NanoRNAs: a class of small RNAs that can prime transcription initiation in bacteria

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SUMMARY

It has been widely assumed that all transcription in cells occurs using NTPs only, i.e. *de novo*. However, it has been known for several decades that both prokaryotic and eukaryotic RNA polymerases (RNAPs) can utilize small (2- to ~5-nt) RNAs to prime transcription initiation in vitro, raising the possibility that small RNAs might also prime transcription initiation in vivo. A new study by Goldman *et al.* has now provided the first evidence that priming with so-called "nanoRNAs", i.e. 2- to ~5-nt RNAs, can, in fact, occur in vivo. Furthermore, this study provides evidence that altering the extent of nanoRNA-mediated priming of transcription initiation can profoundly influence global gene expression. In this perspective we summarize the findings of Goldman *et al.* and discuss the prospect that nanoRNA-mediated priming of transcription initiation represents an under appreciated aspect of gene expression in vivo.

Introduction: a historical perspective on priming of transcription with small RNAs

Multisubunit DNA-dependent RNA polymerases (RNAPs) were first identified in 1960 based on their ability to synthesize RNA transcripts in vitro in the presence of a DNA template and NTPs [1]. In addition to being able to initiate RNA synthesis using NTPs only, i.e. "*de novo*", it was demonstrated in the mid-1960s that RNAP could also initiate transcription by using a 2- to 5-nt RNA oligonucleotide carrying a 3′ hydroxyl group as a primer [2, 3]. Later studies revealed that all multisubunit DNA-dependent RNAPs--bacterial RNAP, eukaryotic RNAP I, RNAP II, RNAP III, and archael RNAP--can use small RNA oligonucleotides to prime promoter-specific transcription initiation in vitro [4–7]. Furthermore, the use of such small RNAs to prime transcription initiation in vitro has become commonplace for researchers studying transcription.

In the mid-1970s it was proposed that priming of transcription with small RNAs occurs in cells and can influence gene expression [8, 9]. In particular, it was proposed that small RNA fragments derived from the processing of longer transcripts could serve as sequence-specific primers that stimulate transcription initiation from specific target promoters [8, 9]. The proposal that small RNA fragments could "activate" transcription in this manner was based upon the empirical observation that yields of RNA transcripts produced in vitro could, in certain cases, be increased by adding a small RNA primer to the transcription reactions [2, 3, 10, 11]. Nevertheless, in the ~35 years since this proposal was made, no

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evidence that small RNAs can actually prime transcription initiation in vivo has been presented. Thus, the generally accepted view has been that all transcription in cells occurs *de novo*.

A new study by Goldman *et al.* has now challenged the conventional paradigm that all cellular transcription occurs *de novo* [12]. In particular, this study has provided evidence that very small RNAs can, in fact, prime transcription initiation in vivo. The findings of Goldman *et al.* also provide evidence that altering the extent to which transcription initiation is primed or occurs *de novo* can significantly impact gene expression. These new findings provide an impetus to revisit the notion that priming of transcription with very small RNAs might be an important contributor to cellular gene expression.

Evidence that nanoRNAs prime transcription initiation in vivo

To demonstrate that very small RNAs can be used by RNAP as primers for transcription initiation in vivo, Goldman *et al.* took advantage of studies investigating the metabolism of small RNAs in bacterial cells. These studies had revealed the existence of a class of enzymes responsible for the degradation of RNAs between 2 and ~5-nt in length [13–17]; such extremely small RNAs have been termed "nanoRNAs" to distinguish them from other classes of small RNAs [16]. In *E. coli* the degradation of nanoRNAs is carried out by an essential 3′ to 5′ exonuclease, Oligoribonuclease (Orn) [15]. Thus, inactivation of Orn in *E. coli* was shown to result in the accumulation of 2- to ~5-nt nanoRNAs [15]. Accordingly, Goldman *et al.* tested whether inactivation of Orn might lead to cellular conditions where a significant fraction of transcription is primed due to the presence of elevated concentrations of 2- to ~5-nt nanoRNAs. Because inactivation of Orn in *E. coli* leads to a loss of viability [15], the authors determined the effect of inactivating Orn in *Pseudomonas aeruginosa*, a Gram-negative organism that remains viable in the absence of Orn function [18].

