AN ANALYSIS OF CIS-ENCODED AND TRANS-ENCODED sRNA METABOLISM IN *ESCHERICHIA COLI* K-12

by

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(Under the Direction of Sidney R. Kushner)

ABSTRACT

In order to survive, bacteria must be able to adapt and persist in a variety of poor growth conditions. To do this, cells undergo physiological and morphological changes that are influenced by changes in gene expression. Although proteins dominate the regulatory landscape, the identification and functional characterization of small RNAs (sRNAs) in diverse bacterial lineages has shown that sRNAs are widely used as potent effectors of post-transcriptional regulation that mediate and integrate numerous physiological responses. Typically, sRNAs affect, either positively or negatively, the translation and/or stability of their target mRNA(s). It is generally believed that most sRNAs function stoichiometrically, i.e. one sRNA molecule regulates one target mRNA molecule because sRNA:target degradation is coupled. Therefore, sRNA decay is, in some respects, a consequence of function.

Although this intimate connection between metabolism and function exists, sRNA metabolism has not been comprehensively or deeply investigated. To further understand sRNA metabolism *in vivo*, this study examined the metabolism of two trans-encoded sRNAs, ArcZ and RprA, and two cis-encoded sRNAs, GadY and SibC. Generally, sRNAs are classified based on the genomic position of the sRNA gene relative to the target gene as this has structural and

mechanistic implications. Cis-encoded sRNA genes overlap, are antisense to their target gene(s) and have the potential to form long, perfect duplex structures with their target(s). In contrast, trans-encoded sRNAs genes do not overlap their target gene(s) and typically form short, imperfect duplexes with their targets. To assess global ribonuclease involvement in sRNA metabolism, we examined an isogenic set of mutants containing loss-of-function alleles of all the known major cytoplasmic endoribonucleases and exoribonucleases of *E. coli* K-12. The results of this study revealed a global involvement of both RNase E and polynucleotide phosphorylase (PNPase) in the metabolism of all four sRNAs. Surprisingly, RNase P appeared to participate in the metabolism of RprA, GadY and SibC, whereas RNase G, RNase III and RNase II were necessary for the metabolism of GadY and SibC. When taken together, our results suggested the existence of multiple, complex metabolic pathways that may involve hierarchical cleavage events, as well as, target-dependent and target-independent metabolic pathways.

INDEX WORDS: sRNA, cis-encoded, trans-encoded, *Escherichia coli*, sRNA metabolism, sRNA processing, sRNA decay, RNase E, RNase G, RNase LS, RNase P, RNase Z, RNase III, PNPase, RNase R, RNase II, Hfq, RppH

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BS, University of Georgia, 2002

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

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December 2010

DEDICATION

To RNA. May you always keep us guessing.

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CHAPTER 1

Introduction and Literature Review

Introduction

To survive living organisms must be able to transition between states (e.g. adaptation to the environment) and transform (e.g. development). Alterations like these are affected by a variety of regulatory processes at the molecular level. In a sense, these mechanisms are independent modules that the cell can employ in enumerative combinations to achieve fine-tuned and precise effects. Proteins often mediate these regulatory processes. They are highly effective, but are, like all things, constrained by their own kinetics. Thus, if proteins solely controlled the regulatory network, there would be vast fissures within the regulatory landscape. RNA helps to fill in these gaps.

Typically, trans-acting regulatory RNAs function catalytically (e.g. eukaryotic miRNAs) or stoichiometrically (e.g. eubacterial sRNAs) to affect the translation and/or stability of a target mRNA (Waters and Storz 2009; Fabian *et al*. 2010). Using a combination of mathematical modeling and *in vivo* experimentation, Levine *et al.* (2007) found that sRNAs exhibit novel characteristics as compared to proteins. Small RNAs have a threshold-linear regulatory effect, meaning that when the amount of the sRNA is less than the target, the regulatory effect is silenced. However, when the sRNA concentration exceeds that of the target, its effect is manifested. Thus, sRNAs can efficiently filter-out random or transient noise by suppressing inappropriate bursts of gene expression and can smooth the transition between states. Additionally, since many sRNAs regulate multiple targets, the amount and binding affinity of

one target will indirectly affect the regulation of another target by titrating the sRNA concentration. This mode of action creates hierarchical cross talk among targets. For these reasons, sRNAs fill a unique niche, in which they act to support and integrate cellular regulatory networks.

Many of the eubacterial sRNAs function analogously to eukaryotic siRNAs/miRNAs, but unlike their eukaryotic counterparts, they are almost characteristically devoid of sequence, length, structural or mechanistic homology (Rivas and Eddy 2000; Rivas *et al.* 2001; Hershberg *et al.* 2003; Waters and Storz 2009; Fabian *et al.* 2010). This makes them one of the more enigmatic categories of RNAs. By far, the sRNAs of the γ-proteobacterium, *Escherichia coli*, are the most extensively studied. This chapter will primarily focus on these 80 annotated sRNAs. There are numerous, excellent reviews that explore in detail various aspects of sRNA biology including: sRNA identification (Vogel and Sharma 2005; Altuvia 2007; Kulkarni and Kulkarni 2007; Livny *et al.* 2008; Pichon and Felden 2008); sRNA function (Gottesman 2004; Storz *et al.* 2005; Aiba 2007; Babitzke and Romeo 2007; Brantl 2007; Gerdes and Wagner 2007; Frohlich and Vogel 2009; Waters and Storz 2009); target identification (Vogel and Wagner 2007; Pichon and Felden 2008); and, the physiological roles and consequences of sRNA action (Masse *et al.* 2003b; Repoila *et al.* 2003; Gottesman 2004; Majdalani *et al.* 2005; Guillier *et al.* 2006; Vogel and Papenfort 2006; Masse *et al.* 2007; Papenfort and Vogel 2009). In an attempt to impart a novel perspective to sRNA biology, this review will describe the life history of sRNAs, from biogenesis to decay. Using sRNA metabolism as the backdrop, it will describe how sRNAs are processed and how these events influence and are influenced by their function.

Biogenesis of sRNAs

Consistent with their role as regulators, many, but not all, of the *E. coli* sRNAs accumulate during specific growth conditions (Argaman *et al.* 2001; Wassarman *et al.* 2001; Tjaden *et al.* 2002; Vogel *et al.* 2003; Kawano *et al*. 2005). They can be induced in stationary phase [ArcZ and RprA (Argaman *et al.* 2001; Wassarman *et al.* 2001)], by oxidative stress [OxyS (Altuvia *et al.* 1997)], low iron concentrations [RyhB (Masse and Gottesman 2002)], outer membrane perturbations [MicA (Johansen *et al.* 2006; Udekwu and Wagner 2007) and RybB (Johansen *et al.* 2006; Thompson *et al.* 2007)], accumulation of glucose-6-phosphate [SgrS (Vanderpool and Gottesman 2004)], low temperature [DsrA (Sledjeski *et al.* 1996)] and anaerobic conditions [FnrS (Boysen *et al.* 2010; Durand and Storz 2010)] (Waters and Storz 2009).

Often, the observed accumulation patterns correlate with changes in the rate of sRNA synthesis, not stability (Vogel *et al.* 2003; Andrade and Arraiano 2008, Papenfort *et al*. 2009), and are affected by a variety of transcription factors. These factors can either enhance or repress sRNA expression. Furthermore, the inhibition of a transcription factor can significantly increase or decrease the abundance of a sRNA *in vivo* (Johansen *et al.* 2008; Reichenbach *et al.* 2009; Boysen *et al.* 2010). In some cases, a sRNA functions as part of the transcription factor's regulon to mediate a particular physiological response (Masse and Gottesman 2002; Vanderpool and Gottesman 2004; Guillier and Gottesman 2006; Shimoni *et al.* 2007; Waters and Storz 2009). Some sRNAs are controlled by a single transcription factor, while others are subject to more complex regulation, e.g. *micF* is controlled by eight factors, four activators and four repressors (Coyer *et al.* 1990; Huang *et al.* 1990; Li and Demple 1994; Ferrario *et al.* 1995; Painbeni *et al.* 1997; Deighan *et al.* 2000; Gillette *et al.* 2000; Kwon *et al.* 2000; Delihas and Forst 2001). Thus, one sRNA can help to integrate multiple stress responses. In some cases, regulation can be more

intricate with sRNAs and transcription factors forming feedback loops (Shimoni *et al.* 2007), e.g. the transcription factor Fur represses RyhB and RyhB inhibits Fur (both Fur and RyhB participate in iron homeostasis) (Masse *et al.* 2007; Vecerek *et al.* 2007).

sRNA transcriptional elements (promoter and terminators)

A survey of the literature suggests many of the sRNA genes are or are predicted to be independently transcribed from a σ^{70} promoter and contain a Rho-independent transcription terminator (Argaman *et al.* 2001; Wassarman *et al.* 2001; Chen and Blyn 2002; Vogel *et al.* 2004; Mandin and Gottesman 2009; Moon and Gottesman 2009; Papenfort *et al.* 2009; Boysen *et al.* 2010). However, this genetic arrangement may reflect a bias imposed by the procedures used to identify many sRNAs. For example, one of the more powerful filters used in the first genome-wide screens for novel sRNA genes was to restrict analysis to intergenic regions and scan for the presence of a σ^{70} promoter and Rho-independent transcription terminator within close proximity to one another (Argaman *et al.* 2001; Wassarman *et al.* 2001; Chen and Blyn 2002). However, some sRNAs employ stress promoters (σ^{24}, σ^{38} or σ^{54}) (Vogel *et al.* 2003; Opdyke *et al.* 2004; Johansen *et al.* 2006; Thompson *et al.* 2007), while a few sRNAs have dual promoters, both a σ^{70} and stress promoter (De Lay and Gottesman 2009; Reichenbach *et al.* 2009). These promoters can overlap such that transcription initiates at the same location, e.g. the σ^{70} and σ^{54} promoters of *glmY* (Reichenbach *et al.* 2009), or can be physically separated, leading to a short form and long form of the sRNA, e.g. the σ^{70} and $\sigma^{70/38}$ promoters of *ssrS* (6S RNA) (Kim and Lee 2004). Dual promoters, which undergo preferential switching, can allow for expression during multiple physiological states.

Although many of the sRNAs have Rho-independent transcription terminators (Argaman *et al.* 2001; Wassarman *et al.* 2001; Chen and Blyn 2002; Vogel *et al.* 2004; Urban *et al.* 2007;

De Lay and Gottesman 2009; Moon and Gottesman 2009; Papenfort *et al.* 2009; Rasmussen *et al.* 2009; Boysen *et al.* 2010), there are currently no reports of a sRNA having a Rho-dependent transcription terminator. Yet, some of the characterized sRNAs lack conical Rho-independent transcription terminators, e.g. the Sib RNAs (SibA-E) (Rudd 1999; Fozo *et al*. 2008). In these cases, the RNA contains a strong stem-loop structure that could cause termination *in vivo* (S. Kushner and B. Mohanty, personal communication). As is the case with multiple promoters, there are instances of sRNA genes containing two, active Rho-independent transcription terminators, e.g. *gcvB* (Urbanowski *et al.* 2000) and *dicF* (Faubladier *et al.* 1990).

sRNA processing

Once synthesized, most non-coding RNAs undergo some kind of post-transcriptional modification and/or processing event(s), e.g. rRNAs, tRNAs, siRNAs/miRNAs (Venema and Tollervey 1995; Carthew and Sontheimer 2009; Deutscher and Ciaran 2009; Heinemann *et al.* 2010; Karijolich *et al.* 2010). These processes serve to generate mature, functional RNAs from precursor transcripts. However, many of the sRNAs bypass this step and are transcribed as the functional form (Masse and Gottesman 2002; Guillier and Gottesman 2006; Papenfort *et al.* 2008). Currently, there are only a few documented cases of sRNA processing.

Of the 80 annotated sRNAs, only three are thought to be processed: 6S RNA (Li *et al.* 1998; Kim and Lee 2004), DicF (Faubladier *et al.* 1990) and ArcZ (Papenfort *et al.* 2009). The σ^{70}-short precursor and the $\sigma^{70\text{-}38}$-long precursor of 6S RNA have both 3' trailer and 5' leader sequences that are removed by ribonucleases. Both 6S precursors have approximately eight additional nucleotides at their 3' ends, which are removed by the 3'→5' exoribonucleases, RNase PH and RNase T, both *in vivo* and *in vitro* (Li *et al.* 1998). It is possible that two additional exoribonucleases, RNase D and RNase II, are involved as well. The short precursor

contains an additional nine nucleotides at the 5' end. These residues are removed endonucleolytically by RNase E, or its homolog RNase G (Kim and Lee 2004). RNase E also removes the 224 nucleotide leader of the long precursor through multiple cleavage events. Regardless of the pathway, the identical functional form of 6S RNA is generated from both the short and long precursors.

Like 6S RNA, there are two DicF precursors. Both contain a 200 nucleotide leader sequence that is cleaved first by RNase III, an endoribonuclease that cuts double-stranded RNAs, and subsequently by RNase E to generate the mature 5' end (Faubladier *et al.* 1990). The DicF gene contains a Rho-independent transcription terminator. Normally, transcription stops there, but occasionally there is read-thru transcription to a more distal but undetermined site. RNase III initially processes the 3' trailer of the read-thru transcript, and then 3'→5' exoribonucleases presumably chew back to the base of the Rho-independent transcription terminator.

ArcZ was identified in one of the first genome-wide screens for novel sRNAs (Argaman *et al.* 2001; Wassarman *et al.* 2001). On Northern blots of wild type cells, there are two discrete, stable forms of ArcZ. Papenfort *et al.* (2009) claimed that the shorter RNA was generated by RNase E-mediated cleavage of the longer form, and that the shorter species was the functional moiety. Consistent with this hypothesis, Soper *et al.* (2010) found that only the short form can bind its target *in vitro*.

Although there are currently only a few documented cases of sRNA processing, this phenomenon may actually be more prevalent. Two of the genome-wide screens for novel sRNAs identified candidate sRNA genes that were encoded either within an operon or the untranslated region (5' UTR or 3' UTR) of an adjacent gene (Vogel *et al.* 2003; Kawano *et al.* 2005). Those genomic contexts suggest these sRNAs could be processed from di- or polycistronic transcripts

or arise by transcriptional attenuation (Henkin and Yanofsky 2002; Vogel *et al.* 2003). Since, none of these RNAs have been functionally characterized, it remains unclear whether they are functional, trans-acting molecules, cis-acting RNAs or stable breakdown products.

However, like ArcZ, there are at least 29 other sRNAs that exist as two or more discrete species in wild type cells (DsrA, GadY, GcvB, GlmY, GlmZ, IsrA, IstR, OmrA, OmrB, PsrN, PsrO, RdlA-D, RprA, RybA, RybB, RyeA, RyeB, RyfB, RyfC, SibA-E, SroC and SroH) (Argaman *et al.* 2001; Rivas *et al.* 2001; Wassarman *et al.* 2001; Chen and Blyn 2002; Kawano *et al.* 2002; Vogel *et al.* 2003; Opdyke *et al.* 2004; Vogel *et al.* 2004; Kawano M 2005; Fozo *et al*. 2008). Multiple stable forms can arise from alternative transcription, i.e. transcription from physically separated promoters [IstR-1 and IstR-2 (Vogel *et al.* 2004)] or terminators [GcvB (Urbanowski *et al.* 2000)]. Although multiple forms of a particular sRNA can be generated by transcriptional events, it is more likely that some arise from ribonuclease mediated cleavage events like those associated with ArcZ. For example, in the case of the three GadY RNAs, three 5' ends and one 3' end were identified by primer extension and RACE analysis (Opdyke *et al.* 2004). Mutation of the putative promoter of the longest form resulted in the loss of all three RNAs. This result suggested the two shorter RNAs were generated from cleavage of the longest form. For the three sRNAs, RyeA, RyeB and PsrN, in the absence of RNase III, longer species accumulated and/or shorter forms disappeared on Northern blots (Argaman *et al.* 2001; Vogel *et al.* 2003).

Based on results observed with tRNAs and rRNAs, it is tempting to describe these types of events as processing. However, in the field of non-coding RNA metabolism, the term processing carries a functional connotation. Normally, precursors are highly unstable and only the processed, mature forms are stable and functional, e.g. rRNAs and tRNAs. However, this

does not hold true for all sRNAs. ArcZ appears to be a classical example of RNA processing, although the precursor is relatively stable and can be detected by Northern blotting (Argaman *et al.* 2001; Papenfort *et al.* 2009; Mandin and Gottesman 2010). In the case of GlmZ, it is the longer form (and not the shorter form) that is capable of target regulation (Reichenbach *et al.* 2008; Urban and Vogel 2008). Similar to GlmZ, the region of RprA that is complementary to its target is located within the 5' end of the RNA (Majdalani *et al.* 2001; Majdalani *et al.* 2002). This region is missing in the shorter RNA, suggesting that it is not functional (Argaman *et al.* 2001; Wassarman *et al.* 2001). Therefore, without accompanying functional analysis, it is unclear whether ribonuclease mediated cleavage represents processing or decay.

Evidence of ribonuclease mediated cleavage is not restricted to the presence of multiple species in wild type cells (Argaman *et al.* 2001; Wassarman *et al.* 2001). Although, no group has specifically focused on this aspect of sRNA biology, experiments done to identify or characterize sRNAs have produced data that are consistent with ribonuclease activity. For example, the presence of a 5' monophosphate on stable RNAs, microarray data showing an increase in sequences flanking the annotated gene, RT-PCR generated sequences containing chromosomally-encoded nucleotides that are extraneous to the annotated sRNA gene, the presence of poly(A) tails within those sequences, additional promoter and/or terminator elements, close proximity to an adjacent gene, and *in vitro* transcription experiments demonstrating that the longer but not shorter forms are produced (Argaman *et al.* 2001; Rivas *et al.* 2001; Wassarman *et al.* 2001; Chen and Blyn 2002; Kawano *et al.* 2002; Tjaden *et al.* 2002; Vogel *et al.* 2003; Kawano *et al*. 2005; Sittka *et al.* 2008; Sittka *et al.* 2009). All these results suggest the action of ribonucleases on various sRNAs, and in total, these data suggest over 50%

of the sRNAs are subjected to some type of ribonucleolytic processing that precedes decay to mononucleotides.

Function of sRNAs

By definition, all of the *E. coli* regulatory sRNAs act *in trans*. This excludes cis-acting RNAs like riboswitches and RNA thermometers (Narberhaus *et al.* 2006; Henkin 2008; Dambach and Winkler 2009). In fact, the function(s) or putative function(s) of approximately half of the 80 sRNAs are known. Most of them act via complementary base-pairing to a target mRNA to affect the translation and/or stability of the target mRNA (Waters and Storz 2009). However, four of the sRNAs antagonize specific proteins through direct binding: 6S RNA, CsrB, CsrC and GlmY.

sRNAs that modulate protein activity through structural mimicry

CsrB, CsrC, 6S RNA and GlmY sRNAs are thought to mimic the structure of a protein's normal nucleic acid substrate (Weilbacher *et al*. 2003; Wassarman 2007; Reichenbach *et al.* 2008; Urban and Vogel 2008). When the sRNA binds to the protein, it effectively titrates the protein away from its usual target, which results in an observed repression of the protein's activity. 6S RNA resembles a open promoter (Wassarman 2007). When 6S RNA accumulates, like in stationary phase, it binds to and sequesters σ^{70}-bound RNA polymerase but not σ^{38}-bound RNA polymerase. This causes a down-regulation of some σ^{70} promoters and an increase in transcription from some σ^{38} promoters (Trotochaud and Wassarman 2004).

The CsrA protein affects the translation and/or stability of target mRNAs by binding to their 5' UTRs (Liu *et al.* 1997; Liu and Romeo 1997; Baker *et al.* 2002). The sRNAs, CsrB and CsrC, each contain multiple CsrA binding motifs (Liu *et al.* 1997; Weilbacher *et al*. 2003), such that when their levels rise, they sequester CsrA, thereby inhibiting CsrA activity (Weilbacher *et*

al. 2003). Similarly, GlmY is thought to function by titrating YhbJ away from its normal substrate, the sRNA GlmZ. In response to decreasing glucosamine-6-phosphate levels in the cell, GlmZ stimulates *glmS* translation (Reichenbach *et al.* 2008; Urban and Vogel 2008). GlmZ is inhibited by YhbJ, which destabilizes GlmZ by an unknown mechanism. GlmY is a sequence and putative structural homolog of GlmZ but lacks a *glmS* binding motif. However, GlmY also causes an increase in GlmS synthesis but does so indirectly. It is postulated that when GlmY binds YhbJ, GlmZ decay is inhibited. Consequently, the GlmZ concentration increases and this then leads to an increase in GlmS synthesis.

Although only four of the 80 annotated sRNAs act directly on protein targets, this paucity may not accurately reflect the prevalence of this type of sRNA mediated regulation. As opposed to sRNAs that regulate at the mRNA level, there is no robust, high-throughput method to identify sRNAs that modulate protein activity (Waters and Storz 2009). Typically, these RNAs have been discovered in the course of a more detailed investigation of a particular pathway or protein (Liu *et al.* 1997; Reichenbach *et al.* 2008; Urban and Vogel 2008). Therefore, it is possible that when better methods are available, more sRNAs of this functional category will be identified.

sRNAs that act through complementary base-pairing to a target mRNA(s)

Unlike those sRNAs that target proteins, most sRNAs physically associate with target mRNAs thorough complementary base-pairing (Waters and Storz 2009). Binding of the two molecules affects, either positively or negatively, the translation and/or stability of the mRNA. This has been experimentally demonstrated numerous times both *in vitro* and *in vivo* (Vogel and Wagner 2007). Possibly the most elegant proof of this mechanism has been provided by compensatory mutagenesis experiments *in vivo* (Lease *et al.* 1998; Vogel and Wagner 2007; Guillier and Gottesman 2008; Papenfort *et al.* 2008; Reichenbach *et al.* 2008; Urban and Vogel 2008; De Lay

and Gottesman 2009; Papenfort *et al.* 2009; Rasmussen *et al.* 2009). When mutations that are predicted to disrupt base-pairing are introduced into either the sRNA or target mRNA, the regulatory effect is abolished, but the effect is restored by compensatory mutations that re-establish base-pairing.

The duplex formed between the sRNA and target mRNA can either be long and perfect or short and discontinuous (Waters and Storz 2009). The difference tends to correlate well with the genomic location of the sRNA gene relative to that of the target gene. Cis-encoded sRNAs overlap and are antisense to their targets, whereas trans-encoded sRNAs are not (Figure 1.1). Since cis-encoded sRNAs are complementary to their targets, these duplexes have the potential to be perfect and span throughout the region of overlap (Figure 1.1.A). However, a complete duplex may not form *in vitro* or *in vivo* (Brantl 2007; Han *et al.* 2010).

The trans-encoded sRNAs do not overlap their target genes, and typically, only short non-contiguous regions of the sRNA are complementary to the targets (Figure 1.1.B) (Waters and Storz 2009). For example, ArcZ binds the *tpx* mRNA through two short separate regions—five nucleotides and six nucleotides separated by seven residues (Figure 1.1.C) (Papenfort *et al.* 2009). For SgrS, there are 23 nucleotides in a 31 nucleotide region that are complementary to *ptsG* mRNA (Vanderpool and Gottesman 2004). However, only six of these nucleotides are essential for *ptsG* regulation *in vivo* (Kawamoto *et al.* 2006). These two examples highlight the fact that binding of the sRNA to the target does not have to result in the formation of a conserved, consensus structure. This inherent flexibility of the structural outcome may be the underlying reason why many of the trans-encoded sRNAs regulate multiple target mRNAs (Papenfort and Vogel 2009). Often, the targets will fall within the same pathway, e.g. RyhB represses several iron-binding proteins in response to low intracellular iron concentrations

(Masse and Gottesman 2002) and RybB downregulates numerous porins and channel proteins when the envelope is perturbed or misfolded porins accumulate (Johansen *et al.* 2006; Thompson *et al.* 2007). Thus, one sRNA can affect gene expression on a physiologically global scale.

