

DEFINING THE NANORNA REGULON AND THE MECHANISM BY WHICH GENE
EXPRESSION IS CONTROLLED AND MANIFESTED

By

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ABSTRACT OF THE THESIS

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Gene transcription is mediated by the enzyme RNA polymerase (RNAP). RNAP is a multi subunit nucleotidyl transferase that polymerizes ribonucleotides at the 3' end of an RNA transcript. Transcription by RNAP typically occurs by *de novo* transcription initiation whereby nucleoside triphosphates (NTPs) initiate transcription. Small RNAs of approximately 2-5 nucleotides have long been known to successfully prime transcription *in vitro*. In published work, the Nickels lab has established that these small RNAs (nanoRNAs) successfully compete with NTPs for transcription priming of some genes *in vivo*. NanoRNA-mediated priming alters the position of transcription initiation and possibly has a vital role in the regulation of gene expression. The complete set of genes whose expression is affected by nanoRNA-mediated priming remains to be elucidated. I propose to define the nanoRNA regulon and the mode by which gene expression is controlled and manifested. I will use a variety of molecular and biochemical *in vivo* and *in vitro* methods to determine the nanoRNA-mediated priming impact on gene expression.

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Chapter 1. Specific Aims

Gene transcription is mediated by the enzyme RNA polymerase (RNAP). RNAP is a multi subunit nucleotidyl transferase that polymerizes ribonucleotides at the 3' end of an RNA transcript. Transcription by RNAP typically occurs by *de novo* transcription initiation whereby nucleoside triphosphates (NTPs) initiate transcription. Small RNAs of approximately 2-5 nucleotides have long been known to successfully prime transcription *in vitro*. In published work, the Nickels lab has established that these small RNAs (nanoRNAs) successfully compete with NTPs for transcription priming of some genes *in vivo*. NanoRNA-mediated priming alters the position of transcription initiation and possibly has a vital role in the regulation of gene expression. The complete set of genes whose expression is affected by nanoRNA-mediated priming remains to be elucidated. I propose to define the nanoRNA regulon and the mode by which gene expression is controlled and manifested. I will use a variety of molecular and biochemical *in vivo* and *in vitro* methods to determine the nanoRNA-mediated priming impact on gene expression. To achieve this, I propose the following specific aims:

Aim 1: Define the nanoRNA regulon.

- a. Identify genes whose expression is affected by alteration in intracellular nanoRNA concentrations using the following parallel approaches:
 1. Measure transcript abundance using high-throughput sequencing
 2. Measure proteomic abundance using mass spectrometry

Aim 2: Investigate how metabolism of nanoRNAs occurs.

- a. Establish whether *E. coli* Orn exhibits substrate specificity *in vitro*
- b. Establish whether *E. coli* Orn exhibits substrate specificity *in vivo*

Aim 3: Establish whether transcripts generated by nanoRNA-priming have altered fates compared to transcripts generated by *de novo* initiation.

- a. Identify transcript turnover patterns of genes possibly stabilized by nanoRNA priming

Chapter 2: Background and Significance

Transcription, the first step of gene expression, is a complex highly regulated series of steps. The multi-subunit DNA-dependent RNA polymerase (RNAP) is the central component in transcriptional regulation. Prokaryotic RNA polymerase core enzyme consists of a β subunit, a β' subunit, two α subunits and a ω subunit. The large β and β' subunit form the active site of the polymerase. The amino terminal domains of the α subunits are necessary for the assembly of the β and β' subunits. The carboxyl terminal domains assist in the binding of DNA in some promoters. The ω subunit is not necessary for transcription to occur, but when present, functions as a chaperone to assist the folding of the β' subunit [1]. Before RNA polymerase begins transcription at a particular promoter, it interacts with a σ subunit to form holoenzyme. The σ subunit recognizes specific promoter sequences to position the RNA polymerase at a target promoter and assists in the unwinding of the DNA duplex near the transcription start site [1].

Another key component in transcription regulation is the promoter. Within the promoter, are sequence elements that are recognized by specific regions of RNA polymerase. Ten bases upstream of the transcription start site is the -10 element that has a consensus sequence of TATAAT. Promoter -10 elements are recognized by σ region 2. There is an extended -10 element made up of ~3-4 bp immediately upstream of the -10 element that has a consensus sequence of TGn and is recognized by σ region 3. Thirty-five bases upstream of the transcription start site is the -35 element that has a consensus sequence of TTGACA. Promoter -35 elements are recognized by σ region 4. Some promoters have upstream promoter regions known

as UP elements located approximately 41 bases upstream of the transcription start site. UP elements can be repeated every 10 bases from -41 upward and are recognized by the carboxyl terminal domains of the α subunits.

Together the -10, -35, extended -10 and UP elements specify the initial binding of RNA polymerase to a promoter to form the RNAP-promoter closed complex (RP_c). After initial binding of the RNA polymerase, DNA strands from approximately -10 to position +2 are unwound in a “bubble” to form the RNAP-promoter open complex (RP_o). Individual NTPs are brought into the active center cleft to base pair with DNA within the transcription bubble. A growing chain of RNA-DNA hybrid is formed in the initial transcribing complex (RP_{itc}). RNA polymerase engages in multiple abortive cycles of synthesis and releases short RNA products less than ~9-11 nt. Once a length of ~11 bp of the RNA-DNA hybrid is stably reached in the transcription bubble, RNAP escapes the promoter region and continues to transcribe the rest of the DNA in the elongation complex (RD_e). Transcription elongation is completed by termination where the RNAP will be released from the DNA.