Using a specially engineered strain of *P. aeruginosa* that allows for the artificial depletion of Orn, Goldman *et al.* first demonstrated that, similar to results obtained in *E. coli*, loss of Orn function in *P. aeruginosa* leads to accumulation of 2- to ~4-nt nanoRNAs. Next, to determine whether increasing the intracellular concentrations of 2-to ~4-nt nanoRNAs leads to the widespread use of nanoRNAs as primers for transcription initiation, the authors used high-throughput sequencing to analyze the 5′ ends of primary transcripts on a genome-wide scale.

In vitro studies indicate that 2- to 4-nt RNAs that are complementary to the DNA template can efficiently compete with NTPs for use as primers during transcription initiation provided the 5′ end of the RNA base pairs with promoter sequences between positions −3 and +1 and the 3′ end of the RNA base pairs with promoter sequences between positions +1 and +3 [11, 12, 19–23]. (Note that by convention the position where *de novo* transcription begins is designated +1.) Thus, use of 2- to 4-nt RNAs to prime transcription initiation in vitro can, in some cases, result in production of transcripts that are extended in length by 1, 2, or 3 nt at the 5′ end compared with transcripts that are produced in reactions performed in the presence of NTPs alone. The authors therefore reasoned that if 2- to 4-nt RNAs could also prime transcription initiation in vivo, increasing the intracellular concentration of 2- to 4-nt nanoRNAs should result in 5′ end alterations for primary transcripts produced from a significant fraction of cellular promoters. Consistent with this prediction, using high-throughput sequencing Goldman *et al.* found that depletion of Orn did, in fact, cause the appearance of significant amounts of primary transcripts that were, on average, 1, 2, or 3 nt longer than those observed in non-depleted cells.

The authors presented several lines of evidence supporting the conclusion that the observed changes in the average distributions of the 5′ ends of primary transcripts upon depletion of Orn are a direct consequence of widespread use of 2- to 4-nt nanoRNAs as primers for transcription initiation. Among these, the two most important are the following. First, depleting Orn in the presence of a heterologous nanoRNA-degrading enzyme, the NrnB protein from *Bacillus subtilis* [17], did not result in global alterations in the average distributions of the 5′ ends of primary transcripts. This finding established that the observed change in the distribution of the 5′ ends of transcripts that occurs upon depletion of Orn was a direct consequence of the accumulation of 2- to ~4-nt nanoRNAs (and not due to an unrelated consequence of Orn depletion). Second, it is well documented that alterations in the intracellular concentrations of NTPs can lead to changes in the position where transcription initiation begins [reviewed in [24]]. (This phenomenon, which is termed NTP-dependent start point switching, is discussed further below.) Thus, it was important to rule out the possibility that the observed changes in the average distributions of the 5′ ends of primary transcripts upon depletion of Orn were not due simply to any alterations in NTP concentrations. To investigate this possibility, Goldman *et al.* compared the average distribution of the 5′ ends of transcripts carrying a 5′ triphosphate group, i.e. those potentially derived from a *de novo* initiation event, with the average distribution of the 5′ ends of all transcripts regardless of the phosphorylation status of the 5′ end. Strikingly, this comparison revealed that whereas depletion of Orn results in a pronounced alteration in the distribution of the 5′ ends of transcripts when all 5′ ends are considered, depletion of Orn results in only a modest change in the average distribution of transcripts carrying a 5′ triphosphate group. These data rule out the possibility that the pronounced changes in the average distributions of the 5′ ends of transcripts are a consequence of alterations in the abundance of NTPs that, in turn, alters the position of *de novo* initiation. Consistent with this assertion, the authors presented evidence that the intracellular concentrations of NTPs were identical in Orn-depleted cells and cells in which Orn was not depleted.

NanoRNA-mediated priming as a means of altering gene expression

Having shown that depletion of Orn leads to widespread nanoRNA-mediated priming, Goldman *et al.* next determined the effect that widespread nanoRNA-mediated priming had on global gene expression. Using DNA microarrays they found that the relative abundance of transcripts associated with ~20% of all genes differed by a factor of 2 of more when comparing Orn-depleted cells with non-depleted cells. Importantly, nearly all of the genes whose expression changed significantly upon depletion of Orn did not exhibit a significant change in expression when Orn was depleted in the presence of a heterologous nanoRNA-degrading enzyme. Thus, the authors concluded that the vast majority of the changes in gene expression that occur upon depletion of Orn are a direct consequence of the accumulation of 2- to ~4-nt nanoRNAs. The expression analysis therefore revealed a correlation between widespread nanoRNA-mediated priming of transcription initiation and widespread alterations in gene expression; these findings raise the intriguing possibility that the balance between *de novo* initiation and nanoRNA-primed initiation may be an important determinant of gene expression.

