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Short Communication

In Vitro Construction of Effective M1GS Ribozymes Targeting HCMV *UL54* RNA Segments

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Abstract Seven sequence-specific ribozymes (M1GS RNAs) derived *in vitro* from the catalytic RNA subunit of *Escherichia coli* RNase P and targeting the mRNAs transcribed by the *UL54* gene encoding the DNA polymerase of human cytomegalovirus were screened from 11 ribozymes that were designed based on four rules: (1) the NCCA-3′ terminal must be unpaired with the substrate; (2) the guide sequence (GS) must be at least 12 nt in length; (3) the eighth nucleotide must be U, counting from the site –1; and (4) around the cleavage site, the sites –1/+1/+2 must be U/G/C or C/G/C. Further investigation of the factors affecting the cleavage effect and the optimal ratio for M1GS/substrate was carried out. It was determined that the optimal ratio for M1GS/substrate was 2:1 and too much M1GS led to substrate degrading. As indicated above, several M1GS that cleaved HCMV *UL54* RNA segments *in vitro* were successfully designed and constructed. Our studies support the use of ribozyme M1GS as antisense molecules to silence HCMV mRNA *in vitro*, and using the selection procedure as a general approach for the engineering of RNase P ribozymes.

Key words ribonuclease P (RNase P); guide sequence; HCMV; DNA polymerase

Ribozymes, which were discovered to have catalytic functions similar to that of protein enzymes, are being developed as promising gene-targeting reagents for the regulation of gene expression [1,2]. Ribonuclease P (RNase P), a type of ribozyme, is a ubiquitous ribonucleoprotein complex responsible for the 5′-end maturation of tRNAs. It catalyzes a hydrolysis reaction to remove the leader sequence of tRNA precursors by recognizing the common structure shared by all tRNAs [3,4]. RNase P from *Escherichia coli* contains a catalytic RNA subunit (M1 ribozyme) and a protein subunit (C5 cofactor). The M1 ribozyme can cleave an mRNA substrate as long as the target sequence hybridizes with its complementary sequence (designated as an 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 [**Fig. 1(A)**] [5]. When

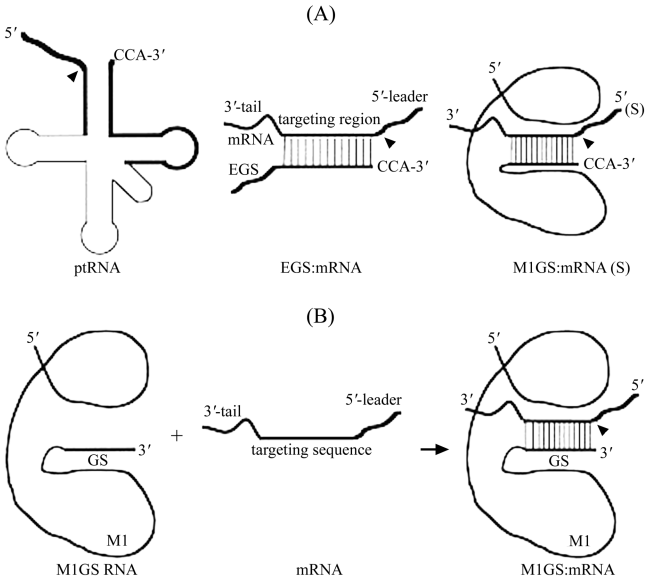

Fig. 1 Scheme summarizing the substrate for pre-tRNA (ptRNA) and ribozyme P from *E. coli* (Kilani *et al.* [5])

(A) Schematic representation of a ptRNA substrate and a small model substrate (*EGS:mRNA*) for ribonuclease P. (B) Schematic representation of an M1GS RNA construct to which a target RNA (*S*) has hybridized. Arrowhead indicates the cleavage site of RNase P or M1 RNA. The mRNA substrate contains three sequence elements: a 5′ leader sequence, a targeting sequence, and a 3′ tail sequence.

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covalently linked with a guide sequence (GS), M1 RNA can be engineered as a sequence-specific ribozyme, M1GS, that can efficiently cleave all target RNA sequences that are base paired with the guide sequence [**Fig. 1(A, B)**] [5].

