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Chapter 7

Evaluating the effect of small RNAs and associated chaperones on Rho-dependent termination of transcription *in vitro*

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Running head: sRNA and chaperone effects on Rho-dependent termination

Abstract

Besides their well-known posttranscriptional effects on mRNA translation and decay, sRNAs and associated RNA chaperones (e.g. Hfq, CsrA) sometimes regulate gene expression at the transcriptional level. In this case, the sRNA-dependent machinery modulates the activity of the transcription termination factor Rho, a ring-shaped RNA translocase/helicase that dissociates transcription elongation complexes at specific loci of the bacterial genome. Here, we describe biochemical assays to detect Rho-dependent termination signals in genomic regions of interest and to assess the effects of sRNAs and/or associated RNA chaperones on such signals.

Key words: Rho; transcription; termination; ring-shaped; helicase; hexamer; sRNA; chaperone.

1. Introduction

In bacteria, transcription termination is triggered by two main types of signals having distinct nucleic acid (NA) and protein cofactor requirements (reviewed in: (1,2)). Intrinsic (Rho-independent) terminators are encoded by a NA signal that is usually sufficient to destabilize the transcription elongation complex. Intrinsic terminators are relatively easy to detect in bacterial genomes as they most often involve the formation of a GC-rich hairpin followed by a run of U residues at the 3' end of the nascent RNA transcript. By contrast, Rho-dependent terminators are characterized by a strict requirement for the protein factor Rho (**Fig. 1**) but have relaxed sequence determinants rendering them difficult to detect in genome sequences (1).

Transcription termination signals are sometimes found in the 5'-untranslated regions (5'-UTRs) of genes or operons where they contribute to the regulation of the gene/operon through conditional attenuation of transcription (3,4). Attenuation mechanisms involving intrinsic terminators are diverse and widespread while, until recently, known instances of comparable Rho-dependent mechanisms were few (5-7). This situation is, however, quickly evolving with the discovery of riboswitches governing Rho-dependent termination (8,9) and, more to the point of the present chapter, of conditional Rho-dependent mechanisms governed by sRNAs and/or RNA chaperones. For instance, a Rho-dependent terminator has been identified recently in the 5'-UTR of the *rpoS* gene of *Escherichia coli* where it is regulated negatively by the binding of sRNA DsrA, ArcZ, or RprA to the *rpoS* mRNA leader (10). A conditional Rho-dependent terminator operating by a distinct mechanism has also been identified recently in the 5'-UTR of the *pgaABCD* operon of *E. coli* (11). In this case, remodeling of the mRNA leader by the CsrA chaperone allows Rho access to a binding *Rut* (*Rho utilization*) site that is otherwise sequestered in a RNA secondary structure (11). This

mechanism contributes to the multilayered regulation of *pgaABCD* by CsrA, which notably includes scavenging of CsrA by CsrB, CsrC, and McaS acting as sRNA sponges (*12,13*).

Conditional Rho-dependent termination can also be triggered by the binding of a sRNA to its mRNA target. In the first case described, binding of the sRNA ChiX to the 5'-leader of the *chiPQ* operon of *Salmonella* prevents *chiP* translation (*14*). This in turn exposes an intragenic *Rut* site within the *chiP* mRNA that is otherwise hidden by translating ribosomes, and triggers Rho-dependent transcriptional polarity within the operon (*14*). Similarly, the sRNA Spf (aka Spot 42) triggers Rho-dependent termination at the end of the *galT* gene within the *galETKM* operon of *E. coli* (*15*). These two known cases suggest that the activation of Rho-dependent transcriptional polarity by sRNAs could be a general mechanism contributing to gene/operon silencing. When exploring this possibility, however, one needs to consider the potential contribution of accessory factors. For instance, the Hfq chaperone can inhibit Rho-dependent termination in a manner that is antagonized by NusG – an essential, multi-role transcription factor (*16*)- or by NA ligands that alter Hfq interaction with Rho and RNA (*17*). Moreover, some Rho-dependent terminators are effective only in the presence of NusG, probably because their *Rut* sites are too weak to recruit or activate Rho by themselves (*14,18,19*).

Here, we describe *in vitro* methods to probe whether transcription termination factor Rho is involved in sRNA-mediated regulation of a given bacterial gene or operon. Together with *in vivo* probing, these methods should prove useful to unravel the potentially complex interplays existing between Rho, sRNAs, and accessory factors such as RNA chaperones.

2. Materials

2.1. Preparation of DNA templates for transcription termination assays

1. 100 μ M stock solutions of DNA primers FWD, REV, and T7A1 (**Table 1**).

2. Genomic DNA or frozen stock of bacterial strain of interest (*see Note 1*).
3. Stock solution of dNTPs (10 mM each).
4. 2 U/ μ L Vent DNA polymerase (*see Note 2*).
5. 10x Vent reaction buffer: 100 mM $(\text{NH}_4)_2\text{SO}_4$, 100 mM KCl, 20 mM MgSO_4 , 1% Triton X-100, 200 mM Tris-HCl, pH 8.8.
6. PCR thermocycler equipped with a heated lid and a thermal block for 0.5 mL microtubes.
7. PCR purification kit (*see Note 3*).
8. Benchtop centrifuge.
9. 1x T₁₀E₁ buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (*see Note 4*).
10. Agarose.
11. Commercial DNA ladder (e.g. #N3233S ladder from New England Biolabs).
12. 6x agarose gel loading buffer: 15% Ficoll-400, 0.1% SDS, 0.1% bromophenol blue, 20 mM Tris-HCl, 66 mM EDTA, pH 8.0.
13. 20x TAE buffer: dissolve 98 g of Tris base in approximately 800 mL of water, then add 22.8 mL of glacial acetic acid and 40 mL of 0.5 M EDTA pH 8.0, and add water to obtain a final volume of 1 L.
14. 1x TAE buffer: obtained by dilution of the 20x TAE stock buffer.
15. Horizontal gel electrophoresis system and power supply.
16. 10 mg/mL ethidium bromide stock solution (*see Note 5*).
17. Microwave oven.
18. UV transilluminator or dedicated gel documentation system.

19. Sephadex G-50 spin columns (e.g. Microspin columns from GE Healthcare).
20. UV spectrophotometer suitable for micro-volumes measurements (e.g. Nanodrop 2000c from Thermo Scientific).

