Dynamic features of gene expression control by small regulatory RNAs

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Small regulatory RNAs (sRNAs) in eukaryotes and bacteria play an important role in the regulation of gene expression either by binding to regulatory proteins or directly to target mRNAs. Two of the best-characterized bacterial sRNAs, Spot42 and RyhB, form a complementary pair with the ribosome binding region of their target mRNAs, thereby inhibiting translation or promoting mRNA degradation. To investigate the steady-state and dynamic potential of such sRNAs, we examine the 2 key parameters characterizing sRNA regulation: the capacity to overexpress the sRNA relative to its target mRNA and the speed at which the target mRNA is irreversibly inactivated. We demonstrate different methods to determine these 2 key parameters, for Spot42 and RyhB, which combine biochemical and genetic experiments with computational analysis. We have developed a mathematical model that describes the functional properties of sRNAs with various characteristic parameters. We observed that Spot42 and RyhB function in distinctive parameter regimes, which result in divergent mechanisms.

gene regulation | mRNA silencing | RyhB | small RNA | Spot42

Small regulatory RNAs (sRNAs) play numerous roles in stress adaptation and development in eubacteria, archeabacteria, and eukaryotes (1, 2). These molecules are generally noncoding and <300 nt. Today, ≈70 sRNA genes have been identified in *Escherichia coli* (3–5). Those characterized are involved in diverse cellular functions such as initiation of DNA replication (6), regulation of transcription (7), regulation of translation (8–12), and mRNA stability (13, 14). Such sRNAs are usually expressed in response to a specific stimulus that signals the cell about a possible threat to survival.

A critical stimulus to which growing cells must respond rapidly is the level of glucose, which regulates genes involved in carbon metabolism such as those belonging to the galactose operon (*galETKM*). In the absence of glucose, D-galactose can induce transcription of all 4 genes of the *galETKM* operon in equimolar fashion, which is required for catabolism of D-galactose. In contrast, when cells grow in the presence of glucose, mainly the first 2 genes of the operon are expressed, which are necessary for providing substrates for biosynthetic glycosylation reactions (15–17). Spot42, a 109-nt-long sRNA, is involved in this glucose-dependent regulation. When glucose is used as a carbon source, Spot42 represses the translation of *galK*, the third gene of the galactose operon that is unnecessary when glucose is present (11, 18). However, when cells grow in the absence of glucose, Spot42 levels decrease significantly, allowing translation of *galK*. It has been suggested that the sRNA only acts at the translation level, because Spot42 expression does not inhibit GalE and GalT synthesis (11).

Another important cellular response mediated by a sRNA is the adaptation to iron (Fe) starvation (19). The sRNA RyhB, normally repressed by the transcriptional regulator Fur (ferric uptake regulator) in presence of sufficient Fe, is expressed in the bacterium *E. coli* specifically during Fe depletion (20). Thus, in conditions of low Fe, RyhB induces the rapid degradation of at least 18 mRNAs, all of which encode nonessential Fe-using proteins, to redirect the metal into essential parts of the cell (19, 21). The rapidity of this sRNA-mediated mRNA degradation (within 2–3 min) suggests that the target mRNAs have to be silenced as soon as possible under low Fe conditions. We recently confirmed this hypothesis by demonstrating an inhibitory growth phenotype of a *ryhB* mutant during Fe starvation (22). Furthermore, data from our group indicates that RyhB plays a role in the regulation of intracellular Fe homeostasis (22, 23). Because Fe is an essential factor that is carefully distributed among the Fe-using proteins, it is likely why many organisms share a RyhB-like mechanism of Fe regulation (21, 24).