Below we consider several potential mechanisms by which altering the extent of nanoRNA-primed transcription at a particular promoter could alter gene expression (Figure 1).

NanoRNA-mediated priming as a means of altering transcription efficiency

As mentioned above, ~35 years ago the proposal that small RNA oligonucleotides might "activate" transcription from specific target promoters was made. In support of this proposal biochemical evidence indicates that the affinity of nanoRNAs for the RNAP-promoter open complex can be substantially higher than that of the initiating mononucleotide. In particular,

the affinity of a 2-nt nanoRNA complementary to positions +1 and +2 for an RNAP-promoter open complex can be ~10-fold higher than the affinity of a mononucleotide complementary to position +1; furthermore, the affinity of a 3-nt nanoRNA complementary to positions +1, +2 and +3 for a RNAP-promoter open complex can be ~100-fold higher than the affinity of a mononucleotide complementary to position +1 [23, 25]. Correspondingly, the half-maximal effective concentration for initiating transcription (K_m) of a 2-nt nanoRNA complementary to positions +1 and +2, or a 3-nt nanoRNA complementary to positions +1, +2, and +3, can be ~10- to 100-fold lower than the K_m of a mononucleotide complementary to position +1. A regulatory mechanism that involves nanoRNAs acting to stimulate transcription initiation may be targeted to promoters that require high concentrations of the initiating NTP (and/or the second NTP added to the transcript) for efficient *de novo* initiation.

We note that, in contrast to nanoRNAs that carry a 3′ hydroxyl group, those that carry either a 3′ deoxy or 3′ phosphate group will not be able to function as a primers for transcription initiation. Such "non-extendable" nanoRNA species could, in principle, function as promoter-specific repressors. In this regard, it has been shown that 3′ deoxy small RNA oligonucleotides complementary to the region around the transcription start point can be potent promoter-specific inhibitors of transcription in vitro [26, 27]. In this case, inhibition of transcription occurs due to the hybridization of the non-extendable oligonucleotide with the exposed single-stranded DNA that is generated during formation of the RNAP-promoter open complex, which traps the transcription complex in an inactive state.

NanoRNA-mediated priming as a means of altering the sequence of the 5′ end of a transcript

As illustrated in the study by Goldman *et al.*, initiation with a nanoRNA primer provides a means by which the sequence of the 5′ end of the transcript can be altered. Specifically, Goldman *et al.* found that increasing the extent of nanoRNA-mediated priming leads to the production of transcripts that are 1, 2, or 3 nucleotides longer than their *de novo* initiated counterparts. In principle, the nanoRNA-mediated addition of 1, 2, or 3 nucleotides onto the 5′ end of a transcript could have a significant impact on gene expression by a variety of mechanisms. For example, the addition of nucleotides onto the 5′ end of a transcript could profoundly influence the secondary structure of the resultant RNA transcript and thereby alter transcript stability. In this regard, unpaired 5′ ends can strongly destabilize transcripts in *E. coli* by increasing the likelihood that the transcript will be cleaved by RNase E, the primary endonuclease in this organism [28, 29]. The nanoRNA-mediated addition of nucleotides onto the 5′ end of a transcript could also influence events that occur during transcription elongation. Indeed, it is well established that changes in the sequence of the 5′ end of a transcript can inhibit the efficiency of an intrinsic terminator located several hundred base pairs downstream [30, 31]. In these cases it is likely that the 5′ end of a transcript, due to its inherent flexibility, is able to hybridize with other regions of the RNA, which, in turn, prevents formation of the terminator hairpin.

Precedent for the model that the nanoRNA-mediated addition of 1, 2, or 3 nucleotides onto the 5′ end of a transcript could influence gene expression also comes from studies of NTP-dependent transcription start-point switching (reviewed in [24]). In particular, the two examples of NTP-dependent transcription start-point switching provided below reveal how the addition of 1 or 2 nt onto the 5′ end of a transcript can significantly influence gene expression by altering either translation efficiency or the extent to which productive transcription occurs.