Mechanism of complementary base-pairing and the role of Hfq

Cis-encoded sRNAs and their targets have the potential for extensive base-pairing and they associate rapidly *in vitro* (Brantl 2007; Heidrich *et al.* 2007; Han *et al.* 2010), whereas trans-encoded sRNAs and their targets tend to bind more slowly (Zhang *et al.* 2002; Rasmussen *et al.* 2005; Kawamoto *et al.* 2006; Arluison *et al.* 2007; Soper and Woodson 2008; Updegrove *et al.* 2008; Rasmussen *et al.* 2009; Soper *et al.* 2010). For trans-encoded sRNAs, complementarity is more limited, and sometimes these areas are sequestered in predicted double-stranded regions, which must unwind to allow for sRNA:target pairing, e.g. OxyS and *rpoS* mRNA (Zhang *et al.* 2002). If unassisted, these interactions would be too slow to account for the rapidity of trans-encoded sRNA-mediated regulation (Zhang *et al.* 2002; Levine *et al.* 2007; Rasmussen *et al.* 2009).

To solve this problem, thirty of the trans-encoded sRNAs form complexes with Hfq, a ubiquitous RNA binding protein (Zhang A 2003; Mandin and Gottesman 2010). In fact, Hfq is often essential for the function of these sRNAs. Hfq is a homolog of eukaryotic Sm and Sm-like proteins and acts as a chaperone to facilitate base-pairing between sRNAs and their targets (Moller *et al.* 2002a; Moll *et al.* 2003b; Geissmann and Touati 2004). *In vitro*, Hfq induces secondary, and likely, tertiary structural rearrangements in the sRNA and/or mRNA target (Moll *et al.* 2003b), thereby significantly enhancing the rate of duplex formation (Zhang *et al.* 2002; Rasmussen *et al.* 2005; Kawamoto *et al.* 2006; Soper and Woodson 2008; Updegrove *et al.* 2008; Rasmussen *et al.* 2009). Using real-time FRET analysis, Arluison *et al.* (2007) described

Hfq-mediated duplex formation between DsrA and *rpoS* mRNA. The data suggested Hfq bound quickly to DsrA and *rpoS*, then promoted the slow melting of an inhibitory element in the 5' UTR of *rpoS*, which facilitated rapid association between DsrA and *rpoS*. The resulting ternary complex can be stable, but Hfq may not be required for maintenance of the duplex and probably dissociates from it (Zhang *et al.* 2002; Afonyushkin *et al.* 2005; Arluison *et al.* 2007; Soper and Woodson 2008; Updegrove *et al.* 2008; Soper *et al.* 2010).

Consequences of base-pairing: repression and activation of transcription initiation

Once bound, most of the sRNAs negatively affect translation initiation of their target mRNA by binding within the 5' UTR or 5' coding region of the mRNA. In fact, many sRNAs bind directly to the translation initiation region (TIR: the area spanning the Shine-Delgarno sequence and start codon) (Gottesman 2004; Storz *et al.* 2005). Compensatory mutagenesis and translational fusions have shown that sRNAs inhibit translation through RNA:RNA interactions, and *in vitro* toeprinting assays have also demonstrated that sRNA binding to the TIR inhibits 30S ribosome loading (Altuvia S 1998; Argaman and Altuvia 2000; Chen *et al.* 2004; Udekwu *et al.* 2005; Darfeuille *et al.* 2007; Vogel and Wagner 2007; Guillier and Gottesman 2008; Papenfort *et al.* 2008; De Lay and Gottesman 2009; Mandin and Gottesman 2009; Rasmussen *et al.* 2009). Recent studies indicate that sRNAs can bind outside of the TIR and still negatively affect translation. This window of inhibition can extend from the fifth codon to several hundred nucleotides upstream of the TIR.

For example, RybB binds to the area between the second and seventh codons of *ompN* and inhibits 30S ribosome binding *in vitro* (Bouvier *et al.* 2008). By adjusting the space between the RybB binding site and start codon, Bouvier *et al.* (2008) found that binding down to the fifth codon effectively inhibited translation. The repression window can also extend far upstream of

the TIR. In this case, GcvB binds the *gltI* mRNA at positions -57 to -45 relative to the start codon in a C/A rich region, which may serve as a translation enhancer element (Sharma *et al.* 2007). IstR-1 binds ~100 nucleotides upstream of the *tisB* TIR (Darfeuille *et al.* 2007). A combination of *in vitro* toeprinting, footprinting and translation assays indicated that there is a "standby" ribosome binding site that is necessary for *tisB* translation, and IstR-1 directly competes with ribosomes by binding to the standby site. OmrA and OmrB downregulate CsgD both *in vivo* and *in vitro* by binding to the 5' UTR of *csgD* mRNA (Holmqvist *et al.* 2010). However, both sRNAs bind so far upstream of the TIR that this inhibition of translation cannot be explained by any of the known mechanisms of sRNA repression, including competition with "standby" ribosomes.

Interestingly, there are five sRNAs (ArcZ, DsrA, RprA, RybB and GlmZ) that stimulate translation of their target mRNAs (Sledjeski *et al.* 1996; Lease *et al.* 1998; Majdalani *et al.* 1998; Majdalani *et al.* 2001; Wassarman *et al.* 2001; Majdalani *et al.* 2002; Reichenbach *et al.* 2008; Urban and Vogel 2008; Mandin and Gottesman 2010; McCullen *et al.* 2010; Soper *et al.* 2010). In these cases, the 5' UTR of the target naturally forms an inhibitory structure that occludes the ribosome binding site (Repoila *et al.* 2003; Reichenbach *et al.* 2008; Urban and Vogel 2008; Mandin and Gottesman 2010). Binding of the sRNA causes structural rearrangements of the 5' UTR, opening the TIR, thus allowing ribosome access (Vecerek *et al.* 2010). For ArcZ, DsrA and RybB, these sRNAs regulate multiple targets either positively or negatively (Sledjeski *et al.* 1996; Lease *et al.* 1998; Majdalani *et al.* 1998; Wassarman *et al.* 2001; Johansen *et al.* 2006; Thompson *et al.* 2007; Papenfort *et al.* 2009; Mandin and Gottesman 2010). These results indicate that how the sRNA functions as a regulator is not due to something inherent to the

sRNA, but rather is the consequence of where the sRNA binds on the target mRNA and the structural rearrangements that occur upon binding.

Consequences of base-pairing: destabilization and stabilization of the target mRNA(s)

Often, but not always, deletion and overexpression of a sRNA results in a coincident change in both the target mRNA and protein levels (Sledjeski *et al.* 1996; Lease *et al.* 1998; Lease and Belfort 2000; Kawamoto *et al.* 2006; Vogel and Wagner 2007; Reichenbach *et al.* 2008; Urban and Vogel 2008; Rasmussen *et al.* 2009). Although, many sRNAs affect translation initiation, steady-state and chemical half-life analysis has shown that sRNAs can also affect the abundance and stability of their target mRNAs (Masse *et al*. 2003a; Vanderpool and Gottesman 2004; Vogel *et al.* 2004; Afonyushkin *et al.* 2005; Antal *et al.* 2005; Morita *et al.* 2005; Rasmussen *et al.* 2005; Kawamoto *et al.* 2006; Morita *et al.* 2006; Guillier and Gottesman 2008; Morita *et al.* 2008; Desnoyers *et al.* 2009). It is commonly believed that inhibiting translation creates "ribosome-free" mRNAs that are more susceptible to ribonuclease attack, while stimulating translation would inhibit ribonuclease activity (Kushner 1996).

In vitro and *in vivo*, SgrS inhibits translation initiation of *ptsG* mRNA and destabilizes *ptsG* mRNA (Vanderpool and Gottesman 2004; Kawamoto *et al.* 2005; Morita *et al.* 2005; Kawamoto *et al.* 2006; Morita *et al.* 2006; Maki *et al.* 2008). Morita *et al.* (2006) found that when translation of *ptsG* was blocked by mutating the ribosome binding site, there was still SgrS-mediated destabilization of *ptsG*. These data indicated that sRNAs can affect the stability of the target independent of its translation initiation effects. This may help to explain the regulatory effect of GadY. GadY binds to the 3' UTR of *gadX* mRNA and stabilizes it (Opdyke *et al.* 2004). In this scenario, it is hard to imagine how binding to the 3' UTR would affect translation. Yet in eukaryotic cells, miRNAs affect the translation of their target mRNAs by

binding to the 3' UTR, even though that mechanism of action is not well understood (Fabian *et al*. 2010).

Of the negative regulators, some sRNAs mediate an RNase E dependent decay of their target mRNAs (Andersen *et al.* 1989; Kimata *et al.* 2001; Masse *et al.* 2003a; Moll *et al.* 2003a; Morita *et al.* 2003; Morita *et al.* 2004; Vanderpool and Gottesman 2004; Kawamoto *et al.* 2005; Morita *et al.* 2005; Morita *et al.* 2006; Aiba 2007; Guillier and Gottesman 2008; Morita *et al.* 2008; Desnoyers *et al.* 2009). In contrast, positive regulators like DsrA and RprA, which stimulate *rpoS* translation, protect *rpoS* mRNA from RNase E degradation *in vivo* (McCullen *et al.* 2010). There may be a physical basis to why several sRNAs act via a RNase E dependent pathway. Morita *et al.* (2005) found that Hfq and sRNAs copurify with RNase E. The copurification requires the C-terminus of RNase E, which is the degradosome scaffolding region (Carpousis 2007). However, other components of the degradosome (enolase, RhlB RNA helicase and PNPase) do not copurify with the sRNA-Hfq-RNase E complexes (Morita *et al*. 2005). These results, in combination with data from other studies, have led to a generalized model of sRNA mediated RNase E dependent degradation of target mRNAs (Masse *et al*. 2003a; Moll *et al.* 2003a; Morita *et al.* 2005; Aiba 2007). In this model, a sRNA associates with a Hfq-RNase E complex, which is recruited to the target mRNA via complementary base-pairing between the sRNA and target (Morita *et al.* 2005; Aiba 2007). Several groups have reported that Hfq-binding sites and RNase E cleavage sites overlap, because both recognize a similar structure, i.e. a single-stranded AU rich region flanked by a stem-loop structure (Brescia *et al.* 2003; Moll *et al.* 2003a; Rasmussen *et al.* 2005). Thus, Hfq binding to the mRNA can inhibit RNase E cleavage, but dissociation of Hfq would expose the cleavage site.

However, only 30 of the 80 sRNAs are known to interact with or require Hfq for their function (Zhang *et al*. 2003; Mandin and Gottesman 2010). Therefore, the Hfq-RNase E based model of target mRNA decay does not account for all instances of sRNA mediated target destabilization/stabilization (Morita *et al.* 2005; Aiba 2007). For these sRNAs, RNase E may also be involved, but in one instance, target degradation appears to be initiated by RNase III. IstR-1 has a 23 nucleotide region of perfect complementarity with its target *tisB* and is not known to require Hfq for its function (Vogel *et al.* 2004). Both IstR-1 and the target are cleaved by RNase III, and the cleavage sites map to the region of complementarity. This results suggests it is the sRNA:target duplex that is cleaved by RNase III.

Decay of sRNAs

Stoichiometric decay versus catalytic decay of sRNAs

Cleavage of the IstR-1:*tisB* duplex by RNase III is a good example of stoichiometric action—the sRNA and target function in a 1:1 manner whereby degradation of the sRNA is coupled to the degradation of the target (Vogel *et al.* 2004). The cis-encoded transposon and plasmid-borne sRNAs, RNA-OUT and CopA, are also known to function in this fashion (Simons and Kleckner 1983; Blomberg *et al.* 1990; Case *et al.* 1990; Ma and Simons 1990; Hjalt *et al.* 1995). Although many of the Hfq binding trans-encoded sRNAs typically lack extensive complementarity with their targets, they can also act stoichiometrically. Masse *et al.* (2003a) provided data that suggested decay of this type of sRNA is dependent upon the target and degradation of the two are coupled. When transcription was blocked by the addition of rifampicin to the medium, RyhB was very stable. However, when transcription was allowed to proceed in the absence of new RyhB synthesis, RyhB was markedly less stable. This same effect was observed for two other

sRNAs, OxyS and DsrA. The authors concluded that these sRNAs were rapidly degraded when target mRNAs were present, implying sRNA decay is dependent upon the target.

To determine if sRNA and target degradation are coupled, Masse *et al.* (2003a) identified the factors that initiated the decay of both RyhB and its target mRNA. As for many of the other trans-encoded sRNAs, Hfq was necessary for the function and stability of RyhB (Masse and Gottesman 2002; Masse *et al*. 2003a; Moll *et al.* 2003a; Morita *et al.* 2005). In the absence of Hfq, the half-life of RyhB was significantly reduced (Masse *et al*. 2003a). However, in a RNase E Hfq double mutant, the half-life was comparable to a wild type control. These results suggested Hfq stabilized RyhB by inhibiting RNase E-mediated decay. Consistent with this observation, RyhB cleavage by RNase E was inhibited by Hfq *in vitro* (Moll *et al.* 2003a). RyhB's target, *sodB* mRNA, was also degraded by RNase E in a RyhB dependent manner (Masse *et al*. 2003a; Moll *et al.* 2003a; Morita *et al.* 2005), and similar to its sRNA regulator, RNase E cleavage of *sodB* was inhibited by Hfq *in vitro* (Geissmann and Touati 2004). Furthermore, the RNase E cleavage site mapped to the Hfq binding site (Afonyushkin *et al.* 2005).

Masse *et al.* (2003a) concluded that since Hfq was necessary for RyhB function and inhibited RNase E cleavage of both RyhB and *sodB*, then decay of the sRNA and the target were likely coupled. Since, sRNA degradation was dependent upon the presence of the target and was coupled to the target, they argued that sRNAs, like RyhB, act stoichiometrically and not catalytically. The C-terminus of RNase E was necessary for the decay of both RyhB and *sodB in vivo* (Masse *et al*. 2003), and RyhB copurified with Hfq and RNase E in a C-terminus dependent manner (Morita *et al.* 2005). Taken together, these data suggest a RyhB:*sodB*-Hfq-RNase E complex exists within the cell and that dissociation of Hfq facilitates RNase E cleavage of both.

This putative complex could be viewed as the physical component underlying a stoichiometric mechanism of action of Hfq binding sRNAs.

Although it has been generally assumed that sRNAs act stoichiometrically, it is possible that some act catalytically. MicM is a trans-encoded sRNA that requires Hfq for its function to negatively regulate *ybfM* by affecting both mRNA and protein levels (Rasmussen *et al.* 2009). Using a two-plasmid inducible system, Overgaard *et al.* (2009) found that the half-life of MicM was unaffected by the presence or absence of its target, whereas *ybfM* was significantly less stable in the presence of MicM than without (4 minutes versus 27 minutes). When *ybfM* clearance was monitored by simultaneous inhibition of *micM* and induction of *ybfM*, molar estimates suggested one MicM molecule mediated the decay of several *ybfM* mRNAs. This result suggested that MicM was acting catalytically. In addition to a different mode of action, MicM displays properties unlike other sRNAs. RyhB, OxyS and DsrA exhibit different half-lives in the presence or absence rifampicin (Masse *et al*. 2003a), while the half-life of MicM determined by Overgaard *et al.* (2009) was similar to that of the rifampicin half-life (Vogel *et al.* 2003). In addition, in the absence of Hfq, MicM accumulated (Rasmussen *et al.* 2009).

Whether a sRNA acts catalytically or stoichiometrically may affect how the sRNA is degraded. For a sRNA that acts stoichiometrically, it is possible that ribonucleases are recognizing structures either within the sRNA:target duplex or are created within the sRNA upon binding. Whereas, catalytic sRNA decay may be dependent upon structures inherent to the sRNA.

Decay of target bound versus unbound sRNAs

MicA is a Hfq binding trans-encoded sRNA that negatively regulates OmpA synthesis in stationary phase by affecting both mRNA and protein levels (Rasmussen *et al.* 2005; Udekwu *et*

al. 2005; Urban and Vogel 2007). In exponentially growing cells, the half-life of *ompA* is 15 minutes, whereas in stationary phase cells it is reduced to four minutes, and the rapid destabilization of *ompA* is initiated by an RNase E cleavage within the Hfq-binding site in the 5' UTR (Melefors and von Gabain 1988; Nilsson *et al.* 1988; Lundberg *et al.* 1990; Mudd and Higgins 1993). In stationary phase, the 3'-5' exoribonuclease polynucleotide phosphorylase (PNPase) and full-length RNase E are necessary for MicA decay *in vivo* (Viegas *et al.* 2007; Andrade and Arraiano 2008). In fact, inhibition of RNase E caused a two-fold increase of the MicA half-life, while inhibiting PNPase caused a three-fold increase (Andrade and Arraiano 2008). The PNPase effect is unique to stationary phase (it is not necessary in exponential phase) when MicA regulates *ompA*. However, the PNPase activity is independent of the target mRNA, since the half-life of MicA in a PNPase OmpA double mutant was similar to that of the PNPase single mutant. Andrade and Arraiano (2008) observed a very slight stabilization of MicA in the absence of *ompA* and the authors concluded that the subtly of the effect was due to the fact that MicA has multiple targets, i.e. *phoPQ* and *ompX* mRNA (Johansen *et al.* 2008; Coornaert *et al.* 2010). In addition to being independent of *ompA*, PNPase mediated decay was separate from RNase E terminus degradation because the effect of combining the alleles was additive. These results suggested two independent pathways of MicA decay in stationary phase: one that is PNPase dependent/*ompA* independent and the other that is RNase E dependent. If MicA is like RyhB, then the RNase E mediated decay of MicA may be dependent upon *ompA* (Masse *et al*. 2003a).

The rapid destabilization of the *ompA* mRNA in stationary phase is consistent with efficient regulation by MicA. Levine *et al.* (2007) demonstrated that sRNAs exhibit a threshold-linear regulatory effect, such that sRNA mediated regulation is effective only when the sRNA

concentration exceeds that of its target. In this case, an excess of MicA would predicate efficient ompA regulation. Consistent with this prediction, MicA levels significantly increase in stationary phase cells because its transcription is controlled by σ^{24} (Argaman *et al.* 2001; Udekwu *et al.* 2005; Johansen *et al.* 2006; Udekwu and Wagner 2007; Viegas *et al.* 2007). Since PNPase decay of MicA is independent of the target, this observation may represent degradation of the unbound, excess MicA, whereas RNase E decay may represent coupled decay of the MicA:*ompA* duplex (Masse *et al*. 2003a).

Motivation for Dissertation

The above literature review has focused on the life history of sRNAs and described the biogenesis, function and decay of the annotated *E. coli* sRNAs. This perspective was chosen to provide the reader with a general understanding of sRNA biology by establishing a unifying base for all sRNAs. All sRNAs have a life history, but beyond this, they share few commonalities. They are characteristically devoid of sequence, base composition, length and structural homology (Rivas and Eddy 2000; Rivas *et al.* 2001; Hershberg *et al.* 2003; Waters and Storz 2009). They are expressed at different times and various growth conditions (Argaman *et al.* 2001; Wassarman *et al.* 2001) because they are regulated by different subsets of transcription factors and employ different sigma factors (Masse and Gottesman 2002; Vanderpool and Gottesman 2004; Johansen *et al.* 2006; Udekwu and Wagner 2007; Reichenbach *et al.* 2009; Waters and Storz 2009). Some sRNAs undergo post-transcriptional maturation events that precede function (Faubladier *et al.* 1990; Li *et al.* 1998; Kim and Lee 2004; Papenfort *et al.* 2009), but most are transcribed in their functional forms (Masse and Gottesman 2002; Guillier and Gottesman 2006; Papenfort *et al.* 2008). They all act *in trans* as post-transcriptional

regulators, but some act on specific proteins, whereas most affect the translation and/or stability of a target mRNA (Waters and Storz 2009). These effects on translation and stability can be separated, but in the context of the cell, they act in concert to reinforce one another (Morita *et al.* 2006; Aiba 2007).

Thus, it may be more appropriate to think of sRNA mechanisms of action as a spectrum, such that the primary mechanism in one instance may be an affect on translation that subsequently affects stability of the target mRNA versus a primary effect on stability that subsequently alters translation (Moller *et al.* 2002b; Opdyke *et al.* 2004). Furthermore, one sRNA can regulate multiple target mRNAs (Papenfort and Vogel 2009). Depending on the target, the regulatory effect can either be positive or negative. This observation demonstrates that the mode of action of the sRNA is not something inherent to the sRNA but is a consequence of how it interacts with its target. Additionally, many sRNAs require Hfq for their function, but not all do (Mandin and Gottesman 2010). Typically, trans-encoded sRNAs associate with Hfq, but there are examples of cis-encoded sRNAs that also form complexes with Hfq *in vivo* (Zhang *et al*. 2003; Opdyke *et al.* 2004).

Most sRNAs are thought to function stoichiometrically, i.e. the sRNA acts in a 1:1 manner with a target mRNA with degradation of the two being coupled (Masse *et al*. 2003; Levine *et al.* 2007). Thus, for many sRNAs, metabolism can be, in part, a consequence of its function. If one sRNA can differentially regulate multiple target mRNAs, then there is the potential for multiple metabolic pathways for a sRNA. For example, GadY has two targets, *gadX* and the dicistronic transcript *gadXW* (Opdyke *et al*. 2004; Tramonti *et al*. 2008). GadY is thought to stabilize *gadX* (Opdyke *et al.* 2004), whereas it induces processing of *gadXW* (Tramonti *et al.* 2008). Therefore, it is possible that the metabolism of GadY when it is bound to

gadX is different from its metabolism when bound to *gadXW*. Likewise, in the case of DsrA, different regions of DsrA are predicted to interact with its different mRNA targets (Lease and Belfort 2000). Lease and Belfort (2000) demonstrated that the conformation of DsrA when it was bound to *rpoS* was different from the structure that it formed when it was bound to *hns*. Therefore, if the structure of DsrA changes, it is possible that when bound to *rpoS*, DsrA is cleaved by one ribonuclease, whereas DsrA could be cleaved by a different ribonuclease when bound to *hns*.

Although an intimate association between sRNA function and metabolism exists, sRNA metabolism has not been extensively or comprehensively investigated. What is known for a limited number of sRNAs has been used to put forth two basic models of sRNA metabolism. The first model hypothesizes that sRNAs that require Hfq for their function will be cleaved by RNase E, since sRNAs, Hfq and RNase E apparently form a complex *in vivo* (Masse *et al*. 2003; Moll *et al* 2003a; Morita *et al.* 2005; Aiba 2007). The second model predicts that sRNAs that share extensive complementarity with their targets and have the potential to form long duplexes with their targets will be cleaved by RNase III (Gerdes *et al*. 1992; Franch *et al.* 1999; Vogel *et al.* 2004). However, sRNA metabolism is probably more complex than either of these two models predict.