Despite the importance of sRNAs, their regulatory features are not yet fully understood. Here, we analyze quantitative properties of regulation where a sRNA binds to target mRNAs, which is the mechanism of action of a large family of sRNAs in bacteria. The pairing between the sRNA and a target mRNA facilitates irreversible inactivation of both RNAs in the complex (in the case of RyhB both the sRNA and mRNA are actively degraded), as illustrated in Fig. 1. sRNA regulation can exhibit ultrasensitivity (25), hierarchy of action (26), cross-talk between downstream targets (19, 23, 27), prioritization of targets (28), fast response against sudden environmental changes (13, 28, 29), and discoordination of translation in a polycistronic mRNA (11, 30). Most of these abilities, except discoordination, are constrained by 2 key parameters: the system's capacity to overexpress the sRNA relative to its target (α) and the speed at which the target is irreversibly inactivated (γ). By performing a computational analysis of the potential functional properties of sRNA regulation, covering all parameters, we show which functions can be achieved for which parameter values. Using 2 well-known sRNA systems, Spot42 and RyhB, we demonstrate different methods for the determination of the above 2 key parameters by combining biochemical and genetic experiments with computational analysis.

Results

Ultrasensitivity and Graded Regulation. Fig. 2*B* shows the steady-state concentration of the target mRNA, m, as a function of α for different values of the degradation rate γ (the units were

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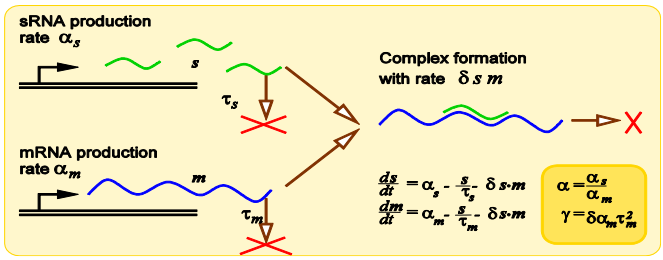

Fig. 1. Schematic mechanism of sRNA regulation. The formation of the sRNA–mRNA complex irreversibly inactivates both RNAs. The behavior is entirely determined by 3 parameters (see *SI Text*): the ratio of the sRNA production rate to mRNA production rate, $\alpha = \alpha_s/\alpha_m$; the dimensionless rate of inactivation of the mRNA via sRNA–mRNA complex formation, $\gamma = \delta\alpha_m\tau_s^2$; and the ratio of the mRNA lifetime to the sRNA lifetime, $\tau = \tau_m/\tau_s$.

chosen as described in *SI Text*). For $\alpha < 1$ there is insufficient sRNA to inactivate much mRNA, whereas when $\alpha > 1$ the mRNA levels are significantly reduced. This reduction is much sharper for a larger γ value, i.e., a small change of α results in a large difference in mRNA concentration m. Such switch-like regulation has been termed "ultrasensitivity" (25). At lower γ values, m declines in a more "graded" manner.

Speed of Response. In addition to steady-state regulation, the speed of the response is also important. Perturbations in the external stimuli sensed by a sRNA will result in changes in the value of α. When α is abruptly increased from 0.01 to a higher value (which we vary from $\alpha = 2$ to 10), then the mRNA level decreases rapidly in response. Fig. 2*C* shows that the time required for m to reach its new steady state is smaller for larger α and larger γ, because less time is needed to produce more sRNA for large α, whereas a large γ results in quick degradation of m. Because increasing α can compensate for lower γ, and vice versa, the behavior of m with $\alpha = 10$ and $\gamma = 10$ looks very similar to the case of $\alpha = 2$ and $\gamma = 100$. Note, however, that the former has a much higher sRNA level in the new steady state.

When α is abruptly decreased from a starting value (which we vary from $\alpha = 2$ to 10) to 0.01, the response is slower than in the case of increasing α, as shown in Fig. 2*D* (note the larger span on the x axis relative to Fig. 2*C*). This difference is because recovery of m level begins only after all of the sRNAs (which are not being produced any more) present are degraded. The larger α is, the larger this initial pool of sRNA, and therefore the longer the time delay before recovery of mRNA level.

In both scenarios (increasing or decreasing α), however, a larger value of γ results in relatively sharper regulation and faster response.

