from immune invasion and modulate intracellular as well as intercellular environments. After HSV infection, the host innate defense mechanism is turned on to prevent viral entry of cells, viral propagation, and spreading between cells. Soon after, host-acquired immune response is activated to clear viral infections effectively. In response, HSV has developed three strategies for immune evasion.

First, HSV can modulate cellular apoptotic conditions to induce pro-apoptotic or anti-apoptotic effects on defender cells. HSV-1 Us12 gene product affects immune

invasion by inhibiting cytotoxic T-lymphocyte recognition[107,145]; Us5 and Us3 gene products function to delay cellular apoptosis to allow complete viral replication by inhibiting Fas-mediated pathway as well as caspase activation.[165-167] HSV-2 ribonucleotide reductase (ICP10) blocks apoptosis in neurons by activating the MEK/MAPK survival pathway.[283,284] There are also other HSV genes (HSV-1 genes $\gamma_1$34.5, ICP27, LAT, and gene encoding gD[9,65,235,285]) involved in these modulation events.

Herpes Simplex Virus can counterattack dendritic cells (DC) by inhibiting DC maturation as well as by inducing apoptosis. DC populations exist throughout the human body, particularly in the interface to the environment (e.g. airways, skin and gut) where they capture antigens to present and activate naïve $CD4^+$ T cells. HSV infection of DCs cause down-regulation of co-stimulatory molecules, including CD1a, CD40, CD80, CD86, the adhesion molecule CD54 (ICAM-1)[249], and major histocompatibility class (MHC) I molecules. Infected DCs also have lower IL-12 production. Together, this down-regulation leads to a weaker stimulatory capacity toward T cells.[288] Although there is much that remains unknown in the mechanism of how HSV infection regulates DC maturation, it is clear that MHC class I molecule expression is inhibited by formation of HSV ICP47 with TAP (transporter associated with antigen presentation) to ICP47-TAP complex which blocks the translocation of the MHC class I peptide complex to the cell surface *in vivo*.[145,169,352] Herpes simplex virus interrupts DC mediated T helper cell responses and antibody production by interfering with MHC II antigen processing. One example is that HSV glycoprotein B (gB) interacts with HLA-DR and HLA-DM polypeptides.[263] As another effective defense strategy, HSV induces apoptosis of attacking DC which can be separated in two phases: anti-apoptotic and pro-apoptotic

phase. In the early stages, HSV infects DC to prevent apoptosis which allows sufficient viral replication. For example, HSV glycoprotein D induces NF-κB activation which thereby protects against Fas-induced apoptosis by the reduction of caspase-8 activity and up-regulation of intracellular anti-apoptotic molecules.[235] In the second phase, HSV induces apoptosis in immature DC by induction of caspase-8 pathway, up-regulation of tumor necrosis factor (TNF)-α, TNF-related apoptosis-inducing ligand (TRAIL) and p53 in combination with a down-regulation of the cellular FLICE-inhibitory protein (c-FLIP).[258] HSV also impairs mature DC migration and function to induce antiviral immune responses.[290]

Finally, the most significant feature of HSV is the ability to establish latency in sensory ganglia where viral protein expression becomes quiescent. By these means HSV hides from host immune system with episodes of periodic reactivation.

Herpes Simplex Virus Infection and Herpes Simplex Virus Keratitis

Along with the development of human society and lifestyles, HSV has become a very common pathogen worldwide. Currently, it is believed that more than 70% of the population worldwide is affected by HSV infection. HSV-1, a widespread neurotropic virus, is one of the best-characterized human pathogens. Infection with HSV-1 is very common and associated with various diseases: oral-facial infections (e.g., gingivostomatitis, pharyngitis, and recurrent herpes labialis), skin infections (e.g., eczema herpeticum, and erythema multiform), and genital infections. HSV-1 infection can cause encephalitis, called herpes simplex encephalitis (HSE), which causes pronounced mortality and morbidity despite of antiviral treatments.[323,324] HSE is the most common cause of non-epidemic, acute fatal encephalitis in the western world.[322] Herpes simplex

virus can also cause severe ocular diseases. In humans, HSV ocular infection generally begins as conjunctivitis, and it can proceed to corneal epithelial keratitis or damage deeper layers.[218]

Herpes Simplex Virus Keratitis

Herpes simplex virus keratitis (HSK) is the most common cause of corneal blindness in the United States[218], and around 300,000 cases of HSV eye infections are diagnosed yearly in the U.S.[388] HSK is caused by HSV-1 infection on the cornea in most cases (in very rare cases it is caused by HSV-2), and it is initiated by a low dose of infectious virus that causes primary infection in corneal epithelial cells. Replication of the virus causes loss of epithelial cells leading to corneal lesions indicated by branching shapes which can be detected using calcein or Rose Bengal staining.[102] These branching lesions are termed dendritic keratitis and more extensive lesions are called geographic ulcers. Herpes simplex virus type 1 viral proteins that are involved in intracellular spreading and host immune responses are believed to be responsible for different ulcer formations that occur in some individuals. Following the initial infection, HSV-1 establishes latency in trigeminal ganglia through neurons innervating the corneal epithelium and stroma. The reactivation of HSV-1 happens spontaneously when individuals are under various conditions of stress. The reactivation often causes asymptotic viral shedding, and attendant clinical symptoms may appear depending on patient's immune status. Herpes simplex virus type 1 reactivations in the cornea caused by latent infections from the trigeminal ganglia or other sites[46,46,122,122,229,229,266,266,267,267,301,301] lead to recrudescent keratitis. During each episode of reactivation, elevated corneal damage can result in stromal scarring and corneal neovascularization which are caused by increasing level of host immunity against the

virus. Theses lead to the loss of clarity of the cornea and, eventually, to corneal blindness.

Human Corneal Anatomy and Contributions to Herpes Simplex Virus Keratitis

The human cornea has unique features and these contribute to the pathogenesis and disease progress of Herpes simplex virus keratitis (HSK). The cornea is the transparent tissue in the front of the eye and is primarily responsible for transmitting light on the retina. Therefore the clarity of the cornea is extremely important to the vision. There are five cell layers comprising human cornea (shown in Figure 1-3), from front (facing light) to back they are epithelium, bowman's layer, stroma, the Descemet's membrane, and endothelium. The epithelium is a stratified squamous, non-keratinizing cell layer about 5 cell-layers thick. Epithelial basal cells have the stem-cell like feature in that they are able to regenerate epithelial layer in 2 to 4 days. Corneal epithelial stem cells are believed to reside in the basal cell layer of limbal epithelium at the transitional zone between the cornea and conjunctiva.[408] Bowman's layer is a thin acellular tissue considered to have no regenerative capacity, and it is believed that epithelial wounds heal quickly over an intact Bowman's layer. The next layer is the stroma which constitutes about 90% of the cornea. The stroma consists mainly of collagen fibrils, ground substance, and keratocyte which is the predominant cell of the stroma but only accounts for about 5% of the dry weight of the cornea. Disturbing the regular, uniform array of collagen will cause loss of clarity, and the ground substance plays a major role in maintaining regular array of collagen fibrils. In response to stromal injury, the keratocytes migrate into the wound area and undergo transformation into myofibroblasts which contribute to the scar formation by proliferation and collagen production. The layer between endothelium and stroma is called Descemet's membrane which is produced by the endothelium. The

endothelium is a monolayer of regularly shaped hexagonal cells which lie posterior on Descemet's membrane. The main function of endothelium is to control stromal hydration which is essential for corneal transparency, and they do not exhibit mitotic activity.

The cornea is believed to contain highest amount of neuron innervations among all the human tissues, and sensory innervations of the cornea are supplied by the ophthalmic branch of the trigeminal nerve. The nerve fibers of the cornea, radially oriented nerve bundles, enter the cornea from the sclera at the middle one third of its thickness. These nerves lose their myelin sheath after traversing 0.5-2.0mm into the cornea and then continue as transparent axon cylinders which contribute to the maintenance of corneal clarity. After passing Bowman's layer, they ramify (send out branches) and end within the epithelium as free nerve endings. The nerve bundles in the sub-basal plexus of the human cornea form a regular dense meshwork with equal density over a large central and mid-peripheral area. These neuron innervations open the gate for HSV transport to trigeminal ganglia where it establishes latency.

Herpes Simplex Virus Keratitis Pathogenesis

Ocular herpes simplex virus (HSV) infections involve direct viral cytopathic effects and the immune response, which both contribute to ocular damage. Primary or acute ocular infection begins with a small amount of HSV infectious viral particles. Although infectious viral load might be higher when conjunctivitis is present, and viral replication is required for herpes simplex virus keratitis (HSK) pathogenesis.[11] It is believed that once HSV infection is initiated, a threshold level of viral replication is required to develop HSK.[36,182] This phenomenon implies that it is not necessary to completely eliminate the viral replication in order to achieve a therapeutic effect.

Host immune response plays a major role in the next stage of HSK. Responding to viral replication, corneal and surrounding cells produce series of pro-inflammatory cytokines as well as chemokines. These include IL-1α, IL-1β, IL-8, IL-6, IFN-γ, TNF-α, MIP-2, MCP-1, IL-12, and MIP1-α.[80,138,175,270,338,339,364,400] Interferon α, β (IFN- α, β) are also released to inhibit viral replication directly, and this effect can be enhanced by IFN-γ.[373] These pro-inflammatory molecules draw neutrophils to the infection sites. Neutrophils attack infected cells through numbers of effector mechanisms including phagocytosis of antibody coated virus particles and release of cytokines.[243,252,350] Langerhans cells are also recruited to the site of infection, particularly the center cornea, where they acquire antigens and travel back to draining lymph nodes to activate T-cells. Eventually, all these events activate and attract T-cells to the infection site.[50,240,335] The T-cell response appears to be a Type IV hypersensitivity response mediated primarily by TH_1 $CD4^+$ cells.[89,100,118,335,406] During these events HSV infection is gradually cleared from the cornea. However, scar tissue also forms in the stroma. The damage in the stroma causes the cloudiness of cornea, eventually resulting in blindness if this happens repeatedly.

There are three factors that have an impact on HSK pathogenesis: the genetic background of the host, the host immune response, and the strain of HSV. The host's genetics make-up, although poorly understood, affects the course of infection through a number of physical factors. These genetic factors consequently affect the severity of corneal infection, given the fact that reducing viral titer even slightly could prevent HSK disease progress. Studies of HSV corneal infection in mice indicated that strains of inbred mice have different susceptibilities to HSK (C57BL/6 mice being most resistant,

DBA/2 mice being most susceptible, and BALB/C mice being intermediate).[240,337] The pattern of resistance parallels with the severity of acute infection and susceptibility of encephalitis.[172,223] While a preponderance of HSK cases occur in males according to series of studies[219], female patients are more likely to have more severe forms of the disease. These suggest a host genetic factor which contributes to HSK disease progression. The presence of a mucin layer on the outer surface of cornea, the secretion level as well as the effectiveness of antiviral molecules (e.g., lactoferrin) in the tear film[109], and the production level of numbers of cellular molecules (e.g., interferon, TNF-α, NO) all contribute to immune resistance, indicating an important role of host genetics to the outcome of corneal infection. There are also other unknown host gene products involved in the progress.[223,394] A recent study indicated that an autosomal dominant resistance locus Hrl (herpes resistance locus) mapped to chromosome 6 of mice[224] affects reactivations and viral replication in the cornea as well as in neuronal cells. It has been suggested that the igh locus on chromosome 12, loci on chromosomes 4, 5, 13 and 14 affect the susceptibility/resistance to HSV, and loci on chromosomes 10 and 17 seem to be specific for ocular disease.[265] Although functions of these gene products as well as the mechanisms of these host genes still remain to be studied, these host factors provide a new perspective for prevention of HSK. Targeting interactions of host factors and HSV for HSK therapies can help to reduce the risk of this blind-causing disease.

Host innate and acquired immunity plays a very important role in the disease progress of ocular HSV infection. On the other hand genetic differences among HSV strains also alter the clinical indications and severity.[125,376,391] Different composition of viral genes involved in DNA replication, e.g., the origin binding protein (U_L9)[35],

processivity factor (U_L42)[35], ribonucleotide reductase (encoded by U_L39 and U_L40)[34] and thymidine kinase[121], all can affect virulence in cornea. Genes encoding viral structure proteins can also be corneal virulence factors, e.g., the gene encoding a host shutoff (vhs) protein (U_L41 gene)[35,336], the gene encoding γ_1 34.5 protein[387] which also has neurovirulence function, and U_L33[35] encoding a protein essential for the cleavage and packaging of concatameric herpesvirus DNA into preformed capsids. HSV viral gene products also serve as targets for immune response, e.g., U_L21, U_L49, and the gene encoding gK can induce antibody-dependent cell-mediated cytotoxicity (ADCC).[118,189] The identification of more immune target genes will be beneficial in modifying treatment strategies for this immunopathological disease.

Overall, HSK pathogenesis involves a complex interaction between host genetic background, host immunity and the constellation of viral genes. A better understanding of these interactions will facilitate the treatment of this disease more efficiently.

Treatments and Emerging Therapies

HSV infection is a significant cause of ocular morbidity. Currently there is no drug or any form of therapy available that will eliminate the causative agent. Detailed classification of various clinical manifestations of ocular HSV infection has facilitated improving treatment strategies.[154,217] According to Herpetic Eye Disease Study (HEDS)[389], appropriate steroid usage should be applied to suppress immune response. Corticosteroid usage has been an important part of successful management of HSK. However, because they are immunosuppressive, the use of corticosteroids is counterindicated early in the infection. In the early stage of HSK, when infection takes place in epithelium and in stroma, active HSV infection can be controlled by topical or systemic antiviral treatments. There are a limited number of antiviral agents available to

treat HSV infection, including idoxuridine (IDU), Vidarabine (Ara-A), trifluridine (Triflurothymidine-TFT), acyclovir, ganciclovir, and Cidofovir. These are nucleoside analogues, and there are also metabolite analogues with antiviral effects.[247]

Idoxuridine (IDU), a thymidine analogue, was the first agent found to be effective in the treatment of HSV keratitis.[173] Although IDU is useful in inhibiting viral replication in epithelial infection, it can cause an allergic reaction. Idoxuridine has poor solubility and low penetration rate, and is rapidly inactivated. As with other antiviral drugs, IDU treatment leads to the emergence of viral resistance. The mechanism of IDU toxicity is that it is incorporated into host DNA, and is the same cause of toxicity as other antiviral drugs (e.g., Vidarabine, trifluridine) which often affect the regenerating epithelium.[210] Adverse effects often cause severe problems in patients (punctate keratopathy[277]) which complicate the antiviral treatment. Idoxuridine, Vidarabine, and trifluridine are mostly used as topical antiviral drugs for HSK. Because of their limitations in solubility, short half-life, and penetration when treating deep stromal diseases and uveitis, they are often found to be inefficient.

Acyclovir (ACV), a purine analog, has made the significant contribution in antiviral therapy of HSV and Varicella-Zoster Virus (VZV) infection. It can be activated by the viral thymidine kinase followed by phosphorylation by two cellular kinases to form an active form with triphosphate. The triphosphate form of ACV is recognized more readily by the viral DNA polymerase than by cellular polymerases. Therefore, it inhibits viral DNA replication specifically[210] and has low toxicity. An oral ACV dose of 400mg, five times daily can provide therapeutic levels in the tears, serum, and aqueous humor.[71] Topical treatment of ACV can be at a dose of 3% ophthalmic ointment five

times daily applied for 10 to 14 days in the case of denditic ulceration. Patients might have to be on ACV for a longer period if geographic ulceration is diagnosed, and often for months in the case of stromal diseases.[70,157] Acyclovir can have side effects of neurotoxicity[131], caused by crystallization of ACV and intratubular obstruction, which are presented as confusion, hallucinations, seizures, and coma. Although rarely encountered, they can often be mis-interpreted as indications of herpes encephalitis.[141] HSV develops resistance to ACV predominantly by alternations in thymidine kinase (TK) and mutations in viral DNA polymerase[181], although polymerase mutations are less frequent. However, problems due to ACV resistant HSV strains almost exclusively affect immune-compromised patients.[14,104,320] The bioavailability of oral ACV is relatively low, only 10-20%, while Valacyclovir and L-Valine ester of ACV has higher absorption rate (50%) which can rapidly convert to ACV in liver.[365] Ganciclovir (Brovinyl Deoxyuridine) acts in a very similar manner as ACV by competitively inhibiting viral DNA polymerase. Cidofovir (3-Hydroxy-2-phosphonyl-methoxypropyl cytosine, an acyclic nucleoside 5'-monophosphate) is a very promising broad-spectrum antiviral agent with longer half-life permitting once a week dosing. However, Cidofovir is available only as intravenous (IV) preparation which has substantial nephrotoxicity.[63,255]

In summary, current antiviral treatments of HSK with nucleoside analogues can control symptoms of disease but cannot cure or prevent the infections. The isolation of drug resistant HSV strains, particularly in immune-compromised patients, has attracted more clinical attention. It has been estimated that about 4-7%[61,62,66,374] of patients experience infection caused by drug resistant HSVs after antiviral treatment with nucleotide analogues. Although in immune-competent patients the incidence of infection

with drug resistant HSV is much lower (about 0.3%)[13,31,69], alternative therapies will be beneficial to overcome limitations of current antiviral drugs for general public health.

Since HSV infections continue to be prevalent, it is important to explore new treatments to improve the management of drug resistant HSV infections, suppress recurrent infections, and ideally eliminate reactivations. There is also a need for treatments that require less frequent dosing. Very often when lesions are more advanced, current medications are no longer efficient. Furthermore, alternative therapies that lack the toxicities of existing medications will be beneficial. Immunomodulating agents, such as resiquimod, can act on the viruses indirectly by inducing host production of cytokines and thereby reduce recurrences of herpes. The new helicase primase inhibitors are the first non-nucleoside antiviral compounds and are being investigated for the treatment of HSV disease. Along with the above progress, development of gene therapy methods may contribute significantly in HSV disease management.

Gene Therapy of Herpes Simplex Virus Infection

The concept of gene therapy arose during the 1970s, along with the development of recombinant DNA technology. Gene therapy has been used to deliver foreign genes to cells for correction of genetic deficits. Furthermore, with the improvement of viral vector delivery, gene transfer can be conducted in a tissue-specific manner. A significant number of studies indicate that gene therapy can provide corrections of phenotypes *in vitro* and *in vivo*, now making it a broadly accepted approach to therapy.[106,369,380]

Gene Targeting

Disease-causing genes can be down-regulated at the post-transcriptional level. Therefore, by reducing or inhibiting gene expressions, disease progress can be suppressed or even reversed. Currently, agents for sequence-specific mRNA inhibition are antisense

oligodeoxynucleotides (ODNs), ribozymes and their DNA counterparts (DNAzymes), and RNA interference (RNAi). These techniques been extensively studied in order to improve the therapeutic effect for these methods, to achieve an efficient delivery, avoid off-target effect, and to locate target sequence.

Antisense oligodeoxynucleotides

As early as 1978, it was demonstrated that an oligodeoxynucleotide (ODN) containing 13 nucleotides complementary to long terminal repeat (LTR) of Rous Sarcoma virus (RSV) could inhibit RSV translation as well as viral replication.[333,403] This initiated the study of mechanism of antisense mediated inhibition. Large scale ODN synthesis and the development of backbone modifications to increase stability as well as effectiveness have permitted antisense ODNs to be developed as drugs and to undergo clinical trials. Vitravene (ISIS pharmaceutical, Carlsbad, CA, USA) is approved by FDA (Food and Drug Administration) for treatment of cytomegalovirus-associated retinitis by targeting IE2 mRNA of cytomegalovirus (CMV). Another ODN, Genasense (Genta, Berkerly Heights, NJ, USA) has finished its phase III clinical trial for metastatic melanoma in conjunction with chemotherapy. The mechanism of antisense ODNs varies depending on the backbone modification.[33,90,332] Generally negatively charged ODNs (e.g., phosphodiesters and phosphorothioates) attract RNase H to cleave mRNA at the DNA-RNA helix. Other backbone modifications (2'-O-methyls, 2'-O-allyls, and peptide nucleic acid) are classified as steric hindrance ODNs, which do not recruit RNase H but block translation, splicing, and nuclear transport. However, the delivery of antisense ODNs is the major limitation for their application in therapy.

Ribozymes

Ribozymes are catalytic RNA molecules with the ability of breaking or forming phosphodiester bonds even in the complete absence of protein. In the ribozyme catalysis event, a 2' oxygen nucleophile attacks the adjacent phosphate in the RNA backbone resulting in cleavage products with 2′,3′-cyclic phosphate and 5′ hydroxyl termini. Ribozymes exist naturally, and they were discovered in group I intron in the large ribosomal RNA of many single-celled eukaryotes and fungal mitochondria, the RNA component of RNase P, group II introns (from fungal and plant mitochondria as well as chloroplasts), plant viroid and virusoid RNAs, hepatitis delta virus, and a satellite RNA from *Neurospora crassa* mitochondria. Ribozymes can be modified to contain a simple catalytic core and guide sequences to locate target RNA (as summarized in Table 1-1). Furthermore, they can be delivered *in trans* by cloning in plasmid or viral vectors for sequence-specific gene knock-down. The biochemical aspect of ribozymes is discussed in Chapter 2.

Hammerhead and hairpin ribozymes, discovered from different plant viroids and virusoids, have been tested as gene therapy agents extensively. Two phase I clinical trials using ribozymes for gene therapy against human immunodeficiency virus 1 (HIV-1) were conducted[5,393] in the U.S. The potential of these ribozymes in antiviral therapy of hepatitis C virus and chronic hepatitis B virus infections has also been recognized. Additional studies have indicated that RNase P also has significant potential for antiviral and cancer therapy.[67,354-360] Moreover, tissue-specific delivery provides promise for ribozymes in gene therapy of diseases caused by dominant genetics mutations.

Chemically modified synthetic ribozymes display improved nuclease resistance compared to RNA. These stabilized synthetic ribozymes, maintaining their catalytic

ability, have shown promising results in targeting RNAs associated with induction or progression of cancer *in vitro* and *in vivo*.[225,278] Direct delivery of stabilized ribozyme RNAs has several advantages (e.g., it can be appropriately dosed and can be stopped, if necessary) and has been evaluated in clinical trials.[366]

Another catalytic nucleic acid is DNAzyme, a small DNA molecule with the ability of site-specific cleavage of RNA target. DNAzymes do not exist in nature and have been developed through *in vitro* selection. Because DNAzymes are inexpensive to synthesize and can be modified chemically which increase their stability, they are useful alternatives to antisense ODN and ribozymes. However, they can only be delivered exogenously and have the same limitation as antisense ODNs with respect to delivery.

RNAi and si/shRNA

RNA interference (RNAi) represents an active organism-defense response against foreign RNA, which demands cellular machinery to initiate the process. In many organisms (such as *C. elegans*, *D. melanogaster* and vascular plants) the silencing signals can be amplified using an RNA-dependent RNA polymerase. In eukaryotic cells, the RNAi pathway also regulates gene expression that determines cell fate such as differentiation stages and cell survival. The physiological inducer of RNAi in cells is double-stranded RNA (dsRNA), which is 21-23nt long and processed by Dicer (a cellular endonulease) from longer dsRNA. This 21-23nt dsRNA contains 3' overhang, and is called siRNA (small interfering RNA). The terminal effector molecule is the antisense strand separated from siRNA which is then incorporated into the RNA-induced silencing complex (RISC complex) and serves as a guide to the complementary sequence in target mRNA. RISC conducts the endonucleolytic cleavage of mRNA within the target sequence which leads to the degradation of mRNA, and then the antisense recycles for

additional mRNA targeting.[27] For gene therapy applications, siRNA can be delivered in the form of hairpin structure with a single stem loop, referred to as short hairpin RNA or shRNA. Short hairpin RNAs are processed by Dicer into siRNAs.

RNAi pathway provides a very powerful gene silencing approach by mRNA degradation, which can be used in gene therapy. Experience from antisense ODN and ribozyme therapies have led to the development of chemically modified siRNA with resistance to endonuclease degradation. In the case that disease-causing gene expression localizes in easily accessed tissue, siRNA can be delivered without transfection reagents or delivery vehicles, e.g., intranasal or intratracheal administration of siRNA in lung gene silencing.[28,405] However, to improve the tissue specific uptake of siRNA and provide long-term effect in mammalian cells, shRNA can be delivered in a DNA vector. Different promoter complexes can be used for conditional regulaton of shRNA function.

A major concern for gene therapy is that siRNA, and other antisense molecules such as ribozymes an oligodeoxynucleotide (ODN), can have "off target" effects caused by partial homology between the intended target RNA and another RNA.[161,310] This problem is worse for siRNA delivered as shRNA, since they can block translation of an RNA by binding to the 3' UTR of an mRNA and acting as a microRNA (miRNA).[57] This inhibition requires as few as 7 base pairs between the siRNA and the 3' UTR. In addition, introducing excess amounts of siRNA could cause saturation of cellular RNAi machinery, consequently interfering with normal cellular functions. Finally un-intentional toxicity of si/shRNA might come from induction of interferon response particularly in specialized sensitive cell lines. When they are used at high concentrations of siRNAs[38,93], inflammatory effects can be induced. These can be avoided by using

siRNAs of high potency so that they are not needed in high concentration. In summary si/shRNA provides a very efficient approach for gene silencing and has been exploited extensively in gene therapy. However, toxicity and off-target effect may cause significant side-effects in clinical applications.

Delivery Systems

Adenovirus vectors

Adenovirus is a 36kb double-stranded DNA virus, originally isolated from adenoid tissue.[302] Many features of adenoviruses make them well-suited for gene therapy. Adenovirus is capable of infecting both actively dividing and quiescent cells, and its genome does not integrate into the host genome, therefore, avoiding the risk of mutagenesis. The high capacity of adenovirus allows insertion of large foreign genes, as the most advanced adenovirus vector can accommodate up to 37kb of transgene. High titers of adenovirus preparations can be obtained easily by propagating virus in 293 cells (human kidney embryonic cells), and the high efficiency of adenovirus transduction also makes it a very attractive vector for gene transfer. The first generation of adenovirus vector (containing E1 gene deletion) triggers an immune-response which leads to the loss of transgene expression within weeks *in vivo*. The second generation of adenovirus vectors incorporates a deletion of the E2 and/or E4 gene in addition to the E1 gene, and the resulting vector is therefore less immunogenic; however, the immune response still exists. Recently, the third generation of adenovirus vectors has been constructed by removal of the entire viral genome except for two ITRs (internal terminal repeats) and the packaging signal, and they are referred to as helper-dependent or “gutless” vectors. Although many problems remain to be resolved for large-scale preparation of helper

dependent adenovirus, the third generation vectors have shown promise for gene therapy applications.[86,96,244,260]

Recombinant adenovirus vectors have been tested extensively in the cornea for gene therapy. Although transgene expression turns on early and lasts for a fairly long time in corneal epithelial cells *in vitro* and in conjunctival epithelium[362] *ex vivo*, a serotype 5 vector failed to transduce corneal epithelial cell *ex vivo*[183,208] and *in vivo*.[362] These results suggested the resistance of corneal epithelium to the adenovirus vector delivery. However, adenovirus vectors are capable of transducing corneal endothelium[208] and keratocytes[49], which showed the promise of using Ad vectors for ocular gene therapy. Since donor corneas are routinely maintained *ex vivo* for an extensive period of time before transplantation, treatment with Ad vectors *ex vivo* offers a selective gene delivery method to the cornea.

Adeno-associate virus vector

Adeno-associated virus is a Dependovirus in the family Parvoviridae.[188] The genome of AAV is a 4.7Kb linear, single-stranded DNA molecule and encodes two large open reading frames (ORFs) flanked by invert terminal repeats (ITRs). The viral capsid is non-enveloped with icosahedral symmetry and a diameter approximately 25nm. This small diameter makes AAV better at diffusing through tissue structures than adenovirus. A characteristic feature of AAV is that infection of a cell in the absence of a helper virus cannot lead to a lytic infection. No known human disease has been associated with AAV infection. Hence, AAV is classified as a defective and non-pathogenic human parvovirus. An adenovirus (Ad), a herpesvirus (HSV-1, HSV-2 and CMV), or a vaccinia virus can supply complete helper functions for fully permissive AAV infection.[40,153,311]

Adeno-associated virus is a human non-pathogenic virus with a broad host range among mammals. AAV latent infection in humans appears to be common, as antibody to AAV2 can be detected in between 50% and 96% of the normal population.[53] However, no human diseases are associated with wild type AAV[29], and there is no immunologic evidence for AAV re-activation upon challenge by a helper virus.[188] In the absence of a helper virus, AAV establishes latency by integrating into the host genome or by forming an episome. In human cells, AAV prefers to integrate in a site-specific manner on human chromosome 19q13.3-qter.[190] In recombinant AAV vectors (rAAV) the *rep* protein is absent, and there is no integration between inverted terminal repeats (ITRs) and the human chromosome 19 locus, however the virus may integrate in a non-site specific manner. Another advantage of using AAV as a gene transfer vehicle is the long-term transgene expression in non-dividing cells.[2,130,280] The maximal transgene expression can be detected in weeks and typically persists for the lifetime of the animal.[170,212,327,328,399] In dividing cells, such as regenerating liver, however, episomally maintained virus could be diluted, and gene expression might decrease over time.[245,380]

There are a number of AAV serotypes and over 100 variants isolated today.[112,113,256,312] Based on the current understanding of AAV serology, AAV1-5 and AAV7-9 are defined as true serotypes. Some serotypes preferentially transduce certain tissues: AAV8 transduces liver with high efficiency; AAV1 works very well in muscle transduction; and AAV7 demonstrates efficiency in transducing skeletal muscles equivalent to that observed with AAV1.[112] AAV1, AAV2 and 5 all can be used to target murine retina, however, AAV1 has earlier onset of transgene expression and has specificity to the retinal pigment epithelium (RPE).[10] In the brain, AAV5 transduces only

neurons as does AAV2[83]; in the CNS, recombinant AAV1 and 5 (rAAV1 and rAAV5) can be used to target the entire hippocampus (HPC)[41], in contrast, transduction by rAAV2 is limited in the hilar region of HPC.[171,184,234] Currently there are at least 20 clinical trials that have been either completed or initiated to evaluate 15 different AAV2-based vectors.[52] A "cross-packaging" system has been developed to produce hybrid AAV vector packaging AAV2 genome while containing capsid proteins of a different serotype (a pseudotype). This provides an unbiased comparison of transduction efficiency of different AAV capsids containing the same transgene expression cassette.[127,292] The development of hybrid AAV vector engineering, (including peptide ligand insertation[261], production of mosaic AAV[136,291] and chimeric AAV[32], and combinatorial AAV vector libraries[230,282]) enables constructions of vectors with improved tropism and increased tissue specificity. Although cross-reactivity of different AAV serotypes appears to be tissue/specie specific and delivery method dependent[398], it is often recognized that *in vivo* administration of one serotype is not affected by pre-existing neutralizing antibodies of the other.[279,398] Alternative gene transfer vectors of different AAV serotypes can be applied when patients have high titers of antibody against one serotype, for example AAV2. Moreover, multiple vectors delivering various genes simultaneously can be applied.[294,316]

Herpes simplex virus vectors

Herpes Simplex Virus (HSV), a neurotropic double-stranded DNA virus, is a promising vector for gene transfer applications. HSV contains a large genome which provides significant capacity to accommodate multiple or large transgene cassettes by replacing dispensable and pathogenic genes. The toxicity of HSV vector can be minimized by eliminating genes necessary for viral replication (IE gene deletions).

These replication-defective HSV vectors can be propagated in cell lines complementarily expressing these gene products.

Because HSV-1 has a broad host range and is able to infect dividing as well as quiescent cells, it can deliver transgenes to a variety of tissues or cell types. By exploiting the ability of HSV-1 to infect neuronal cells and establish latency, HSV-1 viral vector is particularly suitable for long-term transgene expression in the nervous system. As recombinant HSV vector maintains the natural HSV-1 axonal transport mechanism, it can be used to deliver foreign genes to inaccessible tissues. Delivery method can be simplified by noninvasive procedures, e.g., subcutaneous vector inoculation. This allows transgene expression within the nucleus of the inaccessible trigeminal ganglion as well as dorsal root ganglion. As the nervous system is the natural target for HSV-1 latency, latency promoter complex can be used to achieve long-term transgene expression in neurons.

The unique mechanisms of HSV-1 viral entry and transport (retrograde or anterograde transport) have led to the extensive vector development in neurological applications. The natural existence of HSV-1 entry receptors obviates the need to modify viral surface for a broad cell-type targeting, as HSV viral entry has been described in the section of Herpes Simplex Virus (HSV) Biology earlier. In the sensory neurons of periphery nervous system, HveC, a major mediator for HSV entry, is abundantly expressed[233], and thereby HSV vector can be applied to target these cells. However, efficient transduction of peripheral motor neurons cannot be achieved due to low levels of HSV receptor expression, targeting these cells requires alterations of viral glycoprotein(s). Very similar to other viral vector applications, HSV-1 vectors can be

modified to retarget specific cell types. Two criteria must be met for this purpose: first, the natural receptor-ligand interactions of the virus need to be diminished; second, the virus must be redirected to preferred receptors by either alterations of viral surface[206,407] or the addition of adaptors.[8,124]

Herpes simplex virus vectors have also been evaluated to transduce ocular tissues. It has been shown that HSV vector could transduce corneal epithelium *in vivo* after topical application of HSV vector to the mouse cornea.[331] However, corneal scarification on the superficial epithelium before inoculation of viral vector was necessary to induce efficient transgene expression, and transgene expression was limited surrounding the site of scarification. It was also suggested in the same study that by using the topical application, HSV vector could only transduce a few cells of the iris pigmented, trabecular meshwork, and ciliary body. This limited the application of using HSV vector for corneal gene transfer.

Overall, various aspects of HSV basic biology have been exploited to expand the utility of HSV vector as therapeutic vector for diseases in periphery nervous system and central nervous system.

Other methods of gene transfer

A number of delivery methods for gene transfer have been studied extensively, including iontophoresis, electroporation, nanoparticles, cationic lipid-mediated gene transfer, etc. Each of these can be made efficient, but all lead to transient gene expression and, therefore, may not be suited for the long term effect of a chronic disease or recurrent disease. Efficient delivery is one of the keys leading to the success of gene therapy. Different approaches can be chosen depending on factors such as the delivery tissue, the disease mechanism, and the therapeutic effect pursued.

Summary

The ultimate goal of HSV infection therapy is prevention: preventing recurrent herpes simplex virus (HSV) infection and consequent tissue damage. In spite of the development of current antiviral drugs, no available therapy can reach this goal. HSV infection triggers host immune response, downstream events of the disease are affected by the interaction of host and HSV. Herpes simplex virus infection on cornea has significant impact on patients' life. Considering the prevalence of HSV infection among the population, it is a major concern for general public health. Inhibiting HSV replication at the post-transcription level by down-regulating HSV essential gene expression shows promise for antiviral therapy. By establishing surveillance against each episode of reactivation either at the corneal epithelium or in the trigeminal ganglia, HSV viral load can be significantly reduced, therefore preventing subsequent damage to the stroma and corneal blindness. The goal of this study is to test therapeutic ribozymes/siRNAs for their potential in inhibiting viral replication. By testing a proof-of-principal concept, this study provides a guide for future applications using ribozymes/siRNAs in anti-HSV gene therapy, especially in the cornea. Furthermore, this study also provides experience in corneal transgene delivery. Finally while testing antiviral reagents targeting genes from different kinetic classes of HSV-1, a better understanding of HSV-1 biology and interaction of HSV-1 proteins can be achieved.

Table 1-1. Ribozyme activity in nature and therapy.[213]

Ribozyme	Catalytic activity	Relevant role in nature	Therapeutic applications
Hammerhead	Sequence specific ribonuclease	Self-cleaving RNA	Digestion of viral, oncogene or mutant mRNA
Hairpin	Sequence specific ribonuclease	Self-cleaving RNA	Digestion of viral, oncogene or mutant mRNA
RNase P	Structure specific ribonuclease	tRNA processing	Digestion of viral mRNA
Group I intron	RNA cleavage and ligation	Splicing	RNA repair of mutant mRNA or ocogenes
Group II intron	RNA and DNA cleavage and ligation	Splicing and transposition	Gene disruption of viruses and mutant mRNA
Spliceosome	RNA cleavage and ligation	Splicing	Repair of mutant mRNA
DNA enzymes	Sequence specific ribonuclease	None	Digestion of viral, oncogene or mutant mRNA

(Lewin, A.S. and Hauswirth, W.W., 2001)

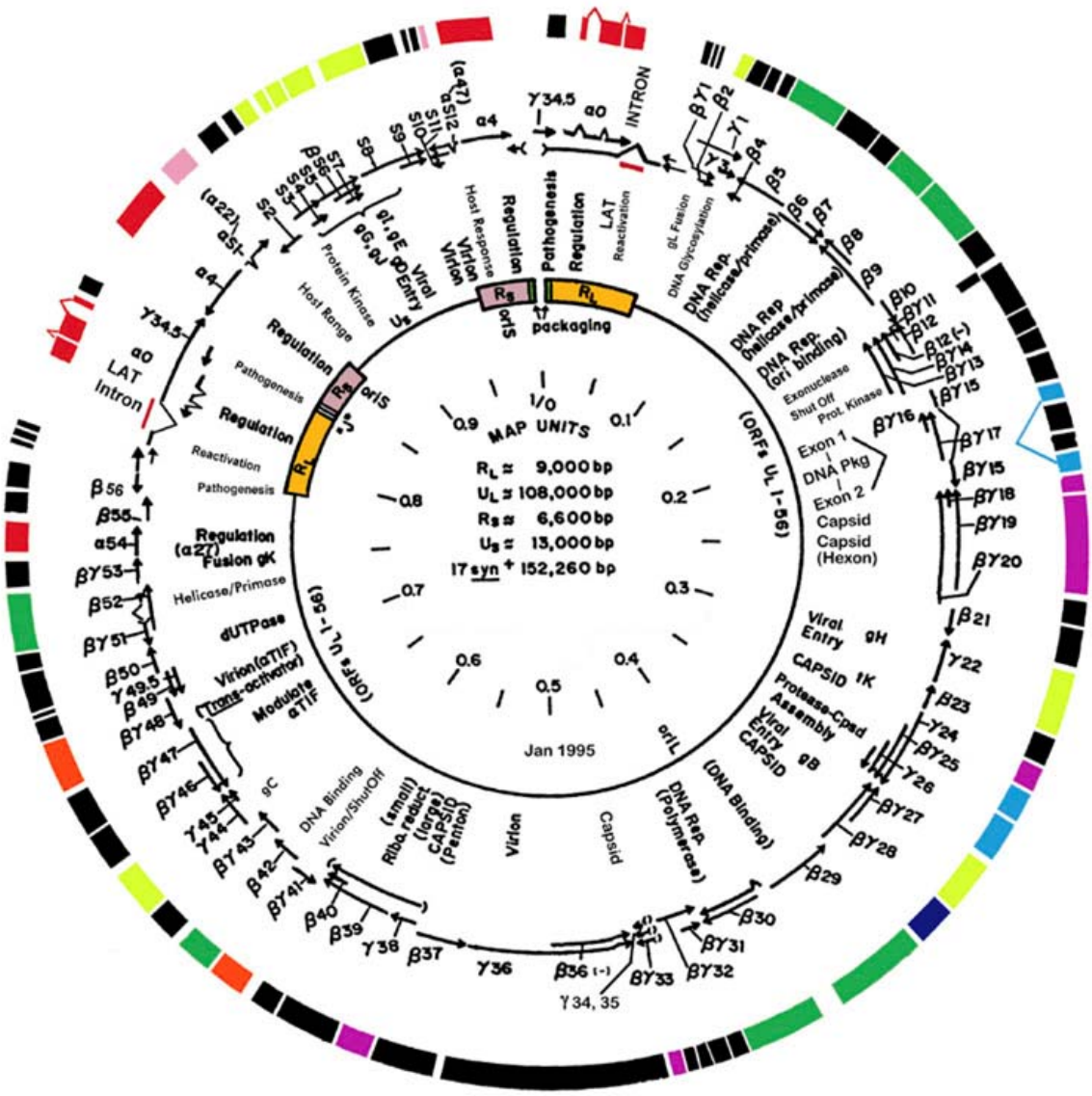


Figure 1-1. Herpes simplex virus type 1 genetic map. (Modified from http://www.dbc.uci.edu/~faculty/wagner/hsvimg04z.jpg) HSV-1 is double-stranded DNA virus. In the virion, viral DNA is packaged in the form that the ends of the genome are in close proximity which appears to be circular. The HSV genome was estimated to be approximately 150 kilobase pairs, and complete sequencing of HSV-1 strain 17 genome describes the genome as 152260 base pairs (accession number X14112).