Traditionally, *in vivo* analysis of eubacterial RNA metabolism has involved the comparison of wild type RNA to that of RNA harvested from a mutant defective in one or more ribonucleases (Kushner 1996; Condon 2007; Mohanty *et al.* 2008). A ribonuclease is implicated in the metabolism of a particular RNA if inactivation of the gene results in the accumulation of precursor forms, accumulation or loss of breakdown products, increases in steady-state levels or the chemical half-life of the RNA (Mohanty *et al.* 2008). As robust as this method has been for

investigating the metabolism of other noncoding RNAs and mRNAs, recent data suggest this approach, as currently being applied, may not be ideal or suitable for sRNA analysis. For example, detection of precursor forms of many of the annotated *E. coli* sRNAs is not possible because the primary transcript is the mature, functional form (Masse and Gottesman 2002; Guillier and Gottesman 2006; Papenfort *et al.* 2008).

Although all RNA species are degraded, few studies have used loss or accumulation of breakdown products as a means to study sRNA metabolism, primarily because, decay products may be very unstable and evade detection (Vogel *et al.* 2004; Afonyushkin *et al.* 2005; Condon 2007). Rather, most studies have relied on changes in steady-state levels or chemical half-life determinations (Masse *et al*. 2003a; Afonyushkin *et al.* 2005; Morita *et al.* 2006; Viegas *et al.* 2007; Andrade and Arraiano 2008; Overgaard *et al.* 2009). In the absence of an activity that is necessary for the metabolism of a RNA, the RNA becomes more stable and can accumulate. Thus, an increase in the steady-state level of a RNA in a ribonuclease deficient mutant can be diagnostic of a defect in metabolism. However, this assumption only holds true if the transcription rate of the RNA is equivalent between the mutant and wild type strains.

However, transcription of many of the studied sRNAs is regulated by one or more transcription factor(s). Deletion of a transcription factor can cause a significant change in the steady-state levels of a sRNA *in vivo* (Johansen *et al.* 2008; Reichenbach *et al.* 2009; Boysen *et al.* 2010). Therefore, if the mutation under investigation affects the metabolism of a sRNA transcription factor, which results in a change in protein concentration, then sRNA expression may be induced or repressed in the mutant as compared to the wild type control. For example, this wrinkle in steady-state analysis is the basis of conflicting reports on the metabolism of RyhB *in vivo* (Masse *et al*. 2003a; Afonyushkin *et al.* 2005). Masse *et al.* (2003a) showed that in the

absence of RNase E activity there was an increase in RyhB steady-state levels relative to a wild type control and concluded that RNase E was necessary for the decay of RyhB. In contrast, Afonyushkin *et al.* (2005) saw a similar increase in RyhB steady-state levels but found that the rifampicin half-life of RyhB was not affected by loss of RNase E activity. From these results, the authors concluded that RNase E was not necessary for RyhB metabolism. They argued that the observed increased in RyhB levels stemmed from changes in the transcription rate.

One possible explanation for the differing observations is that, rifampicin treatment in addition to blocking transcription initiation may affect sRNA function, which in turn affects sRNA metabolism. Masse *et al.* (2003a) found that sRNAs, like RyhB, were very stable when cells were treated with rifampicin to inhibit global transcription initiation, but when transcription was allowed to proceed and sRNA transcription was specifically inhibited, the sRNA was markedly less stable. The authors concluded that sRNAs were destabilized by their target mRNAs, and the elevated half-life in the presence of rifampicin was caused by a rapid depletion of the target pool. This result could mean that ribonuclease mediated aspects of sRNA metabolism that are dependent upon the target may be largely or partially obscured by addition of rifampicin to the medium, i.e. if the target and ribonuclease are part of the same sRNA metabolic pathway, then the half-life of the sRNA in the ribonuclease deficient mutant should not significantly different from the half-life of the sRNA in wild type cells. This idea could explain why Afonyushkin *et al.* (2005) did not observe an increase in the rifampicin half-life of RyhB in the absence of RNase E activity. However, both groups agreed that RyhB was acting stoichiometrically and that RyhB's target, *sodB*, was degraded by RNase E. However, Masse *et al.* (2003a) argued that RNase E cleaved both RyhB and *sodB*, whereas Afonyushkin *et al.* (2005) concluded that RNase III cleaved RyhB and RNase E cleaved *sodB*.

Furthermore, Andrade and Arraiano (2008) found that the rifampicin half-life of MicA was not affected by the absence of its target and postulated that this result was because MicA had multiple targets (Johansen *et al.* 2008; Coornaert *et al.* 2010). Yet, the model put forth by Masse *et al.* (2003a) would predict that the rifampicin half-life of MicA should be essentially the same in a wild type control and a strain where the target had been deleted, because rifampicin treatment causes the rapid depletion of the target pool. Andrade and Arraiano (2008) observed an increase in the half-life of MicA in a PNPase deficient mutant that was also independent of the target. These results raise the possibility that in addition to target-dependent sRNA metabolic events, there are also target-independent metabolic pathways. Furthermore, not all sRNAs act stoichiometrically. For sRNAs that function catalytically, like MicM, the decay of the sRNA is by definition not coupled to that of the target (Overgaard *et al.* 2009).

Given the potential complexity of sRNA metabolism and the possible caveats associated with of steady-state and rifampicin half-life determinations, we decided to take advantage of a feature displayed by over one-third of the annotated *E. coli* sRNAs, namely the presence of two or more stable species of the particular sRNA on Northern blots of wild type RNA (Argaman *et al.* 2001; Rivas *et al.* 2001; Wassarman *et al.* 2001; Chen and Blyn 2002; Vogel *et al.* 2003; Opdyke *et al.* 2004; Vogel *et al.* 2004; Kawano M 2005; Fozo *et al*. 2008). In some cases, these multiple forms arise through alternative transcription initiation or termination events (Faubladier *et al.* 1990; Urbanowski *et al.* 2000; Kim and Lee 2004; Vogel *et al*. 2004), but for most other sRNAs the smaller species are likely derived through ribonuclease mediated cleavage of longer form(s) (Argaman *et al.* 2001; Vogel *et al.* 2003; Opdyke *et al.* 2004; Fozo *et al.* 2008; Papenfort *et al.* 2009). Regardless of the functional outcome of these cleavage events, i.e. maturation or decay, from a RNA metabolism perspective, these shorter forms of sRNAs can be viewed as

trackable markers that are no different from the precursor forms or breakdown products used by others to successfully identify factors necessary for tRNA, rRNA and mRNA metabolism *in vivo* (Kushner 1996; Li and Deutscher 1996; Mohanty *et al.* 2008; Deutscher and Ciaran 2009). However, unlike other precursors and breakdown products, the smaller species are stable enough to be visible in wild type cells.

Therefore, this study was designed to identify genes involved in sRNA metabolism by assaying for the loss of shorter species in ribonuclease deficient mutants. Previous reports have limited their analysis to one or a few ribonucleases (Masse *et al*. 2003a; Afonyushkin *et al.* 2005; Viegas *et al.* 2007; Andrade and Arraiano 2008). Yet, it appears as though sRNA metabolism can involve more than RNase E and RNase III, since Andrade and Arraiano (2008) found that both RNase E and PNPase were necessary for MicA decay *in vivo*. Therefore, to assess global ribonuclease involvement in sRNA metabolism, we examined loss-of-function alleles of all the known major cytoplasmic endoribonucleases and exoribonucleases of *E. coli* K-12 including: RNase E (*rne-1*), RNase G (*Δrng*), RNase LS (*ΔrnlA2*), RNase Z (*Δrnz*), RNase P (*rnpA49*), RNase III (*rnc-14*), PNPase (*pnp-7*), RNase II (*Δrnb*) and RNase R (*Δrnr*). To provide the most valid basis of comparison between the different mutants, all strains were in an isogenic genetic background (MG1693), and RNA was harvested from physiologically equivalent cell populations.

After initial screening of 16 sRNAs, we chose to study the metabolism of four specific sRNAs: ArcZ, RprA, GadY and SibC. Chapter 2 focuses on ArcZ and RprA, which are trans-encoded sRNAs, and Chapter 3 focuses on GadY and SibC, which are cis-encoded sRNAs. Collectively, these sRNAs were selected to provide a more comprehensive understanding of the

different types of metabolic pathways that sRNAs are subject to *in vivo*. Specifically, these sRNAs were picked for the following reasons:

1. In addition to displaying multiple forms in wild type cells, published data suggested the shorter forms were generated through ribonuclease mediated cleavage events (Argaman *et al.* 2001; Opdyke *et al.* 2004; Fozo *et al.* 2008; Papenfort *et al.* 2009).
2. All four sRNAs accumulate in early stationary phase in wild type cells (Argaman *et al.* 2001; Wassarman *et al.* 2001; Opdyke *et al.* 2004; Fozo *et al*. 2008; Papenfort *et al.* 2009; Mandin and Gottesman 2010). It is known that the concentration of a ribonuclease varies with respect to growth conditions, e.g. RNase R levels increase in stationary phase, in minimal medium and under cold-shock (Chen and Deutscher 2005; Chen and Deutscher 2010). Thus, we chose to focus on a single physiological state (early stationary phase) to minimize the variation in results that could arise from different growth conditions. By using the same steady-state RNA for the analysis of all four sRNAs, it was possible to determine if an observed defect was specific to a particular sRNA or was a generalized defect caused by the mutation under investigation.
3. All four sRNAs bind Hfq *in vivo* (Majdalani *et al.* 2001; Wassarman *et al.* 2001; Zhang *et al*. 2003; Opdyke *et al.* 2004).
4. ArcZ and RprA are trans-encoded sRNAs (Majdalani *et al.* 2001; Majdalani *et al.* 2002; Papenfort *et al.* 2009; Mandin and Gottesman 2010), whereas GadY and SibC are cis-encoded sRNAs (Opdyke *et al.* 2004; Fozo *et al*. 2008; Tramonti *et al.* 2008; Han *et al.* 2010). Both ArcZ and RprA share limited, imperfect complementarity with their targets, while GadY and SibC are perfectly complementary to their targets. Therefore, the structures formed between the sRNAs and their targets are fundamentally different. ArcZ

and RprA form imperfect, short duplexes with their targets (Majdalani *et al.* 2001; Majdalani *et al.* 2002; Papenfort *et al.* 2009; Mandin and Gottesman 2010), while GadY and SibC have the potential to form long, perfect duplexes with their targets.

5. These four sRNAs are known to function differently. ArcZ both positively and negatively affects the translation and stability of multiple target mRNAs (Papenfort *et al.* 2009; Mandin and Gottesman 2010). RprA has a single known target, *rpoS*, and positively affects both *rpoS* translation and stability (Majdalani *et al.* 2001; Majdalani *et al.* 2002; McCullen *et al.* 2010). In contrast, GadY stabilizes *gadX* and promotes processing of *gadXW* at two positions (Opdyke *et al.* 2004; Tramonti *et al.* 2008). SibC has a single confirmed target, *ibsC*, and is thought to induce the rapid destabilization of this mRNA (Fozo *et al*. 2008). If the metabolism of the sRNA is dependent, in part, on its function, then because these four sRNAs functionally differently, they may be subject to different metabolic pathways.
6. With respect to function, the metabolic pathway(s) that generate the shorter, stable forms represent either processing or decay. In the literature, it is presumed that the longest forms of RprA, GadY and SibC are the functional RNAs, and the shorter forms represent decay products (Majdalani *et al.* 2001; Majdalani *et al.* 2002; Opdyke *et al.* 2004; Fozo *et al*. 2008; Updegrove *et al.* 2008; Soper *et al.* 2010). Conversely, the shorter form of ArcZ is the functional form (Papenfort *et al.* 2009; Soper *et al.* 2010). Thus, the generation of this species occurs through processing of the longer form. Collectively, analysis of these sRNAs should afford the opportunity to investigate both sRNA processing pathways and sRNA decay pathways.

7. The generation of shorter forms occurs through either 5'→3' cleavage events or 3'→5' cleavage events. For ArcZ, RprA and GadY, the stable species vary with respect to their 5' ends, whereas the SibC RNAs differ with respect to their 3' ends (Argaman *et al.* 2001; Majdalani *et al.* 2001; Wassarman *et al.* 2001; Majdalani *et al.* 2002; Fozo *et al.* 2008; Papenfort *et al.* 2009; Mandin and Gottesman 2010). Since, there are no known 5'→3' exoribonucleases in *E. coli* (Li and Deutscher 2004), it is probable that the metabolism of ArcZ, RprA and GadY RNAs are mediated by endoribonucleases. Yet, endoribonucleases and/or exoribonucleases could be mediating the metabolism of the SibC RNAs.

In summary, ArcZ, RprA, GadY and SibC represent a diverse subset of sRNAs with respect to structure and function. Thus, analysis of their metabolism should provide a basic understanding of the different types of metabolic pathways that sRNAs are subjected to *in vivo*.

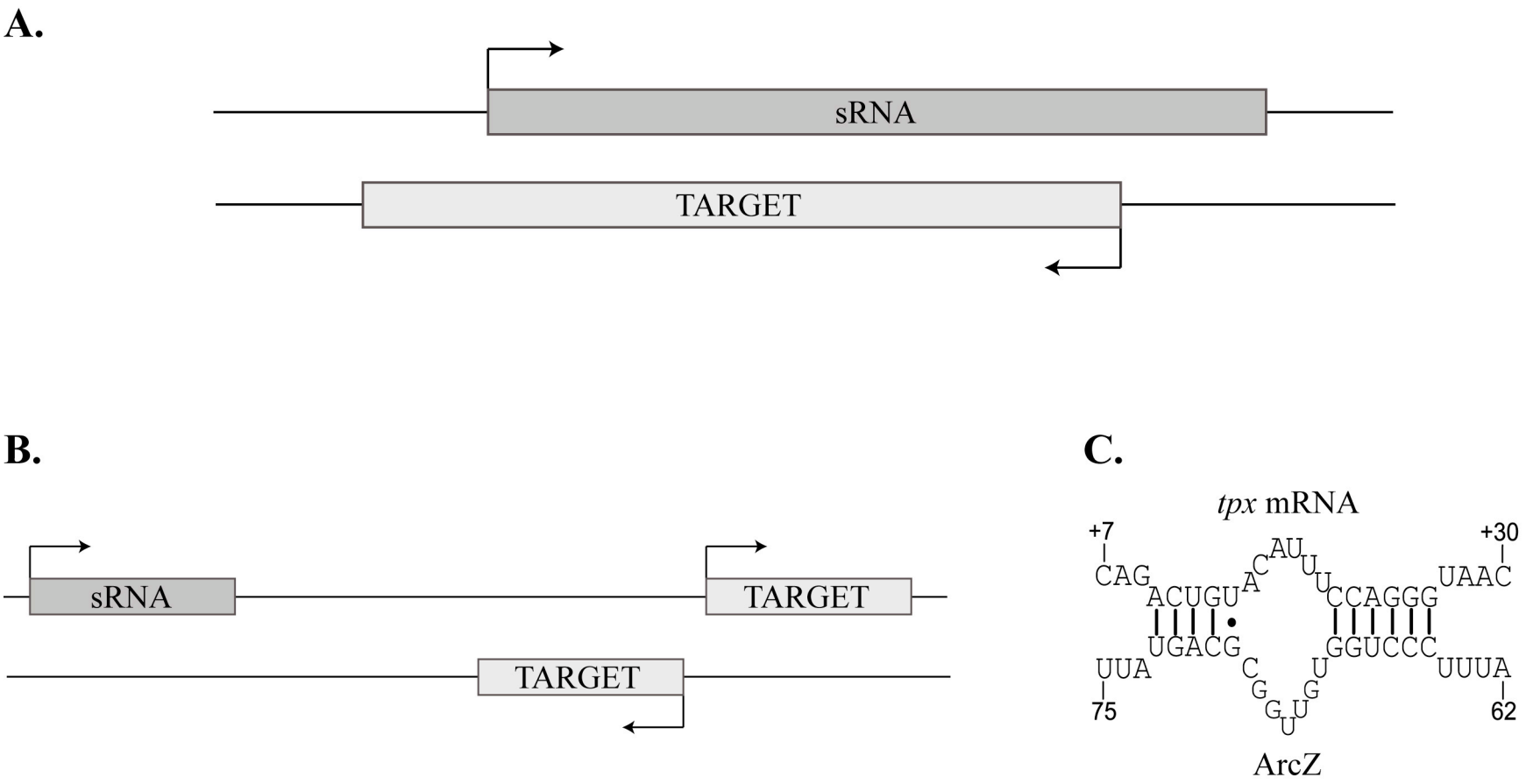

Figure 1.1. Diagrams representing the genomic context of cis-encoded and trans-encoded sRNAs relative to their target genes. (A.) Diagram of a cis-encoded sRNA gene relative to its target gene. (B.) Diagram of a trans-encoded sRNA gene relative to its target gene(s). (C.) The predicted interaction between ArcZ, a trans-encoded sRNA, and its target mRNA, *tpx* (modified from Papenfort *et al*. 2009).

CHAPTER 2

The Metabolism of Trans-encoded sRNAs is Primarily Dependent upon RNase E[1]

[1] Marshburn, S. and Kushner, S.R. To be submitted to *Nucleic Acids Research*.

Introduction

In order to survive, bacteria must be able to adapt and persist in a variety of poor growth conditions. When confronted with stress, cells undergo physiological and morphological changes that are influenced by changes in gene expression (Hengge-Aronis 1996; Huisman and Yanofsky 1996). Although proteins dominate the regulatory landscape, the identification and functional characterization of sRNAs in diverse bacterial lineages has shown that sRNAs are widely used as potent affecters of post-transcriptional regulation that mediate and integrate numerous physiological responses (Masse *et al.* 2003b; Livny *et al.* 2008; Papenfort and Vogel 2009; Waters and Storz 2009). Typically, sRNAs affect, either positively or negatively, the translation and/or stability of target mRNA(s) through complementary base-pairing (Waters and Storz 2009). Many of these sRNAs bind within the 5' untranslated region (UTR) or 5' coding region of the mRNA (Gottesman 2004; Storz *et al.* 2005; Darfeuille *et al.* 2007; Sharma *et al.* 2007; Bouvier *et al.* 2008). Binding can either inhibit 30S ribosome loading or induce structural rearrangements that relieve an inhibitory structure that normally occludes the ribosome binding site (RBS) (Waters and Storz 2009).

Although sRNAs affect target protein levels by regulating translation initiation, Northern blots often show a corresponding change in the steady-state level of the target mRNA as well (Sledjeski *et al.* 1996; Lease *et al.* 1998; Lease and Belfort 2000; Moller *et al.* 2002a; Kawamoto *et al.* 2006; Vogel and Wagner 2007; Reichenbach *et al.* 2008; Urban and Vogel 2008; Rasmussen *et al.* 2009). In fact, stimulating or inhibiting translation can affect the stability of a mRNA by making it more or less accessible to ribonuclease cleavage (Kushner 1996). However, sRNAs can affect the stability of their targets independent of their effects on translation. For example, SgrS stimulates *ptsG* decay in the absence of *ptsG* translation (Morita *et al.* 2006).

Thus, a change in the metabolism of the target mRNA is not necessarily a consequence of a change in translation initiation but is a separate mechanism of sRNA mediated regulation. Although these processes can be separated, in the context of the cell, these two mechanisms can also act in concert to reinforce one another (Aiba 2007).

It is generally believed that most sRNAs function stoichiometrically, i.e. one sRNA molecule regulates one target mRNA molecule because sRNA:target degradation is coupled (Masse *et al*. 2003a; Levine *et al.* 2007). Therefore, sRNA decay can be, in some respects, a consequence of function. Although, this intimate connection between metabolism and function probably exists, sRNA metabolism has not been comprehensively or deeply investigated. Both RNase E and Hfq have been implicated repeatedly in the metabolism of sRNAs (Schmidt and Delihas 1995; Masse *et al*. 2003a; Moll *et al.* 2003a; Baker *et al.* 2007; Brennan and Link 2007; Davis and Waldor 2007; Urban and Vogel 2007; Viegas *et al.* 2007; Andrade and Arraiano 2008; Madhugiri *et al.* 2010). Furthermore, in addition to its role as chaperone, Hfq is thought to stabilize sRNAs by inhibiting RNase E mediated cleavage, because both share seemingly similar substrate specificities (Masse *et al*. 2003a; Moll *et al.* 2003a; Moll *et al.* 2003b). In fact, Hfq and RNase E are thought to form a complex *in vivo*, so once Hfq disassociates from the sRNA, RNase E would be present to initiate decay (Morita *et al.* 2005). Thus, based on the results of a few sRNAs, a generalized model of Hfq dependent sRNA decay has been put forth (Masse *et al*. 2003a; Moll *et al.* 2003a; Morita *et al.* 2005).

This is a well-supported model, but not all of the sRNAs copurify with or require Hfq for their function (Mandin and Gottesman 2010). For example, IstR-1 may bypass a need for Hfq chaperone activity, because it shares extensive complementarity with its target mRNA and can form a long, perfect duplex with *tisB* (Vogel *et al.* 2004). The decay of IstR-1 is initiated by

RNase III (Vogel *et al.* 2004), an endoribonuclease specific for double-stranded RNA with a preference for long, perfect duplexes (Li and Deutscher 2004).

Traditionally, *in vivo* analysis of eubacterial RNA metabolism (processing and decay) involves the comparison of wild type RNA to that of RNA harvested from a mutant defective in one or more ribonucleases (Kushner 1996; Condon 2007; Mohanty *et al.* 2008). A ribonuclease is implicated in the metabolism of a particular RNA if inactivation of the gene results in the accumulation of precursor forms, accumulation or loss of breakdown products, an increase in the steady-state level or chemical half-life of the RNA (Mohanty *et al.* 2008). As robust as this approach has been for investigating the metabolism of other noncoding RNAs (e.g. tRNAs) and mRNAs, recent data suggest these methods, as currently being applied may not be ideal or suitable for sRNA analysis. For example, for many of the annotated *E. coli* sRNAs detection of precursor forms is not possible because the primary transcript is the functional moiety (Masse and Gottesman 2002; Guillier and Gottesman 2006; Papenfort *et al.* 2008).

Although all RNA species are degraded, few studies have used the loss or accumulation of breakdown products as a means to study sRNA metabolism, primarily because decay products are very unstable and evade detection (Vogel *et al.* 2004; Afonyushkin *et al.* 2005; Condon 2007). Rather, most studies have relied on changes in steady-state levels or chemical half-life determinations (Masse *et al*. 2003a; Afonyushkin *et al.* 2005; Morita *et al.* 2006; Viegas *et al.* 2007; Andrade and Arraiano 2008; Overgaard *et al.* 2009). In the absence of an activity that is necessary for the metabolism of a RNA, the RNA becomes more stable and can accumulate. Accordingly, an increase in the steady-state level of a RNA in a ribonuclease deficient mutant can be diagnostic of a defect in metabolism. However, this assumption only holds true if the transcription rate of the RNA is equivalent between the mutant and wild type strains.

In fact, transcription of many of the studied sRNAs is regulated by one or more transcription factors, and deletion of a transcription factor can cause a significant change in the steady-state level of a sRNA *in vivo* (Johansen *et al.* 2008; Reichenbach *et al.* 2009; Boysen *et al.* 2010). Therefore, if the mutation under investigation affects the metabolism of a sRNA transcription factor, which subsequently results in a change in the amount of protein, then sRNA expression may be induced or repressed in the mutant as compared to the wild type control. This wrinkle in steady-state analysis is the basis of conflicting reports on the metabolism of RyhB (Masse *et al*. 2003a; Afonyushkin *et al.* 2005). Masse *et al.* (2003a) showed that in the absence of RNase E activity there was an increase in the steady-state level of RyhB relative to a wild type control and concluded that RNase E was necessary for the decay of RyhB. In contrast, Afonyushkin *et al.* (2005) saw a similar increase in the steady-state level of RyhB but found that the rifampicin half-life of RyhB was not affected by loss of RNase E activity. Thus, these authors concluded that RNase E was not necessary for RyhB metabolism, and that the observed increase in the amount of RyhB stemmed from changes in transcription rate.