Determination of α and Calculation of γ. The parameter α can be measured directly, both in vivo and in vitro by measuring the promoter strengths for the sRNA and target mRNA in steady-state conditions, and computing their ratio. However, direct measurement of γ is difficult: not much is known about the mechanism of Hfq-mediated sRNA–mRNA coupling. Once α is determined, however, the value of γ can be calculated by measuring either (*i*) the timing of sRNA–mRNA degradation or (*ii*) the steady-state level of mRNA and sRNA.

Measurement of α and Estimation of γ for RyhB and Target mRNA Using Degradation Kinetics. For the Fe–Fur system in *E. coli*, it has been observed that 1 of the RyhB targets, *sodB*, is depleted ≈5-fold within 3 min after full induction of the P_{ryhB} promoter (13), which constrains the value of α and γ. We numerically calculated the time required to deplete mRNA 5-fold, T, by changing the value of α from ≈0 to higher values (see Fig. 3). T strongly depends on α and γ. The contour line of $T = 3$ min is the solid green line in Fig. 3. Further, we measured the value of promoter activity of

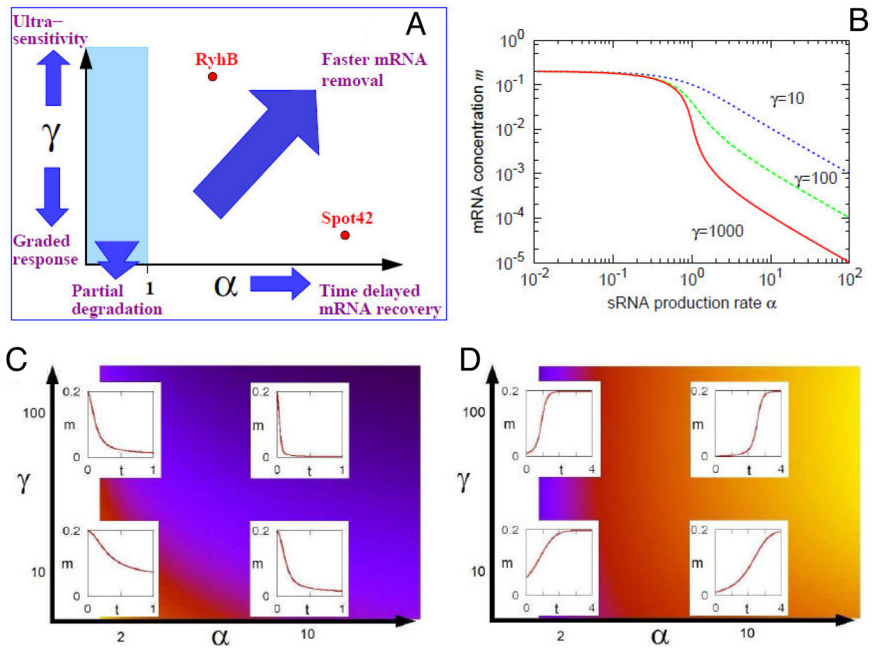

Fig. 2. Capabilities of sRNA regulation. (*A*) Schematic diagram showing functional features observed in the model behavior at different values of the parameters, α and γ. These are shown more quantitatively in *B*–*D*. (*B*) The steady-state level of sRNA as a function of α for $\gamma = 10$ (blue dotted line), $\gamma = 100$ (green dashed line), and $\gamma = 1{,}000$ (red solid line). The value of mRNA concentration m for $\alpha > 1$ is scaled with $1/\gamma$, which results in a ≈10-fold difference between lines. m changes gradually for small γ, and more abruptly when γ is large (termed "ultrasensitivity"). (*C*) Dynamics of m (red solid lines) when the value of α is increased, at time 0, from 0.01 to the value shown on the horizontal axis. The background color indicates the time to reach the new steady state, with darker colors indicating shorter times. (*D*) Dynamics of m when the value of α is reduced, at time 0, from the value shown on the horizontal axis to 0.01. The background color indicates the time to reach the new steady state; darker colors indicate shorter times.