M1GSs have been used as anti-HCMV agents to inhibit the expression of HCMV-essential genes and abolish viral replication. With the M1GS, IE1 and IE2 of HCMV have been shown to cleave their target mRNAs efficiently *in vitro* and are highly effective in inhibiting viral gene expression and replication in cultured cells [6]. However, no reports have been published describing the use of M1GS on the DNA polymerase of HCMV (the *UL54* gene product), which is a key enzyme for virus replication and is related to the resistant virus strains [7].

In the present study, our aim was to construct an M1GS ribozyme to target the mRNA segment of the HCMV *UL54* gene, and to investigate how it cleaves the target mRNA *in vitro*.

Materials and Methods

Materials

pFL117, a plasmid containing the DNA sequence coding for M1 RNA that is driven by the T7 RNA polymerase, was a gift from Dr. Fenyong LIU (University of California, Berkeley, USA). The screened cDNA library, containing all the segments of the HCMV (strain AD169) *UL54* gene, was also kindly provided by Dr. Fenyong LIU. pGEM3z vector was purchased from Gene Company Limited (Guangzhou, China). The primers used in our studies were synthesized by BioAsia Biotechnology Co., Ltd. (Shanghai, China).

M1GS ribozyme and substrate constructs

The DNA sequences that encoded ribozymes M1GS-T1, M1GS-T2, M1GS-T3, M1GS-T4, M1GS-T5, M1GS-T6, M1GS-T7, M1GS-C1, M1GS-C2, M1GS-C4 and M1GS-C6 were constructed by PCR with pFL117 as the template. The 5′ PCR primer was OliT7 (5′-TAATACGACTCACTATAG-3′), and the 3′ primers were 5′-TGGtgcgtcaacgttttTGTGGAATTG-3′, 5′-TGGtgcgccgagaagtcTGTGGAATTG-3′, 5′-TGGtgcgggcaggtgggTGTGGAATTG-3′, 5′-TGGtgcggcaacgcaagTGTGGAATTG-3′, 5′-TGGtgccgcgattttatTGTGGAATTG-3′, 5′-TGGtgcaagaaacgttaTGTGGAATTG-3′, 5′-TGGtgccgtcattaagcTGTGGAATTG-3′, 5′-TGGcgcccgaaaacgtgTGTGGAATTG-3′, 5′-TGGcgcaaggatgacctTGTGGAATTG-3′, 5′-TGGcgcgacgatctgtaTGTGGAATTG-3′ and 5′-TGGcgctgctagacgagTGTGGAATTG-3′, respectively (the sequences in lowercase refer to GS). Based on the targeted sites, four segments (UL54-A, UL54-B, UL54-C and UL54-D) were generated by PCR with the screened cDNA library (containing the *UL54* gene) as the template and were subcloned into the plasmid pGEM3z.

In vitro cleavage by M1GS RNA

All RNAs (M1GS RNAs and all *UL54* segment RNAs) were transcribed *in vitro* by T7 RNA polymerase following the manufacturer's recommendations and further purified on 7 M urea/8% polyacrylamide gels. Subsequently, the α-^{32}P-labeled mRNA substrates were mixed with the M1GS RNAs. The cleavage reactions were carried out at 37 °C for 60 min in 10 μl of buffer B (50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$). The reactions were stopped by the addition of 9 M urea, 0.05% bromophenol blue, and 0.05% xylene cyanol. The cleavage products were separated in 3%–5% denaturing polyacrylamide gels containing 7 M urea and quantified with a Typhoon9200 phosphorimager (Amersham, UK).

Results

Construction of M1GS ribozymes targeting the HCMV *UL54* RNA sequence and the substrate for M1GS ribozymes

GSs were designed according to four criteria: (1) the NCCA-3′ terminal must be unpaired with the substrate; (2) the length for the GS must be at least 12 nt; (3) the eighth nucleotide must be U, counting from the site –1; and (4) around the cleavage site, the sites –1/+1/+2 must be U/G/C or C/G/C. Based on the *UL54* sequence, 11 M1GSs were screened out: M1GS-T1, M1GS-T2, M1GS-T3, M1GS-T4, M1GS-T5, M1GS-T6, M1GS-T7, M1GS-C1, M1GS-C2, M1GS-C4 and M1GS-C6.

