HHS Public Access

Author manuscript

Ann N Y Acad Sci. Author manuscript; available in PMC 2016 October 09.

External guide sequence technology: a path to development of novel antimicrobial therapeutics

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Abstract

RNase P is a ribozyme originally identified for its role in maturation of tRNAs by cleavage of precursor tRNAs (pre-tRNAs) at the 5′-end termini. RNase P is a ribonucleoprotein consisting of a catalytic RNA molecule and, depending on the organism, one or more cofactor proteins. The site of cleavage of a pre-tRNA is identified by its tertiary structure; and any RNA molecule can be cleaved by RNase P as long as the RNA forms a duplex that resembles the regional structure in the pre-tRNA. When the antisense sequence that forms the duplex with the strand that is subsequently cleaved by RNase P is in a separate molecule, it is called an *external guide sequence* (EGS). These fundamental observations are the basis for EGS technology, which consists of inhibiting gene expression by utilizing an EGS that elicits RNase P-mediated cleavage of a target mRNA molecule. EGS technology has been used to inhibit expression of a wide variety of genes, and may help development of novel treatments of diseases, including multidrug resistant bacterial and viral infections.

Background

RNase P, a ubiquitous ribozyme present in all living organisms, was originally identified for its role in the maturation of the 5′-end termini of tRNAs by a single endonucleolytic cleavage of the precursor tRNA (pre-tRNA) (Fig. 1A).[1-5] Further functional studies found that RNase P of different organisms are required for synthesis of other natural RNA molecules, such as the precursors to 4.5S RNA, transfer messenger RNA, some multicistronic mRNAs, phage-related RNAs, small non-coding RNA genes, and others.[4, 6-17]

The RNase P holoenzyme is a ribonucleoprotein composed of the RNA molecule responsible for its catalytic activity [3] and one or more proteins as cofactors with different

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Conflicts of interest

functions, which, in some cases, remain unknown.[8, 18] Bacterial RNase P usually contains one protein, while archaeal and human counterparts include between 5 and 10 proteins.[19] Although structural studies on RNase P holoenzymes are still at an early stage, the structure of a bacterial RNase P in complex to mature tRNA has been resolved at high resolution.[20]

Early applications of external guide sequence technology

The *Escherichia coli* RNase P consists of the 377-nucleotide catalytic RNA subunit M1 and the 119 amino acids cofactor protein C5.[21-23] The holoenzyme recognizes the acceptor stem (Fig. 1A) and, possibly, the T stem-loop regions in pre-tRNAs, which form a particular structure recognized by RNase P.[4, 22, 24-26] Experiments designed to determine domains in a pre-tRNA molecule without abolishing *E. coli* RNase P activity demonstrated that most of the pre-tRNA molecule could be removed; these experiments also showed that any bimolecular complex with the appropriate structure could also be a substrate for RNase P (Fig. 1B).[24, 27-29] Importantly, the (antisense) complementary oligoribonucleotide was the only requirement to guide bacterial RNase P to cleave the target RNA molecule; when the antisense sequence that forms the duplex with the RNA is in a separate molecule, it is called an *external guide sequence* (EGS) (Fig. 1B).[26-28] This fundamental finding led to the development of EGS technology, which consists of inhibiting gene expression by utilizing an EGS that elicits RNase P-mediated cleavage of a target RNA molecule.[25, 26, 30-32]

The general path to selection of EGSs consists of first identifying the regions in the target RNA molecule that are accessible for interaction with an antisense oligonucleotide (or oligonucleotide analog). This can be achieved by different methods, such as RNase H mapping,[33, 34] cleavage assay by random EGSs,[35] dimethyl sulfate *in vivo* mapping, [36-38] or digestion with specific enzymes.[39-41] The results obtained can be further refined using computer prediction of the secondary structure of the RNA molecule using software such as mfold.[42] EGSs are designed to target regions that are identified by one or more methods and then evaluated for their ability to elicit RNase P-mediated cleavage of the target RNA in vitro or in vivo (whole cells or animal models). EGS technology has been used to inhibit the expression of a wide range of genes.[43-46] Early applications of the technology were in animal cell gene expression,[47-51] plant cells,[52, 53] parasites,[54] as well as *Saccharomyces cerevisiae*.[55]

In this review, we briefly summarize illustrative examples of the utilization of EGS technology in development of antibacterial and antiviral compounds. A summary of applications of EGS technology to infectious agents is shown in Table 1.

