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RNase P Catalytic RNA as a Tool for Anti-Viral Therapy



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# RNase P Catalytic RNA as a Tool for Anti-Viral Therapy

By

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Professor Fenyong Liu, Chair
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**Abstract**

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**By**

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**Professor Fenyong Liu, Chair**

Engineered RNase P-based gene-targeting agents derived from the RNA subunit of *E.coli* RNase P, represent a promising approach for anti-viral applications. The focus of the research in this dissertation has been to develop anti-viral therapeutic agents from RNase P-based RNAs, with enhanced efficacy at cleaving targeted human cytomegalovirus (HCMV) mRNAs in cells. This dissertation is organized into six chapters that conclude my research. In Chapters 2 and 3, engineered RNase P-based ribozyme variants V661-IE2 and V718-A, which were generated using an in-vitro selection procedure, were designed to target HCMV mRNAs of the immediate-early protein (IE2), and the overlapping mRNA region of capsid assembly protein (AP) and protease (PR), respectively. Variant ribozyme V718-A showed a nearly 60-fold greater activity than wild type M1 RNA in cleaving target AP/PR mRNAs in-vitro. Additionally, a reduction in AP/PR expression of 98% – 99% and a viral growth inhibition of 50,000-fold was observed in cells expressing V718-A, while a 75% decrease in AP/PR expression and a viral growth reduction of 500-fold was detected in cells expressing M1-A. Variant V661-IE2 showed a nearly 50-fold greater in-vitro cleavage activity than wild type M1-IE2 in targeting IE2 mRNA. Likewise, A nearly 98% decrease in expression of IE2 and a 3,500-fold reduction in viral growth was observed in cells expressing variant V661-IE2, compared to a 75% decrease in IE2 expression and a 100-fold reduction in viral growth in cells expressing wild type ribozyme M1-IE2. These results indicate that the generated ribozyme variants selected to be highly active in-vitro are also more effective in targeting mRNAs in cultured cells, and are a potential effective approach for anti-viral therapeutic applications. In Chapter 4, engineered external guide sequence (EGS) molecules, which were generated from an in-vitro selection procedure, was designed to target mRNAs of the herpes simplex virus 1 (HSV-1) major transcriptional regulator (ICP4). The EGS variant induced human RNase P to cleave ICP4 mRNA in-vitro, with a 60-fold greater activity than EGSs generated from a natural ptRNA substrate. Additionally, a nearly 97% inhibition in ICP4 expression, and a 7,000-fold decrease in viral replication was seen in HSV-1-infected cells expressing the variant EGS, compared to a 75% reduction in ICP4 expression and a 500-fold decrease in replication in cells expressing the EGS derived form a natural ptRNA. These results suggest that engineered EGS variants can effectively block HSV-1 gene expression and viral growth in cells, and show the potential for engineered EGS variants to be developed as effective anti-viral therapeutic agents.

Attenuated strains of Salmonella bacteria represent promising nucleic acid delivery agents. Lastly, in Chapter 5 we constructed a novel strain of attenuated Salmonella for the delivery and expression of antigens hemagglutinin (HA) and neuraminidase (NA) from the H5N1 influenza virus. Here, we showed that the Salmonella vaccine induced a substantial production of anti-HA serum IgG and mucosal IgA, and anti-HA interferon-gamma producing T cells in orally-vaccinated mice. Additionally, we observed orally-vaccinated mice that expressed viral antigens HA and NA were completely protected from H5N1 virus in lethal amounts, while all mice treated with empty expression vector-carrying Salmonella were not protected. These results indicate that Salmonella-based vaccines induces strong humoral and cellular immune responses that are antigen-specific. Thus, Salmonella-based vectors can be designed to provide effective immune protection against multiple influenza virus strains. Moreover, this study highlights the potential in developing attenuated Salmonella strains as new and viable oral vaccine vectors or as effective gene-delivery agents for anti-viral gene-targeting applications in vivo.

*To my wife, my mother and family for their support and love*

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# 1 Introduction

## 1.1 Human Cytomegalovirus

Human cytomegalovirus (HCMV) or human herpesvirus 5 (HHV-5) belong to the Beta-herpesvirinae genus of the Herpesviridae family. The Beta-herpesvirus subfamily includes HCMV and its primate CMV relatives, muromegalovirus (murine cytomegalovirus, MCMV), roseolovirus and proboscivirus [1]. HCMV is a prevalent and opportunistic pathogen that causes mild or subclinical (silent) disease in immune-competent adults, however, may lead to severe morbidity or mortality in neonates and immuno-compromised individuals such as organ transplant patients and AIDS patients [1]. The total prevalence of HCMV infection is estimated to be 58.9% in the US and over 90% in developing countries [1, 35, 36].

A pregnant woman can pass HCMV on to her unborn child by congenital (transplacental) infection with tragic result. The virus is transmitted congenitally in women with primary infection (HCMV-seronegative) which causes a multitude of birth defects including mental problems, vision and hearing impairment, microcephaly, seizures, and is the leading cause of congenital deformities [1, 2]. On average, 33% of women with primary infection transmit the virus trans-placentally while transmission will occur in only 1% of women with re-infection (HCMV-seropositive) during pregnancy [2]. It is estimated that nearly 40,000 children are born with CMV infection each year and about 400 of these cases are fatal [42]. Most congenital infections are asymptomatic given that only 10 – 15% of children with congenital CMV show clinical signs of infection [43]. A large majority (90%) of children with symptomatic infection, and few (10%) with asymptomatic infection will develop one or more long-term CMV-related medical conditions including those mentioned above [43]. Attesting to the disease impact, It is estimated that the incidence (8,000 children) of CMV-related long-term medical conditions that develop each year in the US is far greater than that of more well-known childhood disorders, such as down syndrome (4,000/year), fetal alcohol syndrome (5,000/year) and spinal bifida (3,500/year), designating congenital CMV infection as the most common cause of birth defects and childhood disorders in the US [42].

For immunocompromised individuals, CMV is one of the most common and difficult pathogens because it results from three ways: reactivation of latent virus, reinfection, and primary infection [1]. For AIDS patients, CMV infection can lead to blindness and other complications such as gastrointestinal disease [3-5]. Additionally, CMV seropositivity remains a strong adverse risk factor for survival following partial T-cell–depleted allogeneic stem cell transplantation and other organ transplants [1]. To further complicate care, most infections are not clinically discernible among non-immunocompromised patients with transfusion acquired CMV [1, 37].

### HCMV Genome and Virion Structure

Beta Herpesvirus, as with all herpesviruses have linear double-stranded DNA genomes. HCMV, the largest of all herpesvirus genomes, is approximately 235 kbp in size and encodes for 167 gene products [1, 11, 31]. The HCMV genome displays a class E genome order with terminal and internal repeats flanking unique long (UL) and unique short (US) regions (ab-UL-b'a'c'-US-ca). The terminal and internal a sequences (a and a') contain cleavage and packaging signals that promote isomerization during replication. The b and b' are the terminal repeat long (TRL) and

internal repeat long (IRL) sequences flanking the UL region, while the c' and c are the internal repeat short (IRS) and terminal repeat short (TRS) sequences flanking the US region. The HCMV genome contains an origin of synthesis (oriLyt) centered in the UL region, a position conserved among all betaherpesviruses and is required for DNA replication.

The HCMV virion structure, which is common to all herpesviruses, houses the dsDNA genome inside a 125 nm icosahedral capsid composed of a number of viral core proteins [3, 46]. The capsid is surrounded by the viral envelope which is a lipid bilayer derived from the host cell during replication and contains virus-encoded glycoproteins that function in attachment, fusion, and entry into host cells. The tegument layer of the virion structure, located between the capsid and envelope, is composed of tegument proteins that perform a variety of functions including host-cell conditioning at early stages of infection and mediating later stages of viral assembly. In contrast to the mature infectious virion, particles that are produced with an immature capsid and an absence of viral DNA are termed non-infectious enveloped particles (NIEPs). Additionally, particles produced that are enveloped and contain an abundance of tegument proteins but lack a nucleocapsid and viral DNA are termed dense bodies. A large majority of virus progeny are non-infectious because NIEPs and dense bodies constitute more than 50% of viral preparation, in addition to the instable virion particles that are released from cells [38].

**Viral Capsid**

The CMV nucleocapsid is made up of 150 hexons and 12 pentons, has an icosahedral symmetry (T = 16), and is ~ 200 – 300 nm in diameter [3, 46]. The nucleocapsid houses the 235 kb linear DNA genome of HCMV which is packaged into hexagonal arrangements within the nucleocapsid. Other than the viral DNA and the polyamines present, the interior of the capsid is void of any other proteins coded by the virus or host cell. Procapsids mature into DNA-containing capsids termed C-capsids. Other capsids known as A and B capsids that do not mature into DNA-containing capsids are also found in the cell and are called non-infectious enveloped particles (NIEPs). These NIEPs are observed in EM to be less dense than infectious virion particles due to the lack of encapsidated DNA [46]. The capsid is formed in the host cell nucleus following DNA replication and transcription by way of the CMV essential assembly protein (AP) and the capsid scaffolding protein (CSP) [1, 3].

**Capsid Assembly**

The capsid assembly process starts with five herpesvirus-conserved capsid components (MCP, TR11, TR12, SCP, and PORT) arranging into a procapsid surrounding the assembly protein precursor (pAP) which functions in initiating capsid assembly [39]. In the nucleus, assembled procapsids, mature nucleocapsids, and abnormal capsids accumulate in proximity to replication components and subsequently mature to viral particles and NIEPs.

**Viral Tegument**

The viral tegument is a region of the virion that is formless and joins the capsid and viral envelope. In addition to containing some viral and cellular RNA, this tegument section houses much of the viral proteins, thus termed tegument proteins. Conventionally, components of the virion are considered tegument components if they are not part of the capsid or the viral envelope. Tegument proteins often undergo phosphorylation and have many functional roles during host

infection. Within the tegument section, gene products of UL83 and UL82 which include pp65 and pp71, respectively, are the most abundant proteins [1 ,3, 46]. Tegument proteins have been shown to play important roles in host infection and virion maturation that include tempering of host cell response to infection and impeding host cell defense. Additionally, tegument proteins function in earlier stages including release of viral DNA into the cell nucleus and later stages including encapsidation of viral DNA [1, 39].

**Viral Envelope**

The envelope of mature HCMV particles contains major glycoproteins that are essential for virus attachment, fusion and entry into host cells. These envelope glycoproteins, which are considered a core group across all herpesvirus, form three separate complexes: gB, gH-gL, and gM-gN [1, 3]. Additionally, gH-gL forms a pentamer complex with the gene products of UL128, UL130 and UL131 (gH-gL:UL128-131) which confers specific tropism for endothelial, epithelial, and dendritic cells thus, deletion of this region from repeated passaging results in loss of tropism for these cells [1, 38].

**Replication and Gene Expression**

HCMV show a tightly regulated temporal cascade of gene expression during lytic replication with three main classes of genes expressed: immediate-early, delayed-early, and late genes. This cascade begins immediately after viral entry with expression of immediate early [IE or alpha(α)] genes which code for viral proteins that optimize the host cell for viral gene expression and control transcriptional regulation of delayed-early [DE or beta(β)] genes [1, 3]. Expression of delayed-early genes following immediate-early genes are responsible for viral genome replication and control expression of the following late [L or gamma(γ)] genes. Late genes are expressed following viral genome replication and code for viral structural proteins that are required for viral particle assembly and release of infectious virions. The entire replication cycle of HCMV takes 48 – 72 Hrs. for virus progeny to be released with maximal virus progeny being released at 72 – 92 hours post infection (hpi).

**Host Cell Infection**

HCMV is a ubiquitous virus that is distributed throughout the world and spread by direct exposure to bodily fluids of infected carriers [1]. A common characteristic of all herpesviruses, HCMV remains latent in the body for life following acute infection . Once in latency the virus can be transmitted by occasional shedding by the host. Hematopoietic stem cells (HPC) harbor latent virus, whereas epithelial cells of salivary glands and kidneys are the site of persistent replication, with occasional shedding in body fluids throughout life [40, 41].

HCMV naturally targets a number of cell types including epithelial, endothelial, macrophage, and dendritic cells, as demonstrated with viral antigens and genetic material of patients who died from acute cmv infection [40, 41]. As with all beta-herpesvirus, HCMV replicate relatively slowly and stay cell-associated in cell culture. Human Fibroblast cells are well suited for laboratory propagation of CMV cultured from host cells because the virus can be easily and extensively propagated in fibroblast. Nevertheless, repeated passaging of clinical isolates of CMV can lead to accumulated mutations in that strain [1, 3]. Consequently, many laboratory strains carry large deletions in the genome (i.e. UL128-131) as well as smaller deletions, and some point

mutations as a result of extensive passaging of CMV viral strains. Many of these mutations have a propensity to alter expression of specific genes that control cell tropism for CMV. As a result of accumulated mutations, fibroblasts will retain permissiveness to fibroblast-propagated strains, yet other cell types such as cultured macrophages, dendritic cells, epithelial cells, and endothelial cells are not permissive to these laboratory strains [1, 3]. This results in commonly used and highly passaged laboratory strains (i.e. TOWNE, AD169, Toledo) that are able to replicate robustly in fibroblasts but not very well in endothelial cells, epithelial cells, dendritic cells and macrophages.

**Anti-viral Treatment for HCMV**

Currently, a number of drugs have been approved by the FDA for use in treatment of HCMV, including Ganciclovir (Cytovene), Valganciclovir (Valcyte), Foscarnet (Foscavir) and Cidofovir (Vistide) [1, 3]. These Antiviral drugs have demonstrated ability to restrict or inhibit HCMV in immunocompromised patients, yet are inherently toxic to the cell, thus use of these agents is highly restricted for patients with high disease risk [1]. In addition to the toxicity, other limitations to these antiviral agents include low bioavailability and short half-life (oral administration), or low potency for HCMV. Long-term treatment of immunocompromised patients with antiviral drugs has been linked a rise in HCMV strains with mutations in genes UL97 and UL54, coding for the HCMV phosphotransferase and polymerase enzymes, respectively [1]. Moreover, previous studies have shown that particular mutations in the HCMV genome are related to virulence and HCMV resistance to antiviral agents in-vivo and in-vitro [1]. Antiviral treatment in normal immunocompetent hosts is not indicated due to the characteristic spontaneous resolution to latent infection in most of these patients.

Currently, no vaccine exists for the prevention of HCMV infection and to date, drugs that exist for anti-HCMV therapy are few and not completely effective. Therefore, it is important to develop novel therapies for the treatment of HCMV infections.

## 1.2 RNase P catalytic RNA as a Tool for Anti-Viral Therapy

Nucleic acid-based technologies such as RNAi, antisense oligonucleotides, aptamers and ribozymes that inhibit gene expression by targeting specific RNA sequences, represent promising agents for gene-targeting applications [20-23]. Approaches that target specific viral gene sequences using nucleic acid molecules have great potential as effective therapeutics for the inhibition of HCMV gene expression and viral replication. RNA interference strategies (RNAi) have been used for gene-targeting of several human viruses and were shown to be effective [8]. In addition to siRNAs, ribozymes have been shown to effectively cleave viral mRNAs and inhibit viral replication in human cells [8-10]. In comparison to other gene targeting technologies such as RNAi, the ribozyme has its benefits. siRNAs induce several cellular factors (Drosha, Dicer or Exportin V) which may affect normal cellular function at high levels of siRNA [6, 20-23]. Ribozymes have not been shown to saturate the cellular machinery and as exogenous agents, can be expressed in many organisms to cleave targeted RNAs. Additionally, RNase P ribozymes may enhance their intracellular stability and lifetime by interacting with specific cellular proteins including the RNase P protein subunit [30, 44]. Thus, RNase P-based ribozymes represent a unique and promising class of gene-interfering agents for therapeutic applications.

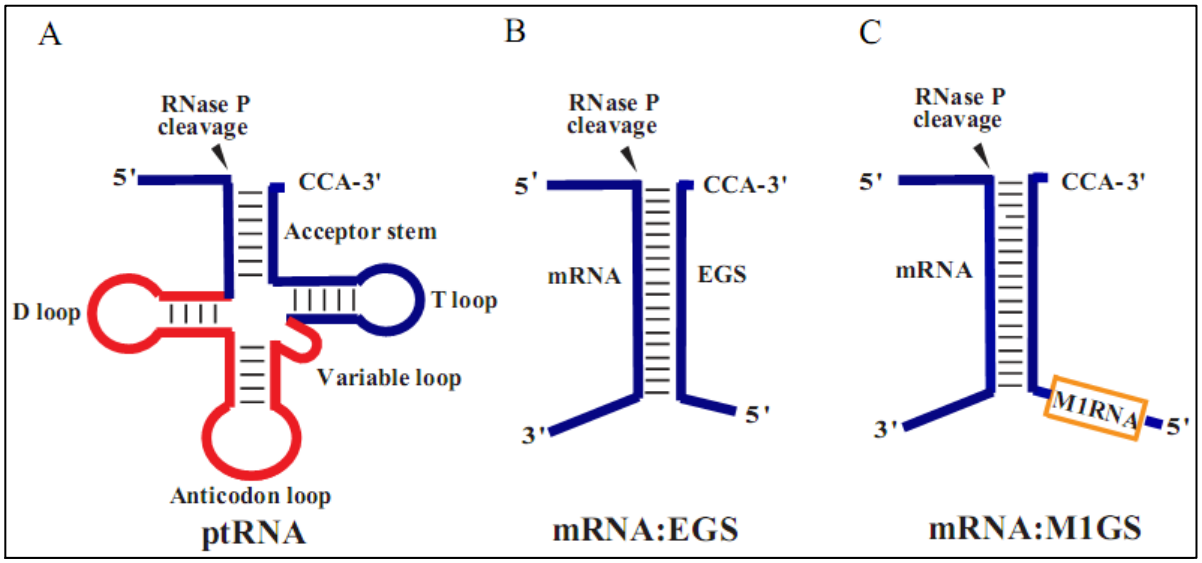


**Figure 1.** Substrates for RNase P and M1 ribozyme. (A) pre-tRNA (ptRNA); (B) complex of EGS and target mRNA; (C) M1GS RNA binding to its mRNA substrate. Arrowhead indicates the cleavage sites.

Ribonuclease P (RNase P) is a ribonucleoprotein complex that is responsible for the 5' maturation of tRNAs [12, 13]. It functions to catalyze a hydrolysis reaction to remove the 5' leader sequence from tRNA precursors (ptRNA) in addition to processing several other small RNAs [12, 31]. RNase P, is one of the most abundant, stable, and efficient enzymes in cells, and complexes have been found in all organisms examined [30, 45]. In E. coli, RNase P consists of a single 400 nt catalytic RNA subunit (M1 RNA) and a single 14kDa small protein subunit (C5) which together comprise the holoenzyme [14]. The RNase P ribozyme is highly active in-vitro at cleaving its pre-tRNA substrate or a target mRNA in the absence of C5 protein at high divalent magnesium cation [$Mg^{2+}$] and salt concentrations [17, 24]. As indicated in previous studies, the ribozyme recognizes its substrate through the folded tertiary structure and not the primary sequence [12, 30]. Thus, any mRNA can be targeted for cleavage by the ribozyme through binding of a complimentary external guide sequence (EGS) to the substrate, forming a structure with features resembling that of ptRNA, which includes the 5' leader sequence, the acceptor stem, and the 3' CCA sequence (Figure 1, A, B) [15, 16].

Recent studies have shown that the targeting efficiency of the M1 RNA ribozyme can be even further enhanced if the EGS is covalently linked to the 3' end of the M1RNA to form a sequence-specific ribozyme (M1GS RNA) (Figure 1C) [18]. We have previously shown that M1GS RNAs can reduce expression levels by more than 80% when targeted to mRNAs of a HCMV essential major transcription regulatory protein IE2, in addition to a 150 – fold reduction in viral

growth in human cells [27]. Thus, M1GS RNase P-based ribozymes can be utilized as promising gene-targeting agents for anti-HCMV therapy.

For the RNase P ribozyme-based approach to be utilized as an effective gene-targeting system it is necessary to enhance the catalytic efficiency of the RNase P ribozyme. We hypothesize that improving the catalytic efficiency of RNase P ribozymes may lead to improved efficacy in inhibiting viral gene expression and viral growth in cultured cells. Previously, we used an in-vitro selection strategy to generate variants of RNase P ribozyme that have greater catalytic activity in cleaving target mRNAs than the wild type M1 RNA ribozyme [28]. With the in-vitro selection procedure, highly active M1GS ribozymes were isolated from an M1 RNA pool that contained ribozymes with random mutations and were shown to have substantially greater cleavage efficiency and reduction in viral gene expression than M1 RNA in cells. Thus, variants of RNase P ribozymes may potentially be used as a powerful gene-targeting approach for anti-CMV applications.

EGS-mediated degradation of mRNAs constitutes a new approach for gene-silencing applications mediated by nucleic acid molecules. This strategy has great potential because a custom-designed EGS can be used to guide intracellular RNase P to cleave any specific mRNA with high efficiency [8, 16]. Thus, the EGS-based strategy, which employs endogenous RNase P for targeted cleavage, is unique and different from the ribozyme-based approach, which introduces an exogenous ribonuclease (RNase p-based ribozyme) for cleavage. Previous studies have shown that the M1 RNA catalytic subunit of Rnase P can cleave its mRNA substrate more efficiently if in the presence EGSs [8, 15, 19]. The EGS directs the RNase P or its M1RNA to cleave the target mRNA when the EGS is hybridized to the target mRNA forming a structure that resembles a portion of the enzyme's natural substrate ptRNA [16]. Recent studies have shown that expression of EGSs alone in tissue culture can inhibit the gene expression of herpes simplex virus and influenza virus, in addition to abolishing viral replication [25, 26]. Furthermore, as evident from previous studies, the levels of EGS-mediated mRNA cleavage and anti-viral efficacy are akin to those observed using RNase P-based ribozymes [32, 33, 34]. Thus, an EGS can be designed to target any RNA for specific cleavage by RNase P or its M1 RNA catalytic subunit.

Moreover, previous studies indicate that the in-vitro selection procedure could represent an effective approach in screening for highly active EGS molecules for gene-targeting applications [32]. Thus, EGS molecules may constitute a novel class of gene-targeting antiviral agents.

One of the fundamental challenges in gene-therapy approaches is the development of efficient delivery systems that are tissue and cell specific, for in-vivo applications. Attenuated strains of Salmonella bacteria represent promising nucleic acid delivery agents as they can be orally administered. We have previously generated attenuated *Salmonella* strains to deliver RNase P ribozyme-based gene targeting agents to cells, leading to effective inhibition of CMV target-gene expression and viral replication [47]. Thus, oral inoculation of *Salmonella* can efficiently deliver an M1GS sequence, leading to expression and effective inhibition of viral systemic infection, and represent promising gene-targeting approaches for in-vivo anti-viral therapeutics.

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# 2 Chapter 2 – RNase P Ribozymes Inhibit the Replication of Human Cytomegalovirus by Targeting Essential Viral Capsid Assembly Proteins (AP).

## 2.1 Introduction

Human cytomegalovirus (HCMV) is a member of the herpesvirus family and is a pathogen of serious consequence that causes life-threatening disease in immunodeficient or immunocompromised individuals including newborn infants, HIV-infected patients and organ transplant patients [1]. HCMV is one of the leading pathogens in causing birth defects in neonates including hearing loss and mental retardation and is the leading cause of congenital deformities [2]. Despite the serious consequences, no vaccine currently exists for the prevention of HCMV infection and not many effective therapeutic agents exist to treat HCMV infection. Therefore, there is an urgent need for researchers to investigate and develop innovative therapeutic approaches for the treatment and prevention of HCMV infections.

RNase P is an RNA-Protein (ribonucleoprotein) complex that functions in processing premature tRNAs (ptRNAs) in the nucleus of cells. Previous studies have demonstrated that the RNA portion of RNAase P (M1 RNA) in E. coli is the catalytically active subunit that can hydrolyze phosphodiester bonds of diverse substrates through the recognition of the substrate tertiary structure [4]. The tertiary structure detected by the M1 RNA (ribozyme) is a stem structure formed by the binding of the substrate and a complimentary external guide sequence (EGS) resulting in a structure that resembles features of the natural substrate (the 5' leader sequence, acceptor stem, and t stem regions of ptRNA). Consequently, there is potential for RNase P ribozyme to cleave any mRNA substrate by designing a modified ribozyme (M1GS) that contains a specific complimentary guide sequence covalently linked to the 3' end of M1 RNA (Figure 1 Ch. 1) [5-8].

Technologies that inhibit gene expression by targeting specific select RNA sequences, such as RNAi, antisense oligonucleotides, aptamers and ribozymes represent promising therapeutic approaches [9-12]. Ribozymes possess some distinctive advantages when compared to RNAi and several other gene-targeting strategies. Because the RNAi technology relies on several cellular components (Exportin V, Drosha, or Dicer) to function, this may have a substantial effect on normal cellular processes [11, 13, 14]. RNase P ribozymes do not employ these cellular factors and is considered exogenous, thus can be expressed in a diverse array of organisms to catalyze the cleavage of specific RNAs of choice [15, 16]. Additionally, in-vitro studies of mutagenized M1 RNA make it easily possible to enhance the catalytic activity and substrate specificity of ribozymes [17, 18]. Therefore, there is tremendous potential in developing RNase P to not only implement as clinical therapeutics, to use in elemental scientific research as well.

To develop the RNase P ribozyme approach into a viable tool it is critical to maximize the catalytic activity of the ribozyme. Using an in-vitro selection procedure, our group has previously assembled ribozyme variants with enhanced targeting activity compared to M 1RNA [19, 20]. In the present study we generated a variant ribozyme (V718-A) that was designed to target the mRNA of HCMV protease (PR) and assembly protein (AP) within their overlapping coding regions of UL80 and UL80.5, respectively. Because both AP and PR are highly conserved genes and are involved in capsid assembly, and thus essential for viral replication, they are ideal genes to target [1, 21, 22]. Moreover, we surveyed the in-vitro enzymatic activity of the generated ribozyme, as well as tested how effective this ribozyme is at inhibiting targeted viral gene expression and viral

replication in living cells. The results of the present study demonstrate that the generated ribozyme variant (V718-A) possesses enhanced activity in reducing AP/PR gene expression and preventing HCMV replication in cells.