2.2 Preparation of sRNAs

1. Items 2-20 of subsection 2.1.
2. 100 μ M stock solutions of DNA primers TOP and BOTT (**Table 1**).
3. 5x Transcription buffer: 0.12 M MgCl₂, 0.4 M HEPES, pH 7.5, 0.1 M DTT, 0.05 % Triton X-100, and 5 mM Spermidine (*See Note 4*).
4. 50 U/ μ L T7 RNA polymerase.
5. 1 U/ μ L RQ1 DNase (Promega).
6. Dry bath incubator with shaking capability.
7. 20 U/ μ L SUPERase-INTM (Thermo Fisher Scientific)
8. Deionized RNA-grade water (*see Note 4*).
9. Set of high-grade rNTPs (100 mM each).
10. 0.5 M EDTA solution, adjusted to pH 7.5 with NaOH (*see Note 4*).
11. Phenol:Chloroform:Isoamyl alcohol (25:24:1) mix, pH 6.7.
12. Diethyl ether.
13. 3 M sodium acetate (NaAc), adjusted to pH 6.5 with acetic acid (*see Note 4*).

14. 20x TBE buffer: dissolve 216 g of Tris base, 110 g of boric acid, 14.9 g EDTA in 1 L of water. Filter on Whatman paper and store at room temperature.
15. 1x TBE buffer: obtained by dilution of the 20x TBE stock.
16. 40% acrylamide:bis-acrylamide [29:1 ratio] commercial stock solution.
17. Denaturing acrylamide solution for sRNA preparation (6% acrylamide:bis-acrylamide [29:1 ratio] and 7 M urea in 1x TBE buffer). Mix 12.6 g of urea, 4.5 mL of 40% acrylamide:bis-acrylamide [29:1 ratio] solution, 1.5 mL 20x TBE and 10 mL of deionized water. Heat the solution to dissolve urea completely. Adjust volume to 30 mL with deionized water and cool down to room temperature. Prepare fresh solution before use.
18. N,N,N,N'-tetramethyl-ethylenediamine (TEMED).
19. 25% (w/v) ammonium persulfate (APS) in water.
20. Vertical electrophoresis system and power supply for DNA sequencing (e.g. adjustable 20x42 cm sequencing kit from CBS scientific).
21. Denaturing loading buffer: 95% formamide, 5 mM EDTA, 0.01% (w/v) bromophenol blue.
22. X-ray intensifying screen or a fluor-coated TLC plate.
23. Hand-held 254 nm UV lamp.
24. 1x Elution buffer: 0.3 M NaAc, 10 mM MOPS, 1 mM EDTA, pH 6.0.
25. 1x M₁₀E₁ buffer: 10 mM MOPS, 1 mM EDTA, pH 6.0 (*see Note 4*).

2.3 *Transcription termination assay*

1. Items 6-21 of subsection 2.2.
2. ‘Protein low binding’ and ‘DNA low binding’ 1.5 mL microtubes.
3. Commercial DNA ladder. Select a ladder made of DNA fragments that are not phosphorylated at 5’-ends (e.g. #N3233S ladder from New England Biolabs).
4. 10U/μL T4 polynucleotide kinase.
5. 10x PNK buffer: 100 mM MgCl₂, 50 mM DTT, and 700 mM Tris-HCl, pH 7.6.
6. γ-³²P ATP at 3000 Ci/mmol [10 mCi/mL].
7. Sephadex G-50 spin columns (e.g. Microspin columns from GE Healthcare).
8. 1.4 μM Rho stock solution (*see Note 6*) in Rho storage buffer (50% glycerol, 100 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 10 mM Tris-HCl, pH 7.9). Preparation of the Rho protein from *E. coli* is detailed in the volume 587 of *Methods in Molecular Biology* (20) (*see Note 7*).
9. 2μM NusG stock solution (*see Note 8*).
10. 2μM stock solution of CsrA or Hfq chaperone (optional) (*see Note 8*).
11. 1U/μL *E. coli* RNA Polymerase, Holoenzyme (New England Biolabs).
12. 5 μM stocks of sRNAs (as prepared in **Section 3.2**). Stocks should include the sRNAs under investigation (i.e. the ones expected to pair with the mRNA target) and at least one negative control (sRNA not expected to bind to the mRNA target). We also like to include a shorter, synthetic oligoribonucleotide that is fully complementary to the mRNA sequence targeted by the sRNA(s) and serves as positive control.

13. 5x transcription termination buffer: 250 mM KCl, 25 mM MgCl₂, 7.5 mM DTT, 0.25 mg/mL bovine serum albumin, and 200 mM Tris-HCl, pH 8.0 (*see Note 4*).
14. 10x initiation mixture: 2 mM ATP, 2 mM GTP, 2 mM CTP, 0.2 mM UTP, 250 μg/mL rifampicin, and 2 μCi/μL ³²P-αUTP in 1x transcription termination buffer. Prepare right before use (*see Note 9*).
15. 0.25mg/mL tRNA stock.
16. 1x resuspension buffer: mix 460 μL of M₁₀E₁ buffer with 40 μL of 0.5 M EDTA, pH 7.5.
17. Denaturing acrylamide solution (termination assay): 7% acrylamide:bis-acrylamide [19:1 ratio] and 7 M urea in 1x TBE buffer (see section 2.2, item 17 for preparation from stocks, using a 19:1 rather than 29:1 acrylamide:bis-acrylamide commercial mixture).
18. Vacuum drying system for electrophoresis gels.
19. Phosphor imager equipped with 35x43 cm phosphor imaging plates and dedicated analysis software (e.g., Typhoon Trio imager and ImageQuant TL software from GE-Healthcare).

3. Methods

3.1 Preparation of DNA templates for transcription termination assays

We prefer working with DNA templates that do not exceed 1000 bp, as longer templates may yield transcription termination products that are not easily separated from runoff transcripts by denaturing polyacrylamide gel electrophoresis. The DNA templates contain the genomic region of interest downstream from the T7A1 promoter (which is efficiently used by the RNA

polymerase from *E. coli*) and are prepared in two successive rounds of PCR amplification (**Fig 2.A**) as follows:

1. Using a sterile loop, scrap and transfer a tiny piece of frozen bacteria (*see Note 1*) into a 1.5 mL microtube. Add 100 μ L of water, incubate for 5 min at 95°C, and then keep on ice (this solution can be stored at -20°C for further use). A diluted solution of purified genomic DNA may be used instead.
2. Transfer 3 μ L of above solution into a 0.5 mL PCR microtube kept on ice. Add 39 μ L of water, 5 μ L of 10x Vent reaction buffer, 0.5 μ L of each 100 μ M solution of primers FWD and REV (**Table 1**), 1 μ L of the stock mixture of dNTPs (10 mM each), and 1 μ L of Vent polymerase.
3. Transfer mixture into a thermocycler and heat it for 3 min at 94°C. Then, perform 30 cycles of PCR amplification using the following cycle parameters: 94°C for 1 min, 50°C for 30s, 72°C for 1 min. Incubate for 10 min at 72°C at the end of the program.
4. Transfer 0.6 μ L of the PCR mixture into a new 0.5 mL PCR microtube kept on ice. Add 41.4 μ L of water, 5 μ L of 10x Vent reaction buffer, 0.5 μ L of each 100 μ M solution of primers T7A1 and REV (**Table 1**), 1 μ L of the stock mixture of dNTPs (10 mM each), and 1 μ L of Vent polymerase.
5. Perform the second PCR round as in step 3. At the end of amplification, add 2 μ L of 0.5M EDTA solution and store on ice.
6. Prepare agarose gel by dissolving 1.5 g of agarose into 100 mL of 1x TAE buffer (perform short heating bursts in microwave oven). Add 1 μ L of ethidium bromide stock solution (*see Note 5*) and pour into gel tray. Once the gel has solidified, install it into a horizontal electrophoresis unit filled with 1x TAE buffer.

