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Engineered external guide sequences are highly effective in inducing RNase P for inhibition of gene expression and replication of human cytomegalovirus

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ABSTRACT

External guide sequences (EGSs), which are RNA molecules derived from natural tRNAs, bind to a target mRNA and render the mRNA susceptible to hydrolysis by RNase P, a tRNA processing enzyme. Using an *in vitro* selection procedure, we have previously generated EGS variants that efficiently direct human RNase P to cleave a target mRNA *in vitro*. In this study, a variant was used to target the overlapping region of the mRNAs encoding human cytomegalovirus (HCMV) essential transcription regulatory factors IE1 and IE2. The EGS variant was ~25-fold more active in inducing human RNase P to cleave the mRNA *in vitro* than the EGS derived from a natural tRNA. Moreover, a reduction of 93% in IE1/IE2 gene expression and a reduction of 3000-fold in viral growth were observed in HCMV-infected cells that expressed the variant, while cells expressing the tRNA-derived EGS exhibited a reduction of 80% in IE1/IE2 expression and an inhibition of 150-fold in viral growth. Our results provide the first direct evidence that EGS variant is highly effective in blocking HCMV gene expression and growth and furthermore, demonstrate the feasibility of developing effective EGS RNA variants for anti-HCMV applications by using *in vitro* selection procedures.

INTRODUCTION

Human cytomegalovirus (HCMV), a ubiquitous herpesvirus, is an important opportunistic pathogen affecting individuals whose immune functions are compromised or immature (1,2). This virus is a leading cause of retinitis-associated blindness and other debilitating conditions such as pneumonia and enteritis among AIDS patients (3,4). Moreover, HCMV causes mental and behavioral dysfunctions in children that were infected *in utero* (2). Development of effective antiviral compounds and approaches is crucial in controlling HCMV infections and preventing HCMV-associated complications.

Nucleic acid-based gene interference technologies represent promising gene-targeting strategies for specific inhibition of mRNA sequences of choice (5,6). For example, ribozymes have been shown to cleave viral mRNA sequences and inhibit viral replication in human cells (7–9). More recently, small interfering RNAs are effective in inducing endogenous RNase of the RNA-induced silencing complex in the RNA interference pathway to inhibit gene expression and growth of several human viruses (5,10,11). Thus, nucleic acid-based gene interference approaches can be used as a tool in both basic and clinical research, such as in studies of tumorogenesis and antiviral gene therapy.

RNase P is a ribonucleoprotein complex and is responsible for the 5′ maturation of tRNAs (12,13). In *Escherichia coli*, this enzyme consists of a catalytic RNA subunit (M1 RNA) and a protein subunit (C5 protein) (14). It catalyzes a hydrolysis reaction to remove a 5′ leader sequence from tRNA precursors (ptRNA). Studies on RNase P substrate recognition revealed that the enzyme recognizes the structure rather than the primary nucleotide sequence of the substrates, and can cleave a model substrate that contains a structure equivalent to the acceptor stem, the T-stem, the 3′ CCA sequence and the 5′ leader sequence of a ptRNA molecule (Figure 1A) (13). Thus, RNase P can cleave an mRNA sequence if the mRNA substrate forms a hybrid complex with a custom-designed sequence [external guide sequence (EGS)] to resemble a ptRNA molecule (Figure 1B–D) (15,16). In this reaction, the EGS binds to its mRNA substrate through base pairing interactions and then guides RNase P to cleave the mRNA

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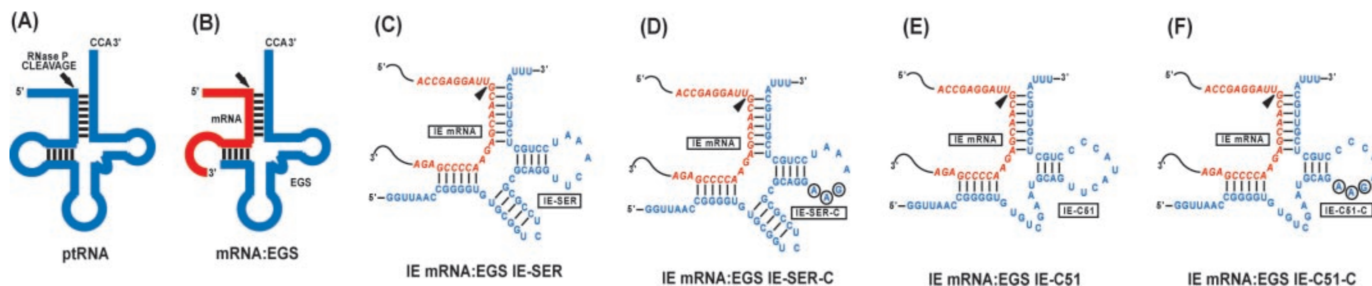

Figure 1. Schematic representation of substrates for RNase P. (**A**) A natural substrate (ptRNA). (**B**) A hybridized complex of a target RNA (e.g. mRNA) and an EGS that resembles the structure of a tRNA. (**C–F**) Complexes between IE mRNA sequence and EGS IE-SER, IE-SER-C, IE-C51 and IE-C51-C, respectively. The sequences of IE-SER and IE-SER-C that were equivalent to the T-stem and loop, and variable region of a tRNA molecule were derived from $tRNA^{ser}$, while those of IE-C51 and IE-C51-C were from EGS variant C51. Only the exact sequence of the IE mRNA around the targeting site is shown (red). The EGS sequence is shown in blue color. The site of cleavage by RNase P is marked with an arrowhead.

substrate. EGS RNAs derived from natural tRNA sequences have been shown to be effective in blocking gene expression in bacteria and in mammalian cells (16–19). We have shown previously that EGSs that were derived from a natural tRNA effectively induced human RNase P to cleave the mRNA of thymidine kinase (TK) of herpes simplex virus 1 (HSV-1) *in vitro* (19,20). A reduction of ~75% in the expression of TK mRNA and protein was observed in HSV-1-infected cells that expressed these functional EGS RNAs.