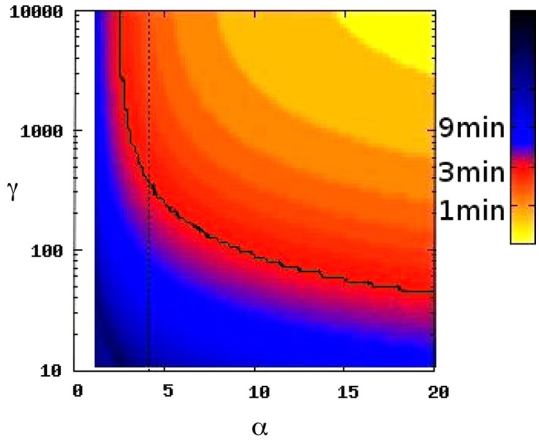

Fig. 3. Measurement of γ from dynamics of target degradation. The color indicates the time T required, in model simulations, to deplete mRNA levels by 5-fold in respond to a sudden increase of α from near 0 to the value on the x axis. The solid line shows the contour corresponding to 3 min, the time observed in RyhB degradation of *sodB* mRNA (13). In the simulation, the lifetimes of *sodB* τ_m = 5/ln2 min and RyhB τ_s = 25/ln2 min were used. The dotted line indicates $\alpha = 4$, the value obtained from our measurements of promoter activities. To calculate α, we assume to have measured all of the target mRNAs of RyhB in Fig. 4. If there are more targets, the computed α value will be less, hence γ will be higher. That is, 4 is an upper bound for α, and 400 is a lower bound for γ. Therefore, our conclusion that RyhB is characterized by low α and high γ is true for *sodB*.

RyhB and major target mRNAs (see Fig. 4) from which we calculate α, the ratio of sRNA production to sum of the production of all its target mRNAs, to be ≈4. For $\alpha = 4$, the $T =$ 3-min contour in Fig. 3 leads to an estimate of ≈400 for the γ value for *sodB*. Therefore, this system represents the low-α, high-γ case, which is consistent with our previous predictions (23). Note that we use one single α, as if all target mRNAs have

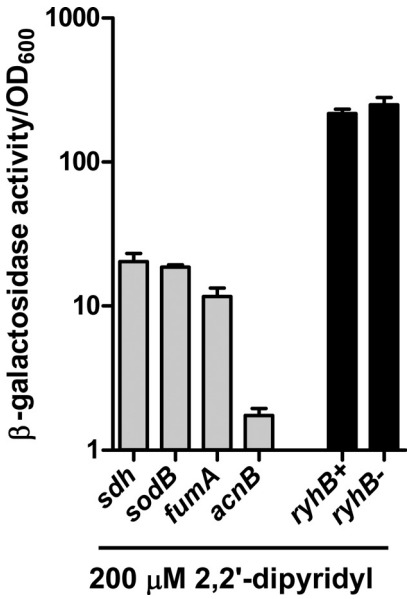

Fig. 4. Specific β-galactosidase activities of promoter fusions of the Fe–Fur system without Fe (in the presence of 200 μM the Fe chelator 2,2′-dipyridyl). In these conditions, the promoter of *ryhB* is fully active. Promoter activities of 4 target mRNAs are indicated by the gray bars, and their sum gives $\alpha_m \approx 20 + 19 + 12 + 2 = 53$. The promoter activity of P_{ryhB} is α_s ≈217(left black bar, *ryhB*+), therefore $\alpha = \alpha_s/\alpha_m \approx 4$. See *SI Text* and Table S1 for experimental details.

similar γ values. The effect of this assumption in determination of γ for *sodB* is discussed in *SI Text*. More careful analysis requires detailed knowledge of the variation of γ across the target mRNAs, as outlined in ref. 28.

