Effective inhibition of herpes simplex virus 1 gene expression and growth by engineered RNase P ribozyme

Phong Trang, Jarone Lee, Ahmed F. Kilani, Joe Kim and Fenyong Liu*

Program in Infectious Diseases and Immunity, Program in Comparative Biochemistry, School of Public Health, 140 Warren Hall, University of California, Berkeley, CA 94720, USA

Received August 13, 2001; Revised and Accepted October 16, 2001

ABSTRACT
Using an in vitro selection procedure, we have previously isolated ribonuclease P (RNase P) ribozyme variants that efficiently cleave an mRNA sequence in vitro. In this study, an M1GS RNA variant was used to target the mRNA encoding human herpes simplex virus 1 (HSV-1) major transcription activator ICP4. The variant is about 15 times more efficient in cleaving the target mRNA sequence in vitro than the ribozyme derived from the wild type RNase P ribozyme. Moreover, the variant is also more effective in inhibiting viral ICP4 expression and growth in HSV-1-infected cells than the wild type ribozyme. A reduction of ∼90% in the expression level of ICP4 and a reduction of 4000-fold in viral growth were observed in cells that expressed the variant. In contrast, a reduction of <10% in the ICP4 expression and viral growth was observed in cells that either did not express the ribozyme or produced a catalytically inactive ribozyme mutant. These results provide direct evidence that RNase P ribozyme variants can be highly effective in inhibiting HSV-1 gene expression and growth and furthermore, demonstrate the feasibility of developing highly effective RNase P ribozyme variants for anti-HSV applications by using in vitro selection procedures.

INTRODUCTION
Herpes simplex virus 1 (HSV-1) is the causative agent of cold sores and may lead to severe morbidity or mortality in neonates and immunocompromised individuals (1,2). The emergence of drug-resistant strains of HSV-1 has posed a need for the development of new drugs and novel treatment strategies. RNA enzymes are being developed as promising gene-targeting reagents to specifically cleave RNA sequences of choice (3–8). For example, both hammerhead and hairpin ribozymes have been shown to cleave viral mRNA sequences and inhibit viral replication in cells infected with human viruses while a ribozyme derived from a group I intron has been used to repair mutant mRNAs in cells (4,9–11). Thus, ribozymes can be used as a tool in both basic and clinical research, such as in studies of developmental processes and in antiviral gene therapy (5,8,12,13).

Ribonuclease P (RNase P) is a ribonucleoprotein complex responsible for the maturation of the 5′ termini of tRNAs (14,15). In bacteria, the RNase P holoenzyme contains a catalytic RNA subunit and a small highly basic protein subunit (e.g. M1 RNA and C5 protein in Escherichia coli), both of which are required for activity in vivo. Under certain conditions in vitro, M1 RNA acts as a catalyst and cleaves pre-tRNAs in the absence of C5 protein (16). One of the unique features of RNase P holoenzyme and its catalytic RNA is their ability to recognize the structures, rather than the sequences of their substrates, which gives them the ability to hydrolyze different natural substrates in vivo or in vitro. Thus, M1 ribozyme can cleave an mRNA substrate as long as the target sequence hybridizes with its complementary sequence (designated as external guide sequence or EGS) to form a complex resembling the portion of a tRNA molecule that includes the acceptor stem, the T-stem, the 3′CCA sequence, and the 5′leader sequence (17,18) (Fig. 1A and B). A sequence-specific ribozyme, MIGS RNA, can be constructed by covalently linking a guide sequence to the 3′terminus of M1 RNA (19–22). We have shown previously that MIGS ribozymes cleaved the mRNA sequence encoding thymidine kinase (TK) of HSV-1 in vitro and inhibited TK expression in HSV-1-infected cells (21,23–26). More recently, an MIGS ribozyme was constructed to target the mRNA coding for HSV-1 ICP4 protein, the major transcription regulatory factor required for the expression of viral early (β) and late (γ) genes (27). A reduction of 80% in the expression of ICP4 and a reduction of 1000-fold were observed in HSV-1-infected cells that expressed the ribozyme.