Targeted cleavage of mRNA by human RNase P provides a unique approach to inactivate any RNA of known sequence expressed *in vivo*. Increasing the *in vitro* efficiency of the EGS-induced RNase P cleavage as well as its efficacy *in vivo* is required in order to develop EGSs 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 recently isolated novel EGS variants that direct RNase P to cleave TK mRNA *in vitro* more efficiently than those derived from a natural tRNA sequence (20). Little is currently known about how these EGS RNA variants increase their activity in directing RNase P to cleave a target mRNA. Equally unclear is whether the EGS RNAs are effective in blocking HCMV gene expression and replication. In this study, one of these EGS variants was used to target the overlapping region of the mRNAs encoding HCMV essential immediately-early (IE) proteins IE1 and IE2, which are the viral major transcriptional activators responsible for activation of viral gene expression (1). We investigated the activity of the EGS in inducing RNase P to cleave the target mRNA and its efficacy in inhibiting HCMV gene expression and growth in cultured cells. The EGS variant, IE-C51, was ~25-fold more active in directing RNase P to cleave the target mRNA than IE-SER, the EGS derived from a natural tRNA sequence. When expressed in cultured cells that were infected by HCMV, IE-C51 was more effective in inhibiting viral gene expression and growth than IE-SER. A reduction of >93% in the IE1 and IE2 expression and an inhibition of at least 3000-fold were observed in cells that expressed IE-C51. In contrast, a reduction of <10% in viral gene expression and growth was observed in cells that either did not express an EGS or expressed EGSs that contained point mutations abolishing their ability to induce RNase P-mediated cleavage. Our results provide the first direct evidence that engineered EGS RNAs are highly effective in blocking HCMV gene expression and growth. These results also demonstrate the potential of generating highly active EGS variants and using them as a research tool and as a therapeutic agent for gene-targeting applications.

MATERIALS AND METHODS

Viruses, cells and antibodies

HCMV (strain AD169) was propagated in human foreskin fibroblasts and astrocytoma U373MG cells in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) as described previously (21,22). The monoclonal antibodies c1202, c1203 and c1207, which react with HCMV proteins UL44, IE1/IE2 and UL99, respectively, were purchased from Goodwin Institute for Cancer Research (Plantation, FL). The monoclonal antibodies against HCMV gH and human actin were purchased from Biodesign Inc (Kennebunk, Maine) and Sigma Inc (St Louis, MO), respectively.

EGS and IE mRNA substrate

The DNA sequence coding for EGS IE-SER was generated by PCR using construct pTK112 (19) as the template with 5′ primer oligoIE31 (5′-GGAATTCTAATACGACTCACTA-TAGGTTAACCGGGGTGTGCGGTCTCC-3′) and 3′ primer oligoIE32 (5′-AAGCTTTAAATGCAACGAGCAGGATTT-GAACCTGCGCGCG-3′). The DNA sequence coding for IE-C51 was generated by PCR using construct pTKC51 (20) as the template with 5′ primer oligoIE41 (5′-GGAATTC-TAATACGACTCACTATAGGTTAACCGGGGTGTGTC-GAATGCAG-3′) and 3′ primer oligoIE42 (5′-AAGCTTTA-AATGCAACGAGCAGGGGTATGAACTGCATTCGA-3′). The DNA sequences coding for EGS IE-SER-C and IE-C51-C were derived from those for IE-SER and IE-C51, respectively, and contained point mutations (5′-TTC-3′ → AAG) at the three highly conserved positions in the T-loop of these EGSs (Figure 1D and F). These EGS DNA sequences were under the control of the T7 RNA polymerase promoter. The DNA template for transcription *in vitro* of RNA substrate ie37 was constructed by annealing the T7 promoter-containing oligonucleotide OliT7 (5′-TAATACGACTCACTATAG-3′) with oligonucleotide sIE1 (5′-CGGGATCCTTTCTCGGGG-TTCTCGTTGCGATTCCCGGTCCTATAGTGAGTCGTA-TTA-3′). RNA substrate ie37 was synthesized *in vitro* from the constructed DNA template using T7 RNA polymerase.

Analysis of *in vitro* binding and cleavage of IE mRNA

Human RNase P was prepared from HeLa cellular extracts as described previously (16,19,20). The EGSs and [^{32}P]-labeled ie37 were incubated with human RNase P at 37°C in buffer A (50 mM Tris, pH 7.4, 100 mM NH_4Cl and 10 mM $MgCl_2$). Cleavage products were separated in denaturing gels and analyzed with a STORM840 phosphorimager (Molecular Dynamics, Sunnyvale, CA). Assays to determine kinetic parameters were performed under multiple-turnover conditions, as described previously (20,23). In brief, the cleavage of substrates was assayed in buffer A at various concentrations of substrates, both above and below the K_m for the substrate. The amount of substrates was in large excess to that of the enzymes in order to assure that saturation with the substrate was achieved in the multiple-turnover conditions. Aliquots were withdrawn from reaction mixtures at regular intervals and analyzed in polyacrylamide urea gels, and the values of $K_{m(apparent)}$ and $V_{max(apparent)}$ were obtained from Lineweaver–Burk double-reciprocal plots (20,23).

The procedures to measure the equilibrium dissociation constants (K_d) of complexes of the EGSs and the substrates were modified from Pyle *et al*. (24). In brief, using a gel-shift approach, various concentrations of EGSs were preincubated in buffer B (50 mM Tris, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 3% glycerol, 0.1% xylene cyanol and 0.1% bromophenol blue) for 10 min before mixing with an equal volume of different concentrations of substrate RNA preheated under identical conditions. The samples were incubated for 10–120 min to allow binding, then loaded on a 5% polyacrylamide gel, and run at 10 W. The electrophoresis running buffer contained 100 mM Tris–HEPES, pH 7.5 and 10 mM $MgCl_2$ (24). The value of K_d was then extrapolated from a graph plotting percent of product bound versus EGS concentration (20). The values were the average of three experiments.