7. Mix 1 μL of PCR reaction mixture with 7.3 μL of 1x T₁₀E₁ buffer, and 1.7 μL of 6x agarose gel loading buffer. Similarly prepare a sample containing $\sim 0.5 \mu\text{g}$ of commercial DNA ladder.
8. Load samples on agarose gel and run the gel for 1 h at 100 Volts.
9. Visualize gel with a transilluminator or dedicated gel documentation system. Only one band migrating at the expected rate for the correct DNA fragment should be visible (**Fig. 2C**).
10. Purify the PCR reaction mixture in two steps, first with a commercial silica-based purification kit (*see Note 3*) and then with a commercial G-50 spin column following manufacturer's instructions.
11. Use a μL -spectrophotometer to determine the molar concentration of the DNA template from the absorbance of the solution at 260 nm, assuming $\epsilon_{260} \sim [13200 \times \text{number of base pairs}] \text{ L/mol/cm}$. Typical yields for a 1000 bp DNA fragment are around 10 pmole.
12. Adjust DNA template concentration to 100 nM with T₁₀E₁ buffer and store at -20°C .

3.2 Preparation of sRNAs

Most sRNAs exceed the size limit of commercial synthetic oligoribonucleotides (~ 80 nts) and need to be prepared by *in vitro* transcription with a phage RNA polymerase using either a dedicated commercial kit or the following protocol:

1. Follow instructions in section 3.1 to prepare the DNA template encoding the sRNA of interest. To introduce a promoter for T7 RNA polymerase (instead of the T7A1 promoter recognized by the RNA polymerase from *E. coli*), use oligonucleotides TOP and BOTT instead of primers FWD (or T7A1) and REV in both rounds of PCR.
2. Gently thaw transcription buffer, DNA template and rNTP stocks on ice. Homogenize each solution by brief vortexing and centrifugation.

3. In a microtube, assemble on ice a mixture of 119 μL of water, 50 μL of 5x transcription buffer, 12.5 μL of each 100 mM rNTP stock, 1 μL of SUPERase-INTM, and 20 μL of the 100 nM DNA template solution.
4. Add 10 μL of T7 RNA polymerase and incubate mixture for 2 hours at 37°C
5. Add 5 μL of RQ1 DNase to digest the DNA template and incubate for 20 min at 37°C.
6. Add 12 μL of 0.5M EDTA and 28 μL of 3M NaAc.
7. Extract with one volume of Phenol:Chloroform:Isoamyl alcohol mix. Vortex and centrifuge briefly to separate phases. Transfer the aqueous (top) phase to a new tube and extract twice with one volume of ether, discarding the top (ether) phase in each case.
8. Add 900 μL of ice-cold ethanol. Incubate overnight at -20°C.
9. Centrifuge for 30 min at 20,000g in a refrigerated centrifuge and discard supernatant. Wash the pellet with 150 μL of ice-cold ethanol and centrifuge for 15 min at 20,000g.
10. Remove the ethanol and leave the microtube open for ~20 min at room temperature to dry the RNA pellet (the process can be sped up by incubating at 37°C in a dry bath) (*See Note 10*).
11. Dissolve pellet in a mix of 20 μL of M₁₀E₁ buffer and 20 μL of denaturing loading buffer. Leave sample on ice while preparing the denaturing polyacrylamide gel for purification.
12. Assemble gel plates and spacers according to manufacturer instructions. We use custom-made 20x20 cm gel plates equipped with 0.8 mm spacers, a 10-teeth comb, and a bottom tape seal.
13. Mix 30 mL of 6% denaturing (29:1) polyacrylamide gel solution with 90 μL of APS and 45 μL of TEMED (volumes may need to be adjusted for commercial sets of plates and spacers). Quickly pour the mixture between the gel plates, and insert comb.

14. Once the gel has polymerized (~30 min), remove the comb and wash the gel wells with 1x TBE using a 5 mL-syringe. Install the gel into an electrophoresis unit and fill the top and bottom tanks with 1x TBE.
15. Set the power supply at 20W and perform a pre-electrophoresis for 20 min.
16. Heat-denature the RNA sample (from step 11) for 2 min at 95°C.
17. Turn off the power supply and flush diffusing urea from gel wells using a syringe containing 1x TBE. Distribute sample into two wells using a flat gel loading tip.
18. Run the gel at 20 W until the band corresponding to bromophenol blue reaches the bottom of the gel.
19. Carefully remove the glass plates and wrap the gel in saran sheets.
20. Place the gel on an X-ray intensifying screen (or a fluor-coated TLC plate) and visualize the band corresponding to the transcript by UV shadowing in a dark room with a hand-held 254 nm lamp (*See Note 11*).
21. Cut the band with a clean scalpel and crush it by passage through a 1 mL-syringe. Soak the gel pieces in 3 mL of 1x elution buffer in a sterile 14 mL-culture tube. Shake the tube overnight at 4°C.
22. Pass the gel slurry through a 5-mL syringe equipped with a glass wool or cotton plug (to retain gel particles) and measure the volume of the resulting solution.
23. Extract the filtered solution with one volume of Phenol:Chloroform:Isoamyl alcohol mix. Vortex and centrifuge briefly to separate phases. Transfer the aqueous (top) phase to a new tube and extract twice with one volume of ether.
24. Add three volumes of ice-cold ethanol and incubate overnight at -20°C.
25. After centrifugation for 30 min at 20,000g in a refrigerated benchtop centrifuge, discard supernatant and wash carefully the RNA pellet with 300 µL of 70% ice-cold ethanol. Centrifuge again for 10 min at 20,000g and discard the ethanol wash.

26. Leave the microtube open for ~20 min at room temperature to dry the RNA pellet (*See Note 10*) and dissolve it in 50-100 μL of M_{10}E_1 buffer.
27. Use a μL -spectrophotometer to determine RNA concentration from the absorbance of the solution at 260 nm, assuming $\epsilon_{260} \sim [10^4 \times \text{number of nucleotides}] \text{ L/mol/cm}$. Typical yields range between 1 and 4 nmoles of purified sRNA for a 250 μL transcription.
28. Store sRNA stock solution at -20°C .

