In Vitro Selection of External Guide Sequences for Directing RNase P-mediated Inhibition of Viral Gene Expression*

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External guide sequences (EGSs) are small RNA molecules that bind to a target mRNA, form a complex resembling the structure of a tRNA, and render the mRNA susceptible to hydrolysis by RNase P, a tRNA processing enzyme. An in vitro selection procedure was used to select EGSs that direct human RNase P to cleave the mRNA encoding thymidine kinase (TK) of herpes simplex virus 1. One of the selected EGSs, TK17, was at least 35 times more active in directing RNase P in cleaving TK mRNA in vitro than the EGS derived from a natural tRNA sequence. TK17, when in complex with the TK mRNA sequence, resembles a portion of tRNA structure and exhibits an enhanced binding affinity to the target mRNA. Moreover, a reduction of 95 and 50\% in the TK expression was found in herpes simplex virus 1-infected cells that expressed the selected EGS and the EGS derived from the natural tRNA sequence, respectively. Our study provides direct evidence that EGS molecules isolated by the selection procedure are effective in tissue culture. These results also demonstrate the potential for using the selection procedure as a general approach for the generation of highly effective EGSs for gene-targeting application.

Antisense technology has been shown to be a promising gene-targeting approach for use in basic research and clinical therapeutic applications. The gene-targeting agents used can be a conventional antisense oligonucleotide, an antisense catalytic molecule (ribozyme or DNA enzyme), or an antisense molecule with an additional (guide) sequence that targets the mRNA for degradation by endogenous RNases such as RNase L and RNase P (1–6). Antisense molecules with guide sequences have several unique features as gene-targeting agents. Targeting with these molecules results in irreversible cleavage and the cleavage can be in a catalytic fashion. Moreover, this targeting approach uses the cellular endogenous RNases (e.g. RNase P) for degradation of the target mRNA and, therefore, assures the stability and efficiency of the targeting enzymes in the cellular environment.

Ribonuclease P (RNase P) is a ribonucleoprotein complex found in all organisms examined. It is one of the highly active enzymes in cells and is responsible for the maturation of 5\’ termini of all tRNAs, which account for approximately 2\% of total cellular RNA (7, 8). This enzyme catalyzes a hydrolysis reaction to remove the leader sequence of precursor tRNA (9). Human RNase P has at least nine polypeptides and a RNA subunit (H1 RNA) (7, 10). One of the unique features of RNase P is its ability to recognize the structures, rather than the sequences, of the substrates, which allows the enzyme to hydrolyze different natural substrates in vivo or in vitro. Accordingly, any complex of two RNA molecules that resembles a tRNA molecule can be recognized and cleaved by RNase P (Fig. 1, A and B) (11, 12). One of the RNA molecules is called the external guide sequence (EGS). In principle, an mRNA sequence can be targeted for RNase P cleavage by using EGSs to hybridize with the target RNA and direct RNase P to the site of cleavage. The EGSs used to direct human RNase P for targeted cleavage resemble three-quarters of a tRNA molecule and consist of two sequence elements: a targeting sequence complementary to the mRNA sequence and a guide sequence, which is a portion of the natural tRNA sequence and is required for RNase P recognition (Fig. 1B) (11, 12). Subsequent studies have shown that expression of EGSs in human cells can reduce the expression of both cellular and viral genes (11–15). For example, we have previously shown that EGSs efficiently direct human RNase P to cleave the mRNA sequence encoding the thymidine kinase (TK) of herpes simplex virus 1 (HSV-1) in vitro (16). A reduction of 50–70\% in the TK mRNA and protein expression was observed in HSV-1-infected cells expressing the EGSs.