E. coli RNA Polymerase was first biochemically identified based on its ability to *in vitro* transcribe from a DNA template using NTPs in *de novo* synthesis [2]. This discovery was followed by evidence that RNA transcription initiation could be primed *in vitro* using 2-5 nt RNAs that possessed a 5' hydroxyl group [3]. Not only was *in vitro* priming possible, but it also seemed to produce more transcripts than *de novo* synthesis [3] thus indicating a possible role in gene expression (Figure 1) [4].

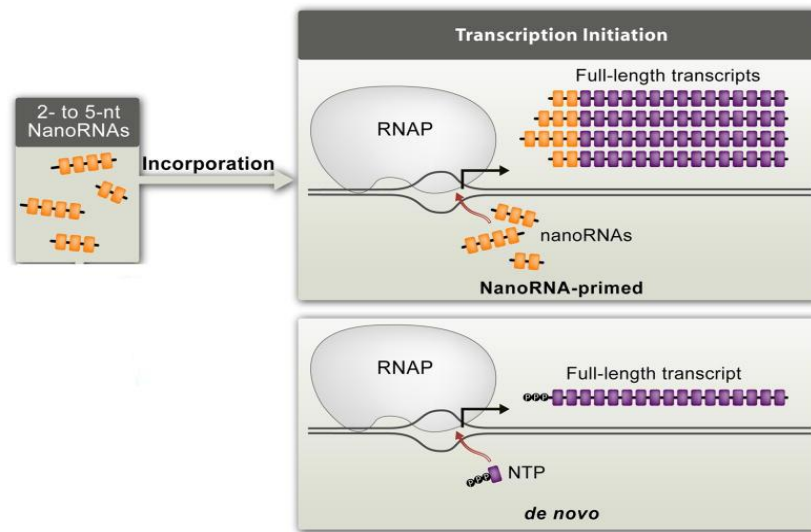


Figure 1: **NanoRNA-mediated priming of transcription initiation alters gene expression.**

Compared with initiation with NTPs only (i.e. *de novo*), initiation with nanoRNAs could, in principle, alter gene expression by increasing yields of transcripts, altering the sequence of the 5' ends of transcripts, or altering the phosphorylation status of the 5' ends of transcripts. Adapted from (Nickels and Dove 2011)

2.1 NanoRNAs prime transcription *in vitro* and *in vivo*

As previously stated, *in vivo* transcription initiation was originally believed to only occur by *de novo* synthesis even though there was abundant evidence that 2-8 nt RNAs were capable priming transcription *in vitro*. Goldman *et al* 2011 proved that small RNAs of 2-~4 nt are capable of priming transcription *in vivo*. To establish that nanoRNAs act as transcription primers, they sought to genetically engineer *E. coli* cells such that they could increase the intracellular concentration of nanoRNAs. The enzyme responsible for the metabolic breakdown of nanoRNAs to mononucleotides in *E. coli* is the exonuclease, Oligoribonuclease (Orn) [5]. Thus the accumulation of intracellular concentrations of nanoRNA is dependent on the depletion of Orn. However, Orn is essential in *E. coli*. *E. coli* ORN inactivation causes an accumulation of nanoRNAs and leads to cell death [5]. The effects of depleting ORN, was therefore studied in the closely related *P. aeruginosa*, which remains viable in the absence of ORN [6].

Similar to *E. coli*, ORN inactivation in *P. aeruginosa* led to the accumulation of 2 to ~5 nt nanoRNAs [6]. *P. aeruginosa* RNAP, similar to *E. coli* RNAP, was determined to effectively use nanoRNAs as primers. Using nanoRNAs as primers resulted in an alteration of the transcriptions start site (TSS). *In vitro* transcription experiments using purified *P. aeruginosa* RNAP and a linear *E. coli* derived promoter (*galP1/cons*) in the presence of a series of 2-4nt RNAs clearly indicated that nanoRNAs could effectively compete with NTPs for use as primers during transcription initiation (Figure2) [6].

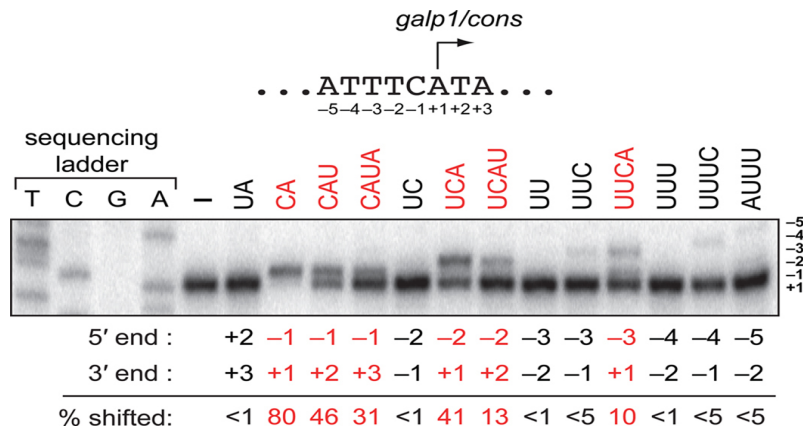


Figure 2: **Priming of transcription initiation with 2- to 4-nt RNAs can alter the TSS *in vitro*.** Top shows *galP1/cons* promoter sequences extending from position -5 to +3 (the *galP1/cons* promoter is a derivative of the *E. coli galP1* promoter with a consensus extended -10 element). Bottom shows results of primer extension analysis of RNA transcripts produced during *in vitro* transcription assays performed using a DNA fragment containing the *galP1/cons* promoter (10 nM). Assays were done using *P. aeruginosa* RNAP (50 nM) in the presence of 100 μ M NTPs in the absence (-) or presence of 100 μ M of the indicated 2- to 4-nt RNA. The position of the 5' and 3' end of each small RNA is indicated below the gel along with the percentage of transcripts shifted by each RNA. Highlighted in red are RNAs that effectively compete with NTPs and shift the TSS of >10% of transcripts initiated from *galP1/cons*. Taken from Goldman *et al* 2011.