Construction of the EGS-expressing cell lines

The DNA sequences coding for the EGSs were subcloned into retroviral vector LXSN and placed under the control of the U6 RNA promoter (25,26). The protocols to construct EGS-expressing cell lines were modified from Miller and Rosman (25). In brief, the retroviral vector DNAs that contained the EGS sequence were transfected into amphotropic PA317 cells using a mammalian transfection kit purchased from Gibco BRL (Grand Island, NY). After 48 h post transfection, culture supernatants that contained retroviruses were collected and used to infect human U373MG cells. At 48–72 h postinfection, neomycin (Gibco BRL, Grand Island, NY) 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.

Viral infection and preparation of RNA and protein extracts

The 1×10^6 cells were either mock-infected or infected with HCMV at a multiplicity of infection (MOI) of 0.05–1 in an inoculum of 1.5 ml DMEM supplemented with 1% fetal calf serum. The inoculum was replaced with DMEM supplemented with 10% FBS after 2 h incubation with cells. The infected cells were incubated for a certain period of time (as stated in Results) before harvesting for viral mRNA or protein isolation. To measure the levels of viral IE transcripts, some of cells were also treated with 100 μg/ml cycloheximide prior to and during infection. Total cellular RNA and protein samples were prepared from the cells as described previously (21).

Studies of the expression of EGS RNAs and viral genes, and inhibition of viral replication

Northern analyses were used to determine the expression levels of EGS RNAs and viral mRNAs. The RNA fractions were separated in 0.8–2.5% agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the [^{32}P]-radiolabeled DNA probes that contained the HCMV or human β-actin DNA sequences, and analyzed with a STORM840 phosphorimager. The radiolabeled DNA probes used to detect EGS RNAs, human β-actin mRNA, HCMV IE 5 kb RNA transcript, IE1 mRNA, IE2 mRNA and US2 mRNA were synthesized using a random primed labeling kit (Boehringer Manheim, Indianapolis, IN).

Western analyses were performed to determine the expression level of viral proteins. The polypeptides from cell lysates were separated on either 7.5 or 9% [vol/vol] SDS–polyacrylamide gels cross-linked with *N*,*N''*-methylenebisacylamide, transferred electrically to nitrocellulose membranes, and reacted to the antibodies against HCMV proteins and human actin. The proteins on the membranes were subsequently stained using a western chemiluminescent substrate kit (Amersham Inc., Arlington Heights, IL) and quantified with a STORM840 phosphorimager. Quantification was performed in the linear range of RNA and protein detection.

To study the EGS-mediated inhibition of viral replication, 5×10^5 cells were infected with HCMV at an MOI of 0.5–2. The cells and medium were harvested at 1 day intervals throughout 7 days postinfection and viral stocks were prepared by adding an equal volume of 10% skim milk, followed by sonication. The titers of the viral stocks were determined by infecting 1×10^5 human foreskin fibroblasts and counting the number of plaques 10–14 days postinfection. The values obtained were the average from triplicate experiments.

RESULTS

In vitro studies of targeting activities of the EGS RNAs

IE1 and IE2 are the major transcriptional regulators required for the expression of viral early (β) and late (γ) genes (1). They share 85 N-terminal amino acids due to alternative splicing and polyadenylation of transcripts initiating at a strong promoter–enhancer (27). Therefore, targeting the overlapping region (e.g. exon 3) of the mRNAs coding for IE1 and IE2 should simultaneously shut down the expression of both proteins and may yield a more effective inhibition of viral replication. Since most mRNA species inside cells are associated with proteins and are present in a folded conformation, it is important to choose a target region that is accessible for EGS binding in order to achieve efficient targeting. We have used an *in vivo* mapping approach with dimethyl sulphate (DMS)

Table 1. Measurement of the kinetic parameters [$V_{max(apparent)}$, $K_{m(apparent)}$ and $V_{max(apparent)}/K_{m(apparent)}$] in the RNase P cleavage of ptRNA^{ser} or ie37 in the presence of different EGSs

Substrate	K_m (μM)	$V_{max(apparent)}$ (pmol min^{-1})	$V_{max(apparent)}$ /$K_{m(apparent)}$ (pmol μM^{-1} min^{-1})	K_d (μM)
ptRNA^{Ser}	0.014 ± 0.003	0.040 ± 0.010	2.9 ± 0.5	
IE mRNA (ie37)				
+IE-SER	0.60 ± 0.09	0.036 ± 0.015	0.06 ± 0.02	1.2 ± 0.3
+IE-SER-C	ND	ND	<0.001	1.3 ± 0.2
+IE-CSI	0.42 ± 0.08	0.63 ± 0.11	1.5 ± 0.3	0.040 ± 0.006
+IE-CSI-C	ND	ND	<0.001	0.045 ± 0.007

Multiple-turnover kinetic analyses to determine the values of $V_{max(apparent)}$ and $K_{m(apparent)}$ were carried out in buffer A (50 mM Tris, pH 7.4, 100 mM NH_4Cl and 10 mM $MgCl_2$) at 37°C, as described previously (20,23,37). The amounts of substrates were in large excess to that of the enzyme in order to assure that saturation with the substrate was achieved in the multiple-turnover conditions. To determine the binding affinity (K_d) between substrate ie37 and EGSs, binding assays were carried out in the absence of human RNase P in buffer B (50 mM Tris, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 3% glycerol, 0.1% xylene cyanol and 0.1% bromophenol blue), using a protocol modified from Pyle *et al.* (24). The values shown are the average derived from triplicate experiments.

(26,28) to determine the accessible regions of IE1 and IE2 mRNAs. A position, 146 nt downstream from the 5′ terminus of exon 3, was chosen as the cleavage site for human RNase P. The targeted region (designated as IE mRNA) is one of the sequences most accessible to DMS modification and is likely accessible to EGS binding (data not shown).