Targeted cleavage of mRNA by RNase P using EGSs provides a unique approach to inactivate any RNA of known sequence expressed in vivo. Further studies aimed at increasing the targeting activity of the EGSs are needed to develop the EGS-based technology as a general tool for use in gene-targeting applications. Although little is known about the rate-limiting step of EGS-targeting reactions in cultured cells, we believe that binding of the EGSs to the target mRNA as well as the efficiency of cleavage are important for the efficacy of the

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1 The abbreviations used are: EGS, external guide sequence; ptRNA, precursor tRNA; TK, thymidine kinase; HSV-1, herpes simplex virus 1; m.o.i., multiplicity of infection.
EGSs. Indeed, recent studies on ribozymes and antisense phosphothioate molecules suggest that binding of these molecules to their target RNAs appears to be rate-limiting in vivo (17–21). In our previous studies, EGS RNAs were used to target a region of TK mRNAs that is accessible to modification by dimethyl sulfate in cell culture and is also accessible to EGS binding (16). Moreover, the EGSs were expressed primarily in the nuclei by using the promoter of small nuclear U6 RNA (12, 16, 22). This design would increase the probability for the constructed EGSs to bind to its target mRNA sequence and co-localize with RNase P, which is localized exclusively in the nuclei. Under such conditions, it is possible that the EGS efficacy in culture cells is dictated by the overall efficiency (\(V_{max}/K_m\)) of the EGS-induced RNase P cleavage. If this is the case, increasing the targeting activity of the EGS may lead to a more effective inhibition of the target mRNA expression in cultured cells.

In the present study, we employed an in vitro selection procedure (23–26) to isolate highly active EGSs from an EGS pool that contained random mutations. One of the selected EGSs exhibited a targeting activity at least 35 times higher than that of the EGS derived from the natural RNA sequence. When the selected EGS was expressed in cells infected with HSV-1, a reduction of 95% in TK expression was observed. These studies demonstrate the feasibility of developing effective EGSs for gene-targeting applications.

**Experimental Procedures**

*Generation of Substrate and EGS RNAs and RNase P Cleavage in Vitro—DNA template coding for substrate tk46 was constructed by annealing oligonucleotide OliT7 (5’-GAATTCATACGACTCACTATAG-3’) with OliTK46 (5’-ACGGCGACGGTCTGCAAGCCACGCTGTTT- GAGCCAGGCGTCTAATGAGCTGTA-3’) and oligo2 (5’-AAGGTTTAAAAGCTCCGAGATTGTG-3’), respectively. DNA sequence coding for TK17 was generated by PCR using the selected substrate construct C17 as the template (27), and was cloned under the control of the T7 RNA polymerase promoter. The 5’ and 3’ primers were oligo1 (5’-GTAAATGTCGGCAAGCTC-3’) and oligo2 (5’-AAACCTTTCAGATCCGCGGCGGT-3’), respectively. DNA sequence coding for TK17 was generated by PCR using the selected substrate construct C17 as the template, and oligonucleotides TK24EGS (5’-AACGTC-3’) and TK-Ser, respectively, and contained point mutations (5

**Kinetic and Binding Analysis and RNase Structural Mapping—**TK-Ser, C-TK-Ser, TK17, and C-TK17 were generated by introducing point mutations into the TK-Ser promoter. The 5’ promoter TK24 was derived as described in the supplemental text (23). In brief, various concentrations of EGSs were preincubated at 35°C for 10 min with the tk46-EGS complexes in buffer A that had been supplemented with 10 pg of bulk tRNA from *Escherichia coli* in a final volume of 10 µl. Cleavage products were separated on 10% polyacrylamide gels that contained 7 M urea. DNA substrates were incubated in buffer A (50 mM Tris, pH 7.4, 100 mM NH₄Cl, and 20 mM MgCl₂) at 37°C with human RNase P and cleavage products were separated on 8% denaturing gels and quantified with a STORM840 PhosphorImager. Assays to determine kinetic parameters were performed under multiple turnover conditions, as described previously (29–31) (see supplemental data available online).

**RNA Kinetics and Binding Analysis**—TK21, the 5’ proximal cleavage product was separated in and isolated from a denaturing 8% polyacrylamide gel, and run at 10 watts. The electrophoresis running buffer contained 100 mM Tris-Hepes, pH 7.5, and 10 mM MgCl₂ (32). TK21 was then washed extensively with cell-free extracts and analyzed by polyacrylamide-urea gels. Values of \(K_m\) (apparent) and \(V_{max}\) (apparent) were obtained from Lineweaver-Burk double-reciprocal plots.