Priming of transcription initiation with 2-4 nt RNAs *in vitro* resulted in a TSS shift to positions -3, -2, or -1. It was then proposed that if the nanoRNAs that accumulate in Orn depleted *P. aeruginosa* can also prime transcription *in vivo*, TSS shifting to positions -3, -2, or -1 should be observed at a significant fraction of promoters in Orn-depleted cells compared to non-depleted cells [6]. High throughput sequencing was used to analyze the sequences of the 5' ends of primary transcripts isolated from Orn-depleted cells and non-depleted cells. TSS shifting to positions -1, -2, and -3

was prominently observed in Orn-depleted cells compared with non-depleted cells (Figure 3A) [6]. The effect observed in Orn-depleted was rescued when wild-type *E. coli* Orn or heterologous *Bacillus subtilis* NrnB was ectopically expressed (Figure 3B) [6]. Thus TSS shifting upon depletion of Orn was confirmed as the result of nanoRNA accumulation.

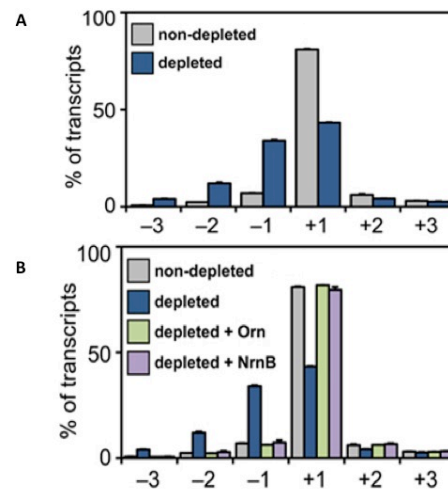


Figure 3. **NanoRNAs prime transcription initiation *in vivo***

Accumulation of nanoRNAs leads to TSS shifting as detected by high-throughput sequencing. Graphs show average percentage of all transcripts) initiated at positions -3 to +3 relative to the primary TSS.

Adapted from Goldman *et al* 2011.

2.2. Growth phase-dependent nanoRNA mediated priming

The findings that nanoRNAs could prime transcription *in vivo* raised the possibility that nanoRNA –mediated priming could alter TSS and affect gene expression during normal physiological conditions in *E. coli*. Thus it was hypothesized that if the concentrations of nanoRNAs under physiological growth conditions in *E. coli* cells were sufficient to prime transcription initiation, an effect on TSS selection would be observed if nanoRNA concentrations were reduced by overproduction of a nanoRNase (Figure 4) [7]. The nanoRNases Orn and NrnB were ectopically expressed and a catalytically inactive form of NrnB, NrnB-DHH was used as a control.

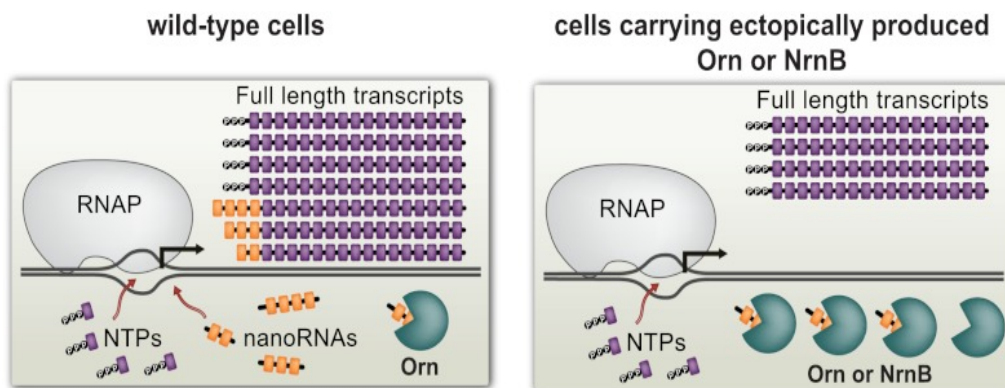


Figure 4. **NanoRNases reduce the concentration of nanoRNAs.** Ectopic production of a nanoRNase (Orn or NrnB) reduces the concentration of nanoRNAs and prevents RNA polymerase (RNAP) from using nanoRNAs as primers for transcription initiation. Taken from Vvendenskaya 2012

High throughput sequencing was used to analyze transcription start sites during the exponential and stationary phases of growth [7]. Ectopic production of either Orn or

NrnB did not result in TSS shifting during the exponential phase of growth (Figure 5A). In contrast, during the stationary phase of cell growth, approximately 5% of promoters were significantly affected by the ectopic production of Orn and NrnB while unaffected by the ectopic production of NrnB^{DHH} (Figure 5B) [7]. Of these, ~50% of transcripts were initiated from position -1, while ~50% of transcripts were initiated from position +1 in cells containing wild type concentrations of nanoRNAs or in cells which NrnB^{DHH} was ectopically produced (Figure 5B) [7]. In contrast, in cells in which Orn or NrnB were ectopically produced ~15% of transcripts were initiated from position -1, while ~85% of transcripts were initiated from position +1 (Figure 5B) [7]. Thus, ectopic production of nanoRNase alters transcription start site selection at a subset of promoters during the stationary phase of growth but not during the exponential phase of growth.

