Artificial regulation of gene expression in *Escherichia coli* by RNase P

(M1 RNA/external guide sequences/ β -galactosidase/alkaline phosphatase/suppressor tRNA)

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ABSTRACT Plasmids encoding various external guide sequences (EGSs) were constructed and inserted into *Escherichia coli*. In strains harboring the appropriate plasmids, the expression of fully induced β -galactosidase and alkaline phosphatase activity was reduced by more than 50%, while no reduction in such activity was observed in strains with non-specific EGSs. The inhibition of gene expression was virtually abolished at restrictive temperatures in strains that were temperature-sensitive for RNase P (EC 3.1.26.5). Northern blot analysis showed that the steady-state copy number of EGS RNAs was several hundred per cell *in vivo*. A plasmid that contained a gene for M1 RNA covalently linked to a specific EGS reduced the level of expression of a suppressor tRNA that was encoded by a separate plasmid. Similar methods can be used to regulate gene expression in *E. coli* and to mimic the properties of cold-sensitive mutants.

The potential value of ribozymes (e.g., RNase P, hammerhead, hairpin) as therapeutic agents is a direct consequence of their ability to catalyze sequence-specific cleavage of targeted RNAs *in vitro* (1). To date, the hairpin and hammerhead ribozymes have also been used with some success to cleave specific mRNAs in tissue culture (2–7). Moreover, RNase P from both *Escherichia coli* and human tissue can inhibit gene expression in mammalian cells in tissue culture (8, 9). In contrast to experiments with eukaryotic cells, mixed results have been obtained for targeted cleavage by hammerhead ribozymes of mRNAs in *E. coli* (10–12). We show here that RNase P can be used to regulate the expression of several genes in *E. coli*.

The main physiological role of RNase P, an essential enzyme (EC 3.1.26.5), is to cleave precursor tRNAs (ptRNAs) to generate the 5' termini of mature tRNA molecules (13). The enzyme in *E. coli* is a ribonucleoprotein in which the RNA moiety (M1 RNA) is the catalytic subunit (14) and the protein subunit (C5 protein) is a necessary cofactor *in vivo* (15). We have shown previously that RNase P can specifically cleave any RNA *in vitro*, provided that the RNA is hybridized to a short oligonucleotide, known as an external guide sequence (EGS), that is complementary to the targeted region (8, 16, 17), but no similar data have been reported for inhibition of gene expression in *E. coli*. The use of an *E. coli* strain that is temperature sensitive for RNase P (18) has allowed us to distinguish between inhibition of targeted gene expression *in vivo* by the action of RNase III or by antisense interactions with targeted RNA and the inhibition that is due to the activity of RNase P.

MATERIALS AND METHODS

Materials. T4 DNA ligase, the Klenow fragment of DNA polymerase I, and restriction endonucleases were purchased

from New England Biolabs; T7 RNA polymerase and RNasin were purchased from Promega; rapid hybridization buffer and radiolabeled chemicals were from Amersham; and Sequenase was from United States Biochemical.

Bacterial Strains. In this study we used BL21 (DE3), F⁻ompT[lon]hsdS_B, r_Bm_B (a strain of *E. coli* B) with DE3, a λ prophage that carries the gene for T7 RNA polymerase (19); BL21(DE3)A49, which is similar to NHY322, Δ (proB-lac), ara, gyrA, thi, zic-501::Tn10, rnpA49 (20, 21); and *E. coli* PP113(A49), recA, lacZ^{am}, rnpA49 (18). T7A49 was constructed by phage P1 transduction with NHY322 as the donor and BL21(DE3) as the recipient, with selection not only for tetracycline resistance (Tet^R) and temperature sensitivity (the donor's markers) but also for β -galactosidase and T7 RNA polymerase activity (the recipient's markers).