## 2.2 Methods

### 2.2.1 Mapping of the AP mRNA Accessible Regions in Cells

Most RNAs exist in highly folded conformations bound to proteins in the cell therefore, to identify the regions of AP mRNAs that are accessible to ribozyme binding, we treated cells with dimethyl sulfate (DMS). An in-vivo mapping procedure was carried out to identify DMS-accessible regions of AP mRNA within the cell, following protocols described previously [17, 25, 26]. Cells infected with HCMV were Initially treated with dimethyl sulfate (DMS) for 5–10 min, then following total RNA extraction, RNAs were used for primer extension assays with radiolabeled oligonucleotides. Finally, primer extension products were separated in 8% denaturing gels for subsequent analysis. The DMS-modification sites represent potential accessible regions for binding to the ribozyme.

### 2.2.2 In Vitro Ribozyme Studies

The DNA template of the 37 nucleotide AP mRNA substrate (ap11) for in-vitro studies was amplified by PCR using pGEM3zf (+) as a template with forward primer AF25 (5'-GGAATTCTAATACGACTCACTATAG-3') and reverse primer AP11 (5'-CGGGATCCGTCCGA GGACGACGACGACGCCGCCGCCCTATAGTGAGTCGTATTA-3') which contains a T7 promoter and the AP mRNA coding sequence, respectively. Plasmids pFL117, pV718, pV718-C and pC102 were used as templates to generate ribozymes M1-A, V718-A, V718-C and M1-C, respectively, and were described in previous studies [19,27]. The forward primer was AF25 while the reverse primer was M1AP11 (5' -CCCGCTCGAGAAAAAATGGTGTCGTCGTCGTCCTCGGATGTGGAATTGTG-3') with the underlined sequence corresponding to the guide sequence. Ribozymes M1-C and V718-C contained the same mutations found in C102 which is a non-functional M1 RNA mutant with point mutations ($A_{347}$ $C_{348}$ → $C_{347}$ $U_{348}$, $C_{353}$ $C_{354}$ $C_{355}$ $G_{356}$ → $G_{353}$ $G_{354}$ $A_{355}$ $U_{356}$). To synthesize the RNA substrate ap11 and ribozyme RNAs, A T7 in-vitro transcription kit (Promega) was used [28]. Kinetic analyses and gel-shift binding assays were conducted following experimental procedures as previously described [19, 20, 29].

### 2.2.3 Construction of Ribozyme-Expressing Cell Lines

Ribozyme-expressing Cell lines were produced as previously described [20, 30, 31]. Briefly, the sequences coding for M1GS were sub-cloned into the LSXN retroviral vector and the constructed LXSN-M1GS vectors were transfected into human amphotropic PA317 cells. At 48 h post-transfection, supernatants were harvested and used to infect human U251 cells. Cells were grown in culture medium containing neomycin (600 μg/mL) at 48–72 h post-infection for the selection cloning of and neomycin-resistant cells [20, 30, 31].

### 2.2.4 Studies of Viral Gene Expression and Growth in M1GS-Expressing Cells

Cells expressing M1GS were either mock-infected or infected at a multiplicity of infection (MOI) of 1 with HCMV. At different time points post-infection, total RNA and protein samples were prepared and studied by Northern and Western blot analyses [31–33]. Samples were harvested at 8 h and 24 h post-infection to detect the expression of IE2 mRNA and protein, respectively. Samples were harvested at 48 h post-infection to measure the expression of US2 and AP/PR mRNAs, [31–33]. Samples were harvested at 72 h post-infection to measure the expression of UL44, UL83, AP, and PR proteins. To measure the expression HCMV 5kb RNA for control expression, samples were harvested at 8 and 48 h post-infection. Samples were harvested at 24 and 72 h post-infection to measure the expression of actin protein as a loading control [31–33]. For the Northern blot analyses, prepared RNA samples (30 µg) were separated in formaldehyde-containing denaturing agarose gels (1%). After separation, RNAs were transferred to membranes and hybridized with the [32P]-radiolabeled probes complementary to coding sequences of HCMV. For Western analyses, the prepared protein samples (50 µg) were separated on SDS-PAGE gels (7.5%), then transferred to membranes and reacted with the antibodies against human β-actin and HCMV proteins and stained using an ECL Western blot detection kit (GE Healthcare) [31, 32].

To measure the decrease in HCMV replication mediated by the ribozyme, M1GS-expressing U251 cells ($n = 1 \times 10^5$) were infected with HCMV (MOI = 1). Viral stocks prepared from the medium and cells were collected at 1-day intervals throughout the 7 days post-infection, then viral titers of the prepared stocks were studied using standard plaque-forming assays [31, 32].

### 2.2.5 Determination of the HCMV DNA Level

DNase I- treated (Encapsidated) and non-DNase I-treated (total) DNA samples were harvested from cells at 96 h post-infection following the protocols described previously [34, 35]. The amount of viral DNA was determined by PCR amplification of the HCMV IE1 sequence (481bp) using primers CMV3 (5'–CCAAGCGGCCTCTGATAACCAAGCC–3') and CMV4 (5'–CAGCACCATCCTCCTCTTCCTCTGG–3'), and PCR-amplified products of the 610 bp actin sequence as the control. Experimental procedures have previously been described in detail [24, 35]. For radiolabeling, PCR reactions were performed with α-[32P]-dCTP, and then products were separated and analyzed in polyacrylamide gels [24, 35].

## 2.3 Results

### 2.3.1 RNase P-mediated cleavage of the HCMV AP mRNA Sequence in Vitro.

In order to achieve high cleavage efficiency, it is necessary to determine a region that is accessible to binding of ribozymes because most intracellular mRNA species exist in highly ordered and folded conformations and are associated with proteins [8, 35]. In the present study an in-vivo DMS mapping strategy was used to identify the most accessible regions of PR/AP mRNA in HCMV-infected cells. The position chosen as the target for the guide sequence was

exceptionally intolerant to DMS treatment and its flanking sequence contained the components required for efficient cleavage by the M1GS ribozyme [29, 38]. This position is 548 nucleotides downstream of the AP translation initiation site and spanned 18 nucleotides in length [36, 37].

Our group has previously produced Rnase P ribozyme variants with increased catalytic activity compared to wild type M1 RNA when targeting the Thymidine Kinase (TK) mRNA of HSV-1 [19, 39]. However, no such comparable studies have been done on the effectiveness of these generated variants at targeting HCMV mRNA and blocking viral replication. Here, we studied the catalytic activity of a ribozyme variant (V718) which has not been previously studies and has two point mutations ( 81A → C and 194G → A)[19]. We show the variant V718 is very efficient in cleaving the mRNA sequence of AP in-vitro (Table 1).

**Table 1.** Kinetic parameters [$(K_{cat}/K_M)$s and $K_d$] of different ribozymes-mediated cleavage with substrate ap11.

| Enzyme | $(k_{cat}/K_m)^s$ ($\mu M^{-1} \cdot min^{-1}$) | $K_d$ (nM) |
|---|---|---|
| M1-A | $0.22 \pm 0.05$ | $0.31 \pm 0.05$ |
| V718-A | $13.5 \pm 2.5$ | $0.35 \pm 0.06$ |
| M1-C | $<5 \times 10^{-6}$ | $0.32 \pm 0.06$ |
| V718-C | $<5 \times 10^{-6}$ | $0.34 \pm 0.05$ |
| M1-TK | $<5 \times 10^{-6}$ | ND |

Values are means of triplicate determinations. $p < 0.02$. "ND", not detected.

The Ribozymes V718-A and wild type M1-A were the only active ribozymes in this study and were assembled from V718 and M1 RNA, respectively. Ribozymes V718-C and M1-C were used as inactive controls due to mutations in the catalytic domain and were constructed from ribozyme mutant C102 in a way similar to the active ribozymes [27, 33]. The same complimentary guide sequence targeting the AP mRNA was incorporated into all of these ribozymes, covalently linked at the ribozyme 3' terminus. To investigate the catalytic activity $(K_{cat}/K_M)^S$ of these ribozymes, In-vitro kinetic analysis was conducted for the different ribozymes in cleaving a substrate (ap11) containing a sequence of 37 nucleotides from HCMV AP mRNA. The results of our In-vitro studies showed that the generated ribozyme variant, V718-A, cleaved the target AP

mRNA sequence efficiently and its activity was about 60-fold higher than that of the wild type ribozyme M1-A ($p < 0.02$). This was opposite to activities of V718-C and M1-C which were $10^4$-fold less active than M1-A in cleaving the ap11 substrate. Binding affinities of these ribozymes to substrate ap11, quantified as the dissociation constant ($K_d$), were determined by gel-shift assays to confirm that differences in cleavage activity were not due to differences in binding affinity to the ap11 substrate. Given that V718-C and M1-C both have a binding affinity for ap11 similar to that of V718-A and M1-A (Table 1), in addition to being catalytically inactive, these ribozymes represent effective negative controls for our M1GS experiments.

### 2.3.2 Ribozymes Expression in Human cells

Retroviral vector LXSN was used to express M1GS RNAs from sub-cloned DNA sequences coding for V718-A, M1-A, V718-C and M1-C as previously demonstrated with success [8, 33, 40]. DNA constructs of LXSN-M1GS were transfected into amphotropic PA317 cells to create M1GS vector particles and harvested particles were used to infect human (U251) cells for cloning of cell lines expressing M1GS. To use as a control ribozyme with an incompatible guide sequence, a cell line expressing the M1-TK ribozyme targeting the Thymidine Kinase mRNA of HSV-1, was also generated [19, 20, 39]. The results of our in-vitro studies show the M1-TK ribozyme lacked any catalytic activity toward the ap11 substrate (Table 1). Northern blot analysis was carried out to measure the expression of M1GS RNAs in cells (Figure 2, lanes 1 – 4) with human ribozyme H1 RNA used as an internal control (Figure 2, lanes 5 – 8). The cell clones with comparable ribozyme expression levels were chosen to be used for subsequent experiments.

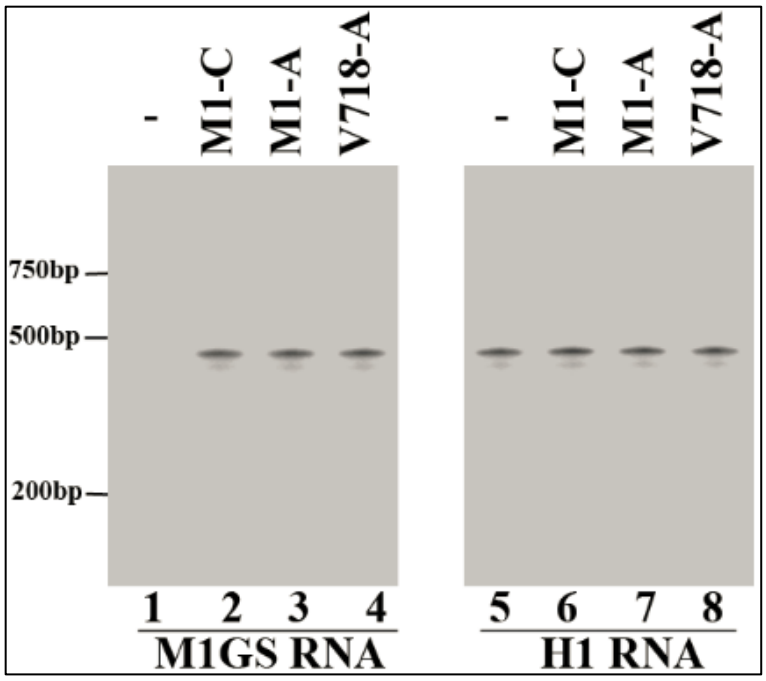


**Figure 2.** Expression levels of ribozymes in cultured cells. Northern blot analyses were performed using RNA samples (30 μg) prepared from parental U251 cells (-, lanes 1 and 5) and cells expressing M1-C (lanes 2 and 6), M1-A (lanes 3 and 7), and V718-A (lanes 4 and 8). Human H1 RNA control (lanes 5–8) (the RNA subunit of human RNase P and a nuclear RNA) [31, 41].

### 2.3.3 Ribozyme-Mediated Reduction of HCMV AP and PR Expression

To measure the expression levels of AP and PR mRNA, Northern blot analysis was carried out with HCMV immediate-early 5 kb RNA used as an internal control (Figure 3, lanes 1 – 4). A reduction of 98%–99% in AP/PR mRNA expression was observed in HCMV-infected cells expressing the variant V718-A, compared to a 75%–78% ($p < 0.04$) reduction in AP/PR mRNA expression in cells expressing the ribozyme derived from the wild type sequence M1-A (Figure 4, lanes 7, 8, 11, 12) (Table 2). Compared to active ribozymes, cells expressing control ribozymes V718-C and M1-C showed no notable reduction (<10%) (Figure 3, lane 6, data not shown) (Table 2). These combined results indicate that ribozyme-mediated cleavage of AP/PR mRNAs was responsible for inhibition of AP/PR expression in cells expressing ribozymes V718-A and M1-A.

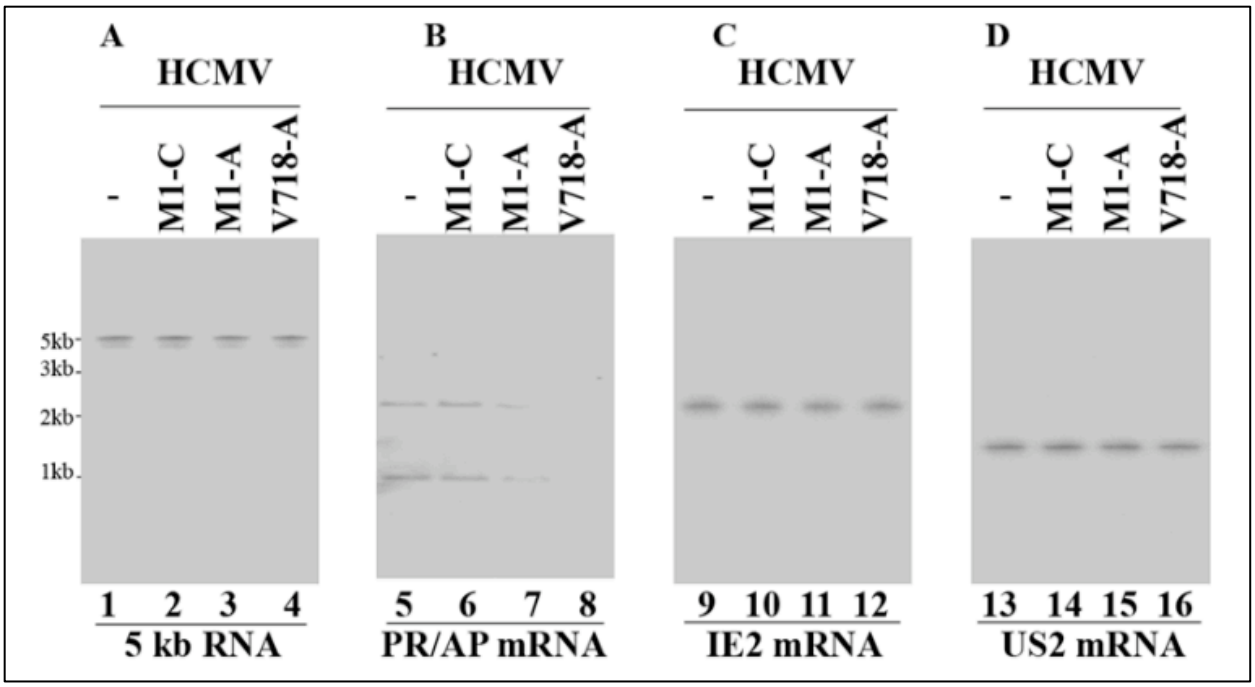


**Figure 3.** Northern blot analyses of the expression levels of HCMV mRNAs. RNA samples (30 μg) were isolated from HCMV-infected parental U251 cells (-, lanes 1, 5, 9, and 13) and cell lines expressing M1-C (lanes 2, 6, 10, and 14), M1-A (lanes 3, 7, 11, and 15), and V718-A (lanes 4, 8, 12, and 16) at 8 h (C) and 48 h post-infection ((A), (B), and (D)). The hybridized hybridized probes were used to detect the HCMV 5 kb RNA (lanes 1–4), AP (upper band) and PR (lower band) mRNAs (lanes 5–8), IE2 mRNA (lanes 9–12), and US2 mRNA (lanes 13–16).

To measure the expression of AP mad PR proteins, western blot analysis was carried out with actin used as an internal control (Figure 4, lanes 1 – 4). A reduction of 98%–99% in AP/PR protein expression was observed in HCMV-infected cells expressing the variant V718-A, compared to a 72%–75% ($p < 0.04$) reduction in AP/PR protein expression in cells expressing the wild type ribozyme M1-A (Figure 4, lanes 7, 8, 11, 12) (Table 2). Cells expressing control ribozymes V718-C and M1-C, in contrast, showed minimal reduction (<10%) (Figure 4, lanes 6, [10, data not shown] (Table 2). Therefore, these combined results showed that inhibition of AP/PR mRNA expression was consistent with the decline in AP and PR protein levels.

**Table 2.** Inhibition Levels of HCMV mRNA and protein expression in the cells with M1GS, as compared with parental U251 cells.

| | Viral Gene Class | Ribozymes | | | | | |
|---|---|---|---|---|---|---|---|
| | | U251 | M1-C | V718-C | M1-A | V718-A | M1-TK |
| IE2 mRNA | α | 0% | 0% | 0% | 0% | 0% | 0% |
| US2 mRNA | β | 0% | 0% | 0% | 0% | 0% | 0% |
| AP mRNA | γ | 0% | 3% | 6% | 78% ± 8% | 99% ± 7% | 0% |
| PR mRNA | γ | 0% | 3% | 7% | 75% ± 7% | 98% ± 7% | 0% |
| IE2 protein | α | 0% | 0% | 0% | 0% | 0% | 0% |
| UL44 protein | β, γ | 0% | 0% | 0% | 0% | 0% | 0% |
| UL83 protein | γ | 0% | 0% | 0% | 0% | 0% | 0% |
| AP protein | γ | 0% | 3% | 6% | 75% ± 7% | 99% ± 8% | 0% |
| PR protein | γ | 0% | 3% | 6% | 72% ± 7% | 98% ± 8% | 0% |

Experiments were carried out in triplicate, and repeated three times. Values are means from these experiments and standard deviations less than 5% were not recorded. Samples were harvested at different time points post-infection as specified in Materials and Methods (pg. 12).

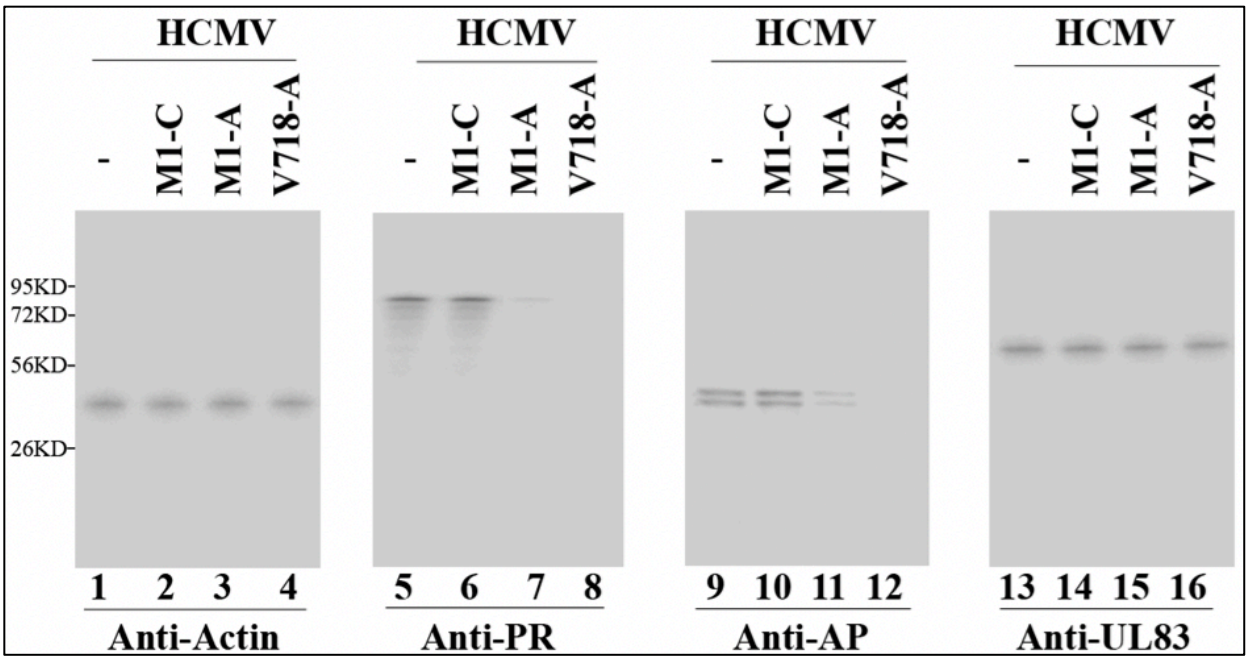


**Figure 4.** Western blot analyses of the expression levels of HCMV proteins. Protein samples (50 μg) were prepared at 72 h post-infection and then separated in SDS-PAGE gels, transferred to membranes and reacted with antibodies anti-AP and anti-PR. The anti-AP antibody detected both full-length AP and its processed form that is cleaved during HCMV capsid assembly [1] (lanes 9–12). Actin control (lanes 1–4).

### 2.3.4 Inhibition of HCMV Replication Mediated by Ribozymes

To study the extent of decrease in HCMV replication in infected cells mediate by M1GS ribozymes, viral plaque assays were conducted. A reduction of 50,000-fold in viral production was observed in HCMV-infected cells expressing the variant compared to a 500-fold reduction in viral production in cells expressing the ribozyme derived from the wild type sequence (Figure 5). In contrast, cells expressing the control ribozyme V718-C, M1-C and M1-TK showed minimal reduction of HCMV growth (Figure 5). These results indicate that inhibition of AP/PR expression mediated by the generated ribozymes prevents HCMV replication in infected cells.

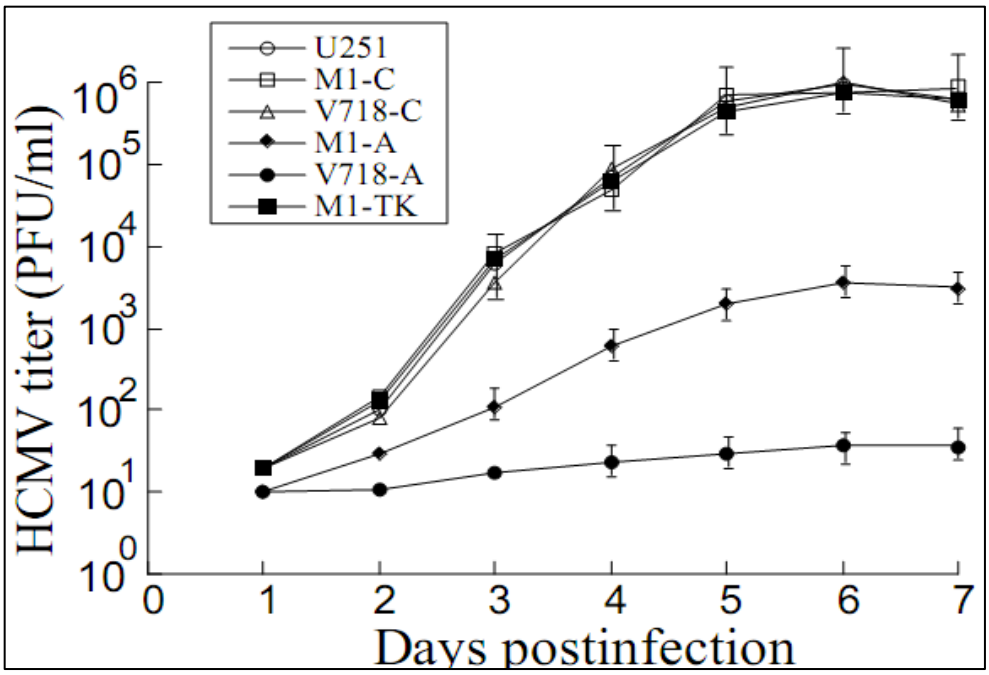


**Figure 5.** HCMV growth in U251 cells and M1GS-expressing cell lines. Experiments were carried out in triplicate, and repeated three times. Values are means of these experiments and the standard deviation is indicated by the error bars.

### 2.3.5 The Antiviral Mechanism of Ribozyme-Mediated Cleavage of AP and PR mRNAs

HCMV assembly protein (AP) and protease (PR) play essential roles in DNA encapsidation during capsid assembly and viral growth [1, 21, 22]. Therefore, we carried out two sets of experiments to study the relationships between the ribozyme-mediated cleavage of the target genes and HCMV lytic replication cycle.

First, we determined whether the generated ribozymes have effect on the expression levels of other viral genes (except AP and PR) in the cells. Northern blot analyses were performed to examine the expression of other viral genes, such as IE2 (an immediate-early (IE) or α gene) and US2 (an early or β gene) mRNA in cells with M1GS (Figure 3, lanes 9–12 and 13–16). Western blot analyses were also carried out to study the expression levels of viral proteins UL44 (an early/late or βγ gene) and UL83 (a late or γ gene) (Figure 4, lanes 13–16, data not shown) (Table 2). No difference was found in the expression levels of these genes in cells with different ribozymes (V718-A, M1-A, V718-C, and M1-C) (Table 2). Thus, V718-A and M1-A specifically inhibited AP and PR expression, and had no effect on overall viral gene expression.

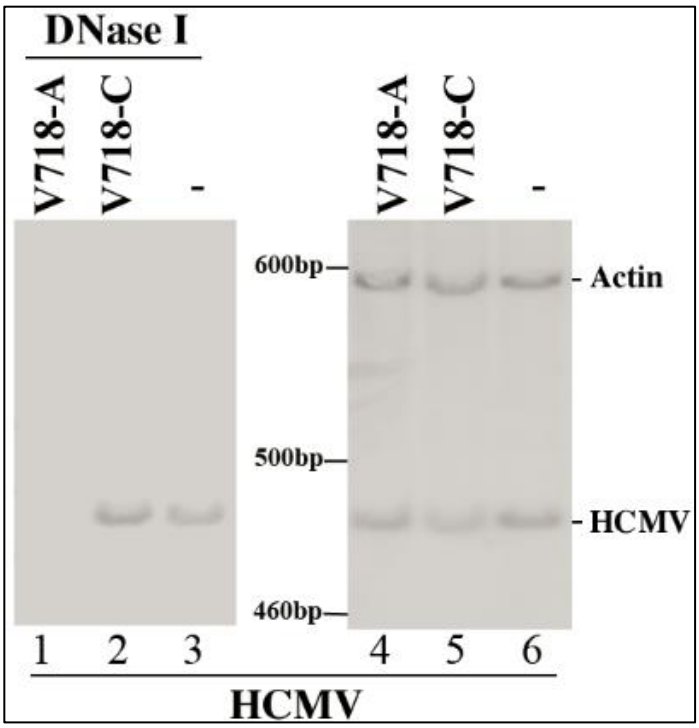


**Figure 6.** The levels of HCMV encapsidated DNA and total intracellular DNA. DNase I-treated DNA samples (lanes 1–3) or total DNA (lanes 4–6) were prepared from cells without ribozyme (-, lanes 3 and 6) or with ribozyme V718-A (lanes 1 and 4) and V718-C (lanes 2 and 5) at 96 h post-infection. Semi-quantitative PCR was performed to determine the levels of viral IE1 sequence and human actin DNA sequence was used as the internal control.