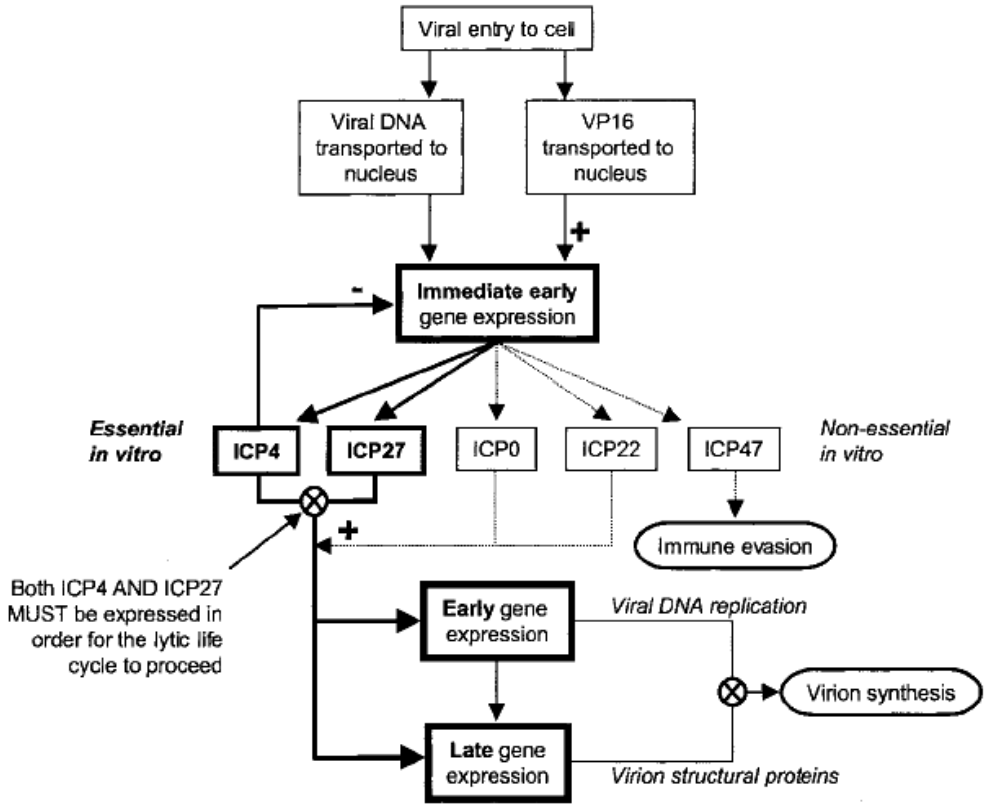


Figure 1-2. Regulation of viral gene expression during lytic infection. Flow chart illustrating the regulation of viral gene expression indicates the important roles of immediate early genes, especially ICP4 and ICP27, in turning on the expression of downstream classes of genes.[43]

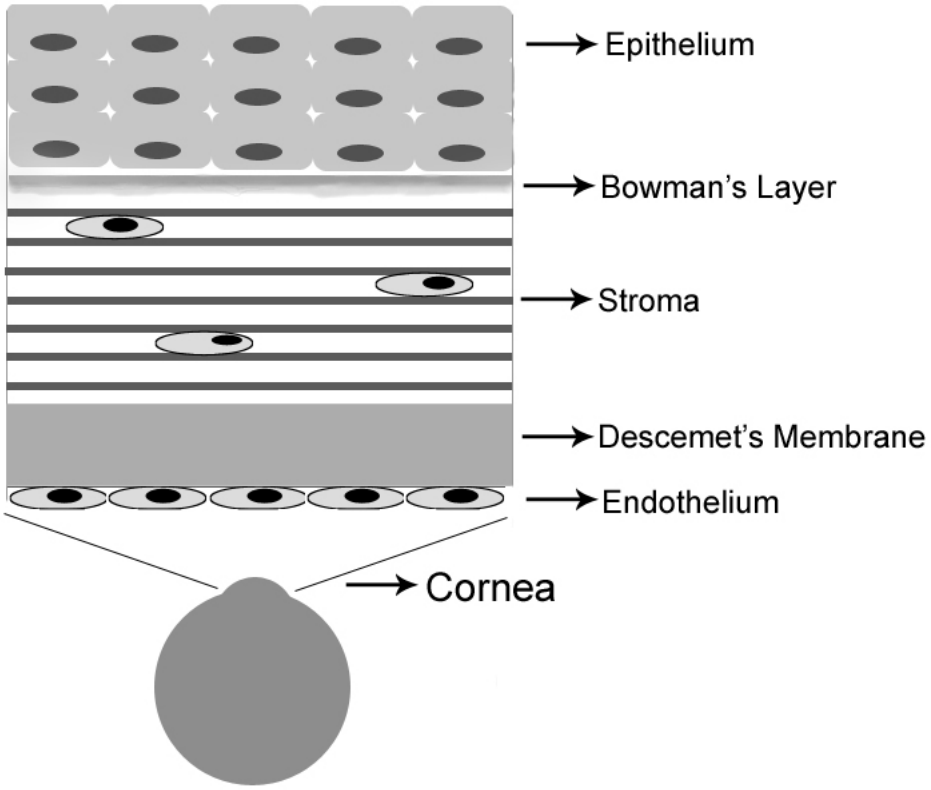


Figure 1-3. Human cornea anatomy.

CHAPTER 2
DESIGN AND IN VITRO KINETIC STUDY OF HAMMERHEAD RIBOZYMES TARGETING MRNA OF HSV-1 ESSENTIAL GENES

Introduction

Ribozymes are catalytic RNA molecules that promote a variety of reactions, often involving splicing of RNA.[347] Naturally occurring ribozymes fall into several classes, including group I introns (from ribosomal RNA of protists and bacteria, and from mitochondrial DNA of fungi), group II self-splicing introns (from yeast, fungal and plant mitochondria as well as chloroplasts[73]), the tRNA processing enzyme RNaseP[129], hepatitis delta virus (HDV) ribozymes[200], the VS ribozyme from *Neurospora crassa* mitochondria[308], and the hammerhead and hairpin ribozymes from single-stranded plant viroid and virusoid RNAs.[44,158,390] The reactions catalyzed by natural ribozymes usually involve breakage and formation of phosphodiester bonds between nucleotides, although they can conduct other biochemical transformations including reactions analogous to the reverse of splicing.[204,313]

From the evolutionary perspective, it has been suggested that self-cleaving ribozymes reflect remnants of the RNA world. The RNA world theory hypothesizes that far before the genetic information flow (from DNA to RNA to protein) formed, functions for life were conducted by RNA.[116] Recent discoveries that self-cleaving ribozymes can associate with protein-coding genes[20,392], raise the question whether self-cleaving ribozymes regulating gene expression may be predated and have been the ancestors of RNA replicons.[22] Salehi-Ashtiani *et al*[304] identified a self-cleaving ribozyme in the first

intron of the cytoplasmic polyadenylation element binding protein 3 (CPEB3), and the association of CPEB3 and CPEB3 ribozyme is actively present in all the mammals but not in other vertebrates.[22] The striking resemblance of the CPEB3 ribozyme to ribozymes in HDV, a pathogenic subviral satellite naturally found only in humans. The fact that HDV has been isolated only from human tissue led to the speculation that this HDV self-cleaving ribozyme may have evolved from modern protein-dominated organisms. Therefore, this may exclude the possibility that HDV ribozyme is a descendant of the RNA world.

The hammerhead ribozyme catalytic motif was first reported in small satellite and viroid RNAs two decades ago[37,345], and it is one of the smallest catalytic RNAs containing around 30 nucleotides active under physiological conditions. The potential of hammerhead ribozymes to catalyze sequence-specific down-regulation of gene expression was realized following the definition of simplified ribozyme catalytic motifs in the late 1980s and early 1990s. With the development of other oligonucleotide-based regulation methods (antisense, DNAzymes, and siRNAs), ribozymes have significant advantages for gene therapy applications. Because of its simplicity and flexibility, the hammerhead ribozyme can be designed to cleave any target RNA independently from cellular pathways and even in the absence of protein, which are different from siRNA/shRNA. The hammerhead ribozyme (and other ribozymes) can be designed against introns and nuclear-specific sequences[248], and this selectivity in intracellular compartmentalization provides it advantages over antisense oligonucleotides, DNAzymes, and siRNAs. In terms of off-target effects, in a comparative study in neurons using an adenoviral delivery, the hammerhead ribozyme showed increased

specificity compared to siRNA[19]; ribozymes are much more sensitive to nucleotide changes at the cleavage site than other methods, and therefore can be used to discriminate between single nucleotide polymorphisms.[94,212]

The essential structural elements of hammerhead ribozyme contain three Watson-Crick base-paired helices; helix I and III are connected by conserved sequences with catalytic potential.[144] *In trans*, the hammerhead ribozyme anneals to its substrate by complementary hybridizing to form helix I and III, and a loop links helix II (shown in Figure 2-1).

Because ribozymes (hammerhead, hairpin ribozymes and RNase P) can down-regulate gene expression by conducting sequence-specific cleavage of target mRNA, they have been extensively used to down-regulate cellular and viral gene expression.[76,177,179,180,355] The hammerhead ribozyme has been used to down-regulate undesirable gene expression: in the dominant-negative gene disorders, where the gene product of mutant allele jeopardizes the normal function (e.g., autosomal dominant retinitis pigmentosa (ADRP); in cancer therapies, e.g., using ribozyme to reduce oncogene expressions (ras[178], bcr–abl[201]); in antiviral therapies, particular anti-HIV.[342,343] The availability of various viral vectors (adenoviral, adeno-associated viral, retroviral, and herpes simplex virus vectors) provides options for tissue specific and long-term delivery.

The concept of using ribozymes as antiviral agents has also been tested. The RNase P ribozyme has been tested *in vitro* against HIV, hepatitis B[409], and hepatitis C virus[216] and herpes viruses.[179,357,358] However, there has been no successful *in vivo* delivery of ribozymes to target herpes viruses for therapy. Recently a liposome mediated

delivery of an siRNA has been used to treat an HSV-2 infection in mice. In this study, I designed hammerhead ribozymes targeting Herpes Simplex Virus type I (HSV-1) to explore a gene therapy approach to inhibit HSV infection.

Herpes simplex virus type 1, a member of Herpesviridae family, is a neurotropic DNA virus with the ability of conducting lytic infection and establishing latency. From the perspective of HSV infection induced pathogenesis, it is the productive viral replication, either from acute infection or reactivation, directly or indirectly causing damage to the host. Thus essential genes of HSV-1 become good targets for antiviral agents, since knocking down an essential gene expression may have significant impact on viral replication cycle, which can limit infectious disease progressing in the host.

Materials and Methods

Target Gene Selection and Determining Target Sequences of Hammerhead Ribozyme

Potential ribozyme target genes were selected from HSV-1 essential genes (the complete HSV-1 genome is in NCBI database with a nucleotide access number of NC_001806) based on their base composition of guanine plus cytosine using software called Vector NTI 8 (1994-2002 InforMax, Inc) and examples are shown in Figure 2-2. GUC, CUC and GUU are the cleavage sites of hammerhead ribozymes that were searched in the potential target gene in order to design corresponding ribozymes. Once the cleavage sites were decided, two hybridizing arms of the hammerhead ribozyme would be developed by using complementary sequences surrounding the cleavage site. A program called MFOLD by Dr. Michael Zuker (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) was used to predict the secondary structure of each designed ribozyme to determine whether they can proceed to

further study. An example of predicted secondary structure is shown in Figure 2-3. The ones with correct secondary folding patterns (catalytic core, conservative stem and free hybridizing arms) will be carried on to *in vitro* kinetic studies to determine their catalytic parameters.

In Vitro Kinetic Studies

In vitro kinetic analysis (including time-course and multi-turnover studies) of hammerhead ribozymes were conducted using commercially synthesized short RNA oligonucletides. Hammerhead ribozymes and corresponding targets were purchased from Dharmacon, Inc (Lafayette, CO) in 0.05μmol scale following the procedure described previously[315]. RNA oligonucleotides were synthesized in a protected form including silyl ethers to protect 5'- hydroxyl (5'-SIL) in combination with an acid-labile orthoester protecting group on the 2′-hydroxyl (2′-ACE). The deprotection procedure was conducted following the manufacturer's manual. In general, oligoes were resuspended to a concentration of 300pmole/μL in RNase- free water as the stock solution, while concentrations of 10pmole/μL and 2pmole/μL were used as working solution of target RNA and ribozyme, respectively. Ribozyme *in vitro* tests started at a reaction condition at 20mM $MgCl_2$, and ribozymes with high catalytic activities were studied under lower magnesium concentration (5mM).

Kinase of RNA oligonucleotides

5' ends of target RNA oligonucleotides were labeled with [γ^{32}P] ATP (MP Biomedicals, Irvine, CA) (10 μCi in 1μL) in a solution with 10μL total volume containing 2μL of RNA oligo (10 pmole/μl; 20 pmole total), 1μL of 10x Polynucleotide Kinase Buffer (Promega, Madison, WI), 1μL of RNasin (Promega, Madison, WI), 1μL of

0.1M Dithiothreitol (DTT) (Sigma, St. Louis, MO), 3µL of RNase-free water, and 1µL of polynucleotide kinase (5 units) (Sigma, St. Louis, MO). The reaction was incubated in 37°C for 30 minutes and 65µL of RNase- free water was added before extracting using 100µL of phenol/chloroform/isoamyl alcohol. The aqueous layer was purified on a pre-packed Spin-50 Mini-column (USA Scientific, Inc., Ocala, FL) according to manufacturer's instructions. Radioactive labeled RNA oligonucleotide can be stored in -20°C for 1 week.

Time-course studies of hammerhead ribozyme cleavage

Time-course reaction was set up as following: 13µL of 400mM Tris-HCl (pH 7.4-7.5) (Fisher, Swanee, GA), 1µL of ribozyme (2pmole), and 70µL RNase-free water were incubated at 65^{o}C for 2 minutes followed by incubating at room temperature for 10 minutes. Meanwhile, a mixture of RNasin and 0.1M DTT in a ratio of 1 to 10 and 200mM $MgCl_2$ were prepared. At the end of the incubation, 13µL of RNasin/0.1M DTT mixture and 13µL of 200mM $MgCl_2$ (final concentration is 20mM and it can be adjusted to final concentration of 5mM as well) were added followed by 30 minutes of incubation at 37°C. 2µL of γ^{32}P-ATP labeled target and 2µL of unlabeled target (20pmole) were added to the reaction. At 0,1,2,4,8,16,32,64, and 128 minutes, 10µL of volume was taken out, and 20µL of formamide dye mix (90% formamide (super pure grade) (Sigma, St. Louis, MO), 50 mM diaminoethanetetraacetic acid disodium salt (EDTA) (pH 8) (Fisher, Swanee, GA), 0.05% bromophenol blue (Sigma, St. Louis, MO), 0.05% xylene cyanol (Sigma, St. Louis, MO)) was added before placed on ice. Samples were denatured at 90°C for 2 minutes before chilled on ice and 6µL of each sample was loaded on 8% polyacrylamide-8M urea gel. The gel was pre-run for 30 minutes before samples were loaded. Wells were rinsed to remove urea before loading the sample. After samples

were run about 2/3 length of the gel, the gel was placed in fixative containing 10% V/V of Methanol (Fisher Scientific, Fair Lawn, NJ), 10% V/V of Acetic Acid (Fisher Scientific, Fair Lawn, NJ), and water for 30 minutes. Dried gels were exposed overnight in storage phosphor screen cassettes and scanned in Storm Phosphorimager (GE Healthcare, Piscataway, NJ) for image quantification. At each time point, the percentage of cut target from total target (the sum of cut and uncut target) was calculated, and a linear range was determined within which the percentage and time form a linear relation. The time it takes to reach 10-20% cleavage of the full length target was decided and was used for multi-turnover kinetic analysis.

***In vitro* multi-turnover studies**

A ribozyme solution of 0.3pmole/μL was prepared and target solutions of 30, 3 and 0.3pmole/μL were prepared as following: to make 150μL of 30pmole/μL solution of target, 15μL of ^{32}P-labeled RNA oligo, 15μL of 300 pmole/μL stock, and 120μL of RNase-free water were mixed together; 1:10 dilution was conducted to make 150μL of 3 pmole/μL solution, and 100μL of 0.3 pmole/μL was made. The experiment set-up is described in Table 2-1, and the concentration of target can be changed depending on the amount of target required to reach saturation in time-course reactions. Target solution was warmed up at 37^{o}C for at least 5 minutes before addition to reactions.

After adding hammerhead ribozyme, tubes were held at 65^{o}C for 2 minutes then at room temperature for 10 minutes. Then they were held at 37^{o}C for 10 to 30 seconds once magnesium was added. Following the addition of target solution, reactions were incubated at 37^{o}C for the time to reach 10-20% cleavage of full-length target (based on the time course experiment) before stopping the reaction with 20μL of formamide dye

mix. Samples were run on polyacrylamide-urea gel which was fixed and dried before exposed in storage phosphor screen cassette for phosphoimager scanning as described in "Time-Course Studies of Hammerhead Ribozyme Cleavage".

A calibration curve was set up by preparing target dilution following the description in Table 2-2. These dilutions were filtered through Hybond N^+ (Positively Charged Nylon Transfer Membrane) (Amersham Pharmacia Biotech, Piscataway, NJ) set in a dot-blot or slot- blot apparatus (BIORAD Life Science Research, Hercules, CA). The calibration curve analysis gave an equation which related target concentration to pixel reading of radioactive intensity of target bands. This led to a quantification of cleavage products in multi-turnover kinetic analysis. By graphing 1/V and 1/S following Lineweaver-Burke kinetics, parameters (V_{MAX}, K_M, and k_{cat}) of respective ribozyme was determined.

Ribozyme Cloning

To proceed to *in vitro* evaluation in cell culture of each chosen hammerhead ribozyme, ribozymes were cloned in the plasmid, pTRUF21-New Hairpin (called p21-NewHP in short), within HindIII and SpeI sites. The map of this plasmid is shown in Figure 2-4. All the ribozyme sequences are listed in Figure 2-5, and single stranded (sense and anti-sense) DNA oligoes (Invitrogen, Carlsbad, CA) were purified using 8% polyacrylamide gel and oligonucleotide bands were cut to elute DNAs in elution buffer (recipe of elution buffer is described in Appendix C). For each ribozyme, sense and anti-sense oligonucleotides were annealed, diluted and ligated in HindIII and SpeI (New England Biolabs, Ipswich, MA) digested p21-NewHP plasmid. SURE® Competent Cells for Unstable Clones (STRATAGENE, La Jolla, CA) were used for transformation of ligation products and plasmid DNA extracted from single colonies were sent for

sequencing (ICBR DNA sequencing core, University of Florida). Plasmids containing correct sequences of respective ribozymes were amplified and DNA extractions were conducted using CsCl gradient purification protocol or Maximum DNA Extraction Kit (Sigma, St. Louis, MO).

Results

Four HSV-1 essential genes (ICP4, ICP27, U_L20, and U_L30 genes) were chosen as targets of hammerhead ribozymes because of their important roles in HSV-1 lytic life cycle (e.g. ICP4 gene) or their low G+C base composition (ICP27, U_L20, and U_L30 genes) (Figure 2-2**)**. ICP4 gene and ICP27 gene (also called U_L54) are immediate early genes, U_L30 gene is an early gene, and U_L20 gene is a late gene shown. Their expression in HSV-1 lytic life cycle is shown in Figure 2-6. For each target gene, among all the potential candidates, at least two hammerhead ribozymes were designed and they were tested *in vitro* for their kinetic parameters using synthesized RNA oligonucleotides (12-nucleotide long target and 39- nucleotide ribozyme). One example of an *in vitro* study including time course cleavage and multiple-turnover analysis is shown in Figure 2-7. Ribozyme 885 targeting ICP4, which has reasonable catalytic activity, is the only functional ribozyme designed for ICP4, and it was cloned in p21NewHP for *in vitro* test (discussed in Chapter 3). Two ribozymes were designed targeting U_L20 gene: although $U_L20$rz-135 was predicted with ideal secondary structure, it has very low catalytic activity at 20mM $MgCl_2$ concentration, as shown by its k_{cat}/K_m ($0.1uM^{-1}$ min^{-1}) (Table 2-3). The second U_L20 ribozyme, $U_L20$rz-154, indicated excellent *in vitro* catalytic activity, with a k_{cat}/K_m of $15.9uM^{-1}$ min^{-1} at a low $MgCl_2$ concentration of 5mM. This ribozyme was tested *in vitro* and *in vivo* described in the Chapter 4 and Chapter 5. Two ribozymes were designed for U_L30 which encodes HSV-1 DNA polymerase. They all showed

reasonable catalytic activity: U_L30rz-933 has a k_{cat}/K_m of 3.6uM^{-1} min^{-1} at 20mM $MgCl_2$ concentration, and U_L30rz-1092 has a k_{cat}/K_m of 1.0uM^{-1} min^{-1} at 5mM $MgCl_2$. U_L30rz-993 was chosen for further test due to its cleavage site located closer to the beginning of the transcript, and it was tested *in vitro* as described in Chapter 4. Ribozyme-825 targeting U_L54 (ICP27 gene) was chosen for further study due to its high *in vitro* catalytic efficiency (a k_{cat}/K_m of 11.7uM^{-1} min^{-1} at 5mM $MgCl_2$), and the other ribozyme targeting U_L54 was discarded due to its low cleavage activity.

Discussions

To design hammerhead ribozymes for gene targeting, there are several criteria that need to be considered: the accessibility of the target sequence, cleavage sites and flanking sequences, and the secondary structure of designed ribozyme. Sequence-specific binding of hammerhead ribozyme to target RNA is the first step for efficient cleavage, thus a good estimate of the accessibility of target site is necessary. Experience with antisense-oligodeoxynucleotide (antisense-ODN) methods has been beneficial, and it has showed that the accessibility of the mRNA to oligonucleotides is restricted by the secondary structure of the mRNA. Although experimental approaches are more reliable in identifying oligonucleotide-accessible sites[95,151,250], computational methods using MFOLD software sometimes give reasonable prediction without time-consuming bench work and high cost. In this study, I eliminated a lot of candidate target genes based on their G+C composition. The rationale is that high level of G+C content very often gives complex tertiary structure which is inaccessible to ribozyme binding. The sequence requirement of the cleavage triplet is any triplet sequence of the NUH type (N: any nucleotide; H: A,U, or C); the catalytic efficiency of hammerhead ribozyme to different cleavage triplets decrease in the following order, GUC>CUC>UUC>GUU, AUA,

AUC>GUA, UUU, UUA, CUA>AUU, CUU.[318] After choosing the target, to decide the ribozyme design, the folding pattern of the ribozyme was estimated using MFOLD. A ribozyme with correct structure of hybridizing arms and helix II without disturbing the catalytic core was tested *in vitro*. There are no general rules for the optimal length of ribozyme hybridizing arm. However, *in vitro* study indicated that short arms, i.e., less than 7 base pairs in each binding sequence, can provide fast dissociation from the cleaved product therefore efficient multiple turnover catalysis.[351] In this study, the length of hybridizing arm is 5 base pairs at the 5' end and 6 at the 3' end.

In order to achieve a successful therapeutic effect using hammerhead ribozyme, target genes need to be carefully selected. To inhibit HSV-1 viral replication, the knock-down of target gene expression should have significant impact on viral life cycle, since HSV-1 genome contains a large number of non-essential genes that have minor influences in initiating and maintaining viral lytic infection *in vitro*. In this study, I chose target gene candidates that were known to be essential for HSV-1 lytic infection.

After designing a hammerhead ribozyme, determination of k_{cat}, K_m, particularly k_{cat}/K_m provide useful descriptions of how efficiently a ribozyme conducts the transesterification of phosphodiester bonds at different substrate concentrations *in vitro*. This may reflect the *in vivo* activity of the ribozyme in which mRNA substrate will exceed ribozyme concentration. However, *in vitro* kinetic studies do not necessarily represent the situation in cells and animals, because cellular proteins can influence RNA conformation and consequently ribozyme catalytic efficiency by forming complexes with ribozyme.[363] The strategy in this study is to clone selected ribozymes into plasmids and

viral vectors to test their biological effects *in vitro* and *in vivo*. This will be described in later chapters.

Table 2-1. Experiment design of in vitro multi-turnover analysis.

Tube(dupes)	water	400mM Tris HCL,pH7.4	Ribozyme	1:10 RNasin: 0.1M DTT	200mM $MgCl_2$	Target	Target solution used Molar ratio Rz:target
1,11	14	2	0	1	2	1	3pm/ul
2,12	10	2	1	1	2	4	3pm/ul 1:40
3,13	8	2	1	1	2	6	3pm/ul 1:60
4,14	6	2	1	1	2	8	3pm/ul 1:80
5,15	13	2	1	1	2	1	30pm/ul 1:100
6,16	12	2	1	1	2	2	30pm/ul 1:200
7,17	10	2	1	1	2	4	30pm/ul 1:400
8,18	8	2	1	1	2	6	30pm/ul 1:600
9,19	6	2	1	1	2	8	30pm/ul 1:800
10,20	4	2	1	1	2	10	30pm/ul 1:1000

All volumes are in microliters. Ribozyme concentration is 15nM.

Table 2-2. Preparation of calibration curve for multi-turnover kinetics analysis.

Tube (dupes)	water	microliters Target	Target solution used	pmole of target
1,13	100	0		0
2,14	99	1	0.3pm/microcliter	0.3
3,15	98	2	0.3pm/microcliter	0.6
4,16	96	4	0.3pm/microcliter	1.2
5,17	94	6	0.3pm/microcliter	1.8
6,18	92	8	0.3pm/microcliter	2.4
7,19	99	1	3 pm/microliter	3
8,20	98	2	3 pm/microliter	6
9,21	96	4	3 pm/microliter	12
10,22	94	6	3 pm/microliter	18
11,23	92	8	3 pm/microliter	24
12,24	90	10	3 pm/microliter	30

Table 2-3. Summary of *in vitro* kinetic analysis of all the hammerhead ribozymes designed against HSV-1.

Kinetic Properties Of Hammerhead Ribozymes With Synthetic HSV RNA Substrates					
HSV Target Gene	Mg^{+2} mM	k_{cat} (min^{-1})	K_m (uM)	k_{cat}/K_m (uM^{-1} min^{-1})	Development Status
ICP4-885	20	15.87	52.83	0.3	Ongoing
ICP4-533	5 & 20	NA	NA	NA	Discarded
U_L20-135	20	0.08	5.64	0.01	Discarded
U_L20-154	5	27.78	1.75	15.9	Ongoing
U_L30-933	20	9.26	2.57	3.6	Ongoing
U_L30-1092	5	22.99	23.59	1.0	Pending
U_L54-233	5	0.91	8.58	0.1	Discarded
U_L54-825	5	51.28	4.44	11.7	Ongoing

NA: No activity. Ribozymes that are labeled as ongoing were cloned in plasmid vector pTRUF21NewHairpin as well as packaged in an adenovirus vector for cell culture and *in vivo* studies; the ones labeled as "Pending" will be used as an alternative for future study.

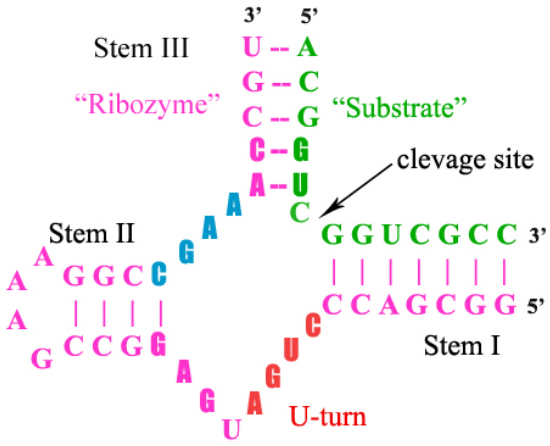


Figure 2-1. Structure of a hammerhead ribozyme. Substrate binding domains of the hammerhead ribozyme bind to target sequence to form Helix I and III (stem I and III), and the length of each hybridizing arm may varies without affecting cleavage efficiency. The catalytic core, the loop area, which is highly conservative, is essential for ribozyme activity (modified from http://www.rwg-bayreuth.de/chemie/chime/rna/frames/hambtx.htm).

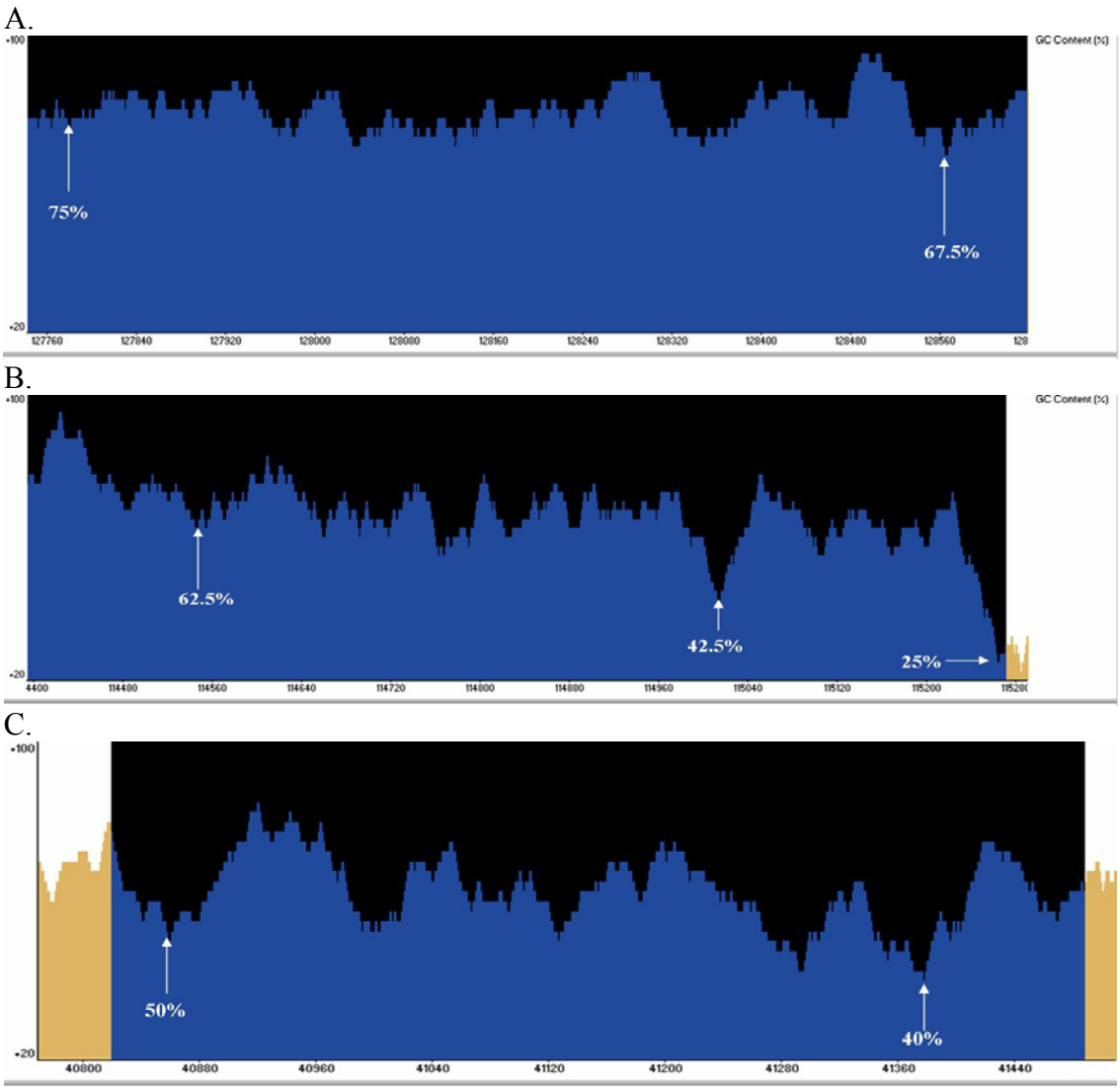


Figure 2-2. The composition of G+C in HSV-1 genes using Vector NTI. Blue area indicates the percentage of G+C content in each sequence investigated; yellow area is the gene sequence flanking the gene of interest; the scale of Y axis in each panel is 100% maximum, and 20% minimum in the composition of G+C; X axis represent the base number of each sequence in HSV-1 genome. Figure A shows a representative sequence from ICP4 gene coding sequence, in which high G+C composition is generally observed, and sequences contain relatively low G+C are labeled as 75% and 67.5% respectively. B: a representative sequence from ICP27 gene coding sequence; C: coding sequence of U_L20 gene; D: a representative sequence from U_L30 gene coding sequence.

D.

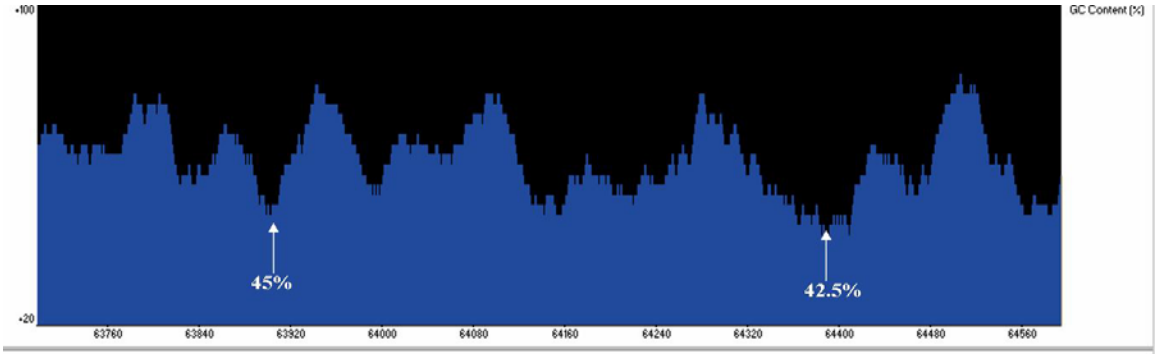


Figure 2-2. (continued.)

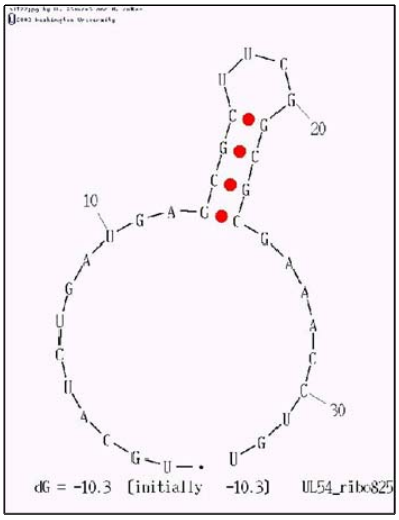


Figure 2-3. Predicted folding pattern for ribozyme U_L54-825 using MFOLD.

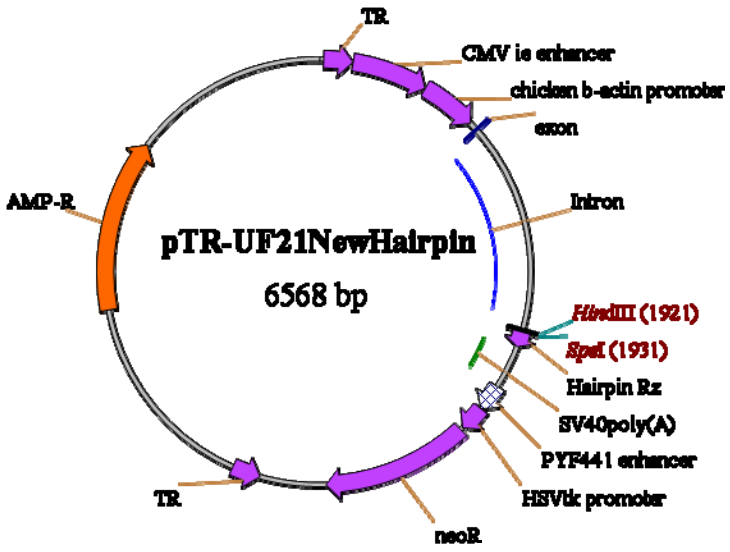


Figure 2-4. The map of plasmid pTR-UF21-NewHairpin for ribozyme cloning.

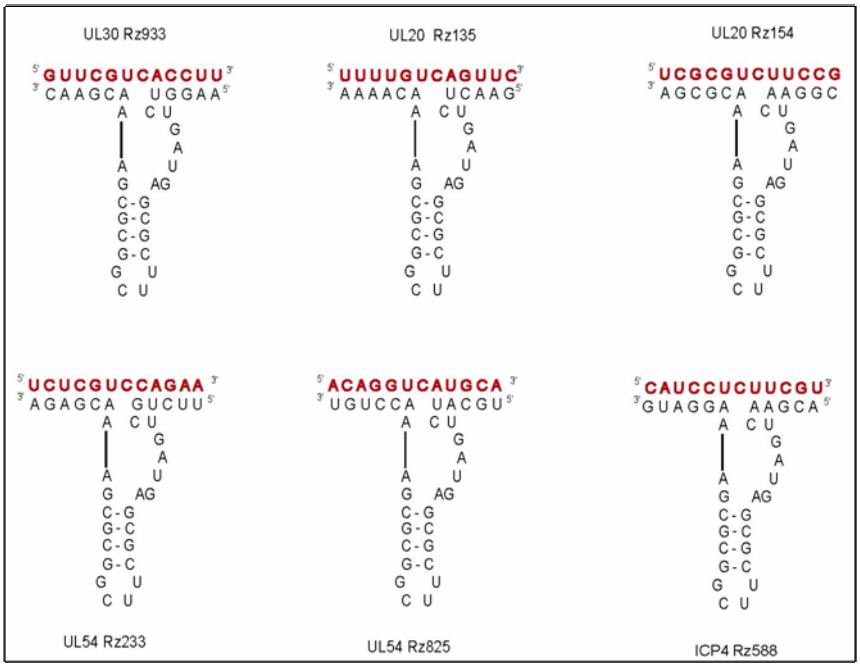


Figure 2-5. Ribozyme sequences and their respective target sequences.

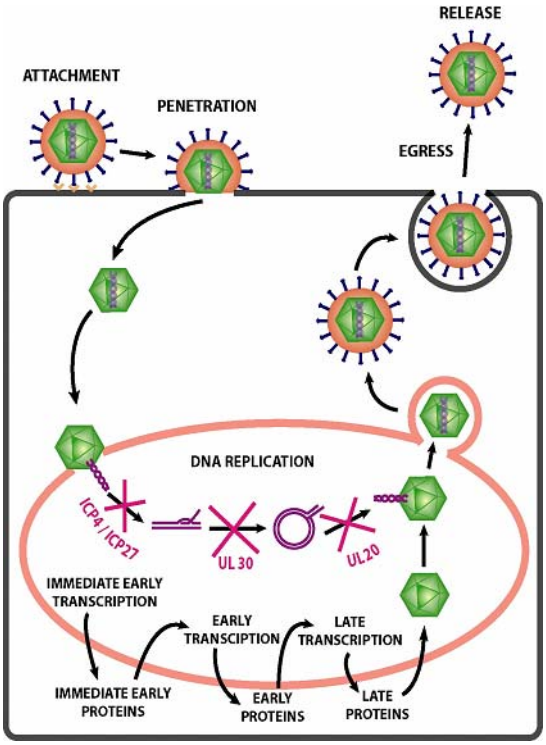
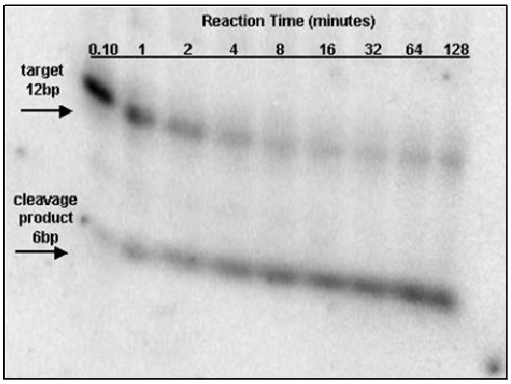
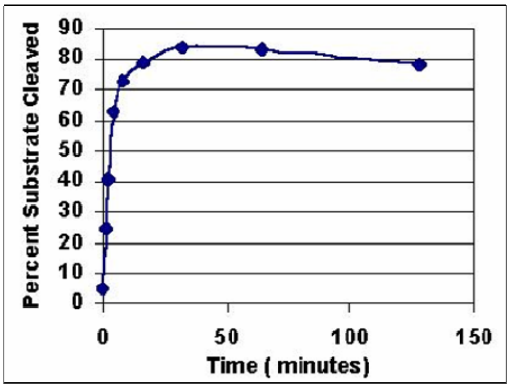


Figure 2-6. Gene targets for hammerhead ribozymes in HSV-1 lytic life cycle. Four HSV-1 essential genes were chosen as targets of hammerhead ribozymes. ICP4 and ICP27 genes are immediate early genes; they have been suggested to be essential to HSV-1 lytic infection *in vitro*, especially ICP4 which is a major transcriptional regulator to basically all the HSV-1 genes. U_L30 gene is an essential early gene which encodes the viral DNA polymerase, and U_L20 gene is a late essential gene. By knocking down the expression of these HSV-1 essential genes, it is expected that a corresponding event (immediate early transcription, early, or late transcription) can be stoped leading to an inhibition viral infection.