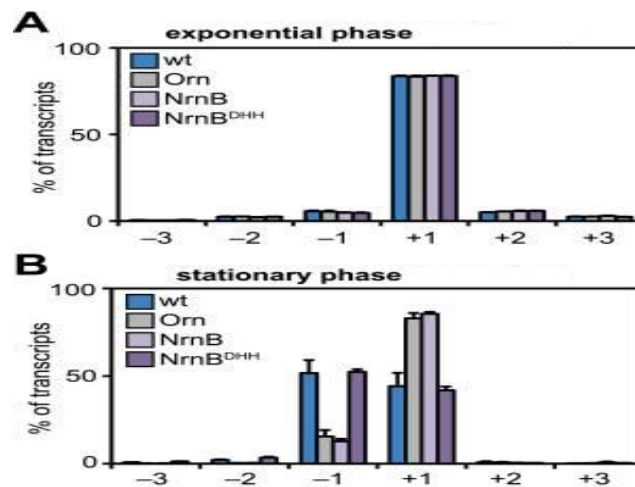


Figure 5. **Effects of ectopic production of a nanoRNase on TSS selection.** (A) Analysis of transcription start sites during exponential growth. (B) Analysis of transcription start sites during stationary phase of growth. Adapted from Vvendenskaya *et al* 2012

Confirmation of the results of the high-throughput sequencing was obtained through primer extension of the two most nanoRNA sensitive promoters, *bhsA* and *tomB*.

Primer extension analysis of transcripts initiated from plasmid-borne copies of the *bhsA* and *tomB* promoters revealed that, for both promoters, essentially all transcripts were initiated from position +1 during exponential phase (Figure 6) [7]. In contrast, during stationary phase, >50% of the transcripts associated with the *tomB* promoter and nearly all of the transcripts associated with the *bhsA* promoter were initiated from position -1 (Figure 6) [7]. Ectopic production of either Orn or NrnB during stationary phase significantly reduced the production of transcripts initiating from position -1 of each promoter, whereas ectopic production of NrnB^{DHH} did not (Figure 6) [7]. It was therefore concluded that nanoRNA-mediated priming regulates transcription start site selection in a growth phase-dependent manner.

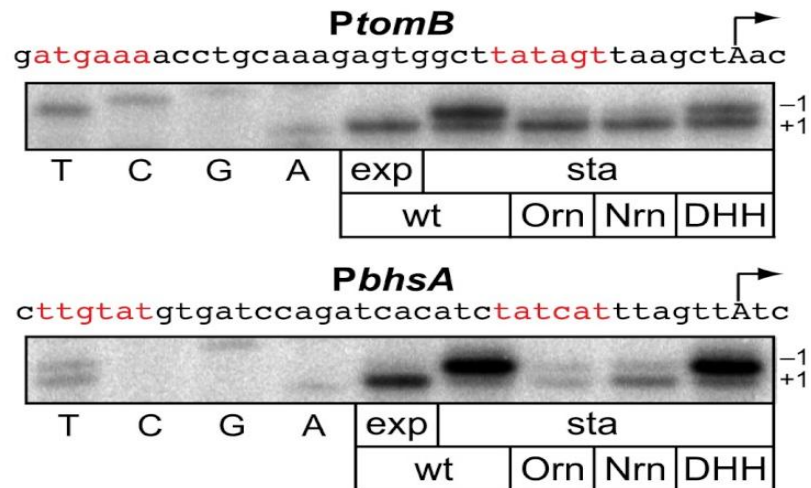


Figure 6. **Primer extension analysis of transcript 5' ends generated during transcription initiation from plasmid- born copies of the promoters associated with *tomB* and *bhsA*.** Analysis was performed using RNA transcripts isolated from *E. coli* cells during exponential phase of growth (exp) or the stationary phase of growth (sta). Cells harbored a plasmid carrying the indicated promoter along with an empty plasmid (wt) or a plasmid that specifies production of *E. coli* Orn, *B. subtilis* NrnB, or NrnB^{DHH}. Putative -10 and -35 elements of each promoter are highlighted in red. Position +1 is indicated by the arrow. Taken from Vvendenskaya *et al* 2012

2.3. Promoter Targeting of Nano RNA-mediated Priming

The nanoRNA sensitive promoters were all identified to carry a T at position -1 and an A at position +1. Conversely, not all -1/+1 TA promoters are targeted by nanoRNA-mediated priming. Only ~ 15% of the -1/+1 TA promoters analyzed were significantly impacted by nanoRNA-mediated priming. It is possible that features aside from -1/+1 TA are responsible for the targeting of a promoter. A model was proposed to account for the differential susceptibility of -1/+1 TA. Nickels 2012 proposed that the concentrations of nanoRNAs during stationary phase are relatively low compared with the concentrations of NTPs and that nanoRNA-mediated priming comprises only a small fraction of the total number of initiation events at all -1/+1 TA promoters (Figure 7) [8]. Thus, in context of the majority of -1/+1 TA promoters transcripts produced by nanoRNA-mediated priming are undetectable because they exhibit similar or lower stability than transcripts generated by *de novo* initiation. In contrast, in the context of a few -1/+1 TA promoters transcripts produced by nanoRNA-mediated priming are significantly more stable than transcripts generated by *de novo* initiation. The model proposes that transcripts generated by nanoRNA-mediated priming are differentially stable compared with those produced by *de novo* initiation. This could also account for the effects of nanoRNA-mediated priming on gene expression.