One possible explanation for the differing observations is rifampicin treatment may effect sRNA function, which in turn effects sRNA metabolism. In fact, Masse *et al.* (2003a) found that sRNAs were very stable when cells were treated with rifampicin to inhibit global transcription initiation, but when transcription was allowed to proceed and sRNA transcription was specifically inhibited, the sRNA was markedly less stable. The authors concluded that sRNAs were destabilized by their targets, and the elevated half-life in the presence of rifampicin resulted from a rapid depletion of the target mRNA pool. This could mean that ribonuclease mediated aspects of sRNA metabolism that are dependent upon the target may be largely or partially obscured by addition of rifampicin to the medium, i.e. if the target and ribonuclease are part of

the same sRNA metabolic pathway, then the half-life of the sRNA in the mutant should not be significantly different from the half-life in the wild type control. Andrade and Arraiano (2008) also reported that the rifampicin half-life of MicA was not affected by the absence of its target. However, they observed an increase in the half-life of MicA in a polynucleotide phosphorylase (PNPase) deficient mutant that was also independent of the target. Their results raise the possibility that in addition to target-dependent sRNA metabolic events there are also target-independent metabolic pathways.

Given the potential complexity of sRNA metabolism and the possible caveats associated with steady-state and rifampicin half-life determinations, we decided to take advantage of a feature displayed by over one-third of the annotated *E. coli* sRNAs, namely the presence of two or more stable species of the particular sRNA on Northern blots of wild type RNA (Argaman *et al.* 2001; Rivas *et al.* 2001; Wassarman *et al.* 2001; Chen and Blyn 2002; Vogel *et al.* 2003; Opdyke *et al.* 2004; Vogel *et al.* 2004; Kawano M 2005; Fozo *et al*. 2008). In some cases, these multiple forms arise through alternative transcription initiation or termination events (Faubladier *et al.* 1990; Urbanowski *et al.* 2000; Kim and Lee 2004; Vogel *et al.* 2004), but for most other sRNAs, the smaller species are likely derived through ribonuclease mediated cleavage of the longer form(s) (Argaman *et al.* 2001; Vogel *et al.* 2003; Opdyke *et al.* 2004; Fozo *et al*. 2008; Papenfort *et al.* 2009). Regardless of the functional outcome of these cleavage events, i.e. processing or decay, from a RNA metabolism perspective, these shorter forms of sRNAs can be viewed as trackable markers that are no different from the precursor forms or breakdown products used by others to successfully identify factors necessary for tRNA, rRNA and mRNA metabolism *in vivo* (Kushner 1996; Li and Deutscher 1996; Mohanty *et al.* 2008; Deutscher and

Ciaran 2009). However, unlike other precursors and breakdown products, the smaller sRNA species are stable enough to be visible in wild type cells.

Therefore, this study was designed to identify genes involved in sRNA metabolism by assaying for the loss of shorter sRNA species in ribonuclease deficient mutants. Previous reports have limited their analysis to one or a few ribonuclease(s) (Masse *et al* 2003a; Afonyushkin *et al.* 2005; Viegas *et al.* 2007; Andrade and Arraiano 2008). To assess global ribonuclease involvement in sRNA metabolism, we examined loss-of-function alleles of all the known major cytoplasmic endoribonucleases and exoribonucleases of *E. coli* K-12, including: RNase E (*rne-1*), RNase G (*Δrng*), RNase LS (*ΔrnlA2*), RNase Z (*Δrnz*), RNase P (*rnpA49*), RNase III (*rnc-14*), PNPase (*pnp-7*), RNase II (*Δrnb*) and RNase R (*Δrnr*). To provide the most valid basis of comparison between the different mutants, all strains were in an isogenic background (MG1693), and total steady-state RNA was harvested from physiologically equivalent cell populations.

Here we have focused on the metabolism of trans-encoded sRNAs. Generally, sRNAs are classified based on the genomic position of the sRNA gene relative to the target gene as this has structural and mechanistic implications (Waters and Storz 2009). Cis-encoded sRNA genes are antisense to their target gene(s), and because they are perfectly complementary to their target at the region of overlap, they have the potential to form long, perfect duplex structures with their target(s). In contrast, trans-encoded sRNA genes do not overlap their target gene(s) and typically form short, imperfect duplexes with their targets. Published studies support the expectation that cis-encoded sRNA metabolism involves RNase III (Gerdes *et al.* 1992; Franch *et al.* 1999; Vogel *et al.* 2004), whereas trans-encoded sRNA metabolism is primarily dependent upon RNase E (Masse *et al*. 2003a; Moll *et al.* 2003a; Morita *et al.* 2005).

In *E. coli*, there are two chromosomally encoded and functionally characterized trans-encoded sRNAs that exhibit two predominant species in wild type cells on Northern blots, ArcZ and RprA (Argaman *et al.* 2001). Both the long and short forms of ArcZ bind Hfq *in vivo* and *in vitro*, and Hfq is necessary for ArcZ mediated positive and negative regulation of multiple target mRNAs (Wassarman *et al.* 2001; Zhang *et al*. 2003; Papenfort *et al.* 2009; Mandin and Gottesman 2010; Soper *et al.* 2010). In *Salmonella typhimurium*, ArcZ negatively regulates *sdaC*, *tpx* and STM3216, which leads to a reduction in both mRNA and protein levels of its target genes (Papenfort *et al.* 2009). Although ArcZ downregulates *sdaC* and STM3216 by binding to the Shine-Delgarno sequence, it downregulates *tpx* by binding to the coding region. The 5' end of *arcZ* is not well conserved, whereas the 3' end is, and it is the well-conserved 3' region of *arcZ* that is complementary to *tpx*, *sdaC* and STM3216 (Papenfort *et al.* 2009).

The 3' end of ArcZ is also complementary to an upstream region in the *rpoS* leader to which two other sRNAs, DsrA and RprA, are also known to bind, and like ArcZ, stimulate translation of *rpoS* (Lease *et al.* 1998; Majdalani *et al.* 1998; Majdalani *et al.* 2001; Majdalani *et al.* 2002; Mandin and Gottesman 2010; Soper *et al.* 2010). The *rpoS* leader normally forms an inhibitory structure that occludes the Shine-Delgarno sequence (Brown and Elliott 1997), and binding of DsrA, RprA and ArcZ to the same upstream region causes a structural rearrangement within the *rpoS* leader such that the Shine-Delgarno sequence becomes accessible (Soper *et al.* 2010). Although, both the long and short forms of ArcZ contain the conserved 3' region that is complementary to its targets, it is thought that the shorter form of ArcZ is the functional form. Papenfort *et al.* (2009) reported that expression of the short form of ArcZ affected the same regulatory response as expression of the full-length gene, from which they concluded that the shorter form and not the longer form was the functional form of ArcZ. Consistent with this,

although the full-length form of ArcZ binds Hfq specifically *in vitro*, only the shorter form was capable of binding the *rpoS* leader *in vitro* and expression of the shorter form stimulated translation of a *rpoS* translational fusion *in vivo* (Soper *et al.* 2010).

In contrast to ArcZ, it is the longer form and not the shorter form of RprA that is thought to positively regulate RprA's only known target, *rpoS* (Majdalani *et al.* 2001; Majdalani *et al.* 2002). The region of RprA that is complementary to *rpoS* is largely absent in the putative shorter form (Argaman *et al.* 2001; Majdalani *et al.* 2001; Majdalani *et al.* 2002). Furthermore, it was the longer form of RprA that was used in *in vitro* experiments with Hfq and *rpoS* (Updegrove *et al.* 2008; Soper *et al.* 2010). Like ArcZ, RprA binds Hfq *in vivo* and requires Hfq for its function (Wassarman *et al.* 2001; Majdalani *et al.* 2002). The results of this study described below indicate that whereas the processing of ArcZ is primarily dependent upon RNase E, the metabolism of RprA is dependent upon both RNase E and RNase P.

Materials and Methods

Strains and growth conditions

The *Escherichia coli* MG1693 wild type strain and isogenic mutant derivatives used in this study are listed in Table 2.1. For RNA isolation purposes, one mL of a standing overnight culture was used to inoculate 25 mL of Luria-Bertani broth supplemented with thymine (50 μg/mL) in 125 mL Klett flasks. Cultures were grown in a water bath with vigorous aeration at either 30°C or 44°C. Strains harboring temperature sensitive alleles (*rne-1* and *rnpA49*) were grown at 30°C and then shifted to 44°C for 15 minutes, 30 minutes or 60 minutes before harvesting. Aliquots were taken when cells entered stationary phase, i.e. the point in the growth phase where the

change in cell density equaled zero. Growth was monitored by a Klett-Summerson colorimeter (green filter, no. 42).

RNA isolation

When cells entered stationary phase, two mL of culture was mixed with eight mL of frozen TM buffer (10 mM Tris pH 7.2, 5 mM $MgCl_2$, 20 mM NaN_3 and 0.4 mg/mL chloramphenicol). Suspensions were spun at 3000 x g for 10 minutes to pellet the cells. Each supernatant was immediately decanted, and the cell pellets were snap-frozen in a dry ice/ethanol slurry. Frozen pellets were briefly thawed at 37°C (for approximately 10 seconds) then immediately resuspended in 1 mL of Trizol© (Invitrogen). RNA was extracted according to the manufacturer's recommendations. After extraction, the RNA was purified and concentrated by sodium acetate precipitation using 80% ethanol during the precipitation and wash. RNA was quantified from A_{260} nm measurements using a Nanodrop 2000c (Thermo Scientific).

Northern blotting

Ten µg of total steady-state RNA was mixed with an equal volume of Gel Loading Buffer II (Ambion) and prepared according to the manufacturer's recommendations. Samples were resolved on prewarmed 8%/8.3M urea polyacrylamide gels in 1 x TBE. RNA was transferred to Nytran SPC membranes (Whatman) at 4°C in a tank blotter in 1 x eTAE at 50V for 60 minutes. After transfer, membranes were baked at 80°C for 30 minutes, then UV crosslinked. Membranes were washed with 1 x SSC/0.1% SDS at 65°C, then prehybridized in PerfectHyb buffer (Sigma) at 50°C before the addition of probe. The oligonucleotides used in this study are listed in Table 2.2. The probes were end-labeled with T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's recommendations. Hybridizations were carried out overnight at

50°C. Membranes were washed twice in 2 x SSC/0.1% SDS at room temperature then twice in 2 x SSC/0.1% SDS at 50°C.

Quantification of Northern blot data

Signals on the Northern blots were captured on a phosphor screen (Molecular Dynamics) and were visualized on a Storm 840 scanner (GE Healthcare). The signal intensities of each band were calculated using ImageQuant TL v7 software (GE Healthcare). For each strain, the relative frequency (α) of each RNA species was calculated (relative frequency = [signal intensity of individual species/total signal intensity of all species]). To approximate the relative quantity (RQ) of ArcZ and RprA RNAs in the mutants relative to wild type at 30°C, the total signal intensity of all species was normalized to 5S rRNA levels in wild type at 30°C and then divided by the total signal intensity of all species in wild type at 30°C. 5S rRNA signal intensities were calculated for each strain from Northern blots prepared in the same way with the same total RNA but using only 500 ng total RNA per lane.

Primer extension and sequencing

The oligonucleotides used for the primer extension and sequencing reactions are listed in Table 2.2. The DNA templates used for the sequencing reactions were prepared by PCR using GoTaq© Green Master Mix (Promega). The templates were purified by polyacrylamide gel electrophoresis. Using 40 fmol of gel-purified template, the sequencing reactions were done with the sequencing reagents provided in the SILVER SEQUENCE™ DNA sequencing system (Promega) according to the manufacturer's recommendations, except the annealing step, which was done at the Tm of the oligonucleotide.

Oligonucleotides were end-labeled using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's recommendations. Primer extension reactions were

done with SuperScript® III RT (Promega) according to the manufacture's recommendations, except 1 pmol of end-labeled oligonucleotide was used per reaction, 5, 10 or 20 μg of total RNA was used per reaction and the reactions were carried out at 55°C for 60 minutes. After heat-inactivation, the samples were treated with RNase I (Epicentre) according to the manufacturer's recommendations then purified by phenol:chloroform:isoamyl alcohol (25:24:1 pH 8, Fisher) extractions. The samples were then purified and concentrated by sodium acetate precipitations using 80% ethanol during the precipitation and wash.

Sequencing and primer extension reactions were mixed with DNA sequencing stop solution (Promega) according to the manufacturer's recommendations. The samples were denatured at 70°C for 3 minutes then snap-cooled on ice prior to loading onto prewarmed 8%/8.3M urea polyacrylamide gels in 1 x TBE. The gels were run at 50W. Gels were subsequently transferred to blotting paper then dried under a vacuum with heat. Signals on the gels were captured on a phosphor screen (Molecular Dynamics) and were visualized on a Storm 840 scanner (GE Healthcare).

Results

Rationale for studying ArcZ metabolism

In *E. coli*, there were two predominate ArcZ RNA species visible on Northern blots of wild type RNA, ArcZ121 and ArcZ55 (the number corresponds to the length of the RNA in nucleotides) (Figure 2.1, lane 1). The electrophoretic mobility of these species was in excellent agreement with the 5' and 3' ends mapped by primer extension and RACE (Argaman *et al.* 2001; Mandin and Gottesman 2010). The two major ArcZ species differ with respect to their 5' ends (Figure 2.2). In *E. coli* MG1655, the 5' end of ArcZ55 maps to position +66 relative to the annotated

start of transcription (Figure 2.2) (Mandin and Gottesman 2010). *In vitro* transcription experiments using *E. coli* RNA polymerase showed that the *arcZ* locus produced a transcript of ~120 nucleotides but not the shorter form (Argaman *et al.* 2001). Since, the ArcZ RNAs differ with respect to their 5' ends and do not arise via alternative transcription initiation, it is probable that ArcZ55 was generated through ribonuclease mediated cleavage of ArcZ121. Thus, ArcZ was an excellent candidate for our analysis.

In MG1655, the two ArcZ RNAs exhibited a growth phase specific accumulation pattern on Northern blots (Argaman *et al.* 2001; Mandin and Gottesman 2010). The ArcZ RNAs were weakly visible in exponentially growing cells but accumulated as cells entered stationary phase. We chose to study ArcZ metabolism in early stationary phase when the ArcZ RNAs have accumulated. During the course of this study, Mandin *et al.* (2010) and Papenfort *et al.* (2009) reported that the stationary phase accumulation of ArcZ RNAs observed in *E. coli* and *Salmonella*, respectively, was due to increased transcription.

Exoribonuclease and endoribonuclease involvement in ArcZ metabolism

Since the ArcZ55 and ArcZ121 species differ with respect to their 5' ends (Figure 2.2) (Argaman *et al.* 2001; Mandin and Gottesman 2010), it was probable that endoribonuclease(s) and not exoribonuclease(s) mediated the processing of ArcZ121 to generate ArcZ55, since all of the known *E. coli* exoribonuclease proceed in a 3'→5' direction (Li and Deutscher 2004). Consistent with this hypothesis, none of the exoribonuclease mutants, PNPase (*pnp-7*), RNase II (*Δrnb*) and RNase R (*Δrnr*), tested in this study affected the α values as compared to a wild type control grown at 30°C (Figure 2.1, lanes 1, 8, 9, 10 and Table 2.3, columns 1, 7, 8, 9). Furthermore, the *Δrnb* and *Δrnr* alleles did not significantly affect the RQ of total ArcZ RNAs, but the *pnp-7* mutation led to a moderate reduction in the RQ. These results suggested that neither PNPase,

RNase II nor RNase R were significantly involved in the processing of ArcZ121 to ArcZ55, because inactivation of these genes did not affect the relative stabilities of ArcZ121 or ArcZ55 as compared to the wild type control. However, PNPase may be necessary for the decay of shorter ArcZ transcripts (<55 nt), because on overexposed images of the Northern blots presented in Figure 2.1, an ArcZ breakdown product accumulated that was not detected in the wild type control (Figure S.2.1, lanes 1, 8).

Based on the exoribonuclease mutant results presented in Figure 2.1 and Table 2.3, we expected that endoribonuclease(s) were required for the processing of ArcZ121. To determine which endoribonuclease(s) were involved, single mutants of all of the known endoribonucleases were tested. These included RNase E (*rne-1*), RNase G (*Δrng*), RNase LS (Δ*rnlA2*), RNase Z (*Δrnz*), RNase III (*rnc-14*) and RNase P (*rnpA49*). The *Δrng*, *ΔrnlA2*, *Δrnz*, *rnc-14* and *rnpA49* alleles did not affect the α values of ArcZ121 or ArcZ55 as compared to the wild type control (Figure 2.1, lanes 1, 3, 4, 5, 6, 7, 12, Table 2.3, columns 1, 3, 4, 5, 6 and Table 2.4, columns 1, 3). These results suggested RNase G, RNase LS, RNase Z, RNase III and RNase P were not involved in the processing of ArcZ121. Although the *Δrng*, *ΔrnlA2*, *Δrnz* and *rnc-14* alleles did not significantly affect the RQ of total ArcZ RNAs as compared to the wild type control grown at 30°C (Figure 2.1, lanes 1, 3, 4, 5, 6 and Table 2.3, columns 1, 3, 4, 5, 6), the *rnpA449* allele caused an approximate two-fold increase in the RQ of ArcZ RNAs as compared to the wild type control at 44°C (Figure 2.1, lanes 7, 12 and Table 2.1, column 3).

In contrast to RNase G, RNase LS, RNase Z, RNase III and RNase P, RNase E had a major impact on the processing of ArcZ121 to generate ArcZ55. The *rne-1* allele encodes a point mutation in the N-terminal catalytic domain of RNase E that leads to an inactive protein at 44°C (Arraiano *et al.* 1988). In the *rne-1* mutant at 44°C, there was a significant increase in αArcZ121

and a decrease in αArcZ55 as compared to the wild type control (Figure 2.1, lanes 2, 12, 14 and Table 2.4, columns 1, 2). These results were consistent with RNase E inactivation leading to the stabilization of ArcZ121, resulting in the loss of production of ArcZ55.

RNase E involvement in ArcZ metabolism

At 30°C, the *rne-1* did not affect the α values or the RQ of ArcZ RNAs as compared to the wild type control (Figure 2.1, lanes 11, 13 and Table 2.3, columns 1, 2). However at 44°C, the *rne-1* allele affected both the α values and the RQ of ArcZ RNAs (Figure 2.1, lanes 12, 14 and Table 2.4, columns 1, 2). Within 15 minutes at the nonpermissive temperature, the ArcZ121 species accumulated in the *rne-1* mutant. With prolonged exposure to the higher temperature, there was a further moderate increase in the amount of ArcZ121 and significant decrease in the amount of ArcZ55 (Figure 2.3). After 60 minutes at 44°C, there was an 80% reduction in the total amount of ArcZ RNAs as compared to wild type cells grown at 30°C (Figure 2.1, lanes 1, 2 and Table 2.4, column 2). However, the higher temperature also caused an approximate 40% reduction in the RQ of ArcZ RNAs in wild type cells (Figure 2.1, lanes 11, 12 and Table 2.4, column 1). Although the *rne-1* allele exacerbated the observed reduction in the total amount of ArcZ RNAs, the time course analysis showed that the reduction largely stemmed from the disappearance of the more abundant ArcZ55 species (Figure 2.3). Additionally, on overexposed images of the Northern blots presented in Figure 2.1, shorter ArcZ transcripts (<55 nt) were largely absent in the *rne-1* mutant (Figure S.2.1).

The results presented in Figures 2.1, 2.3 and Table 2.4 indicated that in the absence of RNase E activity, ArcZ processing was largely inhibited. On the Northern blots presented in Figures 2.1, 2.3 and overexposed images of the Northern blots (Figure S.2.1), in the *rne-1* mutant, a faint smear above ArcZ121 was visible, as well as minor intermediate sized species

and three bands within the ArcZ55 region. Since the processing of ArcZ121 to generate ArcZ55 was occurring through the removal of 5' sequences, these results prompted us to assess the 5' ends of ArcZ in the *rne-1* mutant using primer extension analysis. The results presented in Figure 2.4 demonstrated that the ArcZ55 species was actually three ArcZ RNAs that differ in length by one nucleotide each. These 5' ends mapped to positions +65, +66 and +67 relative to the annotated start of transcription, +1 (Figure 2.2) (Mandin and Gottesman 2010). Additionally in the *rne-1* mutant, numerous 5' ends that mapped to positions between +1 and +65 were present. Some of these 5' ends were also present in wild type cells grown at 30°C, whereas most of the ends appeared to accumulate only in the *rne-1* mutant. Furthermore, three 5' ends that mapped to positions upstream of the annotated start of transcription, -4, -2 and -1, accumulated in the *rne-1* strain (Figure 2.2, 2.4, 2.5). These ends were also weakly visible in the wild type control samples.

The results of our analysis suggested that RNase E was the primary enzyme involved in the 5' processing of ArcZ transcripts. Since RNase E can be inhibited by the presence of a 5' triphosphate group on a primary transcript (Mackie 1998), it was surprising that in the absence of the RNA pyrophosphohydrolase, RppH, which removes a pyrophosphate group from the 5' end of a primary transcript to generate a 5' monophosphorylated end (Deana *et al.* 2008), ArcZ121 processing was not affected (Figure 2.1, lanes 1, 18 and Table 2.3, columns 1, 10). The *ΔrppH* mutation did not affect the α values, which indicated that the relative stabilities of ArcZ121 and ArcZ55 were unaffected as compared to the wild type control, but the mutation did cause an approximate two-fold increase in the RQ of total ArcZ RNAs. These results suggested that RppH mediated removal of pyrophosphate groups from the 5' ends of the ArcZ primary transcripts was not necessary for RNase E processing of ArcZ121.

Since RNase E was necessary for the processing of longer ArcZ transcripts to generate the more abundant, shorter ArcZ RNAs, we chose to examine three RNase E truncation alleles to further investigate the role of RNase E in ArcZ processing. In the *rneΔ91* mutant, which lacks the arginine-rich RNA binding domain (Ow *et al.* 2000), neither the α values nor the RQ of ArcZ RNAs were affected as compared to the wild type control (Figure 2.1, lanes 11, 15 and Table 2.5, columns 1, 3). This result indicated that the RNA binding domain was largely dispensable for ArcZ processing. However, on overexposed images of the Northern blots presented in Figure 2.1, shorter ArcZ products (<55 nt) accumulated, which suggested this domain may be involved in some aspects of either ArcZ decay or accurate cleavage of the ArcZ121 species (Figure S.2.1). In contrast, both the *rneΔ374* and *rneΔ645* caused an increase in αArcZ121, a decrease in αArcZ55 and no significant change to the RQ of ArcZ RNAs as compared to the wild type control at 30°C (Figure 2.1, lanes 11, 16, 17 and Table 2.5, columns 1, 4, 5). The *rneΔ374* allele encodes a RNase E protein that lacks the C-terminal degradosome scaffolding domain, while the *rneΔ645* allele contains only the core N-terminal catalytic domain of RNase E (Ow *et al.* 2000). The *rneΔ645* allele is known to be partially defective for the decay of some mRNAs (Ow *et al.* 2000). The effect on αArcZ121 was greater in the *rneΔ645* mutant than in the *rneΔ374* mutant. These results suggested that although the scaffolding domain of RNase E was necessary for robust processing of the ArcZ121 species, the catalytic domain of RNase E was more important. Additionally, on overexposed images of the Northern blots presented in Figure 2.1, the *rneΔ645* allele caused the accumulation of an ArcZ ~111 nt in length, whereas the *rneΔ374* allele caused the accumulation of smaller, <55 nt, products of ArcZ (Figure S.2.1). These results suggested different roles for the degradosome scaffolding region and the catalytic domain of RNase E in ArcZ metabolism.