A.



B.



C.

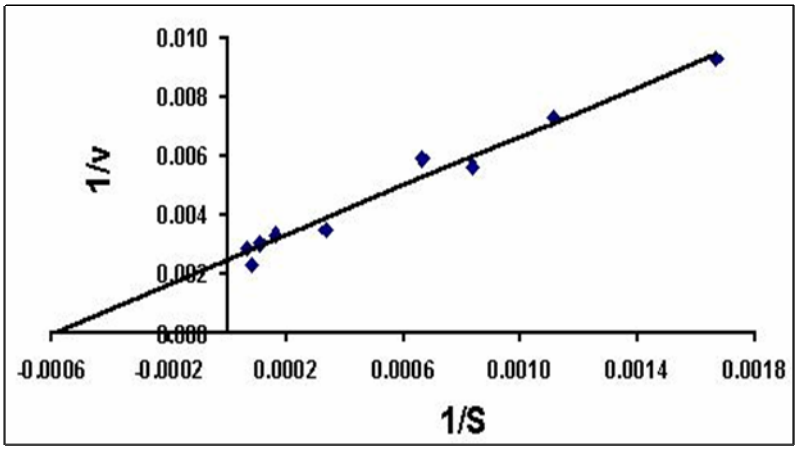


Figure 2-7. *In vitro* kinetic study of hammerhead ribozyme U_L20-154. A) Autoradiogram of the time course of cleavage of an RNA target (end labeled with γ-^{32}P-ATP) by ribozyme U_L20-154 at a magnesium concentration of 5mM. B) The percentage of target RNA cleavage in each time point can be calculated from quantification of cut and uncut target bands in Figure 2-7-A. C) Lineweaver-Burke Plot of Ribozyme U_L20-154 Cleavage of Synthetic HSV RNA Target. Least squares regression analysis generated a best fit line $y = 4.213x + 0.0024$ with correlation coefficient $R^2 = 0.978$. After setting up multiple-turnover analysis of U_L20-154 ribozyme in 5mM Mg^{2+} concentration, the quantitation data was fit in the Lineweaver-Burke plot.

CHAPTER 3
STUDIES OF RNA GENE THERAPY TARGETING ICP4 MRNA OF HERPES SIMPLEX VIRUS

Introduction

Genes of herpes simplex virus (HSV) can be categorized into three kinetic classes: immediate-early (IE or α), early (E or β), and late (L or γ) genes.[155] During the lytic infection, HSV gene product synthesis is regulated in a highly organized cascade manner. Genes from each class contain different components of regulatory elements which define the dynamics of its transcription by cellular RNA polymerase II (pol II) transcriptional machinery.[4,74] The complexity of promoter structures of genes from each class decreases from IE to E to L.[375,384] Five immediate early (IE) genes, ICP4, ICP0, ICP22, ICP27, and ICP47, constitute the first set of genes to be transcribed upon HSV-1 infection and are maximally expressed at approximately 2-4 hours post-infection.[155] These IE genes are expressed with the help of VP16[21,45], a viral transactivator which is contained in the tegument. VP16 associates with cellular Oct-1 and host cell factor (HCF) to bind TAATGARAT elements (where R represents A or G) which are found exclusively in IE gene promoters to activate transcription from them.[110,268] SP1 sites as well as other sites for binding of cellular *cis*-acting factors also contribute to the enhanced transcription of viral IE genes.[114] As a key transcriptional regulator, ICP4 gene of HSV is essential for the expression of virtually all the genes of viral productive life cycle.[187,382]

As an immediate early gene, ICP4 is expressed about 2-4 hours post-infection in the absence of other *de novo* synthesized viral proteins.[299] The same as other α genes,

ICP4 promoter contains consensus sequence 5'-GyATGnTAATGArATTCyTTGnGGG-3' upstream of the cap site[226-228] which binds Oct-1. By binding to a complex of the viral proteins VP16, HCF, cellular Oct-1, and other transcriptional factors, the consensus sequence acts as a response element to promote the expression of α genes.[192-195,246] ICP4 is a large and structurally complex protein: its mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) responds to a molecular weight of 175KDa[75] and it exists in the cells as a homodimer with a strokes radius of 89Å.[242,317] Considering its hydrodynamic properties, this elongated protein can bind to DNA and function as a transactivator of transcription over a long distance. However, ICP4 does not require specific DNA binding sites for its activation, it can activate transcription from a variety of promoters. Of all the α gene products, ICP4 protein, functioning in a poly(ADP- ribosyl)ated form, is absolutely essential for β and γ gene expression beyond α phase of a lytic infection.[72,87,88,91,101] As a transactivator, ICP4 increases the rate of transcription complex assembly on promoters.[126] ICP4 protein also down-regulates α gene expression, including its own, by binding to cognate DNA binding sites located across the transcription initiation sites and interacting with basal transcriptional factors.[128,198]

ICP4 protein functions by interacting with basal transcriptional machinery of RNA polymerase II (RNA Pol II). In the eukaryotic system, structural gene transcription requires the assembly of pre-initiation complex on the core promoter including RNA Pol II and general transcription factors (GTFs) (TFII A, B, D, E, F, H). Although there are different element requirements for a full activity of HSV early and late gene promoters, interactions of TATA box and GTFs are essential for initiating transcription of both

kinetic classes of genes. Binding of Transcription Factor II D (TFIID) to the TATA box via TATA-box binding protein (TBP) is critical for pre-initiation complex assembly. However, efficient responses to cellular and viral trans-activators (SP1 and ICP4) require TBP-associated factors (TAFs). Their interactions with each other, with other GTFs, and with specific DNA sequences (e.g., the initiator element which overlaps the transcription sites) contribute to promoter selectivity.[174] It was suggested that ICP4 interacted with TAF250 of TFIID via its C-terminal domain.[51]

Herpes simplex virus early and late genes have distinct promoter structures which have different requirements in terms of ICP4-specific transcription activation. A study using non-fusion forms of ICP4 linked to either an early gene (*tk*) promoter or a late gene (gD) promoter revealed that ICP4 residues 97 to 109 are required for induction of gD promoter but not for *tk* promoter.[397] It has been suggested that GTF TFIIA is essential for ICP4 activation of HSV early gene transcription but is not required for late gene transcription[402], indicating the elegant regulation of HSV gene expression cascade through ICP4.

Because of the critical role in HSV lytic infection, ICP4 has attracted significant attention as a target for antiviral therapy. Antisense oligonucleotides were explored in cell culture for antiviral effect by targeting the acceptor splice junction of ICP4 pre-mRNA.[176,325] Although they were also tested in BALB/c mice and showed certain inhibitory effect[199], the delivery approach and survival rate of those antisense oligonucleotides were limiting factors for antiviral therapy application. A chemical that can block Sp1 binding (e.g., tetramethyl-O-NGDA (M_4N), a synthetic derivative of the naturally occurring nordihydroguaiaretic acid (NDGA)), which consequently interrupts

ICP4 expression, was demonstrated for its antiviral effect but with limited therapeutic effects.[56] A ribozyme derived from *Escherichia coli* (*E.coli*) RNase P was engineered targeting HSV-1 ICP4 mRNA and *in vitro* it significantly reduced ICP4 expression with certain inhibitory effect against viral replication in cell culture.[355,358] Zinc finger proteins[274] (engineered three or six-finger protein) are very potent suppressors for initiation of transcription. Recently, they have been designed and tested *in vitro* against ICP4 gene promoter. These zinc finger proteins led to certain levels of reduction of ICP4 expression and early/late gene expression level.[274] It was suggested from these studies that targeting only the ICP4 gene might not provide significant effect in inhibiting viral replication. In summary, in these studies *in vitro* systems that were not permissive for HSV-1 viral replication were used to test these antiviral reagents. None of the *in vivo* data obtained indicated a therapeutic effect by knocking down ICP4 expression. No delivery method was suggested or tested for gene therapy purposes. They also suggested that a threshold level of ICP4 gene expression, which may be very low, can provide sufficient function for viral growth. Therefore, it might be very difficult to significantly knock-down ICP4 level to affect HSV-1 lytic infection. However, a therapeutic effect may be achieved from a synergistic effect by targeting multiple targets including ICP4 gene.

In this study, I designed and tested hammerhead ribozymes targeting ICP4 mRNA of HSV-1. These studies were conducted in a permissive *in vitro* system for HSV-1 infection using HSV-1 strains with high infectivity. The application of using siRNA for anti-HSV-2 effect was also explored by targeting ICP4 mRNA of HSV-2.

Materials and Methods

In Vitro Test of Hammerhead Ribozyme ICP4-885 Targeting ICP4 mRNA of HSV-1

Ribozyme ICP4-885 and other ribozymes (mentioned in Chapter 2) were cloned into a plasmid called pTRUF21-New Hairpin between restriction sites of HindIII and SpeI following protocol of ribozyme cloning (Chapter 2) and plasmid construct containing ICP4 ribozyme is called pTR21NewHP-ICP4rz-885 (abbreviation as p21-ICP4rz). The sequences of all the ribozymes and their respective targets are shown in Table 3-1.

Transient transfection of E5 cells with ribozyme ICP4-885 to detect ICP4 mRNA Level

The E5 cell line, African green monkey kidney cell which was constructed to express the ICP4 gene, was used for this study (a generous gift of Dr. Priscilla Schaffer). A transient transfection of pTR-UF11 (GFP containing plasmid, map see Figure 3-1) was conducted using Lipofectamine 2000^{TM} (Invitrogen, Carlsbad, CA) at various ratios of plasmid DNA amount (μg) to Lipofectamine 2000^{TM} reagent (μL) and following the manual of Lipofectamin 2000^{TM}. Ratios of DNA to Lipofectamine 2000^{TM} reagent were: 4μg to 4μL, 4μg to 8μL, 4μg to 12μL, 5μg to 10μL, and 5μg to 15μL. At one day post-transfection, cells were examined for their GFP expression level by fluorescence microscopic observation as well as flow cytometry analysis (FACScan, BD Biosciences, San Jose, CA) to determine the transfection efficiency. The optimal transfection condition was used to conduct further tests. Each well of a 6-well-plate was seeded with $3x10^5$ cells one day before transfection, and for each group, the transfection was conducted in triplicate. There were four groups in this test: mock transfection, pTRUF21 transfection, pTRUF21-ICP4rz, and pTRUF11 (GFP containing plasmid). At 48 hours

post-transfection, two wells of GFP-transfected cells and a well of mock transfected cells were analyzed by flow cytometry analysis to detect transfection efficiency; the remaining cells were harvested using TRIZOL® Reagent (Invitrogen, Carlsbad, CA). Total RNA extraction was performed following TRIZOL® protocol and DNA-free™ (Ambion, Austin, TX) was used to remove DNA contamination. Total RNAs were inspected via the spectrometry (Gene Spec III, MiraiBio Division, Alameda, CA) at a wavelength of 260nm and the quality of RNA was assessed using a ratio of the absorption at 260nm divided by that at 280nm ranging from 1.8 to 2.0. Reverse transcription was conducted using First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) with 1μg total RNA in each reaction. Conventional PCR was conducted using cDNA (1/5 of total reverse transcription reaction for each PCR). HotStarTaq DNA polymerase (QIAGEN, Valencia,CA) was used in PCR at 95°C for 15 minutes (1 cycle); 94°C for 3 minutes, 55°C for 3 minutes, 72°C for 3 minutes (1 cycle); 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute (30 cycles); 72°C for 10 minutes. PCR products were separated on 8% acrylamide gel and stained with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, OR). Images were obtained using Storm Phosphorimager (GE Healthcare, Piscataway, NJ) and quantification was conducted using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Construction of a stable cell line expressing ribozyme ICP4-885

RS cells (rabbit skin cells), maintained in Eagle's minimal essential medium (MEM, Life Technologies) supplemented with 5% calf serum, 250U of penicillin/mL, 250μg of streptomycin/mL, and 292μg of L-glutamine/mL (Life Technologies), were used to construct the stable cell line expressing ribozyme ICP4-885. Each well of a 24-well-plate was seeded with $8x10^4$ of RS cells the day before transfection; Lipofectamine™ and

Plus™ reagents (Invitrogen, Carlsbad, CA) were used for transfection using the recommended conditions (DNA: Plus™: Lipofectamine™ of 0.8μg: 1μL: 3μL). On the second day of the transfection, transfected cells were diluted 5-10 fold and selected in medium containing G418 disulfate (Research Products International Corp., Mt. Prospect, Illinois). The concentration of G418 disulfate began at 600μg/mL and was gradually reduced to 500μg/mL, 400μg/mL, 300μg/mL, and eventually 250μg/mL. After 3 weeks of selection, single colonies were picked to grow in 96-well-plates, and then amplified in 24-well-plates, 6-well-plates and finally $10cm^2$ dishes. Ribozyme expression levels of all the colonies were compared using reverse transcription of total RNA harvested from the same amount of cells followed by conventional PCR. PCR was conducted with an addition of radioactive α^{32}P-dATP (MP Biomedicals, Irvine, CA), and PCR products amplified by ICP4 primers as well as β-actin primers (Table 3-2) were detected on 8% acrylamide gels. Dried gels were exposed overnight in a storage phosphor screen cassette and scanned in Storm Phosphorimager (GE Healthcare, Piscataway, NJ) to detect the radioactive labeled PCR product. ImageQuant™ software (GE Healthcare, Piscataway, NJ) was used to quantify the intensity of PCR product. The colony with highest ratio of ribozyme level to β-actin level was selected to test against HSV-1 infection.

Herpes simplex virus type 1 infection

17*syn*+ (considered a wild-type HSV-1 strain) was used to conduct infection. A series of dilutions of HSV-1 viral stock were prepared in Eagle's minimal essential medium containing 5% calf serum, 250U of penicillin/mL, 250μg of streptomycin/mL, and 292μg of L-glutamine/mL (Life Technologies, Inc., Gaithersburg, MD). One hour incubation at 37°C in 5% CO_2 was allowed for the virus to absorb in a minimal amount (200μL) of medium covered on a monolayer of cells. Infection medium was replaced

with regular serum-containing medium after the incubation. Different times of incubations were allowed before cells were harvested or stained with dye (plaque reduction assay).

Herpes simplex virus type 1 viral stock preparation

The virus was amplified and titrated on rabbit skin cells by using Eagle's minimal essential medium (Invitrogen-Life Technologies, Carlsbad, CA.) supplemented with 5% calf serum (Life Technologies, Inc., Gaithersburg, MD), 292 µg of L-glutamine/ml, and antibiotics (250 U of penicillin/ml and 250 µg of streptomycin/ml). The infection of a monolayer RS cells at an MOI of 10^{-2} was performed when the cells reached 80% confluency. Complete cytopathic effect (CPE) was observed before cells and medium were harvested to pellet the cells at 10,000xg at 4°C in a Sorvall™ GSA rotor (Thermo Electron Corporation, Asheville, NC) for 40 minutes. The cell pellet was resuspended in MEM complete medium containing 5% calf serum and frozen-thawed twice using a -80°C freezer and a 37°C water-bath before the cell lysate was distributed in aliquots. Virus stocks were maintained in 20-100µL aliquots (depending on the purpose) using 2.0mL screw-cap tubes and stored in -80°C freezer. One vial of viral stock was thawed out and titrated before use in animals or cell cultures.

Plaque reduction assay to determine viral titer

RS cells were used for plaque reduction assay (PRA), seeding $1x10^5$ cells per well in each 24-well-plate. 10µL of viral stock was resuspended in 990µL of MEM to make 10^{-2} dilution of infection solution, and from 10^{-2} dilution 1mL of each 10^{-3} to 10^{-9} dilutions were made. For each dilution, infection was conducted in triplicate and 200µL of each dilution were added to each well of cells. One hour incubation was allowed for viral attachment and viral entry. Cells were rinsed by PBS then covered by 2mL of

regular medium containing 0.3% human IgG (Purified Immunoglobulin Technical Grade) (Sigma, St. Louis, MO). For 17syn+ strains, 2 days were required for plaques to develop and for KOS strains plaques show in 3 days.

Transient transfection of pTRUF21-New Hairpin containing ribozyme ICP4-885

E5 cells were seeded in 3.5cm dishes at a density of $2x10^5$ cells per plate one day before transfection. Three groups of transfections were included: mock transfection (MT), control plasmid transfection using pTRUF21NewHairpin (Con), and ribozyme transfection using pTRUF21NewHairpin-ICP4rz-885 (ICP4rz). Transfection of each group was conducted in triplicate using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) at a DNA to Lipofectamine 2000™ ratio of 10μg to 10μL. The transfection procedure followed Invitrogen Lipofectamine 2000™ protocol. E5 cells were maintained in Eagle's minimal essential medium (MEM, Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, GIBCO/ Invitrogen, Carlsbad, CA), 250U of penicillin/mL, 250μg of streptomycin/mL, and 292μg of L-glutamine/mL (Life Technologies, Inc., Gaithersburg, MD). Two days after transfection, E5 cells were infected with KD6 (ICP4 defective HSV-1 strain)[92] at an MOI of 3 for 24 hours before cell lysates were harvested for plaque reduction assay.

***In Vitro* Test of a siRNA ICP4-19 Targeting ICP4 mRNA of Herpes Simplex Virus Type 2**

siRNA ICP4-19 was originally designed by Suresha Rajiguru, a Master student at the University of Florida. The siRNA duplex sequences as well as the target sequence are shown in Table 3-3. HeLa cells were cultured in 10%FBS containing Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 250U of penicillin/mL, 250μg of streptomycin/mL (Life

Technologies, Inc., Gaithersburg, MD). Transfection of siRNA duplex was conducted using Oligofectamine™ Transfection Reagent (Invitrogen, Carlsbad, CA). A scrambled siRNA, kindly provided by Dr. Marina Gorbatyuk, served as the transfection control. Each well of the 12-well-plate was seeded with $1x10^5$ cells one day before the transfection. Transfection was conducted in the presence of serum but no serum was added until duplex-oligofectamine complex formed. OPTI-MEM® I Reduced Serum Medium (GIBCO®, Invitrogen Corporation, Carlsbad, CA) was used during transfection process. 100pmole of siRNA duplex and 2μL of oligofectamine reagent were used for transfecting each well of cells. A four-hour incubation was allowed while in the presence of serum for transfection and the transfection medium was replaced by 10%FBS containing DMEM supplemented with 250U of penicillin/mL, 250μg of streptomycin/mL. After the overnight culture, cells were tested for transgene function.

Infection using HSV-2 (strain HG52) was conducted at an MOI of 3 after transfection of HeLa cells with siRNA duplexes. To evaluate the siRNA effect on HSV-2 ICP4 gene expression level, reverse transcriptions (RT) followed by real-time PCR was conducted to detect the ICP4 expression. Copy DNA (cDNA) from each RT- reaction was diluted 10-fold before the real time PCR assay. Specific primers and a fluorescent probe for either ICP4 (sequences are shown in Appendix B) or RNase P (sequences of primers and probe are not available) were designed and synthesized by ABI system (Applied Biosystems, Foster City, CA) (Assays by Design part no. 4331348) with concentrations recommended by the supplier. Real-time PCR was performed using TaqMan Universal PCR Master Mix, No AmpErase uracil N-glycolase (Applied Biosystems, Foster City, CA). All real-time PCR reactions were performed and analyzed

using ABI Prism 7700 or 7900 sequence detection systems (Applied Biosystems) (ICBR Protein Chemistry Core Facility, University of Florida). Cycle conditions used were as follows: 50°C for 2 min (1 cycle); 95°C for 10 min (1 cycle); and then 95°C for 15 s followed by 60°C for 1 min (45 cycles). Threshold values used for PCR analysis were set within the linear range of PCR target amplification.

Results

Ribozyme ICP4-885 *In Vitro* Test against HSV-1 Target

Effect of transient transfection of ribozyme ICP4-885 to ICP4 expression level in E5 cells

Transient transfection of ribozyme ICP4-885 in E5 cells caused significant reduction in ICP4 expression levels (Figure 3-2A**)**. A semi-quantitative reverse-transcription PCR was conducted to compare ICP4 mRNA level after ribozyme treatment. As shown in Figure 3-2B, ribozyme ICP4 885 reduced the level of ICP4 expression by 42% (compared with a control transfected group). However, the difference in ICP4 levels between ribozyme and control groups of E5 cells was not statistically significant. This is probably because ICP4 expression levels in the cell are already extremely low without HSV-1 infection, since the cell line was constructed to express ICP4 from the original viral promoter.

Transient transfection of pTRUF21-New Hairpin containing ribozyme ICP4-885 in E5 cell line to test against KD6 (ICP4- HSV-1) viral replication

To further investigate ribozyme effect on ICP4 expression level, the ribozyme ICP4-885 was used to transfect E5 cells followed by KD6 infection at an MOI of 3. The rationale for this experiment was that KD6 viral infection is turned on by constitutive expression of ICP4 provided by E5 cells, so the reduction of ICP4 expression will be indicated by a lower level of infectious viral particles in the ribozyme treatment group

than those in control groups. However, transfection efficiency in E5 cells was very low (7% in the optimal condition) and transfected cells could not be enriched by antibiotic selection. (E5 cells were constructed using neomycin resistant gene as selection marker which is the same as ribozyme expressing plasmid.) Although it did not reach statistical significance, there was a mild reduction (20%) of viral yield in the ribozyme treatment group as shown in Figure 3-3.

Cell Line stably expressing ribozyme ICP4-885 tested against wild-type herpes simplex virus type 1 (17*syn*+)

RS cells were stably transfected with ribozyme ICP4-885 and one single colony with highest ribozyme expression level was selected. In Figure 3-4-A, an example of ribozyme expression is shown. Cells from this colony were used to test against wild-type HSV-1 (17*syn*+) infection at an MOI of 10^{-3}. At different time points, cell lysates were used to conduct plaque reduction assay to observe ribozyme effect on multiple rounds of viral replication. A separate group of cells were stained with crystal violet at each time point to observe the plaque forming phenotypes. At an early time point (24 hours post-infection), a significant reduction of viral production level (88%) was observed in ribozyme expressing cells by plaque reduction assay (data not shown). At three days post-infection, significantly reduced plaque production as well as smaller plaque size was observed when cells were stained with crystal violet as shown in Figure 3-4-B. However, when plaque reduction assay was employed to quantify viral yields from cells paralleled to those from Figure 3-4-B, no difference was observed between control cells and ribozyme expressing cells.

Transient Transfection of siRNA Targeting ICP4 mRNA of Herpes Simplex Virus Type 2 in HeLa Cells

An siRNA designed targeting HSV-2 ICP4 mRNA and transfection controls were used to transiently transfect HeLa cells followed by wild-type HSV-2 (strain HG52) infection at an MOI of 10^{-3}. Viral replications at a series of time points (15, 24, 50, and 75 hours post-infection) were compared using a plaque reduction assay to estimate the siRNA effect. Compared with control siRNA treatment, transfection of siRNA-19 significantly reduced HSV-2 viral yield by 63%, 63%, 70%, and 49% respectively at 15, 24, 50, and 75 hours post-infection. However, when HSV-2 ICP4 mRNA level was compared among three groups using reverse transcription and real-time PCR, there was no significant difference observed (data not shown) among three groups (Mock transfection, control siRNA, and siRNA-19 transfected groups). ICP4 mRNA was observed following a very high level of infection (MOI of 3), while siRNA-19 transfection reduced HSV-2 yields at a multiplicity of infection 3000 times lower (an MOI of 10^{-3}).

Conclusions and Discussion

Although HSV-1 and 2 both belong to alpha-herpes family, they are different in a lot of aspects, indicating the difference in virion release. However, the ICP4 gene product for both HSV type 1 and 2 shares not only sequence but functional similarity. They are immediate early genes and function to initiate downstream events. ICP4 has been a very popular target for gene knockdown in the past, but no success was observed from the therapeutic aspect. In this study, ribozyme and siRNA targeting ICP4 were used against wild-type HSVs under rigorous high multiplicity infection conditions that is more extreme than those conditions used in previous studies in the literature in order to select

for candidates for therapeutic purposes. It has been suggested that HSV requires an extremely low threshold level of ICP4 gene product to initiate lytic infection.[3] Therefore, it could be very difficult to block viral replication by reducing expression of this protein. After scanning all the possible cleavage sites in ICP4 mRNA of HSV-1, one hammerhead ribozyme with good kinetic parameters was chosen to test in tissue culture. Although this ribozyme significantly reduced ICP4 gene expression in an ICP4 expressing cell line, when tested against HSV-1 viral replication (either wild-type HSV-1 or ICP4 defective virus in permissive cell line), it did not block infectious viral particle production to a statistically significant level. However, this ribozyme caused some reduction at the very early stage of HSV-1 replication as shown in the ribozyme expressing cells which had the phenotype of smaller plaque size and fewer plaques than control cells infected by HSV-1 (shown in Figure 3-3B**)**. At the later time point, this effect was overcome by active viral replication induced by the accumulation of ICP4. This may explain the phenomenon that no difference was observed in the infectious viral particle production level between control and ribozyme expressing cells.

RNA interference (RNAi) is a conserved biologic response to double-stranded RNA that results in the sequence-specific silencing of target gene expression. Although the siRNA designed against HSV-2 ICP4 mRNA was able to delay viral replication and reduce infectious particle production level, it did not reduce the ICP4 mRNA level implying a complex effect caused by siRNA-19 in the cells: The high level of viral infection might overwhelm the siRNA effect by providing high level of ICP4 expression which implied the limitation of this siRNA effect. On the other hand, siRNA-19 might also function as microRNA targeting either ICP4 mRNA or other gene transcripts,

causing a reduction in viral yield but not leading to a dramatic change in RNA level. The inhibition of viral replication may be both specific and non-specific.

In conclusion, because of the important role of ICP4 in HSV lytic infection life cycle, it is a good target for inhibiting HSV infection if a significant reduction of ICP4 mRNA can be achieved. However, considering that the functional threshold level of ICP4 is extremely low, ICP4 gene by itself might not be an ideal target to eliminate HSV-1 infection. It can be expected that a synergistic effect can be achieved by combining ribozymes/siRNAs targeting other essential genes in addition to ICP4.

Table 3-1. Ribozyme sequences and sequences of their respective targets.

Ribozyme Label	Ribozyme Sequence	Respective Target Sequence
ICP4-885	acgaactgatgagcgcttcggcgcgaaaggatg	catcctcttcgt
ICP4-533	tcgatctgatgagcgcttcggcgcgaaacgccg	cggcgtcatcga
U_L20-135	gaactctgatgagcgcttcggcgcgaaacaaaa	ttttgtcagttc
U_L20-154	cggaactcatgagcgcttcggcgcgaaacgcga	tcgcgtcttccg
U_L30-933	aaggtctgatgagcgcttcggcgcgaaacgaac	gttcgtcacctt
U_L30-1092	cacatctgatgagcgcttcggcgcgaaagcttg	caagctcatgtg
U_L54-233	ttctgctgatgagcgcttcggcgcgaaacgaga	tctcgtccagaa
U_L54-825	tgcatctgatgagcgcttcggcgcgaaacctgt	acaggtcatgca

Table 3-2. Conventional PCR primers.

Primer Label	Primer Sequence
HSV ICP4 sense	5'-CTGATCACGCGGCTGCTGTACACC- 3'
HSV ICP4 anti-sense	5'-GGTGATGAAGGAGCTGCTGTTGCG-3'
Rabbit β-actin sense	5'- AAG ATC TGG CAC CAC ACC TT- 3'
Rabbit β-actin anti-sense	5'- CGA ACA TGA TCT GGG TCA TC- 3'

Table 3-3. siRNA duplex sequences and target sequences.

Name	Sequence
siRNA ICP4-19 Target Sequence	5'- AAGAAGAAGAAGACGACGACG-3'
siRNA ICP4-19 Duplex Sequence	5'- GAAGAAGAAGACGACGACGUU-3' 3'- UUCUUCUUCUUCUGCUGCUGC-5'
Scramble siRNA Target Sequence	CUUCCUCACGCUCUACGUC
Scramble siRNA Duplex Sequence	5'-AACUUCCUCACGCUCUACGUC-3' 3'-GAAGGAGUGCGAGAUGCAGUU-5'

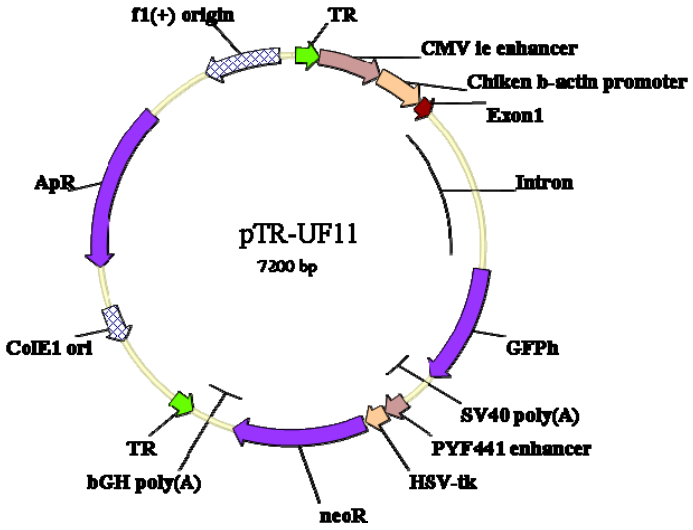
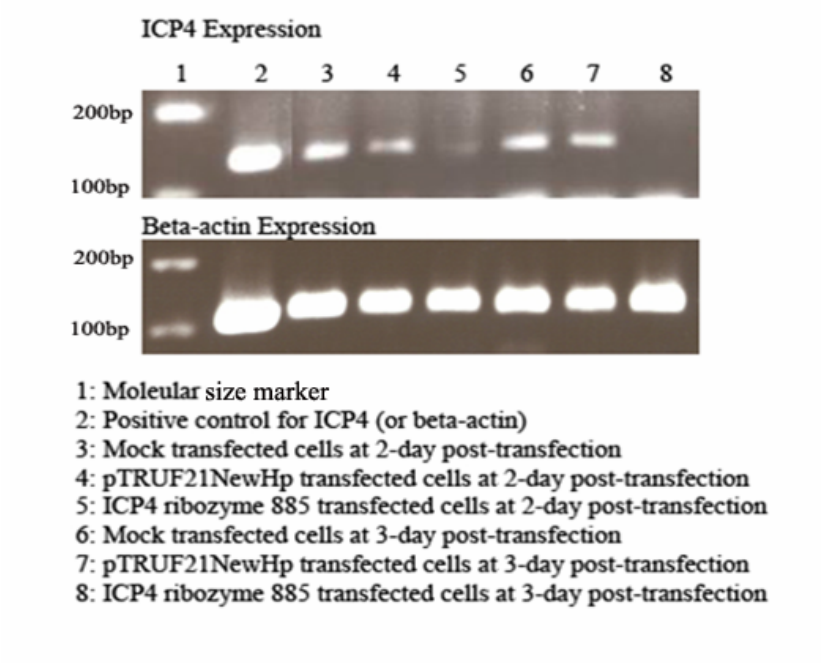


Figure 3-1. Map of plasmid pTR-UF11 generated by Vector NTI.

A.



B.

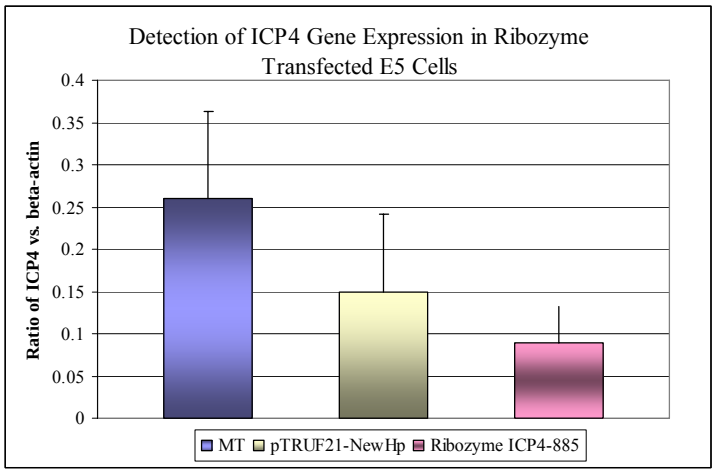


Figure 3-2. Reduction of ICP4 expression level in E5 cells by transient Transfection with ICP4rz-885. A) PCR amplification of reverse-transcribed ICP4 RNA isolated from E5 cells separated on 1.5% agarose gel. Transient transfection of the plasmid containing ICP4rz-855 as well as controls (mock transfection and transfection of plasmid without the ribozyme) was conducted, and total RNAs were harvested at day 2 and day 3 post-transfection for reverse-transcription and PCR. Primers for ICP4 and β-actin were used for PCR. B) Quantification of the PCR product amplified from cDNAs resulted from ICP4 ribozyme treated and control treated E5 cells. Total RNA was harvested from E5 cells treated with ribozyme ICP4-885 or with control treatments at the time point of 2 day post-infection of HSV-1. Reverse-transcription followed by PCR was conducted, and PCR products were separated on 8% acrylamide gel and stained by SYBR® Green nucleic acid dye for quantifications.

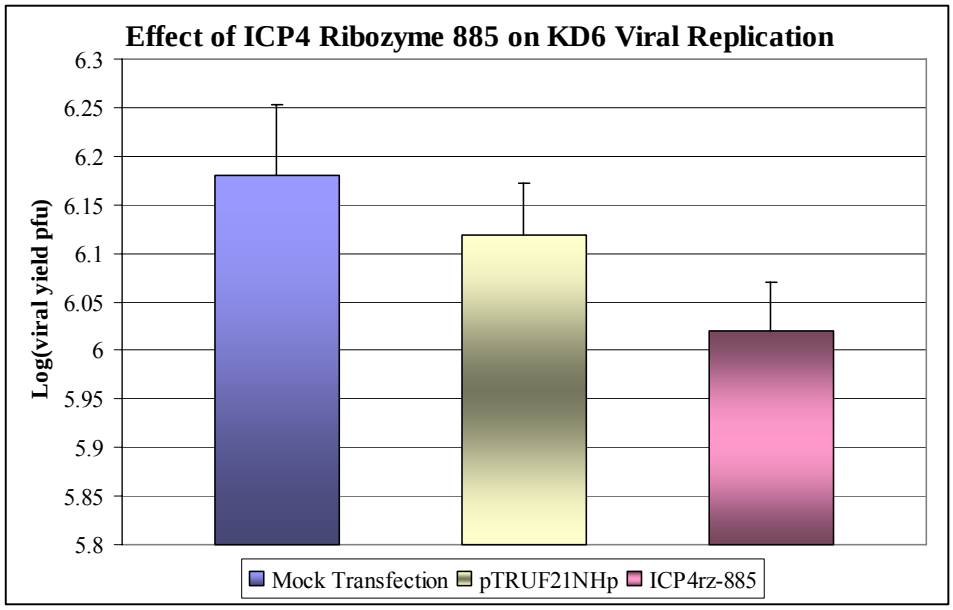
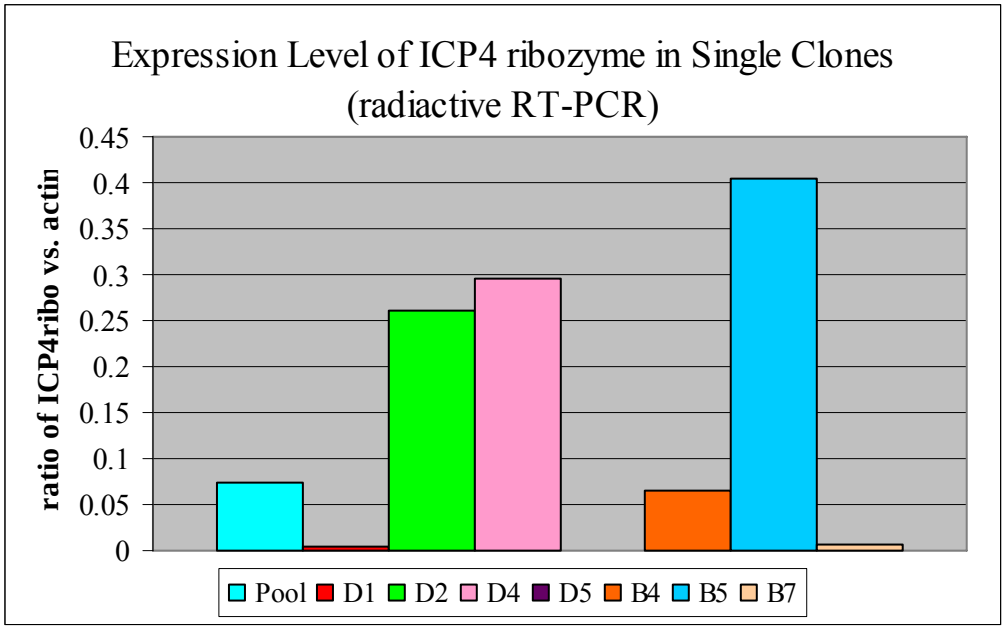


Figure 3-3. Effect of ribozyme ICP4-885 on KD6 viral replication in E5 cell line. E5 cells, constructed to express ICP4 constitutively, were transfected with a plasmid expressing ribozyme ICP-885 followed by infection of HSV-1 strain KD6 which is non-replicating HSV-1 with ICP4 deletion. Mock transfection and transfection using plasmid without ribozyme were used as controls. In this experiment an MOI of 3 was used for KD6 infection. Twenty four hours after HSV-1 infection, cell lysates were harvested for plaque reduction assay on RS cells.

A



B

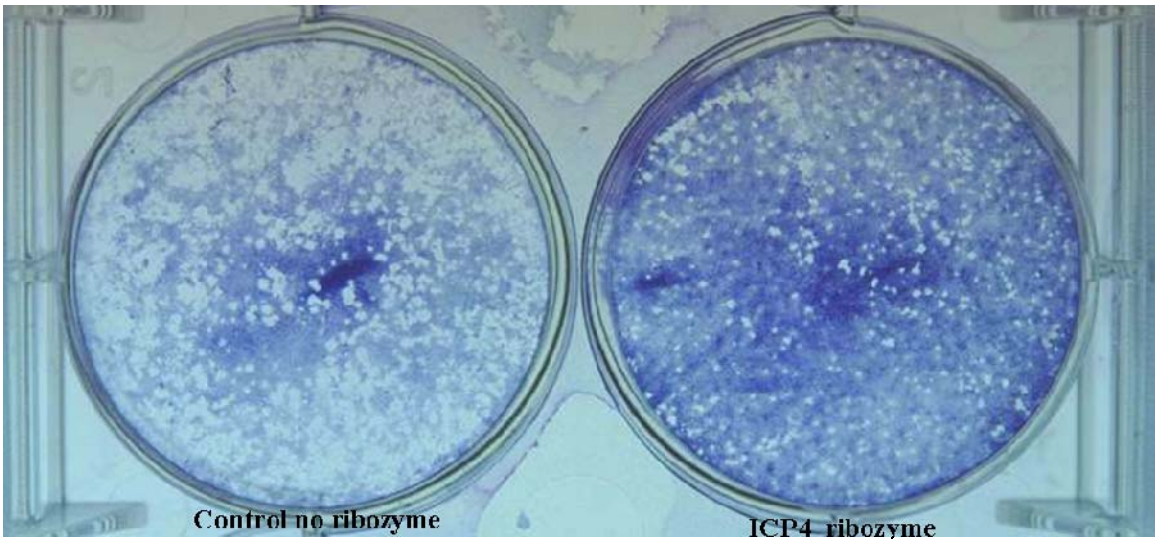


Figure 3-4. Inhibition of wild-type HSV-1 viral replication rendered by ICP4 ribozyme-885 function. A) After selection under G418, 7 single colonies (D1, D2, D4, D5, B4, B5, and B7) were isolated and reverse transcription followed by radioactive labeled PCRs was conducted to compare ICP4 ribozyme-885 expression level. One of the single colony named as B5 has the highest ribozyme expression level, and it was chosen for HSV-1 infection study. ICP4rz-885 expression from the pool of all the positively selected cells was included as a ribozyme expression control (labeled as "pool"). B) RS cells stably expressing ICP4rz-885 had resistance against wild-type HSV-1 infection indicating a phenotype of smaller plaque size and fewer plaques after infection. The infection was conducted at a MOI of 10^{-3} using wild-type HSV-1, and cells were stained using crystal violet at 72 hours post-infection for observation.