2.4. The Role of NanoRNA-mediated priming on gene expression

As previously stated, nanoRNA primed transcripts have been observed to be more abundant than *de novo* transcripts. This was also evident in the screen of the nanoRNA sensitive promoters of *tomB* and *bhsA* (Figure 6) [7]. Ectopic production of nanoRNases resulted in a reduction in transcripts when *tomB* and *bhsA* were expressed on a plasmid (Figure 6) [7]. To investigate the effect of nanoRNA on the abundance of endogenous transcripts, northern blot assays on the expression of *tomB* and *bhsA* were performed in cells containing wild type concentration of nanoRNAs and cells ectopically expressing NrnB. The production of NrnB reduced the abundance of *tomB* and *bhsA* significantly in stationary phase but not in exponential phase (Figure 8A) [7]. The expression of *bhsA* was also evaluated with quantitative RT-PCR analysis. Ectopic production of nanoRNases again significantly reduced the abundance of *bhsA* transcripts during stationary phase (Figure 8B) [7]. Thus it can be concluded that growth phase dependent nanoRNA-mediated priming regulates expression of nanoRNA sensitive genes.

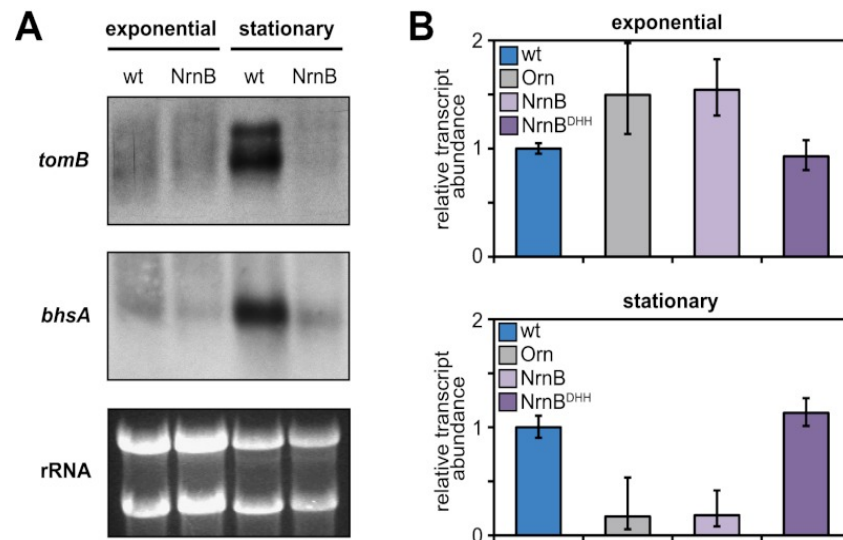


Figure 8. Growth phase-dependent regulation of gene expression by nanoRNA-mediated priming. (A) Northern blot analysis of *tomB* and *bhsA* transcripts during the exponential phase of growth or the stationary phase of growth in cells that harbored an empty plasmid (wt) or a plasmid that specifies production of *B. subtilis* NrnB. The bottom panel shows an ethidium bromide-stained gel of the RNA samples used for Northern blotting. The two most prominent bands correspond to the 23S and 16S rRNAs. (B) Graphs show the abundance of *bhsA* transcripts during the exponential phase of growth or the stationary phase of growth in cells that harbored an empty plasmid (wt) or a plasmid that specifies production of *E. coli* Orn, *B. subtilis* NrnB, or NrnB^{DHH}. Taken from Vvedenskaya *et al* 2012

2.5. Significance

NanoRNAs are a newly identified feature of transcription initiation. Both of the nanoRNA sensitive genes most studied reveal a biochemical relationship in biofilm formation. It is possible that nanoRNAs are utilized to maintain transcriptome states in relation to physiologically diverse conditions that cells are exposed to particularly when stressed. This would possibly implicate multiple biochemical pathways. Despite the theorized possibilities, it is unclear to what extent nanoRNAs regulate global gene expression. Identifying the *E. coli* nanoRNA regulon will not only give insight into the processes of regulation within *E. coli* but could also identify unprecedented modes of gene expression regulation in a variety of organisms.

Chapter 3. Preliminary Data

3.1. Global alterations in gene expression due to nanoRNA priming

To obtain a global gene expression profile based on the abundance of nanoRNAs, microarray analysis has been conducted on *P. aeruginosa* Orn-depleted cells and non-depleted cells. The comparison resulted in the identification of 1158 genes whose expression was significantly altered by a factor of 2 or more due to Orn depletion (figure 9) [6] This is ~20% of all known *P. aeruginosa* transcripts. The extent of the changes due to nanoRNA accumulation was established by ectopically expressing Orn and NrnB. In the presence of nanoRNases the changes in gene expression observed were greatly reduced.