First EGS application in *E. coli*

The first example of EGS-mediated gene-regulation was published by Guerrier-Takada *et al*.[56] EGSs were utilized to reduce expression of β-galactosidase and alkaline phosphatase in *E. coli*. The EGSs used in *E. coli* and other bacterial systems usually include a 13–16 nucleotide antisense molecule complementary to the target region that also has the addition of an RCCA sequence at the 3′ end, which facilitates interaction with RNase P (Fig. 1B). [7, 57] Because RNA molecules are extremely unstable and do not penetrate *E. coli* cells readily, an appropriate recombinant plasmid that expresses the EGS was used. The release of

the EGS sequence was achieved by designing a DNA insert consisting of a T7 promoter, the EGS sequence, a core hammerhead sequence, and a T7 terminator (T7p–EGS–HH–T7t). After induction and expression of the RNA, the core hammerhead ribozyme promotes self-cleavage and releases the EGS into the cytosol.[58] An RNase P temperature-sensitive *E. coli* mutant was transformed with recombinant plasmids that expressed either specific EGSs antisense to regions in target mRNAs or non-complementary sequences as negative controls. Inhibition of gene expression was elicited by the specific EGSs, which did not occur when the cells were cultured at the restrictive temperature, indicating that RNase P mediated the cleavage of the mRNA. Similar experiments were then carried out in which the same strategy was applied except that the EGSs were complementary to bla_{TEM} (the gene that encodes β-lactamase) and *cat* (the gene that encodes chloramphenicol acetyl transferase). In these cases, there was significant inhibition of expression of the respective gene products and thus resistance to ampicillin (in the case of bla_{TEM}-directed EGS) and chloramphenicol (in the case of *cat*-directed EGS).[58]

EGS technology applied to human viruses

Yuan *et al*. [59] first showed that human RNase P can cleave an RNA substrate in the presence of an appropriate EGS that forms a bimolecular complex. However, the characteristics of the human EGSs were different from those designed for use with *E. coli* RNase P.[32, 59] These studies showed that an EGS consisting of the sequence adjacent to a 5-or 6-base pair helix, connected through a bulge with a second 7–8 base pair helix, efficiently elicits cleavage of the target RNA by human RNase P (Fig. 2A).[60, 61] It was later shown that exogenous administration of a 2′-*O*-methyl modified oligonucleotide analog EGS in complex with the transfecting reagent Lipofectin also induced cleavage of a target mRNA by RNase P in cells in culture.[43]

Anti-HIV EGS

Replication of human immunodeficiency virus type 1 (HIV-1) was suppressed by designing EGSs targeting *tat* mRNA and the U5 region of the long terminal repeat (LTR).[62] The Tat protein is a positive regulator of expression of HIV-1 genes at the level of transcriptional elongation.[63] The LTR contains the viral promoter, which is responsible for viral gene expression in eukaryotic cells, and, is present in both early and late viral gene products.[64, 65] Expression of EGSs in tissue culture cells (COS cells, a fibroblast-like cell line from monkey kidney tissue) was carried out by transfecting the cells with plasmids in which the EGS sequences were cloned into the mammalian expression vector pSV2neo, along with either the human $tRNA^{met}$ or human U6 snRNA promoter upstream and the pol III termination downstream of the EGS sequences. Although EGSs specific for several HIV-1 genes efficiently inhibited replication of the virus, the highest inhibitory effect was observed with the EGS that targeted the HIV-1 *tat* gene. [62] Furthermore, no significant differences were found when the EGSs were expressed using either of the two promoters.

Anti-HBV EGS

Another study focused on inhibition of the hepatitis B virus (HBV). [66, 67] The HBV pre-genomic RNA (pgRNA), which is the template for DNA genome synthesis, and the mRNAs

for the viral polymerase and core protein were mapped using the dimethyl sulphate method[37] to identify regions accessible for interaction with EGSs. A region that when digested by RNase P would affect expression of essential viral proteins, as well as the level of viral genomic DNA, was chosen as the target. The bimolecular complex of EGS and target mRNA is shown in Fig. 2B. *In vitro* assays showed that the pgRNA substrate was efficiently cleaved when incubated in the presence of human RNase P and the EGS. The EGS sequence was then cloned into an expression vector under the control of the U6 promoter, along with the gene for green fluorescence protein (GFP). The recombinant plasmid was introduced into the attenuated *Salmonella* strain SL301, developed specifically for gene delivery.[68] The transfected strain was then used to infect human hepatoma HepG2 cells; 24 hours after infection >70% of cells expressed GFP and the EGS at high levels.[66] Experiments to determine inhibition of replication of HBV in infected cells in culture were performed by infecting hepatoma cells with HBV followed by delivery of the EGS using the *Salmonella* delivery strain. After treatment, the hepatoma cells were sorted based on expression of the green fluorescent protein (i.e., the cells that acquired and expressed the EGS-containing plasmid); these cells showed 92% and 93% reduction of the HBV 3.5 kb and 2.4/2.1 kb transcripts, respectively. Further experiments were carried out using an animal model consisting of mice injected with an HBV genomic DNA plasmid that reaches liver tissue and is expressed, leading to secretion of hepatitis B-related antigens.[69] When the transfected mice were treated by oral inoculation of *Salmonella* expressing the EGS, a reduction of ~200,000-fold in viral DNA in the liver and serum was observed.[66] This work was followed by an analysis of different EGS variants to maximize their ability to elicit RNase P-mediated cleavage of the HBV target.[67]