Second, we examined whether the generated ribozymes have an effect on HCMV DNA replication or capsid maturation. DNA was prepared from samples treated either with or without DNase-I since unincapsidated DNA would be susceptible to DNase I treatment, and then the amounts of viral DNA were determined. Results showed that the levels of total intracellular HCMV DNA (no DNase I-treatment) were similar in the cells with different ribozymes (Figure 6, lanes 4–6). In contrast, the levels of "encapsidated" DNA (DNase I-treated) were greatly decreased in the cells with V718-A (Figure 6, lane 1). The results indicate that M1GS-mediated reduction of AP and PR expression has no effect on HCMV genome replication but inhibits DNA encapsidation and capsid formation.

## 2.4 Discussion

In recent years, gene silencing technologies, such as antisense oligonucleotides, RNAi, aptamers, and microRNAs, have been widely used for research studies to inhibit gene expression [9–11]. However, each technology has disadvantages such as specificity, activity, stability and cellular toxicity [13, 18]. Compared to other approaches, RNase P ribozymes represent a promising and attractive approach with high activity and specificity [3, 41, 42]. In this study, M1GS RNAs were generated to target the overlapping region of HCMV PR (UL80) and AP (UL80.5) mRNAs. Further studies showed that the expression levels of PR (UL80) and AP (UL80.5) were greatly decreased (98%–99%), and viral growth was also largely reduced (50,000-fold) in the cells expressing V718-A. In comparison, little reduction (<10%) was found in AP and PR expression and viral replication in the cells with control ribozymes (i.e., V718-C, M1-C, or M1-TK). The results indicate that V718-A is highly active in reducing HCMV gene expression and blocking viral growth.

Evidence presented in this study suggest that the antiviral effects of ribozyme variant V718-A are the result of ribozyme targeting AP/PR mRNAs. First, the generated ribozymes (i.e., V718-A, M1-A) only inhibited the expression of AP and PR; no decrease was found in the expression levels of viral α, β, or γ genes examined (e.g., IE2, US2, UL44 and UL83) (Figures 3 and 4, Table 2, data not shown). Second, viral genomic DNA replication was not affected by ribozyme expression (Figure 6). Third, blocking of capsid maturation and viral growth appeared to be induced by ribozyme-mediated cleavage of AP and PR mRNAs, since encapsidated viral DNA levels as well as AP/PR expression levels were found to be reduced in the cells with V718-A and M1-A but not in the cells with V718-C, M1-C, or M1-TK (Figures 3–6, data not shown). All together, these results suggest that AP and PR play necessary roles in viral capsid maturation and have no effect on HCMV gene expression or genome replication [1, 22, 43].

Further studies on improving the cleavage efficacy of M1GS RNAs are important to develop ribozyme-based approaches [23, 39, 42]. However, no guidelines are currently available about how to generate a highly active M1GS RNA [23, 39, 40]. In this study, functional ribozyme (e.g., V718-A) was designed to target an accessible region of the overlapping region of AP and PR mRNAs. With this design, we hypothesized that the M1GS cleavage efficacy in cells is determined by its catalytic efficiency [$(k_{cat}/K_M)^s$] in-vitro. If this is the case, improving the catalytic activity of ribozymes [$(k_{cat}/K_M)^s$] may lead to more active ribozyme-directed cleavage in cells [23, 39]. As shown in our studies, an M1GS (i.e., V718-A) was about 60 times more active [$(k_{cat}/K_M)^s$] in targeting AP and PR mRNAs in-vitro than the wild type ribozyme (i.e., M1-A). V718-A was also more effective than M1-A in PR expression in cultured cells (e.g., 98%–99% vs. 72%–75%). In comparison, little reduction (<10%) was observed in the expression levels of AP and PR and in viral replication in cells or M1-TK. All of these results support our hypothesis that improving the in-vitro catalytic efficiency [$(k_{cat}/K_M)$ ] may lead to increased cleavage activity of ribozymes in cells. Therefore, providing insights into developing guidelines for the generation of highly active ribozymes.

Our previous studies have shown that ribozymes derived from M1 RNA variants could be constructed to target the mRNAs coding for various cytomegalovirus (CMV) essential proteins including immediate early protein 1 (IE1) and 2 (IE2), AP, and PR [20, 28, 29, 33]. These ribozymes efficiently cleaved the target mRNAs in-vitro and effectively blocked HCMV gene expression and replication in cultured cells. In our current study, we reported the anti-HCMV activity of a ribozyme derived from a M1 RNA variant, V718, which has been generated by an in-vitro

selection procedure and has not been characterized before. V718-A cleaves the AP/PR mRNAs more efficiently than M1-A in-vitro (Table 1). Furthermore, V718-A more effectively inhibited HCMV gene expression and growth than M1-A in cultured cells. The extent of antiviral effects observed in V718-A expressing cells (i.e., the level of inhibition of viral gene expression and growth) is at least 10 times higher than that observed in cells expressing a ribozyme variant targeting HCMV IE2, which was recently reported by our laboratory [33]. Indeed, antiviral effects associated with V718-A are among the best effects observed in our studies using different ribozyme variants that target different CMV targets including AP and PR [20, 28, 29, 33]. The ribozyme variant V718 has two point mutations: 81A → C and 194G → A. How these two mutations increase cleavage activity is not known. Further studies on the mutations found in V718 and other variants will provide insight into the mRNA cleaving mechanism of the ribozymes.

HCMV is a human herpesvirus and causes significant mortality and morbidity in specific human populations [1]. HCMV protease (UL80) and capsid assembly (UL80.5) proteins are necessary for capsid assembly and viral growth, suggesting that these proteins could be used as ideal targets for anti-HCMV development [17, 24]. HCMV is known to infect and replicate in many types of cells and tissues in-vivo [1]. In this study, we showed that V718-A effectively inhibited AP/PR expression and blocked HCMV growth in human astrocytoma U251 cells that were infected with HCMV at a modest multiplicity of infection (MOI). It is important to determine if V718-A effectively inhibits HCMV gene expression and replication in other cells and tissues known to be infected by HCMV in-vivo and under different MOIs. Additional studies on V718 and other ribozymes should provide insights into developing active ribozymes for anti-HCMV therapeutic applications.

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# 3 RNase P Ribozymes Inhibit the Replication of Human Cytomegalovirus by Targeting the Viral Essential Immediate Early 2 (IE2) Gene.

## 3.1 Introduction

Human cytomegalovirus (HCMV) is a common herpesvirus and usually affects individuals with compromised immune system [1]. This virus causes birth defects including mental problems [2]. Furthermore, HCMV can cause debilitating symptoms in AIDS patients, such as blindness and gastrointestinal diseases [3, 4]. New therapeutic approaches are needed to combat and control this important opportunistic pathogen.

Gene targeting approaches using nucleic acid-based molecules to target specific mRNA sequences of choice represent promising therapeutic strategies [5, 6]. One example is small interfering RNAs (siRNAs), which were used to target several human viruses effectively [5, 7, 8]. Ribozymes can also inactivate viral mRNA sequences and diminish viral infection in human cells [9–12].

RNase P functions in cells to process tRNA precursors (ptRNA) into mature tRNAs [13–15]. In Escherichia coli, RNase P forms a holoenzyme containing a protein (C5 protein) and a catalytically active RNA (M1 RNA) [16]. Previous studies have shown that the enzyme recognizes its substrate through the tertiary structure and not the primary sequence. (Figure 1A) [13, 14, 17]. Any mRNA substrate can be targeted and hydrolyzed by M1 ribozyme through the binding of a complementary external guide sequence (EGS) to the substrate to resemble the structure of the tRNA module that includes the 5’ leader sequence, the acceptor stem, the T-stem, and the 3' CCA sequence (Figure 1) [18, 19]. A ribozyme targeting any mRNA sequence of choice, M1GS RNA, can be designed by linking a guide sequence (GS) to the 3' terminus of M1 RNA (Figure 1C) [20, 21]. The guide sequence can basepair with an mRNA target and thus, allows M1GS to bind to the mRNA. Previous studies had designed M1GS ribozymes to target cellular genes and the essential genes of herpes simplex virus 1 (HSV-1) and HCMV [22–24]. The ribozymes were effective in reducing HSV-1 and HCMV growth 10,00-fold and 150-fold, respectively [22, 23].

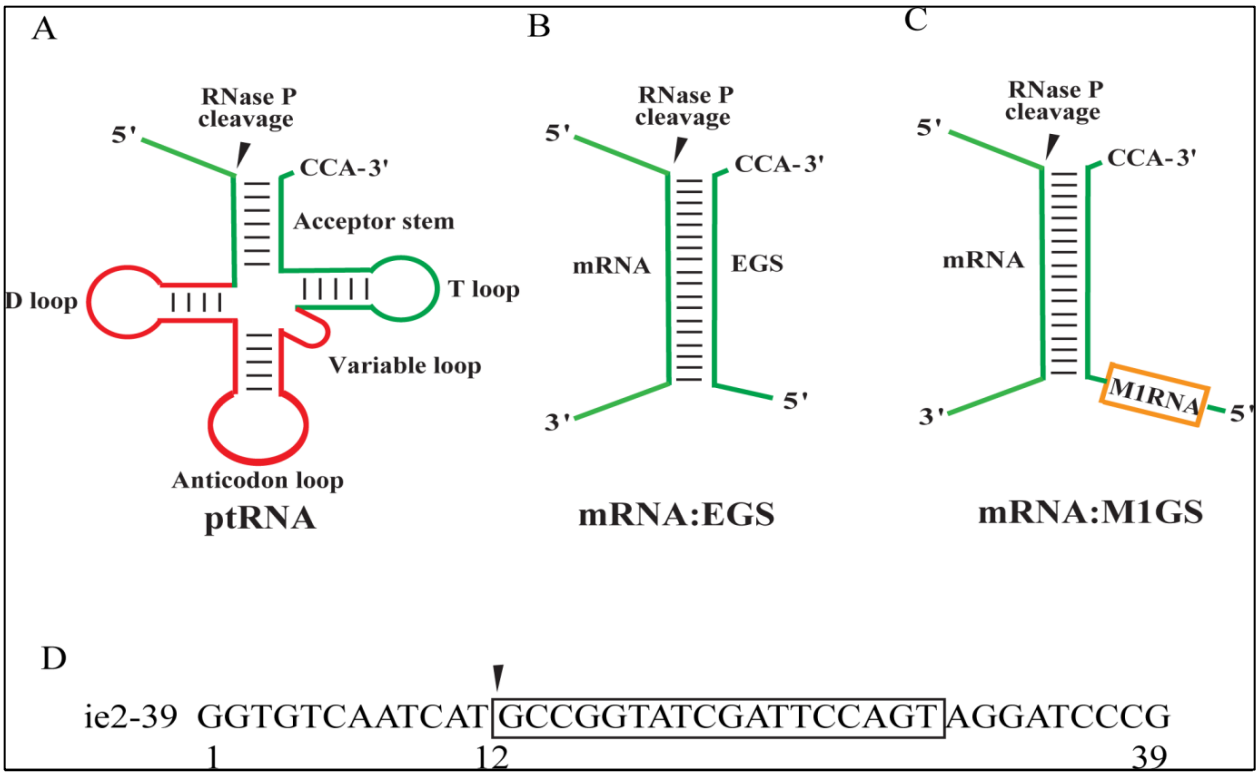


**Figure 1.** Schematic presentation of RNase P/M1GS ribozyme's substrates. (A–C) pre-tRNA (ptRNA), a natural substrate (A); EGS:mRNA complex (B); and M1GS binding to its substrate (C). Filled arrows denote the cleavage sites. (D) Substrate ie2-39 with boxed region representing the complementary sequence to the guide sequence of M1GS.

Compared to other gene-targeting approaches such as RNAi, the M1GS ribozyme has several advantages. Ribozymes have not been shown to saturate the cellular machinery required for their processing while high levels of siRNAs can overwhelm the RNAi machinery that contains various cellular factors and may affect the normal cellular functions of these factors [5, 25–28]. Furthermore, by interacting with specific cellular proteins including the RNase P protein subunits [14, 29], RNase P ribozymes may improve their intracellular stability and catalytic activities in cells. RNase P ribozyme represents a unique and promising class of gene-targeting agents for therapeutic application [30].

Increasing the catalytic efficiency of the RNase P ribozyme is essential for M1GS technology to be used as an effective gene-targeting approach. Previously, we have generated RNase P ribozyme variants through an in-vitro selection system that have better catalytic activity in cleaving an mRNA sequence than the wild type M1 RNA ribozyme [31]. For the study, a highly active ribozyme variant was designed to hydrolyze the exon 5 region of the mRNA encoding HCMV immediate-early 2 (IE2) protein. IE2 encodes a HCMV major transcription regulatory factor and its expression is needed for the expression of viral early (β) and late (γ) genes [1, 32–34].

Thus, IE2 protein expression is essential for HCMV replication in cultured cells. Inhibition of IE2 expression would result in shutting down gene expression and replication of HCMV. We investigated the activity of the constructed ribozyme in generating cleavage of IE2 mRNA sequence in-vitro and in shutting down HCMV infection in cells. Our data showed that the ribozyme variant cleaved the target IE2 mRNA sequence at least 50-fold more efficiently than the wild type ribozyme. More importantly, greater reduction in IE2 expression and HCMV growth was observed in cells expressing the variant. Our results suggest that RNase P ribozyme variants can be used as promising gene-targeting agents for anti-CMV applications.

## 3.2 Methods

### 3.2.1 Mapping of the Accessible Regions of HCMV IE2 mRNA in Cells

A dimethyl sulfate (DMS) mapping procedure was used to map the accessible regions of mRNA and was described in detail previously [36]. Briefly, cells infected with HCMV were incubated with DMS for 5–10 min. Cells were then lysed for total RNA isolation with phenol-chloroform extraction and ethanol precipitation, the, RNA extracts were used for primer extension assays with radiolabeled oligonucleotides to map the DMS-modification sites [21, 35]. Primer extension products were separation in 8% denaturing gels to identify the DMS modification sites that block primer extension. Sites that are amenable to modification by DMS represent potential regions ribozyme binding may be easily accessible.

### 3.2.2 Ribozyme Studies In Vitro

The template DNA of substrate ie2-39 was constructed by annealing oligonucleotide AF25 (5'-GGAATTCTAATACGACTCACTATAG-3') with oligonucleotide sie2-39 (5'-CGGGATCCTACTGGAATCGATACCGGCATGATTGACACCTATAGTGAGTCGTATTA-3'), which contains a T7 promoter and IE2 coding sequence, respectively . Plasmid pFL117, pV661, and pC102, which encode M1 RNA, variant V661, and mutant C102, respectively, have been described previously [31, 37]. C102 contains point mutations ($A_{347}C_{348} \rightarrow C_{347}U_{348}$, $C_{353}C_{354}C_{355}G_{356} \rightarrow G_{353}G_{354}A_{355}U_{356}$), which render it inactive [37]. Ribozymes M1-IE2, V661-IE2, and M1-IE2-C were synthesized using plasmids pFL117, pV661, and pC102, respectively. Plasmid pV661-C, which was used as the template to construct control ribozyme V661-IE2-C, was derived from pV661 and contained the mutations found in C102. The forward primer was AF25 and the reverse primer was M1IE23 (5'- CCCGCTCGAGAAAAAATGGTGCCGGTATCGATTCCAGTTGTGGAATTGTG-3'). The RNA substrate ie2-39 and M1GS Ribozymes were synthesized with T7 RNA polymerase [12]. Kinetic analyses and gel shift assays were carried out as previously described [31, 43, 44].

### 3.2.3 Construction of the M1GS Ribozyme-Expressing Cell Lines

M1GS DNA coding sequences were sub-cloned into retroviral vector LXSN under control of the constitutively expressed U6 promoter [12, 21, 45]. The M1GS-containing retroviral vector DNAs were transfected subsequently into amphotropic PA317 cells. Retroviral particles form the supernatant were used to infect human U373MG cells, followed by selection and cloning of

neomycin-resistant cultured in neomycin-containing media (600 µg/mL) (GE Healthcare, Piscataway, NJ) [12, 21, 45].

### 3.2.4 Studies of Viral Gene Expression and Viral Growth in Cells

M1GS-expressing cells (n = 1 ×$10^6$) were either mock-infected or infected with HCMV at a multiplicity of infection (MOI) of 1. Extraction and isolation of HCMV mRNA or protein samples from infected cells was carried out at various time points as previously described [22, 44]. At 8 and 24 h post-infection, samples were prepared to determine the levels IE2 mRNA and protein expression, respectively. Samples were harvested at 8 h post-infection to measure the mRNA expression of the HCMV immediate-early UL36. At 24 h post-infection, samples were harvested for detection of the HCMV US2 mRNA expression, And 72 h post-infection is when samples were harvested to measure the expression of UL44, UL99, and gH proteins. To use for loading controls, expression of HCMV 5kb RNA was detected from samples harvested at 8 and 24 h post-infection, while expression of actin protein was detected from samples harvested at 24 and 72 h post-infection.

In conducting the Northern blot analysis for mRNA expression levels, RNA samples were prepared and separated in 1% agarose denaturing gels containing formaldehyde [12, 22, 40]. Separated RNA was transferred to membranes, hybridized to [32P]-radiolabeled DNA probes that contained sequence complementarity to the HCMV coding sequences or H1 RNA. [12, 22, 40]. Radiolabeled probes were synthesized with the aid of a random primed labeling kit (Roche Applied Science, Indianapolis, IN). For the western blot analysis, protein samples were prepared and separated on 9% SDS-PAGE gels, transferred to nitrocellulose membranes, reacted with the antibodies against cellular actin and HCMV proteins, then stained with the aid of a Western blot protein detection kit (GE Healthcare) [12, 22, 40].

To detect the reduction in viral growth mediated by M1GS, the M1GS-expressing U373 cells (n = 5 ×$10^5$) were infected with HCMV at a MOI of 1. At 1 day intervals for 7 days post-infection, viral stocks were prepared from cells, and titers of viral stock were assayed by plaque assay as previously detailed [12, 22].

## 3.3 Results

### 3.3.1 Targeting and Cleavage Activities of the M1GS RNAs in Vitro

Since most mRNA species inside cells form complex secondary structures and are associated with proteins, a targeting region must be accessible for ribozyme binding and catalytic cleavage of the target mRNA. We used dimethyl sulphate (DMS) [21, 35] to map the accessible regions of IE2 mRNA in-vivo [36]. To map these regions, we infected human U373MG cells with HCMV and then incubated these cells with culture media that contained DMS [36]. It is expected that DMS would enter the cells and modify the nucleotides of the accessible mRNA regions. We then isolated the total mRNAs. Primer extension assays using reverse transcriptase were used to map the IE2 mRNA regions that were modified by DMS [36]. A position, 30 nucleotides downstream from the 5' terminus of exon 5, was selected as the targeting site. The chosen

targeting region (designated as IE2 RNA) was extensively modified by DMS and was predicted to be accessible for M1GS binding and cleavage (data not shown).

We had previously carried out an in-vitro selection procedure and isolated M1GS RNA variants that have higher catalytic activity in cleaving HSV-1 thymidine kinase (TK) mRNA than the wild type M1 RNA [31]. However, little research has been done to examine whether these variants are also effective in targeting HCMV mRNAs to shut down viral infection. To investigate these issues, we chose variant 661 (designated as V661) for the study because this selected variant is highly active in cleaving target RNAs in-vitro (e.g., IE2 and TK and mRNA) (Table 1) [31]. This variant contains two point mutations (i.e., $A_{94} \rightarrow G_{94}$ and $G_{194} \rightarrow C_{194}$) [31]. Little is known about the contribution of these nucleotides to the activity of M1GS RNAs in cleaving an mRNA substrate. The effect of the point mutations on the activity of RNase P ribozymes has not been studied.

**Table 1.** Overall cleavage rate [(kcat/Km)s] and binding affinity (Kd) of RNase P ribozymes with substrate ie2-39.

| Enzyme | $(k_{cat}/K_m)^s$ ($\mu M^{-1} \cdot min^{-1}$) | $K_d$ (nM) |
|---|---|---|
| M1-IE2 | $0.20 \pm 0.05$ | $0.34 \pm 0.06$ |
| V661-IE2 | $10.5 \pm 0.5$ | $0.30 \pm 0.05$ |
| M1-IE2-C | $<5 \times 10^{-6}$ | $0.33 \pm 0.07$ |
| V661-IE2-C | $<5 \times 10^{-6}$ | $0.32 \pm 0.07$ |
| M1-TK | $<5 \times 10^{-6}$ | ND |

The values shown are the average derived from triplicate experiments. $p < 0.01$. "ND", not determined.

To construct IE2 mRNA-cleaving ribozymes from the variant and wild type M1 sequence, V661-IE2 and M1-IE2, the 3' termini of V661 and M1 RNA were covalently linked with an 18 nucleotides guide sequence that binds to the IE2 mRNA sequence, respectively. We included two control RNase P ribozymes, M1-IE2-C and V661-IE2-C. M1-IE2-C was generated from C102 RNA, an M1 mutant with mutations ($A_{347}C_{348} \rightarrow C_{347}U_{348}$, $C_{353}C_{354}C_{355}G_{356} \rightarrow G_{353}G_{354}A_{355}U_{356}$) at the catalytic P4 domain that rendered it inactive in cleaving a ptRNA [37]. V661-IE2-C was generated from V661-IE2 and contained the mutations found in C102. While sharing identical guide

sequence with M1-IE2 and V661-IE2, both M1-IE2-C and V661-IE2-C were not expected to be functional because of the P4 mutations.

We measured the catalytic activity $(k_{cat}/K_M)^s$ with kinetic analyses for these ribozymes in cleaving substrate ie2-39 of the 39 nucleotide long IE2 mRNA target sequence (Figure 1D). These results, shown in Table 1, indicate that V661-IE2 is about 50-fold more active than M1-IE2 in cleaving substrate ie2-39 ($p < 0.01$). As expected, V661-IE2-C and M1-IE2-C were at least 104-fold less active than M1-IE2 RNA in cleaving substrate ie2-39, possibly because the P4 mutations abolished the catalytic activity of the ribozymes ($p < 0.01$).

Detailed gel-shift assays indicate that the binding affinities of V661-IE2-C and M1-IE2-C to substrate ie2-39, as measured by the dissociation constant (Kd), are similar to those of V661-IE2 and M1-IE2 (Table 1). Since V661-IE2-C and M1-IE2-C are non-functional but contain identical guide sequence complementary to ie2-39 and exhibit similar affinity to ie2-39 as V661-IE2 and M1-IE2, these ribozymes serve as controls for the antisense effect in our experiments.

### 3.3.2 Expression of Ribozymes in Human Cell Culture

The DNA sequences coding for V661-IE2, M1-IE2, V661-IE2-C, and M1-IE2-C were subcloned into LXSN retroviral vector and placed under the constitutively expressed U6 RNA promoter [19, 21, 38]. To construct cell lines that express M1GS RNAs, we generated retroviral vectors that contained the genes for M1GS RNAs by transfecting LXSN-M1GS DNAs into the amphotropic packaging cells (PA317). We subsequently infected human U373MG cells with the retroviruses and generated stable cell lines expressing the M1GSs. A cell line expressing ribozyme M1-TK, which targeted HSV-1 TK mRNA, was constructed to control for M1GS RNA with a mismatched guide sequence [22]. No cleavage of substrate ie2-39 by M1-TK was observed in-vitro (data not shown, Table 1).

Northern blot analysis was performed to assay M1GS RNA expression in individual cell clones (Figure 2, lanes 5–8). Human H1 RNA was used as the loading and internal control (Figure 2, lanes 1–4) [14, 36]. No difference in cell growth and viability was observed for up to two months between cell lines expressing M1GS and a control line with empty vector LXSN DNA, suggesting that ribozymes did not result in significant cytotoxicity (data not shown). For subsequent experiments, only cell lines exhibiting similar ribozyme expression levels were used.

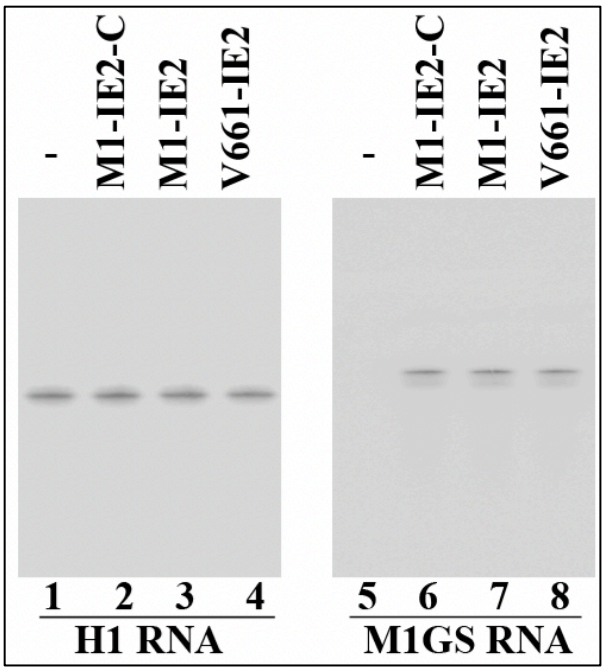


**Figure 2.** M1GS ribozymes expression with Northern blot analysis in parental U373MG cells (-, lanes 1 and 5) and cells with M1-IE2-C (lanes 2 and 6), M1-IE2 (lanes 3 and 7), and V661-IE2 (lanes 4 and 8). H1 RNA control (lanes 1–4).

### 3.3.3 Enhanced Reduction of HCMV IE2 in Ribozyme-Expressing Cells

Cells were infected with HCMV at a multiplicity of infection (MOI) of 1. Total RNAs were then isolated from these cells. HCMV immediate early 5kb RNA, which is expressed from the region spanning viral UL106 and UL111 with its 5' end at nucleotide position 15,9627 and its putative 3’ end at position 15,4829 of the HCMV (AD169) genome [1, 39], was used as an internal loading control for IE2 mRNA expression.

Figure 3 shows northern analysis of IE2 mRNA levels, which are quantitated in Table 2. A reduction of about 98% and 75% ($p < 0.04$) in IE2 mRNA expression was detected in cells expressing V661-IE2 and M1-IE2, respectively (Figure 3, lanes 5 and 8).