Considering that there are 11 candidate cleavage sites on the *UL54* mRNA and the *UL54* gene is too long (3.7 kb) to transcribe *in vitro*, four segments containing the targeted sites with the 11 M1GSs (e.g., UL54-A containing the targeted sites of M1GS-C1 and M1GS-T1, UL54-B containing the targeted sites of M1GS-T2 and M1GS-T3, UL54-C containing the targeted sites of M1GS-T4, M1GS-C2, M1GS-C6 and M1GS-T5, and UL54-D containing the targeted sites of M1GS-T6, M1GS-C4 and

M1GS-T7), with lengths of 413 bp, 488 bp, 525 bp and 726 bp, respectively, were generated and subcloned into the plasmid pGEM3z downstream of the T7 promoter.

In vitro cleavage of the *UL54* RNA segment by M1GS ribozymes

M1GS RNAs were synthesized *in vitro* from the DNA sequences by T7 RNA polymerase. The substrate RNAs—namely, UL54-A, UL54-B, UL54-C and UL54-D—were labeled with α-^{32}P. In the cleavage reaction, the ratio of the concentration of M1GS to the concentration of the corresponding substrate was 2:1 and the substrates were denatured.

In the negative control with the absence of M1 RNAs, no cleavage of the four segment RNAs was observed (**Fig. 2**, lanes 1, 5, 9 and 16).

When the substrate was UL54-A, which contains the targeted sites of M1GS-C1 and M1GS-T1, specific cleavage was observed in the M1GS-T1 group, and the M1GS-C1 group showed unspecific cleavage [**Fig. 2(A)**]. With M1GS-T1, the products were 265 and 148 nucleotides in length.

When the substrate was UL54-B, which contains the targeted sites of M1GS-T2 and M1GS-T3, specific cleavage was observed with M1GS-T3 and no cleavage was detected with M1GS-T2 [**Fig. 2(B)**]. With M1GS-T3, the products were 378 and 110 nucleotides in length.