**Construction of Cell Lines Expressing EGSs—**Constitutively TK-P, pCT-TK-Ser, pTK17, and pCT-TK17 were generated by placing the DNA sequences that coded for EGS TK-Ser, C-TK-Ser, TK17, and C-TK17 under the control of the U6 promoter, respectively (16). Human 143Tk cells were cotransfected with pTK16 (containing the neomycin-resistant gene) and the EGS plasmids, with the aid of a mammalian transfection kit (Stratagene Inc., La Jolla, CA). At 48 h after transfection, neomycin (Invitrogen) was added to the culture medium in a final concentration of 400 µg/ml. Cells were subsequently selected under neomycin for 2 weeks and were eventually cloned. The level of EGS expression in individual cell clone was determined by Northern analysis with probes complementary to EGSs. Only those cell clones that expressed similar levels of EGSs were used for subsequent experiments.

**Virral Infection and Assays for TK Expression—**Approximately 10⁶ cells in a T25 flask were either mock-infected or infected with HSV-1 in 1.5 ml of Medium 199 at a multiplicity of infection (m.o.i.) as specified for “Results.” At 8–16 h after infection, cells were harvested and RNA was isolated as described previously (33). The RNA probes used to detect TK mRNA and the transcripts of the α74, Us10. Us11 genes were synthesized from pTK129 and pTK141, and RNase protection assays were performed as described previously (34). The protected RNA products were separated in 5% urea-polyacrylamide denaturing gels and quantitated with a STORM840 PhosphorImager.
The denatured, solubilized polypeptides from cell lysates were separated on 9% (v/v) SDS-polyacrylamide gels cross-linked with N,N'-methylenebisacrylamide (16). The separated polypeptides were transferred electrically to nitrocellulose membranes and reacted with the antibodies against HSV-1 TK (16) or ICP27 (purchased from Goodwin Institute of Cancer Research, Plantation, FL). The membranes were subsequently stained with a chemiluminescent substrate with the aid of the T7 RNA polymerase. In each round of selection, the substrate tk46 was incubated with TK17 in the presence of the large loop, consists of part of the sequence of the mRNA that encodes HSV-1 TK. The rest of the sequence was designed to hybridize to the TK mRNA-encoding sequence or was randomized. The 25 nucleotides that were randomized are each indicated by N. They are shown in bold type in E and as a bold line in D. The site of cleavage by RNase P is marked with an arrowhead.

RESULTS

In Vitro Selection of EGS RNAs—The RNA substrate tk46 used in the selection experiment contains a 5’ TK mRNA sequence of 46 nucleotides (Figs. 1E and 4A). This sequence has been shown to be accessible to modification by dimethyl sulfate and, presumably, to EGS binding in mammalian cell culture (16). We have previously showed that EGSs derived from tRNA can direct human RNase P to cleave tk46 in vitro and inhibit HSV-1 TK expression in cultured cells (16). However, the cleavage reaction is inefficient compared with the cleavage of a natural tRNA substrate (i.e. ptRNASer). It is believed that the acceptor stem and D stem and loop sequences within a natural tRNA molecule are involved in tertiary interactions with different parts of the tRNA (e.g. variable and T stem and loop regions) and are important for folding of the tRNA and interactions with RNase P (7, 8). The reduced susceptibility of the tk46-EGS complex to be cleaved by RNase P, compared with a natural tRNA, is probably a result of replacing the natural tRNA sequence at the acceptor and D stem regions with the tk46 and its complementary sequence in the complex of tk46 and the EGS. These substitutions may disrupt some of the tertiary interactions that are potentially important in maintaining the proper tRNA-like conformation and in recognition by human RNase P. Restoration of these interactions or introducing additional interactions by changing other parts of the EGS sequence, such as those resembling the variable region, and T stem and loop, may increase the susceptibility of the mRNA-EGS complex to be cleaved by RNase P.