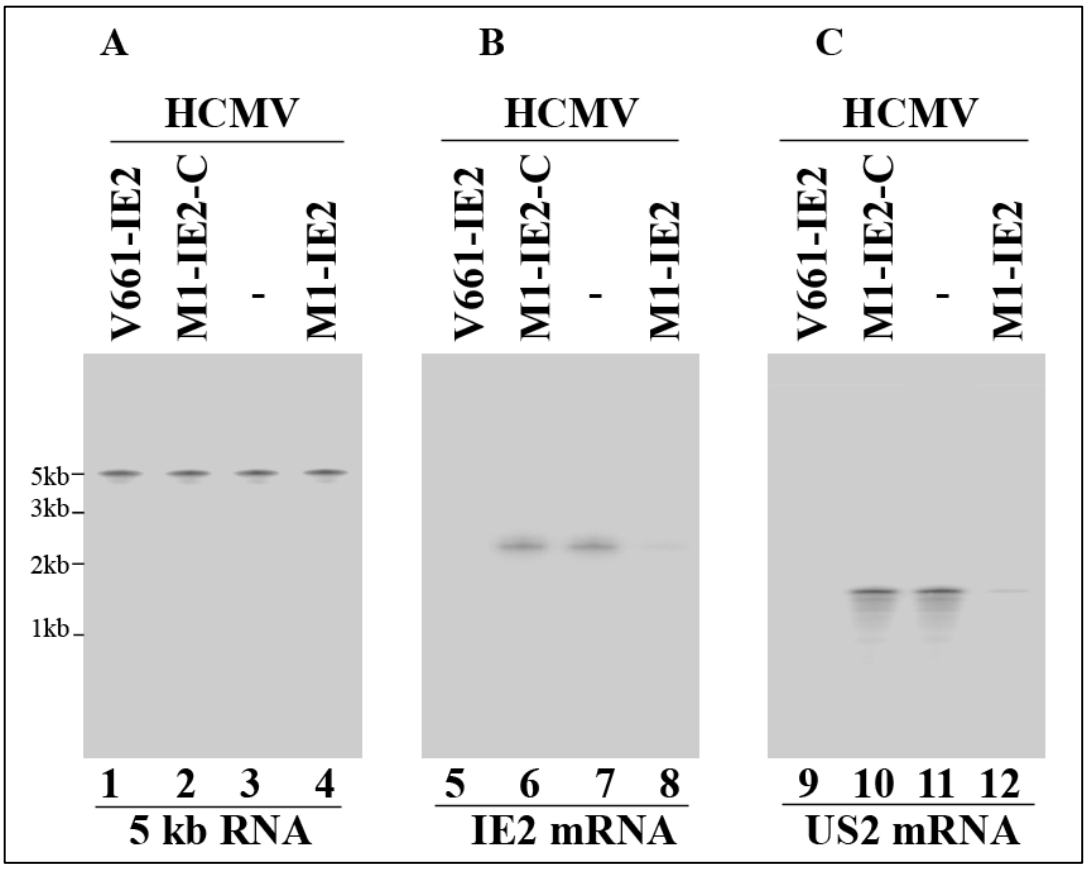


**Figure 3.** Northern analysis of the levels of human cytomegalovirus (HCMV) mRNAs. Cells (n = 1 $\times 10^6$) were infected with HCMV (MOI = 1) and were harvested at either 8 (A–B) or 24 h (C) post-infection. We used RNAs isolated from parental U373MG cells (-, lanes 3, 7, and 11) and cell lines that expressed V661-IE2 (lanes 1, 5, and 9), M1-IE2-C (lanes 2, 6, and 10), and M1-IE2 (lanes 4, 8, and 12). RNA samples were hybridized to a DNA probe coding for HCMV 5 kb RNA (lanes 1–4), IE2 mRNA (lanes 5–8), and US2 mRNA (lanes 9–12).

In comparison, cells that expressed M1-IE2-C and V661-IE2-C showed little reduction (<10%) (Figure 3, lane 6, data not shown) (Table 2). An antisense effect could explain the low level of inhibition found in cells that expressed M1-IE2-C and V661-IE2-C. This is because these control ribozymes contained the same guide sequence as M1-IE2 and V661-IE2 but did not exhibit catalytic activity. These observations suggest that the significant reduction of IE2 mRNA expression in cells that expressed V661-IE2 and M1-IE2 was due to the catalytic cleavage of the target mRNA by these ribozymes. IE2 mRNA cleavage products were not detected in our northern analyses possibly due to degradation by intracellular RNases.

IE2 protein levels in M1GS-expressing cells are expected to decrease due to the corresponding reduction in the level of IE2 mRNA. Proteins were isolated from cells, separated

in gels, and transferred to membranes. The membranes were stained with an anti-IE2 antibody (anti-IE2) (Figure 4, lanes 5–8), and the expression levels of HCMV IE2 protein were determined. Actin was used as an internal and loading control (Figure 4, lanes 1–4). The results are summarized in Table 2 from three independent experiments: 98% and 75% reduction in IE2 protein expression ($p < 0.04$) was found in cells with V661-IE2 and M1-IE2 RNA, respectively. In comparison, little reduction (<10%) was detected in cells with V661-IE2-C, M1-IE2-C, and M1-TK RNAs (Table 2).

**Table 2.** HCMV mRNA and protein expression in cells expressing M1-TK, M1-IE2-C, V661-IE2-C, M1-IE2, or V661-IE2, and cells not expressing any ribozyme (U373MG). The values represent the levels of inhibition of gene expression as compared to the levels of inhibition in U373MG cells.

| | Viral Gene | Ribozymes | | | | | |
|---|---|---|---|---|---|---|---|
| | Class | U373MG | M1-IE2-C | V661-IE2-C | M1-IE2 | V661-IE2 | M1-TK |
| IE2 mRNA | α | 0% | 6% | 5% | 75% ± 7% | 98% ± 7% | 1% |
| US2 mRNA | β | 0% | 1% | 1% | 70% ± 6% | 96% ± 8% | 0% |
| IE2 protein | α | 0% | 4% | 5% | 75% ± 8% | 98% ± 8% | 0% |
| UL44 protein | β,γ | 0% | 1% | 0% | 71% ± 8% | 95% ± 7% | 1% |
| UL99 protein | γ | 0% | 0% | 1% | 72% ± 7% | 94% ± 6% | 0% |
| gH protein | γ | 0% | 0% | 0% | 71% ± 8% | 98% ± 7% | 1% |

The values represent arithmetic means of three independent experiments performed in triplicate. $p < 0.04$.
The values of standard deviation that were less than 5% are not shown.

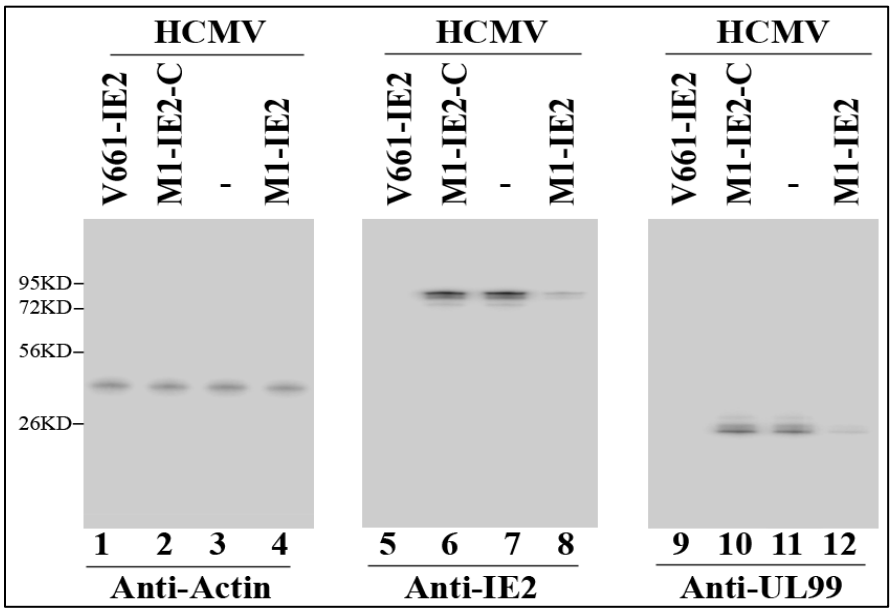


**Figure 4.** Levels of HCMV proteins observed by Western Blot. Actin control (lanes 1–4), IE2 (lanes 5–8), and UL99 (lanes 9–12). Cells (n = 1 $\times 10^6$) were infected with HCMV (MOI = 1) and were harvested at either 24 (lanes 1–8) or 72 h (lanes 9–12) post-infection.

### 3.3.4 Increased Reduction of HCMV Infection by the Ribozymes

The expressions of HCMV β (early) and γ (late) genes are expected to be decreased due to the inhibition of IE2 expression [1, 33]. To explore this, US2 mRNA level (a β mRNA) (Figure 3, lanes 9–12) as well as UL99 protein level (a γ protein) (Figure 4, lanes 9–12) were determined. We used HCMV 5 kb RNA and human actin as the internal loading controls. Our results demonstrated 95%–96% and 71%–73% reduction ($p < 0.04$) in the expression levels of these genes in cells with V661-IE2 and M1-IE2, respectively. Limited inhibition was found in cells with M1-IE2-C, V661-IE2-C, and M1-TK (Figures 3 and 4, Table 2). Similar results were also observed in the expression of HCMV polymerase processivity factor UL44 (a β protein) and viral glycoprotein H (gH) (a γ protein) (Table 2). Thus, V661-IE2 and M1-IE2 expressing cells appeared to exhibit overall viral β and γ gene expression inhibition.

The ribozymes also appeared to inhibit viral growth. After 5 days post-infection, we observed a 3500- and 100-fold reduction ($p < 0.04$) in viral titers in cells that expressed V661-IE2 and M1-IE2, respectively (Figure 5). In comparison, we observed no reduction in cells with control M1-IE2-C, V661-IE2-C, or M1-TK ribozymes (Figure 5, data not shown).

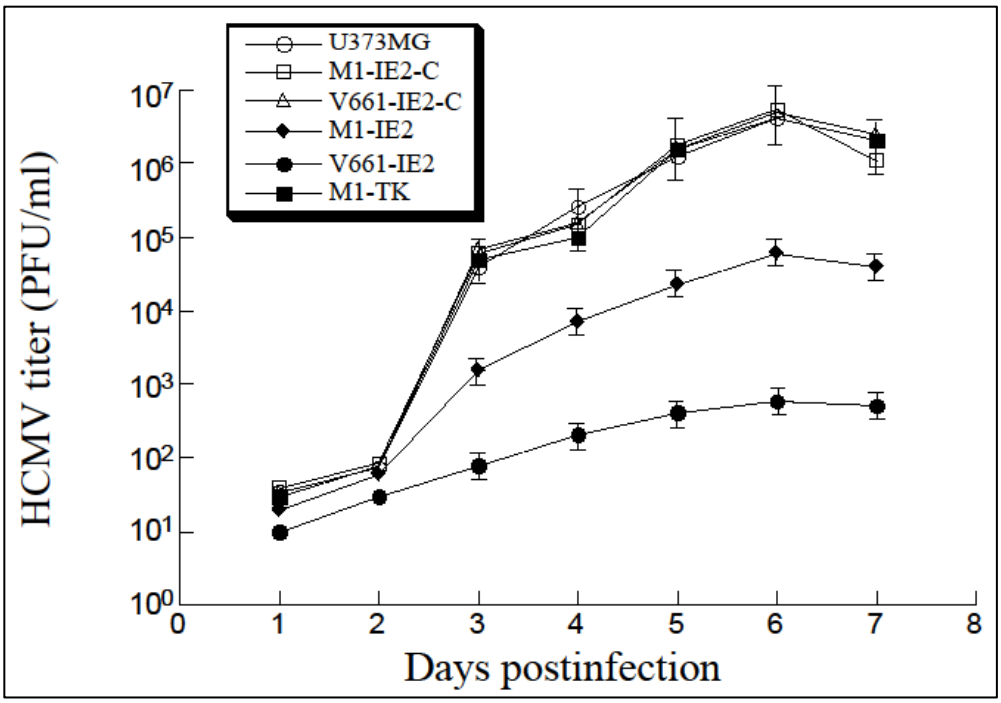


**Figure 5.** HCMV growth in various cell lines expressing M1GS and the parental U373MG cells. These values are the average from three experiments with error bars showing the standard deviation. $p < 0.04$.

### 3.4 Discussion

Ribozyme-based gene targeting represents a promising therapeutic approach [5]. For ribozyme- mediated gene targeting to work efficiently, M1GS needs to be highly specific, catalytically efficient, and easily delivered to the target of choice. We have constructed M1GS RNAs that target the exon 5 region of HCMV IE2 mRNA and have shown that the ribozymes target the substrate efficiently in-vitro. Moreover, 98% reduction in the expression level of IE2 and 3,500-fold reduction in viral growth were observed in cells with a ribozyme variant (i.e., V661-IE2). In comparison, we detected little reduction (<10%) in IE2 expression and HCMV growth in cells with V661-IE2-C and M1-IE2-C. While non-functional, V661-IE2-C and M1-IE2-C contained identical guide sequence and showed similar binding affinity to the targeting mRNA as V661-IE2 and M1-IE2. Thus, our results imply that the overall observed inhibition of HCMV infection with M1-IE2 and V661-IE RNA was primarily due to specific ribozyme-mediated cleavage of the target mRNA.

Limited information is available about what limits M1GS-mediated gene-targeting efficacy in cultured cells [30]. Not much is known about how to improve the efficacy of M1GS RNA in blocking HCMV infection in-vivo. In this study, M1GS was designed to target a DMS-

accessible sequence of IE2 mRNA and was constitutively expressed by the U6 promoter. Our design should enhance the chance for M1GS to find the mRNA target. With the design, we postulated that the efficacy of RNase P ribozyme cleavage in cultured cells is dictated by its catalytic efficiency [$(k_{cat}/K_M)^s$]. It is conceivable that enhancing catalytic activity of M1GS may reduce target mRNA expression more effectively in-vivo. Indeed, V661-IE2, which was more efficient in cleaving ie2-39 in-vitro, blocked IE2 expression and viral growth in cultured cells more effectively than M1-IE2, the wild type M1GS (Tables 1 and 2). The difference between the in-vivo efficacies of V661-IE2 and M1-IE2 (e.g., 98% vs. 75%) is less than the difference of in-vitro cleavage efficiencies (more than 50-fold difference). A possible explanation is that about 1%–2% of the target mRNA substrates are unavailable for ribozyme cleavage because they may be bound by ribosome for translation prior to being bound by the ribozymes. Our work implies that increasing catalytic efficiencies [$(k_{cat}/K_M)^s$] correlates with better ribozyme-mediated effect in reducing HCMV growth, and further suggests that improving the in-vitro catalytic efficiencies of the ribozymes should lead to increased anti-HCMV efficacies in tissue culture. Thus, our study provides a guideline for the generation of highly effective RNase P ribozyme variants.

The antiviral effect of RNase P ribozymes appears to be specifically induced by the cleavage of the IE2 mRNA. First, cells expressing the ribozymes appeared normal with no toxicity for up to two months (data not shown). Second, the antiviral effect of the ribozyme (inhibition of viral growth) appears to be due to the decrease of IE2 expression. We detected overall reduction of viral β and γ gene expression (e.g., US2, UL44, UL99, and gH) in cells expressing functional M1-IE2 and V661-IE2 but not control M1-IE2-C or V661-IE2-C. The levels of reduction of IE2 expression correlate well with the levels of reduction in viral β and γ gene expression. In comparison, we detected no reduction in other viral immediate-early RNA (e.g., 5 kb RNA and UL36 mRNA) expression in these cells (Figure 3A, Table 2, data not shown) [22, 40]. Thus, the antiviral effect of M1GS ribozyme may be induced by the cleavage of the IE2 mRNA.

Our study showed that variant V661-IE2 is more active in cleaving IE2 mRNA sequence than M1-IE2 in-vitro (Table 1). Furthermore, a ribozyme targeting the HSV-1 TK mRNA sequence, V661-TK, was derived from variant V661. V661-TK also cleaved the TK mRNA sequence in-vitro more efficiently than M1-TK, which was derived from the wild type ribozyme [31]. Thus, V661 may be used to construct highly efficient ribozymes to target any specific mRNAs. This variant has two point mutations: 94A → G and 194G → C. Little is known about the roles of these two nucleotides in the M1GS-mediated cleavage of an mRNA substrate. Our study indicated that the point mutations at these two positions increased the overall cleavage rate ($k_{cat}/K_M$) without affecting the binding affinity ($K_d$) to the mRNA substrate (Table 1). These results imply that the mutations may have no effect on the interactions of the ribozyme to the target mRNA. Perhaps these mutations increase the overall cleavage rate by stabilizing the active site and facilitating the folding of the overall structure of the ribozyme. Further biochemical characterization of V661 and other ribozyme variants will elucidate the mechanism by which mutations found in the variants increase the gene-targeting activity of the ribozymes and provide insight into the construction of highly effective ribozymes for gene targeting applications.

HCMV is a member of the human herpesvirus family [41, 42]. Like other herpesviruses, HCMV can actively replicate or enter into latency [1]. When HCMV reactivates from latent infection and initiates lytic infection, IE2 is among the first proteins expressed and is essential for viral replication [1]. To study the functionality of M1GS ribozyme in HCMV latent infection, M1GS

can be delivered into latently infected cells including $CD34^+$ bone marrow progenitor cells. These studies will determine if RNase P ribozymes diminish IE2 expression and block HCMV reactivation. One potential challenge is to develop appropriate vectors for effective delivery and efficient expression of the ribozymes in $CD34^+$ bone marrow progenitor cells. These and further studies should provide insight into the development of M1GSs as potentially effective anti-HCMV therapeutics.

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# 4 Inhibition of Herpes Simplex Virus 1 Gene Expression and Replication by RNase P-Associated External Guide Sequences.

## 4.1 Introduction

Herpes simplex virus 1 (HSV-1), a member of the human herpesvirus family, is one of the leading causes of viral infections in humans and reactivation of the virus from latency can cause herpes simplex encephalitis and corneal blindness in immunocompromised individuals [1]. It is necessary to develop new antiviral compounds and novel approaches because of the emerging drug-resistant strains of HSV-1. Nucleic acid-based molecules represent promising therapeutic strategies for targeting of specific mRNA sequences [2]. Ribonuclease P (RNase P) is a ribonucleoprotein complex which facilitates the maturation of tRNA by catalyzing a hydrolysis reaction to remove the leader sequence of precursor tRNA (ptRNA) (Fig. 1A) [2–4]. It has been shown by previous studies that RNase P recognizes pre-tRNAs (ptRNAs) with their tertiary molecular structures and can cleave a custom-designed substrate which resembles a ptRNA in tertiary structure [5]. In seminar studies by Altman and colleagues, targeted mRNAs were able to be cleaved by recruited RNase P when bound to custom-designed EGSs to form a hybrid resembling a ptRNA molecule (Fig. 1B) [6, 7]. EGSs which were derived from natural ptRNA sequences can be used to inhibit gene expression in both bacterial and mammalian cells [7–10]. Furthermore, EGSs were shown to be effective in inhibiting gene expression and replication of several human viruses including HIV, human cytomegalovirus (HCMV), Kaposi sarcoma-associated herpesvirus (KSHV), and hepatitis B virus (HBV) in human cultured cells [11–14].

The technology of EGS-induced mRNA cleavage by RNase P is distinguished by its simple mechanism from other nucleic acid approaches for mRNA silencing such as RNAi, which involves various cellular factors. Furthermore, the RNase P-mediated cleavage does not generate nonspecific “irrelevant cleavage” which happens in RNase H-mediated cleavage induced by conventional antisense phosphothioate molecules [3, 15]. Therefore, EGSs have the potential to be an effective therapeutic approach for human diseases such as HSV-1 infection [16–18].

Increasing both the in-vitro and in-vivo activity of EGSs can contribute to the efficiency of the reaction of EGS-induced cleavage of target RNA by RNase P. In a previous study, novel EGS variants which were more efficient in recruiting RNase P in the cleavage of HSV-1 thymidine kinase (TK) mRNA were screened out with an in-vitro selection procedure [19]. As of now, little is known about the mechanistic changes in these EGS RNA variants that improve their activity in inducing the cleavage of a target mRNA by RNase P and whether they are also effective in targeting other HSV mRNAs to influence the expression of viral genes and further affect viral growth.

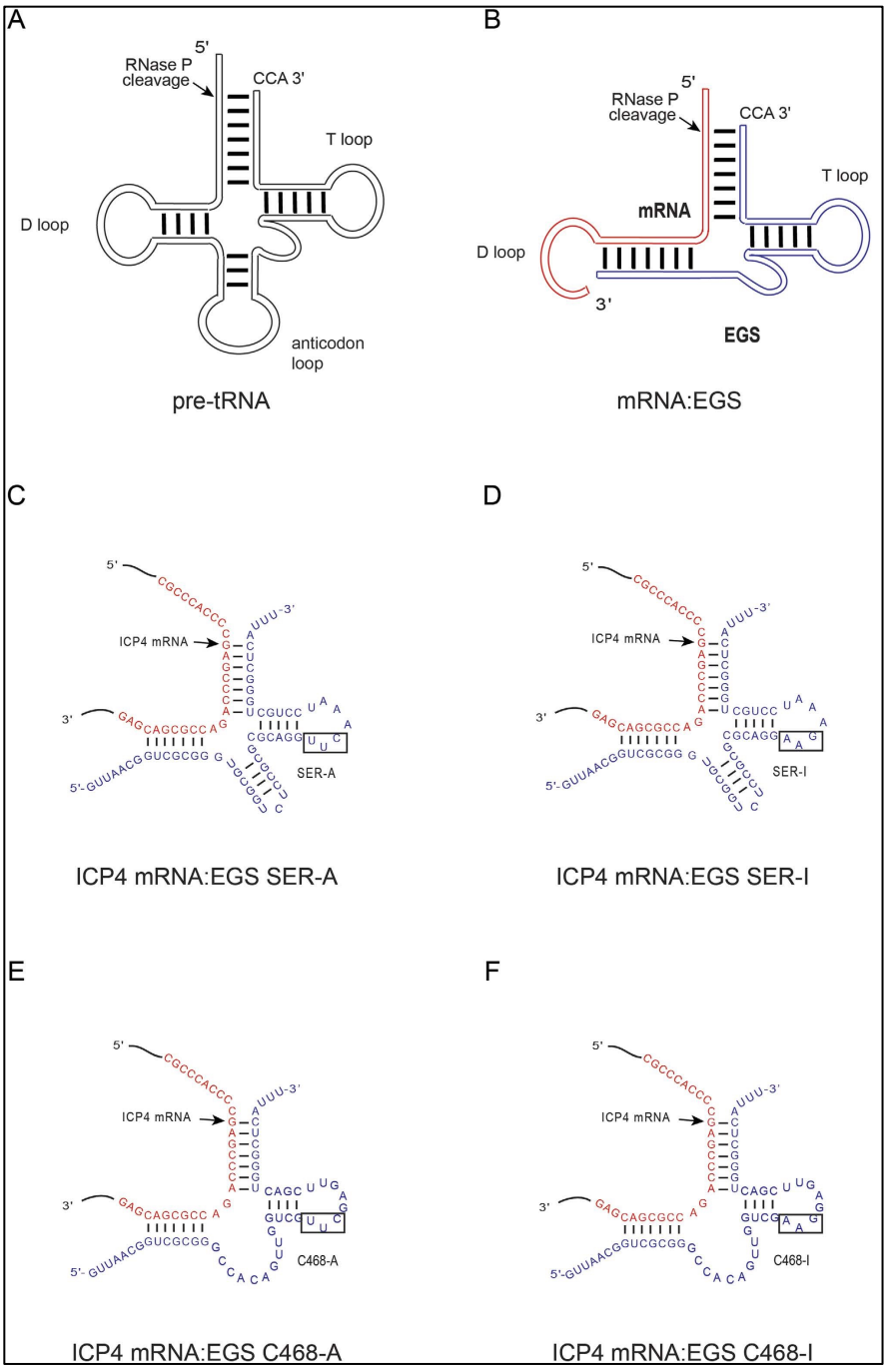


**Figure 1. Schematic presentation of RNase P substrate/synthesized EGS complex.** (**A**) A pre-tRNA structure. (**B**) A hybridized complex of a target RNA and an EGS resembling the T-stem and loop, and variable region of a pre-tRNA structure. (**C–F**) Hybridized complexes of HSV-1 ICP4 mRNA and EGS SER-A, SER-I, C468-A, and C468-I, respectively. The sequences of SER-A and SER-I were derived from natural pre-tRNA$^{SER}$, while those of C468-A and C468-I were from EGS variant C468. The targeting sequences of ICP4 mRNA are shown in red and the EGS sequences are shown in blue, respectively. The site of cleavage by RNase P is marked with an arrowhead. The three mutated positions to inactivate EGS activity are marked in black box.

## 4.2 Methods

### 4.2.1 Mapping of Accessible Regions of ICP4 mRNA in HSV-Infected Cells

HSV-1-infected (MOI = 1) HeLa and Vero cells were incubated with 5 mL of fresh culture media containing 1% DMS for 5–10 min at 6 hours post infection as previously described [20, 21]. Cells were then treated with lysis buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 0.2% NP40) and Total RNA extraction of cell lysates was carried out using the phenol-chloroform extraction method. DMS modification sites were then read out by primer extension assays in 8% denaturing gels, as described previously [20, 21].

### 4.2.2 In Vitro Studies of EGSs and RNase P

The template DNA sequence template for EGS SER-A was amplified by PCR from plasmid pTK11211 using forward (5'GGAATTCTAATACGACTCACTATAGGTTAACGGTCGCGGGTGCGGTCTCC GCGC-3') and reverse (5'-AAGCTTTAAATGAGCCCAGCAGGATTTGAACCTGCGCGCGGAGACCGCAC-3') primers. The DNA template of C468-A was amplified by PCR from plasmid pC46819 using forward (5'-GGAATTCTAATACGACTCACTATAGGTTAACgGTCGCGGGCCACAGUUG–3') and reverse (5'-AAGCTTTAAATGAGCCCAGTCGAACTCGAACGACCAACTGTGGC-3') primers. SER-I and C468-I sequences which were negative controls, were derived from SER-A and C468-A sequence, respectively, and contained a three-nucleotide mutation (5'-TTC-3' to AAG) in their T-loops (Fig. 1C–F). SER-I and C468-I were amplified by PCR using reverse primers oligoSER-I3 (5'-AAGCTTTAAATGAGCCCAGCAGGATTTCTTCCTGCGCGCGGAGACCGCAC-3') and oligoC468-I3 (5'-AAGCTTTAAATGAGCCCAGTCGAACTCCTTCGACCAACTGTGGC-3'), respectively. The 38 nucleotide-long substrate (icp38) sequence was constructed by annealing oligonucleotides AF25 (5'-GGAATTCTAATACGACTCACTATAG-3') and sICP (5'-CGGGATCCCTCGTCGCGGTCTGGGCTCGGGGT GGGCGCCTATAGTGAGTCGTATTA-3'). EGS RNAs or substrate icp38 was synthesized by T7 RNA polymerase using the constructed DNA template sequences. Human RNase P was prepared from HeLa cells as described previously [10, 14, 23]. Kinetic analysis was performed following previously described protocols [13, 19]. Binding affinities were measured in the absence of human RNase P [18, 26].