When the substrate was UL54-C, which contains the

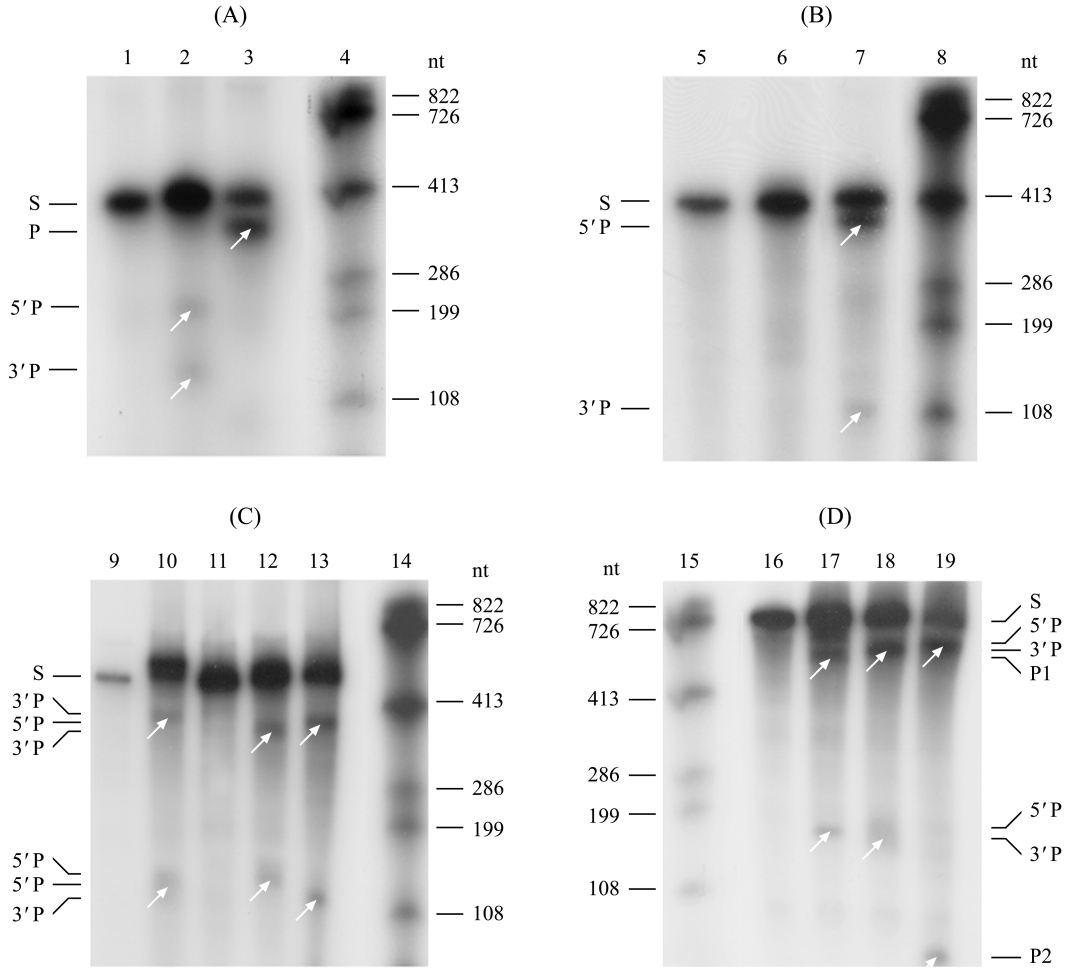

Fig. 2 Cleavage by M1GS ribozymes acting on the substrate

(A) Cleavage by M1GS-T1 and M1GS-C1, with UL54-A as the substrate. 1, control; 2, M1GS-T1; 3, M1GS-C1; 4, RNA marker. (B) Cleavage by M1GS-T2 and M1GS-T3, with UL54-B as the substrate. 5, control; 6, M1GS-T2; 7, M1GS-T3; 8, RNA marker. (C) Cleavage by M1GS-T4, M1GS-T5, M1GS-C2 and M1GS-C6, with UL54-C as the substrate. 9, control; 10, M1GS-T4; 11, M1GS-T5; 12, M1GS-C2; 13, M1GS-C6; 14, RNA marker. (D) Cleavage by M1GS-T6, M1GS-T7 and M1GS-C4, with UL54-D as the substrate. 15, RNA marker; 16, control; 17, M1GS-T6; 18, M1GS-T7; 19, M1GS-C4. S, refers to the substrate; P, 5′ P and 3′ P, refer to the product as indicated by the white arrows.

targeted sites of M1GS-T4, M1GS-C2, M1GS-C6 and M1GS-T5, specific cleavages were observed with M1GS-T4, M1GS-C2 and M1GS-C6, while no cleavage was observed with M1GS-T5 [**Fig. 2(C)**]. With the three specific cleavage M1GSs, the products were 170 and 355 nt, 418 and 107 nt, and 398 and 127 nt in length, respectively.

When the substrate was UL54-D, which contains the targeted sites of M1GS-T6, M1GS-C4 and M1GS-T7, specific cleavages were observed with M1GS-T6 and M1GS-T7, while M1GS-C4 yielded unspecific cleavage [**Fig. 2(D)**]. With the two specific cleavage M1GSs, the products were 189 and 537 nt, and 539 and 187 nt in length, respectively.

As indicated above, seven specific cleavage M1GSs were screened out from the 11 candidate M1GSs; namely, M1GS-T1, M1GS-T3, M1GS-T4, M1GS-C2, M1GS-C6, M1GS-T6 and M1GS-T7.

Effect of the M1GS/substrate ratio

M1GS-T7 was used to determine the optimal ratio of M1GS/substrate. While the concentration of the substrate remained unchanged, the concentration of M1GS-T7 was changed to get three different ratios of M1GS/substrate, which were 1:1, 1:2 and 1:5. Of the three ratios of M1GS versus substrate, the best ratio was 1:2, no cleavage occurred with the ratio of 1:1 and the substrate degraded with the ratio of 1:5 (**Fig. 3**).