We have employed previously an *in vitro* selection procedure to isolate EGS RNA variants that are more efficient in directing human RNase P for cleavage of the TK mRNA sequence than the EGS derived from a natural tRNA sequence (20). However, little is known whether these variants are also highly active when they are used to target another mRNA. Whether the EGS RNAs can be used for effective inhibition of HCMV gene expression and growth has not been reported. To address these issues, we chose variant C51 for the study because the EGS RNAs derived from this variant are among the most active EGSs in inducing RNase P to cleave the TK as well as the IE1/IE2 mRNA sequences *in vitro* (see below, Table 1). By covalently linking the EGS domain of C51 to the targeting sequences that are complementary to the IE mRNA, we constructed EGS IE-C51, which resembles a part of a tRNA structure and contains a T-loop, a T-stem and a variable region but not the anticodon region that is dispensable for EGS activity (Figure 1E). Another EGS, IE-SER, which was derived from the natural tRNA^{ser} sequence, was also constructed in a similar way and included in the study (Figure 1C). The DNA sequences that code for the EGSs and are driven under the promoter of T7 RNA polymerase were generated by PCR using primers that contained the sequences complementary to the targeting region of IE mRNA. EGS RNAs were synthesized *in vitro* from these DNA sequences by T7 RNA polymerase and subsequently incubated with human RNase P and substrate ie37, which contains an IE mRNA sequence of 37 nt. IE-SER and IE-C51 efficiently directed human RNase P to cleave IE mRNA sequence *in vitro* as apparent cleavage of the substrate was observed in the presence of these EGSs (Figure 2, lanes 3–4). In contrast, no cleavage of ie37 by RNase P was observed in the absence of these EGSs (Figure 2, lane 1).

To further study the activity of the EGS in inducing RNase P to cleave IE mRNA sequence *in vitro*, detailed kinetic analyses for the reactions in the presence of different EGSs were carried out, and the values of $K_{m(apparent)}$ and $V_{max(apparent)}$ as well as the overall cleavage efficiency [$V_{max(apparent)}/K_{m(apparent)}$] for the cleavage reactions were determined. These results indicate

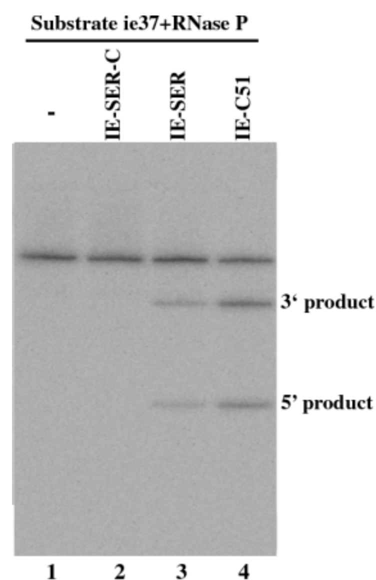

Figure 2. RNase P-mediated cleavage of ie37 in the presence of different EGS RNAs. No EGS was included in the reaction mixture in lane 1. An aliquot of 10 nM (lanes 2 and 3) and 5 nM of the EGS (lane 4) were incubated with ^{32}P-labeled ie37 RNA substrate (10 nM) and either 4 U (lanes 1–3) or 1 U (lane 4) of human RNase P at 37°C in a volume of 10 μl for 45 min in buffer A (50 mM Tris, pH 7.0, 100 mM NH_4Cl and 10 mM $MgCl_2$). Cleavage products were separated on 10% polyacrylamide gels that contained 8 M urea.

that IE-C51 was highly efficient in directing human RNase P to cleave ie37 and was at least 25-fold more active than IE-SER (Table 1). An increase in the cleavage rate of RNase P may be due to additional tertiary interactions that may potentially stabilize the mRNA–EGS complex. If this is the case, it is expected that binding affinity of the EGS variant (i.e. IE-C51) to the IE mRNA sequence may be better than that of the EGS (i.e. IE-SER) derived from the natural tRNA sequence. The binding affinities of EGS IE-C51 and IE-SER to substrate ie37, measured as the dissociation constant (K_d), were determined using gel-shift assays by separating substrate–EGS complexes in polyacrylamide gels under non-denaturing conditions. IE-C51 exhibited about 30 times higher binding affinity to ie37 than IE-SER (Table 1). Given the fact that both IE-C51 and IE-SER have the same antisense sequences (7 and 6 nt, respectively) to ie37 (Figure 1C and E), these results strongly suggest that the increased binding affinity and the stability of the substrate–EGS complex in the presence

IE-C51 is probably due to the additional tertiary interactions introduced by this EGS.

Intracellular expression of EGS RNAs in human cell culture

The DNA sequences coding for IE-C51 and IE-SER were subcloned into retroviral vector LXSN and placed under the control of the small nuclear U6 RNA promoter, which has been shown previously to express EGS RNA and other RNAs steadily (16,26,29). This promoter is transcribed by RNA polymerase III, and its transcripts are highly expressed and primarily localized in the nucleus (16,26,29).

Two additional EGSs, IE-C51-C and IE-SER-C, were also constructed and cloned under the control of the U6 RNA promoter. IE-C51-C and IE-SER-C were derived from IE-C51 and IE-SER, respectively, and contained point mutations (5′-UUC-3′ → AAG) at the three highly conserved positions in the T-loop of these EGSs (Figure 1D and F). These nucleotides were found in most of the known natural tRNA sequences (30) and are believed to be important for the interactions between the tRNA domains and human RNase P (13). Previous studies have shown that EGSs carrying these mutations precluded RNase P recognition and exhibited little activity in directing RNase P-mediated cleavage (20,23,31). Indeed, cleavage of ie37 by human RNase P in the presence of these two control EGSs was barely detected (Figure 2, lane 2 and data not shown) and was at least 1.5×10^3-fold slower than the cleavage in the presence of IE-C51 (Table 1). IE-C51-C and IE-SER-C contained the same antisense sequence to the IE mRNA sequence as IE-C51 and IE-SER (Figure 1D and F), and exhibited similar binding affinities to ie37 as IE-C51 and IE-SER, respectively, when assayed *in vitro* (Table 1). Therefore, IE-C51-C and IE-SER-C can be used as controls for the antisense effect of these EGSs.