Anti-CMV EGS

Human cytomegalovirus, CMV, is a cause of serious disease for immunocompromised patients. Compounding this problem, numerous strains are becoming resistant to available treatments. CMV was the target for the first antisense therapeutic agent, fomivirsen, which was approved by the FDA in 1998 for intravitreal injectable treatment of CMV retinitis.[70] Fomivirsen is a 21-base phosphorothioate oligonucleotide analog that functions by blocking translation of the viral immediate-early gene mRNA.[71]

The application of EGS technology may provide new therapeutic alternatives to prevent replication of CMV. For example, Jiang *et al.* mapped the mRNA encoding the protease (mPR) of murine CMV, whose infection of mice is similar to that of human CMV infection in humans, and selected a location 160 nucleotides downstream from the mPR translational initiation codon to design the EGS.[36] Two identical EGSs were designed, with the exception of three highly conserved nucleotides within the T-loop that are important for interaction with RNase P (Fig. 2C and D). In vitro assays of RNase P cleavage showed that in spite of both EGSs having the same binding activity with the target mRNA, only the EGS with nucleotides corresponding to the $tRNA^{Ser}$ in the T loop was active. Thus, substitution of the three nucleotides rendered the mutated EGS inactive, proving the crucial role of these nucleotides in the action of RNase P.[36]

The active EGS was cloned into an expression vector that was introduced into a *Salmonella* strain for gene delivery in vivo; murine CMV–infected mice were treated orally with *Salmonella* carrying recombinant plasmids expressing either the EGS to be tested or EGSs controls. The infected mice treated with the active, but not control, EGS showed reduced levels of mPR and viral titers, and increased survival.[36]

In other experiments, the mRNA necessary for CMV capsid scaffolding protein expression and assembly, both essential for capsid formation, was targeted.[72] An EGS lacking the anticodon loop was more effective in reducing expression of the proteins and viral growth in human CMV–infected cells in culture compared to an EGS that formed a tRNA-like structure when in complex with the target mRNA. The levels of reduction of protein expression and viral growth by the former EGS were 98% and 7000-fold, respectively, in contrast to 75% and 250-fold, respectively, by EGS that formed a tRNA-like structure.[72]

Both human and murine CMVs were also used to assess the efficiency of M1 guide sequence (M1GS) molecules (RNA molecules consisting of the EGS sequence linked to the 3′ end of the M1 RNA).[73, 74] The overlapping region of the murine CMV mRNA that codes for the M80.5 protein and the viral protease, both essential for viral replication, was used as the target for an M1GS that mediated mRNA cleavage. The DNA sequences of M1GSs were cloned in a plasmid under the control of the U6 promoter and introduced in a *Salmonella* strain for gene delivery in vivo.[73] CMV-infected macrophages and murine CMV–infected mice were treated with the *Salmonella* transformants. Macrophages treated with one of the M1GSs showed a reduction in the synthesis of both M80.5 and protease of about 80–85%, and a 2,500-fold reduction in viral growth, compared to the controls. Oral treatment of infected mice resulted in improved survival, as well as reduced viral titers.[73]

In yet another experiment, a M1GS that targeted the human CMV immediate-early protein 2 mRNA, using the wild-type sequence of the ribozyme, resulted in a 75% reduction in the expression of the protein and a100-fold reduction in viral production in infected human cells in culture. However, when a variant that included two point mutations in the ribozyme component of the M1GS (A94G and G194C), known to confer higher catalytic activity to the ribozyme in vitro,[75] was used, significantly more activity was observed, with reductions of protein expression by 98% and virus production by 3500-fold.[74]

Anti-HSV-1 EGS

The thymidine kinase mRNA from the herpex simplex virus 1 (HSV-1) has been targeted by EGS for inhibition of HSV-1 viral gene expression.[37, 76] The fragment around the translation initiation site of the mRNA was mapped *in vivo* using the dimethyl sulfate technique. The mRNA region selected to target with an EGS would result in cleavage at nucleotide 29 downstream of the translation initiation codon. The EGS designed was 71-nucleotides and formed a tRNA-like structure in complex with the target HSV-1 mRNA. Two additional EGSs were designed and tested, one with a C->G substitution at the highly conserved T-loop sequence and the other with a deletion of a portion equivalent to the anticodon domain of the EGS. The three EGSs were assayed to determine their ability to elicit cleavage of the thymidine kinase mRNA by RNase P. The EGS lacking the anticodon domain cleaved most efficiently, while the EGS with the C->G substitution showed very

low activity. However, the binding affinity to the thymidine kinase mRNA substrate was similar for both EGSs, indicating that the nucleotide change interfered with formation of the adequate structure for the EGS–mRNA complex to be recognized by RNase P.[32] In additional experiments, the DNAs coding for the EGSs were cloned under the control of the U6 promoter and the recombinant plasmids were transfected into human cells in culture that were then infected with HSV-1. A significant inhibition of expression of viral thymidine kinase was observed with the EGS that were shown to be active in the earlier assays.[32] Following these results, a pool of EGSs generated with random mutations was put through an in vitro selection procedure to identify a highly active EGS that reduced expression of thymidine kinase in HSV-1-infected cells by 95%.[37]

