Human cytomegalovirus (HCMV) causes significant morbidity and mortality in immunocompromised or immunologically immature individuals, including neonates and AIDS patients (1, 2). The emergence of drug-resistant strains of HCMV has posed a need for the development of new drugs and novel treatment strategies. RNA enzymes are being developed as promising gene-targeting agents to specifically cleave RNA sequences of choice (3, 4). These ribozymes contain both a catalytic RNA domain that cleaves the target mRNA and a substrate-binding domain with a sequence antisense to the target mRNA sequence. Therefore, these gene-targeting ribozymes bind to the mRNA sequence through Watson-Crick interactions between the target sequence and the antisense sequence in the substrate-binding domain of the ribozyme. Compared with conventional antisense DNA and RNA, a ribozyme may have several unique features as it can cleave its target irreversibly, and multiple copies of its substrate can be cleaved by a single ribozyme molecule. Both hammerhead and hairpin ribozymes have been shown to cleave viral mRNA sequences and inhibit viral replication in cells infected with human viruses, whereas a ribozyme derived from a group I intron has been used to repair mutant mRNAs in cells (5–8). Thus, ribozymes can be used as a tool in both basic research and clinical applications, such as in studies of developmental processes and in antiviral gene therapy (3, 4).

RNase P is a ribonucleoprotein complex responsible for the 5′-end maturation of tRNAs (9, 10). It catalyzes a hydrolytic reaction to remove a 5′ leader sequence from tRNA precursors (pre-tRNA) and several small RNAs. In *Escherichia coli*, RNase P consists of a catalytic RNA subunit (M1 RNA) of 377 nucleotides and a protein subunit (C5 protein) of 119 amino acids (9, 10). Under certain buffer conditions, such as 100 mM Mg²⁺, M1 RNA acts as a catalyst and cleaves pre-tRNAs in vitro in the absence of C5 protein (11). Extensive phylogenetic and biochemical analyses have provided significant insight into understanding the secondary structure (12, 13) and the three-dimensional structure of M1 RNA (14–17). These studies serve as a foundation for identifying the putative active site and substrate-binding site and for investigating the catalytic mechanism of this ribozyme.

Studies on substrate recognition by M1 RNA and RNase P have led to the development of a general strategy in which M1 RNA and RNase P can be used as gene targeting tools to cleave any specific mRNA sequences (18). One of the unique features of RNase P holozyme 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 vitro 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) 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 (18,
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A sequence-specific ribozyme, M1GS RNA, can be constructed by covalently linking a guide sequence to the 3' terminus of M1 RNA (20–23). We have shown previously that M1GS ribozymes cleaved the mRNA sequence encoding the thymidine kinase (TK) of herpes simplex virus type 1 in vitro and inhibited TK expression in herpes simplex virus type 1-infected cells (20, 24–27). More recently, an M1GS ribozyme (25) was constructed to target the mRNA coding for HCMV IE1 and IE2, the major transcription regulatory proteins required for viral gene expression and growth (1, 28, 29). A reduction of 80% in the expression of IE1 and IE2 and a reduction of 150-fold in viral growth were observed in HCMV-infected cells that expressed the ribozyme (25).

Targeted cleavage of mRNA by RNase P ribozyme provides a unique approach to inactivate any RNA of known sequence expressed in vitro. In order to develop this ribozyme for practical use both as a research tool and as a therapeutic agent for gene-targeting applications, further studies are needed to improve M1GS RNA catalytic efficiency in vitro and its efficacy in vivo. By using an in vitro selection procedure, we have recently isolated M1GS 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 (26).

In this study, we used one of these ribozyme variants to target the HCMV IE1 and IE2 mRNA sequence, and we investigated its activity in cleaving the target mRNA in vitro and its efficacy in inhibiting IE1 and IE2 gene expression and viral growth in cultured cells. Our results indicate that the ribozyme derived from the selected variant is at least 90 times more effective in cleaving the target HCMV mRNA sequence in vitro than the ribozyme derived from the wild type M1 RNA sequence. Biochemical characterization of the variant indicates that a point mutation at nucleotide position 80 of M1 RNA \( U^{180} \rightarrow C^{180} \) increases the rate of chemical cleavage, and another mutation at nucleotide position 188 \( C^{188} \rightarrow U^{188} \) enhances substrate binding of the ribozyme. These results provide insights into the catalytic mechanism of how RNase P ribozyme efficiently cleaves an mRNA substrate and generate guidelines for construction of highly active M1GS ribozymes for gene-targeting applications.

The constructed ribozyme variant is also more effective in inhibiting IE1 and IE2 expression and HCMV growth in cultured cells. A reduction of 99% in the expression of IE1 and IE2 and a reduction of 10,000-fold in viral growth were observed in cells that expressed the ribozyme variant. These results provide the direct evidence that engineered RNase P ribozyme variants can be highly effective in inhibiting HCMV gene expression and growth. More importantly, our study demonstrates the feasibility of developing effective RNase P ribozymes for gene-targeting applications, including the treatment and prevention of HCMV infections.

