Rapid selection of accessible and cleavable sites in RNA by \textit{Escherichia coli} RNase P and random external guide sequences

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Contributed by Sidney Altman, December 20, 2007 (sent for review November 16, 2007)

A method of inhibiting the expression of particular genes by using external guide sequences (EGSs) has been improved in its rapidity and specificity. Random EGSs that have 14-nt random sequences are used in the selection procedure for an EGS that attacks the mRNA for a gene in a particular location. A mixture of the random EGSs, the particular target RNA, and RNase P is used in the diagnostic procedure, which, after completion, is analyzed in a gel with suitable control lanes. Within a few hours, the procedure is complete. The action of EGSs designed by an older method is compared with EGSs designed by the random EGS method on mRNAs from two bacterial pathogens.

Selection of accessible sites in target RNAs is critical for efficient mRNA inactivation strategies. Many of the target sequences in cellular RNAs are inaccessible because of the secondary or tertiary structures of the RNA or the binding of proteins to the target RNA \textit{in vivo}. Several approaches for the mapping of accessible sites in target RNAs have been reported. These approaches range from analyses \textit{in silico} to cleavage by complex ribozyme constructs (e.g., 1–6), but they are time-consuming and are not reliable in terms of their efficacy.

In Gram-negative bacteria, down-regulation of gene expression at the RNA level has been achieved by directing external guide sequences (EGSs) to pair with complementary regions in mRNAs from individual genes. The EGS will hydrogen-bond to the target RNA and generate an RNA–RNA stem structure mimicking the natural precursor tRNA (ptRNA) cleavage site (7–9). The target RNA in the complex is cleaved by RNase P (10–14).

EGSs have been designed to successfully alter several bacterial phenotypes (11–14). In mammalian cells, RNase P RNA has been specifically directed to destroy tumor-specific fusion genes created as a result of chromosome abnormalities (15) and to inhibit viral gene expression and growth in cell cultures (16, 17).

Here we describe a rapid method to map directly accessible and cleavable sites in target RNA by the \textit{Escherichia coli} RNase P holoenzyme and a random EGS (rEGS) library.

Results

Design of the rEGSe and rEGSx Libraries. The rEGSe and rEGSx libraries were constructed by using a partially randomized oligonucleotide as a template for PCR (Fig. 1). This oligo contains the T7 promoter sequence upstream of the random 14-nt sequence (N14). The length of the randomized region could be varied, but 14 nts were selected because 13- to 16-nt EGSs were shown to work well in previous studies in bacteria (10–14). In addition, the oligonucleotide contains a cytosine that will base pair to a guanosine in the target RNA sequence and a 3'-ACCA sequence. A guanosine is the preferred nucleotide immediately 3' of the natural ptRNA cleavage site, and a 3'-ACCA sequence in ptRNA is important for cleavage \textit{in vivo} by \textit{E. coli} RNase P. The only difference between the rEGSe and rEGSx libraries is that the former contains all of the nucleotides after the BstNI site (Fig. 1), whereas the latter is digested by BstNI before transcription \textit{in vitro} and so only contains a short 3'-ACCA sequence after the N14C sequence.

Mapping \textit{in Vitro} of Accessible Sites in the vjbR and yscN mRNAs by rEGSs. Analysis of the cleavage products \textit{in vitro} of the vjbR (\textit{Brucella melitensis}) mRNA by the rEGSe and rEGSx libraries resulted in the identification of several sites to be selectively cleaved in the presence of rEGSs (Fig. 2A). The rEGSx library gave better resolution than the rEGSe library. The cleavage products were present when rEGSs and the RNase P holoenzyme were incubated with the target RNA and, importantly, not present when RNA was incubated alone or in the presence of the RNase P holoenzyme alone. We note that the RNase P holoenzyme alone can weakly cleave the vjbR mRNA \textit{in vitro} at a few positions (Fig. 2A) and that these cleavages were inhibited by a large excess of rEGSs. This phenomenon occurs with some RNAs, but does not negate the effect of rEGSs.

The RNase P cleavage of yscN (\textit{Yersinia pestis}) mRNA at the G + 3 position (+1 is defined in Materials and Methods) increased as the amount of rEGSs increased (Fig. 2B). The cleavage at G + 41 decreased as the amount of rEGSs increased. In this case, rEGSs seemed to act as a competitor of RNase P reaction, rather than a specific binding molecule. The G + 3 position also was a strong RNase T1-susceptible site (Fig. 2B). A secondary structure model indicated that this position is hydrogen-bonded at the beginning of a single-stranded region (data not shown).
Fig. 2. Analysis of cleavage products of 5’ end-labeled target RNA generated by RNase P holoenzyme and rEGSe or rEGSx library. The methods are the same for A and B. Reactions were carried out as described in the text. (A) Cleavage of the vjbR mRNA. Internally labeled pSupS1 ptRNA was used as a positive control to check for activity of the reconstituted RNase P holoenzyme. Arrows indicate positions selected for further analyses. The filled triangles represent decreasing concentrations of random EGSs (103-, 102-, and 10-fold molar excess to target RNA). (B) Cleavage of yscN mRNA. The G’ + 3 cleavage site is indicated by an arrow. The G’ + 41 site is marked with an asterisk; 100-, 50-, and 10-fold molar excess of rEGS was used for the RNase P reaction. Lanes con, CS, P, OH, and T1 represent untreated yscN mRNA sample, reaction product with C5 protein, reaction product with RNase P holoenzyme, alkaline ladder, and reaction product of partial RNase T1 digestion, respectively. In lane P, 10 nM M1 RNA and 100 nM C5 protein were used for the reaction in PA buffer. In lane e1, 0.5 µM specific EGS targeting G’ + 13 position was used as a control. Sequencing gels were 40 cm in length and were run at 50°C.