To generate EGSs that are highly active in directing RNase P to cleave tk46, a pool of chimeric, covalently linked tk46-EGS substrates that contain partially randomized sequences was constructed (Fig. 1E) and selected for the ability to be cleaved by human RNase P (Fig. 1D). The chimeric RNA, with its 5’ region consisting of the tk46 sequence, contains the sequences that base pair with tk46 at the regions resembling the acceptor and D stem of a tRNA and, in addition, a randomized sequence of 25 nucleotides at the positions corresponding to the regions resembling the variable and T stem and loop regions (Fig. 1E). The acceptor region, which is dispensable for EGS activity (6), was not included in the chimeric substrate (Fig. 1, C and E). The pool of tk46-EGS chimeric substrates was synthesized in vitro by T7 RNA polymerase. In each round of selection, the pool of RNAs was digested with human RNase P in buffer A (50 mM Tris, pH 7.4, 100 mM NH4Cl, and 20 mM MgCl2) and the 3’ cleavage products were isolated in denaturing gels. cDNA molecules were then synthesized and amplified from these RNA molecules by reverse transcription followed by PCR and used as the templates for synthesis of EGS RNA molecules for the next round of selection. The 5’ primer for the PCR reaction contains the tk46 sequence as well as the T7 promoter sequence and, therefore, allows the restoration of these sequences for the next cycle of selection. The stringency of the selection was increased at each cycle by reducing the amount of human RNase P and the time allowed for the cleavage reaction, such that only those substrates that were rapidly cleaved by the enzyme were selected. The cleavage efficiency of the substrate population of each generation was monitored (Fig. 2A). Moreover, EGSs were also constructed from the selected population of each generation and tested for their activity to direct human RNase P-mediated cleavage (Fig. 2B). These results indicate that the susceptibility of the chimeric substrate as well as the targeting activity of the EGSs increase with each selection cycle (Fig. 2). The selection procedure was repeated nine times, until no apparent enhancement of the cleavage efficiency of the substrate population was observed after a short period of incubation (10 min) (Fig. 2A).

Sequencing and Kinetic Analyses of the Selected EGSs—Twenty-four sequences coding for the EGSs isolated after nine cycles of selection were cloned and determined (Table I). These EGSs were divided into two sets based on their primary nucleotide sequences. Each sequence in set 1 either had the same sequence or extensive homology to other sequences of the same set. In contrast, the three sequences in set 2 did not exhibit significant sequence homology to each other or to those in set 1.

In our selection procedure, the tk46-EGS chimeric substrates were selected for their susceptibility to be cleaved by human RNase P. To determine whether the selected substrates can be cleaved by RNase P, one of the selected substrates, C17, which is the most abundant selected sequence (Table I), was assayed for cleavage by RNase P. Kinetic analyses indicate that human RNase P cleaved substrate C17 as efficiently as ptRNASer (Table II), suggesting that C17 may possess proper tertiary interactions and conformations, which are found in natural tRNA substrate and are required for optimal recognition by human RNase P.

To analyze the relationship between the EGS sequences from the selected tk46-EGS substrates and the capabilities of these EGSs to direct human RNase P for targeting cleavage, an EGS sequence, TK17, was constructed from the sequence of C17. Substrate tk46 was incubated with TK17 in the presence of
The remaining part of each substrate sequence is indicated in sequence similarities. The values of the enzyme to assure that saturation with the substrate was achieved (see supplemental data). The values of $K_{m(apparent)}$ and $V_{max(apparent)}$ as well as the overall cleavage efficiency ($V_{max(apparent)}/K_{m(apparent)}$) were determined (Table II). Under the selection conditions (i.e. buffer A), TK17 was extremely efficient in directing human RNase P to cleave tk46 (Fig. 3, lane 3) and was at least $2 \times 10^4$-fold more active than the EGS molecules (i.e. TK-G0) that were derived from the initial randomized tk46-EGS chimeric substrate pool (G0) (Table II). Moreover, in the presence of TK17, RNase P-mediated cleavage of tk46 was at least 35-fold more efficient than that in the presence of TK-Ser or TK-Ser-D, which is derived from TK-Ser with the deletion of the anticodon domain, and TK-V, which only cleaves at base-paired positions or positions involved in tertiary interactions. Most of these mapping results are consistent with the proposed secondary structure of the tk46-TK17 complex shown in Fig. 4C. The structures of the complex of tk46 with EGS TK-Ser as well as with EGS TK-Ser-D, which is derived from TK-Ser with the deletion of the anticodon domain, were also studied using an RNase mapping approach (Fig. 4). These results indicate that the structures of the acceptor stem, T stem and loop, and the 5’ leader sequence of the tk46-TK17 complex are similar to those of the complexes of tk46 with TK-Ser and with TK-Ser-D. Meanwhile, the structures of the 3’ sequence of tk46 (5’GGA-3’) (equivalent to the D loop region) and the EGS sequence (5’GUG-3’) resembling the variable region in the complexes of tk46 with TK17 appear to be different from those in the complexes of tk46 with TK-Ser or with TK-Ser-D. For example, these two sequences in the tk46-TK17 complex were susceptible to digestion of RNase V, suggesting that they are involved in tertiary interactions. In contrast, the same regions in the complexes of tk46 with either TK-Ser or TK-Ser-D are susceptible to attack by RNase T1 and nuclease S1, an indication that they are in a single-stranded conformation. These results suggest that the presence of TK17 may lead to new tertiary interactions within the complex between tk46 and the EGS, which may enhance the rate of cleavage by RNase P.