Rationale for studying RprA metabolism

There were two predominant RprA species visible on Northern blots in wild type *E. coli*, RprA105 and RprA45 (the number corresponds to the length of the RNA species in nucleotides) (Figure 2.6, lane 1) (Argaman *et al.* 2001). The electrophoretic mobility of RprA105 observed here was in excellent agreement with the annotated ends of this species (Figure 2.7) (Argaman *et al.* 2001; Majdalani *et al.* 2001; Majdalani *et al.* 2002). Since, the ends of RprA45 were not known, a probe designed against the 3' end of RprA105 detected both RprA105 and RprA45 on Northern blots (Argaman *et al.* 2001), whereas a probe designed against the 5' end of RprA105 detected only RprA105 (Majdalani *et al.* 2001; Majdalani *et al.* 2002). These results suggested RprA105 and RprA45 shared 3' sequences and differed with respect to their 5' ends. Similar to ArcZ, *in vitro* transcription assays using *E. coli* RNA polymerase showed that the *rprA* gene produced a transcript of ~105 nt, but not the shorter form (Argaman *et al.* 2001). When these results were combined with the Northern data, it suggested RprA45 did not arise through alternative transcription initiation, rather RprA45 was generated through ribonuclease mediated cleavage of RprA105 via the removal of 5' sequences. This made RprA an excellent candidate for our analysis.

In *E. coli* MG1655, the two RprA RNAs exhibited a growth phase specific accumulation pattern on Northern blots (Argaman *et al.* 2001). The RprA RNAs were not visible in exponentially growing cells but accumulated as cells entered stationary phase. RprA has only one known target, *rpoS* mRNA, which encodes the stationary phase sigma factor (Majdalani *et al.* 2001; Majdalani *et al.* 2002). Since, the RprA RNAs accumulated in early stationary phase, we chose to study the metabolism of the RprA RNAs during this phase of growth.

Exoribonuclease and endoribonuclease involvement in RprA metabolism

The RprA sRNAs exist as two species, RprA105 and RprA45 that appear to differ with respect to their 5' ends (Argaman *et al.* 2001; Majdalani *et al.* 2001; Majdalani *et al.* 2002). Thus, it was probable that endoribonuclease(s) and not exoribonuclease(s) were mediating the processing of RprA105 to generate RprA45. Consistent with this hypothesis, none of the exoribonuclease mutants, PNPase, (*pnp-7*), RNase II (*Δrnb*) and RNase R (*Δrnr*), caused a significant increase in αRprA105 and concomitant decrease in αRprA45 as compared to the wild type control at 30°C (Figure 2.6, lanes 1, 8, 9, 10 and Table 2.6, columns 1, 7, 8, 9). These results suggested neither PNPase, RNase II nor RNase R were involved in the generation of RprA45 from RprA105. However, all three alleles caused reductions in the RQ of total RprA species, with the *pnp-7* allele affecting the greatest reduction, i.e. ~80% as compared to the wild type control (Table 2.6, columns 1, 7, 8,9). Furthermore, in the *pnp-7* mutant there was a moderate reduction in αRprA105 and a small increase in αRprA45. On full-length images of the Northern blots presented in Figure 2.6, there was a significant accumulation of a RprA species <45 nt in the *pnp-7* strain (Figure S.2.2, lane 8). When combined with the relative frequency analysis, these results suggested PNPase may participate in the decay of shorter RprA transcripts.

The data presented in Figure 2.6 and Table 2.6 suggested exoribonucleases were not directly participating in the metabolic pathway converting RprA105 into RprA45. Rather, these results implied one or more of the endoribonucleases mediated this metabolic pathway(s). The *Δrng*, *ΔrnlA2*, *Δrnz* and *rnc-14* alleles did not significantly affect the α values as compared to the wild type control at 30°C (Figure 2.6, lanes 1, 3, 4, 5, 6 and Table 2.6, columns 1, 3, 4, 5, 6). These results indicated RNase G, RNase LS, RNase Z and RNase III were normally not required for the processing of RprA105. However, in contrast to the exoribonuclease single mutants, all

four of these single mutants caused an increase in the RQ of RprA RNAs (Table 2.6, columns 1, 3, 4, 5, 6). The effect of the *Δrnz* allele was the most subtle and only led to a slight increase in the RQ as compared to the wild type control, whereas the *Δrng* allele led to an approximate two-fold increase in the total amount of RprA RNAs.

Both the *rnpA49* and *rne-1* alleles are thermolabile mutations, which render their encoded proteins inactive at 44°C (Arraiano *et al.* 1988; Ow and Kushner 2002). Therefore, a second set of experiments was done at this temperature. When wild type cells were grown at 44°C, both the RQ of RprA RNAs and the observed cleavage pattern were affected as compared with cells grown at 30°C (Figure 2.6, lanes 11, 12, Table 2.6, column 1 and Table 2.7, column 1). At 44°C, there was an approximate three-fold increase in the total amount of RprA RNAs, as well as, the appearance of several intermediate sized RprA RNAs ranging in size from ~68 to ~81 nt. The *rnpA49* allele exacerbated the increase in the RQ, causing a five-fold increase in the total RprA amount (Figure 2.6, lanes 7, 12 and Table 2.7, columns 1, 3). Additionally, this allele resulted in an increase in αRprA105 and decrease to both αRprA68-81 and αRprA45. These results suggested inhibition of RNase P activity caused a moderate stabilization of RprA105 resulting in the loss of production of both RprA68-81 and RprA45. Although RNase P appeared to participate in the metabolic pathway converting RprA105 into RprA45, the results presented in Figure 2.6 and Table 2.7 suggested RNase E was the primary endoribonuclease mediating this metabolic pathway. At 44°C, the *rne-1* allele caused a significant increase in αRprA105 and decrease in αRprA45 (Figure 2.6, lanes 12, 14 and Table 2.7, columns 1, 2). Interestingly, this mutation also caused a very significant increase in αRprA68-81 (Figure 2.6, columns 2, 14). When taken together, these results suggested RNase E was primarily necessary for the generation

of RprA45 and decay of RprA68-81, but that some other endoribonuclease(s) were responsible for the generation of RprA68-81.

Although the effect of the *rne-1* allele on the α values was greater than the effect of the *rnpA49* allele, the opposite was true with respect to the effect on the RQ of RprA RNAs (Figure 2.6, lanes 1, 2, 7, 11, 12, 14 and Table 2.7, columns 1, 2, 3). The *rne-1* allele caused an approximate three-fold increase in RprA RNAs, whereas the *rnpA49* allele lead to a five-fold increase. These results may correlate, in part, with the observed loss of shorter <45 nt RprA RNAs in the *rnpA49* mutant (Figure S.2.2). As compared with the wild-type control at 44°C, the shorter RprA transcripts failed to accumulate in the *rnpA49* mutant. This result suggested RNase P was necessary for the decay of RprA45 and/or generation of these shorter species.

In contrast to what was observed for ArcZ metabolism, in the *ΔrppH* mutant, there was a small increase in αRprA105 and concomitant decrease in αRprA45, which suggested removal of the pyrophosphate group from the RprA105 primary transcript by RppH was necessary for efficient RNase E and/or RNase P mediated processing of RprA105. Both RNase E and RNase P can be inhibited by a triphosphate group (Mackie 1998) (Bowden and Kushner, unpublished results).

RNase E involvement in RprA metabolism

At 30°C, the *rne-1* allele caused a slight increase in αRprA105 and decrease in αRprA45 as compared to the wild type control (Figure 2.6, lanes 1, 2 and Table 2.6, columns 1, 2). This result suggested the *rne-1* strain was marginally defective in RprA metabolism even at the permissive temperature. Interestingly, although RprA metabolism appeared to be partially blocked in the *rne-1* mutant at 30°C, there was a reduction in the RQ of total RprA RNAs in this mutant as compared to the wild type control. However, when the *rne-1* strain was shifted to 44°C, within

15 minutes there was an increase in total RprA RNAs primarily due to the increase in the amount of RprA105 (Figure 2.8, lanes 1, 2, 3, 4). With prolonged exposure to the nonpermissive temperature, both RprA105 and RprA68-81 accumulated. These results were consistent with a stabilization of both RprA105 and RprA68-81 in the *rne-1* mutant.

On the Northern blots presented in Figures 2.6, 2.8, it appeared as though the RprA45 species was at least two different RNAs. In addition, there were multiple species in the range of RprA68-81. Since the metabolic pathway converting RprA105 into RprA45 was occurring through the removal of 5' sequences, we chose to assess the 5' end population of RprA RNAs in both the wild type and the *rne-1* strains by primer extension. The results presented in Figure 2.9 agreed with the annotated +1 end of RprA (Figure 2.7) (Argaman *et al.* 2001; Majdalani *et al.* 2001). Furthermore, numerous 5' ends were detected in the *rne-1* mutant between positions +17 and +39. If the RprA species detected on Northern blots did not differ with respect to their 3' ends, the ends mapped between positions +17 and +39 were in good size agreement with the RprA68-81 RNAs. Additionally, in wild type cells two 5' ends were detected at positions +60 and +61. These products were significantly less prevalent in the *rne-1* mutant. Again, if the RprA species detected on Northern blots did not differ with respect to their 3' ends, then RNAs with a 5' end at position +60 or +61 would be 45 and 44 nucleotides in length, respectively, in excellent agreement with the estimated size of the RprA45 species.

Since RNase E appeared to be the primary endoribonuclease involved in converting RprA105 into RprA45, and the *rne-1* allele already exhibited a slight metabolic defect at 30°C, we chose to investigate three truncation alleles of RNase E at this temperature. The *rne-1*, *rneΔ91*, *rneΔ374* and *rneΔ645* alleles all caused a similar increase to αRprA105 (Figure 2.6, lanes 11, 13, 15, 16 , 17 and Table 2.8, columns 1, 2, 3, 4, 5). These results suggested that the

catalytic region, arginine-rich RNA binding domain and the C-terminal scaffolding region of RNase E were all necessary for the metabolism of RprA105. Interestingly, in the three RNase E truncation mutants the RprA68-81 species were visible. Normally, these species were visible only in cells that were grown at 44°C. Although, only a very small proportion of total RprA RNAs were the RprA68-81 species in the *rneΔ645* mutant, which was more similar to what was observed in the *rne-1* strain, the relative proportion of these intermediate sized species in the *rneΔ91* and *rneΔ374* was more similar to what was observed in wild type cells grown at 44°C. These results suggested the absence of either the arginine-rich RNA binding domain or the scaffolding region was sufficient to cause the change in RprA cleavage patterns such that it more closely resembled what was observed at 44°C.

Discussion

The results presented in Figures 2.1, 2.3 and Tables 2.3, 2.4 indicated that RNase E was the primary endoribonuclease involved in the processing of ArcZ121 to generate ArcZ55. In contrast, the results presented in Figures 2.6, 2.8 and Tables 2.6, 2.7 showed that both RNase E and RNase P were necessary for the processing of RprA105 to generate RprA45. These results represent the first time that RNase P has been implicated in the metabolism of a trans-encoded sRNA. Interestingly, RNase P appeared to participate at multiple points in RprA metabolism, a possible role in the stabilization of RprA105 as well as the stabilization of RprA45 resulting in the loss of shorter (<45 nt) RprA breakdown products. However, the analysis of both sRNAs clearly demonstrated that RNase E was the primary endoribonuclease affecting the metabolism of these two trans-encoded, Hfq-dependent sRNAs. During the course of this study, there were two reports that published findings consistent with our observations. Papenfort *et al.* (2009)

claimed, but did not show, that ArcZ processing in *Salmonella typhimurium* was dependent upon RNase E. Furthermore, Madhugiri *et al.* (2010) showed that the rifampicin half-life of RprA105 was increased in a *rne-3071* mutant as compared to a wild type control.

More importantly, this study has extended our understanding of ArcZ and RprA metabolism by demonstrating that RNase G, RNase LS, RNase Z, RNase III, RNase II and RNase R are probably not necessary for the metabolism of either sRNA at 30°C. In contrast, PNPase appeared to participate in the decay of shorter ArcZ (<55 nt) and RprA (<45 nt) transcripts. PNPase is known to mediate the decay of other trans-encoded sRNAs (Viegas *et al.* 2007; Andrade and Arraiano 2008). When combined with the results of this study, these observations suggest that PNPase mediated decay of trans-encoded sRNAs is a somewhat common feature of sRNA metabolism.

Furthermore, whereas RppH was not necessary for ArcZ121 metabolism, it was necessary for robust RprA105 metabolism. These results suggest removal of a pyrophosphate group from the ArcZ primary transcript(s) by RppH was not necessary for RNase E mediated cleavage of ArcZ121, whereas it was partially required for the RNase E and/or RNase P mediated cleavage of RprA105. Both RNase E and RNase P can be inhibited by the presence of a 5' triphosphate (Mackie 1998) (Bowden and Kushner, unpublished results). This is the first time that RppH has been implicated in the metabolism of a trans-encoded sRNA. Furthermore, these results raise the possibility that there may be another RNA pyrophosphohydrolase in the cell that can remove a pyrophosphate group from these primary transcripts, RNase E was using two different mechanisms when acting on either ArcZ121 or RprA105 (Deana *et al.* 2008; Kime *et al.* 2009), or the presence of the triphosphate affected RNase P mediated cleavage of RprA105 but not RNase E mediated cleavage.

The results from this study clearly demonstrated that a relative frequency analysis was successful in identifying metabolic defects and implicating particular ribonucleases in sRNA metabolism independent of steady-state analysis or chemical half-life determinations. Furthermore, a comparison of the ArcZ and RprA results showed that the changes in α values and the RQs were specific for the sRNA. For example, the *rnpA49* allele affected the α values for RprA RNAs but not ArcZ RNAs, and at 44°C, the RQ of RprA RNAs increased in wild type cells whereas the higher temperature resulted in a reduction in the RQ of ArcZ RNAs. Additionally, the results showed that a change in the RQ of a sRNA did not necessarily affect the α values. For example, in the *Δrng* mutant, there was an approximate two-fold increase in the total amount of RprA RNAs, but the α values were identical to the wild type control. Furthermore, the results demonstrated that significant variation between experiments that affected the RQ values did not result in significant variation in the α values. For example, although the standard deviation of the RQ of RprA RNAs in the *rne-1* mutant at 44°C was ±0.73, the standard deviations for the α values was ≤ ±0.02. These observations indicated that although the absolute amount of a sRNA may vary between experiments, the ratio of the forms remained relatively constant. Thus, this method appeared to be a robust and highly reproducible way to study sRNA metabolism *in vivo*.

Our results for both sRNAs showed additional complexities in sRNA transcription and sRNA metabolism that warrant further investigation. These complexities include: (1) the effect of temperature on the cleavage pattern of RprA; (2) the presence of 5' ends of ArcZ that were upstream of the annotated start of transcription; (3) both the ArcZ55 and RprA45 bands on Northern blots contained multiple species that differed with respect to their 5' ends; (4) numerous RNAs intermediate in size relative to the major forms of the ArcZ and RprA RNAs

that differed with respect to their 5' ends; (5) the decay of RprA45 and ArcZ55; and (6) the role of Hfq in the metabolism of RprA and ArcZ RNAs.

The effect of temperature on the cleavage pattern of RprA

In contrast to ArcZ, the cleavage pattern of RprA was affected at 44°C. When wild type cells were grown at 30°C, only RprA105 and RprA45 were visible on Northern blots. However, when wild type cells were grown at 44°C, in addition to the two major RprA RNAs, intermediate sized RNAs ranging in length from ~68 to ~81 nt accumulated. The results presented in Figure 2.6 and Table 2.7 suggested RNase P was necessary in part for the generation of these intermediate sized RprA RNAs, as well as, RprA45. In contrast, RNase E was necessary for the production of RprA45 and the breakdown but not the production of the intermediate sized RNAs.

Interestingly, in the *rneΔ91* and *rneΔ374* mutants, which were grown at 30°C, these intermediate sized RprA RNAs were also observed. These results indicated that the absence of either the arginine-rich RNA binding domain or the C-terminal degradosome scaffolding domain was sufficient to generate RprA cleavage patterns that were normally observed only at 44°C. However, when these truncations were combined in the *rneΔ645* mutant, the effect was not additive. Rather, the effect of the *rneΔ645* mutation was more similar to what was observed in the *rne-1* strain at 30°C. When combined these results suggested inhibition of RNase E catalytic activity was sufficient to suppress the formation of intermediate sized RprA RNAs at 30°C, but when the catalytic activity of RNase E was unaffected but RNase E lacked either the RNA binding domain or the scaffolding domain, the defect normally observed at 44°C was manifested.

5' ArcZ ends that were upstream of the annotated start of transcription

On the Northern blots presented in Figures 2.1, 2.3, a smear above ArcZ121 was present in the *rne-1* strain at 44°C that was largely absent in the wild type control. The primer extension results

presented in Figure 2.5 showed that the smear corresponded to ArcZ RNAs whose 5' ends were 1-4 nt upstream of the annotated start of transcription (+1) (Argaman *et al.* 2001; Mandin and Gottesman 2010). However, these 5' ends, which mapped to positions -4, -2 and -1, were weakly visible in wild type cells but significantly accumulated in the *rne-1* mutant. Since, the -4, -2, -1 and +1 ends of ArcZ accumulated in the *rne-1* strain, this result suggests transcription of *arcZ* normally initiates at one of four positions and that RNase E was necessary for the metabolism of these species. However, this study did not determine if the -4, -2, -1 and/or +1 ends of ArcZ RNAs contained a 5' triphosphate group or a monophosphate group. This information is absolutely necessary to determine if these ends arose from alternative transcription initiation or RNase E mediated cleavage of the -1 to -4 nt transcription products. In addition, it remains unclear whether RNase E was removing these additional nucleotides to generate the +1 end or if these longer transcripts were normally processed to the shorter, stable forms of ArcZ.

Both the ArcZ55 and RprA45 bands on Northern blots were multiple species that differed with respect to their 5' ends

In the literature there is a one 5' end annotated for ArcZ55, which is located at position +66 relative to the annotated start of transcription (Mandin and Gottesman 2010). However, the results presented in Figures 2.3, 2.4 clearly demonstrated that the ArcZ55 band on Northern blots corresponded to three ArcZ RNAs that differed with respect to their 5' ends. These 5' ends mapped to positions +65, +66 and +67. Since the amount of all three species and ends was reduced in the *rne-1* mutant, it appears that RNase E cleavage specificity is somewhat relaxed. However, it is not clear from the results of this study if these ends represented independent RNase E mediated metabolic pathways, a hypothesis we favor, or if the three ends resulted from

hierarchical cleavage events in one metabolic pathway such that RNase cleaved first at +65, then at +66 and finally at +67.

The results presented in Figures 2.6, 2.8, 2.9 demonstrated that the RprA45 band seen on Northern blots was in fact at least two RprA species that differ with respect to their 5' ends. These ends mapped to positions +60 and +61 relative to the start of transcription (Argaman *et al.* 2001; Majdalani *et al.* 2001). Previous to this study, the 5' ends of the these species were not known. Similar to ArcZ, it remains unclear whether or not RNase E and/or RNase P cleaved independently at positions +60 or +61 or whether these ends result from a hierarchical metabolic pathway.

Numerous RprA and ArcZ RNAs intermediate in size to the major forms of ArcZ and RprA that differ with respect to their 5' ends

On the overexposed images of the Northern blots presented in Figure 2.1, there were bands and smears representing minor ArcZ RNA species intermediate in size between ArcZ121 and ArcZ55 in all of the strains tested by this study (Figure S.2.1). The primer extension results presented in Figure 2.4 showed that some of these minor species in wild type cells corresponded to ArcZ RNA that differed with respect to their 5' ends. In the *rne-1* mutant at 44°C, these ends, as well as other 5' ends not normally seen in wild type cells, accumulated. These observations raised the question: what enzyme was generating these intermediate 5' ends in wild type cells and the *rne-1* mutant? Since none of these intermediate 5' ends observed in wild type cells disappeared in the *rne-1* mutant, this would suggest that some other endoribonuclease was generating these products. Consistent with this hypothesis, in the case of the intermediated sized RprA RNAs (~68 to ~81 nt), a preliminary experiment done in a *rne-1 Δrng* double mutant at 44°C showed the ~68 nt RprA band did not accumulate (data not shown). This result suggested

RNase G was necessary for the production of the ~68 nt RprA RNAs at 44°C. Therefore, to determine which endoribonucleases are responsible for generating the intermediate 5' ends of both ArcZ and RprA RNAs, all combinations of double mutants containing the *rne-1* allele should be assessed by primer extension. Another possibility is that some of these 5' ends were due to residual RNase E activity (Mohanty and Kushner 2007; Mohanty and Kushner 2008; Chung *et al.* 2010). To address this possibility, *in vitro* cleavage assays using wild type RNase E and the mutant RNase E encoded by the *rne-1* gene need to be performed.

The decay of ArcZ55 and RprA45

This study was designed to identify ribonucleases involved in the initial processing of ArcZ121 and RprA105. However, the accumulation or loss ArcZ (<55 nt) and RprA (<45 nt) breakdown products implicated several enzymes that may participate in the decay of ArcZ55 and RprA45. For example, in the *pnp-7* mutant breakdown products of both RprA and ArcZ RNAs accumulated that were not present in the wild type control. Thus, PNPase may participate in the decay of both sRNAs.

In the *rneΔ91* mutant, several shorter (<55 nt) ArcZ RNAs accumulated relative to the wild type control. This result suggested the arginine-rich RNA binding domain of RNase E was involved in decay of shorter ArcZ RNAs as well. An involvement of RNase E in ArcZ55 decay would be consistent with the literature (Masse *et al*. 2003a; Moll *et al*. 2003a, Morita *et al.* 2005), since ArcZ55 binds to and requires Hfq for its function (Wassarman *et al*. 2001, Zhang *et al*. 2003; Papenfort *et al*. 2009, Soper *et al*. 2010). Additionally, there appeared to be a slight accumulation of shorter ArcZ RNAs in the *rneΔ374* mutant. Since PNPase also appeared to be involved in some aspects of ArcZ decay, and PNPase associates with RNase E via the C-terminal

scaffolding domain, these results may suggest that ArcZ decay was dependent in part on a functional degradosome.

Unexpectedly, in the *rnpA49* mutant shorter (<45 nt) RprA RNAs failed to accumulate as compared to the wild type control. This result suggested RNase P may participate in the decay of RprA45. To address which enzymes are necessary for the decay of ArcZ55 and RprA45, the chromosomal copy of these genes would need to be deleted and the shorter forms expressed from a plasmid in the various ribonuclease deficient cells lines. In this way, the decay of ArcZ55 and RprA45 could be assessed independent of their production.

The role of Hfq in the metabolism of ArcZ and RprA RNAs

The data presented in Figures 2.6, S.2.2 showed that in the absence of Hfq, RprA105 was retained whereas RprA45 was absent. This result suggests Hfq was necessary for the stability of RprA45. Although, Hfq is not known to bind RprA45 *in vivo*, these results suggest that it may, because for other sRNAs that bind Hfq, in the absence of Hfq, the steady-state level of the sRNA is dramatically reduced (Masse *et al*. 2003a; Opdyke *et al.* 2004; Papenfort *et al.* 2009). However, it is not clear from the results of this study if Hfq was necessary for the stability of RprA45 or if in the absence of Hfq, RprA45 was never produced.

