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Determination of *in vivo* target search kinetics of regulatory non-coding RNA

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Abstract

Base-pairing interactions between nucleic acids mediate target recognition in many biological processes. We developed a super-resolution imaging and modeling platform that enabled the *in vivo* determination of base pairing-mediated target recognition kinetics. We examined a stress-induced bacterial small RNA, SgrS, which induces the degradation of target mRNAs. SgrS binds to a primary target mRNA in a reversible and dynamic fashion, and formation of SgrS-mRNA complexes is rate-limiting, dictating the overall regulation efficiency *in vivo*. Examination of a secondary target indicated that differences in the target search kinetics contribute to setting the regulation priority among different target mRNAs. This super-resolution imaging and analysis approach provides a conceptual framework that can be generalized to other sRNA systems and other target search processes.

Base-pairing interactions between nucleic acids constitute a large category of target recognition processes such as non-coding RNA-based gene regulation (e.g. microRNAs (1) and long non-coding RNAs (2) in eukaryotes and small RNAs (sRNAs) in bacteria (3)), bacterial adaptive immunity (e.g. the CRISPR system (4)) and homologous recombination (5). Although target search kinetics by transcription factors has been studied *in vivo* (6), the

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SUPPLEMENTARY MATERIALS

Materials and Methods

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Supplementary Movie Captions

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Movies S1 to S3 as separate files

rate constants for target identification via base-pairing interactions *in vivo* are not known for any system. Here, we developed a super-resolution imaging and analysis platform in order to assess the kinetics of base-pairing interaction mediated target recognition for a bacterial sRNA, SgrS. SgrS is produced upon sugar-phosphate stress and its function is dependent on an RNA chaperone protein Hfq. SgrS regulates several target mRNAs post-transcriptionally through base-pairing interactions that affect mRNA translation and stability (7). We combined single-molecule fluorescence *in situ* hybridization (smFISH) (8) with single-molecule localization-based superresolution microscopy (9) to count RNAs and obtain information on subcellular localization. High spatial resolution is required for accurate quantification of the high copy number RNAs and sRNA-mRNA complexes. Here, simultaneous measurements of sRNA, mRNA and sRNA-mRNA complexes together with mathematical modeling allow determination of key parameters describing sRNA target search and downstream co-degradation of sRNA-mRNA complexes.

We first studied the kinetic properties of SgrS regulation of *ptsG* mRNA, encoding a primary glucose transporter. SgrS binds within the 5' untranslated region (UTR) of *ptsG* mRNA, blocks its translation and induces its degradation (10). We induced stress and SgrS production in *E. coli* strains derived from wild-type MG1655 (Table S1) using a non-metabolizable sugar analog, α -methyl glucoside (α MG) (10,11). Fractions of cell culture were taken at different time points post induction and fixed (12). Oligonucleotide probes (Table S2) labeled with photoswitchable dyes, Alexa 647 and Alexa 568, were used to detect SgrS (9 probes) and *ptsG* mRNA (28 probes), respectively, using smFISH (8). We then imaged the cells using two-color 3D superresolution microscopy (9,12) (Fig. 1A; compare to diffraction limited images in Fig. 1B).

In the wild-type strain (Table S1), we observed production of SgrS and corresponding reduction of *ptsG* mRNA over a few minutes (Fig. 1A), consistent with SgrS-mediated degradation of *ptsG* mRNA (10). In a strain producing an SgrS that does not base pair with *ptsG* mRNA due to mutations in the seed region (13,14) and in an Hfq deletion (*hfq*) strain (Table S1), *ptsG* mRNA reduction was not observed (Figs. S1 and S2). To quantify the copy number of RNAs in each cell, we employed a density-based clustering algorithm to map single-molecule localization signal to individual clusters corresponding to individual RNAs (12,15,16) (Fig. 1C and Movies S1 and S2). The absolute copy number quantification was validated by quantitative PCR (12) (Fig. 1D).