First, we consider the case of the *pyrC* promoters of *E. coli* and *Salmonella enterica*, which control expression of a gene involved in pyrimidine biosynthesis. Production of the *pyrC*

gene product is determined by the intracellular concentration of CTP, which dictates whether transcription from the *pyrC* promoter will initiate at one of two template positions that are separated by 2 base pairs (bp) [32, 33]. When CTP concentrations are high transcription initiates from a C located 7 bp downstream of the promoter 10 element (C7), whereas when CTP concentrations are low transcription initiates from a G located 9 bp downstream of the promoter −10 element (G9). Primary transcripts initiating from C7 are not efficiently translated due to formation of an RNA secondary structure that inhibits ribosome binding. In contrast, transcripts initiated from position G9 are efficiently translated because these transcripts cannot adopt a secondary structure that inhibits ribosome binding. Thus, in the case of the *pyrC* promoter, the addition of 2 nt to the 5′ end of the RNA transcript dramatically alters protein synthesis.

Second, we consider the case of the *codB* promoter of *E. coli*, which controls the expression of genes involved in cytosine uptake and utilization. Gene expression from the *codB* promoter is determined by the intracellular concentration of UTP, which determines whether transcription will initiate at one of two template positions that are separated by 1 bp [34]. In particular, when the concentration of UTP is sufficiently high, transcription initiates from an A located 8 bp downstream of the promoter 10 element (A8); this A is followed by a stretch of 6 Ts. (Note that in this case UTP is the second nucleotide added to the nascent RNA transcript and is not the initiating nucleotide.) Initiation at A8 leads to non-productive transcription through a mechanism of UTP-dependent reiterative transcription. Specifically, transcription of the 6 T:A base pairs in the initial transcribed region of *codB* creates a weak RNA-DNA hybrid that is prone to 'slippage'. As a result of transcript slippage at this poly T tract, UTP is repetitively added to the 3′ end of the nascent RNA transcript, ultimately resulting in the release of the transcript. In contrast, when the UTP concentration is relatively low, transcription initiates from a G located 1 bp upstream of A8 and 7 bp downstream of the promoter 10 element (G7). When transcription initiates at G7, UTP-dependent reiterative transcription does not occur and the result is production of full-length transcripts. Initiation at G7 is thought to prevent reiterative transcription due to the addition of a stabilizing G:C base pair in the RNA-DNA hybrid, which anchors the transcript and prevents slippage upon the encounter of the poly-T stretch. Thus, in the case of the *codB* promoter, the addition of a single nucleotide to the 5′ end of the RNA transcript enables productive transcription to occur.

NanoRNA-mediated priming as a means of altering the phosphorylation state of the transcript 5′ end

Goldman *et al.* observed pronounced alterations in the distribution of transcript 5′ ends when all transcripts were analyzed regardless of the phosphorylation status of the 5′ end and not when only transcripts carrying a 5′ triphosphate group were analyzed. These results indicate that the majority of nanoRNAs that prime transcription upon depletion of Orn carry either a 5′ monophosphate or a 5′ hydroxyl group. Furthermore, these results suggest that widespread nanoRNA-mediated priming of transcription results in the production of a significant fraction of primary transcripts that do not carry a 5′ triphosphate.

The phosphorylation state of the 5′ end of primary transcripts is a key determinant of transcript stability [for review see [35]]. In particular, the rate-limiting step in the degradation of many mRNA transcripts in *E. coli* is the conversion of the 5′ end of the transcript from a triphosphate to a monophosphate, which stimulates the activity of RNase E [29, 36]. In *E. coli*, conversion of the 5′ end of the transcript from a triphosphate to a monophosphate can occur through the action of the RNA pyrophosphohydrolase RppH [29]. Thus, in principle, incorporation of a 5′ monophosphate-carrying nanoRNA could provide a means of bypassing the requirement for RppH and accelerating RNA degradation. In contrast, as RNase E does not efficiently cleave RNA transcripts carrying a 5′ hydroxyl end [36–38],

incorporation of a 5′ hydroxyl-carrying nanoRNA could provide a means of stabilizing the resultant RNA transcript.