**EXPERIMENTAL PROCEDURES**

Antibodies, Viruses, and Cells—The monoclonal antibodies against human actin and HCMV gH were purchased from Sigma and Biosciences, respectively. The monoclonal antibodies c1202, c1203, and c1205, which react with the gH/gM protein of HCMV, respectively, were purchased from the Goodwin Institute for Cancer Research (Plantation, FL). Human foreskin fibroblasts and astrocytoma U373MG cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) as described previously (23). HCMV (strain AD169) was obtained from American Type Culture Collection (ATCC) and was propagated in human foreskin fibroblasts and U373MG cells.

**Generation of Ribozyme and Substrate Constructs**—Plasmids pFL117, pR27, and pC102, which contain the DNA sequences encoding M1 RNA, variant R27, and mutant C102, respectively, have been described previously (26, 30). Variant constructs pV80 and pV188 were generated by changing \( U^{180} \rightarrow C^{180} \) and \( C^{188} \rightarrow U^{188} \) in the M1 sequence of pFL117, respectively. C102 contains several point mutations (e.g., A\(^{347}C^{348} \rightarrow A^{347}T^{348}\), C\(^{352}C^{353}G^{354}C^{355}G^{356} \rightarrow C^{352}G^{353}A^{354}T^{355}U^{356}\)) at the catalytic domain (P4 helix) of the M1 RNA sequence (30). The DNA sequences that encode ribozymes M1-IE, R27-IE, V80-IE, V188-IE, and C-IE were constructed by PCR using pFL117, pR27, pV80, pV188, and pC102 as the templates, respectively, with oligoT7 (5‘-TAATACGACT- CACTATAG-3’) as the 5’ primer and M1IE12 (5‘-TGTCGACACGAGAACCTGTGAAT-3’) as the 3’ primer. Similarly, the DNA sequences that encode C-R27-IE, C-V80-IE, and C-V188-IE were generated by PCR using oligonucleotide AP25 (5‘-GAATTCCTGAAATACGACTCATAAGTGAATGATT-3’) as the 5’ primer, oligonucleotide M1IE12 as the 3’ primer, C-R27, C-V80, and C-V188 as the templates, respectively.

The DNA template for the in vitro transcription of RNA substrate is37 was constructed by annealing the T7 promoter-containing oligonucleotide OliT7 with oligonucleotide sIE1 (5‘-CGGGATCCTTCTCCTGAGGTCGGTCAAGC-3’). The DNA that codes for is37-3 was generated by PCR to replace the entire 12-nucleotide-long 3’ tail sequence of is37 with a thymidine tail sequence. The DNA sequences that code for RNA substrates is37-3 and is37-3 were constructed by PCR using oligonucleotide sIE1 as the template, OliT7 as the 5’ primer, and oligonucleotides oligoT7 (5‘-CGGGATCCTTCTGAGGTCGGTCAAGC-3’) and oligoT7 is37 (5‘-GAATTCCTGAAATACGACTCATAAGTGAATGATT-3’) as the 3’ primers, respectively.

**Construction of Retroviral Plasmids and Ribozyme-expressing Cells**—Retroviral constructs RvM1-IE, RvC-IE, RvC-V188-IE, RvC-R27-IE, RvV188-IE, and RvR27-IE were constructed by placing the DNA sequences that code for M1-IE, C-IE, C-V188-IE, C-R27-IE, V188-IE, and R27-IE under the control of the U6 promoter in the LXSN retroviral vector, respectively (20, 31). To generate cell lines containing the retroviral vector DNA sequences that contain the ribozyme sequence were transfected into amphotropic PA317 cells by using a mammalian transfection kit (Invitrogen). At 48 h post-transfection, culture supernatants that contained retroviral vector particles were collected and used to infect human U373MG cells. At 48–72 h postinfection, neomycin (Invitrogen) was added to the culture medium at a final concentration of 600 μg/ml. Cells were subsequently selected in the presence of neomycin for 2 weeks, and neomycin-resistant cells were cloned (20, 31). The level of M1GS RNA expression in individual cell clones was determined by Northern analysis with a probe complementary to the M1 RNA sequence. Only those cell clones that expressed similar levels of ribozymes were used for subsequent experiments.

**Viral Infection and Assays for Viral Gene Expression and Growth**—5 × 10^5 cells were either mock-infected or infected with HCMV at a multiplicity of infection (m.o.i.) of 0.05–1 as described previously (23). The infected cells were incubated for a certain period of time (as stated under the "Results") before they were harvested for viral mRNA or protein isolation. Total cellular RNA and proteins were prepared from the cells as described previously (23, 32). To measure the levels of viral immediate-early (IE) transcripts, some of the cells were also treated with 100 μg/ml cycloheximide prior to and during infection.