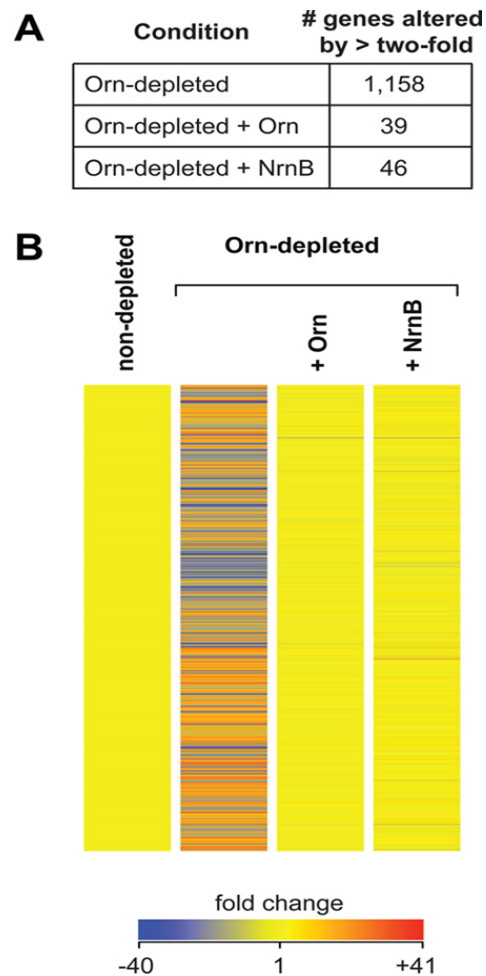


Figure 9. **NanoRNA-Mediated Priming of Transcription Initiation In Vivo Leads to Changes in Gene Expression.** (A and B) Effect of Orn depletion on gene expression in *P. aeruginosa* as determined by DNA microarray. The table in panel A shows the number of genes whose expression changes by a factor of 2 or more upon depletion of Orn, or upon depletion of Orn in the presence of either wild-type Orn, or NrnB. Panel B shows a heat map representation of the 1,158 genes whose expression changes by a factor of 2 or more upon depletion of Orn. Also shown are the corresponding effects on expression of these genes when either wild-type Orn, or NrnB, are supplied ectopically and Orn is depleted. Taken from Goldman *et al* 2011.

Thus, the accumulation of nanoRNAs is responsible for the global alterations in gene expression. By using high-throughput sequencing and mass spectrometry analysis, I aim to identify in *E. coli*, the global alterations in gene expression due to the reduction in the concentration nanoRNAs when nanoRNases are ectopically expressed. This method will help identify the complete set of genes whose expression is directly and indirectly affected by nanoRNA-mediated priming.

3.2. Mechanisms of Promoter Targeting

As previously discussed, the nanoRNA sensitive promoters were all identified to carry a T at position -1 and an A at position +1. These promoters must consequently be primed with nanoRNAs beginning with the sequence UA. To explore the role of UA in priming -1/+1 TA promoters, the *placUV5* promoter was engineered to possess -1/+1 TA and compared with -1/+1 CA. The introduction of -1/+1 into *placUV5* led to growth phase dependent nanoRNA-priming as exhibited through primer extension (Figure 10).

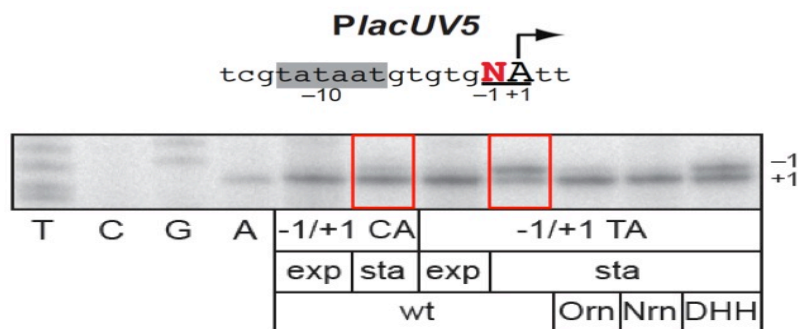


Figure 10. **Introduction of “-1/+1 TA” into *placUV5* leads to growth phase-dependent nanoRNA-priming.** In exponential phase, +1A initiation occurs on both templates, but in stationary phase initiation from -1T occurs only on -1/+1 TA template. Over expression of nanoRNases (Orn and NrnB) eliminates initiation from -1T. Seth Goldman (Rutgers)

To determine which nanoRNAs prime *placUV5* -1/+1 TA, the following nanoRNA species were evaluated through primer extension: UA, UAU, and UAUU. It was determined that only UA is capable of discriminating between *placUV5* -1/+1 TA and -1/+1 CA while UAU and UAUU cannot (Figure 11). Only UA possesses the requisite

template discrimination to restrict nanoRNA priming to -1/+1 TA promoters. Consequently, it was concluded UA is responsible for most, if not all, of the nanoRNA priming observed *in vivo* at all TA promoters.