We note that in vitro analysis indicates that incorporation of NMPs or nucleosides into the 5′ end of the nascent transcript is strongly disfavored. Thus the K_m of an NTP that is complementary to position +1 is, in general, an order of magnitude lower than the K_m for the corresponding NMP or nucleoside [39]. In contrast, nanoRNAs (of identical sequence) that carry a 5′ triphosphate, a 5′ monophosphate or a 5′ hydroxyl group are equally efficient as primers for transcription initiation [23]. Thus, whereas *de novo* initiation events are biased against the incorporation of a 5′ monophosphate or a 5′ hydroxyl group into the resultant RNA transcript, nanoRNA-mediated priming events would not exhibit such a bias. Accordingly, nanoRNA-mediated priming provides a mechanism by which the phosphorylation state of the transcript 5′ end could be readily altered. Furthermore, an initiation event that utilizes a 2-nt nanoRNA complementary to positions +1 and +2, or a 3-nt RNA oligo complementary to positions +1, +2, and +3 could enable the phosphorylation state of the 5′ end of a transcript to change without any alteration in the sequence of the 5′ end.

Potential sources of nanoRNAs

In principle, there are several cellular processes that could generate nanoRNA species carrying a 3′ hydroxyl group that would be suitable for use as a primer.

Intermediates of RNA degradation

As mentioned above, it has previously been proposed that small RNA fragments derived from the processing of longer transcripts could serve as sequence-specific primers that stimulate transcription initiation from specific target promoters. In this regard, nanoRNAs are, in fact, likely generated in vivo as intermediates during mRNA degradation. In particular, the canonical pathway for the degradation of mRNA transcripts in *E. coli* begins with the endonucleolytic cleavage of a full-length transcript by RNase E, which cleaves the full-length transcript several times in single-stranded regions to generate a number of smaller fragments [reviewed in 35, 40–42]. The resulting fragments are subsequently degraded by the processive 3′ to 5′ exonucleases, polynucleotide phosphorylase (PNPase), RNase II and RNase R. In vitro analyses of these exonucleases reveal that they do not efficiently process RNA species less than ~5-nt in length. Specifically, RNase R produces 2-nt and 3-nt terminal fragments in vitro [43, 44], RNase II produces 3- to 5-nt terminal fragments [45, 46], while PNPase produces 2- to ~5-nt terminal fragments [47]. Thus, degradation of a single full-length transcript in vivo likely results in the production of several 2- to ~5-nt nanoRNAs as the terminal products of digestion by PNPase, RNaseII or RNase R. Furthermore, as the processing of the fragments produced initially by RNase E cleavage occurs in a 3′ to 5′ manner, nanoRNAs that result from RNA degradation are likely derived from the 5′ ends of the initial fragments derived from RNase E cleavage. Thus, the positions where RNase E cleaves a particular mRNA transcript would be predicted to specify the 5′end of a specific set of nanoRNAs.

The 3′ fragment resulting from cleavage with RNase E contains a 5′ monophosphate, and so any subsequent nanoRNA derived as a terminal degradation product of this fragment will also contain a 5′ monophosphate. Thus, full-length transcripts initiated with such nanoRNAs would themselves be expected to be good substrates for RNase E, as RNase E exhibits a strong preference for transcripts carrying a 5′ monophosphate [28]. Although RNase E is the principal endonuclease in *E. coli* responsible for the majority of RNA turnover, other endonucleases are present in the cell and some of these generate a 5′ hydroxyl group upon cleavage [reviewed in 48]. Initiation with any subsequently derived 5′ hydroxyl carrying

nanoRNA would result in the production of a transcript that would likely be resistant to subsequent attack by RNase E.

Products of abortive transcription initiation

NanoRNAs are likely generated in vivo during the process of "abortive initiation". In particular, during transcription initiation, the RNAP-promoter initial transcribing complex can engage in tens or hundreds of cycles of synthesis and release of short RNA transcripts between 2 nt and ~15 nt in length [reviewed in [49, 50]]. This process is called abortive initiation and the short transcripts that are released during abortive initiation are termed abortive RNA transcripts. The production of abortive transcripts is well established in reactions performed in vitro. Furthermore, it has recently been established that abortive transcripts are also produced in vivo [51].

Abortive transcripts can be produced in vast stoichiometric excess over full-length transcripts. Thus, a single RNAP-promoter complex could, in principle, produce large quantities of particular nanoRNA species. Short abortive transcripts (i.e. those between 2-nt and ~4-nt in length) would be suitable primers in the absence of any exonucleolytic processing, whereas longer abortive transcripts (i.e. those between ~5-nt and 15-nt) would require processing before becoming a size suitable for use as primers.