Anti-Influenza virus EGSs

Production of influenza virus from infected cells in tissue culture has been inhibited by addition of two EGSs that target two flu virus mRNA molecules coding for the polymerase subunit 2 (PB2) and the nucleoprotein NP.[77] Both proteins are essential for replication and production of viral particles.[78] Target regions were selected by mapping RNase T1 digestion sites within mRNAs. EGSs were designed and cloned into the LSXN cloning vector under the control of the U6 promoter. The recombinant clones were introduced into mouse cells in culture, which were then infected with flu virus at a multiplicity of infection (MOI) high enough to ensure infection of all the cells. Inhibition of protein expression and production of viral particles was observed in those cases where the cells expressed the EGSs from recombinant clones. However, as anticipated, not all EGSs showed the same level of inhibition. The strongest inhibitions, ~90% (MOI = 3) and 50% (MOI = 10), were achieved with an EGS targeting the *PB2* mRNA. Introduction of another recombinant clone consisting of the vector RVY with an EGS that targets the NP mRNA resulted in cells from which particle production was inhibited 90–100% at a MOI = 10.[77] To demonstrate that RNase P mediated cleavage, an EGS was designed with a defective T-loop that prevented RNase P activity;[77] all experiments using this control EGS showed no inhibition of virus replication.

EGS technology applied to bacterial pathogens

Following the pioneering work by Guerrier-Takada *et al.* [56, 58] described above, several groups attempted to overcome antibiotic resistance or target essential genes in a variety of bacterial pathogens (Table 1).

E. coli: GyrA and the C5 subunit of RNase P

McKinney *et al*. successfully designed EGSs that targeted mRNAs coding for GyrA and the C protein (C5) component of RNase P.[79] Two EGSs targeting different regions of either the *gyrA* or the C5 mRNA were cloned in a single DNA sequence with the orientation T7p–EGS1–HH1–HH2–EGS2–HH3–T7t. *E. coli* cells were transformed with the recombinant clones and upon induction a decrease in viability of about 10-fold was observed targeting either the *gyrA* or the C5 mRNA.[79] A recombinant clone that included all four EGSs, i.e., two specific for *gyrA* mRNA and two specific for C5 mRNA, was also tested. The results showed additive, but not synergistic, effects. An analysis of the efficiency of the EGSs

targeting *gyrA* demonstrated that up to three mismatches did not impair the inhibitory effect of the EGS.[79]

E. coli: aac(6′)-Ib and ftsZ

The aminoglycoside 6′-*N*-acetyltransferase type Ib (AAC(6′)-Ib) is a clinically relevant enzyme that confers resistance to several aminoglycosides, including amikacin.[80-82] The gene *aac(6′)-Ib* has been found in numerous plasmids, transposons, and integrons in Gram-negative bacteria.[83, 84]

Available regions for interaction with EGSs were determined by a combination of a computer generated secondary structure and RNase H mapping (Fig. 3A).[33, 34] A battery of EGSs targeting the selected regions was then tested in vitro to assess efficiency in eliciting RNase P-mediated cleavage (Fig. 3B).[85] Those with the higher efficiency were cloned into a vector similar to that used by Guerrier-Takada *et al*. [58] (i.e., T7p–EGS–HH–T7t) and transferred to *E. coli* carrying *aac(6′)-Ib*. Cells with one of the EGSs, EGSC3, showed growth inhibition upon addition of amikacin (Fig. 3C).[85] It is noteworthy that the target region (region C, Fig. 3B and C) is not entirely single-stranded in its predicted structure: four nucleotides are predicted to be in double-stranded form. Nevertheless, it was identified as region accessible for interaction with an antisense sequence by RNase H mapping (Fig. 3B and C). Possible explanations are that either the predicted structure may not reflect the precise structure of the mRNA or the structural complexity of the region permits breathing at the short double-stranded stems, allowing interaction with the EGS.[34, 86]

A strategy similar was used to interfere with normal expression of the *ftsZ* gene. FtsZ is essential for cell division and together with FtsA and ZipA participate in the first event of construction of the divisome, the formation of the proto-ring, which continues with the assembly of other proteins and protein complexes. FtsZ functions as a scaffold for the divisome and generates the constrictive force to initiate cell division.[87-90]

The *ftsZ* mRNA was mapped and EGS targeting regions accessible for interaction with antisense sequences were tested in vitro to determine the best candidates to elicit cleavage by RNase P (Fig. 3D).[91] These assays led to selection of a few EGSs that were promising with respect to interfering with expression of *ftsZ* and disrupting cell division. The selected EGSs were cloned as part of a DNA fragment with the structure T7p–EGS–HH–T7t, and the recombinant clones were introduced in *E. coli*. Upon induction of the T7 promoter, cells expressing specific EGSs showed filamentation, indicating that cell division had been disrupted (see Fig. 3E and F).[91]