Plasmids. To construct genes for EGSs under the control of phage T7 RNA polymerase, inserts were cloned into pUC19 that lacked a Pvu II–Pvu II fragment of 322 bp. DNA oligonucleotides containing the sequences for the T7 promoter, the various EGSs used, and a hammerhead core of 57 nucleotides (CCAGGUCACCGGAUGUGCUUCCGGUCUGAUGAGUCCGUGAGGACGAAACCUGGAUC; the underlined sequence is the 3' terminus of the EGS) were ligated to a BamHI–HindIII fragment that contained the T7 terminator sequence. This DNA fragment was obtained from pET3040 (22). Plasmid DNAs that contained the new inserts were obtained from transformants and subjected to sequence analysis to verify that the expected sequences were present. The following plasmids were obtained: pNT7DS1HH (coding for DS1-EGS, directed against β -galactosidase mRNA); pNT7APHH (coding for AP-EGS, directed against alkaline phosphatase mRNA); and pNT7CIHH and pNT7NHH (coding for EGSs directed against phage λ CI and N mRNAs, respectively). Plasmid pBRCCA was a generous gift from N. B. Reuven and M. P. Deutscher (University of Connecticut Health Center). For use in experiments in which the suppressor tRNA^{Tyr}_{su3} was the target, an EcoRI–HindIII fragment (filled in with the Klenow fragment of DNA polymerase) from pBRCCA was inserted into the HindIII site (also filled in similarly) of pACYC184. The new plasmid was called pACSu₃. All EGSs were also cloned downstream from the gene for M1 RNA (M1-EGSs) that was transcribed under the control of *E. coli* RNA polymerase in pUC19 that lacked a Pvu II–Pvu II fragment of 322 bp.

Culture Conditions. Overnight cultures of T7 A49 cells (transformed with plasmids that harbored different EGSs) in LBC (LB medium supplemented with carbenicillin at 100 μ g/ml) were diluted to OD₆₀₀ = 0.05 in LBC and the cultures were incubated at 30°C for about 3 h. The cells were then harvested and resuspended in phosphate-free P medium (23) supplemented with carbenicillin and isopropyl β -D-thiogalactoside (IPTG) (2 mM, final). One half of each culture was

incubated at 30°C and the other half was incubated at 43°C for 1 further hour. Cell growth was stopped by placing the cultures on ice. Three aliquots were taken from each culture: one for assays of β -galactosidase and alkaline phosphatase activities, one for assays of RNase P, and one for extraction of RNA.

In experiments in which tRNA^{Tyr}su₃⁺ was targeted, *E. coli* PP113(A49) (which contained a *lacZ*^{am} mutation) was transformed with two plasmids: the first, pACSu₃⁺, encoded a functional tRNA^{Tyr}su₃⁺; and the second was chosen to express different M1-EGS (or mutant M1-EGS) RNAs. All RNAs were transcribed by *E. coli* RNA polymerase. Overnight cultures in LBACAP (LB medium supplemented with ampicillin at 100 μ g/ml and chloramphenicol at 50 μ g/ml) were diluted to OD₆₀₀ = 0.05 in LBACAP and IPTG (2 mM, final concentration) and were incubated at 30°C for about 3 h. The cultures were then divided and one half of each culture was incubated at 30°C and the other half at 43°C for 1 further hour. Cell growth was stopped by placing the cultures on ice. Two aliquots were taken from each culture: one for assays of β -galactosidase and RNase P and one for extraction of RNA.

Assays for β -Galactosidase and for Alkaline and Acid Phosphatases. Assays for β -galactosidase were performed at 28°C in Z buffer (60 mM Na₂HPO₄/40 mM NaH₂PO₄/10 mM KCl/1 mM MgSO₄/50 mM 2-mercaptoethanol) as described by Miller (24). Alkaline phosphatase activity was measured at 37°C in 200 mM Tris-HCl, pH 8.0, with the substrate, *p*-nitrophenyl phosphate (NPP), at 0.8 mg/ml. Acid phosphatase activity was measured at 37°C in 200 mM NaOAc, pH 4.0, with NPP as substrate at 0.8 mg/ml. All reactions were terminated by the addition of 1 M Na₂CO₃ (0.75 M, final).

Assays for RNase P Activity. One-milliliter aliquots of cell cultures were harvested and the cell pellets were resuspended with 100 μ l of buffer A (50 mM Tris-HCl, pH 7.5/100 mM NH₄Cl/10 mM MgCl₂) and placed on ice. Then 5 μ l of a solution of lysozyme (10 mg/ml) was added and the samples were kept on ice for about 30 min. Protein (0.1 μ g) from such crude extracts was used for assays of RNase P with the precursor to tRNA^{Tyr} as substrate (25).