To construct cell lines that express EGS RNAs, amphotropic packaging cells (PA317) were transfected with LXSN-EGS DNAs to produce retroviral vectors that contained the genes for EGS RNAs. Human U373MG cells were then infected with these vectors, and cells expressing the EGSs were cloned. To determine whether EGS RNA with an incorrect guide sequence could target the HCMV mRNA in tissue culture, we also constructed an additional cell line that expressed a control EGS TK112, which targeted TK mRNA of HSV-1 (19). The TK mRNA is not found in HCMV and, therefore, is expressed from a promoter different from those of HCMV IE1 and IE2 (1). No RNase P-mediated cleavage of substrate ie37 in the presence of TK112 was observed *in vitro* (data not shown). The level of EGS RNA expression in each individual cell clone was determined by northern analysis with DNA probes that are complementary to the EGSs, using the expression of human H1 RNA as the internal control (Figure 3). Figure 3 shows the result from cloned cell lines that expressed IE-SER, IE-SER-C, IE-C51 and IE-C51-C (lanes 2–5 and 6–10). 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 EGSs did not exhibit significant cytotoxicity. Only the cell lines that expressed similar levels of these EGS RNAs were used for further studies in tissue culture.

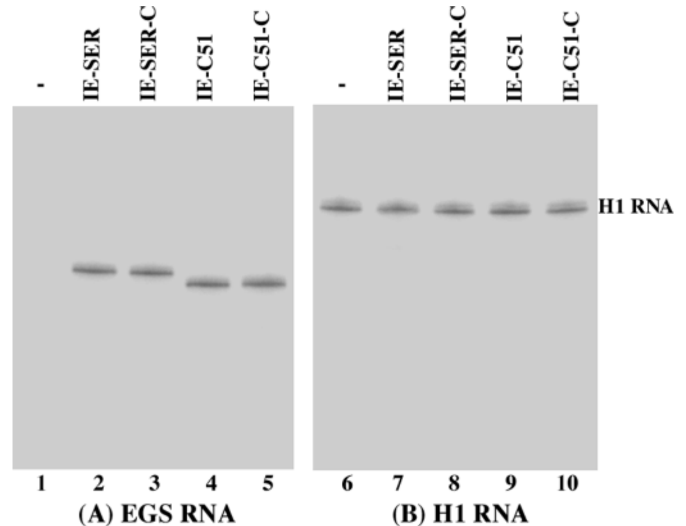

Figure 3. (**A** and **B**) Northern analyses of the expression of EGS RNAs from nuclear RNA fractions isolated from parental U373MG cells (dash, lanes 1 and 6) or a cloned cell line that expressed IE-SER (lanes 2 and 7), IE-SER-C (lanes 3 and 8), IE-C51 (lanes 4 and 9) and IE-C51-C (lanes 5 and 10). Equal amounts of each RNA sample (25 μg) were separated on 2% agarose gels that contained formaldehyde, transferred on to nitrocellulose membranes, and hybridized to ^{32}P-radiolabeled probes that contained the DNA sequence coding for IE-SER and IE-C51 (lanes 1–5) or H1 RNA (lanes 6–10), the RNA subunit of human RNase P and a nuclear RNA (13). The hybridized products corresponding to the full-length retroviral transcripts (∼6 kb), transcribed from the LTR promoter, are at the top of the gel and are not shown.

Inhibition of HCMV IE1/IE2 expression in EGS-expressing cells

To determine if the EGSs inhibited IE1 and IE2 expression, cells were infected with HCMV at a MOI of 0.05–1. Total RNAs were isolated from cells that were pretreated with 100 μg/ml of cycloheximide and then were either mock-infected or infected with HCMV. Under this condition, only viral IE mRNAs would be synthesized (1). The levels of IE1/IE2 mRNAs in the infected cells were determined by northern analyses. The level of the 5 kb long viral immediate-early transcript (5 kb RNA), which expression is not regulated by IE1/IE2 under the assay conditions (1,32), was used as an internal control for the quantification of expression of IE1 and IE2 mRNAs. Figure 4 shows the results (which are summarized in Table 2) of the northern analysis experiments with the IE1 (Figure 4B), IE2 (Figure 4C) and 5 kb RNA probes (Figure 4A). A reduction of ∼93 and 79% (average of three experiments) in the levels of IE1 and IE2 mRNA expression was observed in cells that expressed EGS IE-C51 and IE-SER, respectively (Figure 4, lanes 8–9 and 13–14). In contrast, cells that expressed IE-C51-C and IE-SER-C RNAs only exhibited a reduction of <10% (Figure 4, lanes 7 and 12) (Table 2). The low level of inhibition found in cells that expressed IE-C51-C and IE-SER-C RNAs was probably due to an antisense effect. This is because IE-C51-C and IE-SER-C, with the point mutations at the T-loop (Figure 1D and F), exhibited little targeting activity but bound to IE mRNA sequence as well as IE-C51 and IE-SER (Table 1). Thus, these observations suggest that the significant reduction of IE1 and IE2 mRNA expression in cells that expressed IE-C51 and IE-SER was due to the