### 4.2.3 Construction of EGS-Expressing Cells and Detection of EGS Expression

The EGS-expressing HeLa and human oral keratinocytes (HOK) cell lines were produced as previously described [20, 31]. Briefly, the constructed EGS sequences were cloned into retroviral vector LSXN, and the LXSN-EGS constructs were transfected into amphotropic PA317 cells. Retroviral vectors harvested from cell supernatants at 48 hours post transfection were used to infect HeLa or HOK cells, and resistant cells that grew in neomycin-containing media (600 μg/ml) were selected and cloned. As described previously, Northern blot analysis was conducted using H1 RNA as an internal control, to determine the expression levels of EGS [20, 31].

### 4.2.4 Infection of Cells and Detection of Viral Gene Expression and Growth

EGS-expressing Hela or HOK cell lines were either mock-infected or infected with HSV-1 at a MOI which is specified in the Results section. Total RNA samples were extracted at 4 or 10 hours post-infection to measure viral mRNA expression. Viral protein expression was measured form samples that were extracted 12 hours post-infection.

Radiolabeled probes for the detection of mRNAs ICP4, ICP47, and TK were synthesized by PCR using plasmids pICP4, pICP47 and pTK129, respectively. The expression of proteins ICP4, ICP27, ICP35, gB, and gC were detected using antibodies against each of the respective proteins. Northern blot, Western blot, and RNase protection assays to measure the EGS, mRNA, and protein expression levels were carried out following details as described previously [28, 32].

To measure the EGS-mediated decrease in viral replication, HeLa or HOK cells lines expressing different EGSs were infected with HSV-1 (MOI = 1–1.5). Cell culture supernatants were harvested in intervals of 6 hours for 36 hours post infection for preparing virus stocks, and viral titers were studied by plaque assays of viral stocks [28, 32].

## 4.3 Results

### 4.3.1 EGS-Mediated Cleavage of ICP4 mRNA Sequence by RNase P in Vitro

We determined the accessible regions of ICP4 mRNA in HSV-1 infected cells with a dimethyl sulphate (DMS)-based mapping approach [19–21]. HSV-1 infected HeLa cells were incubated in media containing DMS. DMS can modify the nucleotides of the accessible mRNA regions after entering the cells. Total mRNA from these cells was isolated and primer extension assays with reverse transcriptase were used to determine the regions modified by DMS, which were also the accessible regions in the ICP4 mRNA sequence. A position, 66 nts downstream from the start codon was chosen as the cleavage site for the EGSs since it was among the regions where the ICP4 mRNA was most modified by DMS.

Previously, we carried out an in-vitro selection procedure to isolate EGS variants which were more active in guiding RNase P to cleave HSV-1 TK mRNA than the EGSs derived from a natural ptRNA [19]. The original goal of the research was to construct effective gene-targeting EGSs and find out how EGSs guide RNase P to cleave target RNA by studying those variants. However, how these screened-out EGSs gain enhanced activity in guiding RNase P to cleave target mRNA sequences is still unknown. Among those most active EGSs in guiding RNase P to cleave target mRNA sequences, C468 efficiently recruited RNase P to cleave both HSV-1 TK mRNA and ICP4 mRNA (see below, Table 1). EGS C468-A was designed by attaching the T loop and variable domain of C468 to parts of complementary sequence of the ICP4 mRNA (Fig. 1E). Another EGS, SER-A, which was derived from the natural ptRNA SER sequence, was constructed in the same way as C468-A (Fig. 1C).

| Substrate | $K_m$ (μM) | $V_{max}$ (apparent) (pmol·min$^{-1}$) | $V_{max}$(apparent)/$K_m$(apparent) (pmol·μM$^{-1}$·min$^{-1}$) | $K_d$ (μM) |
|---|---|---|---|---|
| pre-tRNA$^{SER}$ | 0.022 ± 0.071 | 0.069 ± 0.020 | 3.1 ± 0.5 | |
| ICP4 RNA (icp38) | | | | |
| +SER-A | 0.60 ± 0.16 | 0.029 ± 0.010 | 0.048 ± 0.013 | 2.1 ± 0.5 |
| +SER-I | ND | ND | <0.001 | 2.0 ± 0.5 |
| +C468-A | 0.29 ± 0.09 | 0.84 ± 0.36 | 2.9 ± 0.4 | 0.025 ± 0.005 |
| +C468-I | ND | ND | <0.001 | 0.025 ± 0.005 |

**Table 1. Overall cleavage rates ($V_{max(apparent)}/K_{m(apparent)}$) and binding affinities ($K_d$) in EGS-mediated cleavage reactions of pre-tRNA$^{SER}$ or ICP4 mRNA by RNase P.** Kinetic parameters were determined with previously described protocols [13, 19]. Binding affinities were measured without the presence of RNase P. The values shown are the average derived from triplicate experiments. "ND": not determined.

We measured the RNase P mediated cleavage rate of substrate icp38 containing 38 nucleotides of ICP4 mRNA, which represents the target sequence, in the presence of C468-A or SER-A. Efficient cleavage of icp38 by human RNase P was observed in the presence of active EGS SER-A and C468-A (Fig. 2, lanes 3–4). Applying kinetic analyses, we obtained the cleavage efficiency values [Vmax(apparent)/Km(apparent)] for the cleavage reactions. C468-A was 60 times more efficient in guiding human RNase P to cleave icp38 than SER-A (Table 1). It is possible that the variable loop of C468-A contributes to the stability of the mRNA-EGS complex and thus improves RNase P cleavage rate. In that case, the binding affinity of C468-A to the target ICP4 mRNA sequence should be better than that of SER-A. Using gel-shift assays, the binding affinities of C468-A and SER-A to icp38 RNA sequence were measured and represented by the dissociation constant ($K_d$). Despite that C468-A and SER-A share the same complementary sequence of the ICP4 mRNA (Fig. 1C,E), C468-A showed about 80 times higher binding affinity to icp38 than SER-A (Table 1). These results imply that the enhanced binding affinity of C468 A to icp38 may be due to newly introduced tertiary interactions.

C468-I and SER-I, derived from C468-A and SER-A respectively, were designed and used as negative controls. These two EGSs contain the same mutations (from 5'-UUC-3' to AAG) in their T- loop compared with C468-A or SER-A (Fig. 1D, F). The mutated nucleotides are highly conserved and changes at this site inactivate EGS activity3. The two control EGSs showed targeting activities at least 2 × $10^3$-fold slower than C468-A (Table 1; Fig. 2, lane 2). C468-I and SER-I contained the identical complementary sequence to ICP4 mRNA sequence with C468-A or SER-A (Fig. 1C–F), and also showed similar binding affinities to icp38 with C468-A and SER-A,

respectively (Table 1). Thus, C468-I and SER-I, which share the similar structures with C468-A and SER-A respectively but with inactivated EGS activity, can serve as controls to exclude antisense or other unspecific effect of EGSs.

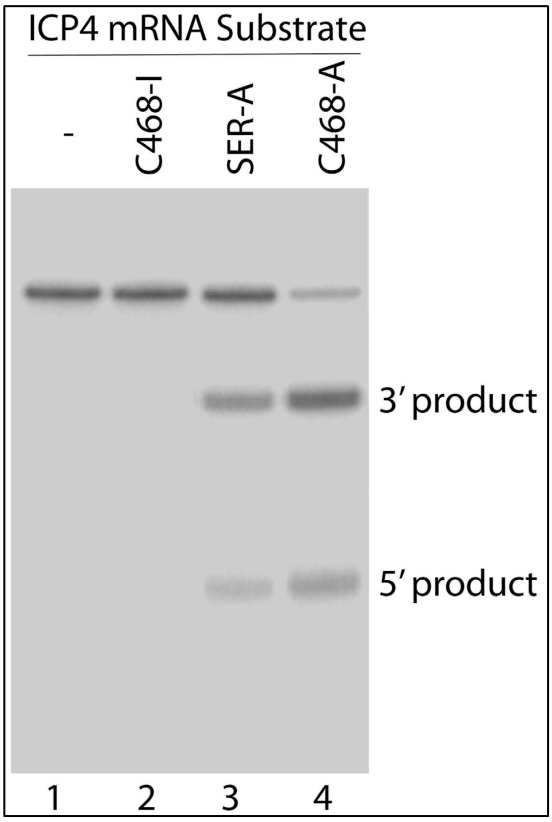


**Figure 2. In vitro cleavage of an ICP4 mRNA substrate by human RNase P.** The reactions were carried out in the absence of an EGS (-, lane 1) and presence of different EGSs (lanes 2–4).

### 4.3.2 Expression of EGSs in Human Cells

To construct HeLa and human primary oral keratinocyte (HOK) cell lines expressing EGSs, DNA sequences encoding SER-I, SER-A, C468-I, and C468-A were cloned into retroviral vector LXSN under the control of the small nuclear U6 RNA promoter and subsequently transfected into amphotropic packaging cells (PA317) [22, 23]. Infection of HOK cells by HSV-1 can be found in-vivo, while HeLa cells represent a commonly used cell model for HSV-1 lytic infection1. Subsequently, HeLa and HOK cells were infected with these vectors and cell lines expressing these

EGSs were screened out. No cytotoxicity was observed in the constructed cell lines for up to one month in MTT assays. Northern blot was used to detect EGS expression in these cells and only cell lines expressing similar EGS RNA levels were chosen for this study (Fig. 3, data not shown).

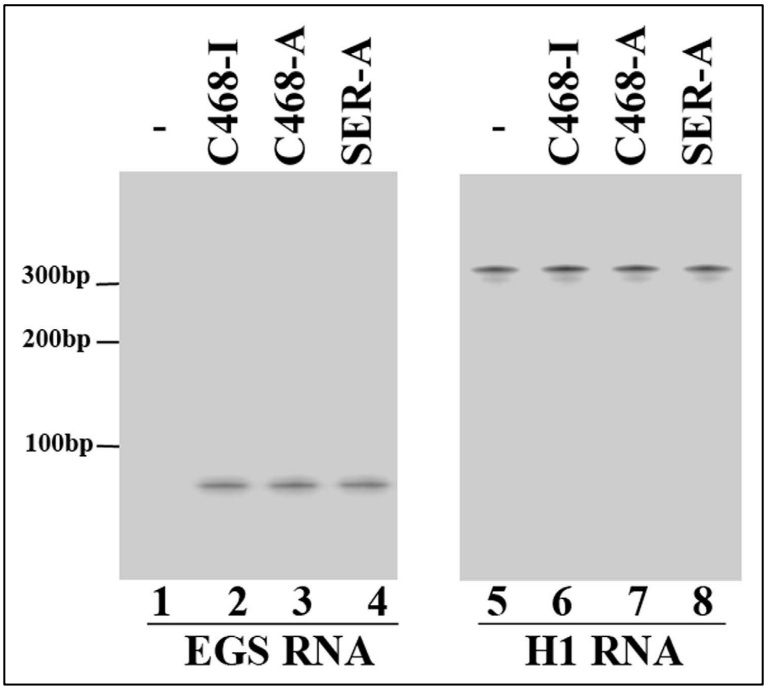


**Figure 3. EGS RNA expression with Northern blot analysis in HeLa cells.** RNA samples (25μg) were isolated from parental HeLa cells (-, lanes 1 and 5) and cells expressing EGS C468-I (lanes 2 and 6), C468-A (lanes 3 and 7), and SER-A (lanes 4 and 8). Human H1 RNA (lanes 5–8) was used as the loading control.

### 4.3.3 EGS-Mediated Inhibition of HSV-1 ICP4 Gene Expression

To determine the EGS-mediated inhibition of ICP4 gene expression, cells were infected with HSV-1 (MOI = 0.05–1). Furthermore, some of cells were treated with 100 μg/ml cycloheximide which can inhibit protein synthesis and only allows the expression of viral IE transcripts but not early or late transcripts [1]. Total RNAs were then isolated. The expression levels of ICP4 mRNA were measured with an RNase protection assay and viral immediate early gene ICP47 mRNA was used as the internal loading control (Fig. 4). A decrease of ~96–97%, 75–77%, 8–9%, and 5–6% (results of experiments in triplicate) in the expression levels of ICP4 mRNA

appeared in cells that expressed C468-A, SER-A, C-468-I, and SER-I, respectively (Tables 2 and 3). Meanwhile, the expression levels of ICP4 protein were assayed with western analysis using human actin as the internal loading control (Fig. 5). A reduction of 97%, 75–76%, 8%, and 5–7% (results of experiments in triplicate) in the expression levels of ICP4 protein appeared in cells expressing C468-A, SER-A, C468-I, and SER-I, respectively (Tables 2 and 3). Results of these two experiments implied that the specific cleavage of ICP4 mRNA induced by the EGSs might be the main cause contributing to the significant decrease of expression level of ICP4 in cells expressing C468-A or SER-A. The far less decrease in the expression level of ICP4 shown in cells expressing C468-I or SER-I was possibly because of the antisense effect as C468-I and SER-I shared the same antisense sequences of icp38 with C468-A or SER-A, but were not able to guide RNase P as the three nucleotides at the T-loop region were mutated (Fig. 1, Table 1). We detected no intracellular cleavage products of ICP4 mRNA, possibly due to the rapid degradation of these RNAs, which lacked either a poly A sequence or a 5′ cap structure.

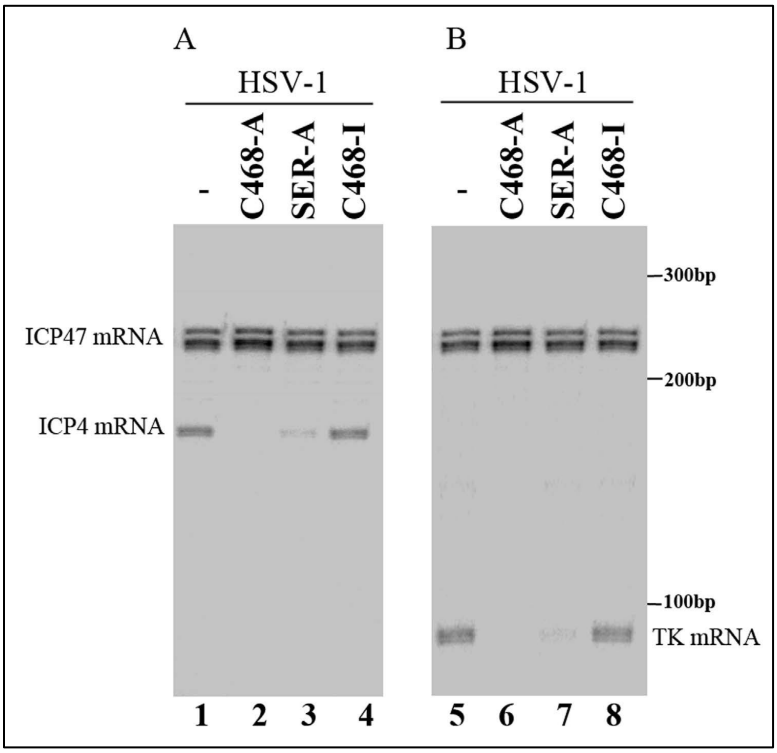


**Figure 4. Expression levels of HSV-1 mRNAs with RNase protection assay.** Parental HeLa cells (lanes 1and 5), cell lines which expressed C468-A (lanes 2 and 6), SER-A (lanes 3 and 7), and C468-I (lanes 4 and 8) were infected with HSV-1(MOI = 0.9). Total RNAs were isolated from those cells 4 hours (**A**) or 10 hours (**B**) post infection. RNA samples (30 μg) were hybridized to the RNA probes containing the sequences of ICP4 and ICP47 (lanes 1–4) and to those containing ICP47 and TK mRNAs (lanes 5–8). The ICP47 mRNA was used as the internal loading control.

### 4.3.4 EGS-Mediated Inhibition of HSV-1 Gene Expression and Growth

The ICP4 protein is one of the viral α or immediate early (IE) genes which is necessary for the activation of the expression of HSV early (β) and late (γ) genes1. The expressions of early and late genes of HSV are expected to be inhibited due to the repression of ICP4 expression. RNase protection and western blot analyses were applied to detect the expression of the TK mRNA (a β gene), ICP27 protein (an α protein), and glycoprotein gC (a γ protein), respectively (Figs 4 and 5). Significant decreases (75–97%) of the expression of TK and gC were detected in cells expressing C468-A or SER-A.

| Viral gene | Gene class | HeLa | SER-I | C468-I | SER-A | C468-A |
|---|---|---|---|---|---|---|
| ICP4 mRNA | IE | 0% | 6% | 9% | 77±7% | 97±8% |
| ICP47 mRNA | IE | 0% | 0% | 1% | 1% | 2% |
| TK mRNA | Early | 0% | 2% | 2% | 78±6% | 96±9% |
| ICP4 protein | IE | 0% | 7% | 8% | 75±7% | 97±8% |
| ICP27 protein | IE | 0% | 3% | 3% | 2% | 1% |
| gC protein | Late | 0% | 1% | 2% | 77±8% | 95±7% |
| ICP35 protein | Late | 0% | 0% | 1% | 77±8% | 95±9% |
| gB protein | Late | 0% | 1% | 2% | 75±7% | 97±8% |

**Table 2. Viral mRNA and protein expression in HeLa cells lines expressing EGS C468-A, C468-I, SER-A, and SER-I, respectively, presented as the percentages of inhibition compared to those in the parental HeLa cells.** The values represent arithmetic means of triplicate experiments and the values of standard deviation which were less than 5% are not shown.

| Viral gene | Gene class | HOK | SER-I | C468-I | SER-A | C468-A |
|---|---|---|---|---|---|---|
| ICP4 mRNA | IE | 0% | 5% | 8% | 75±8% | 96±9% |
| ICP47 mRNA | IE | 0% | 1% | 2% | 1% | 1% |
| TK mRNA | Early | 0% | 1% | 1% | 76±8% | 96±9% |
| ICP4 protein | IE | 0% | 5% | 8% | 76±8% | 97±9% |
| ICP27 protein | IE | 0% | 2% | 2% | 3% | 2% |
| gC protein | Late | 0% | 2% | 2% | 76±7% | 97±8% |
| ICP35 protein | Late | 0% | 1% | 1% | 76±8% | 95±8% |
| gB protein | Late | 0% | 1% | 1% | 76±8% | 97±9% |

**Table 3. Viral mRNA and protein expression in human oral keratinocyte (HOK) lines expressing EGS C468-A, C468-I, SER-A, and SER-I, respectively, presented as the percentages of inhibition compared to those in the parental human oral keratinocytes.** The values represent arithmetic means of triplicate experiments and the values of standard deviation which were less than 5% are not shown.

However, no significant decrease was shown in cells expressing C468-I or SER-I, respectively (Figs 4 and 5). Similar results were also obtained when we assayed the protein expression level of HSV-1 capsid protein ICP35 and glycoprotein gB, two other γ gene products (Tables 2 and 3). Consistent with previous observations that ICP4 does not significantly affect viral IE expression [1], no decrease in the level of ICP27 protein expression was found in cells expressing these EGSs (Fig. 5, lanes 9–12; Tables 2–3). These results show that EGSs C468-A and SER-A specifically inhibit the expression of viral early and late genes but not immediate early (IE) genes.

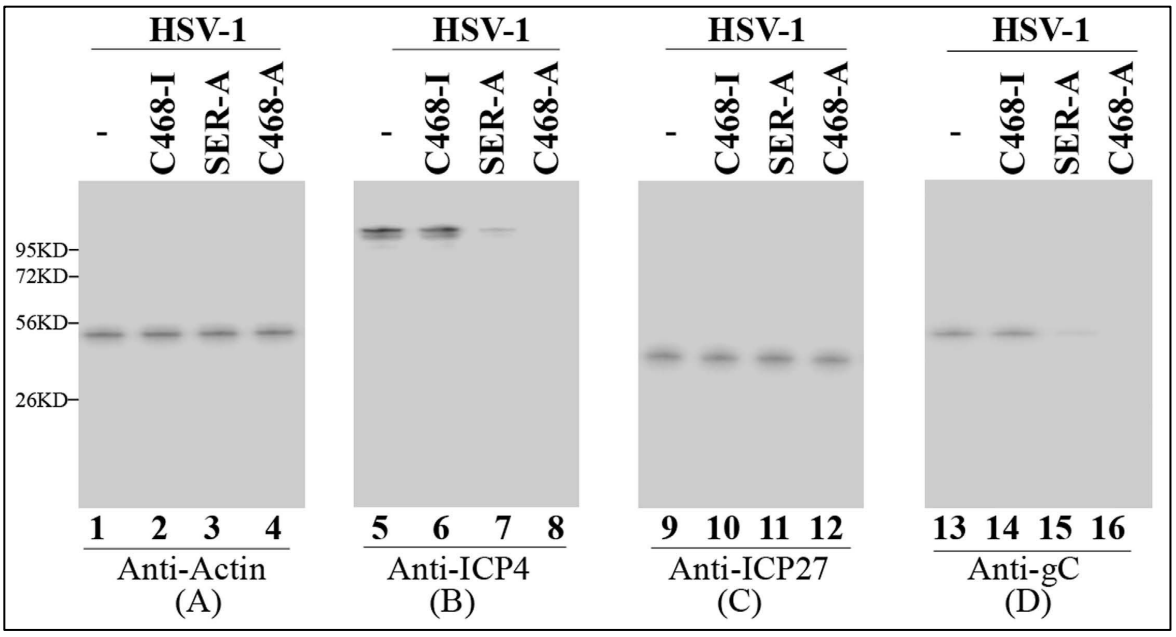


**Figure 5. Expression levels of HSV-1 proteins with western blot analysis.** Parental HeLa cells (lanes 1, 5, 9, 13), cell lines which expressed C468-I (lanes 2, 6, 10, 14), SER-A (lanes 3, 7, 11, 15), and C468-A (lanes 4, 8, 12, 16) were infected with HSV-1(MOI = 0.8). Protein samples were collected from those cells 12 hours post infection and detected with western blot analysis. Human actin (**A**), HSV ICP4 (**B**), ICP27 (**C**) and gC protein (**D**) were detected with specific antibodies.

The EGSs also seemed to inhibit viral growth in HSV-1 infected human cells. Equal number of parental HeLa and HOK cells, and cell lines that expressed SER-I, C468-I, SER-A, and C468-A were infected with HSV-1 (MOI = 1–1.5). Virus stocks were prepared from cells and culture media was harvested at every 6 hours for 36 hours post infection. The viral titer was measured by counting the number of plaque forming units (PFU) on Vero cells.

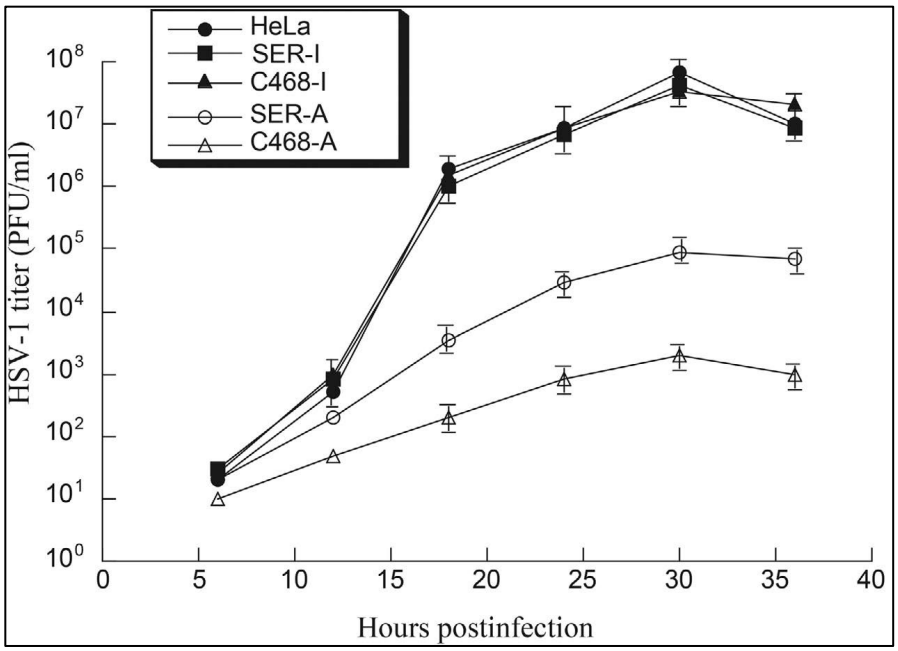


**Figure 6. Growth analysis of HSV-1 in parental HeLa cells and cell lines expressing EGS RNAs.** Equal number ($5 \times 10^5$) of parental HeLa cells and cell lines which expressed SER-I, C468-I, SER-A, and C468-A were infected with HSV-1 (MOI = 1). Virus stocks were prepared from the cells and culture media harvested at every 6 hours for 36 hours post infection. The virus titer of each sample was measured by counting the plaque forming unit (PFU) in Vero cells. The values are the average of three experiments, and the standard deviations are shown by the error bars.

After 18 hours post infection, a decrease of more than 7,000- and 500 folds in virus yield was observed in cells expressing C468-A and SER-A, respectively, compared to parental HeLa or HOK cells expressing no EGS (Figs 6 and 7). In contrast, no significant decrease was observed in those cells expressing C468-I and SER-I. These results support our observations that an inhibition of ICP4 expression in the presence of C468-A or SER-A leads to a significant decrease of expression of the β and γ genes of HSV.