Salmonella enterica serovar Typhimurium

McKinney *et al*. designed EGSs that target the *invC* and *invB* genes found in the *Salmonella enterica* serovar Typhimurium.[39] The *invC* and *invB* genes code for an ATPase required for host cell invasion and a type III secretion chaperone, respectively.[92, 93] Mapping of mRNA was carried out by RNase T1 digestion and four EGSs, three of them targeting *invC*, were designed on the basis of the mapping results. The EGSs were capable of inducing digestion of the target mRNAs in vitro and were cloned into high and low copy number vectors to be

expressed within a *Salmonella* strain. Expression of all four EGSs resulted in a decrease in *invC* mRNA, consistent with the existence of a dicistronic mRNA. Assays to determine the effect on InvC-dependent type III secretion expressing two EGSs, both targeting *invB* or *invC* or one targeting *invB* and the other targeting *invC*, showed a reduction in secretion of the protein SipB and the levels of reduction were dependent on the copy number of the recombinant plasmids used. Expression of two EGSs, one targeting *invC* and the other *invB*, also resulted in inhibition of expression of InvC.

Finally, in cell invasion assays, *Salmonella* expressing EGSs targeting *invB* or *invC* from a high copy number plasmid exhibit about 90% reduction in invasion with respect to the controls.[39] The authors proposed that both *invC* and *invB* are encoded in the same mRNA and the 3′nucleotide of the final codon of *invB* is the 5′nucleotide of the initial codon of *invC*. Then, since all four EGSs resulted in inhibition of expression of *invC*, it is possible that the mRNA is destabilized or functionally disrupted after cleavage at the *invB* location. This result suggests the non-surprising possibility that EGS targeting one region of an mRNA may have deleterious effects on the rest of the molecule.[39]

Yersinia pestis, *Francisella tularensis*, and *Brucella melitensis*

Ko *et al*. [41] applied EGS technology to inactivate the *Yersinia pestis* virulence genes *yscN* and *yscS*, which are part of a group of more than 20 genes that form a Type III secretion system, which is induced upon entry of *Y. pestis* into the mammalian host and when the bacterium makes contact with the mammalian cell it mediates injection of the effectors, known as Yop proteins, through the cell membrane. Once inside the eukaryotic cell, the effectors inhibit bacterial phagocytosis and suppress the production of pro-inflammatory cytokines.[94]

Messenger RNAs for both *yscN* and *yscS* were mapped and EGSs were designed that proved active in in vitro assays. To test their ability to elicit RNase P-mediated cleavage of the mRNAs *in vivo* using *E. coli* as host, the EGSs and the target mRNA were cloned into expression vectors.[41] The results showed that the most effective EGSs reduced the target mRNAs to 37–40% compared to the control condition of expressing the mRNA in the absence of an EGS.

A similar strategy was used to design EGSs that inhibit expression of the global virulence regulator MglB from *Francisella tularensis*,[95] an intracellular pathogen that causes tularemia.[96] The *mglB* mRNA was mapped and EGSs were designed and tested *in vitro* and then recombinant plasmids equivalent to those described for the *Y. pestis* system were constructed and assayed. Two EGSs alone or expressed tethered to M1 RNA reduced mRNA levels significantly.

Likewise, EGSs that reduce levels of the *Brucella melitensis vjbB* gene, which codes for the virulence regulator VjbB, have been identified following the same methodologies.[35, 95]

Modifications of EGSs to aid stability

While the recombinant plasmid based expression of EGSs from within the cells cytosol is appropriate as a general strategy to demonstrate proof of concept, it is not viable for

therapeutic purposes because the EGSs added exogenously must penetrate the cells to exert their action. Furthermore, because oligoribonucleotides are rapidly degraded by nucleases, successful development of EGS technology depends on finding nuclease-resistant analogs that induce RNase P-mediated degradation of the target mRNA.

In the case of *aac(6′)-Ib*, locked nucleic acid (LNA)/DNA co-oligomers in the appropriate configuration efficiently elicited RNase P-mediated cleavage of the mRNA *in vitro*.[97] Interestingly, unlike most oligonucleotides and oligonucleotide analogs that are not internalized, LNA/DNA co-oligomers were able to penetrate *E. coli* cells, but with very low efficiency.[98] Therefore, their ability to interfere with amikacin resistance was assessed using the hyperpermeable *E. coli* AS19 strain.[99] Administration of an LNA/DNA co-oligomer at 50 nM to *E. coli* AS19 cells harboring the *aac(6′)-Ib* gene significantly reduced the levels of resistance to amikacin.[97]

In a different study, phosphorodiamidate morpholino oligonucleotide EGSs conjugated to permeabilizer peptide (PPMO) efficiently inhibited expression of Gram-negative and Gram-positive genes.[100, 101] Interestingly, these experiments showed that a mix of the PPMO that specifically targets an mRNA and permeabilizer peptide that remained in the mix as unreacted substrate during the conjugation reaction act as a powerful antibiotic.[102, 103] The permeabilizer peptide possessed an unspecific antibacterial activity, while the PPMO functioned as a specific agent targeting a unique mRNA.[102]