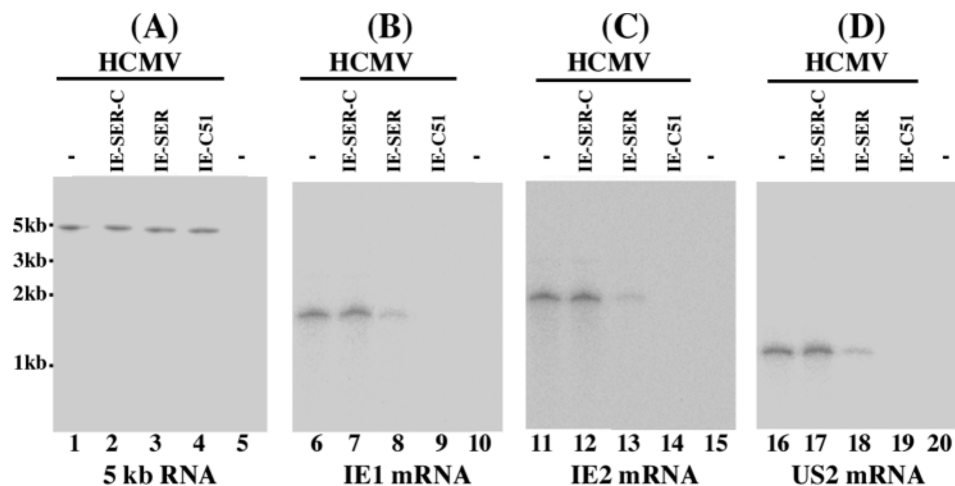

Figure 4. Levels of HCMV mRNAs as determined by northern analysis. Cells (1×10^6) were either mock-infected (lanes 5, 10, 15 and 20) or infected with HCMV (MOI = 1) (lanes 1–4, 6–9, 11–14 and 16–19) and were harvested at either 8 (**A–C**) or 24 h (**D**) postinfection. Viral infection was carried out either in the absence (**D**) or presence (**A–C**) of 100 µg/ml cycloheximide. Northern analyses were carried out using RNAs isolated from parental U373MG cells (dash, lanes 1, 5, 6, 10, 11, 15, 16 and 20) and cell lines that expressed IE-SER-C (lanes 2, 7, 12 and 17), IE-SER (lanes 3, 8, 13 and 18) and IE-C51 (lanes 4, 9, 14 and 19). Equal amounts of each RNA sample (35 µg) were separated on agarose gels that contained formaldehyde, transferred on to a nitrocellulose membrane, and hybridized to a ^{32}P-radiolabeled probe that contained the cDNA sequence of the HCMV 5 kb transcript (lanes 1–5), IE1 mRNA (lanes 6–10), IE2 mRNA (lanes 11–15) and US2 mRNA (lanes 16–20). The hybridized products corresponding to the 5 kb RNA, IE1, IE2 and US2 mRNAs were ~5, 1.9, 2.2 and 1.5 kb, respectively (1).

Table 2. Levels of inhibition of viral gene expression in the cells that expressed EGSs IE-C51, IE-C51-C, IE-SER, IE-SER-C and TK112, as compared with that in the parental U373MG cells that did not express an EGS (U373MG)

	Viral gene class	EGS RNA U373-MG (%)	TK112 (%)	IE-SER-C (%)	IE-C51-C (%)	IE-SER (%)	IE-C51 (%)
IE1 mRNA	α	0	1	6	7	80 ± 6	94 ± 6
IE2 mRNA	α	0	2	5	6	79 ± 7	93 ± 5
US2 mRNA	β	0	1	1	1	75 ± 6	92 ± 7
IE1/IE2 protein	α	0	2	5	6	78 ± 8	93 ± 6
UL44 protein	β, γ	0	1	2	1	74 ± 6	90 ± 5
gH	γ	0	0	1	1	73 ± 6	91 ± 5
UL99	γ	0	2	1	1	75 ± 6	91 ± 6

The values shown are the means from triplicate experiments and the values of SD that were <5% are not shown.

RNase P-mediated cleavage of the target mRNA directed by these EGSs. No products of the cleavage of IE1 and IE2 mRNAs were detected in our northern analyses presumably because these RNAs, which lacked either a cap structure or a polyA sequence, were rapidly degraded by intracellular RNases.

The levels of IE1 and IE2 proteins in the EGS-expressing cells are expected to reduce because of the decreased level of IE1/IE2 mRNAs. Proteins were isolated from cells at 24 h postinfection, separated in SDS–polyacrylamide gels, and transferred to two identical membranes. One membrane was stained with an anti-IE1/IE2 antibody (anti-IE1/IE2) (Figure 5B) and the other was stained with a monoclonal antibody against human actin (anti-Actin) (Figure 5A). The latter serves as an internal control for the quantification of IE1/IE2 protein expression. The results of three independent experiments are summarized in Table 2: a reduction of ~93 and 78% in the level of IE1 and IE2 proteins was observed in cells that expressed IE-C51 and IE-SER, respectively, while a reduction of <10% was found in cells that expressed IE-C51-C, IE-SER-C or TK112.

Inhibition of HCMV overall gene expression and growth in the EGS-expression cells

A reduction of IE1 and IE2 expression is expected to result in an inhibition of the expression of both viral early (β) and late (γ) genes (1). To determine whether this was the case, cells were infected with HCMV for 48–72 h. The levels of the US2 mRNA (an early mRNA) (Figure 4D) as well as the protein levels of UL44 (an early and late protein) and gH (a late protein) (Figure 5C–D) 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 ~90–92% and 73–75% in the expression levels of these genes was observed in cells that expressed IE-C51 and IE-SER RNA, respectively. No significant reduction was detected in cells that expressed IE-C51-C and IE-SER-C (Figures 4 and 5 and Table 2). Similar results were also observed in the study of the expression of UL99, a viral late protein (Table 2 and data not shown). These results suggest an overall inhibition of viral early and late gene expression in the cells that expressed IE-C51 and IE-SER.