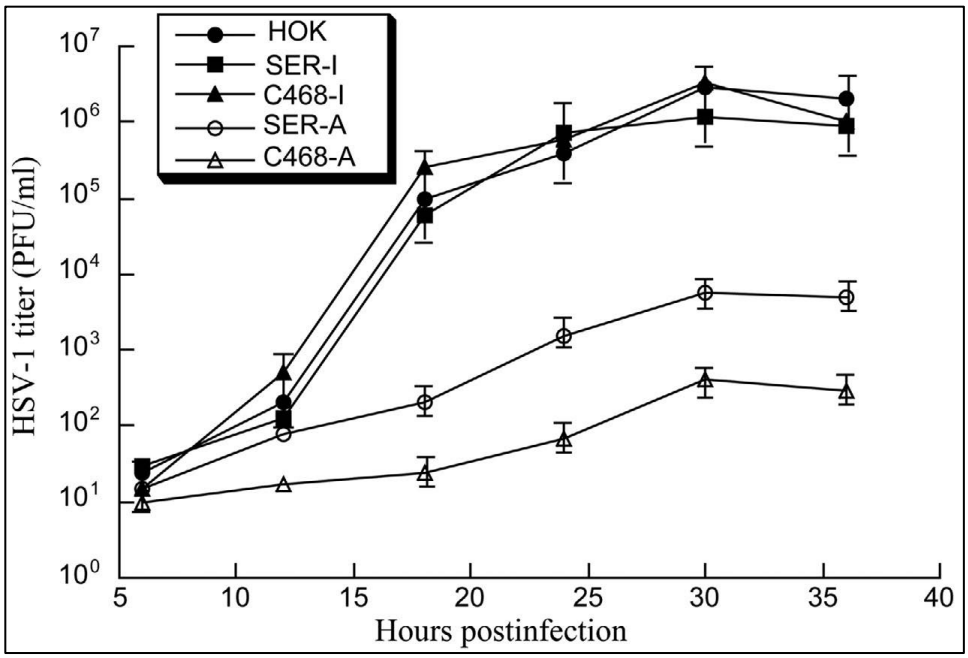


**Figure 7. Growth analysis of HSV-1 in parental human oral keratinocytes (HOK) and HOK cell lines expressing EGS RNAs.** Equal number ($1 \times 10^5$) of parental HOK cells and cell lines which expressed SER-I, C468-I, SER-A, and C468-A were infected with HSV-1 (MOI = 1.5). Virus stocks were prepared from the cells and culture media harvested at every 6 hours during 36 hours post infection. The virus titer of each sample was measured by counting the plaque forming unit (PFU) in Vero cells. The values are the average of three experiments, and the standard deviations are shown by the error bars.

## 4.4 Discussion

EGS-mediated degradation of mRNA represents a new nucleic acid based approach for gene silencing applications. This strategy is a promising approach because intracellular RNase P can be guided with custom-designed EGSs to cleave any target mRNAs efficiently and specifically [3, 24]. However, little is known about if EGS is effective in blocking HSV-1 growth by inhibiting the expression of viral essential genes. Our study presented here provides the direct evidence that engineered EGSs effectively block the replication and growth of HSV-1 in human cells.

It is still not clear how to improve the targeting activity of EGS and much work is needed to understand how to design EGSs exhibiting better efficacy in down-regulating gene expression in cultured cells. Here, making use of dimethyl sulphate (DMS), we determined an accessible region of HSV-1 ICP4 mRNA and constructed several EGSs that target the accessible region. Our results demonstrated that C468-A derived from ESG variant C468 was about 60 times more active in guiding RNase P to cleave target mRNA, more efficient (97% vs. 75%) in inhibiting ICP4 RNA

and protein expression, and about 14 times more effective in reducing viral growth (~7000-fold vs. ~500 fold) than SER-A derived from natural pre-tRNA SER. In contrast, C468-I and SER-I , which share the similar structures with C468-A and SER-A respectively but with inactivated RNase P guiding activity because of mutations of the three conserved positions in the T loop, showed far less efficacy (less than 10%) in the inhibition of ICP4 mRNA and protein expression and exhibited no effect on reducing viral growth. The significant difference in the inhibition of ICP4 expression level and viral growth between C468-A and C468-I or between SER-A and SER-I indicates that the observed reduction is mainly caused by the specific EGS-mediated cleavage of ICP4 mRNA by RNase P rather than the antisense effect or other nonspecific effects of EGSs. The EGS (i.e. C468-A) showing higher activities in guiding RNase P to cleave the ICP4 mRNA sequence in-vitro is also more effective in reducing HSV-1 gene expression and viral growth. These results are consistent with our observations that ESG-mediated inhibition of the expression level of ICP4, which is necessary for the expression of the early and late genes1, can result in inhibition of HSV-1 overall gene expression and viral growth. C468-A, which shows 60 folds higher activity to induce RNase P-mediated cleavage in-vitro than SER-A, is also more effective in reducing ICP4 mRNA (97% vs. 75%) in cultured cells. These results suggest that the in-vitro selection procedure may represent a good approach for the generation of highly effective EGSs for gene-targeting applications. The remaining 3% of the target mRNA in C468-A-expressing cells is possibly due to the expression of this little fraction of the mRNA in specific cellular compartments, which make it less accessible to be degraded by RNase P. Moreover, proper folding of the mRNA-EGS complexes in cells may also affect the efficacy of the EGS approach. Further studies will be needed to address these issues.

Enhanced stability of target mRNA-EGS complex seemed to be one of the main causes for the improved activity of EGS in guiding RNase P to degrade target mRNA. C468-A, which was 60 times more active in guiding RNase P to cleave icp38 sequence, showed 80 times greater affinity with icp38 sequence than SER-A (Table 1). It has been shown that the interaction between the variable and D-loops of pre-tRNA plays an important role in the constitution of the pre-tRNA tertiary structure and in the induction of RNase P-mediated cleavage [3, 25]. In the icp38-C468-A complex, the 3’ region of icp38 is equivalent to the D-loop in a pre-tRNA (Fig. 1A,B,E). The difference between C468-A and SER-A is at their variable loop and T loop sequences. It is conceivable that the additional interactions between 3’ region of icp38 and the variable loop region of C468-A stabilize the mRNA-EGS complex and promote the targeting activity of the EGS. Thus, our study implies that in-vitro selection is an effective approach for screening for highly active EGSs molecules.

In other studies, in-vitro selection procedures have been widely used to generate functional RNA molecules such as ribozymes, aptamers, and EGSs which target mRNA encoding chloramphenicol acetyltransferase (CAT) [18, 26, 27]. In our studies, variant C468, which was selected to target a TK mRNA sequence23, was used to con- struct EGS C468-TK and C468-A that target two different mRNA sequences (i.e. TK and ICP4 mRNA). Our previous [23] and current studies showed that C468-TK and C468-A are highly efficient in inducing RNase P-mediated cleavage of the TK and ICP4 mRNA sequences in-vitro, respectively. It is conceivable that C468 could form unique tertiary interactions with both the targeted TK and ICP4 mRNA sequence in order to achieve efficient targeting activity. These results suggest that an EGS optimized for one target mRNA may exhibit efficient activity in targeting other mRNAs, possible by engineering EGS sequences to form novel and unique tertiary interactions with the targeted mRNA sequences.

Further studies of EGS-based RNA interfering technology should improve the efficiency of EGS-mediated cleavage of target RNA by RNase P. These studies would facilitate the technology as a promising gene targeting approach for use in research and clinical therapeutic applications.

Induced cleavage of the ICP4 mRNA seems to be the main reason for the antiviral effect of EGSs. First, expression of the EGSs was not toxic to cells as cells expressing EGSs show no difference with parental cells expressing no EGSs in terms of cell growth and viability for up to one month (data not shown). Second, the antiviral effect (inhibition of viral growth and viral gene expression) associated with the expression of C468-A and SER-A appears to be caused by a reduction in the expression of ICP4. We observed overall reduction of viral early and late gene expression (e.g. TK, gC, ICP35, and gB) in cells which expressed C468-A and SER-A but not in those which expressed control C468-I or SER-I (Figs 4 and 5, Tables 2 and 3). The extent of inhibition of viral early and late gene expression and viral growth correlates with the extent of inhibition of the expression of ICP4 mRNA and protein. Third, designed ICP4-targeting EGSs specifically inhibit the expression level of ICP4 but not those of other immediate-early genes of HSV-1 like ICP27 and ICP47. No reduction was observed in the expression levels of ICP27 and ICP47 in the HSV infected cells expressing the EGSs (Figs 4 and 5, Tables 2 and 3). These results are consistent with the previous observations that ICP4 protein is required for overall expression of viral early and late genes and does not play a significant role in regulating the expression of viral IE genes [1]. Thus, induced cleavage of the ICP4 mRNA seems to be the main reason for the antiviral effect of EGSs.

In our previously studies, ribozymes derived from the catalytic RNA subunit of RNase P from E.coli were used to target TK and ICP4 mRNAs and were found to inhibit the expression of TK and ICP4 genes in cultured human cells [28, 29]. Furthermore, the expression of anti-ICP4 mRNA ribozymes effectively blocked HSV-1 replication and infection in cultured cells [28]. In our current study, EGS RNAs, which are short RNA molecules, were constructed to bind to an ICP4 mRNA sequence and recruit endogenous RNase P to cleave the target mRNA. Thus, the EGS-based technology, which induces endogenous RNase P for cleavage, is unique and different from the ribozyme-based approach, which introduces an exogenous ribonuclease (i.e. RNase P-based ribozyme) for cleavage. Our results presented in this study provide direct evidence that EGSs are effective in blocking HSV-1 replication and infection in cultured human cells. The levels of EGS-mediated anti-HSV-1 efficacy are comparable to those observed using RNase P-based ribozymes [28, 29]. These results further suggest that EGS may represent a novel class of antiviral gene-targeting agents.

Little is currently known about the stability and half-life of the EGSs in cells. It is conceivable that an EGS may be stable due to the possible association of the tRNA-like domains of the EGS with intracellular tRNA-binding proteins. Consistent with our previous studies detecting ICP47 mRNA [28, 29], two bands representing the RNase-protected products of the RNA probes hybridizing the ICP4 mRNA were detected in our RNase protection experiments (Fig. 4). It is possible that the RNase-protected products exhibited significant secondary and tertiary structures, which may lead to two different conformations that migrate differently in polyacrylamide gels even under denaturing conditions in the presence of urea. Further biochemical studies should be able to clarify this issue and reveal the nature of these two bands.

HSV-1 belongs to the human herpesvirus family that includes several other human viruses such as herpes simplex virus 2, varicella zoster virus, Epstein-Barr virus, cytomegalovirus, and

Kaposi's sarcoma-associated herpesvirus [1]. Like other herpesviruses, HSV-1 can engage in both lytic replication and latent infections. The ICP4 protein is one of the viral α or immediate early (IE) genes and a major transcription activator necessary for the expression of HSV early (β) and late (γ) genes [1]. Further study of EGS for anti-HSV application may focus on delivering EGSs into neuronal cells, where latent infection is established, and may determine if the delivered EGSs can prevent HSV-1 from reactivating from latent to lytic infection [1]. These studies will further promote the development of engineered EGSs for both basic research and clinical therapeutic applications.

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# 5 Oral Delivery of a Novel Attenuated Salmonella Vaccine Expressing Influenza A Virus Proteins Protects Mice against H5N1 and H1N1 Viral Infection.

## 5.1 Introduction

Influenza infection, which is caused by seasonal and pandemic influenza viruses, can potentially lead to large numbers of deaths in humans, animals, and domestic birds as well as significant financial losses [1]. For example, the World Organization for Animal Health highlighted the outbreaks of highly pathogenic avian influenza (HPAI) H5N1 virus, which is associated with a growing number of human zoonotic infections and has been met with intense public health interest [2, 3]. In 2009, a novel swine-origin H1N1 influenza A virus, which was initially identified in Mexico and spread globally, has continued to circulate in humans and may have the potential to develop into the first influenza pandemic of the twenty-first century [4–6]. To control and prevent potential outbreaks of influenza viruses (e.g. the H5N1 avian influenza virus and the 2009 swine-origin H1N1 virus) in the future, effective vaccines against influenza viruses are urgently needed.

Influenza viruses are members of the Orthomyxoviridae family, which have enveloped, segmented, single-stranded negative sense RNA genome [1]. The hemagglutinin (HA) is the most abundant viral membrane protein in the envelope and is responsible for both binding and fusion with host cells. The neuraminidase (NA), another viral membrane protein found in the virion, is pivotal in the release and spread of progeny virions, following the intracellular viral replication process [1]. It has been previously reported that influenza virus infections can be primarily and effectively controlled by vaccines that elicit both humoral and cellular immune responses against the viral surface proteins HA and NA [7–9]. Vaccines being used or explored against influenza viruses include conventional inactivated whole viral antigen vaccines, live attenuated reasserted virus vaccines, recombinant protein vaccines, virus-like particle (VLP) vaccines, and DNA vaccines [8, 10, 11]. DNA vaccine, as a novel vaccine candidate, has been shown to induce effective antibody response and long-term cell-mediated immunity in animal models [12–15]. Furthermore, DNA vaccines, when delivered orally, can induce systemic and mucosal immune as well as cellular immune responses to antigens as compared to vaccines delivered via the parenteral routes [9, 16]. These results suggest that orally delivered DNA vaccines may represent promising novel vaccines against influenza virus.

Oral vaccines are cost effective and operate conveniently because they eliminate the use of syringes and needles and thus are an affordable choice for mass vaccination. Attenuated Salmonella strains have successfully been used as an oral carrier system for delivery of nucleic acid-based vaccines [16, 17]. In previous studies, attenuated *Salmonella* were constructed and transformed with plasmid constructs containing transgenes under the control of an expression promoter [18–20]. In human cells infected by *Salmonella*, plasmid DNA can be released and transported to the nuclei, leading to the expression of the transgene [18, 21, 22]. The genes of Salmonella pathogenicity island 2 (SPI-2), which encode virulence factors and are required for intracellular survival and replication [23–25], are believed to play important roles in gene transfer ability of *Salmonella* vector. Inactivation of these genes, including the components of the type III secretion system (T3SS) encoded by SPI-2, led to better lysis of the bacteria and more efficient gene transfer [7, 18, 21, 26].

*Salmonella* may serve as promising oral vaccine vectors in-vivo [19, 27]. Generation of new *Salmonella* strains with better gene transfer activity and further studies of these strains should facilitate the development of Salmonella as a vaccine vector against infectious pathogens including influenza viruses. In this study, we generated a novel attenuated Salmonella strain, SL368, with a deletion at the spiR gene, which is required for the expression of many SPI-2 genes [28]. Using SL368, we constructed a Salmonella-based vaccine expressing the HA and NA proteins of a human H5N1 influenza virus. We showed that SL368 exhibited efficient gene transfer activity and little virulence, leading to expression of viral antigens in cultured cells and in mice. Using BALB/c mice as the model, we studied the constructed *Salmonella*-based vaccine in comparison to the current commercial H5N1 and H1N1 influenza vaccines. Our study provides direct evidence that the SL368-based vaccine induces the production of anti-HA serum IgG and mucosal IgA, and of anti-HA interferon-γ producing T cells. Furthermore, mice orally vaccinated with the Salmonella vaccine were completely protected from lethal challenge of both highly pathogenic H5N1 and H1N1 influenza viruses. These results suggest that the Salmonella-based vaccine elicits strong antigen-specific humoral and cellular immune responses and provides effective immune protection against multiple strains of influenza viruses. Furthermore, our study demonstrates the feasibility of developing novel attenuated Salmonella strains as new oral vaccine vectors against influenza viruses.

## 5.2 Methods

### 5.2.1 Construction of Recombinant DNA Plasmids

Coding sequences for the HA and NA gene of the avian influenza virus A/Viet Nam/1194/2004 (H5N1) were contained within constructs pMD-5HA and pMD-5NA, respectively [15]. The template for the H5N1 HA gene fragment was amplified from plasmid pMD-5HA by PCR using the forward primer 5HA (5'-GGCAAGCTTCTGTCAAAATGGAGAAAAT-3') and reverse primer 5HA (5'- CGCTCGACTTTAACTACAATCTGAACT-3'). PCR products were restriction digested and cloned into eukaryotic expression vector pVAX1 (Invitrogen, Carlsbad, CA, USA) to generate p5HA. Similarly, the H5N1 NA gene fragment was PCR-amplified from construct pMD-5NA using the forward primer 5NA (5'-GTCAAGCTTACCATGAATCCAAATCAG-3') and reverse primer 5NA (5'-AGCTCGACCTACTTGTCAATGGTGAAT-3'), and then cloned into pVAX1 to produce p5NA.

### 5.2.2 Construction and Growth Analysis of Salmonella Strains

The clinical strain ST14028s of *Salmonella* typhimurium has been described previously [18, 33]. The SL368 strain of Salmonella was derived from the auxotrophic Salmonella typhimurium aroA strain SL7207 (a gift from Bruce A. D. Stocker, Stanford University, CA)[34] by deleting a part of the SpiR coding sequence with the alpha (λ) Red recombinase method [35], as previously described [33, 36]. Analysis of bacterial growth in LB broth was conducted following details previously outlined [33, 36].

### 5.2.3 Expression of Viral Antigens by Salmonella-Mediated Delivery in Cultured Cells

Plasmid constructs p5HA, p5NA, or pVAX1S were used to transform strain SL368 and generate Salmonella vector strains carrying constructs Sal-HA-NA, and Sal-vector, respectively. In gene transfer experiments, we infected HeLa cells and differentiated THP ($1x10^6$ cells) (pretreated with IFN- γ (150 U/mL) (R&D Systems Inc., Minneapolis, MN) for at least 12 hours) with Salmonella at a multiplicity of infection (MOI) of 10–20 bacteria/cell [18, 21, 33]. The infected cultures were then incubated at 37°C in 5% $CO_2$ for 1 hour. Cells were then washed and incubated with fresh medium containing 50 μg/ml of gentamicin, and harvested after 72 hours incubation[18, 21, 33].

Total RNAs were isolated from cells using Trizol (Invitrogen, San Diego, CA) and digested with DNase I for removal of genomic DNA, to assay the expression of the viral mRNAs. Northern blot and Western blot analyses were performed as described in detail previously [18, 21]. In Western blot analyses, proteins were stained using antibodies against influenza proteins and actin, then separated by SDS-PAGE gels and imaged with a STORM840 Phosphor-imager [18, 21].

### 5.2.4 Oral Immunization of Mice

BALB/C mice (specific-pathogen-free females) at 6 weeks old, were obtained from the Laboratory Animal Centre, Yangzhou University (China) and housed in a specific pathogen free facility. Mice were randomly divided into groups (5–10 mice per group) and were immunization on day 0, 14, and 28. In experiments with *Salmonella*-based vaccines, mice were first anesthetized with isoflurane and then intragastrically inoculated with 0.1–0.2 mL phosphate-buffered saline (PBS) containing no *Salmonella* or $1 \times 10^9$ cfu Sal-HA-NA or Sal-vector, using a gavage needle [18, 21, 37]. In experiments with the commercial H5N1 and H1N1 vaccines, mice were intra-muscularly inoculated with cv-H5N1 (Weike Inc, Harbin, China) and cv-H1N1 (Hualan Inc, Henan, China) respectively, following the manufacturers' recommendations. We have closely monitored the mice after the treatment. Even though no apparent side effects have been observed in mice vaccinated with the commercial influenza vaccines and SL368 carrying different plasmid constructs, we still took special precautions to ensure that ample food and water as well as sanitary cage conditions were present in order to maximize the animals' comfort.

### 5.2.5 Enzyme-linked Immunosorbent Assay (ELISA)

Samples were prepared from mice sera at indicated time points after immunization and before viral challenge. Blood was centrifuged at 13,000 rpm for 15 min, and the supernatant (serum) was removed and stored at -80°C. The of serum IgG and mucosal IgA-specific antibody levels were determined by ELISA with the mouse hemagglutinin HA IgG ELISA Kit and the mouse hemagglutinin HA IgA ELISA Kit (Xiangsheng Inc, Shanghai, China) respectively, following the manufacturers' recommendations [7, 15]. Briefly, samples of test serum or mucosal nasal wash were added to triplicate wells, then mixed with diluted horseradish-peroxidase (HRP)-labeled anti-mouse IgG or anti-mouse IgA antibody (50 μl/well) and incubated for 1 hour at 37°C. Following several washes, the plates were incubated for 15 min with 100 μl visualization reagent A and B of the HA IgG and IgA ELISA kits (Xiangsheng Inc, Shanghai, China). The reactions were stopped with 50 μl stop buffer and the absorbance was measured at 450 nm using an ELISA plate reader.

### 5.2.6 Hemagglutination Inhibition (HI) Assay

HI assays were performed to detect the serum neutralization activity as detailed previously [38]. Briefly, blood samples were collected at 0, 16, 32, 42 days post-immunization, and specific receptor-destroying enzyme (RDE)-treated sera were serially diluted and then mixed with inactive influenza A/Puerto Rico/8/34(H1N1) or A/chicken/China/1204/ 04(H5N1). The highest serum dilution that showed 100% hemagglutination inhibition designates the HI titer.

### 5.2.7 IFN-γ ELISPOT Assay

T Cells in a single spleen cell suspension that expressed IFN-γ expressing T cells were counted using the mouse IFN-γ ELISPOT kit (U-Cytech biosciences, Utrecht, Netherlands) as described previously [15]. Briefly, isolates from mice splenocytes ($1\times10^6$) were either mock-stimulated or stimulated with HA533–541 epitope peptide (IYSTVASSL) (5 μg/ml) [32] for 24 hours at 37°C. Phytohemagglutinin (PHA, 4 μg/ml, Sigma–Aldrich, St. Louis, MO, USA) was used as a positive control. After incubation, the cells were removed and incubated with 100 μl/well (0.5 μg/ml) of biotinylated detector antibody (anti-mouse IFN- γ) and 50 μl/well of 2% gold particle-labeled anti-biotin antibody (GABA) at 37°C for 1 hour. Spot-forming cells (SFC) per million cells were analyzed with the aid of a computer-assisted video image analyzer.

### 5.2.8 Studies of Immunized Mice Challenged With Influenza Viruses

In studies of virus challenge in mice, anesthetization of five mice per group was carried out with isoflurane and then mice were infected intranasally with 50 μl PBS (25 μl per nostril) containing a 20M lethal dose of influenza virus strain A/Puerto Rico/8/34(H1N1) or A/Viet Nam/1194R (H5N1) two weeks after the final vaccination. Body Weight was monitored daily to for 16 days and non-lethal endpoints were used in the survival studies. Animals that lost more than 30% body weight or exhibited lethargy, ruffled hair coat, or hunched posture were deemed gravely ill and were euthanized by overdose inhalation of carbon dioxide.

In the histological analyses, lung tissues from euthanized mice were prepared and cut into 5 μm sections. Microscopy was used to examine Hematoxylin and eosin (H&E)-stained sections.

Lung tissues were prepared from infected mice at 3 days and 6 days post-inoculation for viral titer studies. Viral titers, expressed as $\log_{10}TCLD_{50}$/g lung tissue, were calculated employing the Reed-Muench method.

## 5.3 Results

### 5.3.1 Construction of Attenuated Salmonella Vaccine

We have previously shown that attenuated *Salmonell*a strains can be used as gene delivery vectors for expression of ribozymes in both human cells and in mice [18, 21, 39]. In this study, we constructed an attenuated *Salmonella* strain, SL368, as the gene delivery vector for the generation of the influenza vaccines. SL368 was derived from *Salmonella* typhimurium strain SL7207 [34] and in addition, contained a deletion of a part of the coding sequence of the SpiR protein. SL7207 is an attenuated strain with gene delivery activity [27, 34, 39, 40]. The SpiR protein is a part of the two-component transcription complex regulating the expression of the

majority of the SPI-2 encoded genes, which are important for *Salmonella* intracellular survival and virulence in-vivo [28, 41]. To generate *Salmonella* vaccine expressing influenza virus antigens, SL368 was electroporated with plasmid constructs p5HA and p5NA, which contained the DNA sequences coding for the HA and NA genes of a highly pathogenic influenza A H5N1 virus strain that were under control of a eukaryotic expression promoter, respectively.

Four vaccines were included in our study. Vaccine Sal-HA-NA contained strain SL368 carrying plasmid constructs p5HA and p5NA. Vaccine Sal-vector contained SL368 carrying the empty vector construct pVAX1 without any influenza virus sequences and was used as a negative control. We also included the commercial H1N1 and H5N1 vaccines, cv-H1N1 and cv-H5N1, as the positive controls in our study.

### 5.3.2 Gene Delivery and Expression of Influenza Antigens in Cultured Cells and in Mice Mediated by Attenuated Salmonella

In growth analysis experiments, *Salmonella* carrying constructs containing expression cassettes for influenza virus genes grew in LB broth as well as bacteria carrying no constructs or carrying the pVAX1 empty vector (Fig 1). These results indicate that the presence of the influenza virus sequence does not result in an impaired viability of the bacterial carrier.

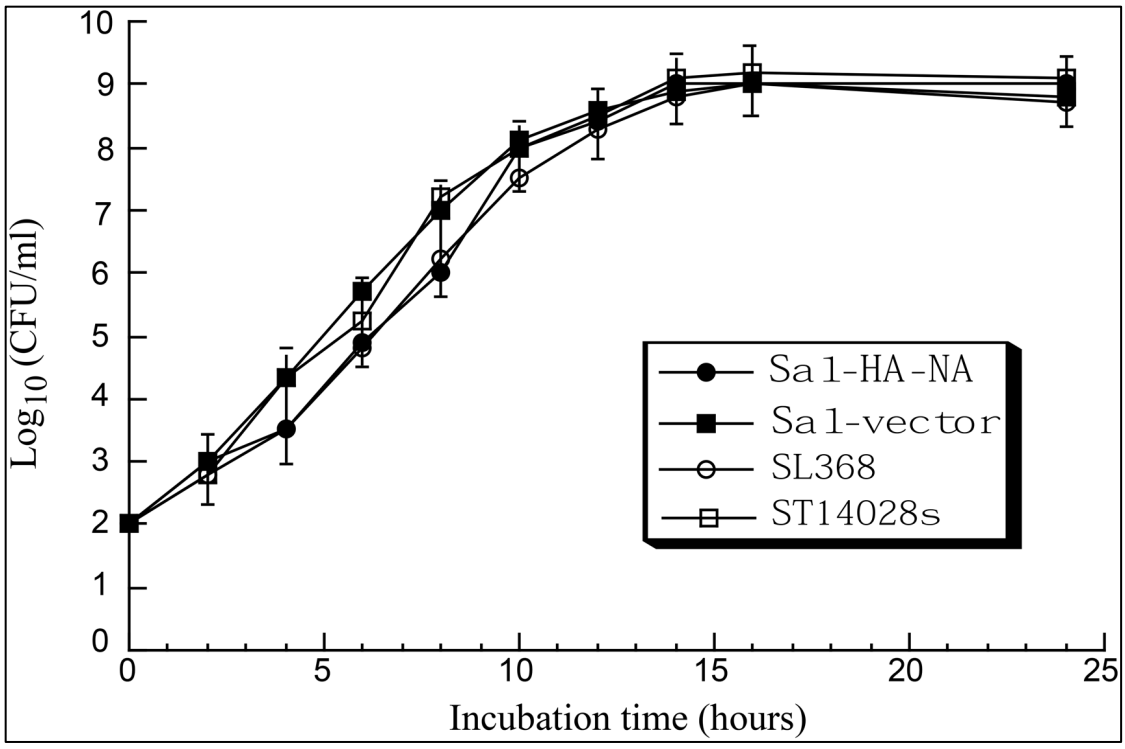


**Fig 1. Analysis of growth in LB broth of *Salmonell*a**. *Salmonella* used in the study include the wild type strain ST14028s, mutant strain SL368, and its derivatives that carry the empty vector construct pVAX1 (Sal-vector), and constructs p5HA and p5NA (Sal-HA-NA). Experimental details can be found in Materials and Methods.