An increase in the cleavage rate of RNase P may also be caused by additional tertiary interactions that may potentially stabilize the mRNA-EGS complex. If this is the case, it is expected that binding affinities of the selected EGSs to the TK mRNA sequence may increase. The binding affinities of EGS TK17 as well as TK-Ser to substrate tk46, measured as the dissociation constant ($K_a$), were determined by separating substrate-EGS complexes in polyacrylamide gels under nondenaturing conditions (Table II). TK17 exhibited 50 times higher binding affinity to tk46 than TK-Ser. Given the fact that both TK17 and TK17-Ser have the same antisense sequences (7 and 4 nucleotides, respectively) to tk46 (Fig. 4, A and C), these results strongly suggest that the increased binding affinity and the stability of the substrate-EGS complex in the presence of 

| Selected substrate | # of clones | The Randomized Region |
|--------------------|------------|-----------------------|
| G0                 |            | [50x514]              |
| Set 1              |            |                       |
| C17                | 17         | TC CTGAG GCTAC CCACT CTCAC CTCAC CTCAC |
| C14                | 3          | A CTGAC GCTAC CCACT CTCAC CTCAC CTCAC |
| C20                | 1          | TTGAG TCAGC GCTAC CTCAC CTCAC CTCAC |
| Set 2              |            |                       |
| C9                 | 1          | TCCTG CGAGC CTCAC CGCTA CTCAC CTCAC |
| C18                | 1          | TGCGA ATGAC AAATG ATGAC CTCAC CTCAC |
| C21                | 1          | AAGAC GCGGC CTCAT GCAGA GAGCC |

Fig. 2. RNase P cleavage of chimeric tk46-EGS substrate (A) and of tk46 substrate in the presence of EGSS derived from the population of randomized substrates (B) during the nine cycles of the selection procedure. Human RNase P was incubated with either the tk46-EGS RNAs after 0, 4, 8, and 9 cycles of selection (A, lanes 1–5) or with substrate tk46 in the presence of EGSS derived from the selected tk46-EGS population after 0, 4, 8, and 9 cycles of selection (B, lanes 6–10). Details are given under “Experimental Procedures.” No significant increase in the rate of cleavage of substrates was observed after eight cycles.
Selection of Gene-targeting External Guide Sequences

TABLE II
Measurement of the kinetic parameters \( V_{\text{apparent}} \), \( K_{\text{apparent}} \), and \( V_{\text{apparent}}/K_{\text{apparent}} \) in the RNase P cleavage of RNA\(^{-\text{ser}}\) or tk46 in the presence of different EGSs

| Substrate | \( K_{\text{apparent}} \) (\( \mu \text{M} \)) | \( V_{\text{apparent}} \) (pmol/min) | \( V_{\text{apparent}}/K_{\text{apparent}} \) (pmol \( \mu \text{M}^{-1} \text{min}^{-1} \)) | \( K_d \) (nM) |
|-----------|-----------------|-----------------|-----------------|--------|
| ptRNA\(^{-\text{ser}}\) | 0.015 ± 0.003 | 0.04 ± 0.007 | 2.7 ± 0.4 | 960 ± 110 |
| C17 | 0.020 ± 0.003 | 0.06 ± 0.008 | 3.0 ± 0.5 | ND |
| TK mRNA (tk46) | | | | |
| + TK-Ser | 0.5 ± 0.09 | 0.03 ± 0.005 | 0.06 ± 0.011 | 960 ± 110 |
| + TK17 | 0.3 ± 0.04 | 0.65 ± 0.01 | 2.2 ± 0.4 | 17 ± 2 |
| + C-TK-Ser | ND | ND | <0.0001 | 950 ± 120 |
| + C-TK17 | ND | ND | <0.0001 | 15 ± 3 |
| + TK-G\(_3\) | ND | ND | <0.0001 | ND |