PPMOs were also successfully used in experimental treatment of *Staphylococcus aureus* wound infection.[104] A PPMO EGS was designed to target the *gyrA* mRNA and it proved effective in interfering with growth of *S. aureus* in liquid cultures.[103] Furthermore, the PPMO was combined with a gel to assess its activity as a topical treatment in a mouse model of *S. aureus* infected skin wounds.[105] Mice that were treated with the PPMO-containing gel showed faster re-epithelization and healing when compared to the controls.[104]

Concluding remarks

Infectious diseases are one of the leading causes of loss of human life. The continuous emergence of new threats and development of resistance to available treatments requires novel approaches to find means to extend the useful life of existing therapies and design new ones. EGS technology is a relatively new strategy that shows promise in the struggle to design novel therapeutic agents. Future research could focus on targeting conserved genes present in viruses or multidrug resistant bacterial pathogens such as efflux pumps, the replication machinery or selected virulence factors genes. Heretofore, success has been demonstrated in a variety of cell processes. Although this bodes well for the future of EGS technology, many challenges still lie ahead before EGS-based therapeutics can be introduced in the clinics. Successful delivery of the drugs to the appropriate targets, achieving high levels of inhibition of gene expression, and low toxicity to the host are just some them. The various strategies and approaches to design efficient EGSs and delivery vehicles described in this review attempt to overcome them.

Acknowledgments

Authors' work cited in this review article was funded by Public Health Service grant 2R15AI047115 (to M.E.T.) from the National Institutes of Health and X-240 Universidad de Buenos Aires (to A.Z.). A.Z. is a career member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). C.D.S. was supported by a fellowship from CONICET. For RAB, the writing of this manuscript was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, the Veterans Affairs Merit Review Program Award 1I01BX001974 and the Geriatric Research Education and Clinical Center VISN 10.

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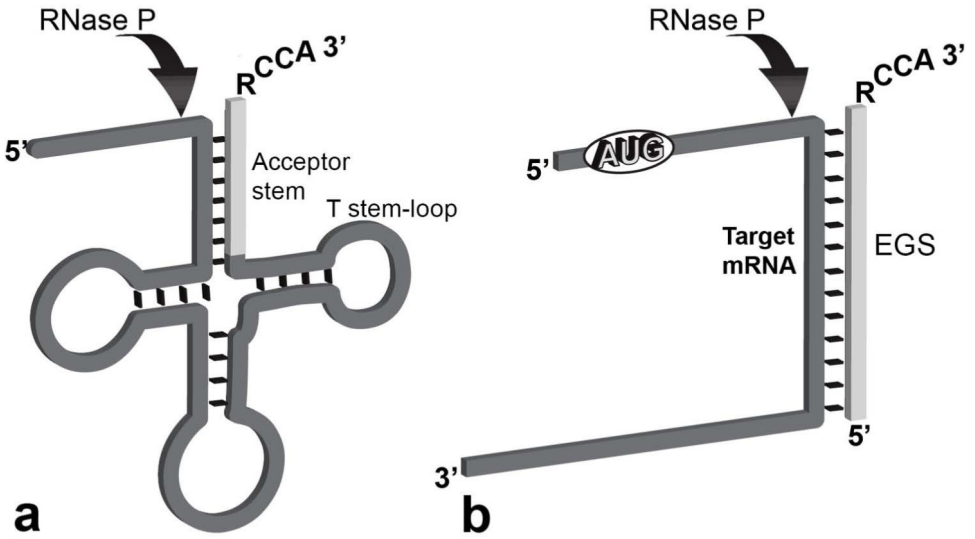

Fig. 1.

Cleavage of a pre-tRNA and an mRNA. (A) The arrow shows the site of action of RNase P on a pre-tRNA. The clear segment is the acceptor stem. (B) Complex between a target mRNA and an EGS that can be recognized as substrate by bacterial RNase P. In this example, the ATG sequence has been added to represent the possibility of using an mRNA as target. The RCCA sequence mimics the 3′-end of the pre-tRNA and facilitates interaction with RNase P. Redrawn from Ref. 4.