In Northern blot analyses, none of the influenza virus RNA transcripts was detected in *Salmonella* carrying constructs with viral sequences (data not shown). Furthermore, Western blot analyses using anti-influenza antibodies showed that neither influenza HA nor NA protein was detected in Salmonella grown in LB (data not shown). These results suggested that the viral proteins, which were under the control of a eukaryotic expression cassette, were not expressed when *Salmonella* grew in culture media outside of human cells.

To determine whether Salmonella can efficiently deliver the influenza virus sequences into human cells, HeLa cells and differentiated macrophage THP-1 cells were infected with *Salmonella* SL368 carrying p5HA, p5NA, and the pVAX1 empty vector, and harvested at 72 hours post-infection. The expression of influenza HA and NA proteins was assayed with antibodies specifically against influenza virus proteins, using actin as the loading control (Fig 2). Western bolt analyses showed that *Salmonella* Sal-vector, in which SL368 carried the empty vector pVAX1, did not express any influenza antigen (Fig 2, lanes 5 and 8). The HA (~64 kD) and NA (~50 kD) proteins were detected in cells infected with *Salmonella* Sal-HA-NA, in which SL368 carried constructs p5HA and p5NA (lanes 6 and 9). These results indicate that SL368 efficiently carried

out gene transfer for delivery of constructs containing DNAs coding for viral antigens and the HA and NA antigens were efficiently expressed following gene transfer of SL368 in human cells.

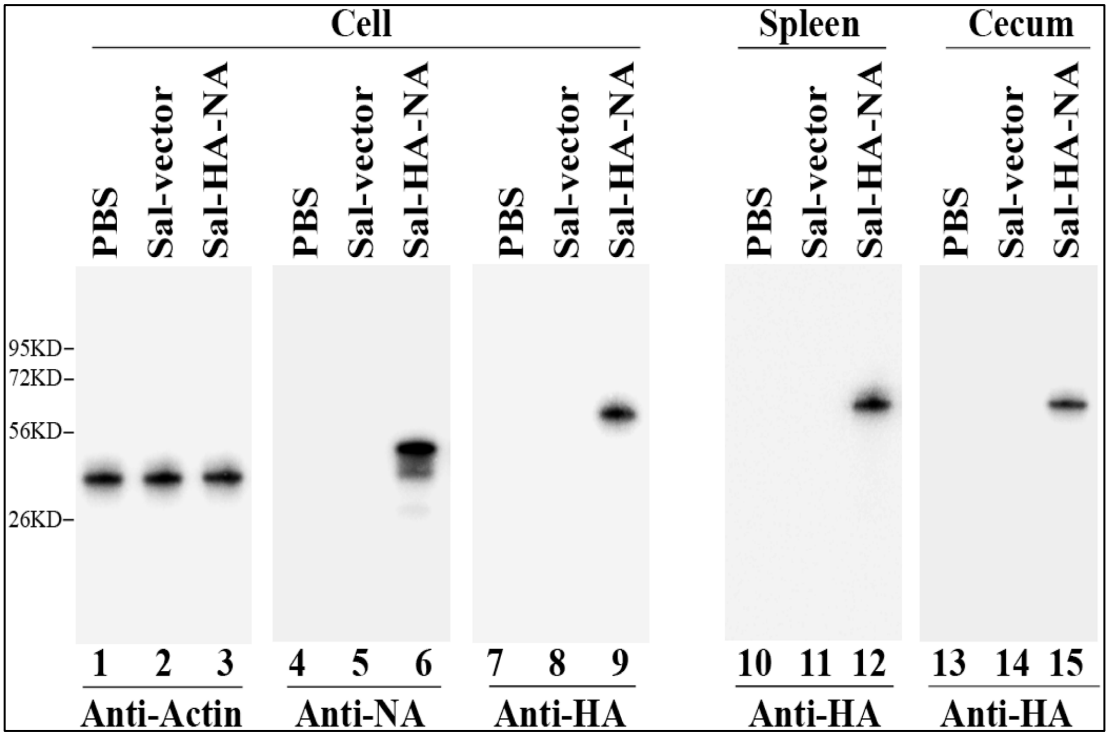


**Fig 2. *Salmonella*-mediated delivery for influenza antigen expression in cells and in mice**. Western blot analyses were performed to study the expression of viral HA and NA proteins in differentiated THP-1 cells (lanes 1–9) and viral HA protein in BALB/C mice (lanes 10–15) treated with phosphate- buffered saline (PBS) only (PBS, lanes 1, 4, 7, 10, and 13), SL368 carrying the empty vector construct pVAX1 (Sal-vector, lane 2, 5, 8, 11, and 14) and constructs p5HA and p5NA (Sal-HA-NA, lanes 3, 6, 9, 12, and 15). Cells treated with PBS or *Salmonella* were harvested at 72 hours post treatment while spleens and cecums were harvested from BALB/C mice that were intragastrically inoculated with PBS or SL368 carrying different constructs at 4 days after inoculation. Protein samples (40 μg) were separated on denaturing gels, transferred to membranes, and stained with antibodies against viral HA (lanes 7–15) and NA (lanes 4–6) or actin (lanes 1–3), which serves as the loading control. Molecular markers are shown on the left.

To study Salmonella-mediated gene delivery for the expression of influenza virus antigens in-vivo, we intragastrically inoculated BALB/c mice with Salmonella Sal-HA-NA and Sal-vector.

Gene delivery mediated by Sal-HA-NA was efficient in-vivo as HA and NA proteins were detected in the intestines and spleens of the Salmonella-treated mice (Fig 2, lanes 10–15, data not shown). Furthermore, SL368 carrying no construct, the viral antigen constructs, and the empty vector pVAX1 exhibited much less virulence in-vivo than the wild-type strain ST14028s. All mice infected with SL368, Sal-HA-NA, and Sal-vector ($1 \times 10^9$ cfu/mouse) remained alive even at 60 days post-inoculation (Fig 3). In contrast, mice inoculated with a much lower dose of ST14028s ($1 \times 10^3$ cfu/mouse) died within 7 days (Fig 3). Thus, the constructed SL368-based vaccine appeared to be efficient in gene transfer and exhibited little virulence and pathogenicity in-vivo.

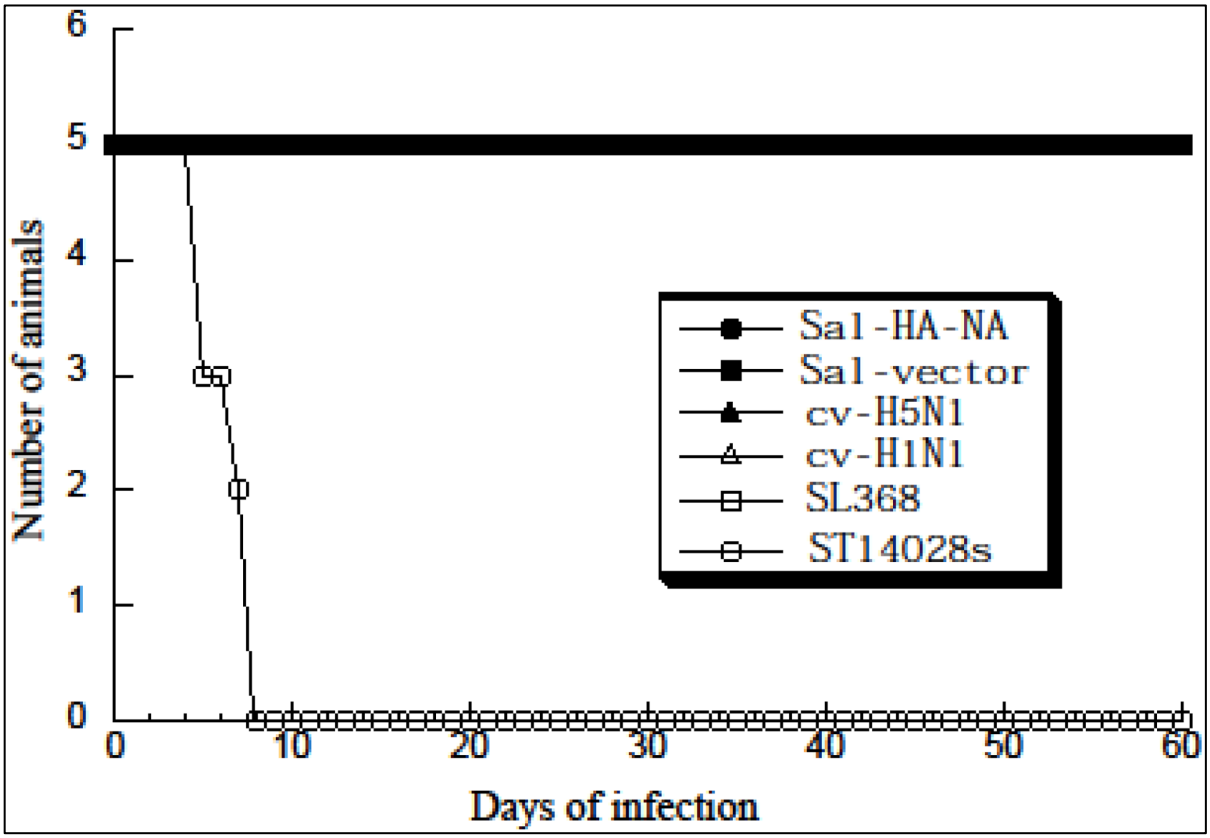


**Fig 3. Toxicity and virulence of different *Salmonella* strains in BALB/c mice**. Mice (5 animals per group) were either inoculated intramuscularly with the commercial H5N1 (cv-H5N1) andH1N1 vaccines (cv-H1N1) or intragastrically with PBS, the wild-type strain ST14028 ($1\times10^3$CFU), and vaccine strain SL368 ($1 \times 10^9$ CFU) carrying the empty vector construct pVAX1 (Sal-vector) and constructs p5HA and p5NA (Sal-HA-NA). The mortality of the animals was monitored for 60 days. Humane (non-lethal) endpoints were used during the experiments and mice were euthanized with overdose inhalational $CO_2$ to limit suffering, when they exhibited weight loss of more than 30%, lethargy, ruffled hair coat, or hunched posture.

### 5.3.3 Humoral Responses Induced by the Vaccines

BALB/c mice were randomly divided into groups and vaccinated intragastrically with the constructed Salmonella Sal-HA-NA and Sal-vector at day 0, 14, and 28. Animals were also treated similarly with phosphate-buffered saline (PBS) in the absence of Salmonella and used as the negative controls. To compare the effectiveness of our generated Salmonella-based vaccines, vaccines cv-H5N1 and cv-H1N1, which were currently commercially available for vaccination in China against H5N1 and H1N1 viral infections respectively, were used as positive controls and injected intramuscularly into mice at day 0, 14, and 28. All animals remained alive at day 42 post immunization (i.e. two weeks after the final immunization) (data not shown), consistent with our observations (Fig 3) that SL368 exhibited little virulence/pathogenicity in mice in-vivo.

To study the effects of *Salmonella* vaccines on humoral responses in BALB/c mice, ELISA was carried out to assay the levels of serum antibodies at 0, 16, 32, 42 day after immunization (Fig 4) [42]. At day 42, mice immunized with vaccine Sal-HA-NA, in which SL368 carried constructs p5HA and p5NA, displayed higher ($p < 0.01$) anti-HA serum IgG levels compared to those immunized with the control vaccine Sal-vector, which carried the empty vector pVAX1 (Fig 4A). Indeed, Sal-HA-NA induced similar anti-HA serum IgG levels as the H5N1 commercial vaccine cv-H5N1 (Fig 4A). The functional activities of the sera from the vaccinated mice were further investigated by determining the antibody titers for hemagglutination inhibition (HI) against influenza virus. Consistent with our results of the serum anti-HA IgG response induced by the vaccines (Fig 4A), mice immunized with Sal-HA-NA also displayed comparable HI antibody titers as those immunized with the commercial H5N1 vaccine, cv-H5N1, and exhibited higher HI antibody titers ($p < 0.01$) than those with control Sal-vector (Fig 4B).

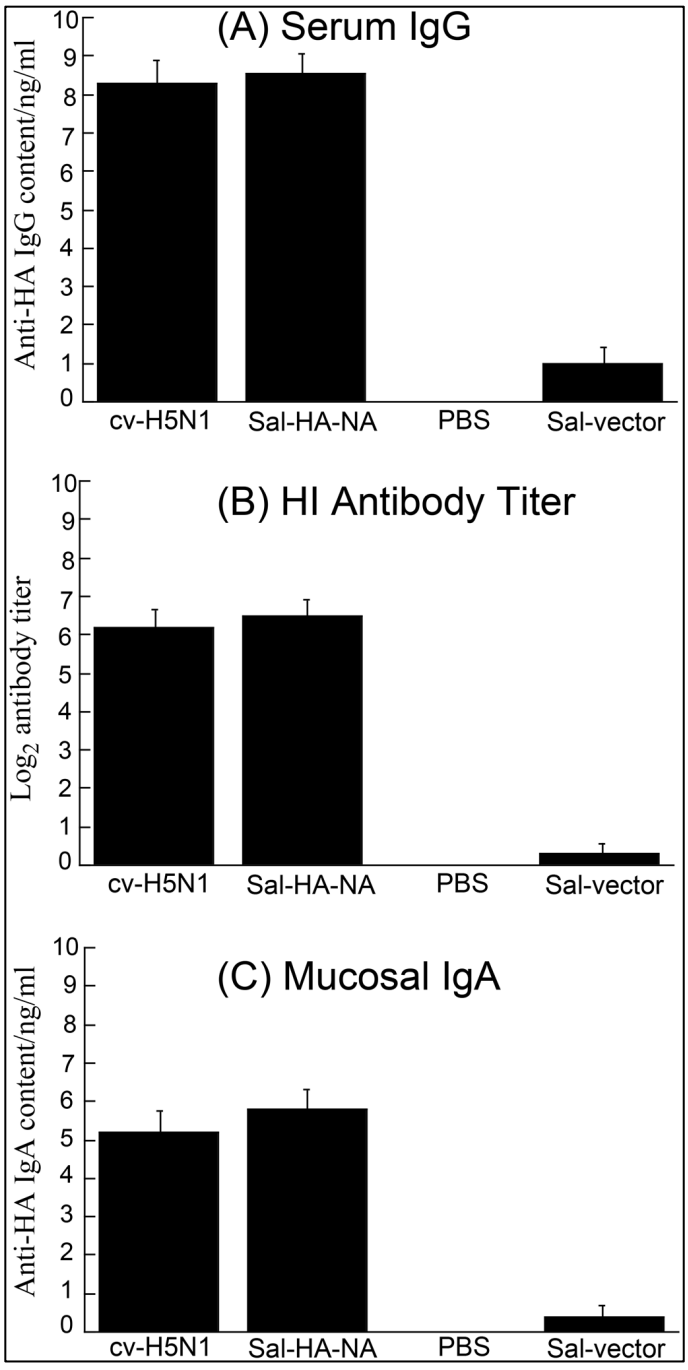


**Fig 4. Antibody titers of anti-HA serum IgG (A), serum hemagglutination inhibition (HI)(B), and anti-HA mucosal IgA (C) detected by ELISA in immunized mice at 42 days after immunization**. Mice were immunized intragastrically at day 0, 14, and 28 with PBS only, the commercial H5N1 vaccine (cv-H5N1), and Salmonella SL368 carrying the empty vector pVAX1 (Sal-vector) and constructs p5HA and p5NA (Sal- HA-NA). Pool serum or mucosal wash samples (n = 5) from mice within a group were assayed and analyzed by two-way ANOVA. *$p < 0.01$. The assays were performed in triplicate and the experiments were repeated three times. The values obtained were the average from these experiments.

To study the effects of Salmonella vaccines on mucosal antibody responses in BALB/c mice, ELISA was also used to assay the anti-HA IgA levels in the mucosal nasal wash at 0, 16, 32, 42 days after immunization [43]. Mice immunized with vaccine Sal-HA-NA displayed higher anti-HA IgA levels ($p < 0.01$) compared to those immunized with control Sal-vector (Fig 4C). Indeed, vaccine Sal-HA-NA induced similar levels of mucosal anti-HA IgA as the H5N1 commercial vaccine cv-H5N1 (Fig 4C). These results suggest that the SL368-based vaccine enhances antigen-specific mucosal IgA as well as serum IgG responses.

### 5.3.4 Antigen Specific T cell Responses Induced by the Vaccines

To study the HA-specific T cell responses induced by attenuated Salmonella vaccines, splenocytes ($10^6$ cells) were isolated from vaccinated mice 42 days after oral administration and stimulated with HA533–541 epitope peptide (IYSTVASSL). This peptide, which is common to most of influenza A virus strains, is considered to be an immunodominant MHC class I epitope (Kd restricted) and can induce $CD8^+$ T-cell responses in BALB/C mice [32]. Salmonella Sal- HA-NA induced higher ($p < 0.01$) anti-HA IFN-γ producing T cell responses than control Sal- vector (Fig 5). Indeed, the level of IFN-γ producing T cell responses induced by Sal-HA-NA was comparable to that by the commercial H5N1 vaccine cv-H5N1 (Fig 5). These results suggest that SL368-based vaccines induce antigen-specific T cell responses.

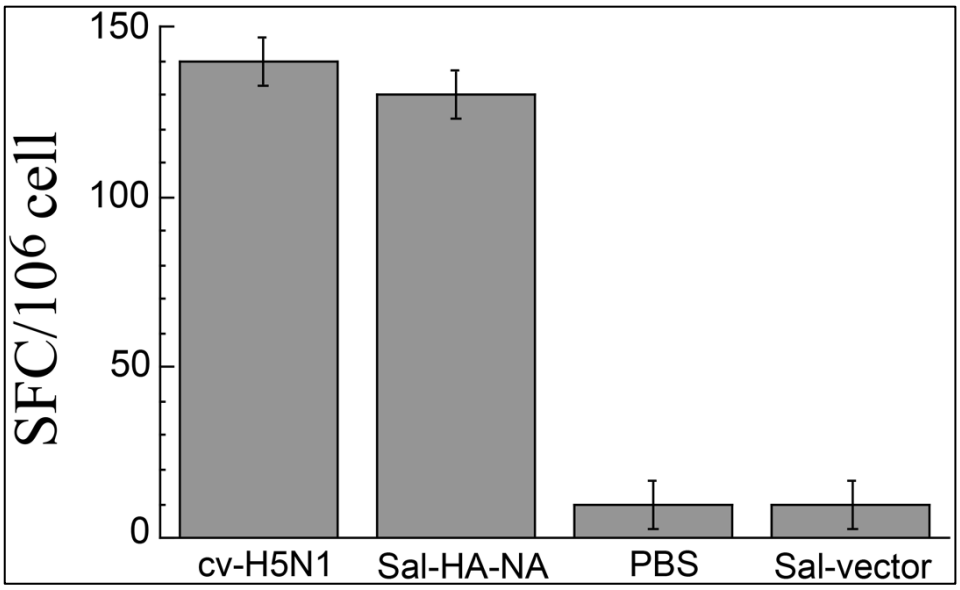


**Fig 5. ELISPOT analysis of IFN-γ production by HA533–541 specific T cells in vaccinated mice at 42 days after immunization**. Mice were immunized intragastrically at day 0, 14, and 28 with PBS, the commercial H5N1 vaccine (cv-H5N1), and Salmonella SL368 carrying the empty vector pVAX1 (Sal-vector) and constructs p5HA and p5NA (Sal-HA-NA). Splenocytes (n = 5) were harvested from immunized mice at 42 days post immunization and stimulated with HA533–541 peptide for 48 hours. The results were expressed as spot-forming cells (SFC) per million cells. The assays were performed in triplicate and the experiments were repeated three times. The values obtained were the average from these experiments. Statistical analysis was performed by two-way ANOVA. *$p < 0.01$.

### 5.3.5 Immune Protection of Mice from Virus Challenge Induced by the Vaccines

Our observations of strong virus specific humoral and cellular responses suggest that vaccine Sal-HA-NA may provide immune protection to challenge of the H5N1 virus and, possibly, H1N1 virus since the NA proteins of both viruses are similar. To determine if the constructed Salmonella vaccines can induce H5N1 virus-specific and H1N1 virus-specific immune protection, mice were immunized with different vaccines at day 0, 14, and 28, and then isoflurane-anesthetized and intranasally challenged with lethal doses of highly pathogenic H1N1 and H5N1 viruses at day 42 (i.e. two weeks after the final immunization). Animals were closely monitored for weight loss and mortality for 16 days post challenge (Fig 6A–6D). Humane (non-lethal) endpoints were used during the survival experiments and mice were euthanized with overdose inhalational $CO_2$ to limit suffering, when they exhibited weight loss of more than 30%, lethargy, ruffled hair coat, or hunched posture. Our results showed that mice vaccinated with the H5N1 commercial vaccine cv-H5N1 and vaccine Sal-HA-NA exhibited no loss of body weight and showed 100% protection against A/Viet Nam/1194R (H5N1) virus challenge after 16 days post challenge

(Fig 6A and 6B). When challenged with A/Puerto Rico/8/34 (H1N1) virus, the mice vaccinated with the H1N1 commercial vaccine cv-H1N1 exhibited no body weight loss and showed 100% protection (Fig 6C and 6D). All mice immunized with Sal- HA-NA survived after challenge with A/Puerto Rico/8/34 (H1N1) virus and regained about 90% body weight by day 16 after challenge (Fig 6C and 6D).

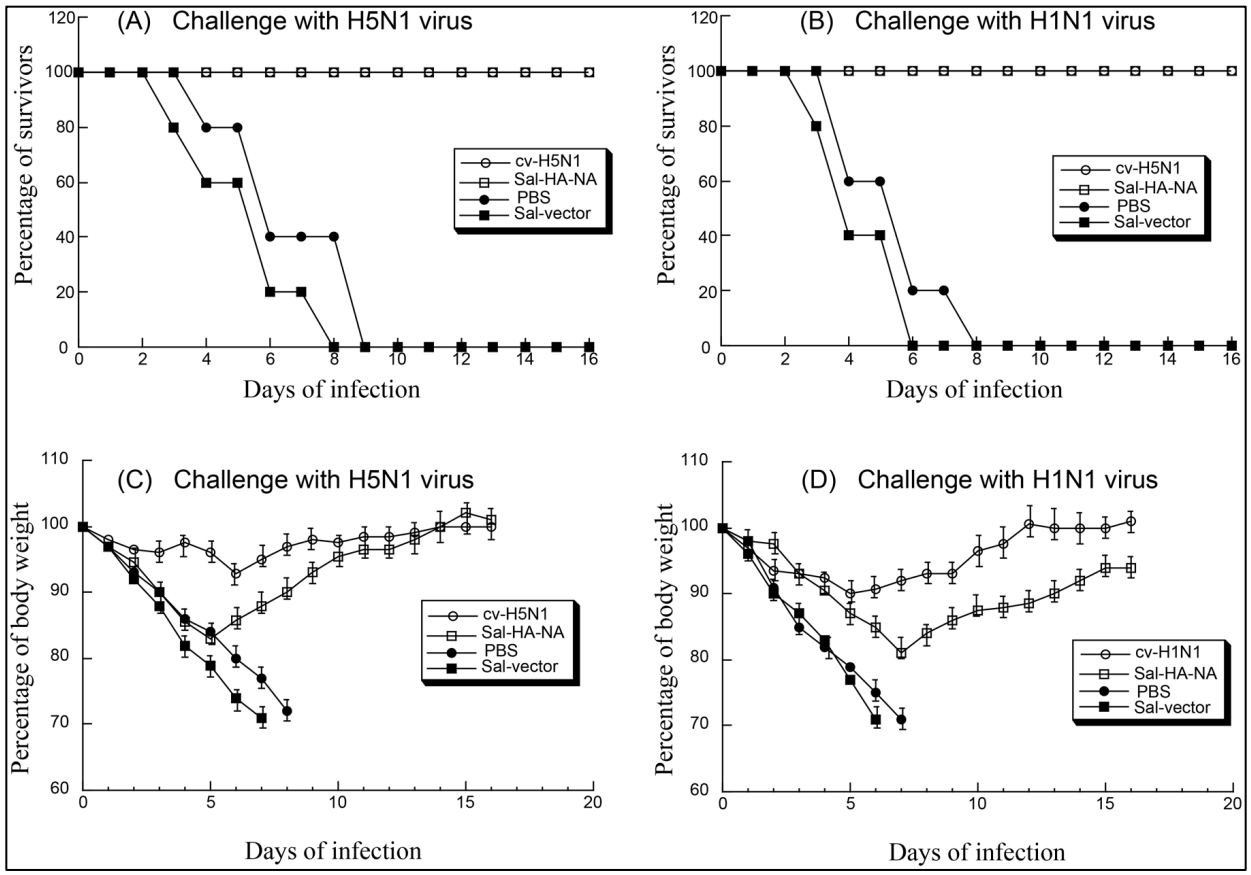


**Fig 6. Immune protection of mice from lethal H5N1 (A and C) and H1N1 viral challenge (B and D)**. Groups of mice (n = 5) were intragastrically immunized three times at day 0, 14, and 28 with PBS only, the commercial H5N1 (cv-H5N1) and H1N1 vaccines (cv-H1N1), and Salmonella SL368 carrying the empty vector pVAX1 (Sal-vector) and constructs p5HA and p5NA (Sal-HA-NA), and then challenged intranasally with 20 $LD_{50}$ of lethal H5N1 (A/Viet Nam/1194R) and H1N1 (A/Puerto Rico/8/34) viruses at two weeks after final immunization (i.e. at 42 days post initial immunization). Mice were monitored for survival (A and B) and weight loss (C and D) throughout a 16 day observation period. The results are presented in terms of percent survival and percent of body weight (at the beginning of the trial) respectively. Humane (non-lethal) endpoints were used during the survival experiments and mice were euthanized with overdose inhalational $CO_2$ when they exhibited weight loss of more than 30%, lethargy, ruffled hair coat, or hunched posture.