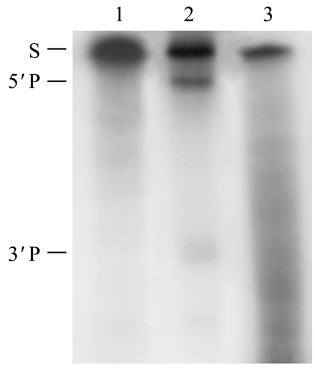

Fig. 3 The cleavages with different M1GS/substrate ratios
1, M1GS/substrate ratio of 1:1; 2, M1GS/substrate ratio of 1:2; 3, M1GS/substrate ratio of 1:5. S, refers to the substrate UL54-D; 5′ P and 3′ P, refer to the products.

Discussion

By covalently linking the external guide sequence (EGS) to M1 RNA, one of the most efficient catalytic RNAs in nature, M1GS RNA, which can hydrolyze RNA, can be created. M1GS RNA represents a distinct class of gene-targeting ribozymes and may be more effective than EGSs in diminishing the expression of a target mRNA. The M1GS-based technique represents an attractive approach for gene inactivation, as it exhibits most of the properties of conventional antisense targeting methods, as well as catalytic and irreversible cleavage of the target RNA [8]. M1GS RNA is more stable than small molecular ribozymes, such as hammerhead ribozymes, hairpin ribozymes and EGSs, although all these ribozymes have been shown to be promising gene-targeting agents for the inhibition of viral gene expression and replication [9,10]. Our study is, to our knowledge, the first to use the M1GS ribozyme for inhibiting the *UL54* gene of HCMV.

In our study, we designed the GS with four criteria: (1) the NCCA-3′ terminal must be unpaired with the substrate; (2) the length of the GS must be at least 12 nt; (3) the eighth nucleotide must be U, counting from the site –1; and (4) around the cleavage site, the sites –1/+1/+2 must be U/G/C or C/G/C. As this enzyme recognizes the structure, rather than the sequence, of its substrates, we constructed the GS when it base-paired with the substrate, which follows the main conformation of pre-tRNA that is recognized and cleaved by M1 RNA. The 3′-CCA terminus is an important factor that allows the reaction to proceed and affects the rate of cleavage [11]. The residues at positions –1/+1/+2/+7 play a role in both cleavage site recognition and the rate of cleavage [12,13]. The length of the GS was larger than 12 nucleotides, which is required for adequate specificity in the total population of RNAs in eubacteria [14]. With the aid of computer technique, 11 suitable oligonucleotides were determined. Using the gun-shot method, we screened seven specific-targeted M1GSs, and obtained two unspecific-targeted M1GSs and two candidate M1GSs without the cleavage function. As the M1GSs turned over slowly during incubation with the target substrate and enzyme *in vitro*, and the binding rate was not 100%, it was necessary to increase the dose of M1GS to get a higher cleavage rate in unit time. However, when a large dose of M1GS was used, the specific cleavage M1GS might change into an unspecific cleavage enzyme, as shown by the results when the ratio was 1:5. Although the substrate was not cleaved completely with the ratio of 1:2, we still consider this ratio to be optimal because studies by Dr. Fenyong LIU show that M1GS-IEs reduce the growth of HCMV by about 150-fold *in vivo* even if the cleavage rate *in vitro* is low. His studies also indicate that the targeted gene that is chosen must be the key gene for

the virus. These results show that the method used for screening the M1GS is effective. Our study provides the foundation for further evaluation of the efficiency of the M1GS ribozyme *in vivo*, and has facilitated the development of M1GS ribozymes as gene-targeting agents for anti-HCMV applications.

Further studies on M1GS ribozymes are underway to target HCMV *UL54* mRNA *in vivo*. More research is needed to confirm that the M1GS ribozymes effectively cleave the *UL54* mRNA *in vivo* before screening the genuine M1GS ribozymes being applied to inhibit HCMV.

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