3.3 Transcription termination assay

3.3.1 Detection of Rho-dependent signals

To probe the effect of a sRNA or chaperone on a known, well-characterized Rho-dependent termination signal, one may skip this section and proceed directly to section 3.3.2.

If the evidence connecting the effects of Rho and a sRNA (or RNA chaperone) on a given gene or operon is vague or indirect, the first step is to determine if this gene or operon contains a Rho-dependent signal. We advise to also check for the potential effect of NusG as this factor can dramatically stimulates Rho-dependent termination at suboptimal *Rut* sites (as was observed for the ChiX-regulated *chiP* terminator of *Salmonella*) (**14**). To undertake these tasks, proceed as follows:

1. To a regular 1.5 mL microtube, add in the following order, 3 μL of deionized water, 1 μL of 10x PNK buffer, 2 μL of DNA ladder (1 $\mu\text{g}/\mu\text{L}$), 3 μL of $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (*See Note 9*), and 1 μL of T4 polynucleotide kinase. Incubate 40 min at 37°C .
2. Add 2 μL of 0.5 M EDTA and 78 μL of T_{10}E_1 buffer. Pass through a G-50 spin column, following manufacturer's instructions. Discard the G-50 column and store the eluate containing the ^{32}P -labeled DNA ladder at -20°C (*See Note 9*). The ^{32}P -labeled ladder can be used for up to ~2 months.

3. Prepare three 1.5 mL microtubes ('Protein low binding' grade) labeled 'T', 'Rho', and 'NusG', respectively. Place tubes on ice.
4. In a 'Protein low binding' microtube prepare a master mix containing 35.2 μL of deionized water H_2O , 10 μL of 5x transcription termination buffer, 3.2 μL of the 100 nM stock solution of DNA template, 0.6 μL of SUPERase-INTM, and 0.9 μL of *E. coli* RNA polymerase.
5. Dispense 15.6 μL of the master mix in tubes 'T', 'Rho', and 'NusG'.
6. Add 2.4 μL of 1x transcription termination buffer to tube 'T'. To tube 'Rho', add 1.4 μL of 1x transcription termination buffer and 1 μL of the 1.4 μM Rho stock. Add 1 μL of the 1.4 μM Rho stock and 1.4 μL of the 2 μM NusG stock to tube 'NusG'. Vortex gently and centrifuge briefly to homogenize each tube solution.
7. Incubate reaction tubes for 10 min at 37°C.
8. Add 2 μL of 10x initiation mixture (*See Note 12*). From this step to the end of the procedure, samples will contain substantial amounts of radioactive material and should be handled accordingly (*See Note 9*).
9. Incubate reactions for 20 min at 37°C. Then, stop reactions by adding, to each tube, 4 μL of EDTA (0.5M), 2 μL of tRNA (0.25mg/mL), 64 μL of deionized water, and 10 μL of 3M NaAc pH 6.5.
10. Extract each tube solution with one volume of Phenol:Chloroform:Isoamyl Alcohol mix. Vortex and centrifuge briefly to separate phases. Transfer the aqueous (top) phases to new tubes (use only 'DNA low binding' tubes from this step) and extract twice with one volume of ether.
11. Add three volumes of ethanol (stored at room temperature) to each tube and incubate for 35 min on ice. Under these conditions, most of the free ³²P- α UTP will not precipitate.

12. Centrifuge for 20 min at 20,000g in a bench centrifuge at room temperature. Discard supernatants and wash carefully the pellets with 150 μ L of 100% ethanol (stored at room temperature). Centrifuge again for 10 min at 20,000g and discard the ethanol washes. Open the tubes and dry the pellets for ~5 min at room temperature (*See Note 10*).
13. Following instructions in steps 12-13 of section 3.2 but using 20 x 40 cm (w x l) gel plates and 0.4 mm spacers, prepare a 7% denaturing (19:1) polyacrylamide gel and install it in a vertical electrophoresis unit.
14. Perform a pre-electrophoresis for 45 min at 45W (gel plates should become warm to the touch).
15. In a 1.5mL microtube, mix 9 μ L of denaturing loading buffer with 1 μ L of 32 P-labeled ladder from step 2 (increase volume of ladder preparation if older than a few days).
16. Dissolve pellets from step 12 in 6 μ L of 1x resuspension buffer (incubate tubes at 30°C for a few minutes to help dissolution) and add 7 μ L of denaturing loading buffer.
17. Heat sample and ladder tubes for 2 min at 90°C. Then, flush diffusing urea from gel wells using a syringe containing 1x TBE and quickly load samples and ladder in the gel wells.
18. Run the gel at 45 W until the band corresponding to xylene cyanol is ~25 cm from the bottom of gel wells.
19. Carefully remove one of the glass plates and replace it with a sheet of Whatman paper. Remove the second glass plate and replace it with saran wrap.
20. Dry the gel in a vacuum gel dryer and expose it overnight to a phosphorimager screen in an exposure cassette.
21. Scan the phosphorimager screen with a dedicated system. The presence of a Rho-dependent signal in the DNA template of interest (**Fig. 3A**) is made apparent by the apparition of fast migrating bands in the samples containing Rho and by the concomitant decrease in the intensity of the band corresponding to the formation of runoff transcripts

(**Fig. 3B**) (*See Note 13*). This trend is usually accentuated in samples containing both Rho and NusG (**Fig. 3B**) (*See Note 14*).

3.3.2 Effects of sRNAs and/or RNA chaperones

Below we describe our procedure to probe the effects of sRNAs on Rho-dependent termination. The protocol can be easily adjusted to probe the effects of RNA chaperones such as Hfq (*17*) or CsrA (*11*). Because Hfq alone can inhibit Rho (*17*), we recommend testing Hfq and sRNAs separately, and using control oligoribonucleotides that form perfect duplexes with the mRNA regions targeted by the sRNAs. To find transcription conditions optimal for sRNA-mRNA pairing, we also recommend to run a few exploratory experiments where the concentration of the tested sRNA or the concentration of KCl is varied. In the case of the *rpoS* template, for instance, we observed that the effect of the sRNA DsrA is optimal in the presence of 75 mM KCl (**Fig. 3C**) and we adjusted the composition of the transcription buffer accordingly.