Measurement of α and Estimation of γ for Spot42 and Target mRNA Using in Vitro Transcription Assays. The Spot42 sRNA specifically binds the *galK* Shine-Dalgarno region of the *galETKM* mRNA and inhibits production of GalK by blocking ribosome binding. Because translation of the upstream regions is not affected, Spot42 action results in discoordinated translation of the *gal* operon genes (11). Transcription of the *spf* gene encoding the Spot42 sRNA is reduced 3- to 5-fold in the presence of cAMP-CRP (31). The *galETKM* operon is transcribed from 2 promoters, $P1_{galE}$ and $P2_{galE}$. Both promoters are repressed in the absence of galactose by GalR. D-galactose inhibits GalR-mediated repression of both promoters. When D-galactose is present, cAMP-CRP can activate $P1_{galE}$ and repress $P2_{galE}$ (32). To determine the relative intensities of sRNA and mRNA transcription we measured the intrinsic activities of the P_{spf} (for sRNA) and $P1_{galE}$ and $P2_{galE}$ (for mRNAs) promoters in the absence of regulators (Fig. 5). Quantitation of band intensities (see *SI Text*) resulted in $\alpha = 18$. The promoter activities measured approximate the conditions in ref. 11 when Spot42 sRNA is expressed at full strength (not repressed by cAMP–CRP) and the *gal* operon is transcribed at the maximum rate possible in the absence of cAMP–CRP and GalR. In cells grown in glucose-rich media the unrepressed P_{spf} promoter activity, approximated in the in vitro transcription assay, results in ≈200 copies of Spot42 sRNA per cell with a half-life of 12–13 min (18). Therefore, by our calculations, the sRNA is produced at a rate of 11 per min and the mRNA, which has a half-life of 4–5 min (33), is produced at a rate of 0.6 per min. This level of Spot42 sRNA reduces the amount of the GalK protein to ≈30% of the level observed in Δ*spf* or glycerol-grown (high cAMP–CRP) cells (11). Because the GalK level is not affected by the 3- to 5-fold reduced Spot42 level in the presence of cAMP–CRP (11), we assume that mRNA–sRNA pairing is negligible when the Spot42 sRNA is present at <50 copies per cell.

In this case, the degradation kinetics is not known, but the amount of sRNA for different values of α and the according fold change of mRNA level has been measured. As can be seen in the plot of the steady state in Fig. 2*B*, for a given value of α, the mRNA level (and also the sRNA level) varies depending on γ, thus once the fold change of mRNA by changing α is known, γ can be calculated. Let m_1 (s_1) denote the concentration of mRNA (sRNA) with P_{spf} unrepressed and m_2 (s_2) denote the concentration of mRNA (sRNA) with P_{spf} repressed. Assuming steady state in both cases, we find, from the equations of Fig. 1, that

$$\frac{m_1}{m_2} = \frac{1/\tau_m + \delta s_2}{1/\tau_m + \delta s_1} \qquad [1]$$

from which γ can be determined:

$$\gamma = \delta\alpha_m\tau_s^2 = \alpha_m\tau_s^2\left[\frac{1 - (m_1/m_2)}{[(m_1/m_2)s_1 - s_2]\tau_m}\right]. \qquad [2]$$

Inserting $(m_1/m_2) = 0.3$, $s_1 = 200$ copies per cell, and $s_2 = 50$ copies per cell, we get $\gamma \approx 2$. This system thus represents a high-α, low-γ case.

Summary and Discussion

Many features of sRNA-mediated mRNA regulation are determined by 2 key parameters: the system's capacity to overexpress the sRNA relatively to its target mRNA (α) and the speed at which the target mRNA is irreversibly inactivated (γ). In this