Targeted cleavage of mRNA by RNase P ribozyme provides a unique approach to inactivate any RNA of known sequence expressed in vivo. Increasing MIGS RNA catalytic efficiency in vitro and its efficacy in vivo is required in order to develop this ribozyme for practical use both as a research tool and as a therapeutic agent for gene-targeting applications. Using an in vitro selection procedure, we have isolated recently MIGS ribozyme variants that are more efficient in cleaving a specific mRNA sequence (i.e. TK mRNA) than that derived from the wild type M1 RNA (25). In this study, we used one of these ribozyme variants to target the HSV-1 ICP4 mRNA sequence and investigated its activity in cleaving the target mRNA.


*To whom correspondence should be addressed. Tel: +1 510 643 2436; Fax: +1 510 643 9955; Email: liu_fy@uclink4.berkeley.edu
**MATERIALS AND METHODS**

**Ribozyme and substrate constructs**

The DNA sequence that encodes substrate icp32 was constructed by PCR using pGEM3zf(+) as a template and oligonucleotides AF25 (5’-GGAATTCTAATACGACTCACTATAG-3’) and IC1P4 (5’-CCGGGATCCGACGCCATCGCGATGGCGGAGCTATGACCATG-3’) as 5’ and 3’ primers, respectively. Plasmids pV6, pFL117 and pC102 contain the DNA sequence coding for V6, M1 RNA and mutant C102 driven by the T7 RNA polymerase promoter (25,28). Ribozyme variant 6 (designated as V6) contains two point mutations (i.e. insertion of an A 5’ immediately adjacent to C228, C235→U235) (25) while mutant ribozyme C102 contains several point mutations (e.g. A347C348→C347U348, C353C354C355G356→G353G354A355U356) at the catalytic domain (P4 helix) (28). The DNA sequences that encode ribozymes V6-ICP4, M1-ICP4 and C-ICP4 were constructed by PCR using the DNA sequences of the ribozymes as the templates and oligonucleotides AF25 and M1-ICP4 (5’-GGGATCCGAGAAA- AAATGGTGACATCGCGATGGCGGAGCTATGACCATG-3’) as 5’ and 3’ primers, respectively.

**Cleavage and binding analysis**

M1GS RNAs and the ICP4 mRNA substrate (i.e. icp32) were synthesized in vitro by T7 RNA polymerase (Promega Inc., Madison, WI) following the manufacturer’s recommendations and further purified on 8% polyacrylamide gels containing 8 M urea. Subsequently, the M1GS RNAs were mixed with the 32P-labeled mRNA substrate. The cleavage reactions were carried out at 37°C in a volume of 10 µl for 30 min in buffer A (50 mM Tris pH 7.5, 100 mM NH4Cl and 100 mM MgCl2) (25). Cleavage products were separated in denaturing gels and quantified with a STORM840 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Assays to determine the observed reaction rate (kob) and the values of the cleavage efficiency [(kcat/Km)’] were performed under single-turnover conditions as described previously (25,28,29). Briefly, analyses were performed with a trace amount of radioactive substrate and an excess of ribozyme. Variants of the amount of substrate did not affect the observed cleavage rate (kob) at a fixed excess ribozyme concentration and the reaction followed pseudo-first-order kinetics. Pseudo-first-order rate constants of cleavage (kobs) were assayed at each ribozyme concentration by the slope of a plot –ln[(Ft – Fe)/(1 – Fe)] versus time using Kaleidagraph program (Synergy Software, Reading, PA). F and Fe represent the fraction of the substrate at time t and at the end point of the experiments, respectively. The values of the overall cleavage rate [(kcat/Km)’]
were calculated by the slope of a least-squares linear regression (Kaleidagraph) of a plot of the values of $k_{eq}$ versus the concentrations of the ribozymes. These values were the average of three experiments. The procedures to measure the equilibrium dissociation constants ($K_d$) of the M1GS-icp32 complexes were modified from Pyle et al. (30) and have been described previously (27). The values of $K_d$ obtained were the average of three experiments.