To determine the mRNA expression using Northern analyses, the RNA fractions were separated in 1–2.5% agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, hybridized with the 32P-radiolabeled DNA probes containing the HCMV DNA sequence, and analyzed with a STORM480 PhosphorImager. The radiolabeled DNA probes used to detect M1GS RNAs, actin mRNA, HCMV immediate-early 5-kb RNA transcript, IE1 mRNA, IE2 mRNA, and US2 mRNA were synthesized from plasmids pFL117, gH/p97/p13 RNA, pCig27, pIE1, pIE2, and pCig68, respectively, by using a random primed labeling kit (Roche Applied Bioscience) (23, 33). The viral 5-kb RNA was transcribed from the region covering HCMV open reading frames between UL106 and UL110 (33). To examine the protein expression using Western analyses, the denatured polypeptides from cell lysates were separated on SDS-polyacrylamide gels cross-linked with N,N’-methylenebisacrylamide, transferred electrically to nitrocellulose membranes, and reacted in an enzyme-linked immunoassay with antimouse IgG conjugated with alkaline phosphatase in addition to the antibodies against human actin and HCMV IE1/IE2, UL44, UL83, and gH. The membranes were subsequently stained with a chemiluminescence detection kit (Amersham Biosciences) and quantitated with a STORM480 Phos...
The values obtained were the average of three experiments. It has been shown that cloned U373MG cells exhibit clonal variability in their susceptibility to HCMV infection (1). To exclude the possibility that the observed inhibition of viral gene expression and growth is due to the variability of the particular cloned U373MG cells used in the experiments rather than the ribozyme-mediated inhibition, two additional sets of plaques that expressed different ribozymes were used to assay the efficacy of the ribozymes in inhibiting HCMV gene expression and growth. Moreover, three additional cloned cell lines that expressed each of the ribozymes were mixed and used as the oligoclonal cell populations. The antiviral efficacies of this set of oligoclonal cell populations were also determined.

Determination of Binding Dissociation Constant (K_D) and Apparent Reaction Rate Constant k_app—The procedures to measure the equilibrium dissociation constants (K_D) of complexes of the ribozymes and the substrates were modified from Pyle et al. (35). In brief, various concentrations of M1GS RNAs were preincubated in buffer E (50 mM Tris, pH 7.5, 100 mM NaCl, 100 mM MgCl2) and single turnover kinetic analyses to determine the values of the observed cleavage rate (k_obs) were performed as described previously (26, 34). Analyses were performed with a trace amount of radioactively labeled substrate RNA preheated under identical conditions. Variations in the amount of substrate did not affect the observed cleavage rate (k_obs) at a fixed excess ribozyme concentration, and the reaction followed pseudo first-order kinetics. Pseudo first-order rate constants of cleavage (k_obs) were assayed at each ribozyme concentration by the slope of a plot -ln(F - F_0)/t (1/F_0) versus time using a Kaleidagraph program (Synergy Software, Reading, PA). F and F_0 represent the fraction of the substrate at time t and the end point (>10 h) of the experiments, respectively. The values of the overall cleavage rate (k_obs/K_D) were calculated by the slope of a least squares linear regression (Kaleidagraph) of a plot of the values of k_obs versus the concentrations of the ribozymes (26, 34). The values obtained were the average of three experiments.

RESULTS

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Engineered RNase P Ribozymes Are Efficient in Cleaving the IE1/IE2 mRNA Sequence—IE1 and IE2, which are the viral immediate early (IE or α) gene products and share 85 amino-terminal amino acids due to alternative splicing and polyadenylation of transcripts initiating at a strong promoter-enhancer (1, 28, 29), function as the major transcriptional regulators and are required for the expression of viral early (β) and late (γ) genes (36, 37). Therefore, targeting the overlapping region (e.g. exons 1–3) of the mRNAs coding for IE1 and IE2 should simultaneously shut down the expression of both proteins and may yield more effective inhibition of viral replication. Choosing a target region that is accessible to binding of M1GS ribozymes is important in order to achieve efficient targeting. This is because most mRNA species inside cells are associated with proteins and are present in folded conformations. We have used an in vitro mapping approach with dimethyl sulfate (20, 38, 39) to determine the accessible regions of IE1 and IE2 mRNAs. A position 39 nucleotides upstream from the 3′ terminus of exon 3 was chosen as the cleavage site for the M1GS ribozyme. The targeted region (designated as IE mRNA), which is in the overlapping region of IE1 and IE2 mRNAs (40), is one of the sequences most accessible to dimethyl sulfate modification and is likely accessible to ribozyme binding (data not shown).

We have used previously (26) an in vitro selection procedure to isolate M1GS RNA variants that are more efficient in cleaving the TK mRNA sequence than the ribozyme derived from the wild type M1 RNA. The objective of the study was to generate highly active RNase P ribozyme variants that can be used for targeting TK mRNA as well as any other mRNAs and to study the variants to understand the catalytic mechanism of RNase P ribozymes in cleaving mRNA substrates. However, little is currently known about the mechanism of how some of these active variants increase their cleavage activity in vitro. Whether these variants can be used for the construction of highly active ribozymes for inhibition of HCMV gene expression and growth has not been extensively studied. In this study, we addressed these issues by studying one of the variants, R27. This variant, as well as its derivative ribozyme (i.e. R27-IE), are among the most active M1GS RNAs in cleaving the TK as well as the IE1/IE2 mRNA sequences in vitro (see below and Table 1). Thus, we chose to characterize the activity of R27 in cleaving IE1/IE2 sequence in vitro and its efficacy in inhibiting IE1/IE2 expression in HCMV-infected cells, and we investigate the mechanism of how the mutations increase its catalytic activity. We hope that these results will provide insight into how RNase P ribozyme efficiently cleaves an mRNA substrate and, furthermore, will generate general guidelines for construction of highly active ribozymes for gene-targeting applications.