We next built a kinetic model containing the following kinetic steps: transcription of SgrS (with rate constant α_S) and *ptsG* (α_P), endogenous degradation of *ptsG* mRNA (β_P), degradation of SgrS in the absence of co-degradation with *ptsG* mRNA ($\beta_{S,p}$), binding of SgrS to *ptsG* mRNA (k_{on}), dissociation of SgrS from *ptsG* mRNA (k_{off}) and RNase E-mediated co-degradation of SgrS-*ptsG* mRNA complex (k_{cat}) (Fig. 1E). We independently measured β_P and the total SgrS degradation rate including endogenous and mRNA-coupled degradation (Table S4, Fig. S3 and Section 1.9 in (12)). Because *ptsG* mRNA levels remained constant in the absence of SgrS-mediated degradation, as observed in the base-pairing mutant strain (Fig. S1), we determined α_P as the product of β_P and *ptsG* mRNA concentration before SgrS induction (Table S4 and Section 1.10 in (12))

To determine k_{on} and k_{off} , it is necessary to count the SgrS-*ptsG* mRNA complexes. Colocalization of *ptsG* mRNA and SgrS at the 40 nm resolution was rarely observed in the wildtype strain (up to ~5%, similar to ~3% colocalization by chance estimated using the base-pairing mutant as a negative control) (Fig. 2). This is possibly because SgrS regulates several other target mRNAs (7) and/or the SgrS-*ptsG* mRNA complex may be unstable due to rapid codegradation or disassembly. In an RNase E mutant strain, in which co-degradation is blocked (17,18) (Table S1), *ptsG* mRNA levels stayed the same as SgrS levels increased (Fig. S4) (17,18), and a fraction of *ptsG* mRNA colocalized with SgrS, increasing over time to reach ~15% (Figs. 2 and S5). A positive control using *ptsG* mRNA simultaneously labeled with two colors (Fig. 2 and Section 1.8 in (12)) showed a high degree of colocalization (~70%), similar to the reported detection efficiency of colocalization by super-resolution imaging (19).

We then applied these measured parameters (α_p and β_p), used total SgrS degradation rate as constraint for $\beta_{S,p}$, and determined the remaining parameters (α_S , $\beta_{S,p}$, k_{on} , k_{off} and k_{cat}) by fitting equations (Fig. 1E) to the six time-course changes of SgrS, *ptsG* mRNA and SgrS-*ptsG* mRNA complex in both the wild-type and the RNase E mutant (Fig. 3A Table S4 and Section 1.10 in (12)). We further validated the model by changing experimental conditions to vary only the transcription rates of SgrS (with lower α_{MG} concentration) and/or *ptsG* mRNA (in the absence of glucose in the growth media), and the model could account for the data with the same set of k_{on} , k_{off} and k_{cat} values (Table S4, Figs. S6–S8 and Section 2.2 in (12)).

We can now quantitatively examine the effect of Hfq, which functions by stabilizing the sRNA or promoting its annealing with the target mRNA (20). In the *hfq* strain, the degradation rate of SgrS increased 20-fold, while the SgrS-*ptsG* mRNA association rate decreased slightly (Table S4, Figs. S1 and S8 and Section 2.2 in (12)). Therefore, for the SgrS-*ptsG* mRNA pair, the primary effect of Hfq in regulation kinetics is in SgrS stabilization.

This *in vivo* determination of base pairing-mediated target search kinetics revealed two important characteristics of SgrS-mediated *ptsG* mRNA degradation. First, the target search process is characterized by slow association ($k_{on} = (2.0 \pm 0.2) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) and fast dissociation ($k_{off} = 0.20 \pm 0.04 \text{ s}^{-1}$), resulting in a dissociation constant ($K_D = k_{off}/k_{on}$) of $1.0 \pm 0.2 \text{ } \mu\text{M}$ (Fig. 3B and Section 1.11 in (12)). In order to get comparable apparent association rate, $k_{a,app}$ ($k_{on} \times [S]$), and k_{off} , about one thousand SgrS molecules per cell need to be produced. The large K_D explains the need for excessive production of sRNA molecules to enable rapid regulation when cells experience high levels of stress. Despite the crowded cellular environment and large excess of non-target RNA molecules, the k_{on} is within the wide range of Hfq-mediated sRNA and target mRNA association rates reported by *in vitro* measurements. In contrast, k_{off} is 1–2 orders of magnitude larger than *in vitro* estimates (21–23). The large K_D for target search *in vivo* is likely due to the limited availability of key players in the cell. For example, Hfq was suggested to be limited in the cell due to the dynamic competition for Hfq among different sRNAs (24,25). Second, k_{cat} and k_{off} are comparable such that both co-degradation and dissociation can occur with similar probabilities upon target binding. Disallowing dissociation by setting k_{off} to zero cannot

explain our experimental data (Fig. S9 and Section 2.1 in (12)). The observed fast k_{cat} ($0.4 \pm 0.1 \text{ s}^{-1}$) may be due to the formation of a ribonucleoprotein complex comprised of SgrS, Hfq and RNase E as suggested by biochemical studies (18); if so, once SgrS-Hfq-RNase E binds the *ptsG* mRNA, RNase E would be readily available for co-degradation.