**Viruses, cells and antibodies**

Vero (African green monkey kidney) cells, PA317 cells and ψCRE cells were maintained, and the propagation of HSV-1 (F) in these cells was carried out as described previously (27). MCA406, the anti-mouse monoclonal antibody against HSV-1 (F) in these cells was carried out as described previously (27). The anti-mouse monoclonal antibodies c1101, c1113 and c1123, which react with HSV-1 proteins ICP4, ICP27 and gB, respectively, were purchased from Bioproduct Sciences Inc. (Indianapolis, IN). The anti-mouse monoclonal antibody c1113 was purchased from Bioproduct for Sciences Inc. (Indianapolis, IN). The anti-mouse monoclonal antibody c1123, which react with HSV-1 proteins ICP4, ICP27 and gB, respectively, were purchased from Goodwin Institute for Cancer Research (Plantation, FL).

**Construction of ribozyme-expressing cells**

The protocols for the construction of ψCRE cells expressing different ribozymes were modified from Miller and Rosman (32) and have been described previously (21). In brief, amphotropic PA317 cells were transfected with retroviral vector DNAs (LXSN-M1-ICP4, C-ICP4, V6-ICP4 and C-V6-ICP4) with the aid of a mammalian transfection kit purchased from Gibco BRL (Grand Island, NY). Forty-eight hours post-transfection, culture supernatants that contained retroviruses were collected and used to infect ψCRE cells. At 48–72 h post-infection, cells were incubated in culture medium that contained 600 µg/ml neomycin. Cells were subsequently selected in the presence of neomycin for 2 weeks and neomycin-resistant cells were cloned.

For northern analyses of the expression of the ribozymes, both nuclear and cytoplasmic RNA fractions from M1GS-expression cells were isolated as described previously (21). The RNA fractions were separated in a 2.5% agarose gel that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the 32P-radiolabeled DNA probe that contained the DNA sequence coding for M1 RNA and finally analyzed with a STORM840 phosphorimager. The separated polypeptides were transferred electrically to nitrocellulose membranes and reacted to the antibodies against HSV-1 ICP4, ICP27 and ICP35. The membranes were subsequently stained with a chemiluminescent substrate with the aid of a western chemiluminescent substrate kit (Amersham Inc., Arlington Heights, IL) and quantified with a STORM840 phosphorimager.

RESULTS

Because most mRNA species inside cells are associated with proteins and are present in a highly organized and folded conformation, it is important to choose a targeting region that is accessible to the binding of ribozymes in order to achieve efficient cleavage. Using dimethyl sulphate (DMS), we employed an in vivo mapping approach (21,33,34) to determine the accessibility of the region of ICP4 mRNA in HSV-1-infected cells (27). A position 5 nt downstream from the ICP4 translational initiation codon (35) was chosen as the cleavage site for M1GS RNA. This site appears to be one of the regions most accessible to DMS modification, and presumably to ribozyme binding. Moreover, its flanking sequence exhibits several sequence features that need to be present in order to interact with an M1GS ribozyme to achieve efficient cleavage. These features include the requirement for a guanosine and a pyrimidine to be the nucleotide 3’ and 5’ adjacent to the site of cleavage, respectively (14,15,36,37). The interactions of these sequence elements with the M1GS ribozyme are critical for recognition and cleavage by the enzyme.

Using an in vitro selection procedure, we have isolated M1GS ribozyme variants that are more efficient in cleaving the TK mRNA sequence than the ribozyme derived from the wild type M1 (25). However, whether these ribozyme variants can be used and are highly effective in inhibiting ICP4 expression has not been demonstrated. We chose V6 for the study because the ribozymes derived from this variant are among the most active M1GS RNAs in cleaving the TK as well as ICP4 mRNAs (Table 1). This variant contains two point mutations (i.e. insertion of an A 5 immediately adjacent to C228, C235→U235) (25).