1. Prepare and label one 1.5 mL ‘Protein low binding’ microtube per planned sample (including ‘minus Rho’ and ‘minus sRNA’ controls) and place tubes on ice.
2. For the n planned samples, prepare a master mix containing $9.4 \times (n+1)$ μL of deionized water, $2.7 \times (n+1)$ μL of 5x transcription termination buffer (containing 375 mM KCl in the case of the *rpoS* template), $(n+1)$ μL of the 100 nM stock solution of DNA template, $0.2 \times (n+1)$ μL of SUPERase-INTM, and $0.27 \times (n+1)$ μL of *E. coli* RNA polymerase.
3. Dispatch 13.6 μL of the master mix into each sample tube.
4. Add 2.4 μL of 1x transcription termination buffer (containing 75 mM KCl in the case of the *rpoS* template) to tube ‘minus Rho’. To each other tube (including ‘minus sRNA’ tube), add 1 μL of the 1.4 μM Rho stock and 1.4 μL of the 2 μM NusG stocks.

5. Mix reactants by gently pipetting up and down and incubate for 10 min at 37°C.
6. Add 2 μ L of 1x transcription termination buffer to tubes ‘minus sRNA’. To each other tube, add 2 μ L of the 5 μ M stock of appropriate sRNA or control oligoribonucleotide (*See Note 15*). Vortex gently and centrifuge briefly to homogenize each tube solution.
7. Proceed as described in steps 8-21 of section 3.3.1
8. Determine the apparent percentage of runoff product (*See Note 16*) using the phosphorimager analysis software. For instance, one may use the analysis toolbox of ImageQuant TL software (GE Healthcare) to box the runoff and termination product bands (rectangle area function; see **Fig. 4**, inset), select the ‘local average’ option for background and ‘percent’ option for view, and obtain percentages of band intensities directly and in an Excel-compatible format.
9. Evaluate sRNA- or chaperone-dependent effects through the comparison of the apparent percentages of runoff product (*See Note 16*) obtained for the different gel lanes (**Fig. 4**, diagram). We recommend using average percentage values obtained from at least three independent experiments to mitigate potential RNA degradation effects and the experimental variability inherent to such complex biochemical setups (*See Note 17*).

4. Notes

1. For the case chosen to illustrate the present chapter (analysis of the *rpoS* region of *E. coli*), we used a 20% glycerol stock of the *E. coli* reference strain MG1655 stored at -80°C . The strain (or genomic DNA) can be obtained from biological repositories such as DSMZ (www.dsmz.de).
2. The Vent DNA polymerase works well for the preparation of most DNA templates having lengths in the 300-1000 bp range. Other high-fidelity DNA polymerases may be used or better when preparing DNA templates longer than 1kB.
3. In our hands, the GeneJET PCR purification kit (ThermoFisher Scientific) works optimally when following manufacturer's instructions.
4. To eliminate bacteria, the major source of RNase contamination, stock solutions and buffers for transcription assays should be prepared with RNA-grade chemicals and water in small amounts (<50 mL) and sterilized with a 0.22 μm filter unit. We routinely obtain RNA-grade water by filtering ultrapure MilliQ (Millipore) water with 0.22 μm bottle-top sterile filter units. We usually avoid DEPC-treated water because harmful contaminants, such as rust particles, are often introduced during the autoclaving step required to remove excess DEPC.
5. Ethidium bromide is toxic. Solutions and waste (including gels) should be handled with great care following current safety regulation. Potentially less toxic DNA stains are available from various commercial sources and may be preferable for inexperienced users.
6. Concentrations of the Rho factor are expressed in hexamers throughout the chapter.
7. It is important to assess the enzymatic activity of every fresh Rho preparation and to verify that it does not decay upon storage at -20°C for extended period of times. The simplest test for Rho activity is the determination of the rate of steady-state ATPase turnover as described in volume 1259 of the series (21).

8. NusG, CsrA, and Hfq proteins are now available from commercial sources (e.g. MyBioSource) which we have not tested. We prepare and use His-tagged versions of NusG (*22*) and CsrA (*11*) and obtained purified Hfq from V. Arluison (*17*).
9. Manipulation of ^{32}P -containing materials should be performed exclusively by individuals who have received proper training and authorization from a radiation safety officer.
10. Over-dried RNA pellets are difficult to dissolve in aqueous buffers.
11. Full-length RNA should be seen clearly as one strong UV-shadow. If several shadows of comparable intensities are visible, one should assume that RNA degradation and/or formation of abortive transcription products have occurred in large proportion. It is then advisable to repeat the transcription/purification procedure with fresh solutions and reactants.
12. Rifampicin is present in the initiation mix to block subsequent rounds of transcription. Heparin cannot be used instead of rifampicin because it will trap Rho (*23*) in addition to free RNA polymerase.
13. Degradation of the transcripts (e.g. upon contamination of the samples by RNases) may yield similar band patterns. To ensure that the apparition of shorter-than-runoff termination products is Rho-dependent, it is advisable to repeat the experiment several times. Moreover, to ensure that neither the Rho nor NusG stock is contaminated by RNases, one may prepare control samples containing $150\ \mu\text{M}$ of the Rho inhibitor bicyclomycin (supplemented in the initial mix of reactants). Rho-dependent bands will disappear at the profit of the runoff band whereas degradation product band patterns will not be affected by the presence of bicyclomycin.
14. Strictly speaking, the assay does not distinguish between truncated transcripts resulting from Rho-induced dissociation of transcription elongation complexes (termination) or from transcription complexes that would be stably paused (or arrested) by Rho along the

DNA template. To the best of our knowledge, however, the second scenario (Rho inducing transcriptional pausing or arrest) is purely hypothetical, without experimental support to date. We thus feel confident that the more complicated transcription assays required to distinguish between termination, pausing and, arrest (24) are not necessary in most cases.

15. When testing the effect of an RNA chaperone, the chaperone is added at this step in the place of the sRNA(s).
16. The apparent percentage of runoff product determined under these conditions should be used only to detect sRNA-dependent effects through the comparison of gel lanes (samples), as shown in the diagram of Figure 4. This is because the apparent percentage of runoff product is not an accurate measure of terminator read-through as transcripts of different lengths contain different numbers of ³²P labels upon their internal labeling with ³²P- α UTP during transcription. Normalization of the intensities of gel bands for the uracil content of the corresponding transcripts is possible only when the sequences (lengths) of the various transcript species are known (25).
17. Because Rho is a nucleic acids binding protein displaying some level of sequence specificity (26), indirect 'sequestration' effects due to sRNA binding to Rho cannot be excluded beforehand, especially when considering the respective concentrations of Rho (70 nM) and sRNAs (500 nM) used in the assay. We strongly recommend testing this possibility by performing control transcription termination experiments with DNA template(s) encoding Rho-dependent terminator(s) unrelated to the original mRNA target sequence.