Hfq may also be necessary for the stability of RprA105, since as compared to the wild type control, the steady-state level of RprA105 was reduced. This result was consistent with the findings of Soper *et al.* (2010) and Madhugiri *et al.* (2010). Soper *et al.* (2010) reported that the steady-state level of RprA105 was reduced in a Hfq deletion strain as compared to a wild type control and Madhugiri *et al.* (2010) observed that in the absence of Hfq the rifampicin half-life of RprA105 was reduced as compared to wild type.

The data presented in Figures 2.1, S.2.1 showed that, like RprA, in the absence of Hfq, ArcZ55 was absent but ArcZ121 was retained. This result was consistent with the results reported by Soper *et al.* (2010). However, whereas Soper *et al.* (2010) showed that the amount of ArcZ121 was the same in a Hfq deletion strain and their MG1655 wild type control, in our study, the amount of ArcZ121 in the *Δhfq* strain was always greater than the amount in MG1693 wild type control, although the actual increase varied between experiments, ranging from approximately two-fold to four-fold. The results presented by Soper *et al.* (2010) would suggest that Hfq was not necessary for ArcZ121 metabolism, but the accumulation of ArcZ121 observed in this study could be consistent with a stabilization of ArcZ121, thus implicating Hfq in the metabolism of ArcZ121. Additionally, our study found that in the *rneΔ374* mutant there was a moderate increase in αArcZ121 and decrease in αArcZ55. These results suggest the C-terminal scaffolding domain of RNase E was necessary for robust ArcZ121 processing. RNase E and Hfq are thought to form a complex *in vivo* via the C-terminal scaffolding domain of RNase E (Morita *et al.* 2005). Thus, an increase in the steady-state level of ArcZ121 in the *Δhfq* mutant, if it resulted from increased stability of ArcZ121, could be consistent with the block in processing observed in the *rneΔ374* mutant. To address this question, the Hfq experiments should be repeated, and the chemical half-life of ArcZ121 should be determined in the *Δhfq* mutant and wild type control.

There may be a possible explanation for the difference in results concerning the steady-state level of ArcZ121 in a Hfq mutant. Mandin *et al* (2010) reported that in the absence of *arcZ*, the rifampicin determined half-life of the *arcB* mRNA was increased relative to a $arcZ^+$ strain, which suggested ArcZ destabilized *arcB*. Additionally, in a $arcZ^+$ strain, the induction of *arcB* caused a reduction in ArcZ121 steady-state levels but not ArcZ55 levels. Thus, these authors

concluded that if ArcZ negatively regulated *arcB* stability, then it was ArcZ121 and not ArcZ55 that was targeting *arcB*. Since ArcZ121 binds Hfq *in vivo* (Wassarman *et al.* 2001; Zhang *et al.* 2003), and RNase E is known to participate in *arcB* degradation (Aiso and Ohki 2003), and this study demonstrated ArcZ121 metabolism is mediated primarily by RNase E, it is possible that ArcZ121 functions stoichiometrically to negatively regulate *arcB* in a Hfq-RNase E dependent manner (Aiba 2007).

Accordingly, in the absence of Hfq, ArcZ121 mediated regulation of *arcB* would be inhibited and both ArcZ121 and *arcB* would be stabilized relative to a wild type control. Both the Soper *et al.* (2010) and Mandin *et al.* (2010) experiments were done with exponentially growing cultures, and under these conditions, *arcB* was not detected in wild type cells even under anaerobic conditions, when ArcB is known to function (Gunsalus and Park 1994). The results from this study were derived from early-stationary phase cells. Since, the cultures used in this study were grown with a 5:1 headspace, it is possible that at the time of harvesting, the cultures were slightly anaerobic. If this was true and there was a moderate induction of *arcB*, then it is possible that the observed increase in ArcZ121 steady-state levels in the *Δhfq* mutant was due to inhibition of *arcB* negative regulation by ArcZ121.

Summary

Consistent with predictions from the literature, this study found that the metabolism of two trans-encoded, Hfq-binding sRNAs was primarily dependent upon RNase E. This dependence upon RNase E was observed in the absence of steady-state analysis or chemical half-life determinations, thus demonstrating that a relative frequency analysis can be an effective way to study sRNA metabolism *in vivo*. However, in addition to demonstrating an involvement of RNase E, this study also implicated PNPase in the decay of both sRNAs and discovered a

significant involvement of RNase P in RprA metabolism. Furthermore, the *Δhfq* allele and the truncation alleles of RNase E did not produce identical defects in ArcZ and RprA metabolism. When taken together, the results of this study clearly demonstrate that sRNA metabolism is more complex than currently acknowledged.

Table 2.1. Bacterial strains used in this study.

Strain Designation	Strain Number	Genotype	Source or Reference
wild type	MG1693*	*thyA715, λ-, rph-1*	*E. coli* Genetic Stock Center, Yale University
rne-1	SK5665	*thyA715, λ-, rph-1, rne-1*	Arraiano *et al.* (1988)
rneΔ91	SK2684	*thyA715, λ-, rph-1, srlD300::*Tn*10, recA56, rneΔ1018::bla, pDHK4* (*rneΔ91*)	Chung and Kushner (unpublished results)
rneΔ374	SK2683	*thyA715, λ-, rph-1, srlD300::*Tn*10, recA56, rneΔ1018::bla, pDHK3* (*rneΔ374*)	Chung and Kushner (unpublished results)
rneΔ645	SK2685	*thyA715, λ-, rph-1, srlD300::*Tn*10, recA56, rneΔ1018::bla, pDHK6* (*rneΔ645*)	Ow *et al.* (2000)
rnpA49	SK2525	*thyA715, λ-, rph-1, rbsD296::*Tn*10, rnpA49*	Ow *et al.* (2002)
Δrng	SK2538	*thyA715, λ-, rph-1, rng::cat*	Ow *et al.* (2003)
ΔrnlA2	SK3170	*thyA715, λ-, rph-1, rnlA2::kan*	Perwez and Kushner (unpublished results)
Δrnz	SK4477	*thyA715, λ-, rph-1, Δrnz::apr*	Maples and Kushner (unpublished results)
rnc-14	SK4455	*thyA715, λ-, rph-1, rncΔ14::*Tn*10*	Stead *et al.* (in press)
pnp-7	SK5691	*thyA715, λ-, rph-1, pnp-7*	Arraiano *et al.* (1988)
Δrnb	CMA201	*thyA715, λ-, rph-1, Δrnb::*Tn*10*	Piedade *et al.* (1995)
Δrnr	SK2059	*thyA715, λ-, rph-1, rnr::kan*	Mohanty *et al.* (2010)
ΔrppH	SK4390	*thyA715, λ-, rph-1, ΔrppH::kan*	Mohanty *et al.* (2010)
hfq-10	SK10246	*thyA715, λ-, rph-1, hfq-10::cat*	Mohanty *et al.* (2004)

*MG1693 is a *thyA715* derivative of MG1655

Table 2.2. Oligonucleotides used in this study.

Oligo Name	Purpose	Sequence
arcZ_Northern_3'	Northern blotting	5' TAGACCGGGGTGCGCGAATA
rprA_Northern_PE_1	Northern blotting and primer extension	5' ATCGTGGGAGATGGGCAAAGACTA
arcZ_SeqTemp_5'	Sequencing	5' AATGGTACGCATCACACATTTAA
arcZ_SeqTemp_3'	Sequencing	5' TCACGACGTAGAAGTGCTGAAA
rprA_SeqTemp_5'	Sequencing	5' AGACGAATCTGATCGACGCCAAAAA
rprA_SeqTemp_3'	Sequencing	5' AAGAGAGTCACAGTATCTTGTGCAA
arcZ_PE_1	Primer extension	5' CTAGACCGGGGTGCGCGAATACT
arcZ_PE_2	Primer extension	5' ATCTTGGCTGCGCCGTAAATTATT

Table 2.3. Analysis of ArcZ levels in various strains of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*Δrng*	*ΔrnlA2*	*Δrnz*	*Δrnc-14*	*pnp-7*	*Δrnb*	*Δrnr*	*ΔrppH*	*hfq-10*
Species											
αArcZ121	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.01 (±0)	1.00 (±0)
αArcZ55	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.99 (±0)	0 (±0)
Relative Quantity (RQ)	1.00 (±0)	0.98 (±0.05)	1.09 (±0.01)	0.91 (±0.01)	1.12 (±0.04)	0.89 (±0.04)	0.73 (±0.03)	0.91 (±0.03)	0.87 (±0.02)	2.16 (±0.13)	0.02 (±0.01)
Column	1	2	3	4	5	6	7	8	9	10	11

The relative frequency (α) of each ArcZ RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total ArcZ RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 2.4. Analysis of ArcZ levels in various strains of *E. coli* at 44°C.

Genotype	wild type	*rne-1*	*rnpA49*
Species			
αArcZ121	0.01 (±0)	0.63 (±0.02)	0.01 (±0)
αArcZ55	0.99 (±0)	0.37 (±0.02)	0.99 (±0)
Relative Quantity (RQ)	0.61 (±0.05)	0.18 (±0.03)	1.39 (±0.01)
Column	1	2	3

The relative frequency (α) of each ArcZ RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total ArcZ RNAs was determined relative to the amount detected in the wild type control (Table 2.3, column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at lest two independent experiments.

Table 2.5. Analysis of ArcZ levels in various RNase E mutants of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*rneΔ91*	*rneΔ374*	*rneΔ645*
Species					
αArcZ121	0.01 (±0)	0.01 (±0)	0.01 (±0)	0.10 (±0.01)	0.32 (±0)
αArcZ55	0.99 (±0)	0.99 (±0)	0.99 (±0)	0.90 (±0.01)	0.68 (±0)
Relative Quantity (RQ)	1.00 (±0)	0.98 (±0.05)	0.94 (±0.09)	0.97 (±0.17)	1.03 (±0.20)
Column	1	2	3	4	5

The relative frequency (α) of each ArcZ RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total ArcZ RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 2.6. Analysis of RprA levels in various strains of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*Δrng*	*ΔrnlA2*	*Δrnz*	*rnc-14*	*pnp-7*	*Δrnb*	*Δrnr*	*ΔrppH*	*hfq-10*
Species											
αRprA105	0.09 (±0.01)	0.14 (±0.01)	0.09 (±0.01)	0.09 (±0)	0.11 (±0)	0.07 (±0.01)	0.05 (±0.01)	0.11 (±0.03)	0.07 (±0.03)	0.14 (±0.01)	1.00 (±0)
αRprA45	0.91 (0.01)	0.86 (±0.01)	0.91 (±0.01)	0.91 (±0)	0.89 (±0)	0.93 (±0.01)	0.95 (±0.01)	0.89 (±0.03)	0.93 (±0.03)	0.86 (±0.01)	0 (±0)
Relative Quantity (RQ)	1.00 (±0)	0.57 (±0.08)	2.40 (±0.40)	1.20 (±0.13)	1.08 (±0.15)	1.51 (±0.24)	0.23 (±0.04)	0.53 (±0.06)	0.86 (±0.02)	2.14 (±0.49)	0.05 (±0.01)
Column	1	2	3	4	5	6	7	8	9	10	11

The relative frequency (α) of each RprA RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total RprA RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 2.7. Analysis of RprA levels in various strains of *E. coli* at 44°C.

Genotype	wild type	*rne-1*	*rnpA49*
Species			
αRprA105	0.09 (±0.02)	0.60 (±0.02)	0.19 (±0.03)
αRprA68-81	0.05 (±0.02)	0.28 (±0.01)	0.01 (±0)
αRprA45	0.86 (±0.05)	0.12 (±0.02)	0.80 (±0.03)
RQ	2.74 (±0.21)	3.51 (±0.73)	5.01 (±0.72)
Column	1	2	3

The relative frequency (α) of each RprA RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total RprA RNAs was determined relative to the amount detected in the wild type control (Table 2.6, column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 2.8. Analysis of RprA levels in various RNase E mutants of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*rneΔ91*	*rneΔ374*	*rneΔ645*
Species					
αRprA105	0.09 (±0.01)	0.14 (±0.01)	0.14 (±0.01)	0.12 (±0)	0.13 (±0.01)
αRprA68-81	ND	ND	0.05 (±0.01)	0.07 (±0.01)	0.01 (±0.01)
αRprA45	0.91 (±0.01)	0.86 (±0.01)	0.81 (±0.02)	0.81 (±0.01)	0.86 (±0.02)
RQ	1.00 (±0)	0.57 (±0.08)	0.96 (±0.17)	1.40 (±0.23)	0.69 (±0.15)
Column	1	2	3	4	5

The relative frequency (α) of each RprA RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total RprA RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments. ND = Not detected.

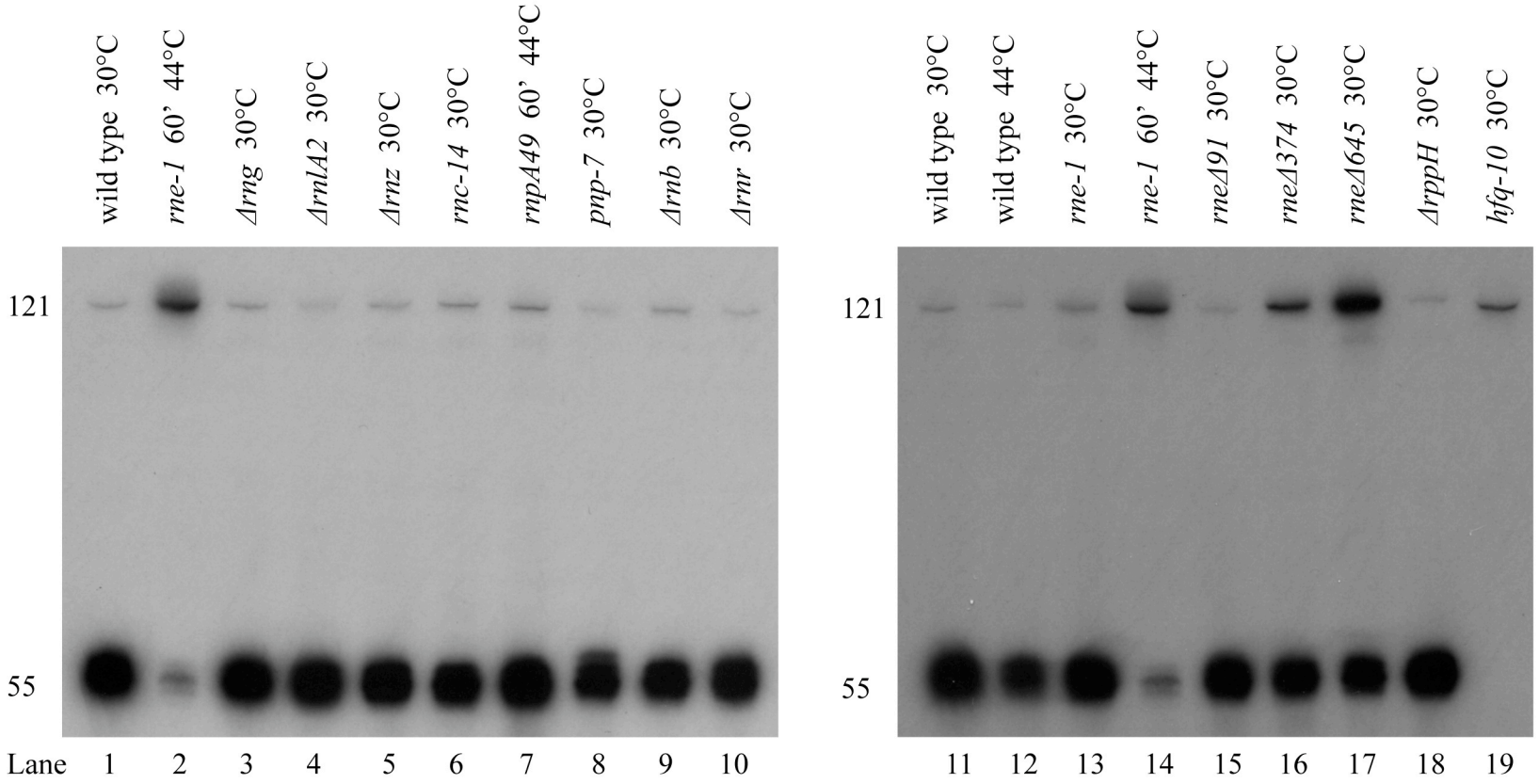

Figure 2.1. Northern blot analysis of ArcZ. All lanes contain 10 μg of total steady-state RNA harvested from early stationary phase cells except for *hfq-10* (30 μg). The sizes of the ArcZ RNAs were estimated from electrophoretic mobility based on the migration of both a RiboRuler low range RNA ladder (Fermentas) and a low molecular weight marker, 10-100 nt (USB). The estimated sizes were in excellent agreement with the ends and lengths reported by Mandin and Gottesman (2010).

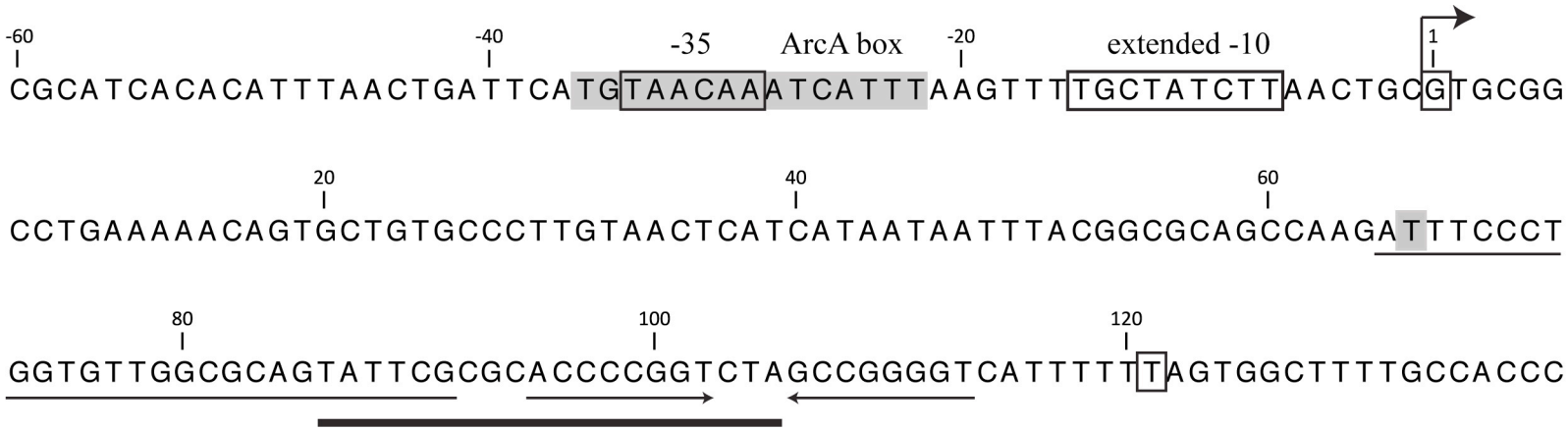

Figure 2.2. The *arcZ* region of the *E. coli* genome. The nucleotide sequence of the *arcZ* gene is annotated with features based on Mandin and Gottesman (2010). The putative promoter including an extended -10 sequence is boxed in. The ArcA box consensus sequence that overlaps the -35 sequence is shaded. The single boxed nucleotides correspond to the annotated ends of the full-length ArcZ, and the box with the bent arrow represents the putative start of transcription. The shaded nucleotide is the 5' end of the short form of ArcZ. The inverted arrows represent the Rho-independent transcription terminator. The thin black line represents the area of ArcZ that is complementary to *rpoS* (Mandin and Gottesman 2010), *tpx*, *sdaC* and STM3216 (Papenfort *et al*. 2009). The thick black line shows the region of ArcZ that the Northern probe was designed against.

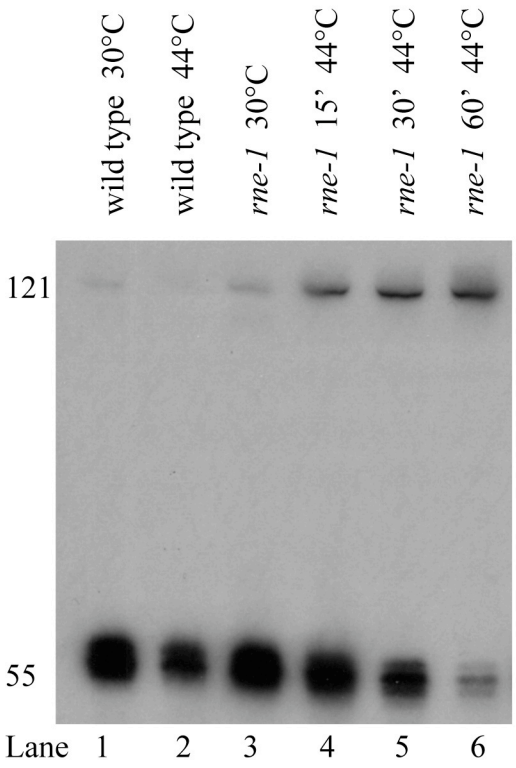

Figure 2.3. Northern blot time course analysis of ArcZ. The gel was loaded as described in Figure 2.1. Samples of the *rne-1* mutant were collected 15, 30 and 60 minutes after a shift to 44°C.

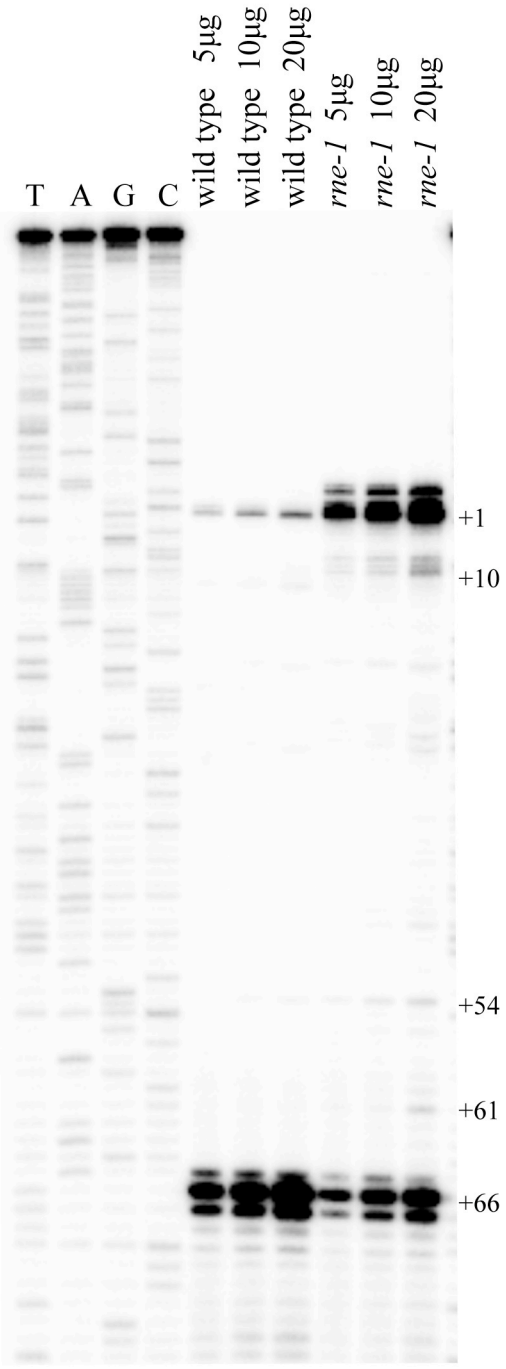

Figure 2.4. Primer extension and sequencing reactions for ArcZ. The primer extension reactions were done with total steady-state RNA harvested from early stationary phase wild type cells grown at 30°C and the *rne-1* mutant that was shifted to 44°C for 60 minutes. Three concentrations (5 µg, 10 µg and 20 µg) of RNA were used to establish concentration dependent ends. The numbers to the right of the primer extension lanes correspond to the nucleotide position relative to the annotated start of transcription (Figure 2.2). The reactions were done with oligonucleotide ArcZ_PE_1 (Table 2.2).