Ribozyme V6-ICP4 was constructed by covalently linking the 3’ terminus of V6 RNA with a guide sequence of 13 nt that is complementary to the targeted ICP4 mRNA sequence. Two other M1GS ribozymes, M1-ICP4 and C-ICP4, were also constructed in a similar way and included in the study. M1-ICP4 was derived from the wild type M1 sequence. C-ICP4 was derived from C102 RNA, a M1 mutant that...
Detailed kinetic analyses were carried out and the overall cleavage efficiency [measured as \( (k_{cat}/K_m) \)] for these ribozymes were determined. These results indicate that V6-ICP4 is about 15-fold more active than M1-ICP4 in cleaving icp32 (Table 1). Experiments with gel-shift assays were also carried out to determine whether the differential cleavage efficiencies observed with V6-ICP4, M1-ICP4 and C-ICP4 were possibly due to their different binding affinities to the ICP4 mRNA sequence. Detailed assays under different concentrations of the ribozymes and icp32 indicate that the binding affinity of C-ICP4 to substrate icp32, measured as the dissociation constant \( (K_d) \), is similar to those of M1-ICP4 and V6-ICP4 (Table 1). As C-ICP4 contains the same antisense guide sequence and similar affinity to icp32 as V6-ICP4 and M1-ICP4 but is catalytically inactive, this ribozyme can be used as a control for the antisense effect in our experiments in cultured cells (see below).

The DNA sequences coding for V6-ICP4, M1-ICP4 and C-ICP4 were cloned into retroviral vector LXSN and placed under the control of the small nuclear U6 RNA promoter, which has previously been shown to express M1GS RNA and other RNAs steadily (21,32,38–40). This promoter is transcribed by RNA polymerase III, and its transcripts are highly expressed and primarily localized in the nucleus (38–40). To construct cell lines that express M1GS ribozymes, amphotropic packaging cells (PA317) (32) were transfected with LXSN-M1GS DNAs to produce retroviruses that contained the genes for M1GS RNA. Subsequently, \( \psi \)CRE cells (41) were infected with these retroviruses, and cells expressing the ribozymes were cloned. The constructed lines and a control line in which cells were transfected with LXSN vector DNA alone were indistinguishable in terms of their growth and viability for up to 2 months (data not shown), suggesting that the expression of the ribozymes did not exhibit significant cytotoxicity.

The level of M1GS RNA in each cell clone was determined by northern analysis with a DNA probe that is complementary to M1 RNA. The M1GS RNAs were exclusively expressed in the nuclei as they were only detected in the nuclear but not the cytoplasmic RNA fractions (data not shown). This is consistent with previous observations in our laboratory as well as others that the transcripts expressed by the U6 promoter are primarily localized in the nuclei (38,40,42,43). Only the cell lines that expressed similar levels of these ribozymes were used for further studies in tissue culture.

To determine the efficacy of the ribozymes in inhibiting ICP4 expression, cells were infected with HSV-1 at an m.o.i. of 0.05–0.5. Total RNAs were isolated from cells that were treated with 100 \( \mu \)g/ml cycloheximide, under which condition only viral IE (\( \alpha \)) mRNAs were synthesized (1). The level of ICP4 mRNA was determined by an RNase protection assay and the level of viral IE \( \alpha 47 \) mRNA was used as an internal control (Fig. 3). A reduction of \(-93 \pm 4, 82 \pm 4, 6 \pm 3\% \) (average of three experiments) in the level of ICP4 mRNA expression was observed in cells that expressed V6-ICP4, M1-ICP4 and C-ICP4, respectively (Table 2). These results suggest that the significant reduction of ICP4 mRNA expression in cells that expressed V6-ICP4 and M1-ICP4 was due to the targeted cleavage by the ribozymes. The low level of inhibition found in cells that expressed C-ICP4 was probably due to an antisense effect because C-ICP4 exhibits similar activity in vitro.