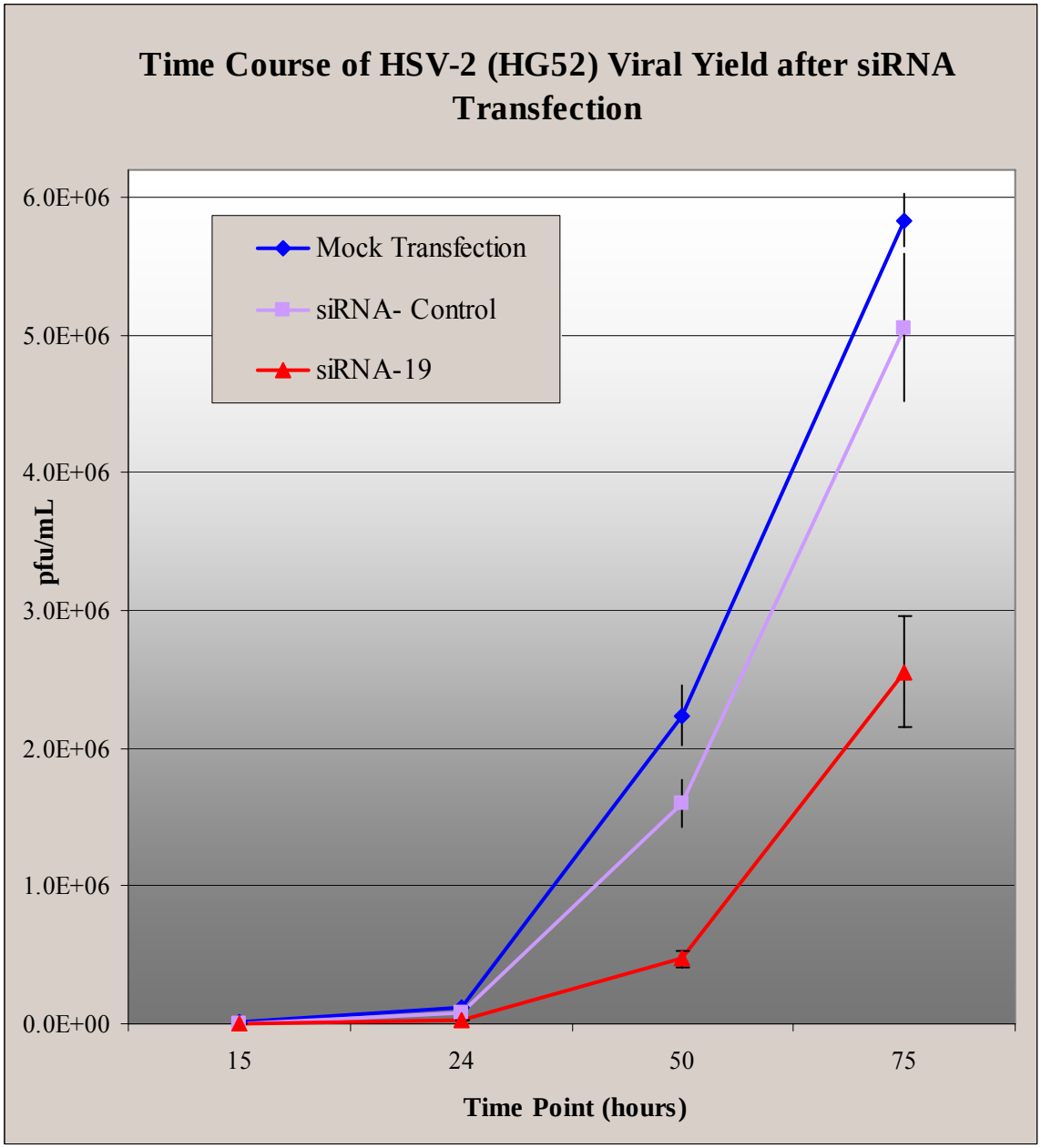


Figure 3-5. Effect of siRNA19 targeting ICP4 mRNA on viral replication of wild-type HSV-2 (HG-52) in HeLa cells. The siRNA targeting mRNA of HSV-2 ICP4 was transfected in HeLa cells followed by HSV-2 infection at an MOI of 10^{-3}. At various time points, viral yields were quantified by plaque reduction assay. Two control groups were mock transfection and scramble siRNA transfection groups. The reduction level of siRNA-19 compared with that of the scramble siRNA control at 15 hours post-infection of HSV-2 is 63%, at 24hours is 63%, at 50hours is 70%, and at 75 hours post-infection of HSV-2 is 49%.

CHAPTER 4
RNA GENE THERAPY FOR HERPES SIMPLEX VIRUS KERATITIS; TARGETING A HSV-1 LATE GENE

Introduction

Herpes simplex virus type 1 (HSV-1), a double-stranded DNA virus, is one of the most well- characterized human pathogens. Infection with HSV-1 is very common and associated with various diseases: oral-facial infections (e.g. gingivostomatitis, pharyngitis, and recurrent herpes labialis), skin infections (e.g. eczema, herpeticum, and erythema multiform), central neural system infection (encephalitis), and disseminated diseases. Herpes simplex virus keratitis (HSK) caused by HSV-1 is the most common infectious cause of corneal blindness in the U.S. The consequence of repeated reactivations lead to cumulative damage; particularly in the case of HSK, patients experience loss of corneal transparency caused by each episode of reactivation which eventually leads to blindness.

Herpes Simplex Virus Keratitis

Currently there is no viable therapy to prevent the recurrent infection despite the availability of systemic and topical antiviral medications, which can shorten the length of infection and reduce the severity of infection. The toxicity of antiviral drugs causes rejection and the failure of clinical treatments. Patents often suffer from both allergic damage and lesions caused by HSV-1 infections which are consistent with *in vitro* toxicity studies.[160,210,211,390] Among all the antiviral chemotherapeutic agents, nucleoside analogs are the most successfully used in clinic, particularly acyclovir (9-(2-

hydroxyethoxymethyl) guanine; ACV). ACV has been commonly used in the systemic treatment of HSV-1 infection with low toxicity. ACV functions by interrupting HSV-1 viral DNA synthesis via HSV-1 thymidine kinase activity.[149,150] The specificity of ACV against HSV is the phosphorylation of ACV to a monophosphate (ACV-MP) which is conducted by HSV thymidine kinase. The large amounts of ACV-MP are then transformed to the diphosphate (ACV-DP) by cellular guanylate kinase. The triphosphate form of ACV, transformed by other cellular enzymes, is the actual inhibitor of viral DNA replication. It functions through its specific binding to viral DNA polymerase. By incorporating into viral DNA, ACV triphosphate leads to premature termination of DNA synthesis.

However, in high risk populations, individuals with compromised immune systems such as AIDS (Aquried Immune Deficiency Symdrome) patients, cancer patients, and patients undergoing organ transplantation, elevated severe recurrence and the generation of drug resistant HSV-1 strains can lead to failure in treatment and even death. ACV-resistant and other nucleoside analogue- resistant strains have been isolated from immune-compromised patients.[77,78] This ability of HSV to readily mutate in response to conventional chemical agents underscores a need to develop novel anti-HSV agents that will substitute for and/or complement ACV and other nucleoside analogues.

U_L20 Gene and Function of Its Gene Product

Although the mechanism of HSV-1 virus maturation and egress to the extra-cellular space has not been fully understood, it has been shown that U_L20 protein, an essential gene product, plays an important role in viral replication in cell culture.[17] HSV-1 U_L20 gene is highly conserved in alphaherpesviruses, e.g., varicella-zoster virus (VZV)[84], bovine herpesvirus-1 (BHV-1)[370] and pseudorabies virus (PRV)[185], as well as in a

gammaherpesvirus MDV-2 (Marek's disease virus type 2)[135], and the U_L20 open reading frame (ORF) is positionally conserved in genomes of different alphaherpesviruses. The U_L20 gene of HSV-1 encodes a 222- amino acid nonglycosylated membrane protein, which is regulated as a γ1 gene and present in the envelope of purified virions.[378] Computer-assisted programs (TMPred[152] and SOSUI[148]) predict that U_L20 protein is a four-time membrane-spanning protein, placing both the amino and carboxyl terminal portions within the cytoplasm of cellular membrane as well as internal to the virion envelope (as shown in Fig. 4-1).[236]

Multiple membrane-associated events are involved in morphogenesis and egress of infectious herpes virions into the extracellular space: Primary envelopment by budding of capsids from the nuclei to the inner nuclear leaflets, de-envelopment by fusion of viral envelopes with the outer nuclear leaflet, re-envelopment of cytoplasmic capsids into Golgi or TGN (trans-Golgi network) derived vesicles, and finally transport of enveloped virus within cytoplasmic transport vesicles to extracellular spaces.[168,241,353] U_L20 protein functions at the step of virion egress from perinuclear space to cytoplasm and to extracellular space by dominantly distributing in nuclear membrane and cytoplasm (the endoplasmic reticulum and the Golgi apparatus). In the absence of U_L20 protein, virions are trapped in perinuclear space as well as in cytoplasmic vesicles. Therefore, no infectious virions are released to extracellular space. It has been shown that deletions of the HSV-1 U_L20 and the PrV U_L20 genes resulted in a reduction of infectious virus production by up to 100 folds compared with their parental wild type viruses.[17,105,108] Although it has been recognized as a membrane protein, U_L20 protein is involved in Golgi dependent glycosylation and cell surface expression of glycoprotein K (gK). gK

and U_L20 gene are required for a phenotype called syncytium during HSV-1 infection. Therefore, U_L20 is also involved in virus-induced cell fusion. However, U_L20 defective HSV-1 is impaired in viral release in a cell-type dependent manner, indicating that certain cellular functions can compensate for U_L20 protein. It has been shown that the integrity of Golgi apparatus is one of the cell factors that have this function. Furthermore, it was suggested that expressing of U_L20 is regulated as a γ1 gene, and impairment in viral DNA synthesis diminished but did not abolish U_L20 production.[378] It is not known whether U_L20 can directly or indirectly regulate viral DNA replication. Considering the important role of U_L20 protein in intracellular virion morphogenesis and virus-induced cell fusion, it is intriguing to know whether defective expression of this gene can affect the pathogenesis phenotype in animals.

Gene targeting of HSV-1 has classically relied on in inhibiting immediate early gene expressions, especially ICP4. The impact of knocking down expression of an essential late gene on the HSV-1 viral life cycle has not been addressed. In this chapter, a hammerhead ribozyme targeting U_L20 mRNA was tested in cell culture against wild-type HSV-1s as well as drug resistant viral strains. As shown from previous *in vitro* kinectic study (Chapter 2), this ribozyme has shown a significant cleavage activity. Further tests of the inhibitory effect at RNA level as well as at viral DNA level were conducted to address the ribozyme effect. Meanwhile, similar approach was used to test another hammerhead ribozyme targeting HSV-1 U_L30 mRNA which encodes viral DNA polymerase.

Materials and Methods

Hammerhead Ribozyme Cloning

Ribozymes with high kcat/Km (higher than $1\mu M^{-1}$ min^{-1}) were selected for cell culture studies (see Chapter 2). Ribozyme sequences along with target sequences are listed in Figure 2-5, and two ribozymes are tested which were named U_L20Rz135 and U_L20Rz154, respectively. U_L20Rz154 was chosen for the cell culture test due to its active catalytic activity (Table 2-3). Ribozymes were cloned in a plasmid (pTR-UF21-New Hairpin) for cell culture transfection experiment. In pTR-UF21-New Hairpin plasmid, ribozyme expression is driven by chicken β-actin promoter and a CMV ie enhancer upstream (shown in Fig. 4-2A). A neomycin gene was included as a selection marker. The ribozyme was also cloned into an adenovirus packaging plasmid, pAdlox, (accession number RVU62024 in NCBI nucleotide database). In this plasmid there are the 3' inverted terminal repeat of adenovirus, a viral packaging signal (ψ), a cDNA expression cassette driven by the cytomegalovirus (CMV) promoter/enhancer, and a *lox*P Cre recombinase recognition sequence. The ribozyme expression was followed by an IRES (internal ribosome entry site)-GFP (green fluorescent protein) element (shown in Fig. 4-2B) for localization purposes. In the ribozyme expression cassette of both pTRUF21-New Hairpin and pAdlox, an internal hairpin ribozyme was located between the hammerhead ribozyme and IRES-GFP element. The hairpin ribozyme conducts self-cleavage in order to free the 3'-end of the ribozyme by releasing downstream sequence.

Test of Transient Transfection of Ribozyme Containing Plasmids against Wild-type Herpes Simplex Virus Type 1

Ribozymes with reasonable catalytic activities were tested in cell culture against wild-type herpes simplex virus type 1 (HSV-1) strain 17*syn*+. Transient transfection of

hammerhead ribozyme was conducted on rabbit skin cell (RSC) using Lipofectamine™ and Plus™ reagent (Invitrogen, Carlsbad, CA). A G418 selection was conducted for 6 to 8 days to enrich transfected cells. An HSV-1 infection using strain 17*syn*+ was performed either at an MOI of 1 for 15 hours or at an MOI of 10^{-3} for 24 hours. Control transfections were conducted using the plasmid without the ribozyme. Viral yields from different transfections were compared using the plaque reduction assay. Ribozymes showing effects in reducing viral yields were packaged in the adenoviral vector for further testing in cell culture.

Adenovirus Vector Packaging

A serotype 5 recombinant adenoviral vector using Cre-lox recombination system, described by Hardy *et al*[134], was used for ribozyme packaging. The protocols of recombination process and recombinant virus preparation were described by Glyn *et al*.[272] Recombinant adenovirus was generated by co-transfection of linerarized pAdlox packaging plasmid with ψ5 adenoviral genomic DNA, which has its packaging sequence flanked by *lox*P sites. The transfection is performed in a 293 cell line called Cre8 cultured in Eagle's minimal essential medium (MEM) 10% Fetal Bovine Serum (FBS), 100 I.U. penicillin/mL, and 100μg/mL streptomycin (Cellgro, Mediatech, Inc., Herndon, VA). Cre8 cells constitutively express Cre recombinase. These cells generate recombinants between the *lox*P sites in the packaging plasmid and the 3' *lox*P site in the ψ5 adenoviral backbone (accession number RVU62024). Propagation of non-recombined ψ5 is negatively selected by deletion of the packaging signal by the Cre recombinase. Plaques isolated from the cotransfected plates were almost exclusively recombinants. Subsequent propagations of the adenovirus in Cre8 cells can eliminate the contaminating ψ5 virus. Two Adenovirus purification methods were used in this study: a

kit called Vivapure AdenoPACK™ 100 (Vivascience AG, Hannover, Germany) was used to purify the recombinant adenovirus for cell culture study and animal experiments, and another method called Cesium Chloride (CsCl) step gradient purification was also adopted.

A detailed procedure of generating recombination adenovirus is recorded as following:

1. Plate a T75 flask of Cre8 cells to 60% confluence in Eagle's minimal essential medium (MEM) supplemented with 10% FBS, 100 I.U. penicillin/mL, and 100μg/mL streptomycin (Cellgro, Mediatech, Inc., Herndon, VA).

2. Digest 4.5- 10 μg of pAdlox plasmid DNA containing ribozyme expression cassette with SfiI (New England Biolabs®, Inc., Ipswich, MA). The DNA was extracted once with phenol: chloroform: isoamyl alcohol followed by ethanol precipitation of the aqueous phase. The DNA was recovered and resuspended in TE (pH8).

3. Cre8 cells were transfected with linear DNA along with ψ5 viral DNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) following product manual. Transfected cells were incubated at 37 °C for 7 to 10 days for plaque formation. Medium (MEM with 10%FBS, 100 I.U. penicillin/mL, and 100μg/mL streptomycin) was refilled depending on cell condition.

4. Two T75 flasks were seeded with Cre8 cells: One was used to prepare a viral stock and the second one was used to extract viral DNA to verify that the virus generated was indeed a recombinant.

5. When prominent cytopathic effects (CPE) were observed throughout the transfected cells (approximately 8- 10 days), the cells and media were harvested from the dishes using a cell scraper. The mixture was transfered to a 50-mL cornical tube. To verify recombinant virus, viral DNA extraction protocol was used followed by appropriate restriction digestions.

6. The harvested cell/ media mixture was frozen and thawed for three times to lyse the cells and release viral particles.

7. To amplify and purify the adenoviral stock, the viral lysate was used to re-infect cells. 0.5 mL of cell lysate and 5 mL of medium were mixed to cover a monolayer of Cre8 cells in a T75 flask. 2-4 hours were allowed for infection.

8. After the incubation, medium containing cell lysate was replaced by fresh medium. Cells were cultured until prominent cytopathic effects (CPEs) were observed throughout the monolayer (approx 1-2 days). Cells and media were harvested, as in

step "4", and they can be stored at -80 °C. After 3 rounds of infection in Cre8 cells, the majority viral population was recombinant virus.

A detailed procedure of CsCl step gradient purification of Adenovirus preparation is following:

1. 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 100 I.U. penicillin/mL, and 100µg/mL streptomycin (Mediatech, Inc., Herndon, VA). Six 75cm^2 tissue culture flasks of 293 cells were prepared to reach confluence.

2. To infect the cells, typically, 5x10^{7}plaque forming units (PFUs) of virus was added to 5mL of Opti-MEM® I Reduced-Serum Medium (Invitrogen, Carlsbad, CA) for each 75cm^2 flask. Three to four hours of incubation was allowed at 37°C in a 5% CO_2 incubator.

3. Viral solution was removed at the end of the incubation and replaced by 15mL of DMEM containing 10% FBS and 1% penicillin-streptomycin (Mediatech, Inc., Herndon, VA). Cell lysates were harvested when prominent cytopathic effects were observed. Typically cell lysates were ready after 2 days.

4. Cells were harvested using a cell scraper and cell lysate was centrifuged at 2000xg at 4°C for 10 minutes. The supernatant can be saved to resuspend the pellet. Usually cell pellets were resuspended in 5mL of media. Cell lysate was frozen and thawed for three times, alternating with a 37°C water bath and -80°C freezer.

5. Cell lysate was treated with Benzonase (Sigma-Aldrich, St. Louis, MO) at 50U/mL at 37°C for 30 minutes. Cell lysate was centrifuged at 2000xg, 4°C for 10 minutes, and the supernatant was saved for CsCl step gradient purification.

6. Polyallomer tubes (Beckman Coulter, Inc., Fullerton, CA) were chilled on ice and a CsCl step gradient contained following components:
    a. 1.4g/mL of CsCl (bottom layer);
    b. 1.2g/mL of CsCl (middle layer);
    c. Viral cell lysate (top layer).

7. The tubes were centrifuged at 40,000xg, 4°C for 1 hour using a swinging bucket rotor (Beckman SW41 Ti Rotor, Beckman Coulter, Inc., Fullerton, CA). Typically, there were two white bands seen near the interface of the 1.2- and 1.4g/mL CsCl layers. The lower band contained the infectious viral particles which were collected using a 20-gauge needle and 3mL syringe. The harvested viral particles were diluted by at least two folds in 10mM Tris Hydrochloride (Tris-HCl) (pH8.0) and mixed well for recentrifugation.

8. The procedure in step 6-7 was repeated for two more times. The purified adenovirus was transferred to dialysis bags and was dialyzed against 500mL of

chilled dialysis buffer for at least 6 hours at 4°C. Two more times of dialysis were conducted. The dialysis buffer was made fresh the same day and stored in 4°C. The recipe of the dialysis buffer can be found in Appendix C. The Adenovirus stock was stored in aliquots at -80°C.

9. The virus particle concentration of adenovirus stock was measured by mixing 15μL of the stock with 285μL of water. The absorption at 260nm (A_{260}) was determined by spectrophotometry. One A_{260} is approximately equal to 10^{12} viral particles per mL. The percentage of infectious virions typically ranges from 1 to 10% of the total number of viral particles.

Preparation of Adenoviral DNA

This procedure was conducted to either amplify ψ5 adenovirus for viral DNA extraction or isolate recombinant viral DNA for restriction digestion analysis.

Culture media were removed from T75 flask of confluent culture (293 cells for ψ5 isolation or Cre8 cells for recombinant viral DNA extraction). Viral lysate (50μL) was mixed with 5mL of serum-free medium (Opti-MEM® I Reduced-Serum Medium, Invitrogen, Carlsbad, CA) and plated on the cells. Two to four hours are allowed for infection. Following incubation, media are supplemented with 10% FBS and 100 I.U. penicillin/mL, and 100μg/mL streptomycin (Cellgro, Mediatech, Inc., Herndon, VA). Cells and media were harvested using a cell scraper when complete cytopathic effect (CPE) was observed (typically when monolayer cells are round-up and begin to detach) which might take 2-5 days before harvesting the cells. Cells were pelleted by centrifugation at 900 rpm at 4°C for 10minutes and resuspended in 400μL of TE pH9 (10 mM Tris-Cl pH9, 1 mM EDTA). The supernatant was discarded and a large volume of undiluted bleach was used to treat the supernatant. DOC lysis buffer (recipe listed in Appendix C) (400μL) was added to the cell resuspension and mixed well by passing through a pipette tip repeatedly. Spermine-HCl (8μL at a concentration of 500mM) was added and mixed well for incubation on ice for 10 minutes. The mixture was centrifuged

at a maximum speed for 4 minutes at 4°C, and the supernatant was transferred to a fresh tube. Ten minutes of incubation was allowed at 37°C after 4μL of RNaseA (10mg/mL) was added. Incubation at 40°C for one hour was followed after adding 60μL of 10% Sodium Dodecyl Sulfate (10% SDS), 20μL of 0.5M EDTA, and 40μL of 50mg/mL pronase (CALBIOCHEM®, San Diego, CA) (see Appendix C for recipe). Phenol/chloroform/isoamyl alcohol (25:24:1) was used to extract viral DNA and the aqueous layer was collected to precipitate DNA. In less than 900μL of collected aqueous solution, 30μL of 5M sodium chloride (NaCl) was added followed by 600μL of Isopropanol (Fisher BioReagents, Fair Lawn, NJ). The DNA pellet was rinsed with 70% ethanol and dried in room temperature. Viral DNA was resuspended in 25μL of TE and BsaB I digestion was conducted to check recombinant Adenoviral DNA. In Ψ5 viral DNA, there are three BsaB I sites producing a series of bands: 11648, 10536, 7723, and 2249 base pairs (bp). When a recombination happened successfully in Cre-loxp system, the 2249bp band would be replaced by another band depending on the insert in recombinant virus.

Herpes Simplex Virus Type 1 Viral Strains and Viral Production

Rabbit skin cells (RSC) were used to propagate wild-type HSV-1 (17*syn*+). Protocols for viral production, purification and plaque reduction assay to estimate viral titer were previous described in Chapter 3.

Cell Culture Tests of the Accumulative Effects of Ribozymes Packaged in Adenoviral Vector against Wild-type Herpes Simplex Virus type 1

To evaluate the viral yields after ribozyme treatment, after 15 hours of delivery of ribozyme as well as control treatments, Herpes simplex virus type 1 (HSV-1) infection was conducted at an MOI of 10^{-3} for either 24 hours or for 6 days. The experiment was

set up in triplicate for each treatment each time point. A plaque reduction assay was performed to compare HSV-1 viral yields. In these early experiments, adenovirus stock was purified using CsCl step gradient purification method, and the infective dose was 800-1000 viral particles per cell. When the ribozyme function was confirmed by at least two independent assays, further evaluation was conducted. Another adenovirus purification method using Vivapure AdenoPACK^{TM} 100 (Vivascience AG, Hannover, Germany) was also adopted.

A dose-response test was performed to observe the ribozyme effect to inhibit viral replication. In this assay, adenovirus was purified using Vivapure AdenoPACK^{TM} 100 (Vivascience AG, Hannover, Germany). RSC were seeded at a density of $2x10^5$ cells per well one day before adenovirus inoculations (control virus was Ad-GFP containing GFP gene instead of ribozyme cassette). A serial of dilutions of recombinant adenovirus (1, 10, 10^2, 10^3, 10^4, 10^5, 10^6 viral particles per cell) were used to conduct infections. Forty eight hours were allowed for accumulation of ribozyme expression followed by HSV-1 (17*syn*+) infection at an MOI of 10^{-3}. 24 hours were allowed before cell lysates were harvested for plaque reduction assay. At each dilution of the recombinant virus the infection was performed in triplicate and the plaque reduction assay was conducted on RSC. An effective dose was used for further observation of either therapeutic effect in cell culture or target mRNA level after treatment.

Real time Polymerase Chain Reaction to Compare Target Levels after the Ribozyme Treatment

To investigate the ribozyme effect in knocking down the target mRNA level, reverse transcriptions were carried out using total RNA extracted from RSC containing ribozyme followed by HSV-1 infection. The real time PCR was carried out to compare

target mRNA levels. Recombinant adenovirus infections were conducted in quadruplicate for 48 hours followed by the infection of 17*syn*+ at an MOI of 3 for 8 hours. Total RNA extraction was performed using TriZol® (Invitrogen, Carlsbad, CA). Contaminated DNAs were cleaned using DNA-*free*™ kit (Ambion, Austin, TX) which is RNase free DNase. Reverse transcription (RT) was conducted using First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK). Total RNA (1μg) and random hexamer primers were used in each reaction. Total RNA was quantified by the spectrometry using a photodiode array detector called Gene Spec III (MiraiBio Division, Alameda, CA) at a wavelength of 260nm and the quality of RNA was controlled with ratio of the absorption at 260nm divided by that at 280nm ranging from 1.8 to 2.0. cDNA from each RT- reaction was diluted in 10 fold before real time PCR assay. Specific primers and a fluorescent probe for either the target or GAPDH (Glyseraldehyde-3-phosphate dehydrogenase) were designed and synthesized by ABI system (Applied Biosystems, Foster City, CA). For each RT- reaction, real time PCR assays were set up in triplicate for both sets of primers and probes. Standard curves for corresponding primers and probes using either RSC genomic DNA or HSV-1 viral genomic DNA (or, as an alternative, using cDNA from infected RSC) as reference DNA were carried out in triplicate. In order to obtain the correlation between the template amount and the cycle threshold (Ct), serial dilutions of reference DNAs (or cDNA) were used to generate standard curves. An absolute template amount resulted from the standard curves based on the Ct was used to compare treatment and control groups. The ratio of target RNA level to GAPDH level was used to compare between ribozyme treatment group and control groups. Meanwhile from the same TRIZOL® extracted samples, DNA

extractions were also conducted following manufacturer's protocol. DNA samples were diluted to 1:10 and used as template for Real-time PCR. Primers and probes for both HSV-1 DNA polymerase and cellular GAPDH were used and their ratio of each sample was compared.

Testing Hammerhead Ribozyme against Drug Resistant Herpes Simplex Virus type 1 Strains

HSV-1 drug resistant strains PAAr5[68,162], tkLTRZ1[82,163] and ACG^{r}4[68] as well as their parental strain KOS were kindly provided by Martha Kramer.

Growth rate study of drug resistance HSV-1 strains and wild-type HSV-1 with or without adenovirus packaged ribozyme treatments

Rabbit skin cells (RSC) were seeded at a density of 10^5 cell per well of each 24-well-plate one day before adenoviral infection. At the second day, Adenovirus infection was conducted at a dose of $5x10^5$ viral particles per cell which was ED_{50} dose (effective dose to reduce viral replication by 50%) from dose response study. On day 3, HSV-1 infection was conducted at an MOI of 10^{-3}; and at time points of 6, 12, 24, 48, 72 hours post-infection, cells were harvested for plaque reduction assay as describe before. Each group at each time point, the experiment was conducted in triplicate.

Acyclovir solution

A 5mM stock of acyclovir (Sigma, St. Louis, Missouri) (ACV) was prepared by resuspending 100mg ACV in 88.8mL H2O and 200uL HCl. The solution was filter sterilized and aliquoted. When preparing the ACV it was important to allow for complete suspension (by warming the sample and vortex mixing).

Acyclovir inhibition threshold for drug resistant HSV-1 strains

RSC was seeded at a density of $1x10^5$ cells per well of 24-well-plates on the first day. On the second day, medium was replaced by ACV-containing medium at various

doses. For each dose, 3 wells of cells were included and HSV-1 (drug resistant strain or wild-type strain) infection was conducted on the third day at an MOI of 10^{-3}. On the fifth day (or at the second day post-infection) infected cells were harvested and cell lysates were stored in -80°C for the plaque reduction assay. The viral yields at each acyclovir dose treatment were plotted against the respective acyclovir dose.

For 17*syn*+ (wild-type HSV-1) and PAAr5, acyclovir doses of 0.1, 0.5, 1, 10, 20, 50,100μM were used to test drug sensitive range. As for tkLTRZ1 and ACGr4 dose range of 0.01, 0.1, 0.2, 0.5, 1, 5, 10μM were tested. A series of acyclovir doses (0.01, 0.05, 0.1, 0.2, 0.5, 1, 5μM) were used to test HSV-1 KOS strain. Cells were seeded at a density of $1x10^5$ per well one day before pretreatment, and acyclovir was added the next day at the doses shown above. On the third day, HSV-1 infection was conducted at an MOI of 10^{-3} for two days before cell lysates were harvested for the plaque reduction assay. Plaque reduction assay was conducted on RSC. Drug inhibition curves corresponding to each strain were plotted by relating ACV doses and corresponding viral yields. The drug inhibition threshold was set at the point that it could distinguish the wild-type HSV-1 (drug sensitive strains) from drug resistant ones.

Testing the hammerhead ribozyme against drug resistant HSV-1 strains

Rabbit skin cells (RSC) were seeded in each well of 24-well-plates at a density of $1x10^5$ cells per well one day before treatments. The next day adenovirus packaged ribozyme or GFP was used at a dose of 10^6 viral particles per cell (viral stock was purified using the Vivascience kit) to infect the cells in 200μL Opti-MEM (GIBCO, Invitrogen Corporation) per well. After 3 hours the media were replaced with 5% bovine serum containing MEM. The same time a no-adenovirus control and an ACV treatments (at a threshold level of 0.1μM) were included using the same media. At 24 hours post-

infection of adenovirus, an HSV-1 (drug resistant strains as well as drug sensitive strain controls) infection was conducted at an MOI of 10^{-3} following HSV-1 infection protocol. Forty eight hours were allowed for HSV-1 infection to develop and cell lysates were harvested for plaque reduction assay at the end. Each treatment was set up in triplicate for all the HSV-1 strains.

Results

Transient Transfection of the Plasmid Expressing Hammerhead Ribozyme Followed by HSV-1 Infection (17*syn*+)

A transient transfection assay in RSC was conducted using pTRUF21 plasmid containing either ICP4 ribozyme-885 or U_L20 ribozyme-154 as well as control transfections (mock transfection and backbone plasmid pTRUF21 transfection). A brief selection using G418-containing medium was followed. HSV-1 infection was conducted using 17*syn*+ strain at a low MOI of 10^{-3} for 24 hours. Cell lysates were harvested for a plaque reduction assay. As shown in Figure 4-3, a nearly two-log reduction in viral production was observed by transient transfection of a plasmid containing U_L20 ribozyme-154. Transfection of a plasmid expressing ICP4 ribozyme did not have any effect on viral production.

Dose-response Assay of Adenovirus Packaged U_L20 Ribozyme-154 against wild-type HSV-1 Viral Replication

Sequences of U_L20 ribozyme-154 and its target are listed in Table 3-1. U_L20 ribozyme-154 was packaged in Adenoviral vector and the ribozyme expression is driven by a CMV promoter. The ribozyme expressing cassette was cloned from pTRUF12 backbone containing hammerhead ribozyme and a downstream hairpin ribozyme. Between the promoter and ribozyme expressing cassette there is a small intron cloned by combining SV40 viral splicing donor and acceptor sites (gta agt tta gtc ttt ttg tct ttt att tca

ggt ccc gga tcc ggt ggt ggt gca aat caa aga act gct cct cag tgg atg ttg cct tta ctt cta g). An IRES-GFP fragment, which was adopted from pTRUF12 backbone, was located downstream from the ribozyme expressing cassette and followed by poly (A) signals. The control for U_L20 ribozyme-154 treatment was an Ad-GFP virus treatment and it contains the GFP coding sequence (CDS) between CMV promoter and poly (A) signals instead. A series of dilutions of Adenovirus (Ad) viral particle numbers were used to treat RSC followed by wild-type HSV-1 (17*syn*+) infection. HSV-1 viral yields from Ad mock infection, Ad-GFP, and Ad-Rz (Ad-$U_L20$rz) treatment were compared. In this assay, Adenovirus preparations were from a commercial adenovirus purification kit. Adenovirus transductions at each dose were observed using a fluorescent microscope for GFP expression as shown in Figure 4-4-A. There was a correlation between increasing levels of Ad-U_L20Rz and decreasing HSV-1 viral production as seen in Figure 4-4-B: when the Ad-U_L20Rz was higher than 1000 viral particles per cell (vp/cell), a >10% reduction was observed, while 10^5vp/cell led to a 56% reduction in HSV-1 viral yield and 10^6vp/cell led to a 93% reduction.

Inhibitory effect of U_L20 ribozyme-154 on Wild-type Herpes Simplex Virus Type 1 Viral Replication

Adenovirus packaged U_L20 ribozyme-154, mock infection and control vector (Ad-GFP) were used to treat RSC. Wild-type HSV-1 infection at an MOI of 10^{-3} was followed for various times (1 to 6 days). The HSV-1 viral yields were compared by plaque reduction assay. As shown in Figure 4-5-A**,** at one day post-HSV-1-infection U_L20 ribozyme-154 treatment significantly reduced HSV viral replication by 83% (compared with Ad vector treated cells $p<0.001$) and this inhibitory effect lasted for 6 days as shown in Figure 4-5-B.

Ribozyme Effect on Viral Target RNA and Wild-type Herpes Simplex Virus Type 1 DNA Replication

In order to evaluate U_L20 ribozyme-154 for its ability to inhibit viral target mRNA expression, RSC were infected with wild-type HSV-1 at an MOI of 3 for 8 hours following ribozyme treatment or control treatments (mock infection and Ad-GFP infection). Data are shown in Figure 4-6. After reverse-transcription followed by real-time PCR, a significant reduction in U_L20 mRNA level by 68% was observed in ribozyme treated cells compared with Ad-GFP treatment ($p<0.0005$). However, a significant reduction in the mRNA level of HSV-1 DNA polymerase (the gene product of U_L30) was also observed (70% reduction). DNA was also collected from the same cell lysate and viral DNA levels were compared to correlate the result came from plaque reduction assay. A significant reduction in viral DNA levels was observed only in ribozyme treated samples which was a reduction of 54% ($p<0.004$).

Ribozyme Effect on Viral Replication of Herpes Simplex Virus Type 1 Drug Resistant Strains

U_L20 Ribozyme-154 was also tested against HSV-1 drug (ACV) resistant viral strains (PAAr5[68,162], tkLTRZ1[82,163] and ACG^{r}4[68]), and their parental strain, KOS, as well as 17*syn*+ were used as controls. To evaluate the therapeutic effect of this ribozyme, a drug control using acyclovir (ACV) was included. A dose response of ACV was tested against wild-type HSV-1 strains and drug resistant strains for their sensitive ranges. A standard concentration of 0.1μM of ACV was chosen, since according to a recent study, at this dose wild-type viruses and drug resistant viruses can be distinguished.[349] However, even at a dose of 1μM, ACV did not have any inhibitory effect on the replications of mutant strains tkLTRZ1 and ACGr4. As shown in Figure 4-7, U_L20 Ribozyme-154 was tested for its ability to inhibit HSV-1 viral replication of not only wild-type strains but

also a series of drug resistant strains that have been well characterized for their resistance mechanisms. PAAr5 has mutation in viral DNA polymerase; tkLTRZ1 has been mutated in thymidine kinase, while ACGr4 contains mutations in both genes. Therefore they are no longer sensitive to ACV treatment. The inhibitory effect caused by ribozyme was compared with that of acyclovir (ACV). When tested against wild-type HSV-1 (both 17syn+ and KOS), ribozyme and ACV showed very similar inhibitory effect on viral replication (shown in Figure 4-7-A**,** B). As expected, ACV did not show any effect on viral replication of drug resistant HSV-1 strains. However, treatment of U_L20 ribozyme-154 led to consistent inhibition in viral replication among all the strains tested (as shown in Figure 4-7-C**,** D**,** E).

Inhibitory Effect of a Hammerhead Ribozyme Targeting U_L30 mRNA in Viral Replication

A ribozyme targeting U_L30 mRNA, U_L30 ribozyme-933, was also packaged in Adenovirus vector to test its ability to knockdown viral replication. Sequences of this ribozyme and its target are listed in Table3-1. A time course test comparing Ad-$U_L20$rz, Ad-$U_L30$rz, and the mixture of both ribozymes, was conducted using an adenovirus dose of 10^6vp/cell for 24 hours. It was followed by HSV-1 infection at an MOI of 10^{-3} for 24 to 72 hours, and cell lysates were harvested for plaque reduction assay. The viral yields were graphed in Figure 4-8-A. Without ribozyme treatment, HSV-1 (17*syn*+) viral production increased from $2.8x10^5$pfu/mL at day 1 post-infection to $1.1x10^8$pfu/mL at the third day; Ad-$U_L20$rz treatment is a positive control that at day 1 post-infection viral yield was $9x10^3$pfu/mL and $6.2x10^5$pfu/mL on the third day; Ad-$U_L30$rz treatment led to $6.2x10^3$pfu/mL HSV-1 yield on day 1 and $4.4x10^5$pfu/mL on day 3; the treatment using a mixture of Ad-$U_L20$rz and Ad-$U_L30$rz (ratio of 1:1) also led to significant reduction (a

viral yield of $3.9x10^3$pfu/mL on day 1,which was a 99% reduction). Although the reduction caused by the mixture of ribozymes led to a slightly higher reduction in HSV-1 production, it was not significantly different from treatment by either ribozyme alone. However, a synergic effect cannot be completely ruled out. Reverse transcription followed by real-time PCR was conducted to study the effect of ribozyme U_L30rz-933 on the target mRNA level. As shown in Figure 4-8-B, a 24% reduction in U_L30 mRNA (encoding HSV-1 DNA polymerase) was detected from ribozyme treated group compared with that from Ad-GFP treatment ($p=0.05$).

Discussion

When considering inhibiting HSV-1 viral replication, very often either immediate early genes, especially ICP4 gene, or early genes such as DNA polymerase (including any DNA synthesis related viral proteins) are the targets for drug development. However, HSV-1 has a relative low expression level of immediate early genes as well as viral DNA polymerase (real-time PCR result showing U_L30 mRNA level), suggesting that their functional thresholds are very low. This makes it difficult to efficiently inhibit viral acute infection by simply knocking down either of these genes alone. On the other hand, at the late stage of HSV-1 replication, a mass of late proteins is expressed for virion packaging, transport and maturation. It can be expected that reduction of an essential protein production at this stage will lead to dysfunctional or decreased release of infectious progeny virions. Therefore the viral infection can be dramatically limited. In this study a hammerhead ribozyme targeting mRNA of HSV-1 U_L20, a γ1 gene, was tested for its inhibitory effect in viral replication. It has been suggested that U_L20 gene encoding a membrane protein is essential for viral intra- and extracellular egression as well as intracellular transport of viral glycoproteins. There is a cell type dependent

phenotype caused by impaired U_L20 expression, indicating that certain cellular function compensates to this viral protein. However, the observation that U_L20 gene is highly conserved among alphaherpesviruses leads to the speculation that this gene may have an important role *in vivo*. As mentioned earlier, a therapeutic effect against HSV-1 infection can be reached *in vivo* without completely restraining the viral replication; thereby a significant reduction in virion production can lead to completely abolishing the clinical indication of infection.