Mice administered with control Sal-vector or PBS exhibited significant body weight loss and exhibited no protection against either H5N1 or H1N1 virus challenge (Fig 6). These results suggest that SL368-based vaccine provides immune protection against both H5N1 and H1N1 influenza viruses.

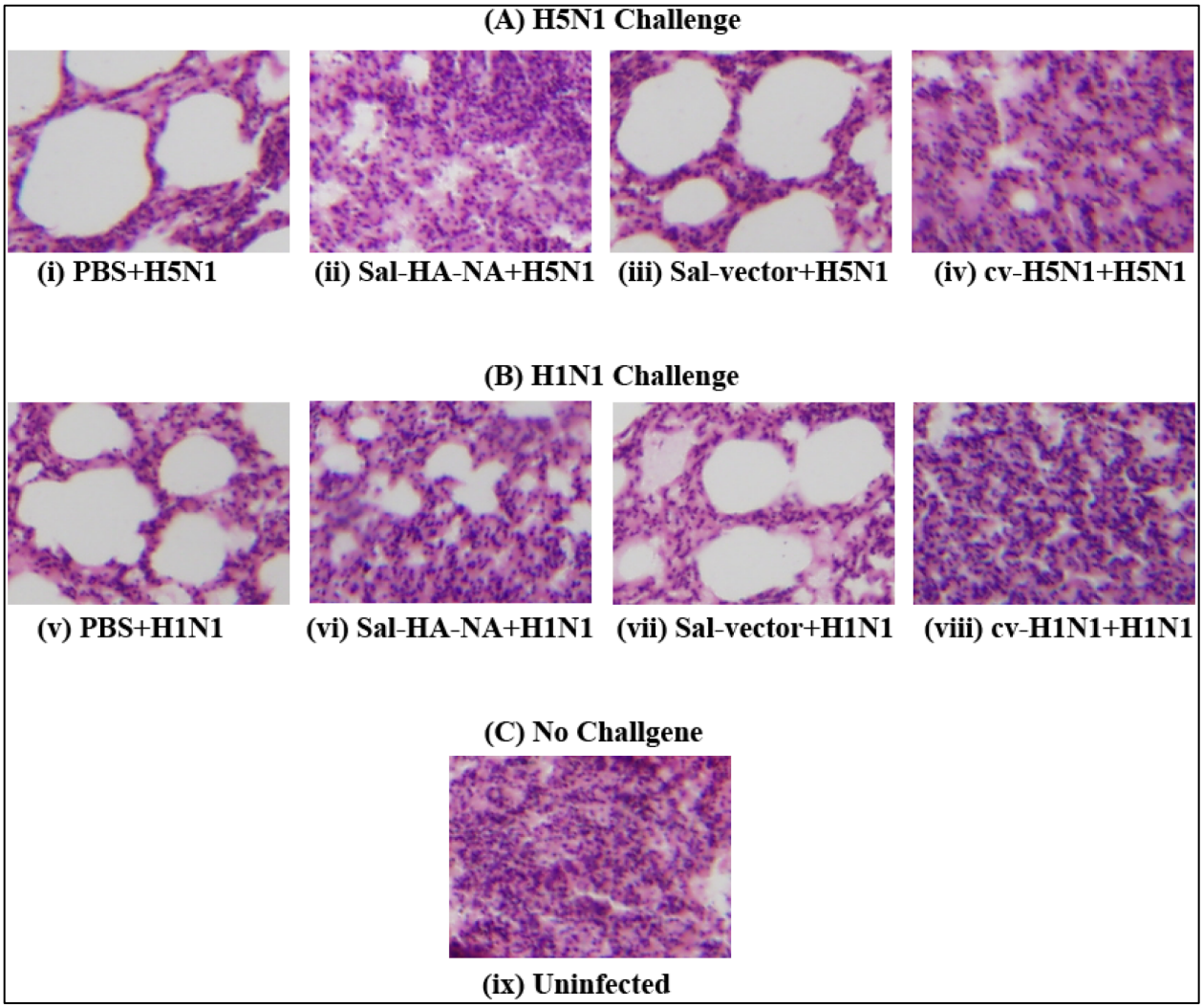


**Fig 7. Photomicrographs of hematoxylin- and eosin-stained lung sections of mice at 7 days post-challenge.** Mice were intragastrically immunized three times at day 0, 14, and 28 with PBS (i and v), the commercial H5N1 (cv-H5N1) (iv) and H1N1 vaccines (cv-H1N1) (viii), and Salmonella SL368 carrying the empty vector pVAX1 (Sal-vector) (iii and vii) and constructs p5HA and p5NA (Sal-HA-NA) (ii and vi), and then challenged intranasally with 20 LD50 of lethal H5N1 (A/Viet Nam/1194R) and H1N1 (A/Puerto Rico/8/34) viruses at two weeks after final immunization. (A) mice challenged with H5N1 virus; (B) mice challenged with H1N1 virus; (C) uninfected mice with no virus challenge.

To further study the effect of the Salmonella vaccines on virus-specific immune protection, histological studies were performed with the mice vaccinated and challenged with A/Viet Nam/ 1194R (H5N1) and A/Puerto Rico/8/34 (H1N1) (Fig 7). At 7 days post-challenge, the lung of the animals were harvested immediately following euthanasia, fixed with 10% formaldehyde, embedded in paraffin and cut into sections. Sections were stained with hematoxylin and eosin, and then examined for histopathologic changes. As expected, no lesion was found in the lungs of uninfected mice (Fig 7C). Mice treated with PBS or control Sal-vector had pulmonary lesions consisting of substantial necrotizing bronchitis and histocytic alveolitis with associated pulmonary edema (Fig 7A(i, iii) and 7B(v, vii)). Mice vaccinated with vaccine Sal-HA-NA, and the H5N1 and H1N1 commercial vaccines (cv-H5N1 and cv-H1N1) lacked lesions in the lung and exhibited minimal bronchitis (Fig 7A(ii, iv) and 7B(vi, viii)). To investigate if immunization with Sal-HA-NA can inhibit local replication of the challenge viruses, lung tissues were harvested from animals at 3 and 6 days post-challenge and the virus titers in these tissues were determined. The titers of H5N1 virus in animals vaccinated with Sal-HA-NA and cv-H5N1 at 6 days post challenge were at least 10,000-fold lower than those in animals administered with PBS or Sal-vector (Fig 8A). Similarly, the titers of H1N1 virus in animals vaccinated with Sal-HA-NA and cv-H1N1 at 6 days post challenge were at least 2,000-fold lower than those in animals administered with PBS or Sal-vector (Fig 8B). These observations are consistent with our immune protection results (Fig 6) and suggest that the SL368-based vaccines can induce effective protection against highly pathogenic H5N1 and H1N1 influenza virus challenge.

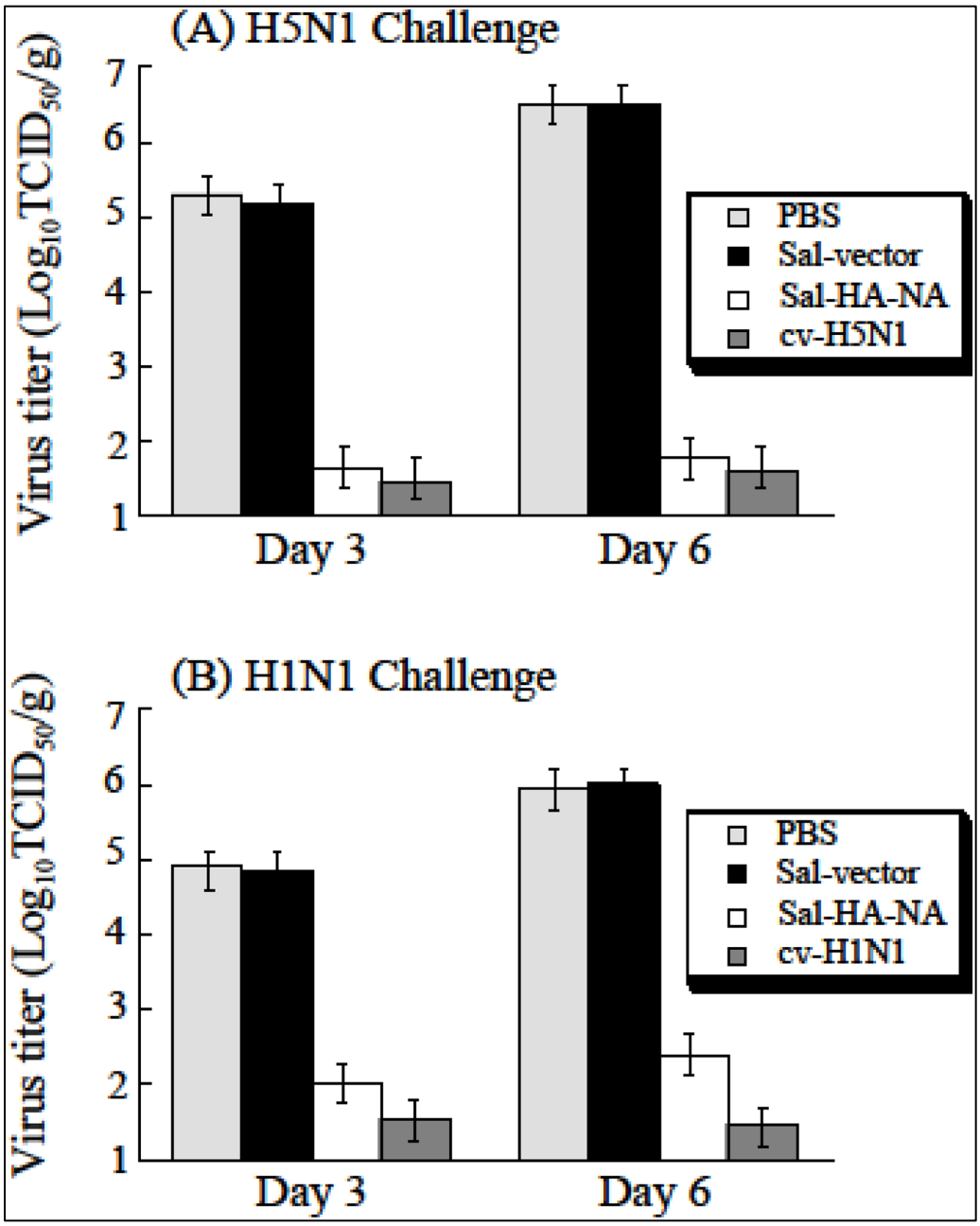


**Fig 8. Virus titers in the lungs of mice challenged with H5N1 and H1N1 viruses.** Mice were intragastrically immunized three times at day 0, 14, and 28 with PBS, the commercial H5N1 (cv-H5N1) and H1N1 vaccines (cv-H1N1), and Salmonella SL368 carrying the empty vector pVAX1 (Sal-vector) and constructs p5HA and p5NA (Sal-HA-NA), and then challenged intranasally with 20 LD50 of lethal H5N1 (A/Viet Nam/1194R) and H1N1 (A/Puerto Rico/8/34) viruses at two weeks after final immunization. Lung tissues were harvested at 3 and 6 days post challenge and viral titers were determined. The experiments were performed in triplicate and were repeated three times, and the standard deviation is indicated by the error bar. The limit of detection was 1.0 $\log_{10}TCID_{50}/g$.

## 5.4 Discussion

Influenza A viruses continue to pose a public health threat to humans and animals [1]. Consequently, there is an urgent need for effective vaccines against these emerging infectious pathogens [44]. Intranasal influenza virus vaccines, while effective, may not be suitable for persons with asthma and certain chronic airway or pulmonary diseases [45]. Oral vaccines may represent an alternative to be used against influenza virus infection. In addition, oral vaccines are cost effective and operate conveniently because they eliminate the use of syringes and needles and thus are an affordable choice for mass vaccination. In this study, a novel attenuated S. typhimurium strain, SL368, was constructed and used as an oral vaccine vector for expressing HA and NA proteins of H5N1 influenza virus. Using BALB/c mice as the model system, we showed that the Salmonella-based vaccine, Sal-HA-NA, elicited anti-HA- specific humoral and T cell responses and induced immune protection against H5N1 and H1N1 virus challenge in immunized mice. These results provide direct evidence to suggest that attenuated Salmonella (spiR-) strains expressing viral antigens represent promising oral vaccines against influenza virus infection.

Attenuated Salmonella represent unique and promising gene delivery tool for developing vaccine against infectious pathogens. First, as vaccines, the Salmonella-based vectors may be safe and can be administered orally. The currently-used antityphoid fever vaccine is derived from an attenuated Salmonella strain [46, 47]. Second, in the poultry industry, attenuated Salmonella vaccines might have unique potential for anti-influenza application, where inexpensive mass immunization can be achieved through drinking water or spray cabinet administration [48]. Finally, Salmonella-based vectors are highly immunogenic in inducing both innate and adaptive immune responses, due to the bacteria-encoded surface antigens such as lipopolysaccharide (LPS), and therefore can enhance the antigen-specific immune responses induced by the expression of the viral proteins [46, 49].

Attenuated Salmonella strains have been shown to function as oral vaccines against influenza virus infections by expressing different viral antigens [7, 26, 50]. For example, the recent seminal studies carried out by Curtiss and colleagues have shown the utility of attenuated Salmonella strains as oral vaccine candidates against influenza viruses [7, 26]. Anti-influenza vaccines were constructed based on an attenuated Salmonella mutant with the deletion of sifA gene [7]. The sifA gene encodes a secreted effector protein of the Type III secretion system (T3SS) of SPI-2 [51]. The SPI-2 proteins are important for Salmonella intracellular survival and virulence in-vivo [23–25]. Salmonella-based vaccines with mutations in the sifA gene have been shown to elicit both humoral and cellular immune responses and induce immune protection against influenza virus infection in mice [7, 26].

In our study, attenuated Salmonella strain SL368 was derived from auxotrophic strain SL7207 [34] and, in addition, contained a deletion of a part of spiR. SpiR is required for the expression of many Salmonella Pathogenicity Island-2 (SPI-2) genes including SifA [28]. Thus, SL368, with a deletion at spiR, is expected to exhibit minimal virulence (Fig 3) and be even less virulent than those mutants with the deletion of a single SPI-2 T3SS factor such as sifA. Moreover, SL368 exhibited excellent gene transfer activity as efficient expression of viral HA and NA proteins was found in cells and in mice treated with the SL368-derived vaccines (Fig 2). Furthermore, the SL368-derived vaccines elicited both HA-specific humoral and T cell immune responses and induced immune protection against influenza virus infection in mice (Figs 4–8).

These results suggest that attenuated Salmonella strains with the mutations at spiR represent novel vaccine vectors against influenza virus infection.

The exact mechanism of how Salmonella carry out gene transfer is not currently understood completely. In our delivery system, attenuated Salmonella were constructed and transformed with plasmid constructs containing transgenes (i.e. HA and NA) under the control of a eukaryotic expression promoter [18–20]. In cells (e.g. macrophages) infected by Salmonella, plasmid DNA can be released and transported to the nuclei, leading to the expression of the transgene [18, 21, 22]. As under the eukaryotic expression promoter, the antigens are only expressed and processed for antigen presentation in Salmonella-infected cells (e.g. macrophages and dendritic cells) and are not exposed to the environment of the digestive tract. Once inside the cells, the attenuated Salmonella will be lysed, and the plasmid DNA will be released and transported to the nuclei. The antigen expression cassette should be in a plasmid instead of the bacterial genome to facilitate its transport to the nuclei. Additional studies on the process and mechanism of Salmonella-mediated gene delivery would facilitate the generation of Salmonella-based vectors for vaccine development.

One of the ultimate goals for prevention of influenza outbreak is to develop a vaccine that can provide cross-protective immunity against many influenza virus strains. It is interesting to note in our results that vaccine Sal-HA-NA, which expressed the HA and NA proteins of an H5N1 virus, also provided strong protection against an H1N1 virus strain in addition to the H5N1 virus. Our results of the cross-protective immunity by Sal-HA-NA suggest that attenuated Salmonella vector simultaneously carrying expression cassettes for various antigens can function as a vaccine candidate against multiple strains of influenza viruses.

Little is currently known about how our vaccine elicited cross-protective immunity. It is possible that the HA protein expressed in Sal-HA-NA, which is from an H5N1 virus, may elicit cross-protective immune responses against the H1N1 virus, because of potential HA sequence homology between H5N1 and H1N1 viruses [52]. Furthermore, it is also conceivable that the cross-protective immunity against multiple influenza viruses induced by Sal-HA-NA may be due to the expression of the NA protein, which is also from an H5N1 virus and which may share sequence homology with the NA proteins from H1N1 viruses. Previous studies have implicated the roles of NA in cross-protective immunity against influenza viruses [53, 54]. A VLP vaccine expressing the N1 protein was found to elicit cross-protective responses against H5N1 and H1N1 viruses while a DNA vaccine designed to generate anti-human N1 response could induce partial cross immunity against H5N1 virus [53, 54]. It will be interesting to deter- mine if Sal-HA-NA vaccine may also elicit immunity against other influenza A viruses expressing HA and NA antigens such as H3N2 and H7N9 viruses. Further studies on the nature of immune responses elicited by Sal-HA-NA and other Salmonella-based vaccines against these viruses should provide insight into developing novel vaccines against influenza virus infections.

We recognize that the BALB/c mice model used in our study has limitations in the evaluation of the constructed vaccines. The immune responses observed in the mouse model may not truly reflect those in humans when immunized with the vaccines and infected with influenza viruses. Additional studies are needed to study the effect of Salmonella-based influenza vaccines in other animal models as well as in humans. Future studies on these issues, as well as on the construction of new attenuated Salmonella strains with novel mutations for expressing

different viral proteins and antigenic regions, should facilitate the development of Salmonella-based vaccines against influenza virus infection.

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# 6 Conclusion and Future Prospective

## 6.1 Using Variants of RNase P-associated Gene-Targeting Agents for Improved Antiviral Efficacy in Cultured Cells

In recent years, gene silencing technologies, including antisense oligonucleotides, RNAi, aptamers, and microRNA molecules have been used extensively for research studies [1–3]. However, each technology has its drawbacks, such as specificity, activity, stability and cellular toxicity [4, 5]. Compared to other technologies Ribozyme-based gene targeting agents represent a promising and attractive approach for anti-viral therapeutics [6]. For ribozyme-mediated gene-targeting to work efficiently, M1GS needs to be highly specific, catalytically efficient, and easily delivered to the target of choice. Currently, limited information exists on what restricts the gene-targeting potency of M1GS ribozymes in cultured cells and there is little understanding about ways to improve the efficacy of M1GS in blocking HCMV infection in-vivo. In the studies presented in Chapters 2 & 3, ribozyme variants of M1GS RNAs were generated by an in-vitro selection strategy to specifically target essential HCMV genes of choice and were highly active in reducing HCMV gene expression and blocking viral growth in cells. In this dissertation research, highly active ribozyme variants were engineered to target DMS-accessible regions of HCMV mRNAs under control of the constitutively expressed eukaryotic U6 promoter. Based on our hypothesis that M1GS cleavage efficacy is governed by its catalytic efficiency ($K_{cat}/K_M$) in-vitro, it is conceivable that enhancing catalytic efficiency of M1GS RNA ($K_{cat}/K_M$) may reduce target mRNA more effectively in cells. Our work implies that enhancing the catalytic efficiencies correlate with a greater efficacy in ribozyme-mediated reduction of HCMV growth in cells, and further suggests that improving catalytic efficiencies of M1GSs should contribute to increasing the anti-HCMV efficacy in-vivo.

RNase P catalyzes the cleavage of a specific phosphodiester bond to yield cleavage products that have a 5’ phosphate and a 3’ hydroxyl end group. This is unlike the catalysis by small ribozymes such as hammerhead and hairpin, that produce a 2’, 3’-cyclic phosphate and a 5’ hydroxyl group [7]. The exact catalytic mechanism of RNase P ribozyme cleavage of a RNA substrate remains to be completely understood. As proposed in a widely accepted model [7], multiple domains within the ribozyme tertiary structure are thought to be involved in catalysis (P4 domain) as well as substrate positioning (J5/15, P15 loop) and the reaction depends on multiple divalent magnesium cations. Evidence from previous work indicate that the P4 domain coordinates with magnesium cations at the cleavage site while the P15 loop and J5/15 domain interacts the 3’CCA and the nucleotide directly 5’ to the cleavage site, respectively [8]. The transition state is proposed to contain at least two divalent magnesium cations involved in a concerted SN2 mechanism [9]. It is proposed that one of the magnesium cations activates a bound hydroxyl for nucleophilic attack on the phosphate bond at the site of cleavage, and the other magnesium cation positions a water molecule for leaving group stabilization by protonation of the 3’ hydroxyl group [9].

The studies presented in this dissertation imply that point mutations in the M1GS or EGS perhaps increased the overall cleavage rate by stabilizing the active site and promoting optimal folding of the ribozyme tertiary structure. Additionally, mutations introduced into M1GS and EGS variants could have increased the intracellular stability possibly through interactions with specific cellular proteins including and RNase P protein subunits and intracellular tRNA-binding proteins,

respectively. Further biochemical profiling of M1GS and EGS variants will expound on the mechanism by which mutations found in variants increase gene-targeting activity and provide insight into the assembly of highly effective RNase P-associated gene-targeting agents. These and further studies should provide guidelines on the development of RNase P-based gene-targeting agents as a potentially promising anti-viral therapeutic.

EGS-mediated degradation of mRNAs constitutes a new approach for gene silencing applications that target nucleic acid molecules. This strategy has great potential because a custom-designed EGS can be used to guide intracellular RNase P to cleave any specific mRNA with high efficiency [6, 7]. Moreover, previous studies indicate that the in-vitro selection procedure could represent an effective approach in screening for highly active EGS molecules for gene-targeting applications [10, 11]. Thus, the EGS-based strategy, which employs endogenous RNase P for targeted cleavage, is unique and different from the ribozyme-based approach, which introduces an exogenous ribonuclease (RNase p-based ribozyme) for cleavage. As evident from studies in Chapter 4 and from previous studies, the levels of mRNA cleavage and anti-viral efficacy mediated by highly active EGS molecules are close in comparison to those observed using RNase P-based ribozymes [12, 13]. Thus, EGS molecules may constitute a novel class of gene-targeting antiviral agents. Further study of EGSs for anti-HSV or anti-HCMV applications may focus on delivering EGSs into neuronal cells, where herpesvirus latent infection is established, and may determine if the delivered EGSs can prevent HSV-1 from reactivating from latent to lytic infection [22]. These studies will further promote the development of engineered EGSs and M1GSs for both basic research and clinical therapeutic applications.

## 6.2 Using Attenuated Salmonella-Based Vectors for Delivery of Oral vaccines or RNase P-associated Gene-Targeting Agents as Anti-Viral Therapeutics

As a promising novel vaccine candidate, DNA vaccines have been previously shown to effectively induce an antibody response with prolonged cell-mediated immunity in animal models [14–17]. Furthermore, when delivered orally, DNA vaccines can be cost-effective and function conveniently because they eliminate the use of needles and syringes thus, are a feasible approach for mass vaccination. Evidence presented in Chapter 5 demonstrate that a novel attenuated strain of Salmonella can be constructed for efficient delivery and expression of antigens from the highly pathogenic H5N1 influenza virus. We established that our generated Salmonella strain carried out efficient gene transfer activity for expression of H5N1 HA (neuraminidase) and NA (hemagglutinin) antigens with minimal cytotoxicity and pathogenicity in mice. We also showed that the Salmonella-based vaccine induced a robust immune response to confer complete protection in mice that were orally vaccinated and expressing viral HA and NA proteins. In our Chapter 5 study, the attenuated Salmonella strain SL368 was derived from the auxotrophic strain SL7207 and also contained a deletion in a part of the spiR gene. SpiR is required for the expression of numerous genes of the Salmonella Pathogenicity Island-2 (SPI-2) including the sifA gene, which encodes a secretory protein of the Type III secretion system (TSS) of SPI-2. Thus, with a deletion in spiR, our generated SL386 Salmonella strain exhibited minimal virulence in carrying out gene transfer in-vivo, as expected.

The exact mechanism of how Salmonella carry out gene transfer remains to be completely understood. In our gene delivery system, attenuated Salmonella were constructed and transformed with transgene-containing plasmid constructs under control of a eukaryotic-specific expression promoter. Upon infection of cells by the attenuated Salmonella vector, plasmid DNA can be released and transported to the nucleus, leading to substantial expression of the transgene [18, 19, 20]. To facilitate optimal construction of Salmonella-based vectors for the development of vaccines, future studies on the process and mechanism of Salmonella-mediated gene delivery would be highly impactful. Additionally, there is a need for future studies on the effect of Salmonella-based vaccines in other animal models including humans. Subsequent studies to address these issues in addition to studies on generating novel attenuated Salmonella strains with new mutations for expressing desired genes should further promote the development of Salmonella-based vaccines against viral infection.

For gene therapy technology, one of the primary challenges is developing effective approaches for the delivery of nucleic acids molecules in-vivo in a tissue and cell specific matter with high efficiency. Salmonella-based vectors show a number of distinctive and attractive features as a gene delivery strategy. Foremost, Salmonella-based vectors are cost-effective, and preparation is relatively simple. Secondly, they can be safely administered orally in-vivo, a rout that has established wide acceptability and shown efficacy in vaccine trials with attenuated Salmonella strains [19, 20]. The attenuated Salmonella-based vector Ty21a, used for the Anti-typhoid-fever is one of the few live vaccines licensed for human use and have been widely used since the 1980s [20]. Additionally, it has been demonstrated that novel attenuated Salmonella strains with different deletion mutations can be easily generated [21]. Lastly, to address the preeminent concern for any gene-delivery agent, Salmonella is not known to be mutagenic and its delivered DNA has not been shown to integrate into host cell genomes.

We have previously generated attenuated Salmonella strains to deliver RNase P ribozyme-based gene targeting agents in cells, leading to effective inhibition of CMV target-gene expression and viral replication [21]. Thus, oral inoculation of Salmonella can efficiently deliver an M1GS sequence, leading to expression and effective inhibition of viral systemic infection. A detailed analysis of the delivery of ribozymes in different tissues in mice should provide more insight into the mechanism of Salmonella-mediated gene delivery of M1GS ribozymes in-vivo. Moreover, studies on the generation of novel and more active M1GS ribozymes through in-vitro selection combined with construction of new Salmonella strains through mutagenesis strategies will facilitate the development of Salmonella-mediated gene delivery of RNase P ribozymes as a promising gene-targeting approach for in-vivo anti-viral therapeutics.

## 6.3 References


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