Cleavage in Vitro of the vjbR and yscN mRNAs by Specific EGSs. Four potential cleavage sites in vjbR mRNA were further examined to compare the cleavage efficiency in the positions predicted by the rEGS strategy with those predicted by the RNase T1 strategy (preparation of the specific EGSs is described in ref. 22). Positions 73 and 127 were identified as accessible by RNase T1 mapping, but were not identified by the rEGS strategy (Fig. 2A). Position 117 was identified as accessible by the rEGS strategy only, and position 166 was identified by both methods (Fig. 2A). EGSs targeting these positions were respectively made and tested in cleavage assays in vitro. Only EGSs targeting positions 117 and 166 were able to cleave the vjbR RNA, whereas positions 73 and 127 were not cleaved (Fig. 3A). The cleavage sites were confirmed by 5’ end-labeling vjbR RNA in cleavage assays in vitro (data not shown).

Specific EGSs (linear EGS or M1 EGS) against yscN mRNA were constructed as listed in Materials and Methods. Assays with M1 RNA alone were carried out in PA100 buffer, which contained 100 mM MgCl₂. The same cleavages were observed as were seen with the rEGSs. When RNase P was used alone, another cleavage site occurred (Fig. 3B). The cleavage site was confirmed as G’ + 3 as expected (Fig. 3C). This site was observed when M1 RNA and a specific EGS were used (Fig. 3B).

Discussion

We have described a method to identify accessible and cleavable sites in target RNAs by using rEGSs and RNase P. Similar studies were reported previously for selecting an EGS from a library that contains a randomized domain of RNA-derived EGSs (23). In these cases, a domain such as the acceptor or D stem was randomized in an EGS that contained three quarters of a tRNA for use in eukaryotes. The difference from the earlier reports is that for the linear EGSs to be used in prokaryotes, we used a fully randomized EGS. Only the 3’ end has a fixed sequence (CACCA). No particular selection procedure was needed in the method described here. After the RNase P reaction, the cleavage product was separated on a polyacrylamide gel, and the cleavage site could be identified by comparing it with a G-specific ladder and a partial alkaline ladder. The regions where rEGSs bind are accessible for hybridization and also will directly indicate that the EGSs bound in those specific locations are able to induce RNase P-mediated cleavage of the target RNA. This observation suggests that the rEGS strategy is much more specific in detecting accessible sites in target RNAs than the older RNase T1 strategy.

However, in some cases, the EGSs that are designed from RNase T1 mapping do not work in vitro most likely because the accessibility of RNase P or EGS to the target mRNA might be different from that of RNase T1. It also is not always possible to get a cleavage location from the rEGS assay. In assaying a fragment of an mRNA, we could not get a candidate site when using rEGS (J.-h.K., unpublished data). RNase P alone cuts the target RNA. The addition of an excess of rEGSs acted as a competitor of cleavage by RNase P alone. However, the total length of the mRNA, several hundred nucleotides, was not completely assayed to determine whether there was any cleavage by the rEGS. The design of an EGS based on the older RNase T1 mapping method on the smaller mRNA fragment was successful.

Binding of an EGS to the target mRNA is believed to be one of the key steps for the EGS activity. Therefore, the tertiary structure of a target mRNA affects the accessibility of EGSs and RNase P. In previous studies, RNase T1 mapping was adopted to determine the secondary structure of the target; based on that premise, an EGS was designed for the sites that looked more accessible. The advantage of the rEGS method is that it rapidly detects accessible and cleavable sites in the target RNA and ensures that the specific EGS designed from this method would be an active EGS in vivo. The function of specific EGSs designed by the rEGS method in vitro has already been demonstrated as successful in inhibiting gene expression in E. coli (J.-h.K. and S.A., unpublished data).