U_L20 protein functions for virion intracellular transport, extracellular release, and intracellular transport of viral glycoproteins. A hammerhead ribozyme targeting U_L20 mRNA significantly inhibited HSV-1 viral replication by sequence-specific cleavage. The ribozyme maintained its inhibitory effect when tested against other HSV-1 strains: it not only reduced wild-type HSV-1 replication (17syn+, KOS) but could also inhibit those of drug resistant HSV-1 strains (PAAr5, tkLTRZ1, and ACGr4). It can be concluded that U_L20 gene product of HSV-1 is essential for viral life cycle *in vitro* (in RSC), and knocking down this gene expression leads to reduction of viral production. This inhibitory effect is probably caused by jeopardizing the egress of virion as well as the transport of glycoproteins which implies a new strategy to inhibit HSV-1 viral replication, to prevent a late event or essential late protein productions of HSV-1.

Although ACV treatment can dramatically prevent wild-type HSV-1 viral replication *in vitro*, it cannot inhibit that of drug resistant viruses (PAAr5, tkLTRZ1, and ACGr4). Even at the dose of 1μM, ACV did not show any effect in viral production of tkLTRZ1 and ACGr4. When patients are infected with drug resistant HSV-1, it will lead to a detrimental result in spite of the availability of drugs. The reason these three drug

resistant HSV-1 strains were chosen is that they represent general drug resistance mechanisms: mutation in thymidine kinase (TK) and in DNA polymerase. Current antiviral drugs for HSV-1 infection are mostly nucleotide analogs. They can either be substrates of TK, indirectly disrupting viral DNA synthesis, or be incorporated in elongated DNA strand leading to pre-mature termination. Because of antiviral treatments in patients especially in immune-deficient patients, drug resistant virus is selected *in vivo* leading to uncontrolled spreading HSV-1 infection, in some cases, this is lethal to patients. It is encouraging that a ribozyme targeting mRNA of HSV-1 U_L20 can overcome this issue. Although nucleotide changes can cause the emergence of resistant escape mutants for U_L20 ribozyme, ribozymes targeting different essential genes of HSV-1 can be combined to guarantee the inhibition, e.g., by combining ribozymes targeting immediate early genes, early and late essential genes. Therefore *in viv*o tests will provide further information concerning the application using the ribozyme as a therapeutic reagent.

Two control adenoviruses were used to indicate the effect of adenoviral vector in the cell during the process of testing adenoviral delivered U_L20 ribozyme; they are adenoviral vector without any transgene insertion (backbone vector or called ψ5 virus) and an adenoviral vector including GFP gene. These two adenoviral vectors showed different effects on HSV-1 lytic viral infection in the cell. Very often there was significant variation when ψ5 virus was used as control. For example, in the experiment shown in Fig 4-5-A, the treatment using ψ5 virus ("Ad vector") led to a 79% reduction in HSV-1 viral replication (at 24 hours post-infection of HSV-1) compared with the viral yield from cells that were only infected with HSV-1 ("No Ad"). In a separate experiment

(shown in Fig 4-5-B), a similar experiment showed a reduction by 23% in HSV-1 viral yield from cells treated with ψ5 virus (Ad vector) compared with that of cells only infected with HSV-1 ("No Ad"). In addition, the efficiency of HSV-1 viral infection in these two experiments varied significantly, indicating the experimental error. It was speculated that Ψ5 virus might cause a non-specific effect which interrupted with cellular machinery; therefore, it indirectly affected HSV-1 viral replication. On the other hand, when the adenoviral vector including GFP gene was used as control, a consistent effect was observed in HSV-1 viral replication level, therefore, the adenovirus packaged GFP vector was chosen as control for the rest of experiments. Another reason to use Ad-GFP as vector control was that an IRES-GFP element was included in the construct of adenovirus packaged ribozyme. Therefore, it is appropriate to use the Ad-GFP to control the effect caused by GFP expression. However, when viral gene expressions (U_L20 and U_L30 mRNA level) were investigated for the ribozyme effect, Ad-GFP treatment led to approximately 50% reduction in viral mRNA level of both U_L20 and DNA polymerase (as shown in Fig4-6-A and B). It is possible that Ad-GFP competed with HSV-1 for the usage of cellular machinery, e.g., RNA polymerase II; therefore it indirectly led to a lower level of viral gene expression in the Ad-GFP treatment group. However, in another experiment (Fig4-8-B), Ad-GFP did not interrupt viral DNA polymerase expression, indicating that there might have been a non-specific effect during the course of experiment. Overall, in spite of above variations, the ribozyme treatment (U_L20 or U_L30 ribozymes) reduced viral target gene expression and inhibited viral replication significantly compared with Ad-GFP treatment. Another observation derived from ribozyme test against drug resistant HSV-1 strains was that although Ad-GFP treatment

did not affect 17syn+, KOS, PAAr5 and tkLTRZ1 viral replication, it significantly interrupted ACGr4 viral replication. ACGr4 is a double-mutant in both TK and DNA polymerase genes generated from a KOS parental strain. This observation suggested that virus with mutations leading to the same drug resistant phenotype may have different behaviors in other aspects. However, when treated with U_L20rz, ACGr4 viral replication was reduced consistent with tests of other HSV-1 strains.

An interaction between DNA polymerase and U_L20 gene expression was observed previously. It was known that when HSV-1 DNA polymerase activity was affected, U_L20 expression would be diminished but not abolished. In this study, when cells were treated with a U_L30 ribozyme to inhibit DNA polymerase expression during HSV-1 infection, a delayed reduction in U_L20 expression was observed by reverse-transcription and real-time PCR (data not shown), although the reduction was not statistically significant compared with those of control treatments. However, when a U_L20 ribozyme was used to treat cells against HSV-1 infection, a synchronized down-regulation in DNA replication level was detected which was statistically significant compared with control treatments. These imply that coordination between U_L20 and U_L30 gene expression exists during lytic infection, and U_L20 expression can provide a feedback signal to viral DNA synthesis.

During the process of packaging ribozymes into the adenoviral vector, a small intron (the sequence is 5'ggg aag tta act ggt aag ttt agt ctt ttt gtc ttt tat ttc agg tcc cgg atc cgg tgg tgg tgc aaa tca aag aac tgc tcc tca gtg gat gtt gcc ttt act tct agg cct gta ccc 3') derived from SV40 SD/SA (splicing donor/acceptor sites) was cloned in between the CMV promoter and the ribozyme expression cassette. The original construct did not

include any intron due to the consideration that an intron would have no effect on the ribozyme level in the cytoplasm after HSV infection. Because HSV-1 infection will shut down the host splicing mechanism, an intron should not provide an advantage for ribozyme transport in the cytoplasm. However, the ribozyme expression from the construct with the intron was significantly higher than the one without it after HSV-1 infection (data not shown). It suggested that the intron led to elevated ribozyme expressions. When the U_L20 ribozyme was tested, the construct containing the intron showed higher inhibition (96% reduction) against HSV-1 replication than that of the construct without the intron (36% reduction). This effect was observed after 6 days of HSV-1 infection in the cell culture (data not shown), although at 1 day post-infection the inhibition levels from both groups were very similar (data not shown). It confirmed that including the intron in the ribozyme cassette can make the expression more efficient. This effect was maintained throughout HSV-1 infection (even with a high moi). Therefore, all the studies related to adenoviral packaged U_L20 ribozyme in this chapter were conducted using the construct with the intron. However, when U_L30 ribozyme was packaged in the adenoviral vector, the intron was not included. It was shown that the U_L30 ribozyme can reduce viral replication *in vitro*, and it can be expected that by inserting the intron in this construct, a more significant inhibition would be observed.

Overall, *in vitro* a hammerhead ribozyme targeting mRNA of U_L20 gene significantly inhibited HSV-1 viral replication of wild-type and drug resistant strains by sequence-specific degradation; it is intriguing to see the therapeutic effect in animal models.

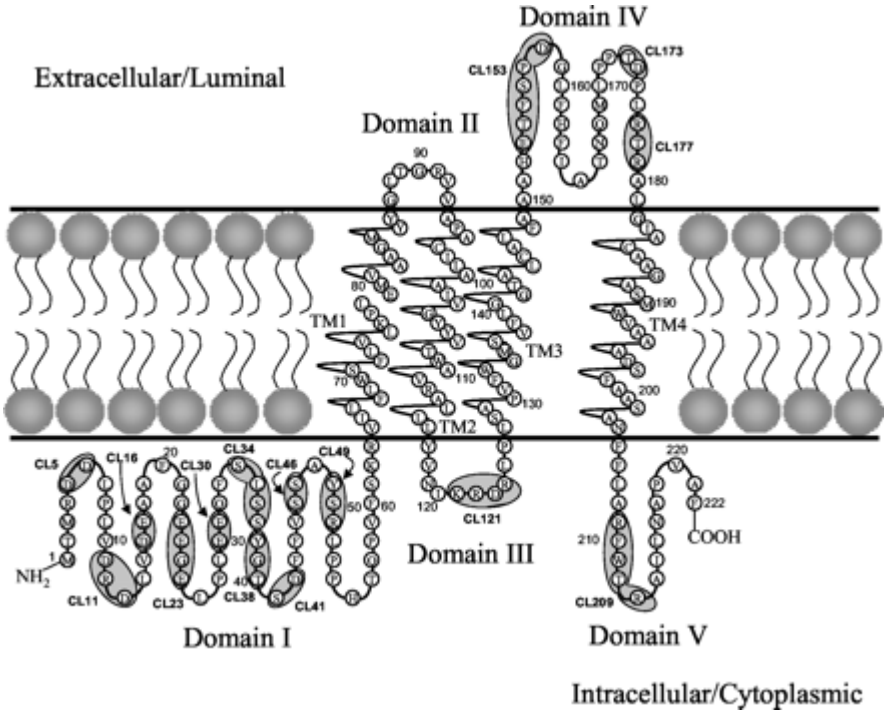


Figure 4-1. Membrane topology of U_L20 protein predicted by the TMPred and SOSUI algorithms.[236]

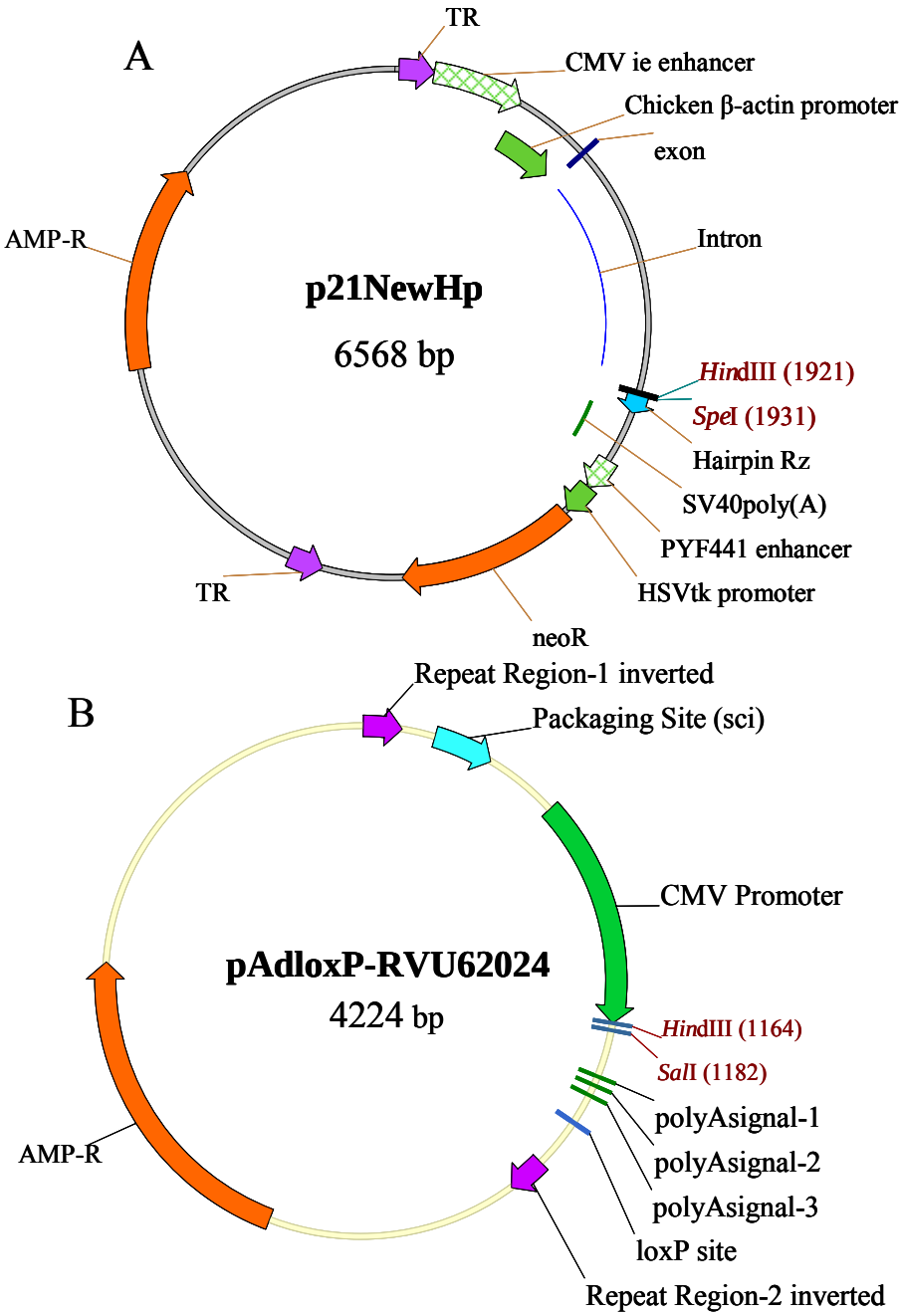


Figure 4-2. Maps of cloning constructs. A) The map of plasmid used for delivery of hammerhead ribozyme by transient transfection. Ribozymes were cloned in between HindIII and SpeI sites. Ribozyme expression is driven by chicken β-actin promoter; and a self-cleavage hairpin ribozyme following hammerhead ribozyme can release down-stream fragment to increase the activity of the hammerhead ribozyme. B) The map of pAdlox designed for adenoviral vector packaging. Hammerhead ribozyme expression cassette was cloned in HindIII and SalI site, and the loxP site was constructed for recombination event.

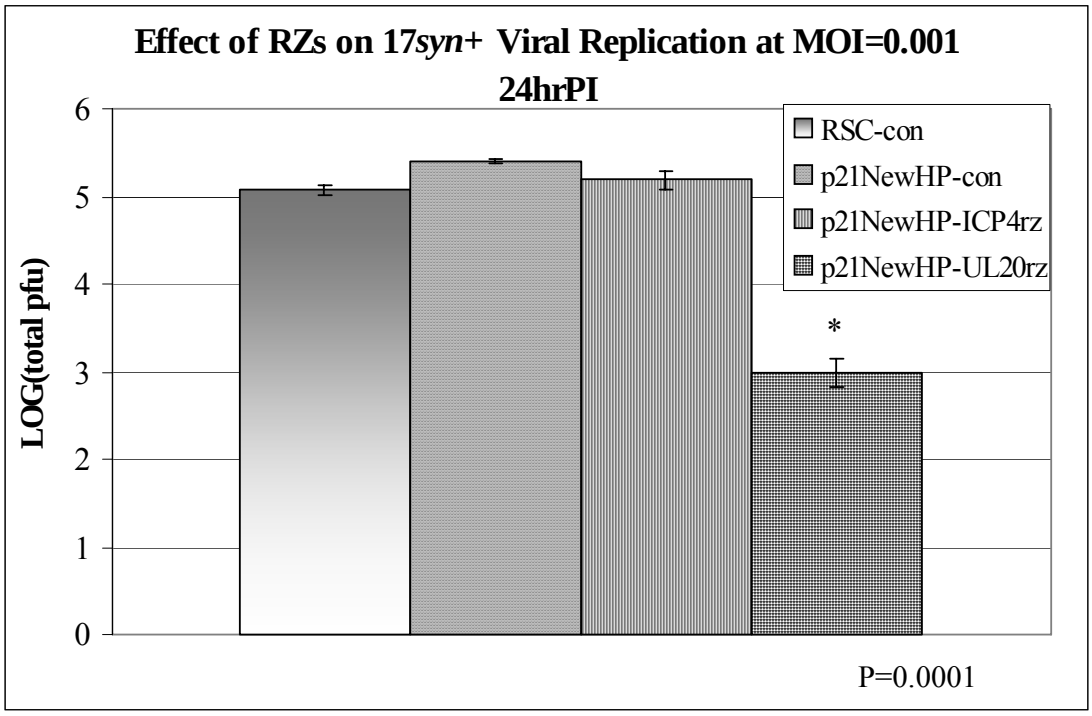


Figure 4-3. Transient transfection of U_L20 ribozyme-154 significantly reduced wild-type herpes simplex virus type 1 (17*syn*+) viral replication. RSC was transiently transfected with a plasmid containing U_L20 ribozyme and the control transfections were a ribozyme targeting ICP4 and a backbone vector without any ribozyme. After brief selection to enrich the transfected cells, cells from each treatment including an untreated RSC group were equally seeded and infected with wild-type HSV-1 (17*syn*+) at a moi of 10^{-3}. Viral production at 24 hours post-infection from each group was compared using plaque reduction assay. There was a 100 fold reduction of viral replication in U_L20 ribozyme treatment group compared with vector only (p21NewHP-con) (with a p value of 0.0001).

A.

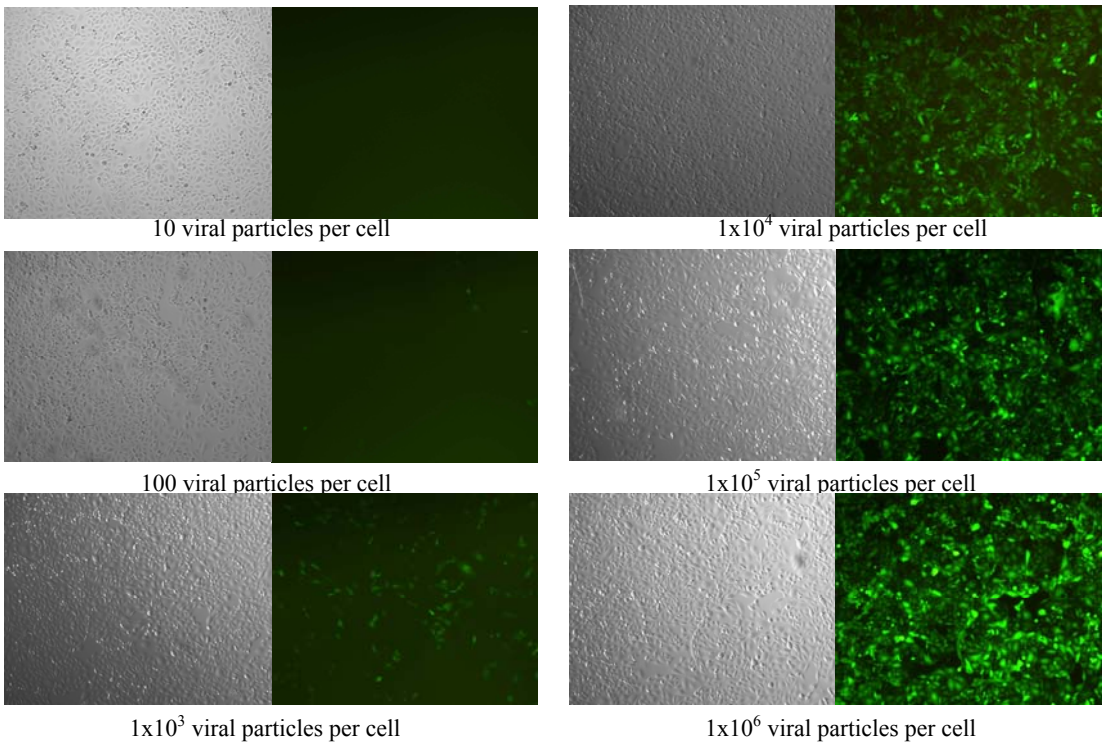


Figure 4-4. Dose-response of adenovirus delivered ribozyme treatments to herpes simplex virus type 1 viral yield. A) Ad-GFP transduction of RS cells at various doses observed through fluorescent microscope. Bright field observations of cells by light microscope were shown on the left, while corresponding doses were labeled under each group of pictures. Pictures were taken at 2 days after adenoviral infection. B) Dose-response indicating an association of increasing level of Ad-Rz (X axis) with a decrease in HSV-1 viral yield (Y axis). Vectored-ribozyme was delivered at various doses showed in X axis, and at 2 days after ribozyme delivery, HSV-1 infection was conducted at a MOI of 10^{-3} for 24 hours before cell lysates were harvested for plaque reduction assay. The infection at each dose of vectored-ribozyme was conducted in triplicate.

B.

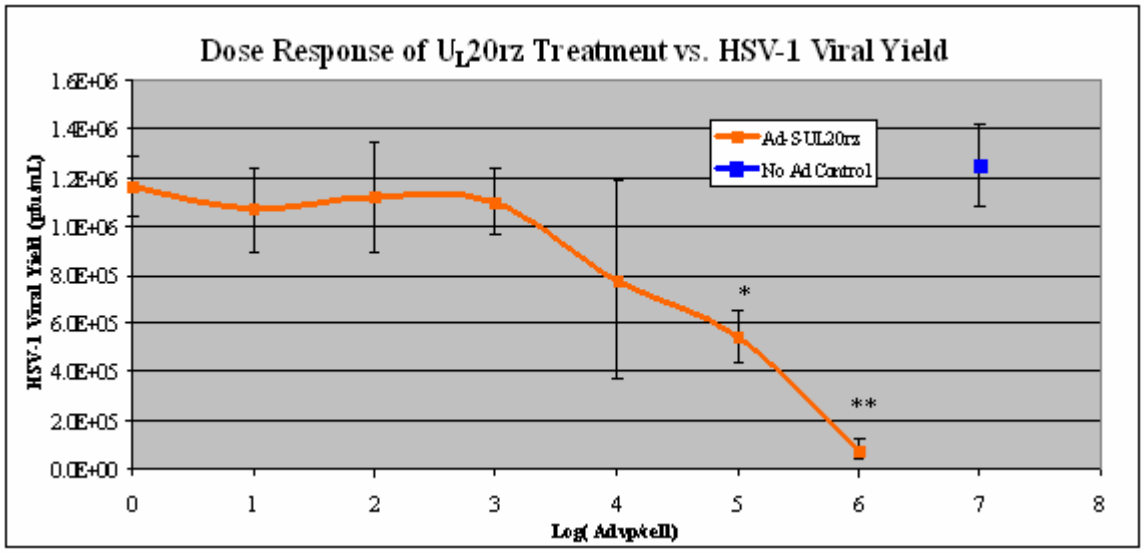


Figure 4-4. (continued)

A.

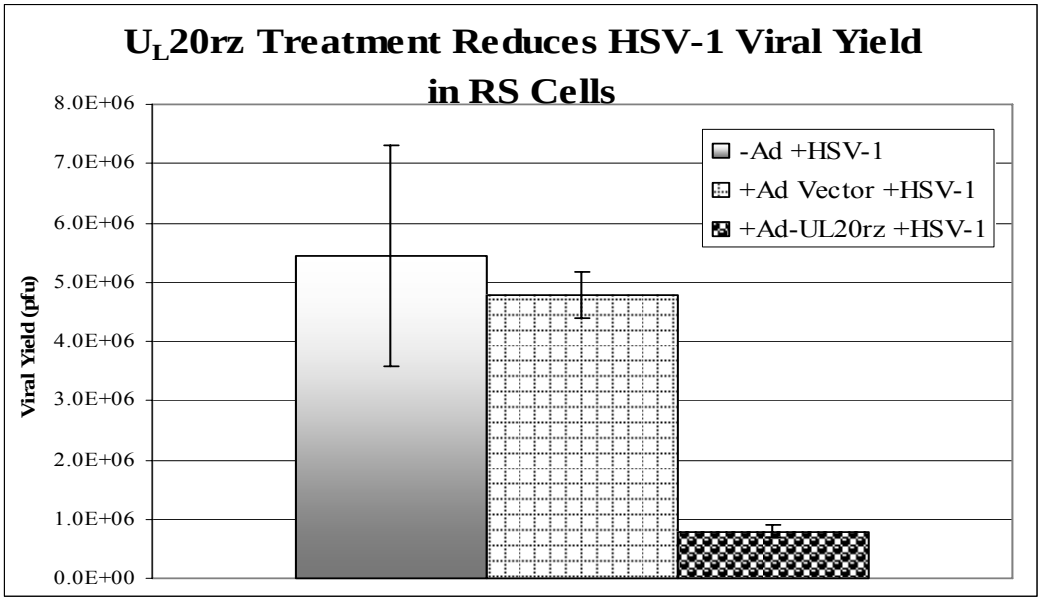


Figure 4-5. Inhibitory effect of U_L20 ribozyme-154 on wild-type herpes simplex virus type 1 viral replication. A) At day one post-infection of HSV-1, U_L20 ribozyme-154 inhibited wild-type HSV-1 viral replication by 83% compared with adenovirus vector (without ribozyme) control treatment ($p<0.001$). Viral yields from "-Ad+HSV-1", "+Ad vector+HSV-1", and "+Ad-Rz+HSV-1" are $5.44x10^6 \pm$(S.D.)$3.24x10^6$pfu/mL, $4.78x10^6 \pm$(S.D.)$6.94x10^5$pfu/mL, and $7.89x10^5 \pm$(S.D.)$1.90x10^5$pfu/mL, respectively. B) A time course study was conducted to address the ribozyme effect on multiple steps of viral replications during longer incubation periods. A comparison of viral productions at day one and six post-HSV infection from ribozyme treatment is shown. Each infection was conducted in triplicate: At 1 day post-infection of HSV-1, viral yield of "No Ad" treatment was $1.01x10^6 \pm$(Standard deviation, S.D.)$1.26x10^5$pfu/mL, viral yields of "Ad vector" and "Ad-Rz" treatments were $7.72x10^5 \pm$(S.D.)$1.98x10^5$pfu/mL and $6.89x10^5 \pm$(S.D.)$6.11x10^4$pfu/mL, respectively. A 10% reduction in viral replication level was observed in ribozyme treated group (Ad-Rz) compared with vector control group (Ad vector) at this time point. At 6 days post-infection of HSV-1, viral yields of "No Ad", "Ad vector", and "Ad-Rz" were $2.20x10^7 \pm$(S.D.)$1.46x10^6$pfu/mL, $1.52x10^7 \pm$(S.D.)$8.33x10^5$pfu/mL, and $5.42x10^5 \pm$(S.D.)$3.63x10^4$pfu/mL, respectively. At this time point, a 96% reduction in viral replication was observed in ribozyme treatment group (Ad-Rz) compared with vector control (Ad vector) ($p<0.00006$).

B.

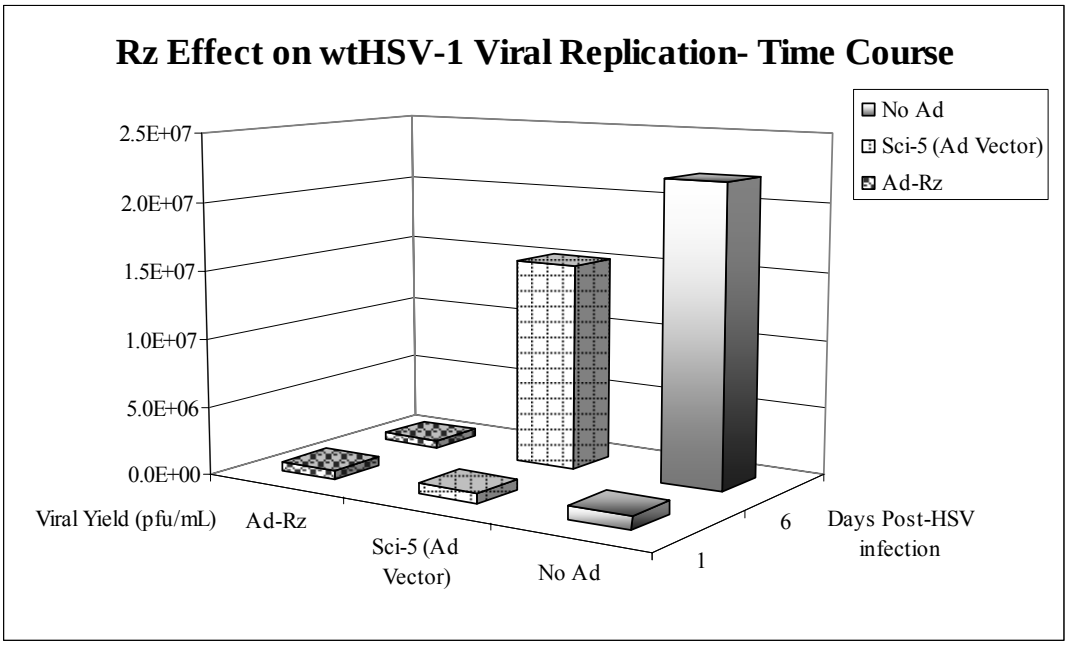


Figure 4-5. (continued)

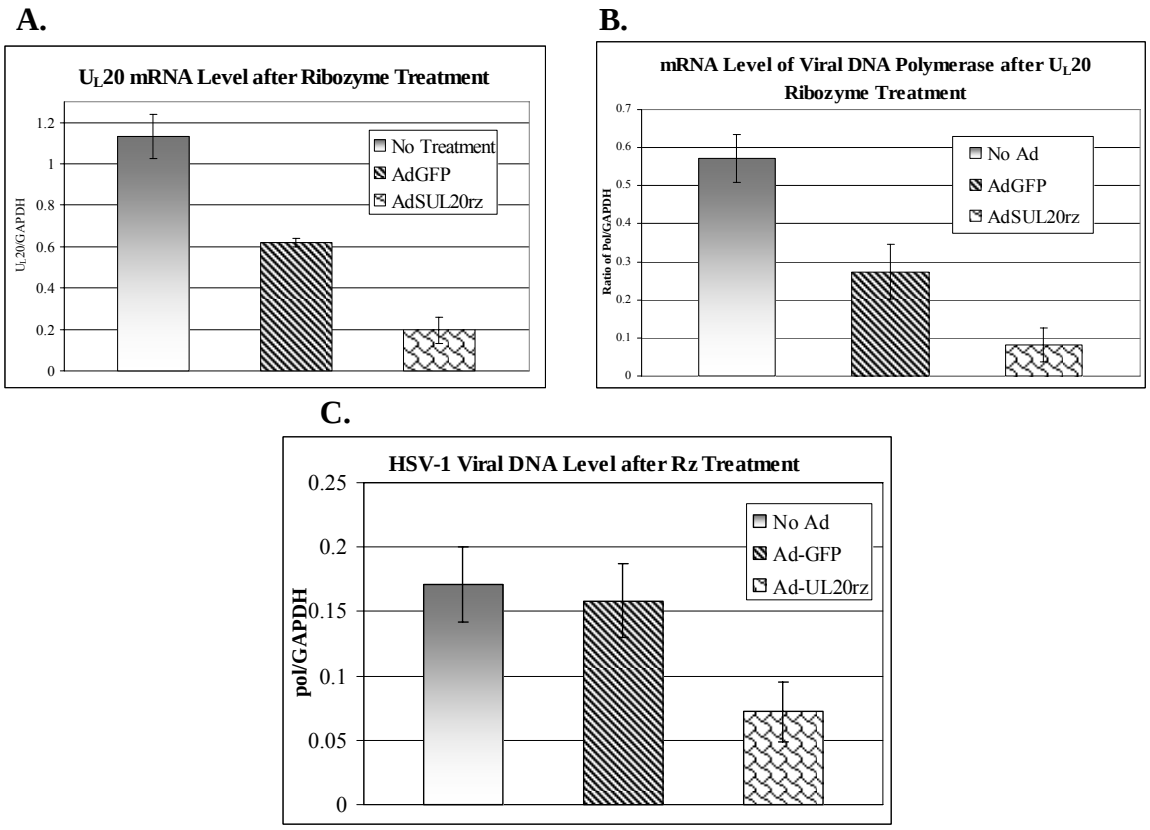


Figure 4-6. Real-time polymerase chain reaction results show the effect of U_L20 ribozyme-154 on viral mRNA and DNA. A) Reverse transcription followed by real-time PCR was conducted to study U_L20 mRNA level. A ratio of viral U_L20 mRNA level to the cellular GAPDH level was used to indicate the abundance of U_L20 mRNA. A 50% reduction in viral U_L20 mRNA level was observed by the "Ad-GFP" treatment compared with that of the "No Ad" control ($p<0.0004$). U_L20 ribozyme-154 treatment (Ad- $U_L20$rz) led to a significant reduction in U_L20 mRNA level compared with Ad-GFP treatment. The reduction is by 68% comparing with Ad-GFP treatment ($p<0.0005$). B) The same set of cDNA was used to studied viral DNA polymerase expression level in each treatment group. A 52% reduction in the expression level of viral DNA polymerase was detected by "Ad-GFP" treatment compared with "No Ad" control treatment ($p<0.006$). Ribozyme treatment led to a significant reduction of 70% in HSV-1 U_L30 expression level, which encodes viral DNA polymerase, compared with "Ad-GFP" treatment ($p<0.0005$). C) A ratio of viral polymerase DNA level to cellular GAPDH level was used to indicate the abundance of viral DNA. There was no significant difference between viral DNA levels from "No Ad" and "Ad-GFP" treatments. The ribozyme treatment ("Ad-$U_L20$rz") led to a 54% reduction in viral DNA level compared with that of the GFP treatment ("Ad-GFP") ($p<0.004$).

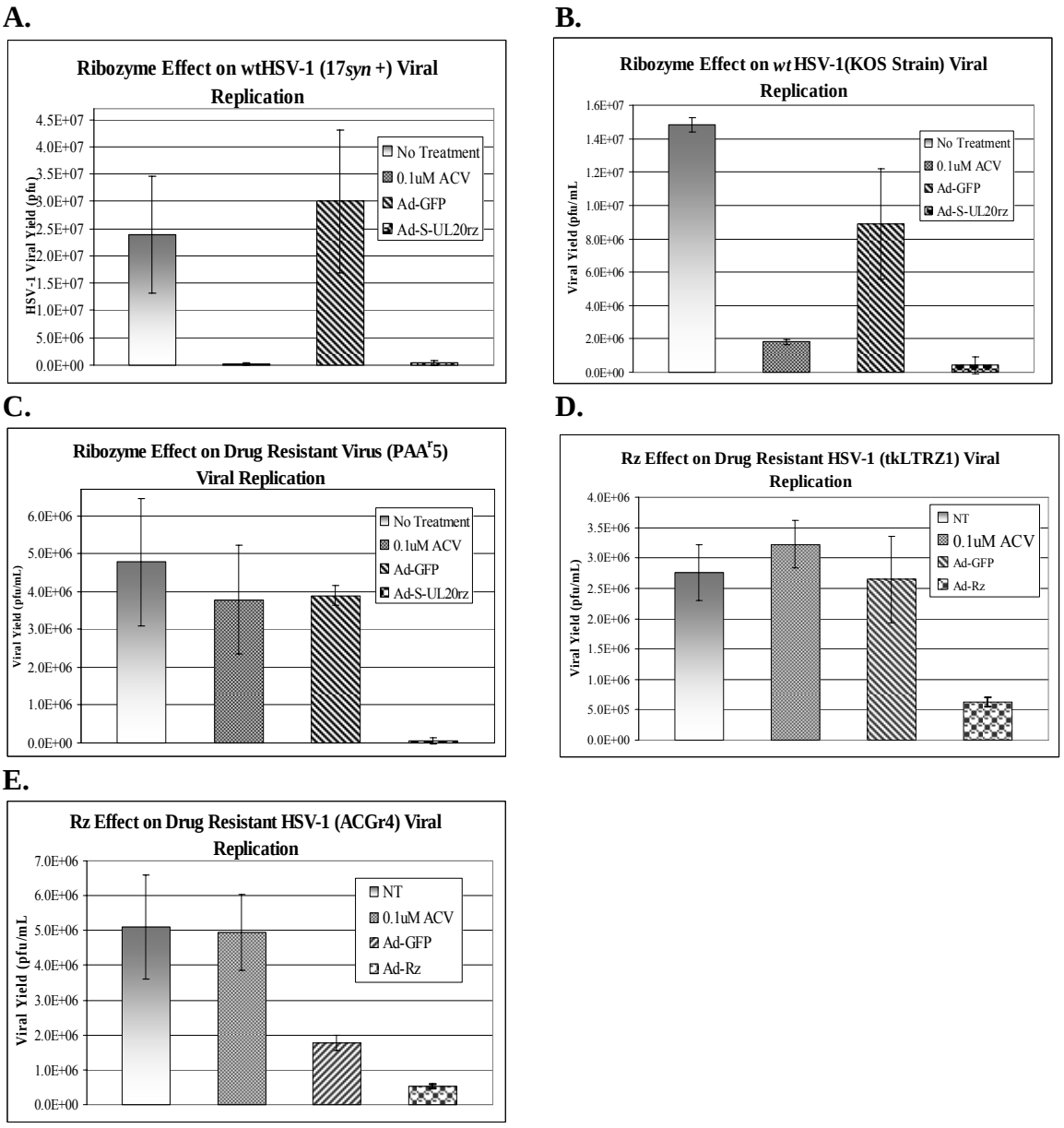
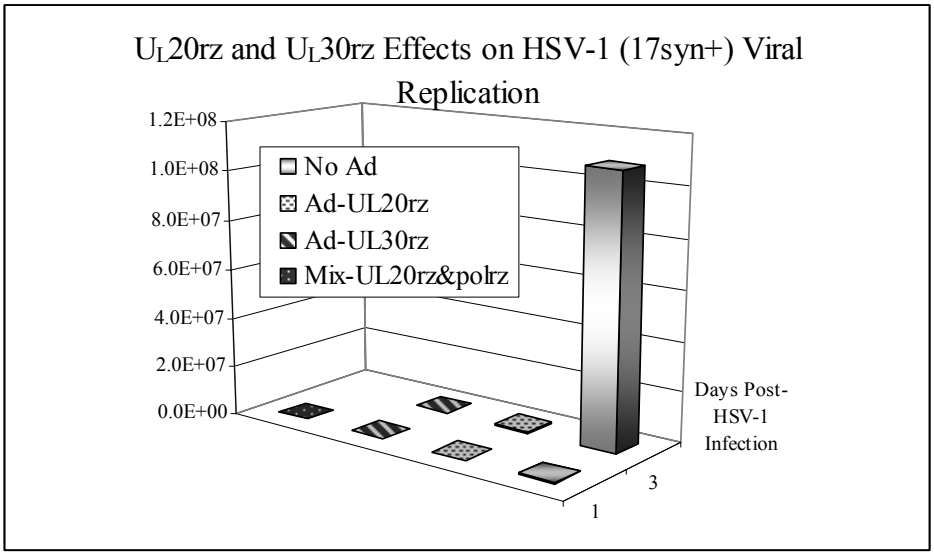


Figure 4-7. U_L20 ribozyme-154 tested against series of herpes simplex virus type 1 strains for inhibitory effects. A) Ribozyme treatment led to a significant reduction (by 98%) in 17*syn*+ viral replication comparing with Ad-GFP treatment ($p<0.002$), while acyclovir treatment had very similar inhibitory effect (99% reduction, $p<0.02$). B) Ribozyme had inhibitory effect on viral replication of HSV-1 strain KOS: 95% reduction was achieved comparing with Ad-GFP treatment ($p<0.05$), while ACV inhibited it by 80% ($p<0.02$). C) HSV-1 drug resistant strain PAAr5 can be inhibited by ribozyme (99% reduction, $p<0.005$) but not by ACV. D) Drug resistant strain tkLTRZ1 viral replication was inhibited by ribozyme by 76% ($p<0.05$), while no effect from ACV. E) Double-mutant ACGr4 viral replication was inhibited by ribozyme by 70% ($p<0.006$), while ACV didn't show any effect.

A.



B.