Two additional EGSs, C-TK-Ser and C-TK17, were also constructed and cloned under the control of the U6 RNA promoter. C-TK-Ser and C-TK17 were derived from TK-Ser and TK17, respectively, and contained point mutations (5'-UC-3' → AAC) at the three highly conserved positions in the T loop of these EGSs (Figs. 4, A and C). These nucleotides were found in most of the known natural tRNA sequences (37) and are believed to be important for the interactions between the tRNA domains and human RNase P. Previous studies have shown that EGSs carrying these mutations precluded RNase P recognition and exhibited little activity in directing RNase P-mediated cleavage (6, 16). Indeed, cleavage of tk46 by human RNase P in the presence of these two control EGSs was barely detected (Fig. 3, lanes 1 and 4) and was at least 2 × 10\(^4\)-fold slower than the cleavage in the presence of TK17 (Table II). C-TK-Ser and C-TK17 contain the same antisense sequence to the TK mRNA sequence as TK-Ser and TK17. Indeed, C-TK-Ser and C-TK17 exhibited similar binding affinities to tk46 as TK-Ser and TK17, respectively, when assayed in \( \text{in vitro} \) (Table II). Therefore, C-TK-Ser and C-TK17 can be used as a control for the antisense effect of the guide sequence.

To construct cell lines that express EGSs, human 143tk\(^{-}\) cells were cotransfected with each of these four EGS DNA constructs and a plasmid containing a neomycin resistance gene (pFL116) (16). These cells were then selected in culture medium that contained neomycin, and cells that exhibited neomycin resistance were cloned. The level of EGS RNA expression in individual cell clones was determined using Northern analysis with a DNA probe that is complementary to TK-Ser (Fig. 5A). The expression of actin mRNA was used as the internal control (Fig. 5B). As expected, the EGS RNAs were exclusively expressed in the nuclei, as they were only detected in the nuclear but not cytoplasmic RNA fractions (16) (data not shown). The constructed cell lines and a control cell line in which cells were transfected with the vector DNA were indistinguishable in terms of cell growth and viability for up to 1 month (data not shown), suggesting that the expression of the EGS RNAs did not result in significant cytotoxicity. Only the cell lines that expressed similar levels of these EGSs (as shown in Fig. 5A) were used for further studies in tissue culture.

Efficacy of the Selected EGS for Inhibition of TK Expression—To determine the efficacy of the EGSs in directing human RNase P for inhibiting TK expression, cells were infected with HSV-1 at an m.o.i. of 0.05–1. Levels of TK mRNA in the infected cells were determined by an RNase protection assay. The levels of the overlapping transcripts coding for viral g47, U510, and U51 proteins were used as the internal controls for quantitation of TK mRNA expression. Fig. 6 shows the results...
Selection of Gene-targeting External Guide Sequences

The proposed secondary structures of complexes of tk46 and EGS TK-Ser, EGS TK-Ser-D (with deletion of the anticodon region), and EGS TK17, which was selected by evolution in vitro. Sites of cleavage by RNase T1 are indicated by solid arrows. Hollow arrows indicate sites of cleavage by nuclease S1. Both RNases T1 and nuclease S1 were used under conditions that allow them to cleave preferentially at G or nucleotides in single-stranded regions. Solid triangles denote sites of cleavage by RNase V, which is specific for regions that are base-paired. (Note the appearance of a few such cleavage sites in the loop structures, an indication that there might be some base-pairing within these loops.) The cleavage site of RNase P was mapped between cytosine of position 20 and guanine at position 21 of the TK mRNA sequence. The circled positions in the T loop of EGS TK-Ser and TK17 represent the nucleotides that are mutated (5'-UUC-3' → AAG) to generate C-TK-Ser and C-TK17.