Table 1. Measurement of overall cleavage rate \( (k_{cat}/K_m) \) and binding affinity \( (K_d) \) in cleavage reactions of icp32 with RNase P ribozymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( (k_{cat}/K_m) ) (( \mu )m(^{-1}) min(^{-1}))</th>
<th>( K_d ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1-ICP4</td>
<td>0.30 ± 0.09</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>V6-ICP4</td>
<td>4.6 ± 0.8</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>C-ICP4</td>
<td>&lt;5 ( \times 10^{-5} )</td>
<td>0.22 ± 0.05</td>
</tr>
</tbody>
</table>

Single-turnover kinetic analyses to determine the values of \( (k_{cat}/K_m) \) were carried out in buffer A (50 mM Tris–HCl pH 7.5, 100 mM NH\(_4\)Cl, 100 mM MgCl\(_2\)) as described previously (25). Binding assays were carried out in buffer D (50 mM Tris–HCl pH 7.5, 100 mM NH\(_4\)Cl, 100 mM CaCl\(_2\)), using a protocol modified from Pyle et al. (30). The values shown are the average derived from triplicate experiments.
The level of ICP4 protein is expected to reduce in cells that expressed V6-ICP4 and M1-ICP4 because of the decreased binding affinity to the target sequence as V6-ICP4 and M1-ICP4 but is catalytically inactive.

The expression of viral mRNAs, as detected by an RNase protection assay. Total RNA fractions were isolated from parental ψCRE cells (P, lanes 1 and 5) or from cell lines that expressed C-ICP4 (lanes 2 and 6), M1-ICP4 (lanes 3 and 7) and V6-ICP4 (lanes 4 and 8). Cells were infected with HSV-1 (m.o.i. = 0.3) and were harvested at either 4 (lanes 3 and 7) and V6-ICP4 (lanes 4 and 8). Cells were infected with HSV-1 (m.o.i. = 0.3) and were harvested at either 4 (A) or 10 h (B) post-infection. Viral infection was carried out either in the absence (B) or presence (A) of 100 µg/ml cycloheximide. Equal amounts of each RNA sample (30 µg) were hybridized to the RNA probes that contained the sequence of ICP4 and α47 mRNAs (A), and α47 and TK mRNAs (B).

Table 2. Levels of inhibition of the expression of viral genes in cells that expressed V6-ICP4, M1-ICP4 and C-ICP4, as compared with the levels of inhibition in cells that did not express a ribozyme (ψCRE)

<table>
<thead>
<tr>
<th>Viral gene class</th>
<th>Ribozyme</th>
<th>ψCRE (%)</th>
<th>C-ICP4 (%)</th>
<th>M1-ICP4 (%)</th>
<th>V6-ICP4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP4 mRNA α</td>
<td></td>
<td>0</td>
<td>6</td>
<td>82</td>
<td>93</td>
</tr>
<tr>
<td>TK mRNA β</td>
<td></td>
<td>0</td>
<td>5</td>
<td>81</td>
<td>90</td>
</tr>
<tr>
<td>ICP4 protein α</td>
<td></td>
<td>0</td>
<td>7</td>
<td>77</td>
<td>92</td>
</tr>
<tr>
<td>ICP35 protein γ</td>
<td></td>
<td>0</td>
<td>5</td>
<td>81</td>
<td>90</td>
</tr>
<tr>
<td>GB protein γ</td>
<td></td>
<td>0</td>
<td>4</td>
<td>80</td>
<td>91</td>
</tr>
</tbody>
</table>

The values shown are the means from triplicate experiments and the values of standard deviation were <5%.

DISCUSSION

Ribozymes have been shown to be promising antiviral agents for the inhibition of viral gene expression and replication (8,13). The M1GS-based technology represents an attractive approach for gene inactivation as it generates catalytic and irreversible cleavage of the target RNA by using M1 RNA, a highly active RNA enzyme found in nature (14,15). Several criteria must be satisfied if successful targeting with M1GS ribozyme is to be achieved. Among these are high efficiency of cleavage, sequence specificity of the ribozymes and efficient delivery of the reagents.