Ribozyme R27-IE was constructed by covalently linking the 3′ terminus of R27 RNA to a guide sequence of 13 nucleotides that is complementary to the targeted mRNA sequence. R27 contains point mutations U186 to C186 and C188 to U188 (Fig. 1C) (26). Little is known about the functional role of U186 in M1GS RNA cleavage, whereas our recent nuclease footprint analyses and UV cross-linking studies suggest that C188 is in close proximity to the 3′ tail sequence of an mRNA substrate and is probably involved in binding the mRNA substrate (24, 25, 27). In order to study the function of these two mutations in the cleavage of the IE mRNA sequence, we constructed two additional ribozyme variants, V80-IE and V188-IE, which were derived from M1 RNA and contained the mutations U186→C186 and C188→U188, respectively. Two other M1GS ribozymes, M1-IE and C-IE, were also constructed by linking the guide
sequence to the 3’ termini of the wild type M1 RNA sequence and mutant C102 RNA sequence, respectively. C102 RNA is derived from M1 RNA and contains several point mutations at the P4 catalytic domain and is at least 1 × 105-fold less active than M1 RNA (30). Cleavage of RNA substrates by ie37, which contains the targeted HCMV mRNA sequence of 37 nucleotides (Fig. 1B), was observed in the presence of R27-IE, V80-IE, V188-IE, and M1-IE (Fig. 2, lanes 3 and 4, and Table I). In contrast, cleavage of the same substrate by C-IE was barely detected due to the point mutations at the catalytic center region (Fig. 2, lane 5). To determine whether the variant is more active in cleaving the IE mRNA sequence, cleavage of ie37 by the ribozymes was assayed, and the overall cleavage rate (k_{cat}/K_m) of the reactions was determined using kinetic analyses under single turnover conditions. Ribozyme R27-IE, V80-IE, and V188-IE were about 90, 8, and 16 times more active than M1-IE that was derived from the wild type M1 RNA sequence (Table I). These observations indicate that ribozyme variants selected for their efficient activities in cleaving TK mRNA are also very active in cleaving IE mRNA in vitro, suggesting that the selected R27 domain can be used for the construction of ribozymes that are highly efficient in cleaving other mRNAs. Ribozyme R27-IE still exhibited at least 80-fold higher cleavage activity than M1-IE when a 450-nucleotide-long substrate of IE2 mRNA sequence was used (data not shown). This substrate includes the ie37 sequence and, in addition, contains a 150-nucleotide-long 5’ leader and a 287-nucleotide-long 3’ tail sequence. These results suggest that R27-IE may also cleave the full-length IE1 and IE2 mRNAs more efficiently than M1-IE.

Ribozyme Variant Increases Its Activity by Enhancing the Rate of Cleavage.—The values of (k_{cat}/K_m) obtained under single turnover conditions reflect the rates of substrate binding and chemical cleavage of the phosphodiester linkage. Accordingly, experiments were carried out with the variant to determine whether a change in the rates of these steps contributed to the increased (k_{cat}/K_m) values. To determine whether the mutations affect the rate of chemical cleavage, ie37 was allowed to form active complexes with the ribozymes in the presence of divalent ions, and the apparent reaction rate constant, k_{app}, for the complexes was measured. CaCl_2 was used instead of MgCl_2 as the source of divalent ions, in order to reduce the rate of cleavage while allowing proper folding of the ribozymes and substrates and preserving the interactions between the ribozyme and the substrate in an active ribozyme-substrate complex. In the presence of CaCl_2, M1 RNA cleaves a pre-tRNA substrate at least 50 times slower than it does in the presence of MgCl_2 (26, 27, 41, 42). Moreover, the rate of cleavage of ie37 was at least 200-fold slower in the presence of CaCl_2 than in the presence of MgCl_2 (data not shown). Meanwhile, nucleosceptide footprint analyses and UV cross-linking studies indicate that the regions of the ribozymes that potentially interact with the substrates in the presence of Ca^{2+} ions are similar to those found in the presence of Mg^{2+} ions, suggesting that the interactions between the substrates and ribozymes are similar in the presence of these different divalent ions (26, 27, 41, 42). 

To assay the values of k_{app}, equimolar amounts of ribozymes and ie37 were mixed in the presence of CaCl_2, and the ribozyme-substrate complexes were separated from the unbound substrates using G-50 Sephadex columns. The ribozyme-ie37 complexes were then diluted in different concentrations and further incubated in the presence of 100 mM MgCl_2 to allow cleavage. The k_{app} values were independent of the concentrations of the complexes within the tested range of 2–70 nM (Table II). These results suggest that substrate cleavage takes place predominantly in the bound complexes (cis-cleavage) as observed previously (25, 26). The values of k_{app} for the selected variants were at least 1 × 10^5-fold higher than those of C-IE (Table II). Moreover, the values of k_{app} for V188-IE were similar to those of M1-IE, suggesting that this variant exhibits a similar rate of chemical cleavage as M1-IE. Meanwhile, the values of k_{app} for R27-IE and V80-IE were about 10 times higher than those of M1-IE (Table II). V80-IE only contains the mutation U_{80}→C_{80}, which is also found in R27-IE. These results suggest that this mutation is responsible for the increased cleavage activity of V80-IE. A “rescued” ribozyme, rV80-IE, was derived from V80-IE, in which C_{80} was changed back to U_{80} and the wild type sequence was restored. Ribozyme rV80-IE exhibited similar activity as M1-IE (data not shown), suggesting that the mutated C_{80} functions to enhance the rate of cleavage (k_{app}) and is indeed responsible for the increased activity of V80-IE.