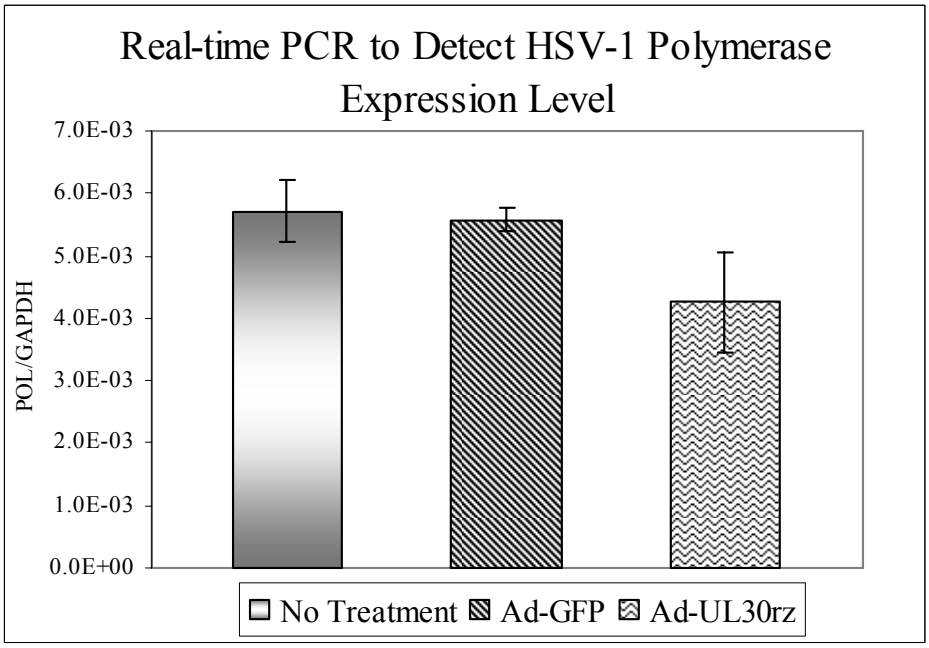


Figure 4-8. Inhibitory effect of U_L30 ribozyme-933 on herpes simplex virus type 1 (17*syn*+) viral replication. A) U_L30 ribozyme-933 treatment led to significant reduction in HSV-1 viral production by 98% ($p<0.01$) at day 1 post-infection and this effect maintained until day 3. $U_L30$rz-933 has very similar effect as $U_L20$rz-154, since $U_L20$rz-154 treatment is a positive control in this assay. Furthermore, a synergistic effect was achieved by combining both ribozymes. B) $U_L30$rz-933 treatment led to a 24% reduction in U_L30 mRNA level ($p=0.05$).

CHAPTER 5
STUDIES OF DELIVERY VECTORS FOR HSK GENE THERAPY

Introduction

Herpes simplex virus keratitis (HSK) is a chronic infection of the cornea by Herpes simplex virus (HSV), which continues to be an important cause of unilateral blindness. Despite considerable progress in the understanding of the virus at cellular and molecular levels, the prospect of prevention still appears to be a long way off.[344] Although it would be ideal to inhibit recurrent infections, probably by selectively targeting latent infected ganglion, or, by establishing surveillance against active viral replication during reactivation, it is difficult to deliver therapeutic agents to neurons and maintain long term protection. However, a therapeutic agent can provide a protection effect at the corneal epithelium to prevent HSV replication. The development of non-toxic topical antiviral agents has been an important step forward in HSK management. Different from traditional antiviral drugs, a gene therapy approach may provide better controlled therapeutic effects.

Viruses that can be considered as gene therapy vectors for HSK are adeno-associated virus (AAV), herpes simplex virus (HSV), adenovirus (Ad). In addition, direct delivery of small molecules using electroporation/iontophoresis is an option.

Adeno-associated Virus Vectors

Adeno-associated virus (AAV) has significant advantages in gene transfer application: It is non-pathogenic, able to transduce dividing and non-dividing cells, and establishes long-term gene transfer in non-dividing cells. However, the small genome

size (approximately 4.7kb) limits packaging capacity. AAV vectors containing capsids from different serotypes can be applied for tissue-specific gene transfer.

Herpes simplex virus (HSV) infection of the cornea, either from primary infection or reactivation, initiates in corneal epithelium. An antiviral agent can be delivered to corneal epithelium to ameliorate active viral replication in order to prevent following damage. Because human epithelium regenerates itself in 10-14 days, a long term gene transfer effect can be achieved by transducing epithelial stem-cells. AAV vectors have the ability to transduce dividing and non-dividing cells which provides an advantage for corneal gene transfer. A gene therapy study using AAV-2 vectors in skin, where cell regeneration has a very similar pattern as to the corneal epithelium, indicates the promise in developing gene transfer in cornea using AAV.[2] However, there are very limited studies to compare the gene transfer efficacy of AAV vectors in cornea. Until 2003, a study of *in vivo* gene delivery to corneal stroma using AAV vector was conducted by Mohan *et al*.[253] This study provided information that AAV can transduce stromal keratocytes, and later unpublished data suggested that AAV5 has better efficacy of gene delivery in stroma than AAV2 (Mohan RR, Schultz GS). For the purpose of a corneal gene therapy, it is important to evaluate the ability of different AAV serotypes to transduce each cell layer of cornea. In this study, a comparison of different serotypes of AAV (AAV1,2,5,7, and 8) was conducted. It showed that AAV vectors could transduce the corneal epithelium, stroma, and endothelium in a very short period of time (7 days). This result indicates that in addition to treating the cornea in patients *in situ*, it might be possible to pre-treat corneal allografts with AAV vectors encoding therapeutic agents in order to prevent disease development after transplantation.

Herpes Simplex Virus Vectors

Herpes Simplex Virus (HSV) is a promising vector for gene transfer applications in nervous system. HSV contains a genome at a size of 152kb which provides an extremely high capacity to accommodate large transgene insertions. Replicating HSV vectors are not suitable for gene therapy due to the toxicity of massive viral gene expression. By eliminating genes necessary for viral replication, the toxicity of the vector can be minimized. Replication defective HSV-1 vector can transduce neuronal cells, and maintain the similar transport behavior as wild-type HSV. When inoculated in peripheral tissues (using subcutaneous inoculation or microinjection in corneal stroma), replicating-defective HSV-1 vectors can undergo retrograde transport to enter the nuclei of neurons. Viral DNA can be maintained as a latency-like episome. By this means, a HSV-1 viral vector can persist and provide long-term transgene expression. As shown in unpublished data from Dr. Bloom's laboratory in Fig. 5-1, an HSV-1 vector containing the LacZ gene was delivered by intrastromal injection in rabbits. At 72 hours post-injection, blue reaction product can be observed in numerous cell bodies (Fig. 5-1-A) and axons in the trigeminal ganglion (Fig. 5-1-C red arrow). In addition to the ability to transduce ganglion cells, HSV-1 vectors can also transduce cells that appear to be corneal limbal cells as shown in Fig. 5-1-B and D. Because of these special characteristics of the HSV-1 vector, it has been employed extensively in gene therapy for neuronal disorders. It may be also applied in preventing HSK by delivering an HSV vector containing the therapeutic transgene to latently infected neurons. It was speculated that by constitutively expressing the therapeutic gene, a protection function might be established to prevent reactivation. However, this hypothesis remains to be tested, given that the mechanism of how previous HSV infection reduced the efficiency of super-infection of

neuronal cells is still unknown. This selective targeting of neuronal cells by HSV vector also requires the bypass of existent immune surveillance from previous HSV infection.

Adenoviral Vectors

Adenoviruses were first isolated from primary cells derived from human adenoid tissue.[147,302] These viruses belong to Adenoviridae family which is divided in to two genera, Aviadenovirus (limited to bird viruses) and Mastadenovirus (including viruses infecting human, simian, etc).[188] Adenoviruses contain a protein capsid surrounding a DNA core, which is composed of the linear double-stranded DNA and four viral proteins. The carboxyl-terminal domain of adenoviral fiber protein is responsible for the binding of the cellular receptor for the step of adsorption. The coxsackievirus and adenovirus receptor (CAR), a member of immunoglobin family, is a high-affinity receptor for human adenoviruses (except for subgroup B).[296] Cellular integrins serve as binding paterners of penton base proteins of adenoviruses which mediate the internalization of virions. The cellular receptors of viral fiber and penton proteins determine the tropism of adenoviruses. Adenoviral genomes contain early and late viral genes: Activation of early gene expressions leads to the S phase entry of host cells, the protection against host antiviral responses, and the preparation for viral DNA replication. These functions are achieved majorly by E1A gene product which is the first gene expressed after the viral genome enters the nucleus. At the onset of adenoviral DNA replication, late genes are expressed efficiently which is controlled by the major late promoter through a strong activation of E1A proteins. Late gene products block the cellular mRNA transport and lead to the preferential translation of viral mRNA; meanwhile they set the stage for virion assembly. Studies of adenoviral infection have led to the molecular understanding of many fundamental cellular events, including transcription[12] and splicing[26], and they also led to

identifications of regulatory proteins for cell cycles. In addition, adenoviruses have been developed as vectors for gene therapy applications.

The first generation adenoviral (Ad) vector contains E1-deletion. Although it is replication defective, it still expresses viral genes in the infected cells. Transduction of tissues with the first generation vectors lead to a rapid development of immune response which drastically limits the transduction efficiency and the duration of transgene expression. The pre-existing immunity against adenovirus vectors in majority of human population also has impeded their clinical application. In animal studies using E1-deleted adenovirus vectors, it has been shown that high systemic dose induced acute inflammatory responses. The cornea is an immune-privileged tissue, but transduction of corneal cells using adenovirus vector may not be an ideal approach due to high immunogenicity of the first generation Ad vector. Corneal epithelium cells cannot be transduced by adenovirus without physical damage of superficial cell layer, while intrastromal injection of adenovirus vectors may induce a severe immune response.

To test the *in vivo* effect of U_L20 ribozyme, a mouse foot-pad model for HSV-1 infection was adopted for this study. Adenovirus vector was used to deliver U_{L20} ribozyme expression in footpad by both subepidermal injection and topical application. A minimal inflammatory response was expected due to the low density of blood vesicle in mouse foot-pad. Wild-type HSV-1 foot-pad infection in outbred Swiss ND4 mice leads to the transport of replicating virus through nerve termini to dorsal root ganglia and to central nervous system (CNS). When a LD_{50} dose of wild-type HSV-1 is applied, in 8 to 14 days post-infection indication of HSV-1 replication with CNS involvement can be observed. Clinical syndromes including hind-limb paralysis, huddled behavior, lethargy

and ruffled fur can also be detected. Mice need to be euthanized when theses symptoms are more pronounced. If HSV-1 viral replication can be reduced by ribozyme, mice will show reduced clinical indications of CNS involvement.

Iontophoresis Delivery of Oligonucleotides

Iontophoresis is a non-invasive delivery method in which ionized drug molecules penetrate in tissue enhanced by a small electric current A low voltage (typically 10 V or less) or continuous constant current (typically 0.5mA/cm^2 or less) is applied to push a charged drug into tissue. This technique has been used clinically to facilitate drug penetration. A very similar technique, called electroporation, applies a higher voltage pulse (typically higher than 100V) for a very short (μs-ms) period of time to permeate the tissue, very often skin, and it is under intense study for clinical applications.[18] Iontophoresis in the ocular field was extensively studied and used during the first 60 years of the twentieth century. However, ocular iontophoresis was not initially accepted as a standard procedure for drug delivery due to the paucity of toxicity data and the lack of carefully controlled trials. Recently the development and optimization of the technology has led to the safe delivery of high drug concentrations by ocular iontophoresis.[262]

The transport mechanism of iontophoriesis includes three parts: "*Nernst-Planck effect*", "*electroosmotic flow*", and "*damage effect*". The "*Nernst-Planck effect*" represents the central tenet of iontophoresis that charged substances are driven into the tissue by electrorepulsion. At the anode, positively charged drug is repelled while at the cathode, negatively charged molecules are pushed into tissue. Electroosmotic flow, first demonstrated by Gangarosa and Burnatte[42,111], is the bulk fluid flow which can deliver neutral species when a voltage difference is imposed across a charged membrane.

"*damage effect*", the third mechanism, is the effect caused by electric current to the tissue which increases the permeability, indirectly enhancing drug penetration.[287] The iontophoresis device contains a direct current power and two electrodes, and there are two approaches for drug retaining. The most common approach is to use an eye cup continuously infused with drug solution, while another component holds the electrode and aspirates air bubbles that disrupt the current. The ground electrode is attached to patient body, in animal very often to the ear, as close to the former electrode as possible to reduce the resistance. There have been various devices developed in this matter.[23,137,371] The second approach is the use of a drug saturated gel in direct contact with the cornea; however, this approach was abandoned due to side effects caused by agar-gel residue. In the last a few years, the development of drug-loaded hydrogel for ocular iontophoresis leads to applications of novel applicators using drug-saturated gel approach, e.g., OcuPhor™ hydrogel (Iomed Inc., Salt Lake City, UT) for transscleral iontophoresis[275,372], Visulex™ (Aciont Inc., U.S.A), and a poly acrylic-porous hydrogel designed by Eljarrat-Bin-stock and Frucht-Pery for transcorneal and transscleral iontophoresis.[97-99,293]

In vivo delivery of oligonucleotides represents the frontier in drug development with the elevated therapeutic applications using antisense, ribozyme or siRNA. Stability is always the major concern when considering delivery of oligonucleotides in tissue as a routine treatment. The industrialization of oligonucleotide production allows the generation of synthetic oligonucleotides with chemical modifications which lead to improved stability against cellular degradation. In this study, chemical modified ribozyme RNA molecules were applied for the *in vivo* study.

Hammerhead ribozymes have a catalytic motif containing 15 conserved nucleotides from which three helices with variable length radiate. Mutations in this motif will impair the catalytic function of the ribozyme. Certain chemical modifications have deleterious effect on ribozyme function by disturbing the structures (noncanonical base pairs, hydrogen bonds, tertiary structures, and aromatic stacking interactions) essential for cleavage action.[30] A large collection of data has been generated to provide references for modified nucleotide substitutions, which can be categorized in three groups: modifications on base group, on the 2'-hydroxyl, and phosphate oxygen. Although there have been detailed systemic studies defining relation between function and modifications[30], most of the chemical modifications and positions of the modification in this study came from experimental experience. According to data of Beigelman et al, 1995[24], ribose residues essential for ribozyme catalytic activity are located at the purine sites G5, A6, G8, G12, and A15.1 (as shown in Fig. 5-3). Therefore, no 2'-ribose modification was recommended at those sites. It was reported that substitution of U4 and U7 by 2'-amino nucleotides can maintain the wild-type catalytic level while improving the nuclease resistance.[139] Finally, the addition of an abasic nucleoside at the 3'-end and phosphorothiaotes in the 5'-end in conjunction with 2'-sugar modifications served as stabilizing elements without substantial effect on catalysis.[139,140]

Materials and Methods

Establishing a Rabbit Model for HSV Ocular Infection

New Zealand white rabbits were used to establish HSV-1 acute or latent infections. The animals were anesthetized by isofluorane inhalation; the corneas were numbed with topical proparacaine drops and were scarified with a needle tip to make breaks in the epithelium. The cornea was then inoculated with 25 μL of the 17 *Syn*+ strain of HSV-1.

At various time points during the study, the rabbits were examined at the slit lamp to detect the infection of the cornea during the primary infection or the reactivation. For this purpose, the rabbits were placed in a standard rabbit restraint box. Their eyes were anesthetized with 2 drops of proparacaine (0.5%, Alcon, Ft. Worth, TX) and a lid speculum was used to retract the eyelids. A standard clinical slit lamp that is used to examine human eyes was used to examine rabbit corneas. Conjunctival cultures were also obtained by swabbing with cotton tipped applicator to detect viral shedding.

After the acute infection subsided, the ability to reactivate the latent virus was tested by induction of shedding by iontophoresis using dilute epinephrine (1:1000). Rabbits were sedated with isofluorane inhalation. The iontophoresis was done by placing electrodes on the cornea and ear of the rabbit and passing a low voltage electric current of 0.8mA for 8 to 10 minutes (as shown in Fig. 5-2). Once the iontophoresis procedure was finished, the animals were given oxygen to recover from sedation and returned to their cages. Two to three days after iontophoresis, recurrent HSV-1 infection could be observed.

Study of Corneal Tropism of AAV Vectors

Delivery of adeno-associated virus vectors to rabbit cornea

New Zealand white rabbits were anesthetized by isofluorane inhalation, and rabbit corneas were treated with topical proparacaine drops followed by an excimer ablation (superficial circular or crosshatched abrasion) of the epithelium (partial thickness to about 25 microns). $2x10^{11}$ AAV particles were applied on the surface of cornea through an eye cup (as shown in Fig. 5-2B) for 10 minutes. This preliminary study was conducted on 6 rabbits with one serotype per animal including one untreated control, and the treatments are listed in Table 5-1. Rabbits were returned to their cages after they

woke up. Seven days were allowed for transgene expression, and rabbits were sacrificed to collect corneas. Rabbit corneas were fixed in 4% paraformaldehyde then embedded in Tissue Tek OCT Compound Embedding medium (Sakura Finetek, Torrance, CA) and frozen by dipping into isopentane cooled by liquid nitrogen. Tissue sectioning was performed with a Microm H550 cryostat (Microm, Walldorf, Germany) and 10-12μm sections were mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) for immunohistochemistry in OCT for cryostat (frozen sectioning). Tissue sectioning was conducted at a thickness of 10-12μm and prepared for immunohistochemistry studies.

Immunohistochemistry analysis of adeno-associated virus vector tropism in the cornea

Frozen sections were dried at the room temperature before fixation for 1 minute in fixative containing a 1:1 ratio of acetone and methanol (Fisher Scientific, Fair Lawn, NJ). After rinsing 3 times with Phosphate-buffered Saline (PBS, the recipe see Appendix C), slides were pretreated with 0.3% hydrogen peroxide (diluted from 3% hydrogen peroxide in methanol) for 30 minutes followed by PBS rinse for 4 times. Sections were treated using R.T.U. Vectastan Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA) following manufacturer's protocol. Sections were circled using a Liquid-repellent Slide Marker Pen to reduce the area exposed to antibodies. After treated with serum for 20 minutes at room temperature, sections were rinsed with PBS for one time meanwhile the primary antibody was prepared. The primary antibody, Chk X GFP (Chemicon International, Inc., Temecula, CA), was diluted to 2000 fold using PBS in the presence of 0.1% BSA (LabScientific, Inc., Livingston, NJ), 0.05% Triton. A control solution without first antibody was prepared as well. Slides were placed in slide mount and with

extra water in the chamber to keep them moist; the primary antibody dilution was added to cover each section as well as control treatment groups (no AAV and no primary antibody groups), and 30 minutes to 2 hours were allowed for the primary antibody binding. Slides were washed with PBS three times, while the secondary antibody was diluted. Anti-chicken IgY (Promega, Madison, WI) was diluted 1000 fold in PBS. Secondary antibody was added to cover each section and incubated for 2 hours at room temperature before rinsing three times with PBS. Sections were treated with stabilized Elite ABC reagent (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature before another PBS rinse. Nova Red staining (Vector NovaRed SUBSTRATE Kit for Peroxidase, Vector Laboratories, Burlingame, CA) was conducted following product instructions, and sections were dehydrated in an ethanol series of 75%, 80%, 90% and absolute ethanol then air dried. At the end, sections were mounted using mounting medium (Vectashield Hard+set^{TM} Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA). Sections were observed under Zeiss Axioplan2 Fluorescence microscope or Morphometric microscope (MCID).

Because endogenous hydrogen peroxidase was detected even after the treatment of 0.3% hydrogen, an alternative immunostaining protocol, which uses alkaline phosphatase system, was also conducted. Vectastain ABC-AP Kit, Alkaline Phosphatase Substrate Kit, Vector RED, and Levamisole solution (Vector Laboratories, Burlingame, CA) were used for immunostaining. A confocal microscope (Leica TCS SP2 AOBS Spectral Confocal Microscope, Leica Microsystems, Inc., Bannockburn, IL) was used to observe the fluorescence, and images were processed using software LCS Version 2.61 Build 1537.

Progress in Testing HSV Vector for Delivery in Cornea and Trigeminal Ganglion

Delivery of non-replicating herpes simplex virus type 1 vector in rabbit cornea

New Zealand white rabbits were anesthetized by isofluorane inhalation, and rabbit corneas were treated with topical 1% proparacaine drops. Scarification was done with a sterile needle, and a double-hatch on the epithelium was made with care to avoid wounding the stroma. $2x10^5$ pfu of wild-type HSV-1 (17*syn*+) was applied directly on the corneal surfaces of both eyes while the rabbit eyes were held open by pulling on the eyelids. Then the eye were closed and gently rubbed. Corneal infection could be observed by slit lamp biomicroscopic examination on the second day and ocular inflammation was observed on third day post-infection. Establishment of latency was confirmed by epinephrine iontophoresis as described above. At 4-month post-infection, a second HSV-1 infection was conducted using a non-replicating HSV-1 vector and intrastromal injection in the right eye. This HSV-1 vector, called $8117/43^{92}$, was constructed by removing ICP4 genes and inserting a LAT/LTR promoter driven LacZ gene fragment into the original ICP4 region. Four days post-infection, rabbits were euthanized and corneas were harvested and fixed in 4% paraformaldehyde for β-galactosidase staining. In this experiment, the left eye of each animal was the negative control for β-galactosidase staining. Blue color formation following incubation with X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) indicated a successful delivery of transgene expression by HSV-1 vector (8117/43).

Protection from previous ocular infection against subsequent herpes simplex virus type 1 super-infection

To study the effect of previous HSV-1 ocular infection on following herpes simplex virus type 1 (HSV-1) superinfection, another set of assays were conducted.

Instead of infecting the animal with wild-type HSV-1, a replication-defective HSV-1 vector, KD6[92,314], constructed by ICP4 deletion, was used for the first infection. A group of four rabbits were included in this experiment. KD6 was delivered to the left eye by intrastromal injection into the rabbit cornea. The existence of KD6 in trigeminal ganglia can be proved by PCR using primers complementary to ICP4 deletion junction. After 2.5 months of inoculation of KD6, the second infection of replicating HSV-1, dUTPase/LAT[164], was applied to both eyes by simply applying virus solution to the scarified rabbit cornea. The dUTPase/LAT was constructed based on 17*syn*+ parental strain by substitutional insertion of the dUTPase promoter and LacZ reporter construct into the region of LAT promoter and 5' region of LAT. Rabbits were terminated on the fourth day of post-infection and corneas were harvested for β-galactosidase staining. The purpose of this assay was to study the effect of HSV-1 vector on subsequent infections.

Antibody neutralization assay

This assay was developed to detect antibodies directed against HSV-1 in serum samples. One day before the assay, Rabbit Skin Cells (RSC) were plated in 24-well-plates to reach ~90% confluency on the day of usage. Blood samples were drawn from naïve (negative control) and HSV-1 infected rabbits, and blood samples were clotted followed by serum removal and complement "inactivation" by heating at 56ºC for 30 minutes in a water bath. These serum samples can be stored indefinitely at -80ºC. A HSV-1 virus stock was diluted to a concentration of $1x10^6$pfu/mL, and 0.1mL of the dilution was used in the neutralization mixture. Neutralization dilutions were set up according to the anticipated range of HSV-1 neutralizing titers. There were three groups of rabbits: naïve (negative control), rabbit ocularly infected with wild-type HSV-1 (17*syn*+) as a positive control, and rabbits ocularly infected with KD6 (non-replicating

HSV-1 vector). Serum samples were diluted as 1:5, 1:10, 1:100, and 1:1000 in MEM supplemented with 5% Calf Serum and antibiotics, and the final volume of all dilutions of serum was 0.9mL. 0.1mL of the HSV-1 stock dilution was added to each of the serum dilutions, which were incubated at 37ºC for 1 hour after mixing well. At the same time a 4.95mL of cold regular medium (5% Calf Serum containing MEM) was prepared for each sample and placed on ice. At the end of the incubation, 50µL of each reaction was diluted in corresponding 4.95mL of cold medium and mixed thoroughly to end the neutralizing reaction. The rest of reactions were stored at -80ºC. After removing the culture media from the RSC in the 24-well-plates, 0.2mL of each diluted neutralizing reaction was plated in each well and for all the samples this was done in triplicate for each of the samples. One hour at 37ºC was allowed for absorption as in a standard plaque reduction assay, and plates were gently rocked at every 30 minutes. After discarding the infection solution, RSCs were overlaid with 2mL of warm media (5% Calf Serum MEM with antibiotic and 0.3% Human Gamma Globulin). Two to three days were allowed for plaque development and cells were stained with crystal violet.

Proof of Principal Experiment: Testing Adenoviral Vector Packaged Ribozyme in an HSV-1 Acute Infection Model in Mice

Ribozyme inoculation and HSV-1 infections in HSV-1 mouse footpad model

Four to six-week-old female Swiss mice (ND-4) were used for footpad experiments. Three groups of 10 mice each were treated with either PBS (mock), Adenovirus-control (an adenovirus packaged GFP control treatment), or adenovirus packaged- U_L20 ribozyme. Mice were first anesthetized with Halothane™ or isofluorane by inhalation and Flunixin meglumine (1.1 mg/kg IM) was administered to alleviate any pain associated with the procedure. To minimize the amount of abrasion and facilitate

efficient uptake of HSV-1 infection, both footpads of each mouse were injected subepidermally with 25-50μL of 10% saline 4 hours prior to HSV-1 infection. For adenoviral vector-treated groups (Ad-GFP or Ad-ribozyme), each saline injection contained $1.4x10^{10}$viral particles. The saline pre-treatment was necessary to establish an efficient and uniform infection of the sensory ganglia. In addition, it reduced the amount of abrasion that was needed to be performed on the foot prior to infection. It is also believed that saline pre-treatment reduces discomfort. At the time of HSV-1 infection, the mice were anesthetized by intramuscular (IM) injection of 0.010 – 0.020 mL of a cocktail of acepromazine (2.5 – 3.75 mg/kg), xylazine (7.5 – 11.5 mg/kg), and ketamine (30 - 45 mg/kg). The ketamine/xylazine/acepromazine cocktail treatment was important for the success of HSV-1 infection, as it provided enough time for virus to absorb (30 minutes for 80 – 90% efficiency) before mice recovered and moved around. Both rear footpads of the anesthetized mice were lightly abraded with an emery board to scratch the keratinized layer of the skin to allow the virus to adsorb efficiently while a same volume of each treatment was applied to the dorsal surface of the footpad (10μL of PBS, Ad-GFP, or Ad-ribozyme solution respectively were used, if adenoviral vector was applied, $1.4x10^{10}$viral particles were included). Then the anesthetized mice were rested on their backs, and 50μL of the virus dilution containing 10^4pfu of HSV-1 strain 17*syn*+ was placed on the footpad using a pipette. After 45- 60 minutes mice recovered from anesthesia and were returned to cage for observations. For a survival study, mice were checked for illness and death everyday up to 12 days.

Quantitative real-time polymerase chain reaction to estimate viral replication level

To evaluate the protection effect of the ribozyme, a time-course study was set up. By sacrificing 4 mice per treatment (ribozyme or GFP treatment) per time point (2, 4, and

6 days post- HSV infection), tissues were collected from spinal cord (SC), dorsal root ganglia (DRG) and feet.

Tissues were ground for homogenization using different tools. For large tissue (e.g., both feet, average weight is 0.7mg), a mortar was used, while different sizes of glass homogenizers were used for neuron or brain tissues, e.g., 1mL glass homogenizer was used for DRG, and SC.

Total RNA was extracted using Trizol® Reagent (Invitrogen™, Carlsbad, CA) and was prepared for reverse-transcription (see Material and Method in Chapter 4) followed by real-time PCR to compare viral gene expression as well as viral DNA level. Real-time PCR primers were designed by ABI system (Applied Biosystems, Foster City, CA). HSV-1 U_L20 expression, U_L30 (viral polymerase) DNA levels, and U_L54 DNA levels were compared between the ribozyme treatment group and the controls at each time point. Taqman primers and probe were designed by ABI system for mouse GAPDH gene expression which can also be used for quantification of genomic DNA.

The DNA was also isolated from the same samples from Trizol® extraction after taking away the aqueous layer which contained total RNA. 150µL of solution containing 0.1M Tris-Cl (pH7.5) and 0.1% of sodium N-lauroyl sarcosine (Sigma, St. Louis, MO) was added to the tube which contained the interphase and organic layer of Trizol® reagent. The tube was vortexed for 30 second followed by centrifugation at 9000xg for 1 minute. The supernatant was collected in a fresh tube, and this step was repeated for two more time. The supernatant from these steps was combined and treated with 5µL of proteinase K (20mg/mL) at 37°C overnight. The next day, 3 times of PCI (Phenol/Chloroform/iso-amyl alcohol at a ratio of 25:24:1) extractions were conducted

followed by ethanol extraction to pellet DNA. The DNA pellet was rinsed using 70% ethanol and air-dried before resuspending in de-ionized water. A conventional PCR was conducted for these DNA sample using a set of PCR primers designed for mouse β-actin (Primer sequences are: Sense primer: 5'-TGAGACCTTCAACACCCCAGCC-3'; anti-sense primer: 5'-TGGCCATCTCCTGCTCGAAGTC-3'.). The PCR was conducted as following condition: 94°C for 5 minutes; 25 cycles of 30 seconds of 94°C, 30 seconds of 55°C, and 30 seconds of 72°C; 72°C for 10 minutes; finally 25°C infinitely. This step was to confirm the quality of DNA sample. The DNA samples were used for real-time PCR to detect viral DNA level (using Taqman primers and probe designed for HSV-1 polymerase) and the DNA level of Xist gene (X inactive specific transcript) or GAPDH was also detected as internal control (all the mice in this study were female).

Standard curves for each set of primers and probe were plotted by using a series of dilutions (10ng, 1ng, 10^{-1}ng, and 10^{-2}ng, etc) of the reference DNA sample. The reference DNA was extracted from the spinal cords of 6 Swiss ND4 mice which had been infected with wild-type HSV-1 strain 17*syn*+. Mouse spinal cords were dissected and each was ground in 0.2mL of ice cold TES (10mM Tris, pH 7.4; 0.1M NaCl; and 1mM EDTA). Homogenized tissue then was treated at 50°C overnight with sodium dodecyl sulfate (SDS) to a final concentration of 1% and Proteinase K to 1 mg/ml. The next day, samples were extracted using PCI for three times followed by ethanol extraction to precipitate the DNA from the aqueous fraction. DNAs were resuspended and combined in de-ionized water, DNA concentration was estimated by spectrometry of the absorbance at 260nm wavelength. Serial dilutions of the reference DNA were stored in -80°C in aliquots.

Iontophoresis of Chemical Protected Synthetic RNA Molecules in an Acute Ocular HSV-1 Infection Model in Rabbits

Design of chemical modifications in hammerhead ribozyme RNA molecule

Natural ribozyme RNA molecules are not stable *in vivo.* In order to achieve a successful delivery of ribozyme molecule independent of delivery vehicles, chemical modifications can be applied to synthetic ribozyme to increase the stability while retaining its catalytic activity. The design of chemical modifications in hammerhead ribozyme needs to maintain the integrity of the catalytic core. Not only mutations in this motif will impair the catalytic function of the ribozyme, but certain chemical modifications have deleterious effect on ribozyme function.[30] According to references from a large collection of data generated for modified nucleotide substitutions, the chemical modifications and positions of the modification in this study were determined. As shown in Fig. 5-4, most of nucleotides have 2'-O-methyl modifications except for G5, A6, G8, G12, and A15.1, while U4 and U7 have 2'-amino residues. An abasic nucleoside was added to the 3'-end and the 5'-end contained series of phosphorothiaotes. By replacing G5 to C5, an inactive hammerhead ribozyme was generated as a control for catalytic activity.

Iontophoresis of synthetic chemical protected ribozyme for treatment of herpes simplex virus type 1 infection in rabbit

Chemically modified active and inactive hammerhead ribozymes for U_L20 were synthesized by Dharmacon RNA Technologies (Lafayette, CO) at a scale of 0.2μmol. Sequences of ribozymes and the design of modifications are shown in Fig. 5-4. A superficial crosshatched abrasion of the epithelium (partial thickness was about 25 microns with a pattern of three lines vertically and three lines horizontally) was conducted followed by iontophoresis of the ribozymes into the cornea. Each rabbit was

treated with active ribozyme in right eye and inactive control in the left with a total amount of 100μg of oligonucleotides in 1mL of solution per eye. Oligonucleotides were de-protected according to the manufacturer's protocol and were resuspended in deionized water (DI water). A small current of 0.8mA was applied for 8 minutes to deliver chemical modified RNA into corneal tissue, and the set up of iontophoresis apparatus is shown as in Fig 5-2-A and B. Since the oligonucleotide used in this study was negatively charged, the cathode was placed in the eye cup. 10^5pfu of HSV-1 replicating virus containing the LacZ gene (dUTPase/LAT^{164}) was applied on rabbit eyes half an hour later. 4 days were allowed for HSV-1 infection to develop before harvesting rabbit corneas for X-gal staining. Images were analyzed using SigmaScan® Pro (Systat Software, Inc., Point Richmond, CA) to quantify the areas of blue staining.

Results

Adeno-associated Virus Vector Tropism in Cornea

All serotypes (type 1,2,5,7, and 8) of adeno-associated virus led to GFP expressions in the rabbit cornea at 7 days post-inoculation as indicated by the immunostaining using GFP antibody. Images were taken from each section using morphometric microscope. The intensity of GFP staining in each section was compared using MCID program. Representative images of GFP staining from rabbits' eyes treated with different AAV serotypes as well as control eyes are shown in Fig. 5-4 A to F. Fig.5-4-G shows the intensity comparison of GFP staining on corneal epithelium from different AAV serotypes treated rabbits. In this assay, no GFP expression could be visualized directly under a fluorescent microscope from any AAV treated eyes, indicating a low level of transgene expression at day 7. AAV1, followed by AAV8, had a slightly higher transduction level on corneal epithelium compared with the others. Although AAV5 did

not show strong staining of the epithelium, when observed for GFP staining across a corneal section, it showed strong penetration of GFP expression even in the monolayer of endothelial cells (as shown in Fig. 5-5-D). However, the small sample number (one rabbit two eyes) in each treatment may limit the representation of the observation. Future experiments to observe AAV transgene expression at a longer period will provide more information. A time course study will be recommended to follow the transgene expression. In addition, since self-complementary AAV has been show to lead to earlier expression of passenger genes; it might be useful to test these vectors in the cornea.

Herpes Simplex Virus Vector Delivery to Cornea and Trigeminal Ganglion

Four months following bilateral infection of rabbit corneas using wild-type HSV-1 (17*syn*+), re-infection of the right eye using a non-replicating HSV-1 expressing LacZ (strain 8117/43[92]) did not lead to formation of dendrites when they were stained for β-galactosidase activity, as shown in Fig. 5-6-A (negative control) and B. Although one or two dots of blue staining were observed in super-infected (the right) cornea (Fig. 5-6-B**)**, at the infection dose of $2x10^5$pfu per eye, it was obvious that the second infection did not lead to efficient transduction, compared to the positive control of a naïve rabbit using the same virus (shown in Fig. 5-1-B**)**. The second experiment was set up using an opposite sequence of infection: Primary infection was conducted in the left eyes by intrastromal infection using a non-replicating HSV-1 strain KD6, and two and half months later, the second infection of replicating HSV-1 containing LacZ (dUTPase/LAT) was performed bilaterally. Four days were allowed for viral replication before corneas were processed. X-gal staining results for detection of β-galactosidase activity in this experiment are shown in Fig. 5-6-C and D. The left eye which was treated previously with KD6 followed by dUTPase/LAT had dramatically reduced blue staining compared with the

right eye which was infected only with dUTPase/LAT. This indicated a protection effect against the later infection of a different viral strain rendered by the previous infection of HSV-1 vector. However, this protection was not systemic: First, as shown in Fig. 5-6-C and D, previous infection in the left eye only protected the same eye from second infection, while the right eye had massive dendrite forming caused by second infection. Second, an antibody neutralization assay was conducted to detect the level of systemic antibody against HSV-1 after the first infection of KD6. This was to confirm whether this ocular protection was related to circulating antibody. The antibody neutralization assay showed (Table 5-2) that the ocular inoculation of non-replicating HSV-1 (KD6) only led to mild production of antibody less than 10 fold higher than a naïve rabbit, while a wild-type HSV-1 (17*syn*+) ocular-infected rabbit had 100 fold higher level of antibodies to HSV than that of the naïve one.

Adenovirus Vector Delivery of a Ribozyme targeting HSV-1 U_L20 mRNA in a Mouse Footpad HSV-1 Infection Model

A survival assay was conducted to evaluate the ribozyme effect on HSV-1 replications in the mouse footpad model. Three treatments with 10 mice per group were set up for footpad infection of wild-type HSV-1 (17*syn*+). Mice received treatments (PBS, or adenovirus packaged GFP, or adenovirus packaged U_L20 ribozyme) followed by wild-type HSV-1 (17*syn*+) infection. The HSV-1 infection dose was 10^4pfu per footpad, and it should be addressed that a LD_{50} dose of 500pfu had been previously determined in this mouse strain using HSV-1 17*syn*+ (data not shown). The infections and treatments were performed in a blinded manner so that investigators had no knowledge of the type of injection (Ad-ribozyme, Ad -GFP or PBS), and two independent studies were performed. Videos were taken to analyze behavior indications of encephalitis. In Fig. 5-

7A where a combined survival rate from two experiments is shown, U_L20 ribozyme delivered by adenovirus vector in mouse footpad protected mice from lethal HSV-1 infection by 90%. Mice in Ad-GFP treatment group had a 40% survival rate, and compared with ribozyme treatment group, a chi-square statistic was 8.25 with associated P-value of 0.0041 using Kaplan-Meier survival analysis; PBS control groups had 45% survival rate with a chi-square statistic of 10.11 which associated with P-value of 0.0015, when it was compared with the ribozyme treated animal group. These indicated a significant protection effect provided by the U_L20 ribozyme treatment in mice against the lethal dose of HSV-1 infection. On the 6^{th} day after HSV-1 infection, mice from control groups (GFP and PBS treatments) showed signs of encephalitis, including hind-limb paralysis, hunched posture, ruffled fur, ataxia, and weakness. Mice were euthanized when severe CNS involvement of infection was observed. At this stage pronounced anorexia, lethargy, and ruffled fur were detected. However, mice from ribozyme treatment group maintained healthy and active at the 6^{th} day post-infection of HSV-1, although two deaths were observed and one mouse showed mild paralysis in one hind limb in a much later time-point. Mouse death at each day was recorded to plot the survival curve. Interestingly, in the ribozyme treatment group the first death was delayed by one day compared with Ad-GFP treated group, and by two days delay compared with PBS treatment. Mice in the ribozyme treatment group showed a phenotype of milder HSV-1 infection than those from control groups, although a HSV-1 infection dose of 20 times higher than the LD_{50} dose was used in this study, indicating a protective effect by U_L20 ribozyme.

To evaluate the ribozyme effect in reducing viral replication, a time-course study was conducted. At day 2, 4, and 6 post-infection of HSV-1, four mice per group (Ad-GFP or Ad-Rz) were sacrificed to collect tissues (footpads, dorsal root ganglia, and spinal cord). Viral DNA level was estimated by normalizing against the DNA of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH). At day 4 post-infection of HSV-1, in ribozyme treated animals a 44% reduction in viral DNA levels in the mouse footpad (data not shown), a 78% reduction of viral DNA levels in the DRG (shown in Fig 5-7B), and a 44% reduction of viral DNA levels in the spinal cord (data not shown) were observed. At day 6 post-infection, viral DNA level in the ribozyme treatment group was reduced by 86% in the spinal cord. However, the differences in viral DNA levels of treatments from footpad, DRG or spinal cord did not reach statistical significance using a nonparametric test for independent samples.

Analysis of the Effect of Iontophoresis of Chemically Protected Hammerhead Ribozymes in Rabbit Corneas in Limiting HSV-1 Infections

An *in vitro* kinetic analysis of ribozymes, including unmodified U_L20 ribozyme, modified active and inactive ribozymes, was conducted to evaluate their catalytic abilities. There was no detectable activity observed for modified inactive ribozyme even in high magnesium concentration (20mM Mg^{2+}). At 5mM$Mg^{2+}$the modified active U_L20 ribozyme exhibited a kcat of 2.2min^{-1}, a K_M of 6.0μM, and kcat/K_M of 0.37$min^{-1}\mu M^{-1}$. As shown before, when the unmodified U_L20 ribozyme 154 was tested at 5mMMg^{2+} it had a kcat of 27.8 min^{-1}, a K_M of 1.8μM, and kcat/K_M of 15.9 $min^{-1}\mu M^{-1}$. Therefore, a 40 fold reduction in kcat/K_M was observed in modified ribozyme compared with that of its unmodified counterpart, indicating a reduction in the catalytic efficiency. However, this modified ribozyme targeting U_L20 mRNA provided significant protection against HSV-1

replication resulting in fewer dendritic lesions forming (as shown in Fig. 5-8-A), particularly in the central cornea than those treated with the control (inactive) ribozyme (shown in Fig. 5-8-B). Fewer lesions were observed around the scarified area in the active ribozyme treated eyes compared with control ribozyme treated eyes. The blue staining on each eye, indicating the active viral expression of β-galactosidase, was quantified using software (SigmaScan Pro) and a significant reduction of 57% ($p<0.02$) in dendrite forming was detected (as shown in Fig. 5-8-C).