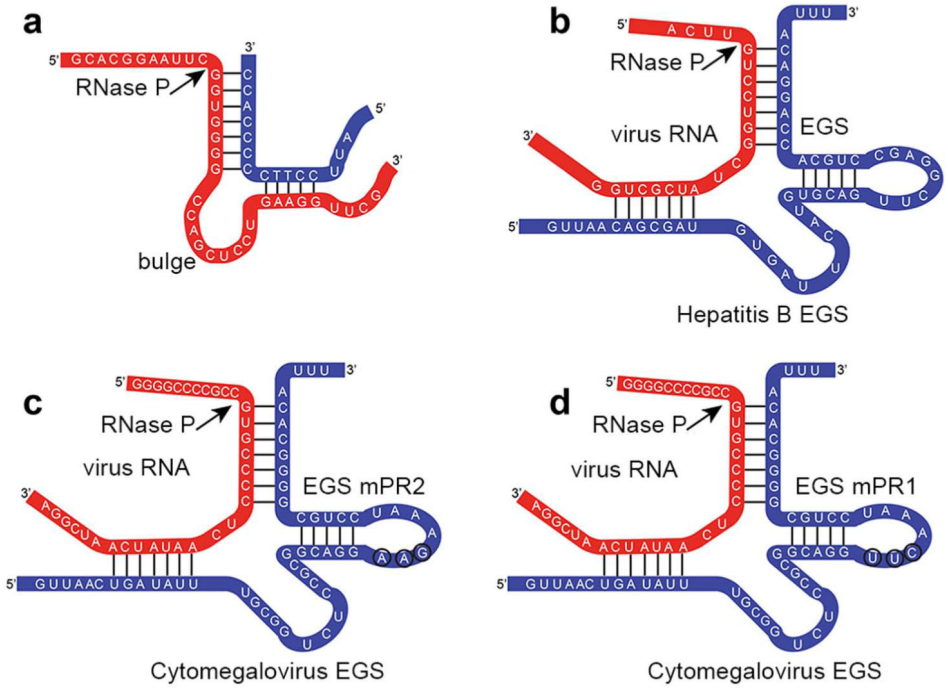

Fig. 2.

Schematic representation of complexes target RNA:EGS. (A) Bimolecular substrate of human RNase P. (B–D) Complexes between the hepatitis B pgRNA sequence (red),B, the mPR mRNA (red), C and D, and the respective EGSs (blue). The nucleotides substituted between EGSmPR1 and EGSmPR2 are circled. The arrow indicates the point of action of human RNase P. Redrawn from Refs. 36, 60, 66.

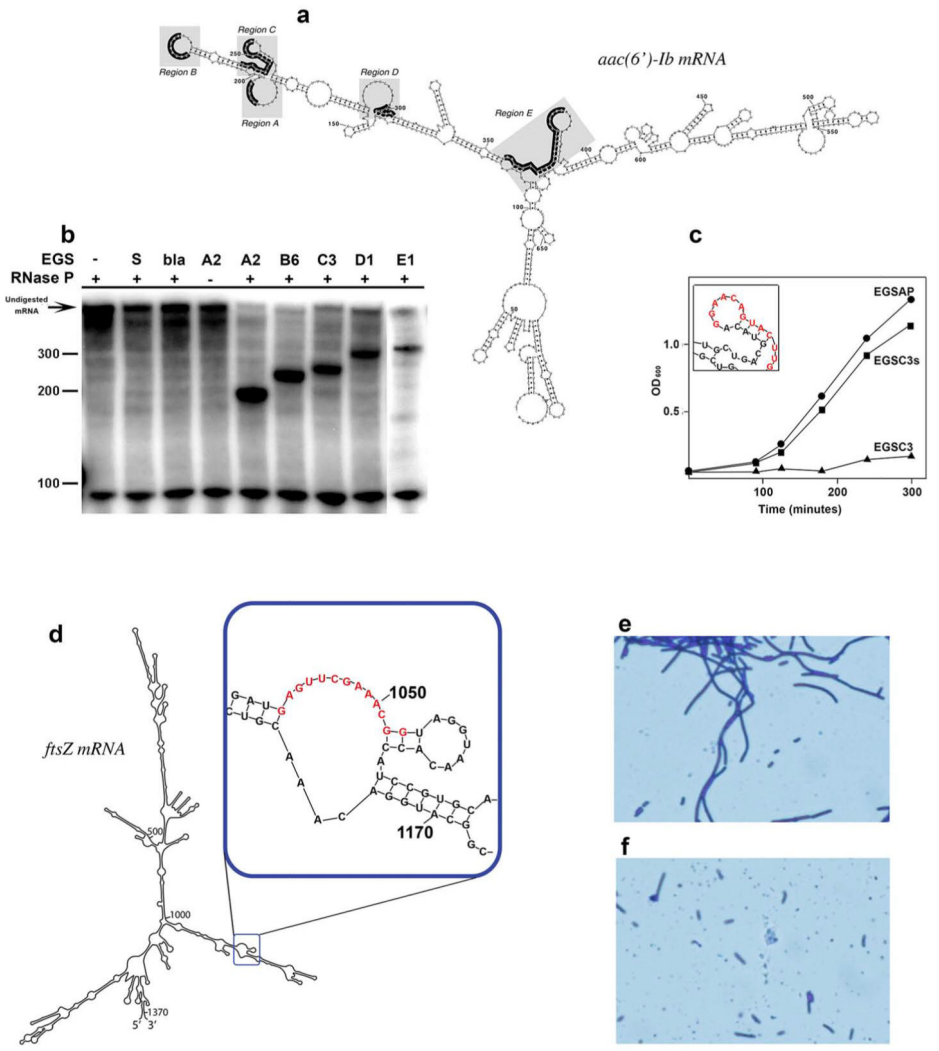

Fig. 3.