Northern Blots and Dot Blots. Total RNA extracted from cells was subjected to electrophoresis on an 8% polyacrylamide gel that contained 7 M urea. Bands of RNA were stained with ethidium bromide and the gel was soaked in transfer buffer (10 mM Tris acetate, pH 7.8/5 mM sodium acetate/1 mM EDTA) for about 20 min. The bands of RNAs were then electrottransferred to a nylon membrane over the course of 12–15 h at 250 mA (26). After transfer, the RNA was crosslinked to the membrane with a Stratalinker (Stratagene). Hybridization was performed in rapid hybridization buffer (Amersham) by the protocol from Amersham. Northern blots were used to measure the steady-state levels of EGSs *in vivo*. Levels of mRNA in cells were determined by dot-blot assays, as described by Yarchuk *et al.* (27). To correct for dot-to-dot variations, 5S RNA was used as the internal control. RNA samples were placed on nylon membranes and crosslinked to membranes with the Stratalinker. The hybridization procedure was similar to that used for Northern blots.

RESULTS

Strategy for Construction of Strains. To distinguish inhibition of gene expression due to RNase P activity from inhibition due to antisense oligonucleotide interaction or RNase III activity, we transformed *E. coli* strains that were temperature-sensitive for RNase P with plasmids that expressed specific and nonspecific EGSs. If RNase P activity were primarily responsible for inhibition of the expression of the targeted gene, no inhibition in cells incubated at 43°C, a treatment that inactivates RNase P, would be observed. All EGSs were cloned under control of the phage T7 promoter control and each EGS sequence was followed by a hammerhead (HH) sequence and

a T7 terminator, as described in *Materials and Methods*. The hammerhead sequence was inserted to ensure that, after transcription, the long transcripts would self-cleave and generate EGSs that contained only four nucleotides after the 3'-terminal CCA sequence. The short EGSs would then function to guide the cleavage of specific mRNAs. (The efficiency of cleavage of substrates by RNase P is significantly reduced when the substrate contains a long 3'-flanking sequence.)

BL21(DE3)A49 (21, 28), a thermosensitive derivative of BL21(DE3) that carried the temperature-sensitive A49 mutation in the *rnpA* gene, was designated as a recipient of different plasmids that contained genes for EGSs. The mRNA for the *lacZ* gene was chosen as a target because it allowed a direct comparison of the results obtained *in vivo* with those previously obtained *in vitro* (17). In preliminary experiments we found, however, that BL21(DE3)A49 did not have the *lacZ*⁺ marker or the gene for T7 RNA polymerase. Accordingly, to generate transcripts of the genes for EGSs in the *rnpA* background, we constructed the strain T7A49 (see *Materials and Methods*), in which both the *lacZ*⁺ gene and the gene for T7 RNA polymerase were present. The EGSs in T7A49 targeted not only the mRNA for β -galactosidase but also the mRNA for alkaline phosphatase. [Unlike the induction of β -galactosidase, the induction of the synthesis of EGS was independent of the induction of mRNA for alkaline phosphatase. Alkaline phosphatase is a reasonably stable enzyme and can be easily assayed colorimetrically (23).] Controls included plasmids with EGSs targeted to phage λ N (pNT7NHH) and CI (pNT7CIHH) mRNAs.

Inhibition of Gene Expression by Chromosomal RNase P. Inhibition of alkaline phosphatase or β -galactosidase activity *in vivo* by using the EGS technology was dependent on the activity of RNase P (Tables 1 and 2). As expected, the extent of inhibition was much lower in cells incubated at nonpermissive temperature (0–11%) as compared with permissive temperature (50–60%). To confirm that the inhibition observed was specific to the targeted message, levels of acid phosphatase activity were also measured and compared to the results for alkaline phosphatase and β -galactosidase. Untargeted acid phosphatase activity remained at about the same level in all the cultures examined. In fact, the levels of inhibition were similar when either cell density or acid phosphatase activity was used as a standardization factor. The results summarized in Tables 1 and 2 show that RNase P was capable of reducing the level of expression of fully induced genes specifically but not down to the basal levels of gene expression.