The kinetic model suggests that the overall rate of *ptsG* mRNA degradation is limited by its association with SgrS: at early time points after SgrS induction, when the copy number of SgrS is on the order of tens per cell, $k_{\text{a,app}}$ is about two orders of magnitude smaller than the codegradation rate k_{cat} . The non-homogenous spatial distribution of the sRNA and its target mRNA may also contribute to the slow target search. We observed membrane localization of *ptsG* mRNA, whereas SgrS is primarily localized in the cytoplasm (Fig. S10). Further modeling incorporating the spatial information and stochastic gene expression may improve the kinetic analysis.

Regulation prioritization among multiple targets by one sRNA was suggested by computational modeling (26,27) and experimental observation (28). However, how the kinetic prioritization is achieved remains to be elucidated. We propose that the combination of k_{on} , k_{off} and k_{cat} is characteristic of a specific sRNA-mRNA pair and determines the regulatory outcome. While k_{cat} may reflect the regulatory mode (co-degradation vs. translation repression), target search kinetics (k_{on} and k_{off}) could contribute to the regulatory specificity and priority among many targets. To test this possibility, we examined *manXYZ* mRNA, which encodes a general sugar transporter for mannose and glucose, and is also negatively regulated by SgrS. Compared to *ptsG*, *manXYZ* mRNA showed slower degradation kinetics (28) (Figs. S11–S13). The prioritization of *ptsG* over *manXYZ* by SgrS is consistent with the observation that SgrS regulation of *ptsG* (but not *manXYZ*) is absolutely essential for continued cell growth under most stress conditions (29). Using the RNase E mutant strain we found that formation of SgrS-*manXYZ* mRNA complexes is slower than SgrS-*ptsG* mRNA complex formation (Fig. S13C). The K_D for SgrS binding to *manXYZ* mRNA, $2.3 \pm 0.2 \text{ }\mu\text{M}$, was also higher than $1.0 \pm 0.2 \text{ }\mu\text{M}$ for SgrS binding to *ptsG* mRNA (Fig. 3B and Section 1.11 in (12)). This result indicates that the slower regulation kinetics observed for *manXYZ* may, at least partially, originate from the differences in target search kinetics.

Overall, our results indicate that the formation of sRNA-mRNA complexes is reversible and highly dynamic in the cell, providing additional layers for regulating individual targets. Our kinetic model highlights the importance of target search kinetics on regulation prioritization. This super-resolution imaging and analysis platform provides a conceptual framework that can be generalized to other sRNA systems and potentially to other target search processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Super-resolution imaging and analysis

(A) 3D Super-resolution images of SgrS and *ptsG* mRNA labeled by smFISH projected in 2D planes. (B) Diffraction-limited fluorescent images of SgrS and *ptsG* mRNA. Cell boundaries imaged by DIC in (A) and (B) are depicted by white solid lines. (C) Examples of clustering analysis with comparison of raw data (left) and clustered data (right). (D) Comparison of average RNA copy number per cell measured by super-resolution imaging and qPCR. (E) Kinetic scheme of SgrS-induced *ptsG* mRNA degradation. Kinetic steps are

described in the main text. $[p]$, $[S]$ and $[Sp]$ are the concentrations of *ptsG* mRNA, SgrS and their complex, respectively, in the mass-action equations.

Figure 2. Colocalization analysis of SgrS-*ptsG* complex

(A) Example of colocalization under various conditions. (B) Quantification of colocalized fraction of *ptsG* mRNA in cases (ii), (iii) (at 10 minutes post-SgrS induction) and (iv) (at 10 minutes post-SgrS induction). Error bars are standard deviations from three to eight images. (C) Time-course changes in the fraction of colocalized *ptsG* mRNA with SgrS. Error bars are standard errors from 200–600 cells from two independent measurements.

Figure 3. Estimation of kinetic parameters

(A) Modeling of time-course changes of SgrS, *ptsG* mRNA and SgrS-*ptsG* complexes.

Average copy numbers per cell are plotted as a function of time. Rate constants and weighted R^2 for modeling are reported in Tables S4 and S5. (B) Extraction of K_D for SgrS-mRNA complex formation. The ratio of mRNA in complex with SgrS to free mRNA is plotted against average SgrS copy number and the slope of the linear fitting reports $1/K_D$. Error bars in (A) and (B) report standard errors from 200–600 cells from two independent measurements.