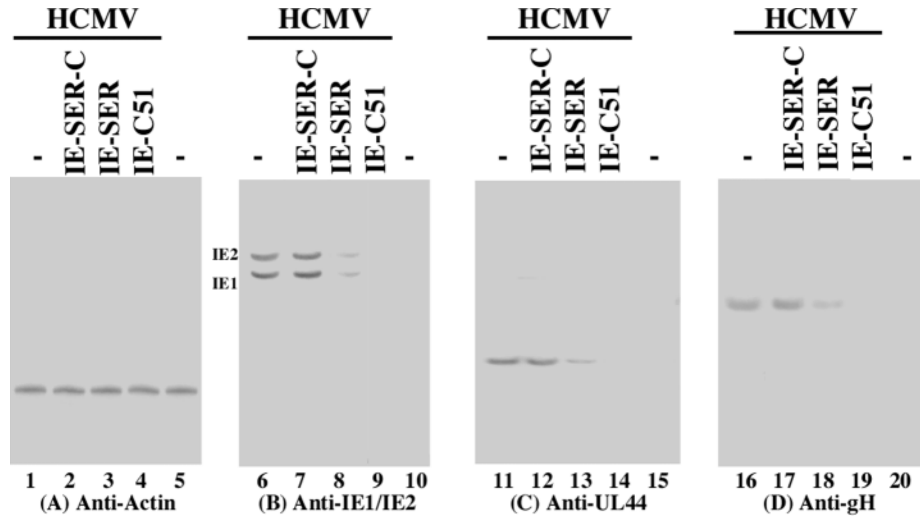

Figure 5. Levels of human actin and HCMV proteins as determined by western blot analysis. Protein samples were isolated from cells that expressed specified EGSs and either mock-infected (lanes 5, 10, 15 and 20) or infected with HCMV (MOI = 0.5–1) (lanes 1–4, 6–9, 11–14 and 16–19) for 36 h (**A** and **B**) or 48 h (**C** and **D**), separated in SDS–polyacrylamide gels, and then transferred on to membranes. One membrane was allowed to react with a monoclonal antibody (Anti-actin) against human actin (**A**) while the others were stained with the monoclonal antibody (Anti-IE1/IE2, Anti-UL44 and Anti-gH) against HCMV IE1/IE2, UL44 and gH proteins, respectively (B–D).

To determine whether viral growth was also inhibited in the EGS-expressing cells, cells were infected with HCMV at an MOI of 0.5–2. Virus stocks were prepared from the infected cultures at 1 day intervals through 7 days postinfection and the plaque forming unit (PFU) count was determined by measuring the viral titer in human foreskin fibroblasts. After 5 days postinfection, a reduction of ∼3000- and 150-fold in viral yield was observed in cells that expressed IE-C51 and IE-SER, respectively (Figure 6). No significant reduction was found in those that expressed the control EGSs IE-C51-C, IE-SER-C or TK112 (Figure 6 and data not shown).

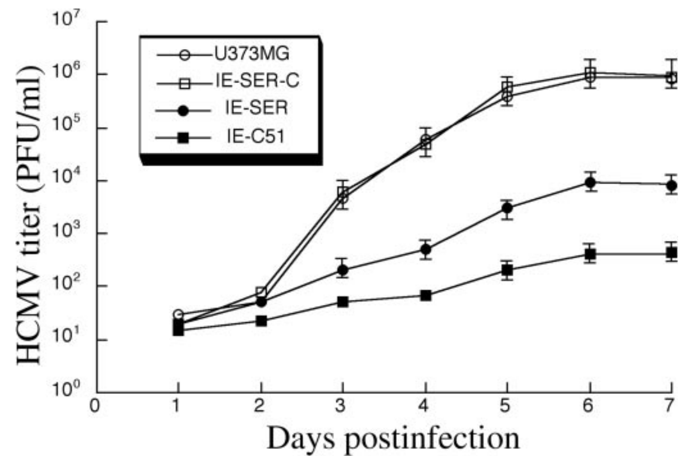

Figure 6. Growth analysis of HCMV in parental U373MG cells and cell lines that expressed IE-C51, IE-SER and IE-SER-C. Cells (1×10^5) were infected with HCMV at an MOI of 1. Virus stocks were prepared from the infected cells at 1 day intervals through 7 days postinfection and the PFU count was determined by the measurement of the viral titer on human fibroblasts. These values are the means from triplicate experiments. SD is indicated by the error bars.

DISCUSSION

RNase P is one of the most ubiquitous and essential enzymes found in nature as it is responsible for processing of all tRNA molecules, which accounts for 2% of total RNA species within a single cell (12,13). The EGS-based technology represents an attractive approach for gene inactivation since it utilizes endogenous RNase P tos generate highly efficient and specific cleavage of the target RNA. Moreover, RNase P-mediated cleavage directed by EGSs is specific and does not generate ‘irrelevant cleavage’, which is usually observed with RNase H-mediated cleavage induced by conventional antisense phosphothioate oligonucleotides (13,33). In order to develop the EGS technology for practical gene-targeting applications, further studies are needed to study how to improve the efficacy of the EGSs in inhibiting gene expression.

Little is known about the rate-limiting step of EGS-targeting reaction in cultured cells. Equally unclear is whether the efficacy of the EGSs can be improved, and if so, how it can be improved. In the present study, EGS RNAs were constructed to target an accessible region of IE1 and IE2 mRNAs. Moreover, EGSs were expressed by the small nuclear U6 RNA promoter. This design would increase the probability for the EGS RNAs to bind to their target mRNA sequence and co-localize with human RNase P, which is exclusively localized in the nuclei (13). Under the described settings, we hypothesized that the efficacy of EGS technology in cultured cells is dictated by the catalytic efficiency (V_{max}/K_m) of RNase P-mediated cleavage directed by the EGS. If this is the case, increasing the activity of EGS in directing RNase P cleavage may lead to more effective inhibition of the target mRNA expression *in vivo*.