Our previous studies indicated that M1GS RNAs are effective in inhibiting gene expression of HSV-1 in cultured cells by 75–80%. Understanding the mechanism of ribozyme cleavage...
Using a RNase P ribozyme variant selected from a pool of M1GS RNAs. Cells (5 × 10^5) were infected with HSV-1 at a m.o.i. of 2. Virus stocks were prepared from the infected cells at 6 h intervals through 36 h post-infection and the p.f.u. count was determined by measurement of the viral titer on CRE cells. These values are the means from triplicate experiments. The standard deviation is indicated by the error bars.

Figure 4. Expression of viral proteins, as detected by western blot analysis with a chemiluminescent substrate. Total protein fractions were isolated from parental ψCRE cells (P, lanes 1, 5 and 9) or from cell lines that expressed C-ICP4 (lanes 2, 6 and 10), M1-ICP4 (lanes 3, 7 and 11) and V6-ICP4 (lanes 4, 8 and 12). Cells were infected with HSV-1 (m.o.i. = 0.2) and were harvested at 12 h post-infection. Equal amounts of protein samples (40 µg) isolated from cells were separated in SDS–polyacrylamide gels. One membrane was allowed to react with a monochlonal antibody (anti-ICP27) against HSV-1 ICP27 protein (A) while the others were stained with the monochlonal antibodies against viral ICP4 and ICP35 proteins (B and C).

Figure 5. Growth of HSV in ψCRE cells and cell lines that expressed M1GS RNAs. Cells (5 × 10^5) were infected with HSV-1 at a m.o.i. of 2. Virus stocks were prepared from the infected cells at 6 h intervals through 36 h post-infection and the p.f.u. count was determined by measurement of the viral titer on Vero cells. These values are the means from triplicate experiments. The standard deviation is indicated by the error bars.

and improving its efficacy is essential to develop M1GS ribozyme for practical anti-HSV-1 applications. However, little is currently known about the rate-limiting step of M1GS RNA cleavage reaction in cells. Equally unclear is whether the efficacy of the ribozymes can be improved, and if so, how it can be improved. In the present study, we showed that an RNase P variant, V6-ICP4, exhibited a 15 times higher rate of cleavage in vitro in cleaving ICP4 mRNA than the ribozyme (i.e. M1-ICP4) derived from the wild type M1 RNA sequence. Moreover, V6-ICP4 inhibited ICP4 expression in cultured cells by >90% and was more effective in cultured cells than M1-ICP4, which reduced ICP4 expression by ~80%. A reduction of ~4000-fold in viral growth was observed in the V6-ICP4-expressing cells while a reduction of ~1000-fold was observed in M1-ICP4-expressing cells. In contrast, a reduction of <10% in the ICP4 expression level and viral growth was observed in cells that expressed C-ICP4. C-ICP4 exhibited similar binding affinity to icp32 as V6-ICP4 and M1-ICP4 but was catalytically inactive due to the presence of the mutations at the catalytic domain (Fig. 2, Table 1). These results suggest that the overall observed inhibition with V6-CIP4 and M1-ICP4 was primarily due to targeted cleavage by these ribozymes as opposed to the antisense effect of the ribozyme sequences. Moreover, the ribozyme (V6-ICP4) that exhibited higher cleavage activities [([k_{cat}/K_m])] appeared to be more effective in cell culture. These results strongly suggest that increasing the catalytic efficiency of RNase P ribozymes may lead to an improved efficacy in inhibiting HSV-1 gene expression and growth in cultured cells. The difference between the in vivo efficacies of the selected variant and M1-ICP4 (e.g. 92 versus 77%) appeared to be more limited than that of the in vitro cleavage efficiencies (>15-fold difference). One of the possible explanations is that ~5–10% of the target mRNA may not be accessible to ribozyme binding, possibly due to its rapid transport to the cytoplasm.