RNase III can cleave double-stranded RNA complexes formed by mRNAs and the corresponding EGSs (17). In our present studies, the inhibition of gene expression due to cleavage by RNase III, as well as inhibition due to antisense interaction, was seen to be negligible when a comparison was made of the values obtained from cultures grown at permissive and nonpermissive temperature. Indeed, if the observed inhibition had been due to antisense and/or RNase III activity, the extent of inhibition should have been much higher at 43°C than at 30°C because the amounts of EGSs produced at 43°C were 5- to 8-fold higher than those produced at 30°C (see Fig. 1 and Table 3). Although our assays indicate that no RNase P activity was detectable in extracts made of cells that had been incubated at 43°C, a low level of inhibition was observed at this temperature in a few experiments. This inhibition might have been due to residual RNase P activity present when the cells were transferred from 30°C to 43°C, since earlier experiments (28, 29) showed that cells with the *rnpA49* mutation, when transformed with plasmids that harbored the gene for M1 RNA, were able to grow at nonpermissive temperatures. However, when crude extracts of cells grown at 30°C and 43°C were assayed for RNase P activity *in vitro*, virtually no activity was found in the extracts of cells grown at the nonpermissive temperature, even in the presence of high concentrations of

Table 1. Effects of different EGS constructs on alkaline phosphatase activity

Temp.	Plasmid	Units of alkaline phosphatase/OD ₆₀₀						Units of alkaline phosphatase/ units of acid phosphatase			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Average	Exp. 1	Exp. 2	Exp. 3	Average
30°C	pNT7NHH	0.8	0.7	0.8	0.6	0.8	0.7	0.9	0.8	0.8	0.8
	pNT7APHH	0.5	0.3	0.4	0.5	0.4	0.4	0.5	0.4	0.4	0.4
	pNT7DS1HH	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	pNT7CIHH	0.9	0.4	1.2	0.7	1.2	0.9	1.0	1.7	1.3	1.3
	Average for nonspecific EGSs						0.9 ± 0.1				1.0 ± 0.2
Inhibition of alkaline phosphatase activity, %							55				60
43°C	pNT7NHH	0.9	1.3	1.1	0.7	1.1	1.0	0.8	1.0	1.0	0.9
	pNT7APHH	0.8	1.0	1.0	0.8	1.0	0.9	0.7	0.9	0.9	0.8
	pNT7DS1HH	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	pNT7CIHH	0.9	0.7	0.8	0.6	0.8	0.8	0.9	0.9	0.9	0.9
	Average for nonspecific EGSs						0.9 ± 0.1				0.9 ± 0.1
Inhibition of alkaline phosphatase activity, %							0				11

Alkaline phosphatase activity is normalized against cell density in the five experiments on the left. Alkaline phosphatase activity is normalized against acid phosphatase activity in the three experiments on the right. The values obtained were normalized against the values obtained in cultures that overexpressed DS1-EGS (pNT7DS1HH). The inhibition of alkaline phosphatase activity was calculated relative to the average of values obtained after overexpression of nonspecific EGSs. Construction of and nomenclature for EGSs are given in *Materials and Methods*.

M1 RNA. Parallel experiments indicated that overproduction of C5 protein rescued RNase P activity at the nonpermissive temperature (data not shown).

Expression of EGSs *in Vivo*. To study the relative steady-state levels of EGSs required for inhibition of gene expression, levels of EGSs were estimated by Northern blot analysis. As shown in Fig. 1 *Right*, the efficiency of self-cleavage of the transcript of the EGS targeted to the gene for alkaline phosphatase (AP-EGS) by the hammerhead ribozyme was very high because most of the EGS detected on Northern blots had the mobility of the EGS without the HH sequence. Similar results were obtained with the β -galactosidase-specific EGS (DS1-EGS; data not shown).

M1 RNA could not be used as an internal control to calculate the amounts of EGSs produced by the *rnpA* cells because the amount of M1 RNA produced at the nonpermissive temperature was much lower than that produced by the cells at 30°C (Table 3). In wild-type cells, such a decrease was not observed (data not shown). Therefore, the intracellular amount of 5S RNA (30) was used as an internal control. The amount of 5S RNA produced was about the same in cultures that overexpressed AP-EGS RNA (Fig. 1 *Left*) at both temperatures.