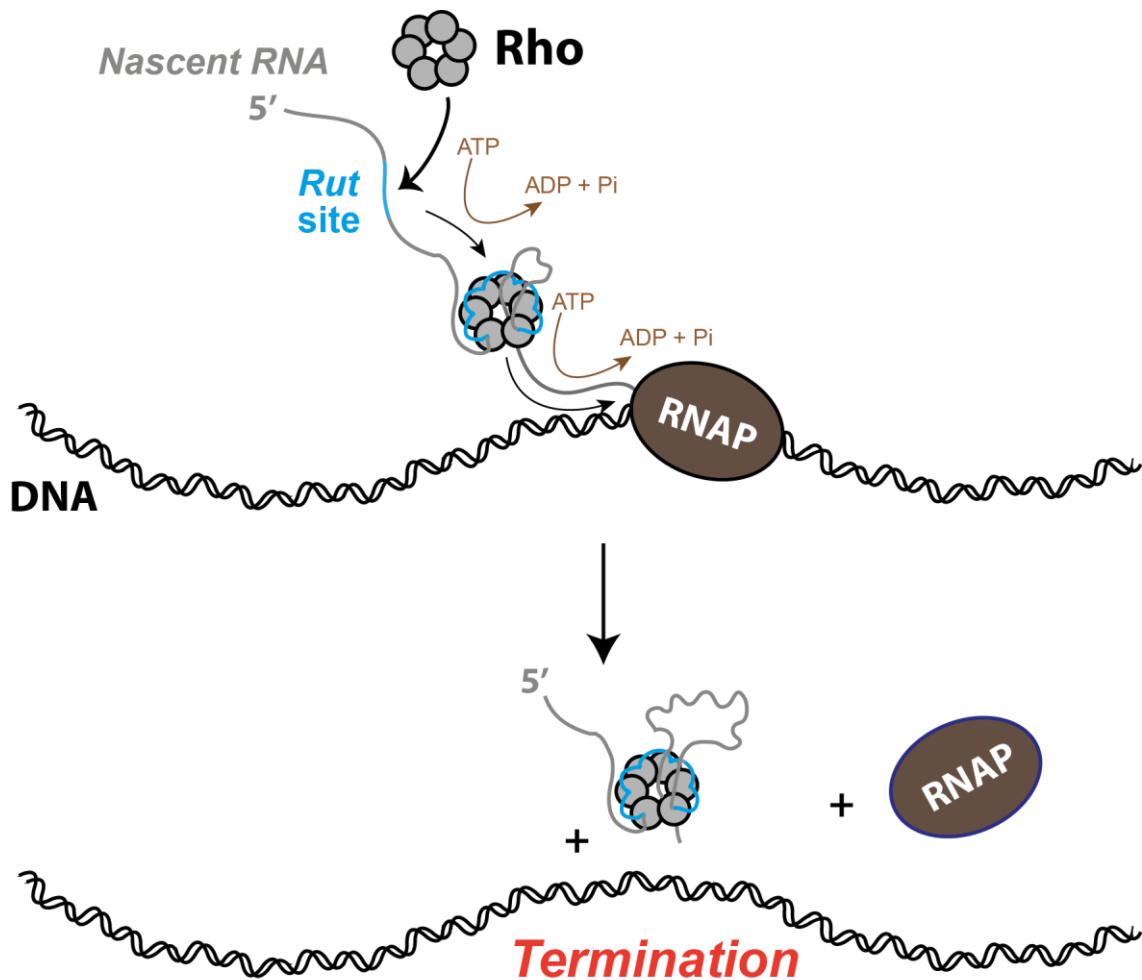
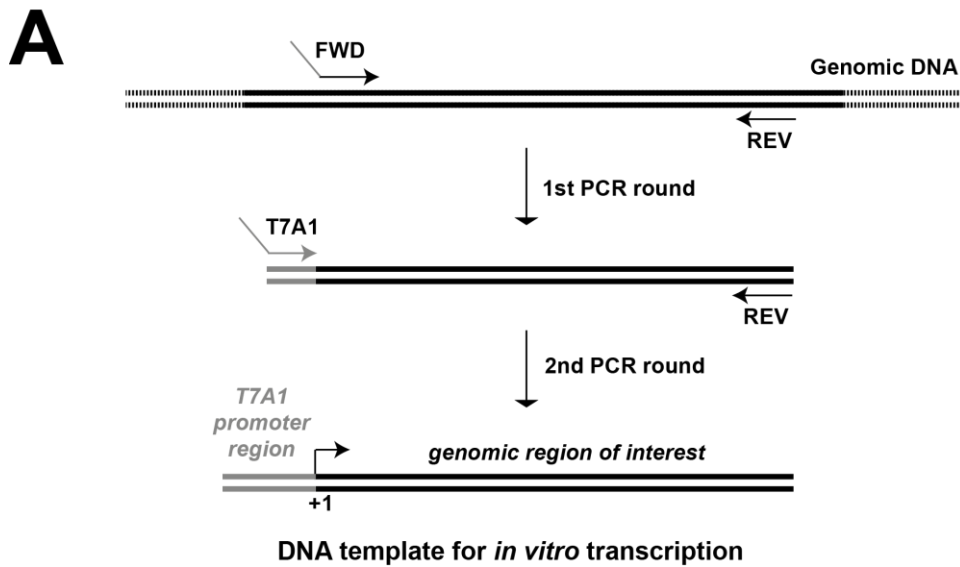


Figure 1: Rho-dependent termination of transcription. The Rho hexamer binds a *Rut* site within a naked (i.e. untranslated) portion of the nascent transcript. Rho anchoring to the transcript activates the ATP-dependent translocation of RNA within the hexamer central channel (note that the initial interaction with the *Rut* site is maintained throughout) (27). Then, Rho triggers dissociation of the transcription elongation complex upon catching up with the RNA polymerase (RNAP). Various mechanisms whereby sRNAs and RNA chaperones regulate Rho-dependent termination of transcription have been described (not shown on the figure). These mechanisms include formation of an Hfq-dependent antitermination complex (17), modulation of Rho access to a *Rut* site through inhibition of translation (14) or structural remodeling of the nascent RNA by CsrA (11) as well as less clear modes of structural interference by sRNA:mRNA complexes (10,15).