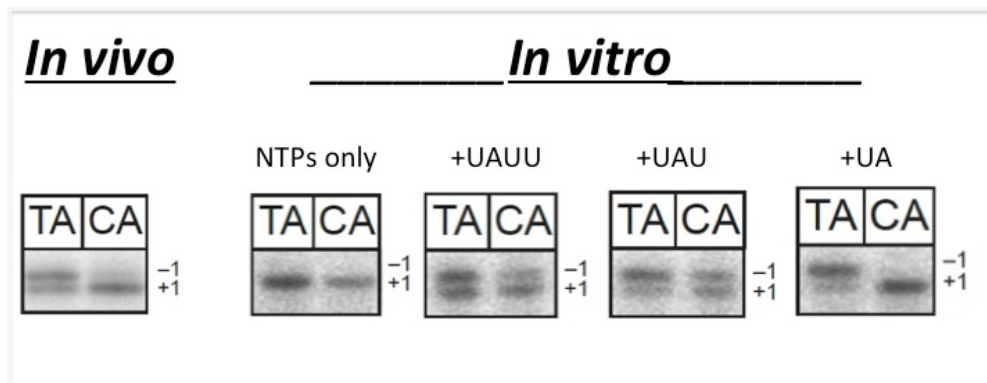


Figure 11. **Only UA possesses the requisite template discrimination to restrict nanoRNA priming to -1/+1 TA promoters.** *In vivo*, nanoRNA-priming does not occur at *placUV5* -1/+1 CA. *In vitro*, UAU and UAUU are incapable of discriminating between *placUV5* -1/+1 TA and -1/+1 CA. Only UA is capable of the discrimination required to restrict nanoRNA priming to -1/+1 TA promoters.

Seth Goldman (Rutgers)

Since nanoRNA is growth phase dependent, it was proposed that the linear dinucleotide UA must accumulate in stationary phase to prime -1/+1 TA promoters. HPLC-MS/MS was employed to directly detect levels of nanoRNAs. The results indicated a direct correlation between levels of nanoRNAs and start site selection (Figure 12).

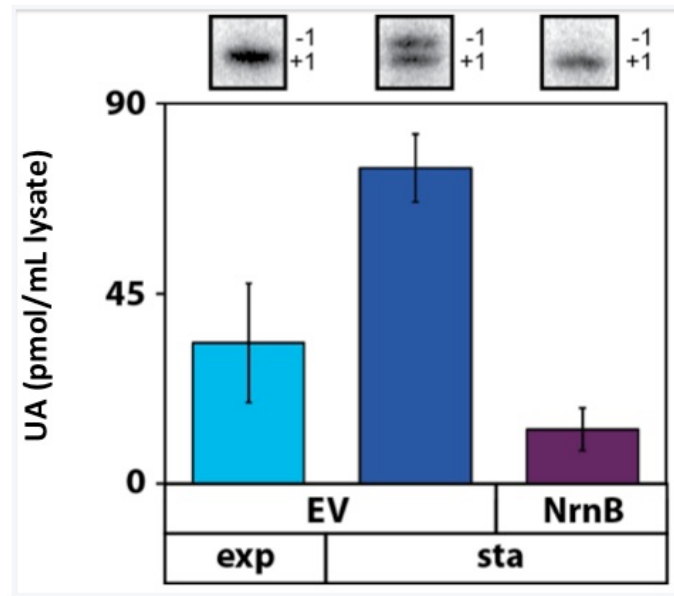


Figure 12. **Direct Correlation between levels of nanoRNAs and start site selection.** The UA dinucleotide accumulates within a cell in a growth phase dependent manner indicating that nanoRNA-priming at -1/+1TA promoters occurs as a consequence of growth-phase dependent accumulation of UA.

The dinucleotide UA was identified to be present in significant amounts capable of impacting priming. Hence, nanoRNA-priming occurs as a consequence of growth-phase dependent accumulation of UA. This raises a key question, which will be addressed in my second aim. Why does UA dinucleotide preferentially accumulate? It has been proposed that they are produced as terminal products of RNA degradation [8]. Using a diverse population of substrates, I aim to explore the substrate specificity behavior of Orn as a possible avenue of growth-phase dependent UA dinucleotide accumulation.

3.3. Distinguishing characteristics of transcripts generated by nanoRNA

priming versus transcripts generated by *de novo* initiation

Transcripts produced as a result of *de novo* synthesis all possess 5'-triphosphate ends. Based on the model that most nanoRNAs are the result of RNA degradation, it is likely those transcripts without 5'-triphosphate ends are the result of nanoRNA mediated priming; particularly since degradation products tend to result in 5'-hydroxyl ends. Previous studies have shown that, in fact, the transcripts produced as a result of nanoRNA-mediated priming possess 5'-hydroxyl ends [7]. *In vivo* studies on transcript stability, indicated that transcripts with hydroxylated 5' ends have a half life that is six times longer than transcripts with triphosphorylated 5'ends [10]. It has also been shown that shorter mRNAs are degraded at a faster rate [11]. These data suggest that, the reason nanoRNAs affect gene expression could be by altering transcript stability. Longer transcripts and 5'-hydroxyl ends could confer a change in the fates of transcript. To determine whether transcripts generated by *de novo* initiation and those generated by nanoRNA-mediated priming have distinct fates, I will utilize high-throughput methods to examine RNA turnover.

Chapter 4. Research Design and Methods

4.1 Define the nanoRNA regulon.

Specific Aim I: Define the nanoRNA regulon

Microarray analysis has indicated the accumulation of nanoRNAs is possibly responsible for global alterations in gene expression. To identify genes whose expressions are altered by the production of nanoRNAs, I will use high-throughput sequencing methods. We already possess pBr-derived plasmids with each nanoRNase (Orn, NrnB, and NrnBDHH) under the control of a Lac promoter. *E. coli* strain MG1655 will be transformed with these plasmids. A vector lacking any of the nanoRNases will also be transformed as a control and for reference. Selection of successfully transformed strains will be based on gentamycin resistance. Successfully transformed strains will be cultured in LB containing gentamycin and IPTG to induce expression of the nanoRNases. Cells will be harvested when they have reached an OD₆₀₀ of ~0.5 to collect samples in exponential phase and after 23 hours to collect samples in stationary phase. RNA from these cultures will be isolated through a phenol chloroform extraction method. They will then be enriched for high molecular weight RNA through selection on a silica gel based membrane to exclude RNAs smaller than 200 nt, which comprises mostly the tRNAs. The ribosomal RNAs will be removed through a magnetic capture hybridization approach. The samples will then be sequenced using RNAseq technology. In parallel, I will construct libraries to assess transcription start site shifting. The 5' ends of the enriched RNA will be converted to monophosphate ends. These will then be ligated with 5' adapters and reverse transcribed with a 3' adapter to create a cDNA library.