Discussion

Adeno-associated Virus Vector Tropism in the Cornea

This preliminary study was done in a small set-up with one rabbit (two eyes) per serotype treatment. The purpose was to select one or several candidates to test the corneal tropism of AAV in a large scale. Because the rabbit corneal epithelium has a turnover half-life very similar to that of the human cornea, probably 7 to 14 days, 7 days were allowed for transgene expression. This was a very challenging task, since AAV delivered transgene expression often takes a long time. AAV is a single stranded DNA virus, and transcription of the transgene requires the synthesis of the second DNA strand. As expected, GFP expression was not detectable directly under the fluorescence microscope. Because the original AAV transduction in the corneal epithelium is diluted while time passes, a more sensitive assay is required for transgene detection. Although immunostaining using the biotin-avidin detection system (Vector Laboratories, Burlingame, CA) can amplify the signal extensively, it very often has background that interferes the result from histochemistry staining depending on the enzymatic detection system used, e.g., peroxidase system. As shown in Fig. 5-4-G, even in the presence of pretreatment to inactivate the endogenous peroxidase, a significant background was

detected using microscope. The alkaline phosphatase system (Vector Laboratories, Burlingame, CA) was proved to have a lower background level than using peroxidase system. Therefore, in the future it is recommended for this purpose. A larger sample number is also important. Considering the experimental error, a group of 3 or more rabbits per treatment per time point is recommended.

The other issue raised by this study is whether AAV is able to exist in the corneal epithelium for extensive period of time. To persist, AAV must transduce corneal stem cells predicted to be resident in corneal limbal area. This can be tested by delivery of AAV1 or AAV8 to rabbit cornea for a time-course study. If GFP expression can persist for months in the cornea, AAV would have to have transduced corneal stem cells. This will lead to a future application using AAV vector in the cornea, particularly in *ex vivo* culture of corneal allograft before corneal transplantation. If AAV can be used to deliver therapeutic genes in donor corneas, it will provide a broader application for disease prevention in transplantation recipients.

Herpes Simplex Virus Vector for Ribozyme Delivery into the Cornea and Trigeminal Ganglion

The original purpose of this study was to establish a delivery model for therapeutic ribozyme in a recurrent HSV-1 ocular model. A well-documented HSV-1 ocular infection model in rabbit[146] was employed. A HSV-1 strain 17*syn*+, which is a neuroinvasive and neurovirulent strain, was used for primary infection. The reactivation of HSV-1 infection can be induced by epinephrine iontophoresis and can reach >90% success among experiment animals. However, when a non-replicating HSV-1 vector containing LacZ gene (strain 8117/43[92]) was delivered, no transgene expression was observed, which led to a failure in our attempt to use HSV-1 vector for therapy against

recurrent HSV-1 infection. The second experiment was set up to confirm this phenomenon. A non-replicating HSV-1 vector (KD6) was inoculated in left eyes 2.5 months before the second infection. The second infection was conducted by infecting rabbits bilaterally with a replicating HSV-1 containing LacZ (a recombinant HSV-1 called dUTPase/LAT. Interestingly, there was unilateral protection rendered by the previous single-eye infection of KD6. This result confirmed observation that previous HSV-1 infection can inhibit later infection caused by the same virus but not necessary the same strain. However, this local (ocular) immune protection against super-infection of HSV is very intriguing. First of all, it has been known that vaccination is not beneficial in preventing HSV infection. A systemic immunization cannot provide full protection against future HSV-1 infection. The antibody neutralization assay (Table 5-2) to compare KD6 infected rabbits with wild-type HSV-1 infected showed that an ocular inoculation with either replication-defective or wild-type virus did not induce significant level of antibody production. Therefore, a non-systemic mechanism was driving this protective effect in cornea, which is an immune privileged tissue. Second, in both assays the primary HSV-1 infection (either with wild-type HSV-1 or with KD6) was conducted a long period of time before the second infection, 4 months and 2.5 months, respectively. One can speculate that without a boost of immunization, cellular immune response could not standby for such long times if a T cell response would be the explanation for the viral resistance in the cornea. There may be other immune mechanisms leading to this phenomenon. However, to test the importance of cytokines, chemokines and other cellular immune response involvements, a different animal model needs to be adopted due to the short supply for antibodies against cellular factors in the rabbit and the

diversity of the rabbit genetic background. Another explanation for resistance to replication by the second virus is the existence of corneal latency that might modulate the cellular environment leading to non-permissiveness for later HSV-1 super-infection in the cornea. However, this hypothesis remains to be tested.

Adenovirus Vector Study

Adenoviral vectors have been shown to possess significant advantages for gene delivery, and in this study a first generation adenoviral vector was used. A high vector production could be achieved, as in this study the Adenovirus vector was generated using a Cre-lox recombination system.[134] Adenoviral vectors can provide high transduction efficiency in quiescent and dividing cells, which leads to significant therapeutic benefits. However, first-generation adenoviral vector is highly immunogenic due to the expression of massive viral proteins, which causes the short-term transgene expression. It has been shown that the transgene expression delivered by an adenoviral vector was not detectable after 2 weeks.[159] Furthermore, it has been shown that adenoviral vector has a cell-specific transduction pattern in cornea according to a series of studies *ex vivo* and *in vivo*[49,183,208,362] related to corneal tropism: Adenoviral vector transduced corneal endothelium, conjunctival epithelium and keratocytes, but it was not efficient in transducing corneal epithelium. A preliminary study in rabbit cornea was conducted during the time of this dissertation research to study the efficacy of epithelial gene transfer. The result suggested that after topical application of an adenoviral vector on the cornea, transgene expression was only limited within areas containing the physical damage in corneal epithelium, e.g., needle scarification or crosshatched abrasion of 25micron of the thickness (data not shown). Therefore, for the purpose of long-term gene transfer in corneal epithelium, adenoviral vector is not an ideal option.

An *in vivo* study was designed to test the U_L20 ribozyme delivered by the adenovirus vector, and a mouse footpad model of HSV-1 acute infection was used to evaluate the efficacy of this ribozyme. Mouse footpad model offers an efficient approach to study HSV-1 viral neuroinvasion, neurovirulence and latency. In this study, ribozyme effect to the initial replication in the footpad epithelium was monitored. HSV-1 viruses applied on the abraded keratinized epithelium initiated viral replication, meanwhile, viruses undergo retrograde transport to the DRG.[334] At an inoculum dose of 10^4pfu of wild-type HSV-1 (20 fold of a LD_{50} dose), without ribozyme treatment, the viral replication in the footpad epithelium leads to severe damage in CNS which causes death. In this study, pre-treating the mouse footpad with adenovirus packaged ribozyme led to a significant protection (90% survival rate) against the lethal dose of HSV-1 infection. Eighty percent of animals in this group remained healthy throughout the study; one mouse showed mild paralysis in the rear limb, but remained active after the ending point. In contrast, in both of the control groups, death and indications of severe damage in CNS were observed. An independent repeat of the survival assay was conducted, and showed similar result in the ribozyme treatment group (90% survival rate); however, a higher survival rate was observed in GFP treatment group than that of PBS group, which was different from the observation in the first test. It is speculated that GFP and PBS treatment will give similar response when a larger amount of repeats are conducted, assuming that the sample number (N=10) in each group is representative.

To answer the question whether this protection effect of ribozyme was from inhibiting viral replication, viral DNA level was quantitated using real-time PCR. Four animals per group at each time point were sacrificed to collect tissues, and quantitative

real-time PCR was conducted using DNA samples from each individual animal. Although there was a 78% reduction of mean level of viral DNA in the DRG since day 4 post-infection of HSV-1, the variation within each group did not lead to a statistically significant difference between the ribozyme and GFP control treatment. The same result was observed in the viral DNA level from the spinal cord: An 87% reduction in viral DNA level from the ribozyme treatment was detected without statistical significance. Notice that the viral DNA detected among animals in one group the same time point fell in a very wide range (as much as 2000 fold difference), a sample size of 4 might not be sufficient to demonstrate a normal distribution of the data. The viral DNA amount present in the tissue might have been below the limits of detection threshold of this method, thus optimizing the procedure to increase the DNA recovery from the sample may lead to a better result. Finally the viral DNA and mRNA levels in the footpad may give a clearer answer since the footpad harbored the initial viral replication.

Effect of Iontophoresis of Chemically Protected Hammerhead Ribozymes in Rabbit Cornea in Limiting Herpes Simplex Virus Type I Infection

Iontophoresis has been broadly applied in clinical applications for transdermal drug delivery. However, it has not been developed as a standard procedure for ocular applications due to toxicity and the lack of carefully controlled trials. The efficient drug penetration provided by this technology has encouraged numerous studies to exploit and optimize this application in the last several years.

In this study, the ability of chemically modified ribozymes to degrade mRNA of an essential gene (U_L20) of herpes simplex virus type 1 (HSV-1) was tested for its therapeutic effect by iontophoretic treatment. The advantage of this approach is to avoid the usage of topical antiviral drugs, since the toxicity of current drugs often leads to

allergy effect which is detrimental for patients. At the same time, switching to other antiviral drugs may resolve the problem temporarily, but patients develop allergy to other drugs eventually. On the other hand, it has been suggested that transcorneal iontophoresis has very few complications even when frequent treatment is required.[25]

The corneal epithelium is the target location for ribozyme delivery in this study, since HSV-1 viral replication in epithelium is an early event that leads to clinical indications of severe HSV-1 infection. By ribozyme delivery to anterior cornea which prevents the active viral amplification and spreading, further damage in stroma can be avoided. From previous *in vivo* studies using adenovirus packaged U_L20 ribozyme a significant anti-HSV-1 effect was confirmed. To take advantage of this therapeutic ribozyme for future application, this study was designed to evaluate the iontophoretic approach in delivering this ribozyme as a potential treatment to prevent HSV-1 ocular infection. It is very promising that a single dose of ribozyme delivery rendered significant reduction in HSV-1 viral replication (shown in Fig. 5-8). It is noticeable that an HSV-1 infection of 10^5pfu was employed on the rabbit cornea in this study. The inhibitory effect from the chemically modified ribozyme seemed very impressive considering that HSV-1 ocular infection in human begins with very low dose of virus load. Although the exact amount of HSV-1 has not been revealed to be efficient to initiate ocular damage, it is known that at the peak of HSV-1 ocular infection 10^5pfu can be detected.

However, chemical modifications in the ribozyme significantly reduced the catalytic efficiency as indicated from *in vitro* kinetic comparison of modified and unmodified ribozyme. A nearly 40 fold reduction in catalytic activity was observed. It

could be expected that a higher level of inhibition can be achieved by vector delivery of ribozyme through exogenous expression.

In this study the iontophoresis was conducted by applying a current of 0.8mA for 8 minutes. Another experiment was conducted using the same condition but led to damage in rabbit eyes. Therefore, further optimization of the protocol is necessary for future application to provide consistent delivery. A better evaluation of the iontophoretic approach is to induce reactivation in latently infected rabbits followed by U_L20 ribozyme delivery, since recurrent infection from latent virus is more relevant to human clinical on-set of the disease than the model tested in this study.

Table 5-1. Treatment code for the tropism study of adeno-associated virus vector.

Rabbit Tattoo#	Treatment
FL32	Iontophoresis with UF11 plasmid (35ug/ml in a total of 2ml dH2O, 8min at 8mA); both eyes; no abrasion- intact corneal epithelium.
FL33	Untreated rabbit
FL28	Partially abraded the corneal epithelium; AAV UF11 (Type 1); stock ref: C458; $2.0x10^{11}$ p per eye.
FL27	Partially abraded the corneal epithelium; AAV UF11 (Type 2); stock ref: 448; $2.0x10^{11}$ particle per eye.
FL23	Partially abraded the corneal epithelium; AAV UF11 (Type 5); stock ref: C339; $2.0x10^{11}$ particle per eye.
FL29	Partially abraded the corneal epithelium; AAV UF11 (Type 7); stock ref: C414; $2.0x10^{11}$ particle per eye.
FL30	Partially abraded the corneal epithelium; AAV UF11 (Type 8); stock ref: C512; $2.2x10^{11}$ particle per eye.

Note: FL32 was harvested approximately 36hrs post-iotophoresis.

Table 5-2. Antibody neutralization assay to detect systemic antibody against herpes simplex virus type 1 (HSV-1) following non-replicating HSV-1 (KD6) infection.

Each rabbit serum dilution was incubated with 10^5pfu of 17*syn*+ (*wt*HSV-1)		
Rabbit label	Treatment	Serum dilution
VTS	Naïve	<1/5
J69	17*syn*+ inoculated	1/100
J89	KD6 (ICP4 defective virus) injected	1/10
J90	KD6 (ICP4 defective virus) injected	1/10
J91	KD6 (ICP4 defective virus) injected	1/10
J92	KD6 (ICP4 defective virus) injected	1/5

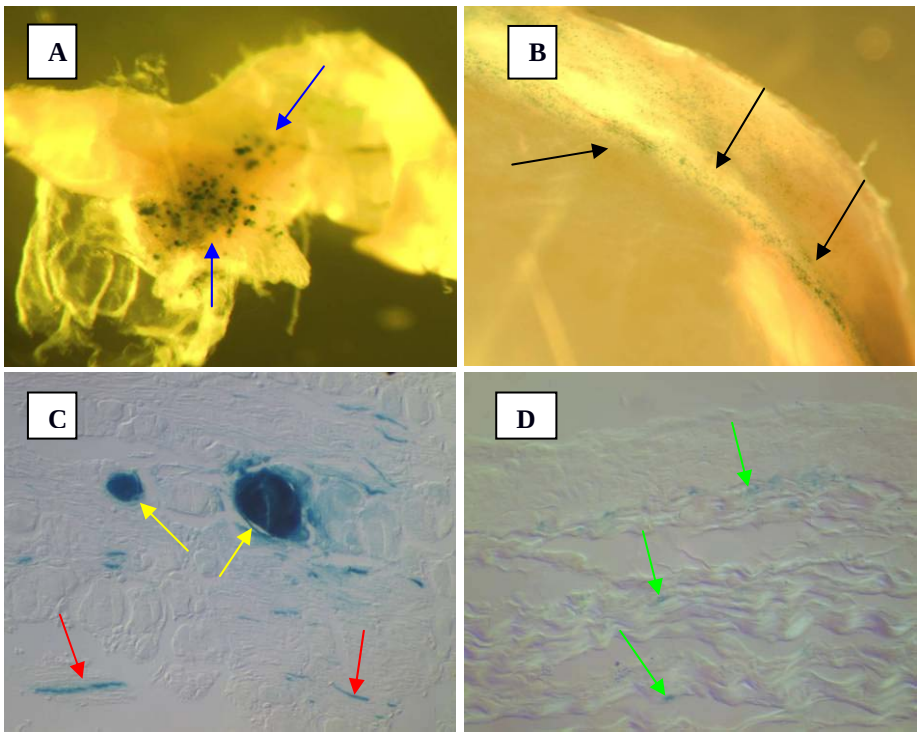


Figure 5-1. Trigeminal ganglia transduced by LacZ packaged herpes simplex virus vector. β-Galactosidase staining of trigeminal neurons (panels A and C) and corneas (panels B and D) in rabbits following HSV-LacZ Infection. Low power photographs (panels A and B) show extensive labeling of ganglia in trigeminal nerve track (blue arrows) and in the limbal region of the cornea (black arrows). Nomarski contrast interference micrographs (panels C and D) show labeled axons (red arrows) and nerve bodies in trigeminal neurons (yellow arrows) and corneal fibroblasts (green arrows).

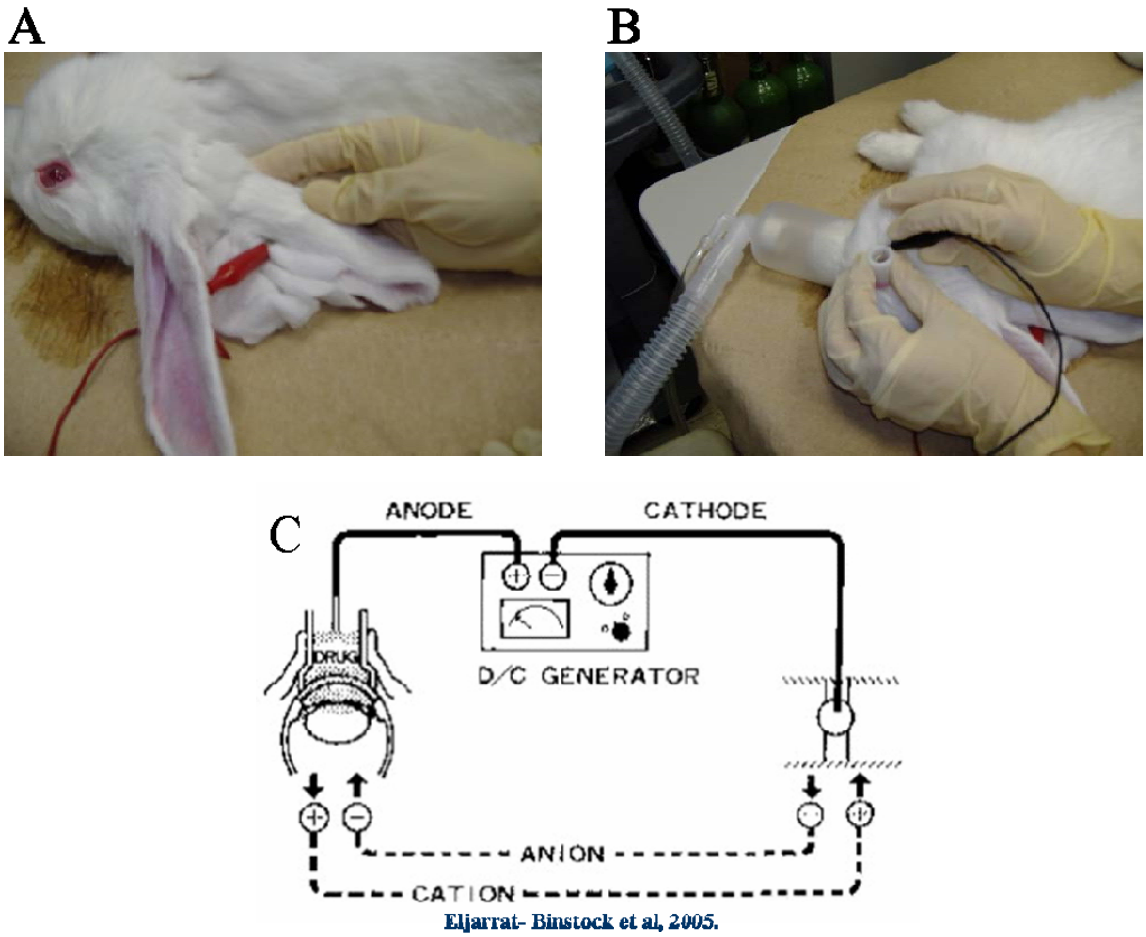


Figure 5-2. Iontophoresis treatment in rabbits. A and B show the apparatus set-up for iontophoresis in this study, the anode was connected to rabbit ear while the cathode was placed in the eye cup which held solution. Rabbits were under anesthesia by isofluorane inhalation and rabbit corneas were treated with topical 1% proparacaine drops before iontophoresis. C shows a diagram of the overall set-up[97]: when drug molecule is positively charged, the anode is placed in the eye-cup which contains drug solution, while the cathode is connected to the ear.

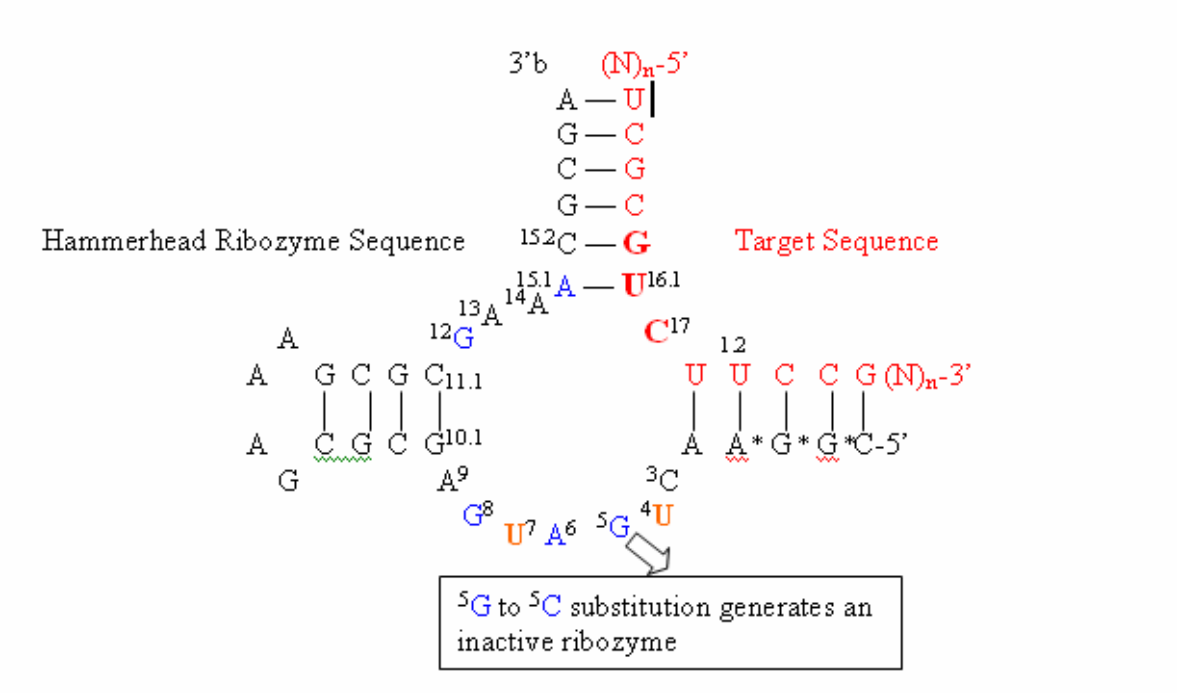
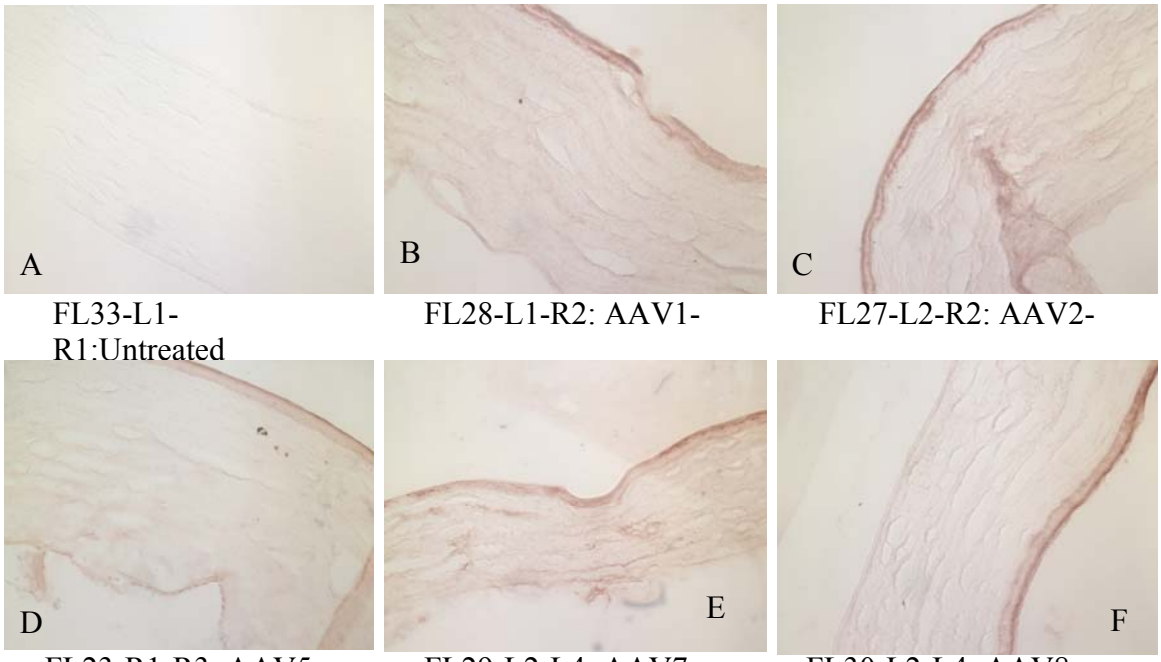


Figure 5-3. Design of chemically modified hammerhead ribozyme targeting U_L20 mRNA of herpes simplex virus type 1. G^5, A^6, G^8, G^{12} and $A^{15.1}$: ribonucleotide; b: 3'-Inverted abasic; U: 2'-Amino-uridine; *: Phosphorothioate; remaining nucleotides: 2'-O-Methyl nucleotides.



G

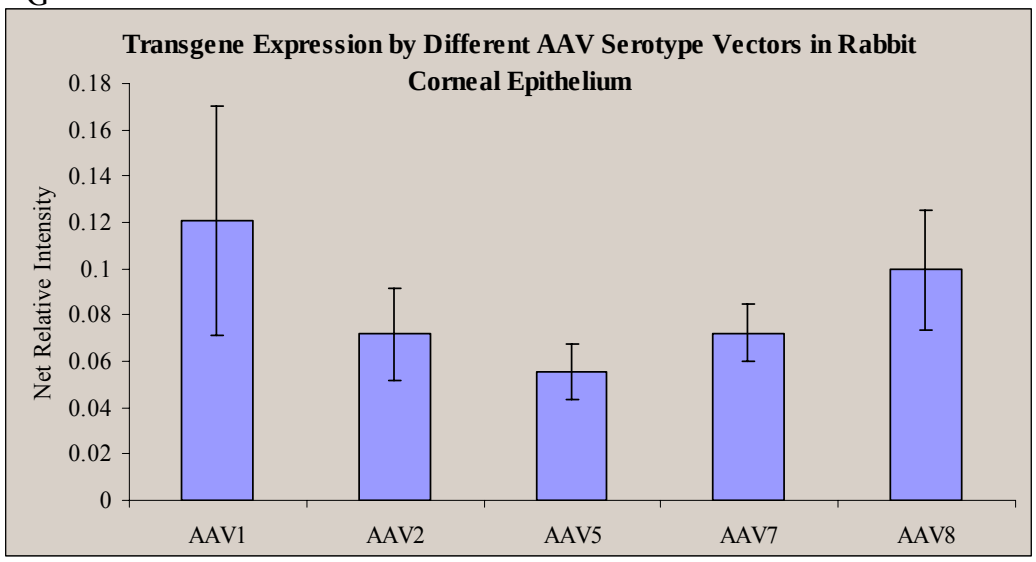


Figure 5-4. Immunostaining of rabbit cornea for green fluorescent protein expression delivered by different serotypes of adeno-associated virus vectors. A) No AAV control; B) AAV1; C) AAV2; D) AAV5; E) AAV7; F) AAV8. G) Quantification of the intensity of staining in corneal epithelium.

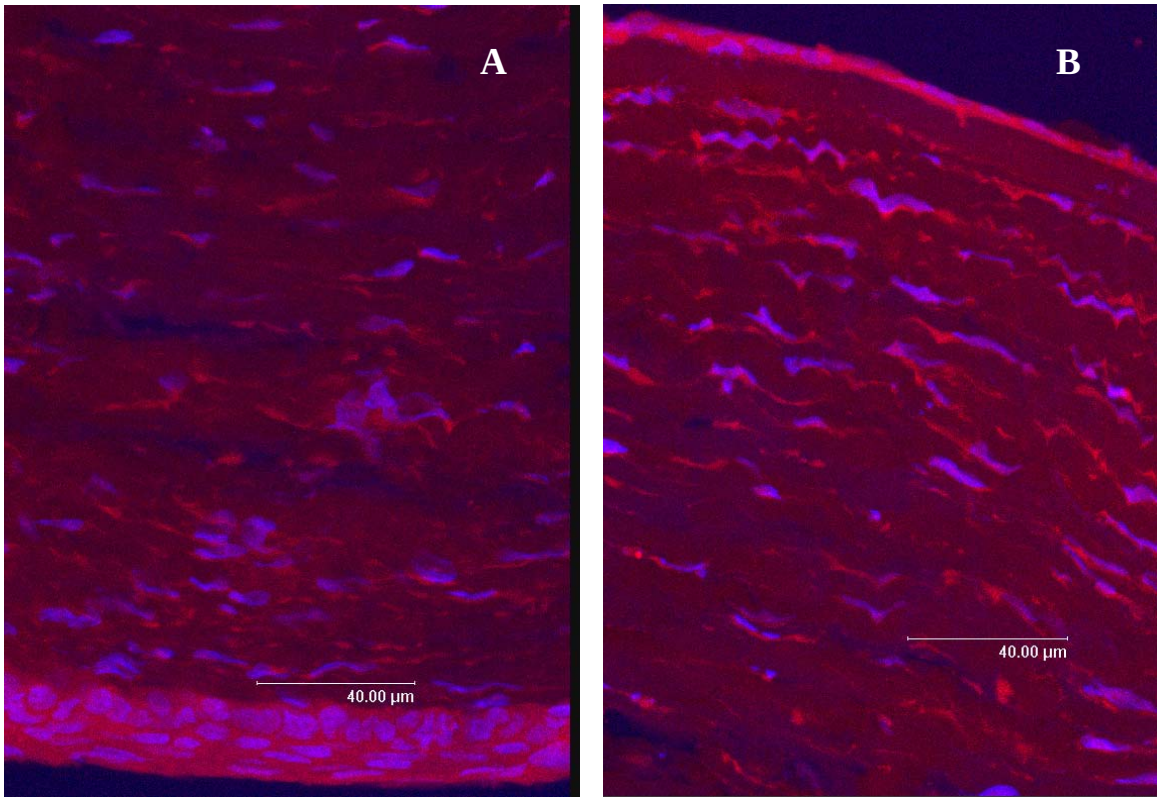


Figure 5-5. Confocal microscope observation of green fluorescent protein using alkaline phosphatase detection system. Red fluorescence: immune staining of GFP expression; blue staining: DAPI staining showing nuclei. A and B: show rabbit FL27 section L1 which was treated with AAV2 packaged GFP; A) (picture taken at 63X magnification) tissue layers from the top to bottom are stroma and epithelium; B) (63X) tissue layers from top to bottom are endothelium and stroma; C, D: pictures were taken at 63X magnification from rabbit FL23 treated with AAV5 and section R1; C) from the top are stroma and epithelium; D) from the top are endothelium and stroma; E, F: pictures were taken at 63X from rabbit FL33 which is untreated and section R1; E: from top to bottom are stroma and epithelium; F: from the top to bottom are endothelium and stroma

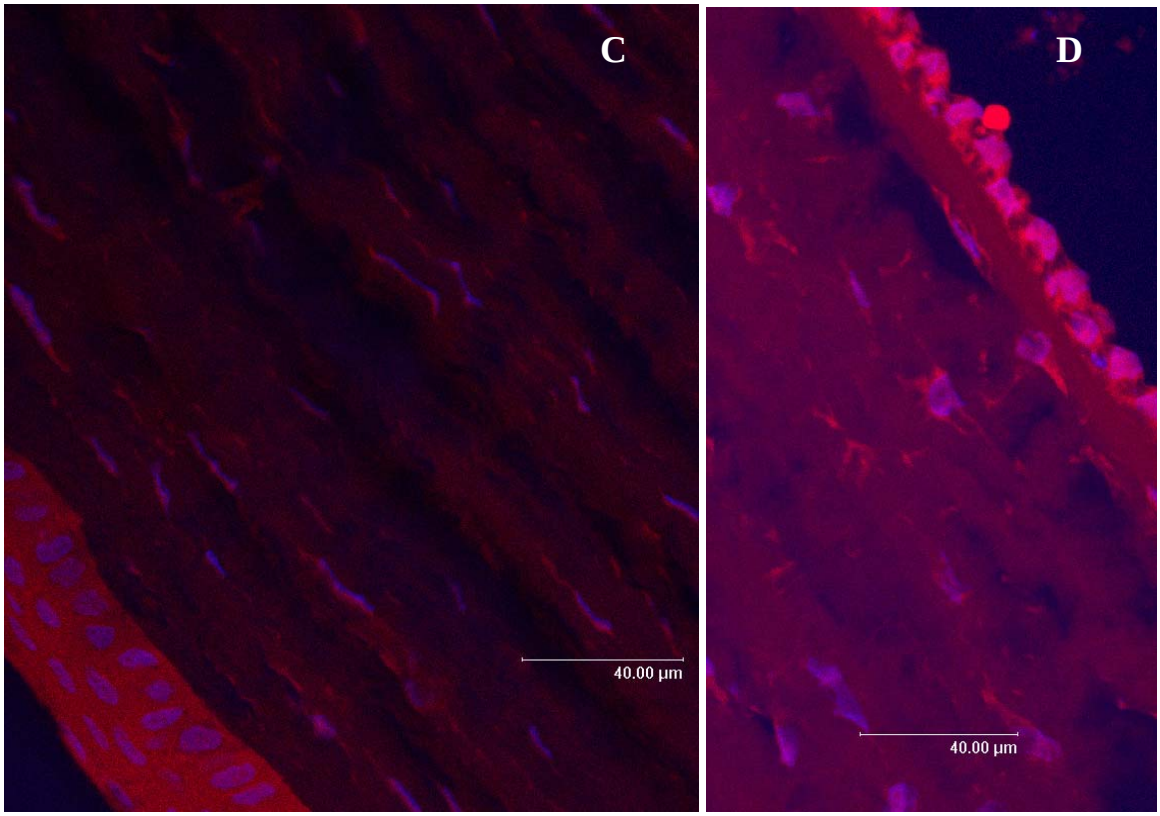


Figure 5-5. (continued)

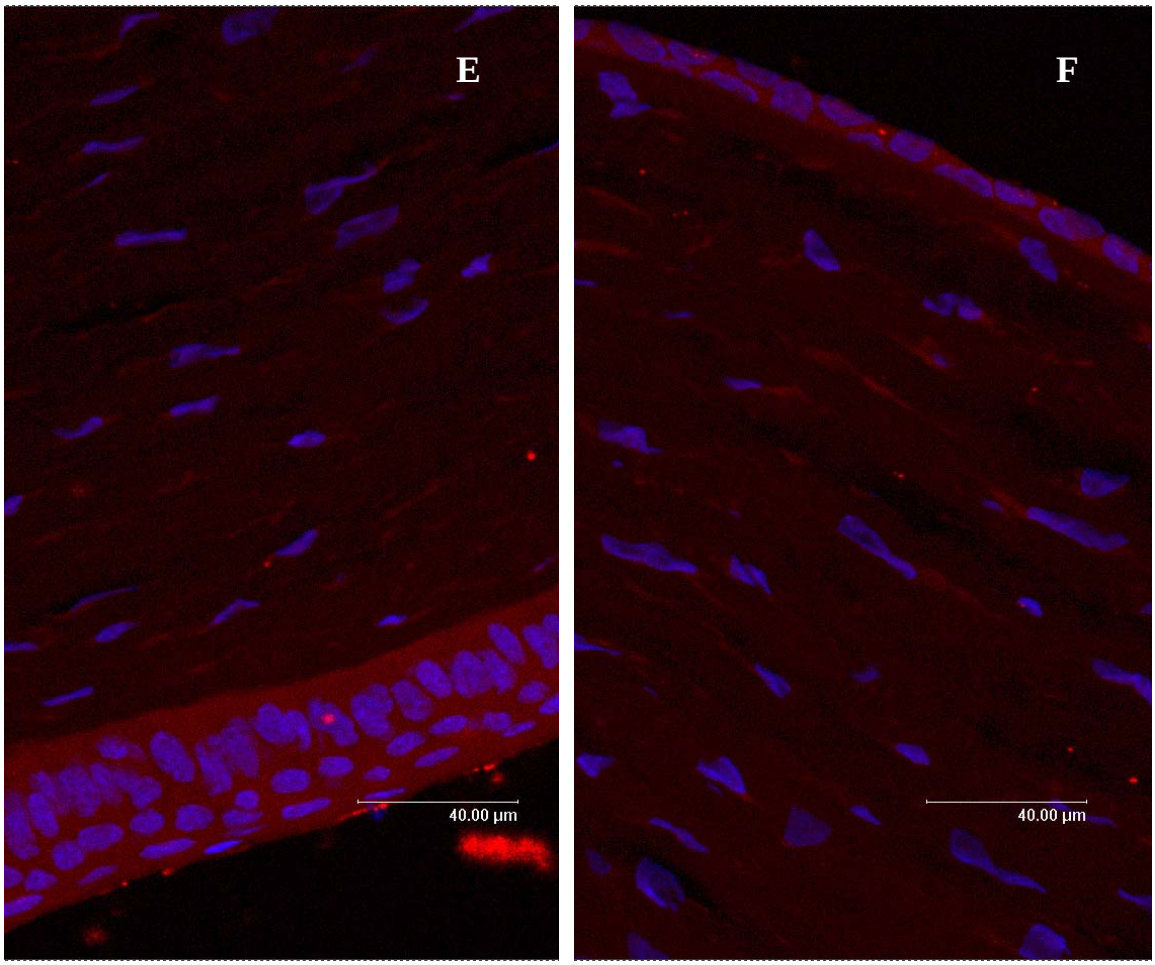


Figure 5-5. (continued)

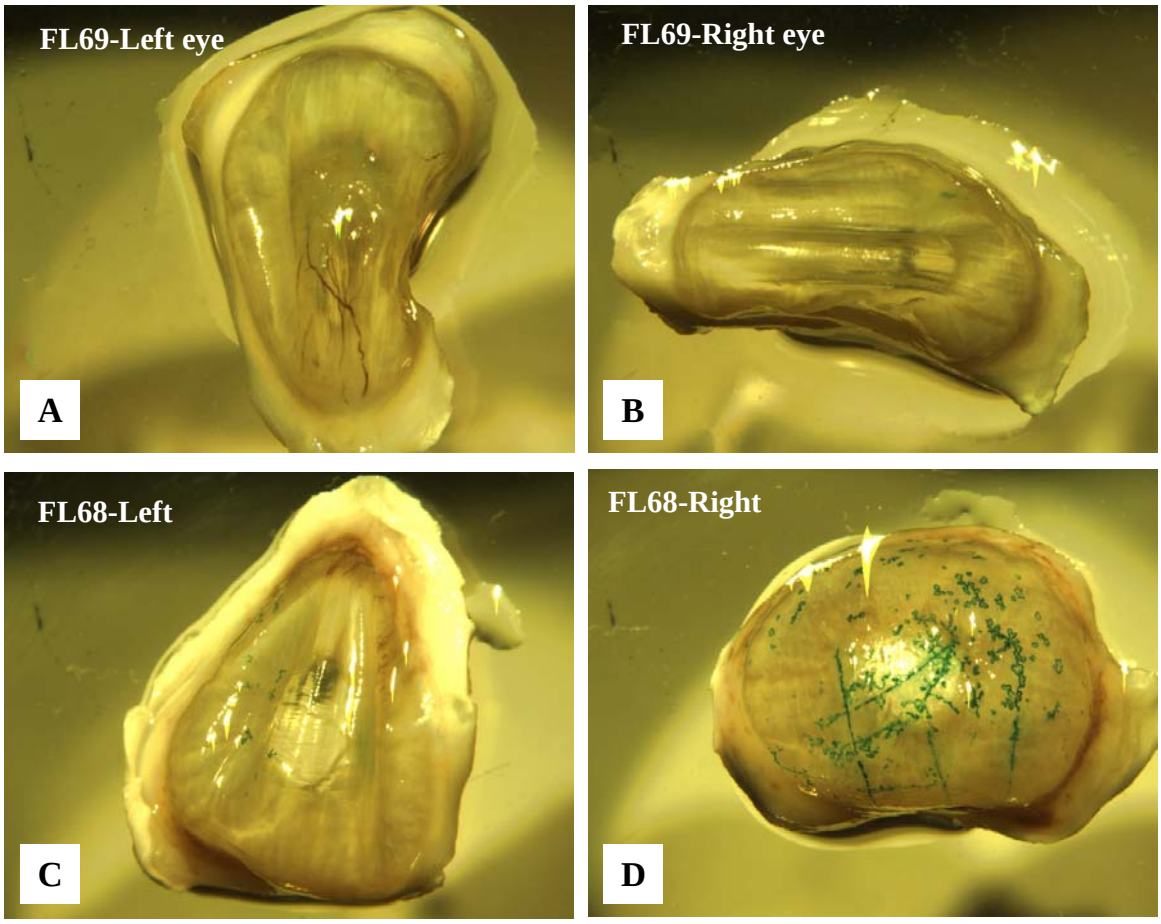


Figure 5-6. Delivery of LacZ gene expression using HSV vector in the cornea of New Zealand white rabbits. Rabbits from group I were ocular inoculated in both eyes with wild-type HSV-1 (17syn+) at a dose of $2x10^5$pfu/eye to establish latency for 4 months before non-replicating HSV-1 vector (8117/43) was delivered in only right eyes. Both eyes of each rabbit were harvested at 4 days after second HSV-1 infection and eyes were fixed for β-Galactosidase staining. A and B are representative pictures of both eyes from rabbit-FL69 taken at low power: A is an image of left eye, and B is right eye. Group II rabbits were first inoculated with a non-replicating HSV-1 vector, KD6, only in the left eye for 2.5 months. A second infection was conducted in both eyes using dUTPase/LAT, which is a replicating HSV-1 vector containing LacZ gene. 4 days after second infection, rabbit eyes were collected for β-Galactosidase staining. C is a low power photograph taken from the left eye of rabbit FL68, and D is taken from the right eye of FL68.