Table I

| Enzyme | Substrate | (k_{cat}/K_m)_s | K_a |
|--------|-----------|----------------|-----|
| M1-IE  | ie37      | 0.30 ± 0.07    | 33  |
|        | ie37-3    | 0.14 ± 0.04    | 98  |
| R27-IE | ie37      | 28.2 ± 3.5     | 0.005 ± 0.001 |
|        | ie37-3    | 1.1 ± 0.2      | 0.99 ± 0.21  |
| V80-IE | ie37      | 2.5 ± 0.4      | 0.32 ± 0.06  |
|        | ie37-3    | 1.2 ± 0.2      | 1.1 ± 0.2   |
| V188-IE| ie37      | 4.8 ± 0.8      | 0.006 ± 0.001 |
|        | ie37-3    | 0.16 ± 0.04    | 1.0 ± 0.2   |
| C-IE   | ie37      | <5×10^{-6}     | 0.35 ± 0.04 |
|        | ie37-3    | <5×10^{-6}     | 0.006 ± 0.002 |
| C-R27-IE| ie37     | <5×10^{-6}     | 0.006 ± 0.002 |

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ribozyme, rV80-IE, was derived from V80-IE, in which C_{80} was changed back to U_{80} and the wild type sequence was restored. Ribozyme rV80-IE exhibited similar activity as M1-IE (data not shown), suggesting that the mutated C_{80} functions to enhance the rate of cleavage (k_{app}) and is indeed responsible for the increased activity of V80-IE.

Ribozyme Variant Increases the Substrate Binding by Enhancing the Interactions to the 3’ Tail Sequence of the mRNA Substrate.—An increase in the overall cleavage rate (k_{cat}/K_m) of the variants may also be due to additional tertiary interactions between the ribozyme and the substrate. These interactions may result in better binding and docking of the substrate to the active site of the ribozyme. To determine whether this is the case, the binding affinities of the ribozymes to the IE mRNA sequence were determined by a gel-shift binding assay, in which ribozyme-substrate complexes were separated in polyacrylamide gels under non-denaturing conditions. To determine the binding affinity of the ribozymes to the substrate rather than to the product, the binding assays were carried out under the conditions that contain Ca^{2+} but not Mg^{2+}. During the binding assays in the presence of CaCl_2, the cleavage products were barely detected, although substantial amounts of products were found under the optimal cleavage conditions (e.g. 100 mM MgCl_2) (data not shown) (26, 27). The binding affinities of the ribozymes to the substrates, measured as the dissociation constant (K_d), were determined and are shown in Table I. V188-IE and R27-IE exhibited about 65-fold higher binding affinity to ie37 than M1-IE. Since V188-IE only contains mutation C_{188}→U_{188}, which is also found in R27-IE, it is likely that this mutation is responsible for
the observed increased affinity and activity of V188-IE. A ribozyme, rV188-IE, was derived from V188-IE, in which U188 was changed back to C188, and the wild type sequence was restored. Ribozyme rV188-IE exhibited similar affinity and activity as M1-IE (data not shown), suggesting that the mutated U188 functions to enhance substrate binding and is indeed responsible for the increased activity of V188-IE.

Nuclease footprint analyses and UV cross-linking studies showed previously (24, 25, 27) that different regions of the ribozymes potentially interact with specific parts of an mRNA substrate. To determine further which part of the substrate is important for the increased binding affinity between the substrates and the ribozymes, binding assays were carried out using substrates ie37-3 and ie37-5, which were derived from ie37 by deleting the 5'H11032 leader and 3'H11032 tail sequence, respectively (Fig. 1B). The binding affinity of M1-IE to ie37-3 was three times weaker than its affinity to ie37 (Table I). The binding affinities of V188-IE and R27-IE to ie37-3, however, were about 190 times weaker than those to ie37. Indeed, the $K_d$ values of V188-IE and R27-IE to ie37-3 were similar to those of M1-IE. Meanwhile, the 5' leader sequence appears to be not important for the increased binding affinity of the variants since R27-IE and V188-IE still exhibit 60 times higher binding affinity to ie37-5 than M1-IE (data not shown). These results suggest that the presence of the 3' tail sequence contributes to the increased binding affinity, and deleting this sequence results in the loss of the increased affinity.