The activity of M1GS ribozymes also appears to be specific. More importantly, the increased efficacy of the selected variant in inhibiting viral gene expression and growth in cultured cells is due to its increased activity in cleaving the ICP4 mRNA sequence in vitro. First, the expression of the ribozymes did not exhibit significant cytotoxicity as cells expressing ribozymes are indistinguishable from the parental cells in terms of cell growth and viability for up to 2 months (data not shown). We found no difference in the expression level of actin mRNA in the ribozyme-expressing cells and the parental ψCRE cells. Moreover, the antiviral effect of the ribozyme (inhibition of viral growth) appears to be due to the reduction of the ICP4 expression. This is because the expression of the viral early and late genes examined, including TK and ICP35 as well as gB, was found to be significantly reduced in cells that expressed V6-ICP4 and M1-ICP4, but not in those that expressed C-ICP4 (Figs 3 and 4, Table 2, data not shown). The extent of the observed inhibition of the expression of these viral early and late genes correlates with that of the inhibition of the ICP4 expression. Meanwhile, no reduction in the expression levels of other viral IE genes examined (e.g. α47 and ICP27) was found in M1GS-expressing cells (Figs 3 and 4, data not shown). Thus, M1GS ribozyme is highly specific in inhibiting the expression of its target mRNA, and increased in vitro cleavage activity leads to the improvement of its efficacy in inhibiting ICP4 gene expression, overall expression of viral early and late genes, as well as reducing viral growth.

Ribozyme technology represents an attractive approach for gene inactivation as it exhibits most of the properties of conventional antisense-targeting method and in addition, catalytic and irreversible cleavage of the target RNA. In vitro selection (45–47) has been widely used to generate either new nucleic acid-based catalysts or more efficient variants from known ribozyme molecules (48,49). For example, this procedure has been extensively used to generate efficient group I intron, hammerhead and hairpin ribozyme variants (50–54). Using a RNase P ribozyme variant selected from a pool of M1
molecules containing randomized sequences, we, in this study, provide the direct evidence that RNase P variant with increased cleavage activity in vitro also exhibits improved efficacy in inhibiting HSV-1 gene expression and growth in cultured cells. Thus, our study provides a direction for the engineering of highly active and effective RNase P ribozyme variants by carrying out selection procedures in order to improve the efficacy of the M1GS-based technology. Further characterization of the cleavage reactions of this as well as other RNase P variants both in vitro and in cultured cells should provide insights into the mechanism of how an RNase P ribozyme efficiently cleaves an mRNA substrate and develop guidelines for construction of effective gene-targeting ribozymes.

HSV-1, a member of human herpes virus family that includes seven other different viruses such as herpes simplex virus 2, varicella Zoster virus, Epstein–Barr virus, cytomegalo-virus and the recently discovered Karpors sarcoma-associated herpes virus 1.55 can, can engage in lytic replication as well as establish latent infections. HSV-1 ICP4, the major viral transcription regulator, is among one of the first viral proteins expressed during lytic infection (1.44). Future challenges to the use of M1GS for anti-HSV application are to determine whether the ribozyme can be delivered in neuronal cells, which are latently infected by the virus (1.2), and whether the delivered ribozyme can prevent HSV reactivation from latent to lytic infection. These studies will further facilitate the development of M1GS ribozymes for general gene-targeting applications.

ACKNOWLEDGEMENTS

We are grateful to Diane Kawa, Jianing Zhu and Edward Nepomuceno for excellent technical assistance on construction of ribozymes and cell lines, kinetic analyses and determination of viral titers in ribozyme-expressing cells. P.T. is partially supported by Block Grant Predoctoral Fellowships (UC-Berkeley). A.F.K. was a recipient of a President’s Graduate Dissertation Fellowship from University of California. F.L. is a Pew Scholar in Biomedical Sciences and a Scholar of Leukemia and Lymphoma Society. The research has been supported by March of Dimes National Birth Defects Foundation, American Heart Association and NIH.

REFERENCES