A.

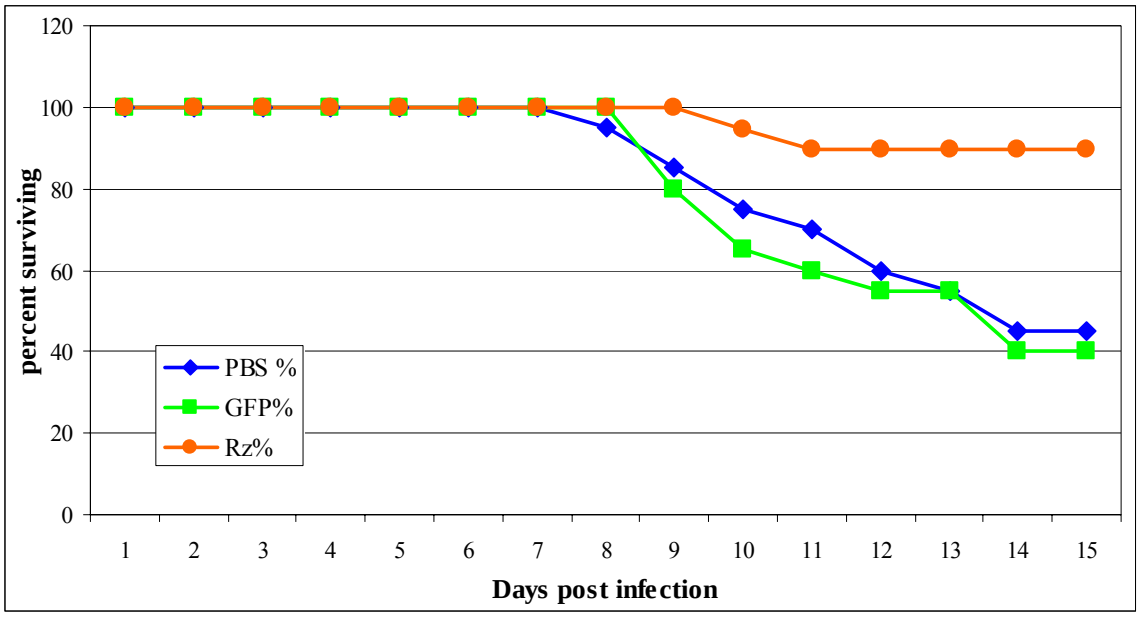
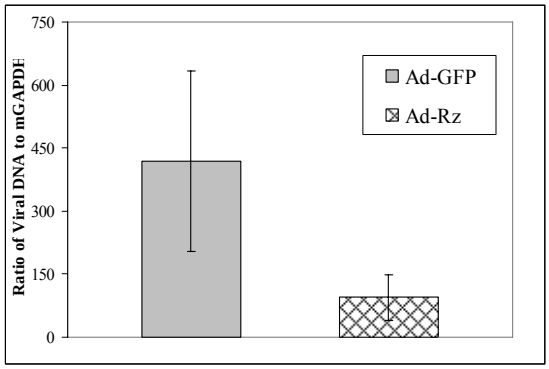


Figure 5-7. Survival assay to observe protection effect of U_L20 ribozyme. A) U_L20 ribozyme effect on lethal infection of HSV-1 in mouse footpad. Ten Swiss ND4 mice were used per group in this study. Pretreatment (using PBS, Ad-GFP, or Ad-$U_L20$rz) was conducted by subdermal injection in mouse rear footpad. Four hours after pretreatment, abrasion was introduced on the footpad followed by another topical treatment (using PBS, Ad-GFP, and Ad-$U_L20$rz, respectively). Twenty minutes later, HSV-1 infection was conducted at a dose of 10^4pfu per footpad. Mice were returned to their cage when they recovered from anesthesia. Observation of behavior was conducted twice a day. The number of mice survived from lethal HSV-1 infection was recorded everyday in order to plot in the survival curve. The percentage of surviving animals from each treatment group was an overall effect calculated by combining two experiments. B) The comparison of viral DNA levels in dorsal root ganglion from Ad-GFP or Ad-U_L20Rz treatment group after 4 days post-infection of HSV-1 in mouse footpad. At day 4 post-infection, four animals per group were sacrificed to collect dorsal root ganglion, and DNA was extracted for quantitative real-time PCR, and a 78% reduction of mean viral DNA level was observed in ribozyme treated mice. The y-axis represents the ratio of viral DNA to mouse glyseraldehyde-3-phosphate dehydrogenase (GAPDH). C) The comparison of viral DNA level in spinal cord from Ad-GFP and Ad-U_L20Rz treatments at 6 days post-infection in mouse footpads. Four mice per group were sacrificed to collect spinal cord for DNA extraction. Quantitative real-time was conducted to compare viral DNA levels. An 86% reduction of mean viral DNA level was observed in ribozyme treated animal. The Y-axis represents the ratio of viral DNA to mouse GAPDH.

B.



C.

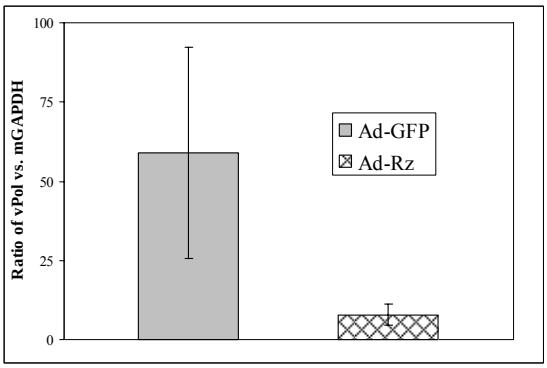


Figure 5-7. (continued)

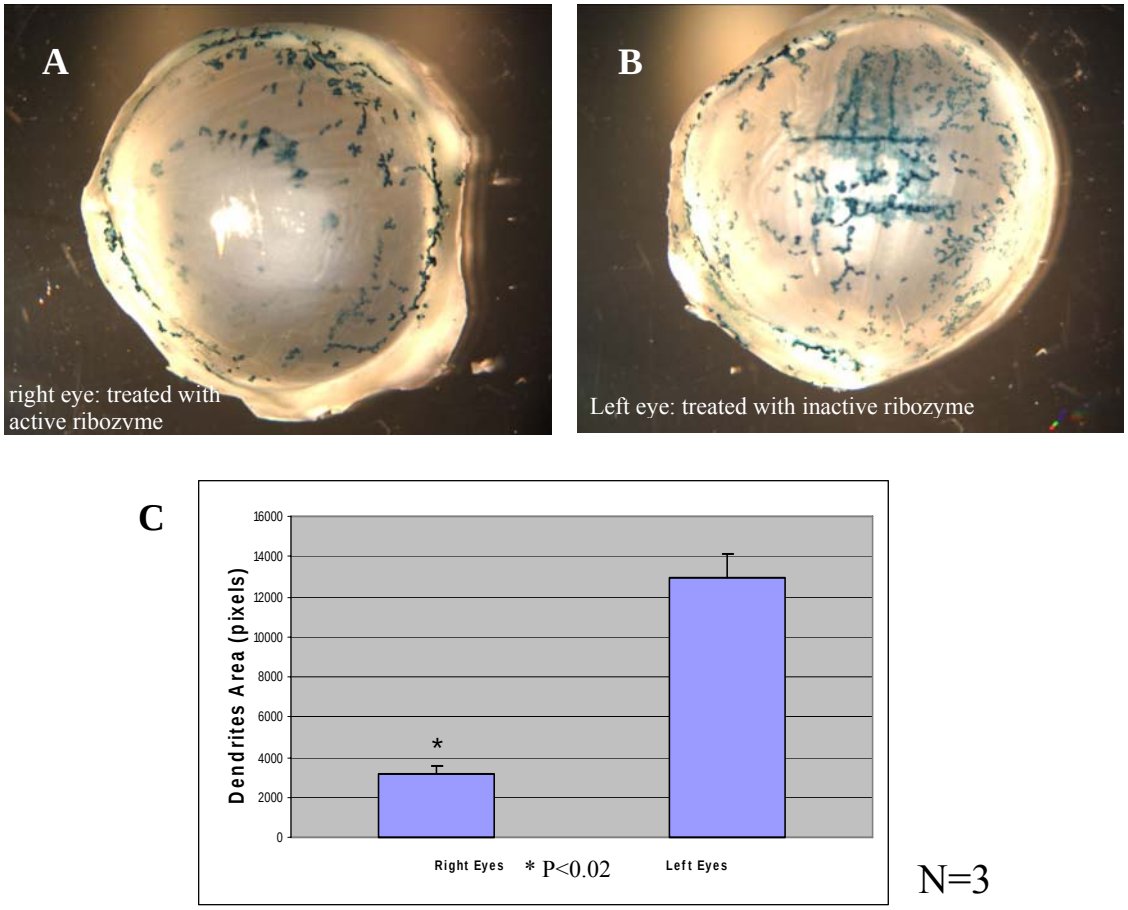


Figure 5-8. Delivery of chemically modified ribozyme reduced dendrite formation in rabbit cornea caused by herpes simplex virus type 1 infection. Three New Zealand white rabbits were used in this study. Chemically modified ribozyme RNA molecules were designed and synthesized from Dharmacon (as shown in Fig 5-3). 100µg of oligonucleotide were used for iontophoresis of each eye and the right eye was treated with active ribozymes while the left eye of each rabbit was treated with the inactive ribozymes. A replicaing HSV-1 strain (8117/43) containing LacZ gene was used for infection in both eyes following iontophoresis. Four days after infection, rabbits were sacrificed and corneas were harvested for β-galactosidase staining to look for viral replication patterns on the cornea. Figure A is a representative picture of a rabbit right cornea which had been treated with the active ribozyme (U_L20 ribozyme-154); B is the control eye from the same rabbit in A, which had been treated with the inactive ribozyme. The area with positive staining indicating the β-galactosidase activity, which showed the viral replication level in each eye, was measured using software called SigmaScan. The staining levels from ribozyme treated eyes and control eyes were compared in Figure C.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

The original design for this study was to use a nucleic acid-based gene therapy approach to inhibit Herpes Simplex Virus type 1 (HSV-1) infection in the cornea. Hammerhead ribozymes and siRNA were designed particularly for targeting the ICP4 gene, since it is the major transcriptional regulator for the expression of all the other viral proteins. An ICP4 defective HSV-1 vector, was designed for ribozyme (or siRNA) delivery. The hypothesis of using HSV vector for delivery was that the HSV vector, although defective, still maintains the behavior for neuronal transport, which can deliver therapeutic agents into sensory neurons.[106] By these means, when latently infected HSV-1 initiates its reactivation, a hammerhead ribozyme functions to inhibit viral lytic infection. Therefore, HSV-1 reactivation could be blocked. The assumptions for this hypothesis were that a therapeutic agent can prevent HSV-1 replication, and the HSV-1 vector can transduce the same neurons which have already been latently infected with HSV-1.

Hammerhead Ribozyme Targeting ICP4

The rationale of targeting ICP4 mRNA to inhibit HSV-1 was that the ICP4 protein is the key activator of HSV-1 lytic infection[188]; therefore, knocking down ICP4 transcripts might eliminate viral replication. After scanning ribozyme cleavage sites in the ICP4 coding sequence (CDS) (nucleotide accession number NC_001806), secondary structures of potential ribozymes were predicted using a computational tool, MFOLD by Dr. Michael Zuker (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi). At

the same time, other HSV-1 essential genes were studied, including U_L20, U_L30, and U_L54. Only two hammerhead ribozymes (ribozyme-885 and ribozyme-533) were chosen against ICP4 mRNA. However, ribozyme-533 was inactive according to *in vitro* analysis of kinetic parameters. Ribozyme-885 was tested in the cell culture for ICP4 mRNA knock-down. Interestingly, this ribozyme reduced target gene expression by 42% in ICP4 expressing cells, but it could not inhibit wild-type HSV-1 viral replication. We speculated that although the ribozyme efficiently cleaved ICP4 mRNA, a threshold level of ICP4 protein can still be reached in order to turn on HSV-1 lytic infection. However, it is possible that a more efficient ribozyme may provide a better inhibition effect. Therefore, an optimized selection approach is required, and an *in vivo* mapping approach (e.g., using dimethyl sulfate or DMS) is recommended to determine the accessibility of mRNA and RNA structure[7,221,404] in addition to computational methods.

There have been a series of ribozyme studies targeting mRNA of HSV-1 ICP4. Although they showed impressive reductions by ribozyme treatment (close to 1000 fold), a non-permissive HSV-1 infection system was applied.[355,358] The tests in this dissertation were conducted in rabbit skin cell (RSC) using17*syn*+ (wild-type HSV-1) that permits a 200-400 times higher viral replication than the cells used in the earlier papers. On the other hand, ICP4 gene is an immediate early gene which is turned on right after viral lytic infections, and at 2 hours post-infection, ICP4 expression level drops to a baseline level.[3] It was also suggested that the abundance of ICP4 expression is very low even at the early time point of HSV-1 lytic infection.[3] Another study using siRNA to inhibit ICP4 expression of HSV-2 was conducted, as HSV-2 ICP4 has a very similar role in lytic life cycle to HSV-1 ICP4. Although an efficient reduction in viral replication was observed

in the cells treated with ICP4 siRNA, no effect was detected at the ICP4 mRNA level. It is possible that the siRNA targeting ICP4 has off-target effect[161], which interrupts not only ICP4 but cellular gene expression. Therefore, an siRNA has to be evaluated very carefully before being used for gene-specific knock-down.

Overall, it is a very difficult task to knockdown HSV-1 ICP4 gene expression, since it seems to only be important at the very first round of viral replication, and the baseline level later on is sufficient to support subsequent rounds of replications. Therefore, ICP4 gene might not be an ideal target for gene therapy purposes unless a complete inhibition of ICP4 expression can be achieved as early as viral entry to the cell nucleus.

Another immediate early gene, U_L54 (or ICP27 gene), was also targeted for in order to inhibit HSV-1 replication. One hammerhead ribozyme designed for this gene had excellent *in vitro* kinetic activity (as shown in Chapter 2), and was tested against wild-type HSV-1 (17*syn*+) replication. No significant effect in reducing viral production was detected (data not shown). HSV-1 U_L54 is an essential immediate early gene with a distinct role in post-translational modulations in viral and cellular transcriptional regulators.[161,186] Its functions include the impairment of host splicing[305], promoting viral transcription[81], and the enhancement of viral mRNA translation.[209] In addition, U_L54 protein also counteracts the early innate immune response.[237] However, there is a definite tolerance of the delay of U_L54 expression. It is speculated that an early time window of U_L54 expression is preferred in the HSV-1 lytic life cycle. In addition, a prolonged expression of U_L54 protein maintains the favorable condition for viral replication. The observation in this study was consistent with the finding that U_L54 (ICP27 gene) expression is not critical to HSV-1 viral replication *in vitro* and *in*

vivo.[326,340,341] The U_L54 gene might not be a good target for anti-HSV-1 therapy, since U_L54 expression is essential for immediate early gene expression at the early time point, but has no effect on the efficiency of viral replication at the later stage.[341]

Knocking down immediate early genes of HSV-1 did not provide a satisfying result in inhibiting viral replication, based on our experience of ICP4 and U_L54. These data supported the argument that HSV-1 immediate early genes are important within a limited set of physiological conditions. A different strategy is needed to eliminate HSV-1 infection *in vitro* and *in vivo*.

Ribozyme Targeting mRNA of Herpes Simplex Virus Type 1 Early/Late Essential Genes

The second stage of this study focused on testing ribozymes targeting genes from kinetic classes other than immediate early genes. Two ribozymes, targeting U_L20 and U_L30, respectively, were chosen for the gene therapy study, due to their high *in vitro* kinetic activities. An adenoviral vector system was adopted to deliver ribozymes in tissue culture because of its high transduction efficient.

Interestingly, the ribozyme targeting a late essential gene, U_L20, achieved the most significant therapeutic effect against HSV-1 infection *in vitro* and *in vivo*. It was suggested from a recent study that suppressing early or late gene expression of HSV could achieve therapeutic effect *in vivo*.[271] In the same study, siRNAs were designed against HSV-2 U_L27 and U_L29, which encodes an envelope glycoprotein and a DNA binding protein, respectively.[298] The study by Palliser *et al.*[271] further supported my observation that knocking down early/late genes of HSV might have a greater impact on the viral lytic life cycle than inhibiting the expression of immediate early genes. Therefore, significantly inhibiting viral protein production in a later kinetic class (early or

late genes encoding structural proteins, functional proteins in DNA replication and virion maturation) might give a more profound inhibitory effect *in vivo*. The ribozyme test was extended to study the antiviral effect against HSV-1 strains with drug resistant phenotypes. This study was the first to show that a nucleic based therapeutic agent (U_L20 ribozyme-154 particularly targeting late gene mRNA) could inhibit viral replication of drug resistant HSV-1 strains consistently and reduce the severity of wild-type HSV-1 ocular infection in rabbits (Chapter 5). Although iontophoretic delivery of chemically modified ribozymes is still under evaluation to determine the preventative effect against recurrent HSV-1 infection, this study pointed towards a future direction of the therapeutic application using nucleic-based agents. A topical drug form of U_L20 ribozyme-154 could be very helpful in preventing ocular herpes, and a standard clinical treatment could be developed. Currently patients with ocular herpes often suffer side-effects caused by the toxicity of anti-HSV nucleotide analogs. A different approach using ribozymes provides an alternative for those patients. In addition, since multiple ribozymes targeting mRNAs of different HSV essential genes can be combined to prevent the generation of escape mutant viruses, ribozyme therapy has a great potential for infectious diseases caused by HSV, especially in immune-compromised patients.

Another observation in this ribozyme study was that there might be a regulation between expression of HSV-1 viral DNA polymerase and U_L20. The important role of U_L20 protein in HSV-1 life cycle has been addressed previously (Chapter 4), and it functions not only for intracellular transport of virions and glycoproteins, but also for extracellular release of virions. It was suggested by Ward *et al*[378] that U_L20 expression can be down-regulated by abolishing DNA replication of HSV-1. During the study of

ribozyme targeting U_L20 mRNA, a significant reduction in U_L20 mRNA level was detected, which led to an equivalent knock-down in the mRNA level of U_L30 which encodes HSV-1 DNA polymerase. Meanwhile, when the ribozyme targeting U_L30 mRNA was tested in cell culture, a delay of U_L20 expression at the transcriptional level was observed, correlating with a significant reduction in DNA polymerase expression. Therefore, a question was raised whether a feedback between U_L20 and HSV-1 DNA polymerase exists. If this relation does exist, what could be its significance in viral lytic infection? It would also be interesting to see whether disrupting this interaction can lead to any change in viral pathogenesis. For this purpose, a recombinant HSV-1 virus can be constructed by switching the promoter of U_L20 from its original leaky late class to the strict late class. Therefore, a delay in U_L20 expression can be established. If any essential interaction exists between U_L20 and viral DNA polymerase, a phenotype can be expected compared with the wild-type parental strain. A growth rate comparison of the recombinant virus and its parental strain can be conducted in cell culture. The modification in pathogenesis of the recombinant virus, if any, can be tested in the acute infection model of HSV-1, a mouse footpad infection model. Since the viral replication is required for its pathogenesis *in vivo*, the inefficiency of lytic infection may lead to a phenotype.

The Establishment of an Ocular Delivery System Using Herpes Simplex Virus Type 1 Vector

A question was raised during the study whether HSV-1 vector could be used for ribozyme delivery in the host that had been latently infected with HSV-1. It is known that the maximum amount of virus present in the trigeminal ganglia is independent of the inoculum dose, during not only acute but also recurrent infections.[269] Therefore, only a

fraction of trigeminal ganglia can be infected during a primary infection or during re-infection, and the percentage of infected neurons may be different among individuals depending on their genetic backgrounds and physical conditions (immune status). How to selectively target neurons that had been latently infected with HSV-1 was the major barrier for an efficient delivery using an HSV-1 vector carrying ribozymes. Currently this issue cannot be resolved, and because of the restricted transduction efficiency in neurons, increasing HSV-1 vector dose would not promote a higher delivery level. However, modifications of envelop proteins of recombinant HSV vectors in order to conduct receptor-specific binding can potentially lead to a selective targeting of infected cells.

Regions within gC(glycoprotein C) and gB (glycoprotein B) are known to contribute to 80% of heparin sulfate (HS) binding.[207] In addition, domains within gD that specifically interact with HveA (Herpes virus entry protein A) or HveC (nectin-1, a nomophilic cell adhesion molecule) have been defined.[48,196,197,231,264,385,386] The understanding of HSV-1 binding and entry led to the development of HSV vectors engineered with the ability to target distinct cell populations.[206] Although it has been generally accepted that in latency, the HSV genome is quiescent in protein expression, allowing latent viruses to hide from host immune system, recent studies have provided evidence against this concept. By employing more sensitive detection methods, the expression of HSV IE (immediate early), E (early), and L (late) genes was observed in latently infected neurons in mice.[59,60,103,191] *In vivo* data also supported HSV antigen expression in trigeminal ganglia (TG) in latently infected mice.[103,309] Together, these

results indicated that HSV vectors may be engineered to target HSV infected cells by recognizing HSV viral antigens on the cell surface.

The study of HSV vector ocular delivery also led to a very interesting finding that not only did the previous HSV-1 infection protect the cornea from a sequential super-infection, but that this protection was unilateral and not related to systemic immune response. This study was conducted in a rabbit model of ocular infection. The acute infection in rabbit cornea imitates clinical indications of human ocular herpes infection; the reactivation of HSV-1 can be induced efficiently by epinephrine iontophoresis[202], and the indication of infection also resembles the recurrent HSV-1 infection in human patients. The protective effect was detected two and a half months after the inoculation of replicating-defective HSV-1, and the same effect was observed at four months after the primary infection of wild-type HSV-1. If the replication-competent virus reactivated spontaneously during the four-month period, this might repeatedly boost the acquired immunity, and eventually protect rabbits from the super-infection. The protection effect on the cornea from a primary infection of a replication-defective HSV-1 (KD6) cannot be explained since the virus could not undergo lytic infection, although it could enter a latency-like stage in the trigeminal ganglia.

T-lymphocytes, especially $CD8^+$ T cell, have been recognized to play a very important role in control of HSV-1 acute infection[205] and latency.[85,289] It was suggested that both $CD4^+$ and $CD8^+$ T cells were enriched and retained in the mouse ganglion for life, and this effect was observed after HSV-1 corneal infection.[222,319] In human trigeminal ganglia (TG), $CD8^+$ T cells were localized in the neuron body of patients with a history of recurrent HSV-1 infections.[348] In addition, studies have shown that T cell-

derived anti-viral cytokines (e.g., IFN γ, TNF α, and RANTES, a T cell chemoatractant) can be detected more than 180 days after HSV-1 corneal infection in mouse TG.[47,58,132,222] These facts suggested that an immune surveillance was established to control HSV-1 latency and reactivation. However, they also led to a speculation that the existence of T lymphocytes (particularly $CD8^+$ T cells) and T cell-derived effectors may re-shape neuronal immunity to prevent a future invasion from another HSV-1 strain. It still remains for further investigation whether this immune surveillance exists for a prolonged period of time in the cornea, which can provide answers to the ocular protection rendered by the previous infection of replication incompetent HSV-1.

The HSV-1 vector study led to a conclusion that HSV-1 vector might need future modifications before it can be used for ribozyme delivery in gene therapy of HSV-1 infections. Further investigation of the interesting phenomenon of local immune protection elicited by a non-replicating HSV vector is currently on the way. A mouse ocular HSV-1 model is being used to study the mechanism since various cellular factors related to the immune response can be probed in the mouse system. In mice, modifications in cellular environment caused by HSV-1 infection can be studied, because antibodies are available to cytokines and to proteins involved in intracellular signal transduction. In addition, corneal infection of nude mice will indicate whether a T cell response is important in the localized immunity we have observed.

The effect in the cornea is especially interesting in that it will provide a better understanding that could lead to new vaccine-based approaches to prevent recurrent ocular HSV infection. $CD8^+$ T cell and IFN-γ can be studied for their roles in this phenomenon. If T cells or T cell-derived factors can be attracted by infection of non-

replicating HSV-1, it will be intriguing to see how long they are retained in the eye. Because the protective effect was induced by infection of replication-incompetent HSV-1, a very sensitive immune response must have been triggered. Therefore, HSV-1 antigens (especially gB and gD) may have an essential role in initiating this process. To avoid complications in the study, cell-free HSV-1 viral stocks[79,381] should be used, since a regular HSV-1 viral preparation often contains excessive amounts of viral proteins and cellular debris which could induce inflammation. Although an ICP4 defective HSV-1 vector was used in the preliminary study, it may be more informative to include another replication incompetent HSV-1 vector with disruptions in other essential genes (e.g., ICP8 or ICP27). A previous study by Morrison *et al*[257] indicated that after subcutaneous immunization of an HSV-1 capable of partially completing replication ($ICP8^-$ virus can still express α and β gene, while $ICP27^-$ virus can express α, β, and γ_1 genes) provided a better protection effect than a complete replication defective virus ($ICP4^-$ HSV-1). The protection effect led to a low clinical score of herpes keratitis in mice when they were super-infected with replicating HSV-1. Although systemic immune response is not a major consideration in the study of this dissertation, using different replication-defective HSV-1 viruses can help to define HSV-1 antigens that have the most potential to induce a persistent immune surveillance.

Viral Vectors for Corneal Gene Transfer

In this study, the potential uses of different viral vectors were explored for gene transfer in the cornea. As discussed in Chapter 5, adeno-associated virus, adenovirus, and herpes simplex virus vectors have their unique advantages. Iontophoresis of chemically modified ribozyme RNAs could be an alternative approach, although further evaluation is required. Another group of viral vectors which have not been studied in this

dissertation research are derived from retroviruses. The advantage of using retroviral vector for corneal gene delivery is to provide long-term transgene expression.

The retrovirus family, the Retroviridae, represents a unique group of viruses that contain a genomic RNA which is a dimer of linear, positive-sense, single-stranded RNA. The genomic RNA monomer is 7 to 13kb in size. After virion internalization and uncoating of viral envelop, the genomic RNA is reverse transcribed into double-stranded DNA. The unique feature that retroviruses share is that they can permanently integrate the viral genome into the host chromosomal DNA, and the integrated form of the virus (provirus) functions as the template for viral gene expression and production of viral progeny. This reverse flow of genetic information from RNA to DNA defines the hallmark of retroviruses. The genomic RNA of retrovirus virions is associated with the viral nucleocapsid protein and this complex is contained in a capsid (or nucleoid) which is surrounded by a spherical layer of protein matrix. Outside of the matrix is envelop consisting of a lipid membrane bilayer, whose surface is studded by projections of an envelop glycoprotein. The genera of the family Retroviridae have formalized by the International Committee on Taxonomy of Viruses (ICTV): The alpha-, beta-, and gamma-retroviruses are considered “simple” retroviruses, meanwhile the delta-, epsilon-retroviruses, lentiviruses, and spumaviruses are considered “complex”. Simple viruses encode the Gag, Pro, Pol, and Env proteins, whereas the complex viruses encode additional regulatory proteins. The entry of different retroviruses relies on their distinct receptors located on the surface of host cells.

Replication-defective vectors derived from retroviruses have been studied for the delivery of therapeutic genes and offer advantages of long-term expression, large package

capacity, and tissue specific tropism. The long-term expression of transgene by retroviral vector can be achieved by permanently integrating into host chromosomal DNA. One key step for the integration is the entry of viral DNA into the nucleus. Lentiviruses have an active nuclear transport mechanism[39,214,303,383], thus they can infect dividing and non-dividing cells efficiently, which made them very attractive gene delivery vectors. Although all current integrating gene-transfer vectors carry the risk of insertional mutagenesis[215], lentiviral vectors have the advantage in safety concern over their oncoretroviral counterparts.[361,395]

For corneal gene therapy, lentiviral vectors have great potential that they can provide prolonged transgene expression efficiently. Wang *et al*[377] tested a lentiviral vector encoding enhanced green fluorescent protein (eGFP) in human keratocytes *in vitro* as well as corneal epithelium and endothelium *ex vivo*. The GFP expression in corneal epithelium was visualized under fluorescent microscope at 3 days post-infection and up until 60 days. In this study, however, a requirement of virus-cell contact was observed for efficient transduction which might limit the application in corneal delivery. Another study by Bainbridge *et al*[16] suggested that in mice lentiviral vector could transduce corneal endothelium by anterior chamber injection and transduce retinal pigmental epithelium (RPE) by sub-retinal injection, and transgene expression was observed up to 6 weeks. A similar effect was also observed by Takahashi *et al*[346] using a different lentiviral vector, and up to 20 weeks after transduction the transgene expression still could be detected.

During the study of this dissertation research, an antiviral therapy was under development to deliver the transgene (therapeutic ribozymes) to the corneal epithelium.

Lentiviral vectors have significant advantages for this purpose. Corneal epithelial cells are highly differentiated and the cell layer regenerates within a relatively short period of time. With the ability of transducing the non-dividing cells, the lentiviral vector may transduce epithelial cells to express anti-HSV ribozymes. Therefore, a temporary protection against HSV infection can be expected. Considering the transgene expression can be turned on fairly quickly after lentiviral delivery (as early as 3 days), a therapeutic effect could be observed within the half-life of corneal epithelium, which is 10-14 days. In addition, lentiviral vectors may transduce limbal stem cells which are responsible for the renewal of the epithelial cell layer. HIV-1 based lentiviral vectors have led to long term expression of marker genes in the outer layers of the skin, suggesting that epidermal stem cells had been transduced.[15,117] Transduction of stem cells will render the expression of therapeutic ribozymes in the progenitor cell population. Consequently a long-term protection against HSV lytic infection would be provided. Lentiviral vectors may be delivered through topical application. However, similarly to AAV, HSV, and adenoviral vectors, a superficial abrasion on the corneal epithelium may be required for efficient transduction. As the intrastromal injection of lentiviral vectors only provided the transgene expression at the injection sites (unpublished data by Mohan, Schultz, and Wilson, 2003), the area of gene delivery could be very limited. A more efficient application of transgene delivery using lentiviral vector may be the *ex vivo* treatment of allogeneic corneas for transplantations[259], although the virus-cell contact is still the major factor for efficient transduction.[377]

APPENDIX A
ABBREVIATIONS

AAV	Adeno-associated virus
ACV	Acyclovir
Ad	Adenovirus
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ara-A	Vidarabine
ASLV	Avian sarcoma leucosis virus
CAR	Coxsakievirus and adenovirus receptor
cDNA	Copy DNA
CDS	Coding sequence
c-FLIP	cellular FLICE-inhibitory protein
CMV	cytomegalovirus
CNS	Central nervous system
CPE	Cytopathic effect
CS	Calf serum
DC	Dendritic cell
DI water	Deionized water
DMEM	Dulbecco's Modification of Eagle's Medium
DRG	Dorsal root ganglion
dsRNA	Double-stranded RNA
E.coli	*Escherichia coli*

EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
FADD	Fas associated dead domain
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FLICE	FADD-like ICE
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
GTF	General transcription factors
HCF	Host cell factor
HEDS	Herpetic Eye Disease Study
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen (or Human lymphocyte antigen)
HPC	Hippocampus
Hrl	Herpes resistance locus
HSE	Herpes Simplex Encephalitis
HSV	Herpes Simplex Virus
HSK	Herpes Simplex Keratitis
IDU	Idoxuridine
IE gene	Immediate early gene
IL-12	Interleukin 12

IM	Intramuscular
INF	Interferon
ITR	Internal terminal repeat
IV	Intravenous
kb	kilo bases
LAT	Latency-associated transcript
LTR	Long terminal repeat
MEM	Eagle's minimal essential medium
MHC	major histocompatibility class
MOI	Multiplicity of Infection
MT	Mocking Transfection
MuLV	Murine Leukemia Virus
ms	milli-second
NaCl	Sodium Chloride
NO	Nitric oxide
NPC	Nuclear Pore Complex
ODN	Oligodeoxynucleotide
ORF	Open Reading Frame
PCR	Polymerase chain reaction
PNS	Periphery nervous system
RISC	the RNA-induced silencing complex
RNAi	RNA interference
RNA Pol II	RNA polymerase II

RPE	the retina pigment epithelium
RSC	Rabbit Skin Cell
RSV	rous sarcoma virus
SDS-PAGE	Sodium Dodecyl Sulfate- polyacrylamide gel electrophoresis
shRNA	Small hairpin RNA
siRNA	Small interference RNA
SC	Spinal cord
TAP	Transporter associated with antigen presentation
TBP	TATA-box Binding Protein
TFIID	Transcription Factor II D
TG	Trigeminal ganglion
TGN	Trans-Golgi network
TK	Thymidine Kinase
TNF-α	Tumor Necrosis Factor α
TRAIL	TNF-related apoptosis-inducing ligand
Tris-HCl	Tris Hydrochloride or 2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
U_L	Unique Long region
U_S	Unique Short region
μs	micro-second
VSV	the vesicular stomatitis virus

Xist	X (chromosome) inactive specific transcript
wt	Wild-type

APPENDIX B
REAL-TIME PCR PRIMERS AND PROBES

The following appendix provides a comprehensive summary of design and sequences of primers and probes of real-time polymerase chain reaction used in this dissertation.

Table B-1. Real-time PCR primers and probes

DNA Target	Sequence	Accession Number. (Nucleotide Number.)
HSV ICP4	5’ CAC GGG CCG CTT CAC 3’ (forward) 5’ GCG ATA GCG CGC GTA GA 3’ (reverse) 5’ CCG ACG CGA CCT CC 3’ (probe)	X14112 (130208-130292)(147941-148025)
HSV-1 U_L20	5’ CCA TCG TCG GCT ACT ACG TTA C 3’ (forward) 5’ CGA TCC CTC TTG ATG TTA ACG TAC A 3’ (reverse) 5’ CCC GCA CCG CCC AC 3’ (probe)	X14112 (41118..41187)
HSV DNA Pol (U_L30)	5' AGA GGG ACA TCC AGG ACT TTG T 3' (forward) 5' CAG GCG CTT GTT GGT GTA C 3' (reverse) 5' ACC GCC GAA CTG AGC A 3' (probe)	X14112 (65880-65953)
U_L54 (ICP27)	5' GCC CGT CTC GTC CAG AAG 3' (forward) 5' GCG CTG GTT GAG GAT CGT T 3' (reverse) 5' CAG CAC CCA GAC GCC 3' (probe)	X14112 (113945-114034)
Mouse Xist	5’ GCTCTTAAACTGAGTGGGTGTTCA 3’ (forward) 5’ GTATCACGCAGAAGCCATAATGG 3’ (reverse) 5’ ACGCGGGCTCTCCA 3’ (probe)	NR001570 (857-925)

APPENDIX C
RECIPE OF SOLUTIONS

1. Dialysis Buffer for Adenovirus Purification:
   It contained 10mM Tris-HCl (pH7.5), 200mM Sodium Chloride (NaCl), 1mM Ethylenediaminetetraacetic acid (EDTA), and 4% (weight/volume) sucrose. To make 2L of the dialysis buffer, 20mL of 1M Tris-HCl (pH7.5), 80mL of 5M NaCl, 4mL of 0.5M EDTA, and 80g of sucrose were mixed and brought up to the final volume with autoclaved de-ionized water. This solution should be made fresh and cooled at 4°C before use.

2. Elution buffer for the ribozyme cloning protocol: (a total volume of 6mL)
   a. 5M Ammonium acetate 600µL
   b. 1M Magnesium acetate 60µL
   c. 0.5M Ethylenediaminetetraacetic acid (EDTA) 120µL
   d. 10% Sodium Dodecyl Sulfate (SDS) 60µL
   e. Sterile water 5,160µL
3. DOC Lysis Buffer for Adenovirus DNA Mini-prep:
   - 20% of total volume of absolute ethanol
   - 100mM Tris-HCl, pH9.0
   - 0.4% solium deoxycholate
4. PBS (Phosphate-buffered Saline)
   - 137mM NaCl
   - 2.7mM KCl
   - 10mM Na_2HPO_4
   - 2mM KH_2PO_4

   Dissolve 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24g of KH_2PO_4 in 800mL of distilled H_2O. Adjust the pH to 7.4 with HCl. Add H_2O to 1L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15psi ($1.05kg/cm^2$) on liquid cycle or by filter sterilization. Store the buffer at room temperature.

Note that the recipe presented here does not include divalent cations. If necessary, 1mM $CaCl_2$ and 0.5M $MgCl_2$ may be supplemented.

5. 50mg/mL Pronase
Pronase was purchased from CALBIOCHEM (San Diego, CA) and resuspended in sterile de-ironed water to a final concentration of 50mg/mL. The solution was incubated at 37°C for 30 minutes to inactivate other enzymes before use.

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BIOGRAPHICAL SKETCH

Ms. Jia Liu was born in December 23rd, 1977 in one of the four municipalities, Tianjin, the third largest city in China. As the only child of Mr. Yuji Liu and Mrs. Hong Zhang, Ms. Jia Liu grew up in a very loving and caring family surrounding with Chinese traditional virtues. They instilled the virtues in her philosophy for life: diligence, honesty, integrity, courage, and passion. She is very talented in various ways: Ms. Jia Liu was always fascinated by science, especially in mathematics. At the age of 13, she won the No. one prize in mathematics competition in the city of Tianjin. She was also simultaneously engaged in art and Chinese literature. At the age of 16, Ms. Jia Liu was admitted in Yaohua High School, the top high school in Tianjin. Out of more than 2000 applicants, Ms. Jia Liu ranked the top 49th in the open entry examination of Yaohua High School. With highly accomplished scores in the national entry examination and an excellent scholastic background, Ms. Jia Liu was admitted to Nankai University in 1996. Nankai University is considered one of the most prestigious universities in China, and there she majored in biochemistry. Due to her passion in science, she wanted to continue working on a project related to biomedical sciences. Ms. Jia Liu was then recruited and trained by Dr. Chunzheng Yang, a highly respected and productive scholar in the field of pharmacology. When she finished her college education with a bachelor degree in biochemistry, Ms. Jia Liu continued working on a project of antibody engineering for cancer therapy in the state key laboratory in Chinese Academy of Medical Sciences. Becoming an academic scholar with extensive contributions to society has always been

her ultimate goal for life. In 2001, Ms. Jia Liu decided to travel oversea to the United States of America, to continue her dream of science education. She was accepted in the interdisciplinary program (IDP) in University of Florida, College of Medicine, and pursued a Ph.D. degree major in genetics in the department of molecular genetics and microbiology. After nearly five years of diligent work, Ms. Jia Liu was awarded degree of Doctor of Philosophy in the fall 2006. In her tradition Chinese family, Ms. Jia Liu was the first female to achieve the Ph.D. degree in her generation. She is going to pursue a post-doctorate training in the field of virology.