Our results showed that an EGS variant, IE-C51, is about 25 times more active [$V_{max(apparent)}/K_{m(apparent)}$] in directing RNase P to cleave IE mRNA sequence *in vitro* than the EGS (i.e. IE-SER) derived from the natural $tRNA^{ser}$ sequence. Moreover, IE-C51 inhibited IE1/IE2 expression in cultured

cells by >93% and was more effective than IE-SER, which reduced IE1/IE2 expression by ∼80%. A reduction of ∼3000-fold in viral growth was observed in the IE-C51-expressing cells while a reduction of ∼150-fold was observed in cells that expressed IE-SER. In contrast, a reduction of <10% in the IE1/IE2 expression level and viral growth was observed in cells that expressed IE-C51-C, IE-SER-C or TK112. IE-C51-C and IE-SER-C exhibited similar binding affinity to ie37 as IE-C51 and IE-SER, respectively, but were inactive in directing RNase P-mediated cleavage due to the presence of the mutations at the T-loop that precluded RNase P recognition (Figures 1 and 2 and Table 1). These results suggest that the observed reduction in viral gene expression and inhibition of viral growth with IE-C51 and IE-SER is primarily attributed to the specific targeted RNase P-mediated cleavage induced by these two EGSs as opposed to the antisense effect or other nonspecific effects of the EGSs. Moreover, the EGS (i.e. IE-C51) that is more active [$V_{max(apparent)}/K_{m(apparent)}$] in inducing RNase P to cleave IE mRNA sequence *in vitro* is also more effective in inhibiting HCMV gene expression and growth in cultured cells. These results strongly support our hypothesis that increasing the activity of EGS in directing RNase P cleavage may lead to improved efficacy in inhibiting gene expression in cultured cells. The difference between the *in vivo* efficacies of IE-C51 and IE-SER (e.g. 92 versus 80%) appeared to be more limited than that of the *in vitro* cleavage efficiencies (∼25-fold difference). One of the possible explanations is that about 5–10% of the target mRNA may not be accessible to EGS binding or RNase P cleavage, possibly due to its specific nuclear localization and its rapid transport to the cytoplasm.

HCMV, like all human herpesviruses, expresses its mRNAs and replicates its genome in the nuclei (1). Indeed, the viral transcription and replication occurs at specific compartments within the nuclei, possibly including the nucleolus. It is also shown that HCMV transcription and replication may result in changes of certain nuclear structures (1). RNase P is localized in the nucleolus (13). Furthermore, transcripts expressed from the U6 RNA promoter, such as the EGSs in our study, are expected to be expressed in the nuclei, and possibly primarily in the nucleolus (16,26,29). Our results indicate that EGSs and RNase P can effectively target HCMV IE1/IE2 mRNAs in nuclear compartments, although the exact location for synthesis of IE1/IE2 mRNAs in the nucleus or whether it is in the nucleolus is currently unknown.

Several lines of evidence presented in our study indicate that the EGS is highly specific. First, the presence of EGSs did not exhibit significant cytotoxicity as cells expressing EGSs are indistinguishable from the parental cells in terms of cell growth and viability for up to 2 months (data not shown). Moreover, the antiviral effect associated with the expression of IE-C51 and IE-SER RNAs (inhibition of viral growth) appears to be due to the reduction of IE1 and IE2 expression. This is because the expression of all the viral early and late genes examined, including US2, UL44, gH and UL99, was found to be significantly reduced in cells that expressed IE-C51 and IE-SER but not in those that expressed IE-C51-C, IE-SER-C and TK112 (Figures 4 and 5, Table 2). The extent of the observed inhibition of the expression of most of these viral early and late genes correlates with that of the inhibition of the IE1 and IE2 expression. Meanwhile, no reduction in the levels of other viral IE transcripts (e.g. 5 kb RNA and UL36 mRNA) was detected in the EGS-expressing cells (Figure 4A and data not shown). Thus, EGS is highly specific in inhibiting the expression of its target mRNA.

Our results suggest that the enhanced stability of the mRNA–EGS complexes may possibly contribute to the increased targeting activity of EGS IE-C51. IE-C51 bound to substrate ie37 at least 25-fold better than IE-SER (Table 1). Previous studies on tRNA molecules indicated that tertiary interactions between variable region and D-loop are important for maintaining the tRNA conformation and RNase P cleavage (12,13). Given the fact that, in the ie37–IE-C51 complex, the 3′ region of ie37 can be considered equivalent to the D-loop in a tRNA (Figure 1E), it is conceivable that the additional interactions between ie37 and IE-C51 stabilize the mRNA–EGS complex and result in an enhanced binding affinity and increased targeting activity of the EGS.

In vitro selection has been widely used to generate highly active ribozymes and functional RNA molecules that have increased activity (34–36). Furthermore, this procedure has been used to generate novel RNA molecules that can serve as the substrates for RNase P and its catalytic RNA subunits (23,37,38). *In vitro* selection was also used to generate EGS molecules that direct human RNase P to cleave the mRNA encoding chloramphenicol acetyltransferase (CAT) (23). However, whether these selected EGSs exhibit higher efficacies in targeting CAT mRNA in tissue culture has not yet been extensively studied. Using an EGS RNA variant selected from a pool of EGS molecules containing randomized sequences, we, in this study, provide direct evidence that an EGS selected *in vitro* with increased targeting activity also exhibited improved efficacy in inhibiting HCMV gene expression and growth in cultured cells. Thus, our study provides a direction for the engineering and generation of highly active and effective EGS molecules by carrying out selection procedures and manipulation of the EGS domain to interact with the mRNA substrates. Further characterization of the cleavage reactions of this as well as other EGS variants both *in vitro* and in cultured cells should provide insights into the mechanism of how an EGS RNA efficiently directs RNase P to cleave an mRNA substrate and develop guidelines for construction of effective gene-targeting EGSs.

HCMV, a member of the human herpesvirus family that includes seven other different viruses such as HSV and Epstein–Barr virus, can engage in lytic replication as well as establish latent infections (1,39,40). HCMV IE1 and IE2 are among the first viral proteins expressed during lytic infection. Their homologous proteins have been found in every other herpesviruses and are among those that are major targets for drug development. To further evaluate the anti-HCMV activity of EGS RNA, the EGSs can be delivered into the monocyte/macrophage-lineage cells where HCMV is believed to establish latent infections (1). These experiments will determine whether the EGSs can abolish the IE1/IE2 expression in these cells and prevent HCMV reactivation from latent infection into lytic replication. These studies, as well as the *in vitro* studies on how to construct highly active EGSs, will facilitate the development of EGSs as exciting novel gene-targeting agents for antiviral applications.

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