The results from kinetic analyses of the cleavage of ie37-3 by the ribozymes further support the notion that the variants strengthen their substrate binding by enhancing their interactions with the 3' tail sequence of the substrate. V188-IE did not exhibit higher activity (($k_{cat}/K_m$) than M1-IE in cleaving ie37-3, whereas this variant was about 15 times more active in cleaving ie37 than M1-IE (Table I). Cleavage of this substrate by V188-IE and R27-IE was about 26 times slower than their engineer...
The ribozyme-ie37 complexes were separated from the unbound substrates by Sephadex G-50 gel filtration columns and diluted in different concentrations before the rate of cleavage was assayed. The cleavage products were separated on 15% polyacrylamide gels and quantitated with a STORM840 PhosphorImager.

| Concentration | C-IE | M1-IE | V80-IE | V188-IE | R27-IE |
|----------------|------|-------|--------|---------|--------|
| nM             |      |       |        |         |        |
| 70             | $<5 \times 10^{-4}$ | 0.41 ± 0.11 | 4.1 ± 0.7 | 0.47 ± 0.10 | 4.4 ± 0.8 |
| 48             | $<5 \times 10^{-4}$ | 0.45 ± 0.12 | 4.2 ± 0.6 | 0.42 ± 0.10 | 4.7 ± 0.9 |
| 12             | $<5 \times 10^{-4}$ | 0.38 ± 0.11 | 3.6 ± 0.7 | 0.40 ± 0.11 | 4.0 ± 0.7 |
| 6              | $<5 \times 10^{-4}$ | 0.42 ± 0.10 | 4.1 ± 0.7 | 0.39 ± 0.08 | 4.2 ± 0.8 |
| 2              | $<5 \times 10^{-4}$ | 0.40 ± 0.09 | 3.9 ± 0.6 | 0.43 ± 0.10 | 4.5 ± 0.7 |

The apparent rate constants ($k_{app}$) of the ribozyme-substrate complexes

The highly active R27-IE and V188-IE as well as M1-IE and C-IE were cloned into the retroviral LXSN vector and placed under the control of the small nuclear U6 RNA promoter. This promoter, from which transcripts are highly expressed and placed under the control of the small nuclear U6 RNA promoter, has been shown previously to be expressed and primarily localized in the nucleus, is transcribed by RNA polymerase III and has been shown previously to be transcribed from the region of the IE1 and IE2 mRNAs (Fig. 4).

To determine the efficacy of the ribozymes in reducing IE1/IE2 expression, cells were infected with HCMV at an m.o.i. of 0.05–1. Total RNAs were isolated from cells that were pretreated with 100 µg/ml cycloheximide and then were either mock-infected or infected with HCMV. Under this condition, only viral IE mRNAs were synthesized (1). The levels of the IE1 and IE2 mRNAs (Fig. 4B) in the infected cells were determined by Northern analyses, using the level of the 5-kb-long viral immediate-early transcript as the internal control (Fig. 4A). The viral 5-kb RNA is transcribed from the region covering HCMV open reading frames between UL106 and UL110 (33). The expression of this RNA is not regulated by IE1/IE2 under the assay conditions (33). A reduction of about 99, 93, and 80% (average of three experiments) in the levels of IE1 and IE2 mRNA expression was observed in cells that expressed R27-IE (lanes 2 and 7), V188-IE (lanes 3 and 8), M1-IE (lanes 4 and 9), and C-IE (lanes 5 and 10). Equal amounts of each RNA sample (30 µg) were separated on 1 (A) or 2.5% (B) agarose gels that contained formamide, transferred to a nitrocellulose membrane, and hybridized to a 32P-radiolabeled probe that contained the DNA sequence coding for actin (A) or M1 RNA (B). The actin mRNA expression (A) was used as the internal control.

The levels of IE1 and IE2 proteins in M1GS-expressing cells are expected to be lowered due to reduced levels of IE1/IE2 mRNAs. Proteins were isolated from cells at 24–48 h post-infection, separated in SDS-polyacrylamide gels, and transferred to membranes. One membrane was stained with an anti-IE1/IE2 antibody (anti-IE1/IE2) (Fig. 5B) and another membrane with a monoclonal antibody against human actin (anti-actin) (Fig. 5A). The latter serves as an internal control for the quantitation of IE1/IE2 protein expression. The results of three independent experiments are summarized in Table III; a reduction of about 98, 93, and 80% in the level of IE1 and IE2 proteins was observed in cells that expressed R27-IE, V188-IE, and M1-IE RNA, respectively. In contrast, a reduction of less than 10% was found in cells that expressed C-IE, C-V188-IE, and C-R27-IE RNAs.

![Expression of M1GS ribozymes in human cells detected by Northern analysis.](image-url)
The inhibition of viral early and late gene expression in the cells (Fig. 4, Figs. 4 and 5 and Table III). These results suggest an overall inhibition of viral early and late gene expression in the cells that expressed C-IE, C-V188-IE, C-R27-IE, M1-IE, V188-IE, or R27-IE, as compared with the levels of inhibition in cells that did not express a ribozyme (U373MG).

The values shown are the means from triplicate experiments. The values of standard deviation for these results are less than 5%.