DISCUSSION

The EGS-based technology represents an attractive approach for gene inactivation because it utilizes endogenous RNase P to generate highly efficient and specific cleavage of the target mRNA. In particular, RNase P is among the most ubiquitous and active enzymes found in nature, as it is responsible for processing of all tRNA molecules (7, 8). Recent studies have shown that expression of EGSs in human cells can reduce the expression of both cellular and viral genes (11–15). Moreover, RNase P-mediated cleavage directed by EGSs is highly specific and does not generate “irrelevant cleavage,” which is usually observed with RNase H-mediated cleavage induced by conventional DNA-based oligonucleotides (7, 14). Thus, EGS molecules represent promising general gene-targeting agents that can be used in both basic research and clinical applications.

Further studies on increasing the efficacy of the EGSs in inhibiting gene expression are needed to develop the EGS technology for practical gene-targeting applications. Little is known about the rate-limiting step of EGS-targeting reaction in cultured cells. Meanwhile, there is currently no comprehensive guideline about how to construct a highly active EGS molecule.

In the present study, EGS RNAs were targeted to an accessible region of TK mRNA and 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 (7, 8). 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
ated 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.

Novel EGS RNAs that exhibited higher targeting activity ($V_{\text{max(apparent)}}/K_{\text{m(apparent)}}$) than that derived from a natural tRNA sequence were isolated from a pool of EGS RNA sequences that contained random mutations. EGS RNAs that exhibited better substrate binding and directed more efficient cleavage by RNase P were selected. EGS TK17 was highly active in vitro and inhibited TK expression in cultured cells by more than 95%. Indeed, this EGS was more effective in cul-

**FIG. 5.** The expression of EGSs in cultured cells. Northern analyses were carried out using nuclear RNA fractions isolated from parental 143tk<sup>-</sup> cells (P; lanes 6 and 12) and cell lines that expressed TK-Ser (lanes 1 and 2 and lanes 7 and 8), C-TK-Ser (lanes 3 and 9), TK17 (lanes 4 and 10), and C-TK17 ribozymes (lanes 5 and 11). 30-μg (lanes 2–6 and 8–12) and 60-μg RNA samples (2x, lanes 1 and 7) were either separated on 0.8% (B) or 2.5% (A) agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, and hybridized to a 32P-radiolabeled probe that contained the DNA sequence coding for TK-Ser (A) or actin DNA sequence (B). The actin mRNA expression (B) was used as the internal control.

**FIG. 6.** Expression of TK mRNA, as detected by an RNase protection assay. RNase protection assays were performed as described previously (16). At 8 h after infection, total RNA was isolated either from parental human 143tk<sup>-</sup> cells (P; lanes 1, 2, and 6) or from cell lines expressing EGS TK17 (TK17, lane 5), TK-Ser (TK-Ser, lane 4), and C-TK17 (C-TK17, lane 3). The cells were either mock-infected (lane 1) or infected with HSV-1 (m.o.i. = 0.5) (lanes 2–6). 40-μg (lanes 1–5) and 80-μg RNA samples (2x, lane 6) were used in the analyses. The protected products corresponding to TK mRNA (TK mRNA) and the overlapping transcripts of α47, Us10, and Us11 mRNA (α47 mRNA) were approximately 90 and 180 nucleotides long, respectively. RNA probes were used in great excess of the detected RNA species.

Novel EGS RNAs that exhibited better substrate binding and directed more efficient cleavage by RNase P were selected. EGS TK17 was highly active in vitro and inhibited TK expression in cultured cells by more than 95%. Indeed, this EGS was more effective in cul-

### FIG. 5.

- **A** EGS RNA
  - TK-Ser (2x)
  - TK-Ser
  - C-TK-Ser
  - TK17
  - C-TK17
  - P

- **B** Actin mRNA
  - TK-Ser (2x)
  - TK-Ser
  - C-TK-Ser
  - TK17
  - C-TK17
  - P

### FIG. 6.

- **A** EGS RNA
  - TK-Ser (2x)
  - TK-Ser
  - C-TK-Ser
  - TK17
  - C-TK17
  - P