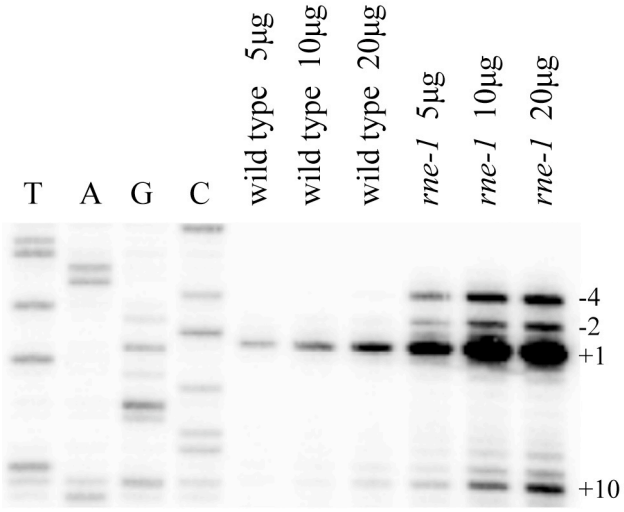

Figure 2.5. Primer extension and sequencing reactions for ArcZ. The primer extension reactions were done as described in Figure 2.4. The numbers to the right of the primer extension lanes correspond to the nucleotide position relative to the annotated start of transcription (Figure 2.2). The reactions were done with oligonucleotide ArcZ_PE_2 (Table 2.2).

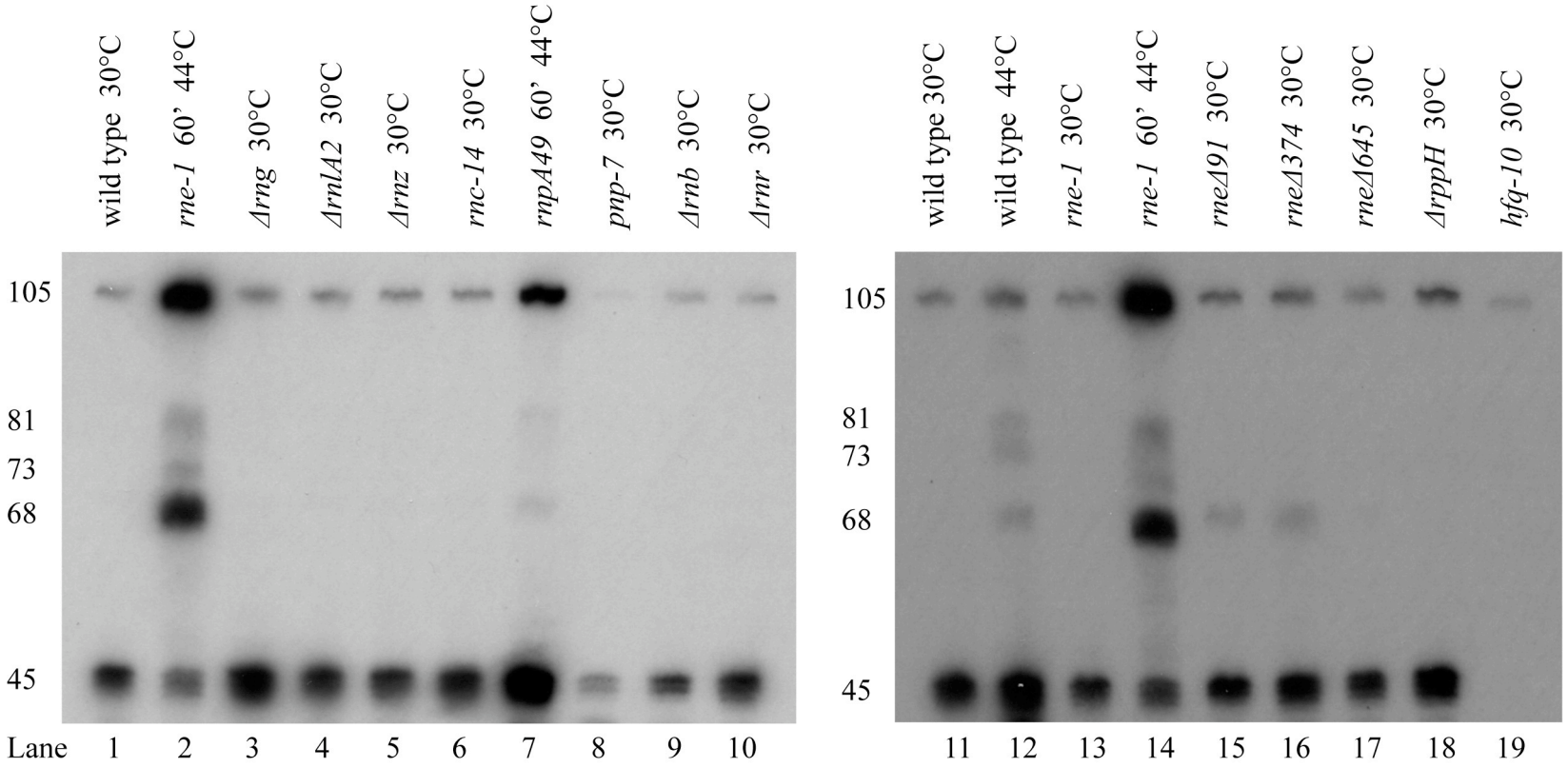

Figure 2.6. Northern blot analysis of RprA. The gels were loaded as described in Figure 2.1. The sizes of the RprA RNAs were estimated from electrophoretic mobility based on the migration of both a RiboRuler low range RNA ladder (Fermentas) and a low molecular weight marker, 10-100 nt (USB).

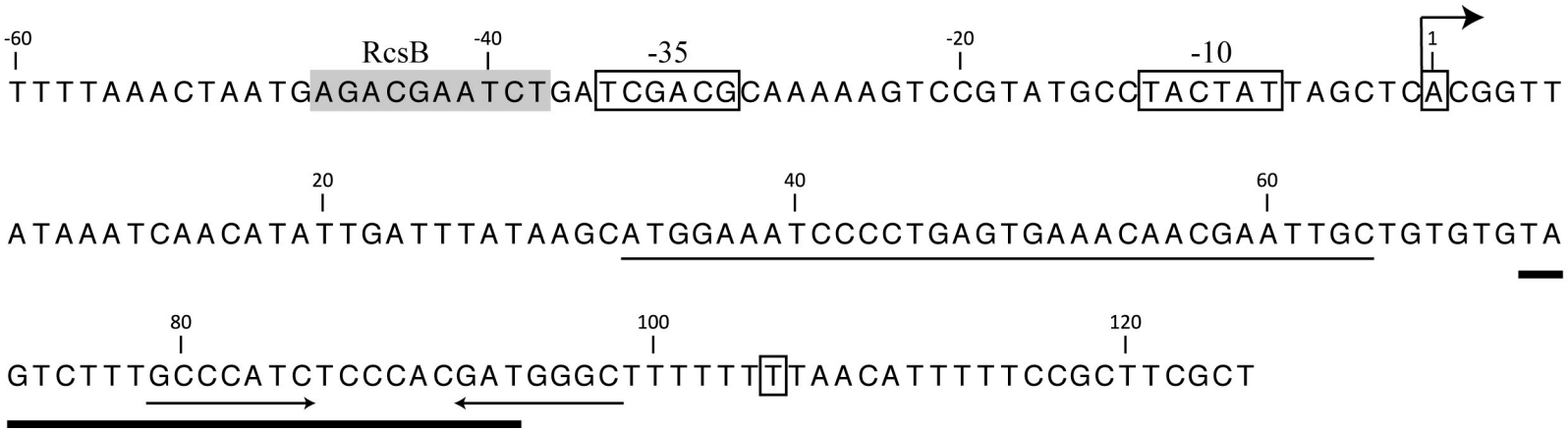

Figure 2.7. The *rprA* region of the *E. coli* genome. The nucleotide sequence of the *rprA* gene is annotated with features based on Majdalani *et al*. (2001) and Majdalani *et al*. (2002). The putative promoter is boxed in. The RcsB transcription factor binding site is shaded and labeled. The single boxed nucleotides represent the annotated ends of *rprA*. The box with the arrow represents the putative start of transcription. The inverted arrows represent the Rho-independent transcription terminator. The thin black line represents the area of RprA that is complementary to *rpoS*. The thick black line shows the region of *rprA* that the Northern probe and the oligonucleotide used in the primer extension reactions was designed against.

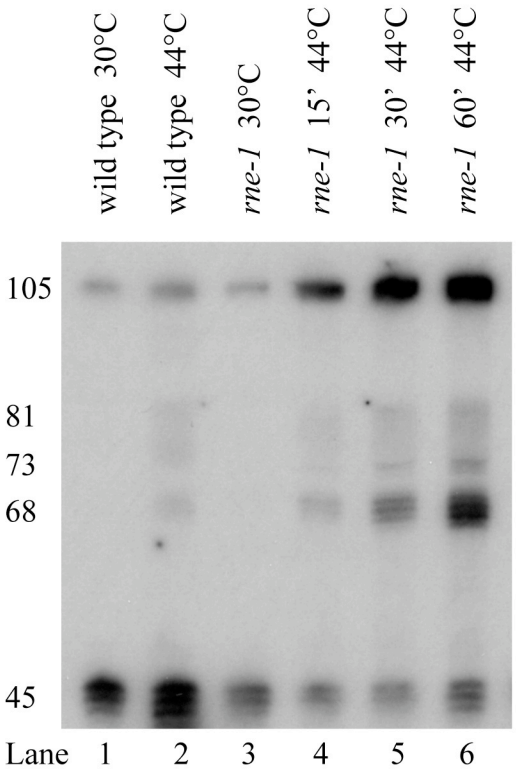

Figure 2.8. Northern blot time course analysis of RprA. The gel was loaded as described in Figure 2.1 and Figure 2.3.

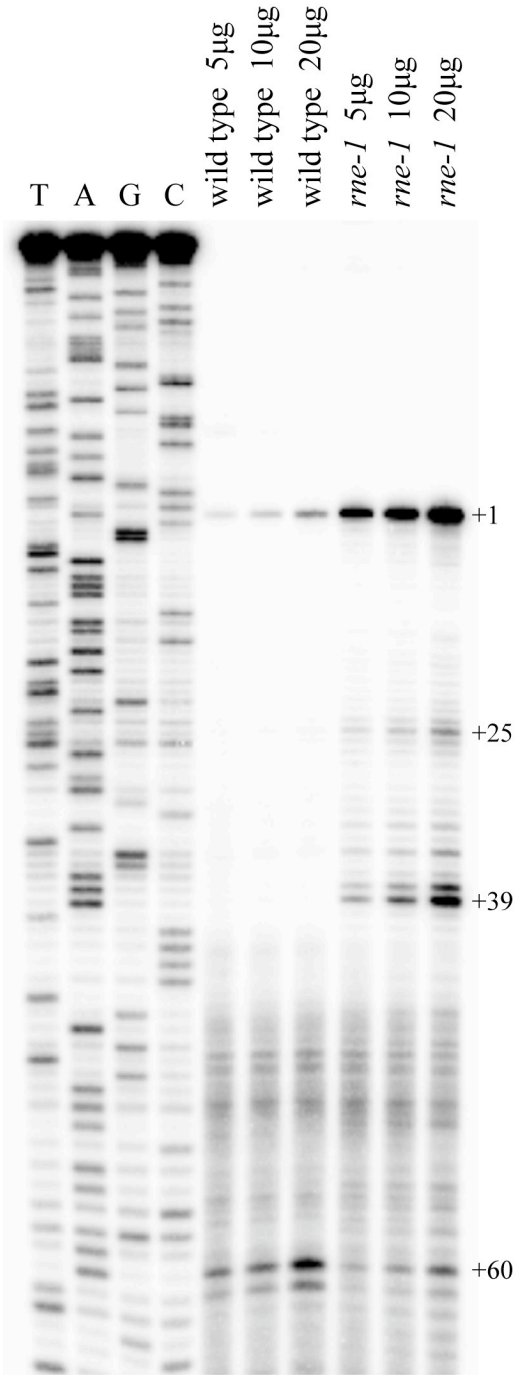

Figure 2.9. Primer extension and sequencing reactions for RprA. The primer extension reactions were done as described in Figure 2.4. The numbers to the right of the primer extension lanes correspond to the nucleotide position relative to the annotated start of transcription (Figure 2.7). The reactions were done with oligonucleotide RprA_Northern_PE_1 (Table 2.2).

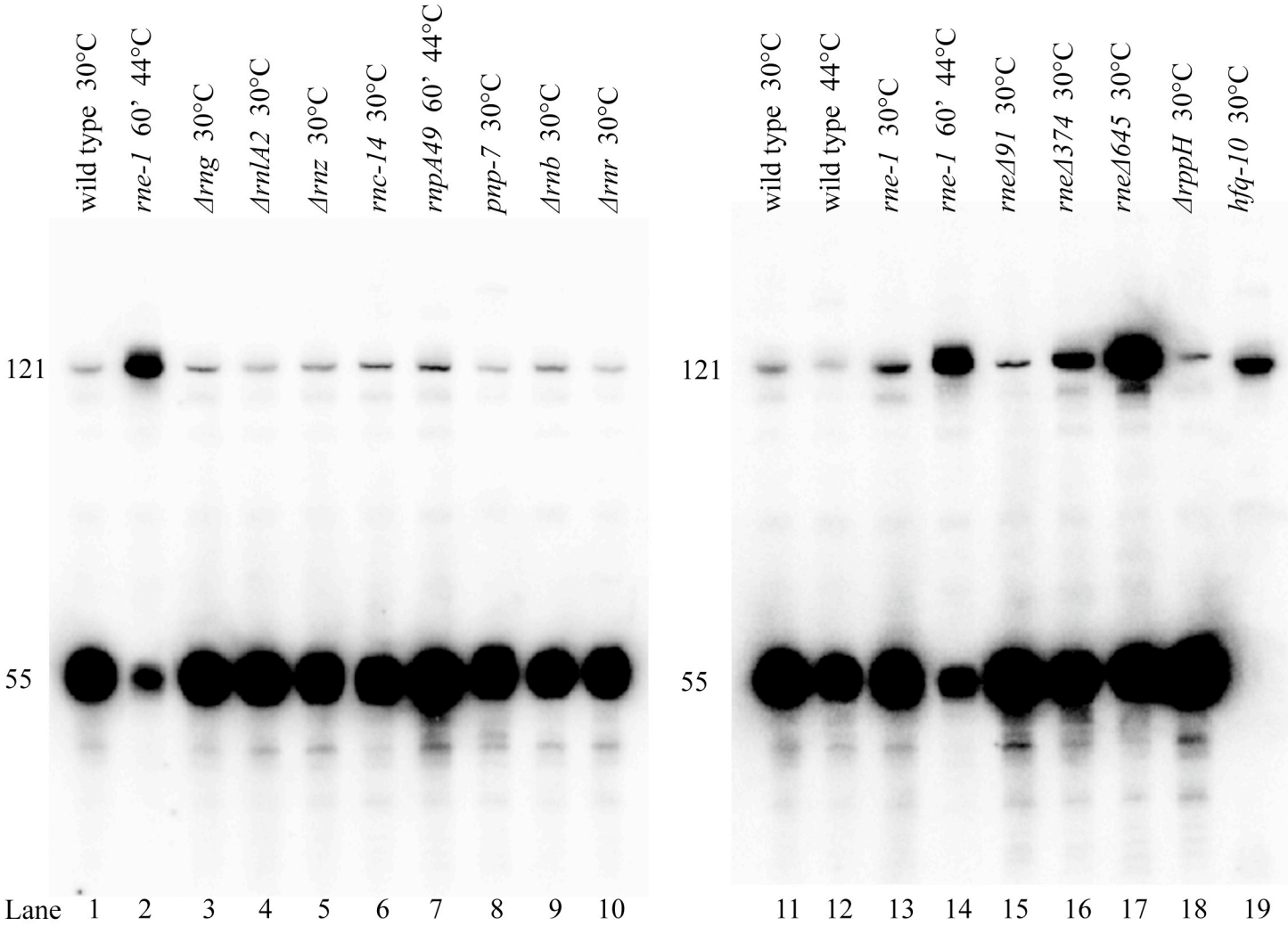

Figure S.2.1. Overexposed Northern blots of ArcZ shown in Figure 2.1.

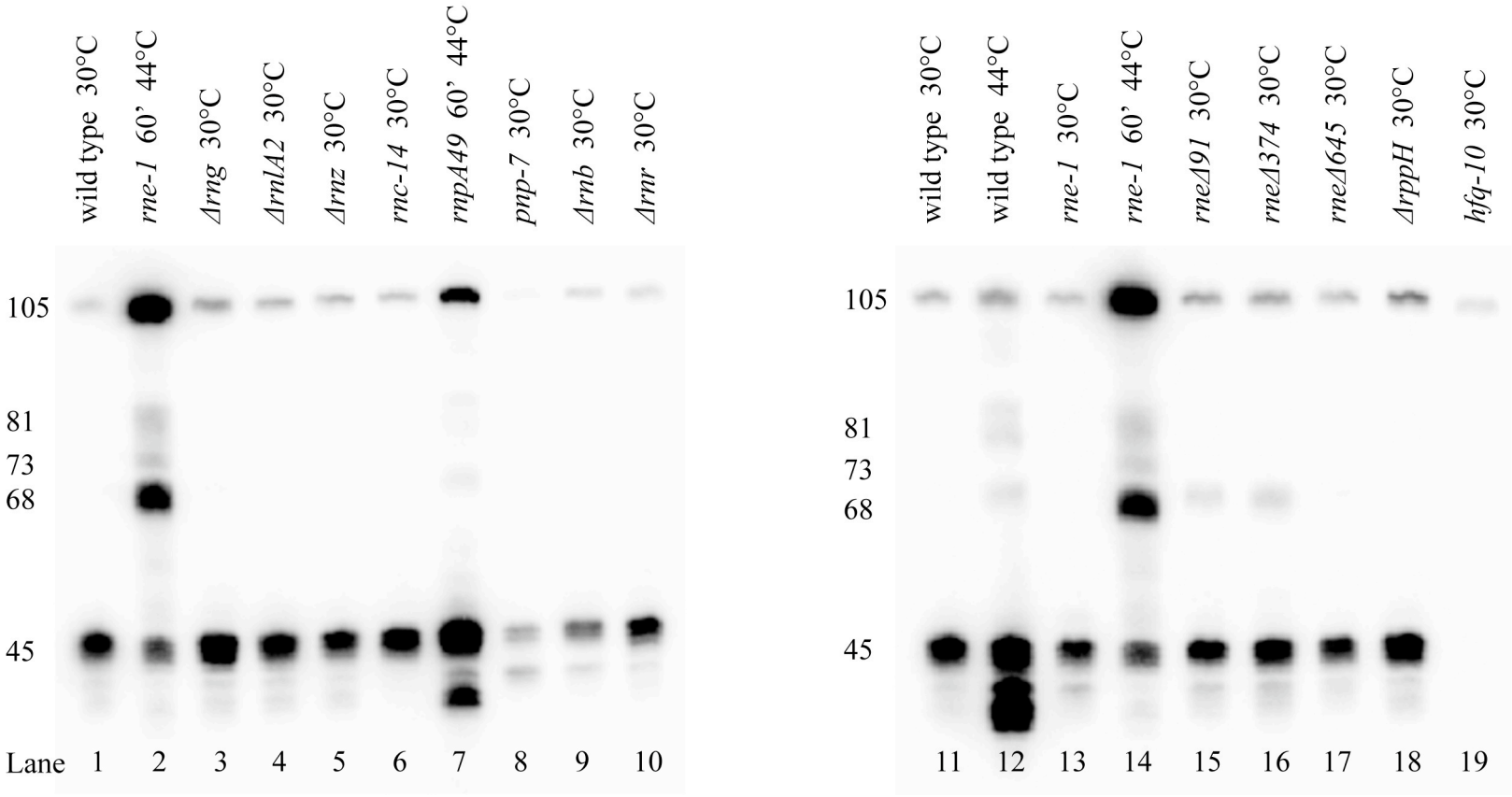

Figure S.2.2. Overexposed Northern blots of RprA shown in Figure 2.6.

CHAPTER 3

Multiple Pathways for the Metabolism of Cis-encoded sRNAs[2]

[2] Marshburn, S. and Kushner, S.R. To be submitted to *Nucleic Acids Research*.

Introduction

Using the same rationale and approach as described in Chapter 2, here we have focused on the metabolism of cis-encoded sRNAs. Generally, sRNAs are classified based on the genomic position of the sRNA gene relative to the target gene as this has structural and mechanistic implications (Waters and Storz 2009). Cis-encoded sRNA genes are antisense to their target gene(s). Because they are perfectly complementary to their target at the region of overlap, they have the potential to form long, perfect duplex structures with their target(s). In contrast, trans-encoded sRNA genes do not overlap their target gene(s) and typically form short, imperfect duplexes with their targets. Published studies support the expectation that cis-encoded sRNA metabolism involves RNase III (Gerdes *et al.* 1992; Franch *et al.* 1999; Vogel *et al.* 2004), whereas trans-encoded, Hfq-bound sRNA metabolism is primarily dependent upon RNase E (Masse *et al.* 2003a; Moll *et al.* 2003a; Morita *et al.* 2005).

In *E. coli*, there are two chromosomally encoded and functionally characterized cis-encoded sRNAs that exhibit multiple forms on Northern blots in wild type cells, GadY and SibC (Opdyke *et al.* 2004; Fozo *et al*. 2008). The *gadY* gene overlaps the 3'UTR of *gadX* and stabilizes *gadX* by presumable inhibiting ribonuclease mediated decay (Opdyke *et al.* 2004). The *gadX* gene lacks a conical Rho-independent transcription terminator and on Northern blots a *gadXW* dicistronic transcript has been observed in wild type cells (Tramonti *et al.* 2008). GadY is also complementary to this dicistronic RNA and is thought to induce processing at two positions within the intracistronic region. Thus, GadY can act as a stabilizer or promote cleavage depending on the target.

SibC is thought to cause the rapid destabilization of its target *ibsC* mRNA (Fozo *et al*. 2008). In wild type cells, *ibsC* is not visible on Northern blots. However, in the absence of *sibC*

ibsC accumulates, which is consistent with SibC promoting the rapid decay of *ibsC*. For these sRNAs, the shorter forms are also complementary to their targets and could theoretically be functional forms. However, it is assumed in the literature that the longer forms of both these sRNAs are the functional moieties and the shorter species represent stable breakdown products (Opdyke *et al.* 2004; Fozo *et al*. 2008, Han *et al*. 2010). The results described below show an involvement of RNase E, RNase III, RNase G, RNase P, RNase II and PNPase in the metabolism of SibC and GadY.

Materials and Methods

Strains and growth conditions

The *Escherichia coli* MG1693 wild type strain and isogenic mutant derivatives used in this study are listed in Table 3.1. For RNA isolation purposes, one mL of a standing, overnight culture was used to inoculate 25 mL of Luria-Bertani broth supplemented with thymine (50 μg/mL) in 125 mL Klett flasks. Cultures were grown in a water bath with vigorous aeration at either 30°C or 44°C. Strains harboring temperature sensitive alleles (*rne-1* and *rnpA49*) were grown at 30°C and then shifted to 44°C for 30 minutes or 60 minutes before harvesting. Aliquots were taken when cells entered stationary phase, i.e. the point in the growth phase where the change in cell density equaled zero. Growth was monitored by a Klett-Summerson colorimeter (green filter, no. 42).