Products of RNA cleavage during transcription elongation

During transcription elongation RNAP can "backtrack". Backtracking is a process of reverse translocation during which the RNAP catalytic center slides back relative to the 3′ end of the nascent transcript [reviewed in [52, 53]]. Backtracking can occur due to (i) the presence of a weak RNA-DNA hybrid, (ii) an encounter between RNAP and a protein roadblock, or (iii) after the misincorporation of a nucleotide into the nascent transcript. For elongation to resume the backtracked complex must either reposition the 3′ end of the nascent transcript in the catalytic center by reversing the backward movement of the enzyme or generate a new RNA 3′ end in the active center via endonucleolytic cleavage. Endonucleolytic cleavage of the nascent transcript, which can be catalyzed by the RNAP active center alone or stimulated by the Gre proteins, will liberate a small 5′ monophosphate containing RNA transcript from the elongation complex. For example, in the case of misincorporation of a nucleotide, backtracking by one base pair precedes cleavage of the penultimate phosphodiester bond, liberating a dinucleotide [54]. Therefore endonucleolytic cleavage of nascent transcripts associated with backtracked elongation complexes, in principle, may provide another potential source of nanoRNAs in vivo [55].

Products of cyclic dinucleotide degradation

Cyclic di-guanosine monophosphate (c-di-GMP) is a second messenger that has attracted considerable attention of late because of its ability to control both biofilm formation and motility in many different bacteria [56–58]. C-di-GMP can influence many different cellular processes either directly or indirectly through binding specific receptor proteins or RNAs [58]. For example, the nucleotide can influence gene expression by binding directly to several different transcription regulators [59, 60]. Synthesized by proteins containing a so-called GGDEF domain, c-di-GMP is degraded by EAL domain-containing phosphodiesterases to the dinucleotide pGpG [61], which could function as a nanoRNA to prime transcription initiation. A second class of phosphodiesterases containing a so-called HD-GYP domain can degrade c-di-GMP to pGpG as well, but these enzymes can also degrade pGpG further to two molecules of GMP [62]. Most bacteria contain genes encoding GGDEF and EAL domain-containing proteins but the number of genes specifying each of these family members can vary considerably between organisms. Because certain GGDEF and EAL domain-containing proteins are predicted to contain signal input domains, it is possible that the synthesis and

breakdown of c-di-GMP by these particular proteins only occurs in response to specific environmental signals. However, there are known instances in which phenotypic and genotypic variants arise within a bacterial population that exhibit increased intracellular c-di-GMP concentrations [63–66]; it is conceivable that in these instances the pGpG dinucleotide breakdown product of c-di-GMP is also more abundant and consequently that priming of transcription by pGpG is particularly pronounced. Although cyclic di-adenosine monophosphate (c-di-AMP) is produced by some bacteria [67], it remains to be learned whether this cyclic dinucleotide is broken down to pApA in the cell and could therefore serve as a source of nanoRNAs.

NanoRNA-degrading enzymes as key regulators of nanoRNA-mediated priming

Goldman *et al.* found that depletion of Orn in *P. aeruginosa* led to widespread use of nanoRNAs to prime transcription initiation. Thus, this study indicates that one major role of Orn, and presumably other nanoRNA-degrading enzymes, is to suppress nanoRNA-mediated priming of transcription. Furthermore, the studies suggest that nanoRNA degrading enzymes play a key role in regulating gene expression by influencing the extent to which transcription initiation occurs using NTPs only or a nanoRNA primer.

Different bacteria appear to have evolved distinct strategies to degrade nanoRNAs [for review see [42]]. For example, *E. coli* appears to carry only a single nanoRNA-degrading enzyme [15], whereas *Bacillus subtilis* carries several proteins (all of which are unrelated to Orn) that can degrade nanoRNAs [17]. The presence of one (or more) enzymes dedicated to the degradation of nanoRNAs, in principle, provides a facile way of altering the degradation of nanoRNAs without affecting other cellular processes.

It remains to be learned why some bacteria carry one nanoRNA-degrading enzyme whereas other bacteria carry multiple enzymes. One potential explanation may be that nanoRNA-degrading enzymes have differing substrate specificities. Thus, in the case of cells carrying multiple nanoRNA-degrading enzymes the bacteria may be able to alter the composition of the cellular nanoRNA pool by down- or up-regulating the activity of one of these enzymes while the activity of the other(s) remains unchanged. Although a systematic analysis of the substrate specificities of the currently identified nanoRNA-degrading enzymes has yet to be reported, some information regarding the substrate specificity of *E. coli* Orn is available. Interestingly, when tested against a series of dinucleotides, *E. coli* Orn was least active on a GpG dinucleotide [68], which as mentioned above, may accumulate in cells under conditions where c-di-GMP levels are elevated.