**TABLE III**

| Viral gene class | Ribozyme |
|------------------|----------|
|                  | U373MG   | C-IE     | C-V188-IE | C-R27-IE | M1-IE | V188-IE | R27-IE |
| IE1 mRNA         | α        | 0        | 5         | 8        | 8     | 80      | 94     | 99     |
| IE2 mRNA         | β        | 0        | 4         | 9        | 7     | 80      | 93     | 99     |
| US2 mRNA         |          | 0        | 2         | 2        | 2     | 75      | 92     | 97     |
| IE1/IE2 protein  | α        | 0        | 2         | 7        | 7     | 80      | 93     | 98     |
| UL44 protein     | β,γ      | 0        | 0         | 2        | 1     | 76      | 92     | 98     |
| US2 protein      | γ        | 0        | 0         | 0        | 0     | 74      | 91     | 98     
| Glycoprotein     |          | 0        | 0         | 0        | 0     | 75      | 92     | 98     |

Ribozyme Variants Are More Effective in Inhibiting Viral Gene Expression and Growth—Inhibition of IE1 and IE2 expression is expected to result in a reduction of the expression of both viral early (β) and late (γ) genes (1, 36, 37). To determine whether this is the case, cells were infected with HCMV at an m.o.i. of 0.1–1 for 48–72 h. The level of the US2 mRNA (an early mRNA) (Fig. 4D) as well as the protein levels of UL44 (an early and late protein), UL83 (a late protein), and γH (a late protein) (Fig. 5, C and D, data not shown) were determined. The level of the HCMV 5-kb transcript and the protein level of human actin were used as the internal controls. A reduction of about 97–98%, 91–92, and 74–76% in the expression levels of these genes was observed in cells that expressed R27-IE, V188-IE, and M1-IE RNA, respectively. No significant reduction was detected in cells that expressed C-IE, C-V188-IE, and C-R27-IE (Figs. 4 and 5 and Table III). These results suggest an overall inhibition of viral early and late gene expression in the cells that expressed M1-IE, V188-IE, and R27-IE.

To determine whether viral growth was blocked in the ribozyme-expressing cells, cells were infected with HCMV at an m.o.i. of 0.5–2. Virus stocks were prepared from the infected cultures at 1-day intervals through 7 days postinfection, and the count of plaque-forming unit was determined by measuring the viral titer in human fibroblasts. After 5 days of postinfection, a reduction of about 10,000-, 1000-, and 150-fold in viral yield was observed in cells that expressed R27-IE, V188-IE, and M1-IE, respectively (Fig. 6). No significant reduction was found in those cells that expressed the control ribozymes C-IE, C-V188-IE, or C-R27-IE (Fig. 6, data not shown).

It has been shown that cloned U373MG cells exhibit clonal variability in their susceptibility to HCMV infection (1). To exclude the possibility that the observed inhibition of viral gene expression and growth is due to the variability of the particular cloned U373MG cells used in the experiments rather than the ribozyme-mediated inhibition, two additional sets of cloned cells that expressed different ribozymes were used to assay the efficacy of the ribozymes in inhibiting HCMV gene expression and growth. Moreover, three additional cloned cell lines that expressed each of the ribozymes were mixed and used as the oligoclonal cell populations. The antiviral efficacies of this set of oligoclonal cell populations were also determined. The levels of inhibition of viral gene expression and growth in these cell lines are similar to the levels observed in the set of cloned cell lines described in Table III and Fig. 6. These results indicate that the observed inhibition of viral gene expression and growth is due to the ribozyme-mediated cleavage but not the clonal variability of the susceptibility of the U373MG cells to HCMV infection.
DISCUSSION

Ribozymes have been shown to be promising antiviral agents for inhibition of viral gene expression and replication (3, 4). The M1GS-based technology represents an attractive approach for gene inactivation since it generates catalytic and irreversible cleavage of the target RNA by using M1 RNA, a highly active RNA enzyme found in nature (9, 10). M1 RNA may further increase its activity in cultured cells by interacting with the cellular proteins (9, 20). These properties, as well as the simple design of the guide sequence, make M1GS an attractive and unique gene-targeting agent that can be used for antiviral as well as other in vivo applications.

Our previous studies indicate that expression of an M1GS RNA derived from the wild type M1 RNA sequence inhibits HCMV gene expression by 75–80% and reduces viral growth by 150-fold in cultured cells (23). Understanding the mechanism of ribozyme cleavage and improving its efficacy is essential in developing the M1GS ribozyme for practical anti-HCMV applications. However, little is currently known about the rate-limiting step of the 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, R27-IE, exhibited 90 times higher rate of cleavage in vitro in cleaving the IE1/IE2 mRNA sequence than the ribozyme (i.e. M1-IE) derived from the wild type M1 RNA sequence. Moreover, R27-IE inhibited the expression of IE1 and IE2 in cultured cells by more than 98% and was more effective in cultured cells than M1-IE, which reduced IE1 and IE2 expression by about 80% (Table III). A reduction of about 10,000-fold in viral growth was observed in the R27-IE-expressing cells, whereas a reduction of about 150-fold was observed in M1-IE-expressing cells (Fig. 6). In contrast, a reduction of less than 10% in the levels of IE1/IE2 expression and viral growth was observed in cells that expressed C-IE, C-V188-IE, or C-R27-IE. Ribozymes C-IE, C-V188-IE, and C-R27-IE had the identical guide sequence and
exhibited similar binding affinity to ie37 as M1-IE, V188-IE, and R27-IE, respectively, but was catalytically inactive due to the presence of the mutations at the catalytic domain (Fig. 2 and Table I). Moreover, similar levels of inhibition of viral gene expression and growth were observed among different sets of clonal cell lines and oligoclonal cell populations. These results suggest that the overall observed inhibition with R27-IE, V188-IE, and M1-IE was primarily due to targeted cleavage by these ribozymes as opposed to the antisense effect or other nonspecific effects of the guide sequence or the variability of susceptibility of the clonal U373MG cells to HCMV infection.