RNase P-mediated inhibition of amikacin resistance and cell division by endogeneously produced EGSs. (A) Secondary structure of the *aac(6′)-Ib* mRNA as determined by utilizing a combination of mfold software[42] and RNase H mapping.[33] Reproduced from Ref. 34. (B) Cleavage reactions carried out in vitro in the presence of several EGSs targeting different regions in the mRNA (A2, B6, C3, D1, and E1). Controls lacked EGS or RNase P, or included EGSs targeting a different gene (*bla*) or carrying the sense nucleotide sequence. The RNase P components, M1 RNA and C5 protein, were incubated with end-labeled *aac(6′)-Ib* mRNA and the EGSs. The products were analyzed on 6% denaturing polyacrylamide gel electrophoresis. Reproduced from Ref. 85. (C) *E. coli* BL21(DE3) harboring the *aac(6′)-Ib* gene and recombinant plasmids coding for the EGSs indicated in the figure were cultured in the presence of 15 μg of amikacin/ml. Growth was monitored by measuring the OD_{600}. The EGSs were: EGSC3, targets the sequence shown in red in the inset, EGSAP, targets the alkaline phosphatase gene, and EGSC3s has the sense sequence. The inset shows the region targeted by EGSC3 (nucleotides in red). Reproduced from Ref. 34. (D) Secondary structure of the *ftsZ* mRNA as determined by mfold software. The boxed

region is shown in detail and the sequence in red is the target of the most efficient EGS tested. Redrawn from Ref. 91. (E and F) *E. coli* BL21(DE3)(pLysS) harboring recombinant plasmids coding for the EGS that targets *ftsZ*, E, or the complementary sequence, F, under the control of the T7 promoter were induced by addition of isopropyl-β-D-thiogalactopyranoside, incubated for 60 minutes at 37 °C and examined by microscopy.

Table 1

EGS technology applied to infectious diseases

Organism	Disease[a]	Target	Chemical nature of EGS	Proof-of-concept system	Reference
HIV-1	AIDS	tat and LTR	RNA[b]	COS cells (mouse)	62
Hepatitis B virus	Hepatitis	pgRNA, S mRNA, pre-S/L mRNA	RNA[b,c]	HepG2 and HepG2.2.15 cells (human)[b] Mice[c]	66, 67
Murine cytomegalovirus	Cytomegalovirus infection	mPR	RNA[c]	J774[c], human U373MG[b] cells (human) Mice[c]	36, 72, 74
Herpes simplex virus 1	Herpes	thymidine kinase	RNA[b]	143 tk- cells (human)[b]	37, 76
Influenza virus	Flu	polymerase subunit 2 (PB2), nucleoprotein (NP)	RNA[b]	C127 cells (mouse)[b]	77
E. coli	NA	*phoA, lacZ,*	RNA[b]	*E. coli* cells in culture	56
E. coli	NA	*bla, cat*	RNA[b]	*E. coli* cells in culture	58
E. coli	NA	*gyrA, rnpA*	RNA[b]	*E. coli* cells in culture	79
E. coli	NA	aac(6′)-Ib	RNA[b], LNA/DNA[d]	*E. coli* cells in culture	85, 97
E. coli	NA	*ftsZ*	RNA[b]	*E. coli* cells in culture	91
S. aureus	Wound infection	*gyrA*	PPMO[e]	*S. aureus* cells in culture Murine cutaneous wound model	104
E. coli	NA (tularemia)	*F. tularensis mlgB*	RNA[f]	*E. coli* cells harboring heterologous gene in culture	95
S. enterica	*Salmonella* infection	*invB, invC*	RNA[b]	*S. enterica* invasion of Henle-407 cells (human)	39
E. coli	NA (plague)	*Y. pestis yscN and yscS*	RNA[f]	*E. coli* cells harboring heterologous gene in culture	41

Organism	Disease[a]	Target	Chemical nature of EGS	Proof-of-concept system	Reference
E. coli	NA (brucellosis)	*B. melitensis vjbB*	RNA[f]	In vitro cleavage of mRNA	35
E. coli *B. subtilis* *E. faecalis*	NA	*gyrA*	PPMO[e]	Bacterial cells in culture	100
P. aeruginosa *S. aureus*	NA	*gyrA*	PPMO[e]	In vitro cleavage of mRNA	100
E. coli *B. subtilis* *E. faecalis*	NA	*rnpA*	PPMO[e]	In vitro cleavage of mRNA	100
E. coli	NA	*cat*	PPMO[e]	Bacterial cells in culture	100
Plasmodium falciparum	Malaria	*gyrA*	PPMO[e]	*P. falciparum* cells in culture	54

[a] In those cases where the inhibition of expression of the gene(s) in question was done in *E. coli* harboring the gene(s), the disease caused by the bacterium providing the gene(s) is indicated in parenthesis. NA, not applicable.

[b] Recombinant clones coding for EGSs were introduced in cells (prokaryotic or eukaryotic) as described in the text.

[c] The EGS was in a recombinant clone harbored by a *Salmonella* strain constructed for delivery.

[d] LNA/DNA, oligomer composed of LNA and deoxynucleotide residues.

[e] PPMO, phosphorodiamidate morpholino oligonucleotide EGS conjugated to permeabilizer peptide.

[f] A recombinant plasmid expressing both the EGS and the target gene was introduced in *E. coli* to test the EGS activity on gene expression.