The cDNA will be PCR amplified, and sequenced using Illumina sequencing technology. The sequencing results will be analyzed using software specifically designed for this purpose within the Nickels lab. The RNAseq approach should present a global picture of genes whose expression is altered by the decrease of nanoRNAs in the cell. Identified genes will be validated through a β -galactosidase assay. The Illumina sequencing approach should aid in the identification of transcripts generated by nanoRNA-mediated priming. Identified genes will be validated through primer extension methods and northern blot analysis.

To further explore the effects of growth phase dependent gene expression control by nanoRNAs proteomic methods will be used to identify proteins whose expressions are positively or negatively regulated. Some of the harvested culture samples will be subjected to lysis and analyzed through mass spectrometry. Identified proteins will be validated through western blot analysis. The proteomic analysis should give an indication of the downstream effect of the regulation.

4.2 Investigate how metabolism of nanoRNAs occurs.

Specific Aim II: Investigate how metabolism of nanoRNAs occurs.

RNA turnover in *E. coli* cells is mediated by various ribonucleases, but the degradation of nanoRNAs to mononucleotides is accomplished through the action of the essential oligoribonuclease, Orn [5]. Thus the accumulation of specific nanoRNA species could be the result Orn's substrate specificity. Orn activity assays conducted upon its discovery utilized oligomers of 3-5 repeats of the same nucleotide as substrates [9]. Orn *in vitro* assays typically have employed substrates with minimal diversity [9][5]. These substrates are not very representative of the pool of available substrates for Orn *in vivo*. Preliminary data has shown that the dinucleotide UA accumulates in *E. coli* cells. I will assess the substrate specificity of *E. coli* oligoribonuclease, Orn, as a possible source of the specific nanoRNA species accumulation.

His-tagged Orn will be ectopically expressed and purified using affinity column chromatography with His binding talon resin. Orn specificity would be assayed using a diverse population of specifically designed DNA oligonucleotides. The DNA oligonucleotides will be *in vitro* transcribed using radiolabelled NTPS. To validate Orn specificities as the mechanism that underlies gene expression, the by-products of the Orn *in vitro* assay would be used in primer extension assays on known TA promoters. To determine the mechanism *in vivo*, Orn will be artificially depleted in *P. aeruginosa* and *E. coli* Orn ectopically expressed. Differential degradation and effect on nanoRNA population will be identified through mass spectrometry methods.

4.3 Establish whether transcripts generated by nanoRNA-priming have altered fates.

Specific Aim III: Establish whether transcripts generated by nanoRNA-priming have altered fates compared to transcripts generated by *de novo* initiation.

NanoRNA-mediated priming provides transcripts with 5'hydroxylated ends. Previous studies have shown that change in phosphorylated state at the 5' end to a hydroxylated state could change the fate of a transcript. To determine whether transcripts generated by *de novo* initiation and those generated by nanoRNA-mediated priming have distinct fates, I will utilize high-throughput methods to examine the total pool of transcripts compared to the pool of nascent transcripts in an attempt identify transcripts that persist longer before being degraded.

E. coli with chromosomally flag tagged β' subunit of RNA polymerase will be developed to ectopically express nanoRNases. As mentioned previously, we already possess pBr-derived plasmids with each nanoRNase (Orn, NrnB, and NrnB^{DHH}) under the control of a Lac promoter. *E. coli* with chromosomally flag tagged β' subunit of RNA polymerase will be transformed with these plasmids. A vector lacking any of the nanoRNases will also be transformed as a control and for reference. Selection of successfully transformed strains will be based on gentamycin resistance. Successfully transformed strains will be cultured in LB containing gentamycin and IPTG to induce expression of the nanoRNases. Cells will be harvested when they have reached an OD₆₀₀ of ~0.5 to collect samples in exponential phase and after 23 hours to collect samples in stationary phase.

These cells will be lysed and the flag tagged RNA polymerase with bound nascent RNA will be purified by affinity chromatography using anti-flag resin. Nascent RNA will then be isolated through a phenol chloroform extraction method. In parallel, total RNA will be also be isolated through a phenol chloroform extraction method. They will then be enriched for high molecular weight RNA through selection on a silica gel based membrane to exclude RNAs smaller than 200 nt, which comprises mostly the tRNAs. The ribosomal RNAs will be removed through a magnetic capture hybridization approach. The 5' ends of the enriched RNA will be converted to monophosphate ends. These will then be ligated with 5' adapters and reverse transcribed with a 3' adapters to create a cDNA library. The cDNA will be PCR amplified and sequenced using Illumina sequencing technology. The sequencing results will be analyzed using software specifically designed for this purpose within the Nickels lab to identify nascent expression profiles. All together the information will be analyzed to identify transcript turnover patterns of genes possibly stabilized by nanoRNA priming.

Chapter 5. References:

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