Materials and Methods

Templates for Transcription. Brucella melitensis DNA was obtained from the Armed Forces Institute of Pathology (AFIP) (Washington, DC). All molecular techniques were performed by standard methods (18). A 5’ fragment of the B. melitensis gene vjbR was amplified by PCR using primers vjbR F (5’-GCG GGT ACC TAA TAC GAC TCA TTA AGG TTC TCT CTT CTA AAG CGA TAC TGT-3’) and vjbR R (5’-GCG GGA TCC AGC GGG CGG CGA TGC TGA GGA TTA-3’), which contained restriction sites for KpnI and BamHI, respectively. The vjbR primer also contained the T7 promoter (underlined) for transcription in vitro. The resulting DNA fragment was digested by the restriction enzymes KpnI and BamHI and was inserted into pUC19 digested with the same enzymes to give the plasmid pT7 vjbR. pT7 vjbR was digested with StyI, ethanol-precipitated, dissolved in distilled water, and subsequently used as the template for tran-
Plate for transcription

Ultrafree-MC columns (Millipore). This DNA fragment was used as the template for transcription of yscN mRNA by T7 RNA polymerase (Roche Diagnostics). The resulting PCR product was purified by using Ultrafree-MC columns (Millipore). The PCR product was either transcribed directly to yield a rEGS library with an extended 3′ product was either transcribed directly to yield a rEGS library with an extended 3′ end (rEGSx). or treated with BstNI. BstNI digestion products were separated by agarose gel electrophoresis, and the 5′ end-labeled vjbR or yscN mRNA in alkali buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 100 mM NH₄Cl) by mixing E. coli RNase P Cs protein (21) with E. coli M1 RNA in a 1:10 molar ratio. Preparation of RNase P. E. coli RNase P holoenzyme was reconstituted in vitro in PA buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 100 mM NH₄Cl) by mixing E. coli RNase P Cs protein (21) with E. coli M1 RNA in a 1:10 molar ratio. Partial RNase T1 digestion products were separated by using Ultrafree-MC columns (Millipore). This DNA fragment was used as the template for transcription in vitro to generate the rEGS RNA library with a shortened 3′ end (rEGSx). 

Construction of the rEGS Library. The rEGS library was constructed by PCR amplification of a partial random oligonucleotide (5′-CCG GAA TTC CGC TAA TAC GAC TCA 3′) and map R (5′-ATA CTG AAC AAT CCC TGG TG-3′) by PCR. The PCR product was cut with XhoI and EcoRI and cloned into the sites between XhoI and EcoRI of the pBluescript II SK(−) plasmid. The plasmid was named pBSK-yscN. The pBSK-yscN contained the region from +29 to +1,352 of yscN. The first nucleotide of the translation start codon of yscN is set as +1. The pBSK-yscN was cut with PvuII and BstNI and was used as the template for transcription of yscN mRNA by T7 RNA polymerase (Materials and Methods). The resulting RNA contained the region from +29 to +120, as well as 31 nts originating from pBluescript II SK(+).

Transcription in Vitro. T7 RNA polymerase (Promega) was used for the vjbR and yscN EGSs and E. coli RNase P subunit, M1 RNA. SP6 RNA polymerase (Promega) was used for transcription in vitro of ptRNA5′eo from the plasmid pSupS1. Transcription reactions were carried out as described previously (19, 20). The transcripts were purified by using Sephadex G-50 or G-25 Quick Spin columns (Roche Diagnostics). For 5′ end-labeled, the transcription reaction was first dephosphorylated with 0.01 units of calf intestinal phosphatase (Roche Diagnostics)/pmol RNA, and then 5′ end-labeled by T4 polynucleotide kinase (New England Biolabs) in the presence of [α-32P]ATP (GE Healthcare). The substrate ptRNA5′eo (pSupS1) and target RNA used for cleavage assays in vitro were internally labeled by transcription in vitro with [α-32P]GTP (GE Healthcare). The labeled RNAs were purified on 4% polyacrylamide/7 M urea gel (20 cm in length) at room temperature.
Assays for Cleavage of mRNAs by rEGS. An aliquot of rEGSSs was incubated with 10 nM 5' end-labeled yscN or 30 nM vjbR mRNAs in PA buffer for 5 min at room temperature. Subsequently, 20 nM M1 RNA and 200 nM C5 protein were added to the mixture and further incubated for 30 min at 37°C. The randomized EGS RNAs were added up to a 1,000-fold molar excess compared with the target RNA. Specific EGS RNAs were added in 100-, 50-, or 10-fold molar excess. Reactions were performed at 37°C for 30 min and were terminated by adding 8 M urea dye. The mixture was run on 8% polyacrylamide/7 M urea gels (20 cm in length) at room temperature, together with the alkali ladder of yscN or yscN mRNAs and the partial RNase T1-digestion products of vjbR and yscN mRNAs, respectively. After PAGE, the gels were dried and exposed to BioMax film (Kodak). Cleavage site locations were determined by comparing those observed to the partial alkaline ladder and partial RNase T1 cleavage products.

Acknowledgments. We thank the members of our laboratory for helpful discussions and advice and Dr. Mina Izadjoo for stimulating this research project. This work was supported by Defense Threat Reduction Agency Grant W81XWH-06-2-0066, the Fulbright Foundation (E.W.L.), Research Council of Norway grants (to E.W.L.), and Northern Norway Regional Health Authority Medical Research Program grants (to E.W.L.).