To correlate the overproduction of EGSs with the extent of inhibition of the targeted enzyme, EGS was induced for 30 min

before induction of the gene for alkaline phosphatase: the extent of inhibition at 30°C varied from 54.7% (obtained with simultaneous induction of EGS and alkaline phosphatase) to 68%. In cultures incubated at 43°C, the extent of inhibition after 30-min preinduction of EGS was 0% (it was 4.7% for coinduction of EGS and alkaline phosphatase). These experiments could not be performed with β -galactosidase because both the EGS and the mRNA were induced by IPTG. Estimates of the steady-state copy numbers of EGSs, ranging from about 100 to 600 and depending on the growth temperature, are shown in Table 3.

mRNA Copy Number. To determine intracellular levels of mRNAs, dot-blot assays were performed, using specific complementary EGSs as probes, with the foreknowledge that both products of degradation and intact mRNA would be detected in this way. 5S RNA was used as the internal control to normalize dot-to-dot variation. When DS1-EGS was used as a probe in the case of *lacZ* mRNA, the amount of mRNA was about 50% lower at 30°C than at 43°C, in agreement with the results obtained for β -galactosidase activity. However, in the case of mRNA for alkaline phosphatase (with AP-EGS used as the probe), no such reduction was observed (Table 3). A possible explanation for these results is that these two targeted RNAs, either before or after cleavage by RNase P, had different kinetics of degradation at 30°C and 43°C when in a

Table 2. Effects of different EGS constructs on β -galactosidase activity

Temp.	Plasmid	Units of β -galactosidase/OD ₆₀₀			Units of β -galactosidase/ units of acid phosphatase		
		Exp. 1	Exp. 2	Average	Exp. 1	Exp. 2	Average
30°C	pNT7NHH	1.0	1.3	1.2	1.1	1.2	1.2
	pNT7APHH	1.0	1.0	1.0	1.0	1.0	1.0
	pNT7DS1HH	0.6	0.3	0.5	0.7	0.4	0.6
	pNT7CIHH	1.2	1.0	1.1	1.4	1.2	1.3
	Average for nonspecific EGSs			1.1 ± 0.1			1.2 ± 0.1
Inhibition of β -galactosidase activity, %				55			50
43°C	pNT7NHH	1.5	1.0	1.3	1.5	1.1	1.3
	pNT7APHH	1.0	1.0	1.0	1.0	1.0	1.0
	pNT7DS1HH	0.9	1.1	1.0	1.0	1.6	1.3
	pNT7CIHH	0.8	1.4	1.1	1.0	1.5	1.3
	Average for nonspecific EGSs			1.1 ± 0.1			1.2 ± 0.1
Inhibition of β -galactosidase activity, %				9			0

β -Galactosidase activity is normalized against cell density in the two experiments on the left. β -Galactosidase activity is normalized against acid phosphatase activity in the two experiments on the right. The values obtained were normalized against the values obtained in cultures that overexpressed AP-EGS (pNT7APHH). The inhibition of β -galactosidase activity was calculated relative to the average of values obtained after overexpression of nonspecific EGSs.

FIG. 1. Northern blot analysis of intracellular EGS RNA. Samples from cells that overexpressed AP-EGS were analyzed. Total RNA was loaded on the inner lanes and a mixture of 0.1 pmol of 5S RNA and 0.1 pmol of an oligonucleotide with the same sequence as AP-EGS RNA was loaded on the outside lanes. (Left) The filter was probed with an oligonucleotide complementary to 5S RNA. (Right) The filter was probed with an oligonucleotide complementary to AP-EGS. A longer RNA transcript corresponding to AP-EGS-HH is visible. The largest RNA might be due to inefficient termination of transcription.

complex with an EGS. Under conditions where maximum inhibition of alkaline phosphatase was achieved, the copy numbers of AP-EGS and alkaline phosphatase mRNA were about equal (Table 3).