B

5' .TTATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCCCTTCGGGTGAACAGAGTGCTA
 AAAAAATGTTGCCGAACAACAAGCCAACCTGCGACCACGGTCACAGCGCCTGTACGGTACCAACAGCAAGCACAA
 CCGAGCCGACTGTCAGCAGTACATCAACCAGTACGCCTATCTCCACCTGGCGCTGGCCGACTGAGGGCAAAGTGA
 TCGAAACCTTTGGCGCTTCTGAGGGGGCAACAAGGGGATTGATATCGCAGGCAGCAAAGGACAGGCAATTATCG
 CGACCGCAGATGGCCGCGTTGTTTATGCTGGTAACGCGCTGCGCGGCTACGGTAATCTGATTATCATCAAACATA
 ATGATGATTACCTGAGTGCCCTACGCCCATACGACACAATGCTGGTCCGGGAACAACAAGAAGTTAAGGCGGGGC
 AAAAAATAGCGACCATGGGTAGCACCAGCAACAGTTCAACACGCTTGCATTT **TGAAATTTCGTTACAGGGGAAAT**
CCGTAAACCCGCTGCGTTATTTGCCGCGAGGATAAATCGGCGGAACCAGGCTTTTGCTTGATGTCCGTCAAGG
 GATCAGGGGTAGGAGCCACCTT **atg**AGTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGCGGAATTTGA
 TGAGAACGGAGTTGAGTTTTTGACGAAAAGGCCTTAGTAGAACAGGAACCCAGTGATAACGATTTGGCCGAAGA
 GGAAGTGTAT . 3'

C

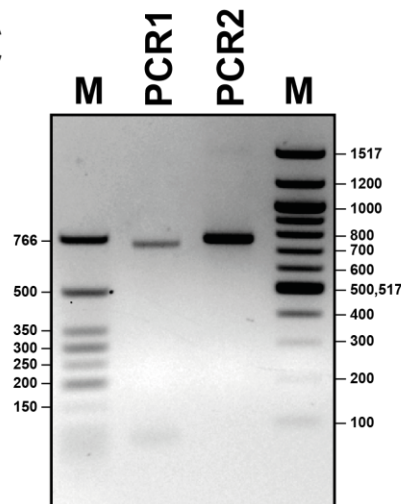


Figure 2: Preparation of DNA templates for *in vitro* transcription. (A) Outline of the method. (B) Sequence of the *rpoS* template used as example in the present chapter. The template contains the 5'UTR as well as the first 139 nucleotides of the *rpoS* coding region. The sequence of the T7A1 promoter is underlined while the region recognized by sRNAs ArcZ and DsrA (28) and the ATG start codon are in bold. (C) Representative 1.5% agarose gel of the DNA fragments obtained after rounds 1 and 2 of PCR amplification of the *rpoS* template using primers FWD and REV (see **Table 1**).

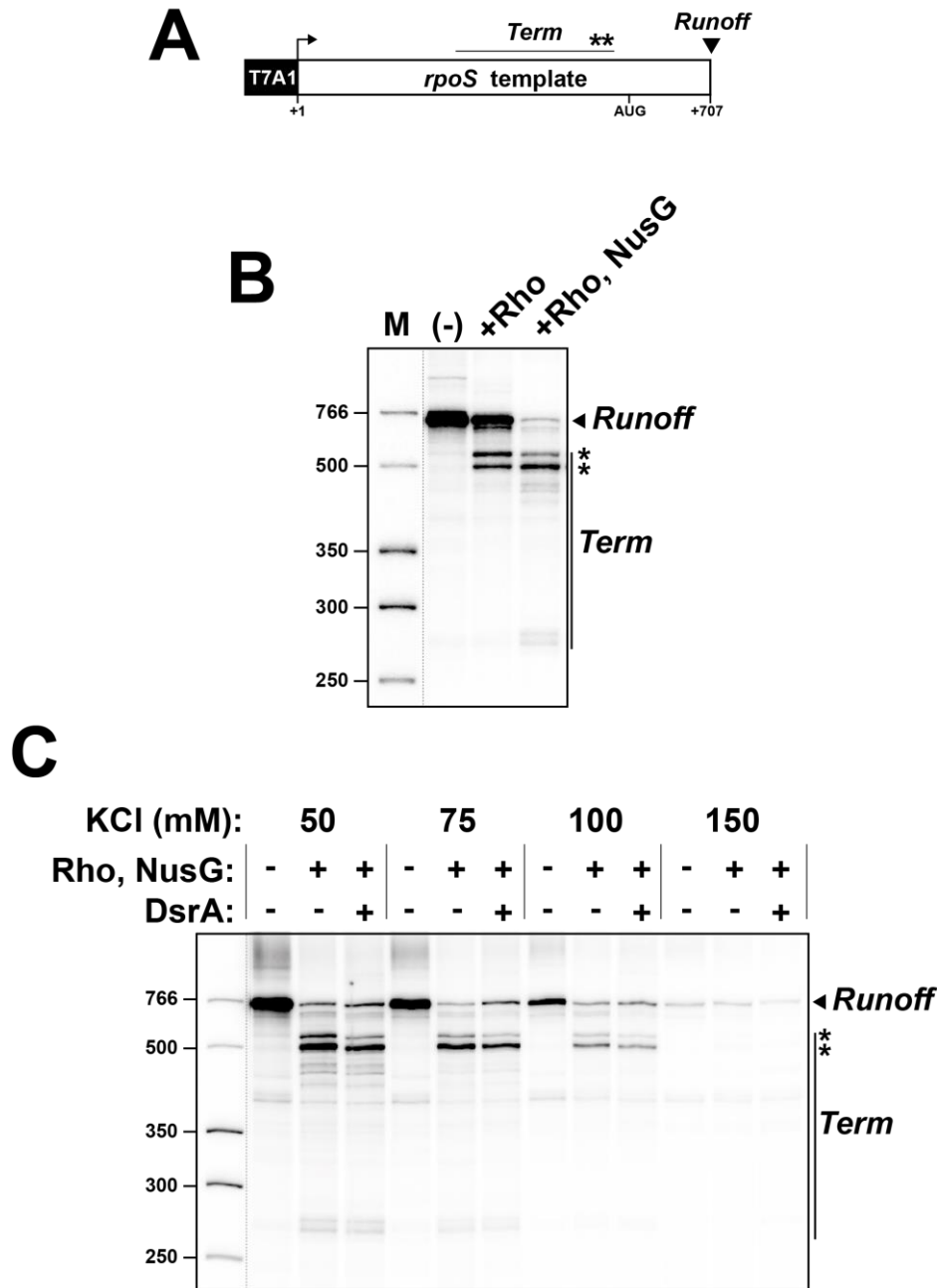


Figure 3: Representative exploratory transcription termination experiments performed with the *rpoS* template. **(A)** Schematic of the *rpoS* template and transcription termination features. **(B)** Initial detection of Rho-dependent signals within the *rpoS* template. **(C)** Analysis of the effect of KCl. In the example shown, 75 mM KCl represents the best compromise between the effects of the salt on transcription initiation, Rho-dependent termination, and DsrA efficiency. The KCl concentration was increased right from the start of the reaction assay but one may change it at a later stage (to limit the inhibitory salt effects on transcription initiation such as shown on the present gel) by adjusting the composition of the 10x initiation mixture rather than the 5x transcription termination buffer. Note that the contrast of the DNA ladder lane has been adjusted separately as indicated by the dotted line separating it from other gel lanes.