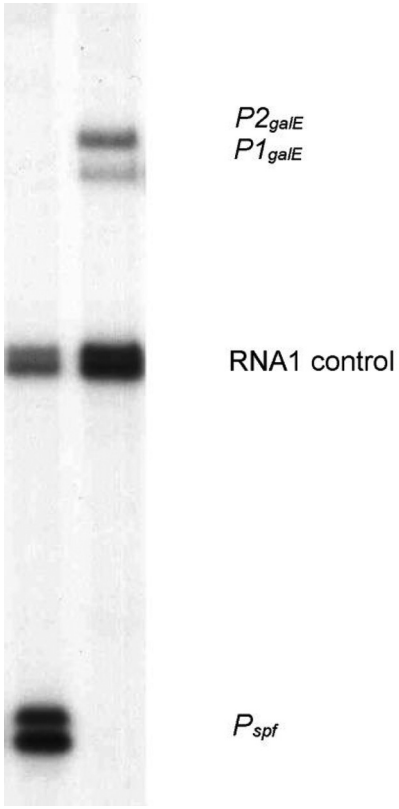

Fig. 5. In vitro transcription assays were performed on pSA850spf (P_{spf}, *Left*) and pSA850 ($P1_{galE}$ and $P2_{galE}$, *Right*) supercoiled plasmid DNA templates in the presence of σ70 RNA polymerase. The *RNA1* transcript was used as an internal control between lanes. See *SI Text* for experimental details.

article, we used a mathematical model to analyze how the regulatory behavior changes depending on these parameters. We then determined α experimentally in 2 sRNA systems, RyhB and Spot42, using 2 different approaches to estimate γ. For the sRNA RyhB, we used the time course of depletion of target mRNA upon sudden derepression of the sRNA promoter, from ref. 13. Using our model simulations of the depletion process and our measurement of α to be 4 or less, we estimate that γ is at least 400 for the target *sodB*. For Spot42, we measured α to be $\approx$18. From the relation between concentrations and model parameters at 2 different steady states, we estimated γ to be $\approx$2. The results indicate that sRNA–mRNA systems can function in different parameter regimes.

We suggest that evolution of sRNA–mRNA systems functioning at different parameter regimes has a physiological significance. In our description of sRNA–mRNA systems, RyhB represents sRNAs that recognize target mRNAs very efficiently (high γ) but where sRNA excess (α) is low. According to our model predictions, this parameter regime optimizes the system for efficient and fast inactivation of target mRNAs in case of stress and, at the same time, a slightly time-delayed recovery of target mRNA levels after the sRNA synthesis is stopped (when stress is removed). Indeed, the RyhB system is responsible for the quick rearrangement of the intracellular channeling of available Fe. RyhB inhibits translation of nonessential Fe-using proteins, allowing a larger fraction of the limited Fe to be available for essential Fe proteins (22). To achieve recovery in the absence of stress, the sRNA expression is rapidly stopped, making the sRNA level decline, which after some time results in production of target genes, coding for nonessential Fe-using proteins. The length of the associated delay depends on how much α exceeds 1 and could be seen as buffering time to confirm that stress is persistently absent. However, because of high γ, the mRNA level quickly recovers after the time-delay period is over. Our results are in agreement with the previously observed in vivo expression of RyhB (13). This type of regulation demonstrates a "dual" response where the cell adapts rapidly to substrate deprivation and more slowly when the substrate becomes newly available.

As opposed to RyhB, the sRNA Spot42 is expressed at higher levels relative to the target mRNA (α large). Nevertheless, the pairing between Spot42 and the *gal* mRNA is inefficient (γ small). Modeling predicts that the activation of Spot42 gives similar fast decline of its target gene. However, turning off of the Spot42 system should give a more graded response with a long time delay. In a recent article by Mehta et al. (34) in which the stochastic response of sRNA regulation was characterized, they suggest that graded response by sRNAs may be limited by transcriptional noise (transcriptional burst). Nevertheless in the case of Spot42, our data indicate that having a low γ would allow filtering of the noise. Thus, even though transcriptional bursts may occur for Spot42, the effect is reduced by the low binding capacity of Spot42 on the target mRNA. Unlike RyhB, Spot42 modulates the amphibolic utilization of the sugar D-galactose by discoordinating the synthesis of the enzymes involved in galactose metabolism (11, 35). Theoretically, partial regulation can be achieved by having low α (even $<$1) and high γ or having high α and low γ. In the case of Spot42, we observed the latter case presumably implemented by weak pairing between Spot42 and *galK* mRNA. The possible advantage of this parameter regime is that the mRNA level is less sensitive to any noise in the sRNA level. If Spot42 were expressed at a lower abundance than the *galETKM* message ($\approx$0.6 per min), however, the noise level would be quite significant and might interfere with efficient functioning.