- **B** Actin mRNA
  - TK-Ser (2x)
  - TK-Ser
  - C-TK-Ser
  - TK17
  - C-TK17
  - P
substrate tk46 at least 50-fold better than TK-Ser (Table II). Second, nuclease mapping studies suggest that the 3’ region of tk46 downstream from the targeting region as well as the variable region of TK17 are probably involved in additional tertiary interactions that are not found in the complexes between tk46 and TK-Ser. 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 (7, 8). Given the fact that, in the tk46-EGS complex, the 3’ region of the tk46 can be considered equivalent to the D loop in a tRNA (Fig. 4C), it is conceivable that these additional interactions stabilize the tk46-EGS complex and result in an enhanced binding affinity and increased targeting activity of the EGS.

In vitro selection (23, 25, 40–42) has been widely used to generate highly active ribozymes and functional RNA molecules that have increased activity (43–48). For example, this procedure has been used to generate novel RNA molecules that can serve as the substrates for RNase P and its catalytic RNA subunits (6, 31, 49). In vitro selection was also used to generate EGS molecules that direct human RNase P to cleave the mRNA encoding chloramphenicol acetyltransferase (6). However, whether these selected EGSs exhibit higher efficacies in targeting chloramphenicol acetyltransferase mRNA in tissue culture has not yet been extensively studied. In this study, we provide direct evidence that EGSs selected in vitro are highly effective in directing human RNase P to cleave a target mRNA in cultured cells. Moreover, our results suggest that improvement of the in vitro targeting efficiencies of EGSs should lead to increased efficacies of the EGS approach in tissue culture. 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.

The increased targeting activity of TK17 appears to be independent of the primary nucleotide sequence of the targeted mRNA. When an EGS was derived from TK17 to target the IE1 mRNA sequence of human cytomegalovirus that is different from the TK sequence, the constructed EGS also exhibited better binding affinity as well as higher activity in directing RNase P-mediated cleavage than that derived from TK-Ser (data not shown). These results suggest that the domain of the selected TK17 sequence enhances binding of the EGS to the mRNA substrate possibly by interacting with the structural features (e.g., the 2’ hydroxyl groups) of the mRNA sequence rather than the bases of the nucleotides. More importantly, these observations suggest that the domains of the selected EGS molecules can be generally used to construct highly active EGSs to target any mRNA sequence. Further studies of these selected EGSs and their interactions with the mRNA substrates should provide insight into the mechanism of how RNase P cleaves the mRNA substrate in the presence of the EGSs and develop guidelines for constructing effective gene-targeting EGSs.

FIG. 7. Levels of expression of TK protein as determined by Western blot analysis with a chemiluminescent substrate. At 16 h after infection, protein samples were isolated from parental human 143tk− cells (P; lanes 1 and 2, 6, 7 and 8, and 12) or from cell lines expressing EGS TK17 (TK17, lanes 5 and 11), TK-Ser (TK-Ser, lanes 4 and 10), and C-TK17 (C-TK17, lanes 3 and 9). The cells were either mock-infected (lanes 1 and 7) or infected with HSV-1 (m.o.i. = 0.5) (lanes 2–6 and 8–12). 20-µg protein samples (2x, lanes 6 and 12) were used in the analyses. Protein samples were separated in two identical SDS-polyacrylamide gels and transferred electrochemically to two identical membranes. One membrane was allowed to react with a monoclonal antibody (Anti-ICP27) against HSV-1 immediate-early-protein ICP27 (A), whereas the other was stained with the polyclonal antibody (Anti-TK) against HSV-1 (P) TK protein (B). Both antibodies were used in great excess of the detected antigens.

FIG. 8. Schematic representation of the expression levels of HSV-1 TK mRNA and protein in virus-infected cells (m.o.i. = 0.5) that did not express an EGS (P(143tk−)) or expressed EGS C-TK-Ser (C-TK-Ser), C-TK17 (C-TK17), TK-Ser (TK-Ser), and TK17 (TK17). The RNA and protein samples were isolated from cells at 8 and 16 h after infection, respectively. The values shown are the averages from three independent experiments. Open bars, TK mRNA; solid bars, TK protein.
Selection of Gene-targeting External Guide Sequences

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