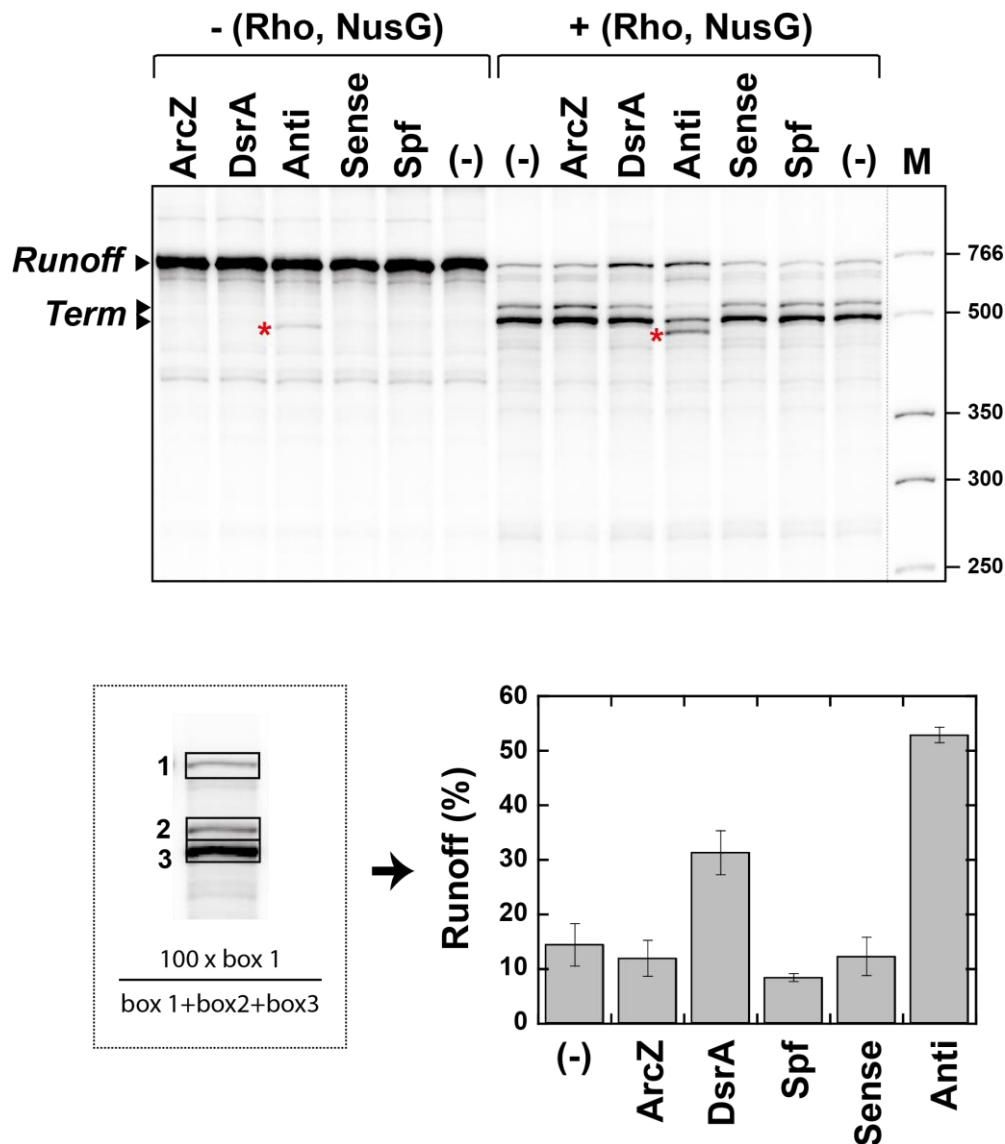


Figure 4: Effects of sRNAs and control oligoribonucleotides on the *rpoS* terminator. Reactions were performed in the presence of 75 mM KCl and 500 nM of sRNA or control oligonucleotide ‘Sense’ (5’ACUUUAAGCAAUGUCCCCUUUAGGC) or ‘Anti’ (5’CGGAUUCCCCUUGUAACGAAUUUCA). The bands identified by asterisks correspond to a Rho-independent product that is formed only in the presence of the ‘Anti’ strand, possibly because pairing of the oligonucleotide to the mRNA triggers intrinsic termination (29). Runoff percentages shown in the diagram are average values obtained from 3 to 6 independent experiments (See Note 16). The ArcZ sRNA has no significant effect under the present conditions whereas it inhibited Rho-dependent termination at a higher concentration (1 μ M) in a slightly different experimental setup (10). This is consistent with the observation that ArcZ forms a less stable hybrid with the *rpoS* mRNA than does DsrA (28).

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Table 1: Sequences of the oligonucleotide primers ^a .		
Template	Primer	Sequence ^b
rpoS ^d	FWD _(rpoS)	5'- <u>GTCTAACCTATAGGATACTTACAGCCCTTCGGGTGAACAGAGTGCTAACAAAATG</u>
	REV _(rpoS)	5'-ATAACAGCTCTTCTTCAGCCAGGTCGTTATCAC
	T7A1 ^c	5'- <u>TTATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCC</u>
DsrA ^e	TOP _(DsrA)	5'- <u>TAATACGACTCACTATAGGAACACATCAGATTTCTGGTGTAAACG</u>
	BOTT _(DsrA)	5'-AAATCCCGACCCTGAGGGGGTCTGGGATGAAAC
ArcZ ^e	TOP _(ArcZ)	5'- <u>TAATACGACTCACTATAGGGGTGCGGCCTG</u> AAAAACAGTG CTGTGCCCTT G
	BOTT _(ArcZ)	5'-AAAAAATGACCCCGGCTAGACCGGGGTGCGC
FnrS ^e	TOP _(FnrS)	5'- <u>TAATACGACTCACTATAGGCAGGTGAATGCAACGTCAAGCGATGGGC</u>
	BOTT _(FnrS)	5'-AAAAAGCCGACTCATCAAAGTCGGCGTCGTACGAATCAATTGTGCTATG
Spot 42 ^e (Spf)	TOP _(Spf)	5'- <u>TAATACGACTCACTATAGGTAGGGTACAGAGGTAAGATGTTCTATCTTTC</u>
	BOTT _(Spf)	5'-TAAAAAACGCCCCAGTCATTACTIONACTGACTGGGGCGG

^a Primers used to study sRNA effects on Rho-dependent termination in the 5'-leader region of *rpoS*.

^b Promoter sequences are underlined. Other oligonucleotide regions are chosen based on which genomic region needs to be amplified (**Fig. 2A**).

^c T7A1 is a universal primer used only in the second round of PCR amplification.

^d For transcription termination assays with RNA polymerase from *E. coli*.

^e For preparation of indicated sRNA by transcription with T7 RNA polymerase.