Inhibition of Gene Expression by an M1 RNA-EGS Construct. No RNase P activity was detected in extracts of strains carrying the *rnpA49* mutation at nonpermissive temperatures, presumably because of the temperature-sensitive mutation in the *rnpA* gene. We expected, however, to be able to restore RNase P activity at 43°C by transforming strains carrying *rnpA49* with a plasmid that harbored the gene for M1 RNA or M1 RNA covalently linked to an EGS (M1 RNA-EGS), since an excess of M1 RNA is known to complement the *rnpA49* mutation (29). Under such conditions, inhibition of targeted mRNA expression should be possible at 43°C. Additionally, recent studies (refs. 9 and 31; Y.L. and S.A., unpublished experiments) have indicated that M1 RNA covalently linked to an EGS can cleave targeted mRNA *in vitro* even in buffers that contain low concentrations of Mg^{2+} . Accordingly, we determined whether such a construct encoded in a high-copy-number plasmid could be used to inhibit the expression of a

suppressor tRNA gene that was encoded by a separate low-copy-number plasmid.

Two groups of three M1-EGS constructs each were made: each group was directed at a different site in tRNA^{Tyr}su⁺. In each group, two of the M1 RNA derivatives were known to be temperature-sensitive (32), but one of these groups, directed at nucleotide 31 in the tRNA (Table 4), was also known to be inactive *in vitro*, and this group served, therefore, as a negative control set (nucleotides in the variable loop of the tRNA are more accessible than those in the anticodon loop for hybridization to an EGS oligonucleotide). Strain PP1113(A49), which contained a *lacZ*⁻ amber mutation, was transformed with the appropriate plasmids and was used as an indicator of suppressor function. The set of M1-EGS constructs directed at nucleotide 43 in the tRNA (Table 4) successfully inhibited the expression of the suppressor. Furthermore, the temperature-sensitive derivatives G65 and G106 exhibited the expected phenotypes at 43°C (Table 4). At the permissive temperature, G65/43-EGS inhibited β -galactosidase activity more efficiently than the wild-type M1/43-EGS. At the nonpermissive temperature, only M1/43-EGS significantly reduced the enzymatic activity. Neither the set directed at nucleotide 31 nor the nonspecific M1-DS construct, directed against *lacZ* mRNA, inhibited suppressor function. The highest levels of inhibition were similar to those observed with EGSs and chromosomal M1 RNA.

Table 3. Levels of expression of mRNAs, EGS RNAs, and M1 RNA

Temp.	Plasmid	RNA, no. of copies per cell*			
		M1 [†]	<i>lacZ</i> [†]	Alkaline phosphatase [†]	AP-EGS [‡]
30°C	pNT7APHH	490	890	180	137
	pNT7DS1HH	376	575	156	0
	pNT7CIHH	406	1050	79	0
43°C	pNT7APHH	67	613	145	754
	pNT7DS1HH	75	1234	197	0
	pNT7CIHH	56	1111	157	0

Total RNAs were extracted, and 2 μ g of RNA was used in dot-blot assays and 10 μ g was used for Northern blot analysis. Standard concentrations of 5S RNA were used in both experiments, and the numbers of copies of different RNAs per cell were estimated relative to the amount of 5S RNA.

*Calculations were made relative to levels of 5S RNA, which was estimated to be 18,700 copies per cell (28).

[†]From dot-blot assays.

[‡]From Northern blot analysis.

DISCUSSION

RNase P, in combination with EGSs, can be used to inhibit gene expression *in vivo*. A significant advantage of this method, compared with methods that employ other ribozymes, is that RNase P is an essential enzyme found in all organisms. Furthermore, since turnover of EGSs occurs *in vitro* (17) and since they also accumulate *in vivo*, as shown by Northern blot analysis, they can be used repeatedly in targeting a specific message. The use of strains of *E. coli* with a temperature-sensitive RNase P allowed us to control gene expression by changing the temperature, and the effects resembled the regulatory effects achieved in cold-sensitive mutants. Using mutants of RNase P, we were also able to distinguish between inhibition due to antisense interaction or RNase III cleavage and inhibition due to RNase P activity. In the experiments

Table 4. Effects of overexpression of different M1-EGS RNAs on β -galactosidase activity