Little is currently known about the rate-limiting step of M1GS RNA in achieving optimal anti-CMV efficacy in vivo. In this study, we constructed M1GS RNAs to target an accessible region of IE1 and IE2 mRNAs. Moreover, M1GS RNAs were expressed primarily in the nuclear compartment by the small U6 nuclear RNA promoter. This design would increase the probability for the ribozymes to locate and bind to their target mRNA sequence. Under the described settings, we hypothesized that the efficacy of RNase P ribozyme cleavage in cultured cells is dictated by its catalytic efficiency (kcat/Km). If this is the case, increasing the cleavage activity of RNase P ribozymes may lead to a more effective inhibition of the expression of the target mRNA in vitro. Our results indicate that V188-IE and R27-IE, which are more efficient in cleaving IE mRNA in vitro, are also more effective in inhibiting IE1/IE2 expression and viral growth in cultured cells than M1-IE, the ribozyme derived from the wild type M1 sequence. Moreover, the ribozyme (R27-IE) that exhibited higher cleavage activities (kcat/Km) appeared to be more effective in cell culture. These results suggest that substrate binding and chemical cleavage are the rate-limiting steps for M1GS ribozyme in cell culture and that increasing the catalytic efficiency of RNase P ribozymes, by enhancing the substrate binding and/or the rate of cleavage, may lead to improved efficacy in inhibiting gene expression in cultured cells. The difference between the in vitro efficacies of the selected variant and M1-IE (e.g. 99 versus 80%) appeared to be more limited than that of the in vitro cleavage efficiencies (about 90-fold difference). One of the possible explanations is that about 1–2% of the target mRNA may not be accessible to ribozyme binding, possibly due to its rapid transport to the cytoplasm.

The targeting activity of M1GS ribozymes appears to be highly specific. More importantly, the increased efficacy of the selected variant in inhibiting HCMV gene expression and growth in cultured cells is due to its increased activity in cleaving the IE1/IE2 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 3 months (data not shown). We found no difference in the expression level of actin mRNA in the ribozyme-expressing cells and the parental U373MG cells. Second, the antiviral effect of the ribozyme (inhibition of viral growth) appears to be due to the reduction of the IE1 and IE2 expression. This is because the expression of the viral early and late genes examined, including US2, UL44, UL83, and gH, was found to be significantly reduced in cells that expressed R27-IE, V188-IE, and M1-IE but not in those that expressed C-R27-IE, C-V188-IE, and C-IE (Figs. 4 and 5 and Table III, and 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 IE1 and IE2 expression. Meanwhile, no reduction in the expression levels of other viral immediate-early genes examined (e.g. 5-kb RNA and UL36) was found in M1GS-expressing cells (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 IE1 and IE2 expression, overall expression of viral early and late genes, as well as reducing viral growth.

Our results provide the first direct evidence that mutation U188→C188 increases the rate of chemical cleavage of an mRNA substrate. We also show that mutation C188→U188 enhances the binding of the 3′ tail sequence to the ribozyme. C188 is not in the conserved ribozyme regions (9, 10), and very little is currently known about its function in RNase P ribozyme catalysis (Fig. 1C) (14–17). Several lines of evidence presented in our study strongly suggest that the presence of U188 enhances the binding of the ribozyme to the 3′ tail sequence of the substrate. First, V188-IE but not a rescued mutant was more active in cleaving ie37 than M1-IE. This ribozyme variant also exhibited higher affinity to ie37 but not a higher apparent rate of cleavage (kcat) than M1-IE (Tables I and II). Thus, the mutation (C188→U188) appears to be responsible for the increased substrate binding and cleavage efficiency (kcat/Km). Moreover, cleavage and binding assays with substrate ie37-3 suggest that the presence of the 3′ tail sequence contributes to the increased binding affinity and cleavage activity (kcat/Km), and deleting this sequence results in the loss of the increased affinity and cleavage efficiency (Table I). Finally, nuclease footprint analyses and UV cross-linking studies suggest that C188 is in close proximity to the 3′ tail region of an mRNA model substrate (25, 27). Thus, it is likely that this nucleotide enhances binding of the ribozyme to the 3′ tail sequence of the ie37 substrate.

Ribozyme technology represents an attractive approach for gene inactivation since it exhibits most of the properties of conventional antisense targeting methods and, in addition, catalytic and irreversible cleavage of the target RNA. In vitro selection (47–49) 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). By using an RNase P ribozyme variant selected from a pool of M1 molecules containing randomized sequences, in this study we provide the direct evidence that RNase P variants with increased cleavage activities in vitro also exhibit improved efficacy in inhibiting HCMV gene expression and growth in cultured cells. Thus, our study may provide a direction for engineering highly active RNase P ribozyme variants and improving the efficacy of the M1GS-based technology by carrying out selection procedures. 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 the construction of highly effective ribozymes for gene-targeting applications, including the treatment and prevention of HCMV infections.

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