In principle γ can be regulated by global changes in the cellular physiology (e.g., affecting the level or activity of the RNA chaperone Hfq). However, because those changes are difficult to quantify we did not consider them in our equations. Nevertheless, our analysis does indicate ways in which global changes can affect the system, especially through the parameter α, which can be specifically regulated by changes in gene expression of the sRNA and/or the target mRNA. For example, in the galactose utilization network of *E. coli*, transcription of both the *spf* gene and the *gal* operon is simultaneously regulated by cAMP–CRP (11, 35). When cAMP–CRP level is increased, *spf* transcription is inhibited, while *gal* transcription is activated, therefore removal of Spot42 sRNA can be facilitated, resulting in a faster but still graded recovery of GalK.

In this work although we focused on 2 particular sRNAs, we assume that our findings can be extended to most, if not all, sRNAs using antisense pairing to regulate their target mRNAs. As demonstrated with RyhB and Spot42, the determination of both α and γ parameters in other sRNAs would indicate the type of physiological response corresponding best to a specific signal. One could imagine a sRNA that acts with a high α and γ, both parameters being necessary high for a rapid and efficient inactivation of a highly expressed target mRNA. Another example is low α and low γ, which would only slightly modulate the target mRNA expression, thus allowing a more graded response. In such a case however, a protein regulator, which allows greater quantitative adjustments than sRNAs, may be more appropriate (34). Indeed unlike protein regulators, the regulation by sRNAs was shown to work best in a situation where a large change in signals is observed (34).

In addition to silencing target mRNAs, some sRNAs activate their target mRNAs by making a complex that can be readily translated into proteins. For example, the sRNAs RyhB, DsrA, and GlmY bind to the 5′ UTR of *cis*-repressed mRNAs such as *shiA*, *rpoS*, and *glmS*, respectively, to activate translation initia-

tion (10, 26, 36–38). In such cases, the sRNA–mRNA complex formation is also considered irreversible, and the response speed will depend on the lifetime of this complex and α and γ. The model can be easily extended to such an activation scenario, which in addition opens for regulation with α substantially smaller than 1.

Our findings are also applicable to synthetic RNA molecules that are engineered to regulate gene expression in vivo (reviewed in ref. 39). Indeed, the rational design of synthetic base-pairing RNAs with specific α and γ will help to achieve tunable and programmable effects on the cell. For example, a single RNA molecule combining multiple binding domains, each with a different γ, could allow a concerted and complex response. This type of molecule could also be used as a tool to disrupt several genes at once to study a complex genetic network.

The combined use of genetic experiments and mathematical calculation allowed us to determine fundamental differences in the regulatory mechanisms of 2 well-characterized sRNAs. We showed that the RyhB system has an ultrasensitive response, whereas the response in the Spot42 system is more graded. It would be of interest to investigate similar parameters for other sRNAs, which may open up additional perspectives on these sophisticated regulatory molecules.

Materials and Methods

Mathematical Model of sRNA and mRNA Dynamics. We model the coupling between an sRNA (s) and its target mRNA (m) using the equations in Fig. 1 (23, 25, 27–29). In *SI Text* we describe how the number of parameters can be decreased by appropriately choosing units of time and concentrations. Three dimensionless parameters then determine the model behavior. The 2 most important are: the sRNA production rate relative to the mRNA production rate, $\alpha = \alpha_s/\alpha_m$, and the mRNA inactivation rate $\gamma = \delta\alpha_m\tau_s^2$. Each individual sRNA–mRNA pair would have a specific value of γ; in general, one would expect that more basepair matching between the sRNA and its target would correlate with a higher value of γ. The third parameter, τ, is the passive degradation time of the mRNA relative to the sRNA. Typically, sRNAs have longer natural half-lives than mRNAs, i.e., $\tau < 1$, partly because Hfq can protect the sRNAs (11, 13, 40). For *sodB*, a target of RyhB, $\tau = 0.2$ (20), and we use this as a default value, unless otherwise specified.

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