Loss of Orn function in *E. coli* leads to a loss of viability [15]. This loss of viability can be rescued by the introduction of a heterologous nanoRNA-degrading enzyme into cells [16, 17]. These findings suggest that the accumulation of nanoRNAs is toxic in *E. coli*. The basis for the toxic effects of nanoRNA accumulation in *E. coli* remains to be determined. However, one possibility, as proposed by Goldman *et al.*, is that the toxic effects of nanoRNAs are a consequence of altered gene expression due to nanoRNA-mediated priming.

In contrast to the situation in *E. coli*, Orn is apparently not essential in either *P. aeruginosa* [18] or *Streptomyces* [69] (although loss of Orn in *Streptomyces* does lead to a growth defect). One possible explanation for the fact that *P. aeruginosa* and *Streptomyces* remain viable in the absence of Orn function is that these organisms may carry some as of yet unidentified nanoRNA-degrading enzyme that allows for survival in the absence of Orn. However, this explanation seems unlikely given the demonstration that nanoRNAs

accumulate in *P. aeruginosa* upon depletion of Orn. An alternative, and perhaps more likely explanation, is that the accumulation of nanoRNAs is not lethal in these organisms.

Summary/future prospects

Goldman *et al.* demonstrate that artificial depletion of Orn allows widespread nanoRNA-mediated priming of transcription coupled with global alterations in gene expression to occur in bacterial cells. These findings raise the possibility that nanoRNAs represent a new class of regulatory small RNAs that can influence gene expression through a novel mechanism—by being incorporated directly into a target transcript as opposed to hybridizing to it. Nevertheless, the extent to which nanoRNA-mediated priming occurs in wild-type cells of *P. aeruginosa*, or in cells of any other bacterium, is still an unknown. Furthermore, if nanoRNA-mediated priming does occur in wild-type cells, it need not necessarily play a specific regulatory function; rather, it may influence the expression of certain genes in a stimulus-independent manner, thus helping to shape the transcriptome. However, because the production or activity of nanoRNA-degrading enzymes such as Orn could in principle be modulated, and because the potential sources of nanoRNAs could differ depending on the particular growth conditions, it is conceivable that nanoRNA-mediated priming of transcription could be more prevalent under certain growth conditions than others, or more prevalent in some bacteria, than in others.

The study by Goldman *et al.* demonstrates that at least one diagnostic feature of nanoRNA-mediated priming is an extension at the 5′ end of a transcript, or, put another way, a change in the transcription start point to a more upstream position. In principle, the high-throughput sequencing approaches outlined in the study by Goldman *et al.*, which relied upon the analysis of the 5′ ends of all transcripts regardless of their phosphorylation status, could be used to investigate the extent of nanoRNA-mediated priming in wild-type cells under different growth conditions. Importantly, the study by Goldman *et al.* also suggests that any approach for the identification of transcription start points that relies upon the analyses of only those transcripts carrying a 5′ triphosphate [see, for example, [70, 71]] might miss a significant portion of transcripts whose synthesis is primed with a nanoRNA.

In conclusion, the study by Goldman *et al.* provides considerable impetus for addressing the extent to which nanoRNA-mediated priming occurs in bacteria and, if so, whether nanoRNA-mediated priming plays a specific regulatory role or serves simply to maintain the expression state of a given set of genes under all conditions.

Acknowledgments

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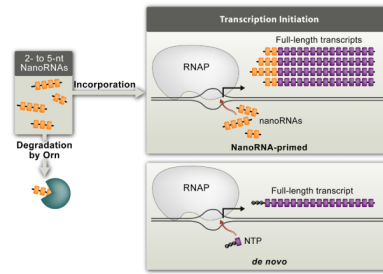
Figure 1.
NanoRNA-mediated priming of transcription initiation alters gene expression. NanoRNAs (2- to 5-nt RNAs) can either be degraded by Oligoribonuclease (Orn) or used by RNAP as primers to initiate transcription. Compared with initiation with NTPs only (i.e. *de novo*), initiation with nanoRNAs 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.