Temp.	Plasmid and EGS	Units of β -galactosidase/OD ₆₀₀		
		Exp. 1	Exp. 2	Average
30°C	pACSu ₃ ⁺ + M1/31-EGS	1.1	1.3	1.2
	pACSu ₃ ⁺ + M1/43-EGS	0.6	0.7	0.7
	pACSu ₃ ⁺ + M1/DS1-EGS	0.9	0.8	0.9
	pACSu ₃ ⁺ + G65/31-EGS	1.0	0.7	0.9
	pACSu ₃ ⁺ + G65/43-EGS	0.4	0.4	0.4
	pACSu ₃ ⁺ + G65/DS1-EGS	1.1	0.7	0.9
	pACSu ₃ ⁺ + A106/31-EGS	1.0	0.8	0.9
	pACSu ₃ ⁺ + A106/43-EGS	1.1	0.5	0.8
	pACSu ₃ ⁺ + A106/DS1-EGS	1.0	0.7	0.9
	pACSu ₃ ⁺ only	1.0	1.0	1.0
Average for nonspecific EGSs (31, DS1)				1.0 $\pm$ 0.1
Inhibition of β -galactosidase activity by M1/43-EGS, % 30				
43°C	pACSu ₃ ⁺ + M1/31-EGS	0.9	0.6	0.8
	pACSu ₃ ⁺ + M1/43-EGS	0.3	0.4	0.4
	pACSu ₃ ⁺ + M1/DS1-EGS	0.8	0.6	0.7
	pACSu ₃ ⁺ + G65/31-EGS	0.6	0.7	0.7
	pACSu ₃ ⁺ + G65/43-EGS	0.9	0.7	0.8
	pACSu ₃ ⁺ + G65/DS1-EGS	0.8	0.7	0.8
	pACSu ₃ ⁺ + A106/31-EGS	0.7	0.7	0.7
	pACSu ₃ ⁺ + A106/43-EGS	1.0	0.8	0.9
	pACSu ₃ ⁺ + A106/DS1-EGS	0.7	0.8	0.8
	pACSu ₃ ⁺ only	1.0	1.0	1.0
Average for nonspecific EGSs (31, DS1)				0.8 $\pm$ 0.1
Inhibition of β -galactosidase activity by M1/43EGS, % 60				

β -Galactosidase activity was assayed and the values obtained were normalized against the values obtained for cells that contained only one plasmid, pACSu₃⁺. The average for nonspecific EGSs was calculated from the values obtained from cultures that overexpressed not only M1/31-EGS and M1/DS1-EGS but also G65/31-EGS, G65/DS1-EGS, A106/31-EGS, and A106/DS1-EGS.

reported here, a maximum of about 60% reduction in the levels of fully induced enzymatic expression was observed. Our results indicate also that, if constitutive production of EGSs could be coupled to production of RNase P, the inhibitory potential of our method could be enhanced (see below).

An M1 RNA-EGS construct inhibited the expression of a suppressor tRNA encoded on a low-copy-number plasmid to about the same extent as that achieved with EGSs alone targeted to the mRNAs for alkaline phosphatase and β -galactosidase. By contrast, such a construct has been found capable of inhibiting gene expression in mammalian cells in tissue culture by more than 80% (9). The inability to achieve complete inhibition of gene expression in *E. coli* may be due to several factors, among which are a limit to the steady-state copy number per cell of EGSs in our constructs and the inaccessibility of a fraction of every population of mRNA due to the tight coupling of transcription and translation in *E. coli*. Eukaryotic mRNAs expressed in the bacterial cells (10) may not be subject to this tight coupling because of cross-species differences in ribosome-binding sites.

Alkaline phosphatase and β -galactosidase activities are stimulated in cells that overexpress M1 RNA (data not shown), an indication that RNase P (and M1 RNA specifically) could play a role in transcription or translation, possibly by cleavage of mRNA with consequent more efficient translation. Such a phenomenon has been observed with mRNA of the histidine operon in *Salmonella typhimurium* (33). Overproduction of M1 RNA in T7A49 also increases the level of RNase P and could cause the biosynthesis of larger amounts of mature tRNA, thereby increasing the efficiency of translation. In BL21(DE3),

which is wild-type for RNase P, such a stimulation of translation should be negligible. Indeed, in the wild-type background, the levels of β -galactosidase activity were similar for all controls, regardless of the presence or absence of overproduced M1 RNA (data not shown), an indication that, in T7A49 cells, even at the permissive temperature, RNase P activity can be limiting. Our general method, however, may be useful for altering levels of resistance to antibiotics and other drugs, as well as for studies of the basic function of genes in prokaryotes.

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