RNA isolation

When cells entered stationary phase, two mL of culture were mixed with eight mL of frozen TM buffer (10 mM Tris pH 7.2, 5 mM $MgCl_2$, 20 mM NaN_3 and 0.4 mg/mL chloramphenicol). Suspensions were spun at 3000 x g for 10 minutes to pellet the cells. Each supernatant was

immediately decanted, and the cell pellets were snap-frozen in a dry ice/ethanol slurry. Frozen pellets were briefly thawed at 37°C (for approximately 10 seconds) and then immediately resuspended in one mL of Trizol© (Invitrogen). RNA was extracted according to the manufacturer's recommendations. After extraction, the RNA was purified and concentrated by sodium acetate precipitation using 80% ethanol during the precipitation and wash. The RNA concentrations were quantified from A_{260} nm measurements using a Nanodrop 2000c (Thermo Scientific).

Northern blotting

Ten µg of total steady-state RNA was mixed with an equal volume of Gel Loading Buffer II (Ambion) and prepared according to the manufacturer's recommendations. Samples were resolved on prewarmed 8%/8.3M urea polyacrylamide gels in 1 x TBE. RNA was transferred to Nytran SPC membranes (Whatman) at 4°C in a tank blotter in 1 x eTAE at 50V for 60 minutes. After transfer, membranes were baked at 80°C for 30 minutes, the UV crosslinked. Membranes were washed with 1 x SSC/0.1% SDS at 65°C, then prehybridized in PerfectHyb buffer (Sigma) at 50°C before the addition of probe. The oligonucleotide probes used in this study were GadY: 5' TAGGGGACCGGGAAGAGGATAGT, SibC: 5' TCAGTCTCAGGGGAGGAGCAAT and IbsC: 5' ATTGCTCCTCCCCTGAGACTGA. The probes were end-labeled with T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's recommendations. Hybridizations were carried out overnight at 50°C. Membranes were washed twice in 2 x SSC/0.1% SDS at room temperature then twice in 2 x SSC/0.1% SDS at 50°C.

Quantification of Northern blot data

Signals on Northern blots were captured on a phosphor screen (Molecular Dynamics) and were visualized on a Storm 840 scanner (GE Healthcare). The signal intensities of each band were

calculated using ImageQuant TL v7 software (GE Healthcare). For each strain, the relative frequency (α) of each RNA species was calculated (relative frequency = [signal intensity of individual species/total signal intensity of all species]). To approximate the relative quantity (RQ) of the GadY and SibC RNAs in the mutants relative to the wild type control at 30°C, the total signal intensity of all species was normalized to 5S rRNA levels in the wild type control at 30°C and then divided by the total signal intensity of all species in the wild type control at 30°C. 5S rRNA signal intensities were calculated for each strain from Northern blots prepared in the same way with the same total RNA but using only 500 ng of total RNA in each lane.

Results

Rationale for studying GadY metabolism

In wild type *E. coli*, we observed three GadY RNA species on Northern blots: GadY105, GadY90 and GadY59 (the number corresponds to the length of the RNA in nucleotides) (Figure 3.1, lane 1). The electrophoretic mobilities of these species were in excellent agreement with the 5' and 3' ends mapped by primer extension and RACE (Opdyke *et al.* 2004). All three species have the same 3' end but different 5' ends (Figure 3.2). Mutation of the putative -10 sequence located six nucleotides upstream of the most proximal mapped 5' end resulted in the loss of all three forms (Opdyke *et al.* 2004). This result suggested the production of GadY90 and GadY59 was dependent upon the synthesis of GadY105. When combined with the end-mapping data, these data indicated that the generation of GadY90 and GadY59 from GadY105 proceeded in a 5' to 3' direction. Since the GadY RNAs did not arise via alternative transcription initiation, and the shorter GadY RNAs likely arose through cleavage events progressing in the 5' to 3' direction, GadY was an excellent candidate for our analysis of sRNA processing/decay.

Relative abundance of GadY RNAs in wild type and mutant strains

In MG1655 the three GadY RNAs exhibited a growth phase specific accumulation pattern on Northern blots (Opdyke *et al.* 2004). The three forms were largely absent in exponentially growing cells but accumulated as cells enter stationary phase. As the cells progressed through stationary phase, there was a dramatic decline in the amount of GadY105 and GadY59 but no apparent change in GadY90 levels. Transcription of *gadY* is dependent upon RpoS, the stationary phase sigma factor, and is positively regulated by one of GadY's targets, GadX (Opdyke *et al.* 2004; Tramonti *et al.* 2008). Since *gadY* was transcribed in early stationary phase and both GadY90 and GadY59 were present, these observations suggested GadY105 was cleaved at this time. Thus, we chose early stationary phase to investigate the metabolism of the GadY RNAs.

On Northern blots, the relative quantities (RQ) of the GadY RNAs were significantly different in the strains used in this study (Figure 3.1, Tables 3.2-3.4). This variation was affected by both temperature and the mutations tested. When wild type cells were grown at 44°C, there was an approximate 86% reduction in the total amount of GadY RNAs relative to cells grown at 30°C (Figure 3.1, lanes 11, 12 and Table 3.3, column 1). However, there were only minor changes in the α values, which suggested the relative stabilities of the three GadY RNAs were not significantly altered by the change in temperature (Tables 3.2, 3.3).

In addition to temperature, some of the mutations affected the RQ of the GadY RNAs in a temperature independent manner. At 30°C, the *rne-1*, *hfq-10*, *rneΔ645*, *pnp-7*, *rnc-14* and *rne-1 rnc-14* mutants all exhibited reductions in the RQ (Figure 3.1, lanes 1, 6, 8, 11, 13, 15, 16, 21, 23, Table 3.2, columns 2, 6, 7, 8, 12 and Table 3.4, columns 2, 5). The effects observed in the *rne-1* and *hfq-10* mutants were consistent with published reports (Figure 3.1, lanes 11, 13, 23 and Table 3.2 columns 2, 12) (Opdyke *et al.* 2004; Takada *et al.* 2007). Both the *rne-1* and *rneΔ645*

single mutants exhibited similar reductions in GadY levels, 59% and 70%, respectively (Figure 3.1, lanes 11, 13, 21 and Table 3.4, columns 2, 5). The *rne-1* allele is a thermolabile point mutation in the N-terminal catalytic domain of RNase E that is partially defective at 30°C (Arraiano *et al.* 1988). The *rneΔ645* allele is a complete truncation of the C-terminal scaffolding domain and the arginine-rich RNA binding domain (Ow *et al.* 2000). This allele is partially defective in the decay of some mRNAs (Ow *et al.* 2000). In contrast, the *rneΔ374* allele (Ow *et al.* 2000), which lacks only the C-terminal degradosome scaffolding region, did not cause a reduction in GadY RNAs (Figure 3.1, lanes 11, 20 and Table 3.4, column 4). Therefore, the defect observed in the *rne-1* and *rneΔ645* mutants likely stemmed from a deficiency in RNase E catalytic activity.

Interestingly, a deficiency in polynucleotide phosphorylase (PNPase) activity also affected the RQ of GadY RNAs (Figure 3.1, lanes 1, 8 and Table 3.2, column 8). PNPase physically interacts with the scaffolding domain of RNase E to form the degradosome (Carpousis 2007). However, the effect observed in the *pnp-7* mutant was apparently independent of degradosome assembly, since a similar reduction was not observed in the *rneΔ374* strain (Figure 3.1, lanes 1, 8, 11, 20, Table 3.2, column 8 and Table 3.4, column 4). Both the *rne-1* and *rnc-14* alleles caused a reduction in the total amount of GadY RNAs (Figure 3.1, lanes 11, 13, 15 and Table 3.2, columns 2, 6). However, these effects appeared to be independent, because the effect of combining the alleles was additive leading to a 77% reduction in the RQ (Figure 3.1, lanes 11, 13, 15, 16 and Table 3.2, columns 2, 6, 7). The effect of the *rne-1* and *rnpA449* alleles was also independent of the defect observed at 44°C, since at the higher temperature, these two alleles exacerbated the reduction in the RQs of GadY RNAs (Figure 3.1, lanes 7, 12, 14 and Table 3.3,

columns 2, 3). The *rnpA49* allele is a well-studied thermolabile allele of the protein component of RNase P (Ow and Kushner 2002).

Not all of the mutants negatively affected GadY levels. In some cases, the changes, whether positive or negative, were not significant, i.e. in the *rneΔ91*, *ΔrppH*, *rneΔ374*, *ΔrnlA2*, *Δrnz*, *Δrnb* and *Δrnr* single mutants (Figure 3.1, lanes 1, 4, 5, 9, 10, 11, 19, 20, 22, Table 3.2, columns 4, 5, 9, 10, 11 and Table 3.4, columns 3, 4). The largest observed increase was in the *Δrng* mutant with approximately two-fold more total GadY RNAs than the wild type control (Figure 3.1, lanes 1, 3 and Table 3.2, column 3).

When taken together, the RQ values showed that the total amount of the GadY RNAs was affected by both temperature and specific ribonucleases, but that these changes did not correlate with cellular growth rates. For example, there was a decrease in GadY levels at 44°C, although cells grew faster at 44°C than at 30°C, whereas there was a small increase in GadY amounts in the *rneΔ374* strain, although the strain grew more slowly than the wild type control (data not shown).

Exoribonuclease involvement in GadY metabolism

If GadY90 and GadY59 are generated by ribonuclease mediated cleavage of GadY105, and all three GadY RNAs have the same 3' end, then exoribonucleases were not expected to mediate these metabolic events, since all of the known *E. coli* exoribonucleases proceed in a 3'→5' direction (Li and Deutscher 2004). However, these facts do not necessarily mean that exoribonucleases could not mediate some aspects of GadY metabolism. Exoribonucleases could affect GadY105, GadY90 and/or GadY59 decay pathways that do not result in the production of the shorter, stable species.

In the absence of either RNase R (*Δrnr*) or RNase II (*Δrnb*), the relative frequencies, αGadY105, αGadY90 and αGadY59, were essentially the same as in wild type cells grown at 30°C (Figure 3.1, lanes 1, 9, 10 and Table 3.2, columns 1, 9, 10). These results suggested the stabilities of the three GadY RNAs were not affected by the absence of either RNase R or RNase II. In contrast, inactivation of PNPase (*pnp-7*) led to significant changes in the α values such that αGadY105 decreased, αGadY90 marginally increased and αGadY59 increased, while the RQ of all GadY species decreased 54% (Figure 3.1, lanes 1, 8 and Table 3.2, columns 1, 8). The increase in αGadY59 suggested GadY59 was stabilized. If that were the case, an increase in αGadY59 should have caused a decrease in both αGadY90 and αGadY105, since these are relative frequencies that by definition sum to one, but only αGadY105 was reduced. The very modest increase (and not decrease) in αGadY90 may indicate that PNPase was involved in GadY90 decay. The significant reduction in αGadY105 could have resulted from the increase in both αGadY90 and αGadY59 or may have resulted from a destabilization of GadY105. On overexposed images, GadY breakdown products (<59 nt) accumulated (data not shown), again implicating PNPase in GadY metabolism.

Endoribonuclease involvement in GadY metabolism

If GadY105 is cleaved to generate GadY90 and GadY59 through removal of 5' sequences, then one or more of the endoribonucleases tested here was expected to mediate these events. To identify which endoribonucleases were involved in GadY105 metabolism, single mutants defective for RNase E (*rne-1*), RNase P (*rnpA49*), RNase G (*Δrng*), RNase Z (*Δrnz*), RNase LS (*ΔrnlA2*) and RNase III (*rnc-14*) were assessed for metabolic defects. We predicted that a complete block in processing would cause αGadY105 to approach one and the combined α values for GadY90 and GadY59 to approach zero.

Based on the data presented in Figure 3.1 and Table 3.2, it was unlikely that RNase LS or RNase Z participated in GadY RNA metabolism, since the αGadY105, αGadY90 and αGadY59 values in these mutants were not significantly different from the α values for wild type cells grown at 30°C (Figure 3.1, lanes 1, 4, 5 and Table 3.2, columns 1, 4, 5). In contrast, significant changes in the α values were observed in the *Δrng*, *rnpA49*, *rnc-14* and *rne-1* single mutants (Table 3.2, columns 1, 2, 3, 6 and Table 3.3, columns 1, 2, 3), suggesting roles for RNase G, RNase P, RNase III and RNase E in GadY RNA metabolism. In the *Δrng* single mutant, there was an increase in αGadY105, a decrease in αGadY90 and no apparent change to αGadY59 (Figure 3.1, lanes 1, 3 and Table 3.2, columns 1, 3). These results were consistent with a stabilization of GadY105 and a loss in production of GadY90. The stabilization of a GadY RNA of ~96 nt in the *Δrng* mutant was also in agreement with RNase G participating in the metabolism of longer GadY RNAs (Figure 3.1, lane 3).

In the case of RNase P, there was a significant reduction in the RQ of GadY RNAs in the *rnpA49* mutant (Figure 3.1, lanes 7, 12 and Table 3.3, columns 1, 3). However, RNase P may be involved in GadY RNA metabolism because αGadY105 increased and both αGadY90 and αGadY59 decreased (Figure 3.1, lanes 7, 12 and Table 3.3, columns 1, 3). These results may be interpreted as a stabilization of GadY105 resulting in the loss of formation of both GadY90 and GadY59. The role of RNase P in the metabolism of GadY RNAs may extend beyond the involvement in GadY processing, since on overexposed images of the Northern blots shown in Figure 3.1, smaller GadY RNAs (<59 nt) accumulated in this strain (data not shown).

Similar to what was observed in the *rnpA49* mutant, in both the *rnc-14* and *rne-1* single mutants there was a reduction in the RQ of GadY RNAs coupled with changes in the α values, which suggested both RNase III and RNase E were involved in GadY RNA metabolism (Figure

3.1, lanes 1, 2, 6, 11, 12, 13, 14, 15, Table 3.2, columns 1, 2, 6 and Table 3.3, columns 1, 2). RNase III has been implicated in the metabolism of other sRNAs that share extensive complementarity with their target mRNAs (Gerdes *et al*. 1992; Franch *et al.* 1999; Vogel *et al.* 2004). Consistent with this, in the *rnc-14* mutant there was an increase in αGadY105 along with decreases in both αGadY90 and αGadY59 (Figure 3.1, lanes 1, 6, 11, 15 and Table 3.2, columns 1, 6). When RNase E was inactivated at 44°C, there was no change to αGadY105, but a significant increase in αGadY90 and decrease in αGadY59 (Figure 3.1, lanes 2, 12, 14 and Table 3.3, columns 1, 2), which was consistent with a stabilization of GadY90 resulting in the loss of GadY59. In the *rne-1* mutant, RNase III, RNase G and RNase P were present, which may explain why a change in αGadY105 was not observed.

However, the role of RNase E in GadY metabolism may be more complex than the results at 44°C indicated, since even at 30°C the *rne-1* allele affected GadY metabolism. There was an increase in αGadY105, a decrease in αGadY90 and an increase in αGadY59 (Figure 3.1, lanes 11, 13 and Table 3.2, columns 1, 2), which suggested a stabilization of both GadY105 and GadY59 and loss in production of GadY90. When the *rne-1* and *rnc-14* alleles were combined there was an additive effect on the α values, which implied that these enzymes are functioning independently, but together are the primary enzymes necessary for the production of the GadY90 and GadY59 species. In the *rne-1 rnc-14* double mutant at 30°C, there was a greater increase in αGadY105 and decrease in both αGadY90 and αGadY59 than compared with either single mutant (Figure 3.1, lanes 11, 13, 15, 16 and Table 3.2, columns 1, 2, 6, 7). This effect was further amplified at 44°C and resulted in αGadY105 approaching one (Figure 3.1, lanes 11-18 and Table 3.2, columns 1, 2, 6, 7 and Table 3.3, columns 1, 2, 4, 5). Although this processing block occurred within 30 minutes, it was slightly exacerbated by prolonged exposure to the

higher temperature consistent with a role of RNase E in GadY metabolism (Figure 3.1, lanes 17, 18 and Table 3.3, columns 4, 5). Furthermore, in the *rne-1 rnc-14* double mutant there was a loss in GadY species <59 nt suggesting that both RNase E and RNase III were necessary for the further degradation of GadY RNAs (Figure S.3.1).

The relative frequency analysis suggested RNase G, RNase P, RNase E and RNase III participated in GadY metabolism. Consistent with RNase E and RNase P being involved in GadY105 metabolism, in the *ΔrppH* single mutant there was an increase in αGadY105, a decrease in αGadY90 and a marginal decrease in αGadY59 (Figure 3.1, lanes 11, 22 and Table 3.2, columns 1, 11). RppH is a RNA pyrophosphohydrolase, which removes a pyrophosphate group from the 5' terminus of a primary transcript to generate a 5' monophosphate group (Deana *et al.* 2008). Both RNase E and RNase P activity can be inhibited by a 5' triphosphate and may explain why GadY105 was stabilized in the *ΔrppH* mutant (Mackie 1998) (Bowden and Kushner, unpublished results).

Although the GadY105, GadY90 and GadY59 species were lost in the absence of Hfq (*hfq-10*) (Figure 3.1, lane 23), a ~85 nt GadY RNA accumulated. This species was also present in wild type and all of the mutants tested (Figure S.3.1). Accordingly, relative frequency calculations were done including this ~85 nt RNA, but its inclusion did not significantly effect the majority of the results presented in Tables 3.2-3.4

The detection of GadY RNAs separate from GadY105, GadY90 and GadY59 revealed additional complexities in GadY RNA metabolism. In addition to the three major species, in the *rnc-14* mutant, there was a smear above GadY105, representing GadY RNAs ranging in size between ~106 and ~110 nt (Figure 3.1, lanes 6, 15). This smear was also present in the *rne-1 rnc-14* double mutant at 30°C (Figure 3.1, lane 16). Although these longer forms existed,

GadY90 and GadY59 were still visible in these mutants (Figure 3.1, lanes 6, 15, 16). However, in the *rne-1 rnc-14* double mutant at 44°C, GadY105, GadY90 and GadY59 were absent, but GadY106-110, GadY93 and GadY62 were visible (Figure 3.1, lanes 17, 18 and Figure S.3.1, lanes 7, 8). These species were used to calculate the α values presented in Table 3.3. The shift up in size of the major GadY RNAs revealed an additional aspect of GadY metabolism that was manifested when normal metabolism was significantly impaired.

The effect of RNase E alleles on GadY metabolism

Since the *rne-1* allele appeared to have a significant effect on GadY metabolism at 30°C, three truncation alleles of RNase E were investigated for their role in GadY processing. In the absence of the arginine-rich RNA binding domain of RNase E (*rneΔ91*) (Ow *et al.* 2000), there was a modest change in the α values (Figure 3.1, lanes 11, 19, Table 3.4, columns 1, 3). This result suggested the RNA binding domain was largely dispensable for normal GadY metabolism, but might have led to a slight reduction in the formation of GadY90 and the stabilization of GadY59.

In the *rneΔ374* mutant there was a decrease in αGadY105, an increase in αGadY90 and no observed change in αGadY59 (Figure 3.1, lanes 1, 20 and Table 3.4, columns 1, 4), which was consistent with a stabilization of GadY90. It is difficult to interpret the reduction in αGadY105 in the *rneΔ374* mutant. As in the case of the *pnp-7* mutant, the reduction could represent destabilization of GadY105 or the reciprocal change due to the increase in αGadY90. PNPase does associate with RNase E via the scaffolding domain that is absent in the *rneΔ374* mutant (Carpousis 2007). Therefore, it is possible that the observed reduction in both mutants was related. There was no observed change in αGadY59 in the *rneΔ374* strain, although GadY90 appeared to be significantly stabilized. If some proportion of GadY59 came from cleavage of GadY90, then a decrease in αGadY59 was expected. Alternatively, it is possible that the *rneΔ374*

allele also caused a stabilization of GadY59 that masked a loss in production. If the *rneΔ374* mutant caused a stabilization of GadY59, this may suggest the stabilization effect of GadY59 observed in the *pnp-7* strain was dependent upon a functional degradosome.

The α values in the *rne-1* and *rneΔ645* single mutants were similar (Figure 3.1, lanes 13, 21 and Table 3.4, columns 2, 5). Since this pattern was different from what was observed in the *rneΔ374* mutant, these results suggested a defect in RNase E catalytic activity supersedes the structural component of RNase E in GadY metabolism.

Rationale for the analysis of SibC

Since the data described in Figure 3.1, Figure S.3.1, Table 3.2 and Table 3.3 showed a complex relationship among RNase E, RNase III, RNase P, RNase G, PNPase and RppH in GadY metabolism, we decided to examine the metabolism of a second cis-encoded sRNA, in particular, the *sib* genes (*sibA-E*), a family of five homologous repeats in MG1655 (Rudd 1999; Fozo *et al*. 2008). They share 59% identity at the nucleotide level and are highly similar both in length and predicted structure (Fozo *et al*. 2008). On Northern blots, each *sib* locus produced two Sib RNAs, ~140 nt and ~110 nt in length (Fozo *et al*. 2008). The *sib* genes are expressed constitutively, but accumulate when cells enter stationary phase and then decrease in late stationary phase. Similar to GadY (Opdyke *et al.* 2004), in late stationary phase, the shorter Sib RNAs disappeared, whereas the longer Sib RNAs were retained. An alignment of the *sib* genes showed a conserved σ^{70} promoter with 17 nucleotide spacing between the predicted -35 and -10 elements (Fozo *et al*. 2008). The two SibC RNAs have the same 5' end but different 3' ends (Fozo *et al*. 2008) (Figure 3.3). The *sib* genes do not contain a conical Rho-independent transcription terminator. However, the most distal 3' end is 2-4 nt downstream of a stable conserved stem-loop structure (Figure 3.3.B) (SibC: $\Delta G = -19.39$), which could cause

termination *in vivo* (B. Mohanty and S. Kushner, personal communication). The more proximal 3' end maps to a predicted 7-11 nucleotide single-stranded region located 5' to the stem-loop structure predicted to cause termination *in vivo*. There is not a conical Rho-independent transcription terminator upstream of the most proximal 3' end. There is a weak stem-loop structure (SibC: $\Delta G = -5.80$) upstream of this 3' end, but it is probably not sufficient to cause termination *in vivo*. Therefore, it was likely that the shorter form of SibC arose through ribonuclease mediated cleavage of the longer form. This made the Sib RNAs suitable candidates for this study. We focused on SibC, since it is the only functionally characterized *sib* gene (Fozo *et al*. 2008).

Relative abundance of the Sib RNAs in various strains of *E. coli*

Using the same rationale as for GadY, SibC metabolism was assessed in early stationary phase because it accumulated at this time and the shorter form was readily visible on Northern blots (Fozo *et al*. 2008). Similar to what was observed for the GadY RNAs, the RQ of the SibC RNAs varied among the strains and temperatures tested in this study (Figure 3.4, Tables 3.5-3.7). Since the same steady-state RNA was used for both the GadY and SibC experiments, a comparison of the RQs demonstrated that the increases or decreases observed for either GadY or SibC were specific for each of the RNAs and were not generalized defects caused by temperature or the allele tested. For example, the total amount of SibC RNAs increased significantly at 44°C (Figure 3.4, lanes 11, 12 and Table 3.6, column 1), while the total amount of GadY RNAs decreased at 44°C (Figure 3.1, lanes 11, 12 and Table 3.3, column 1).

The *rnpA49*, *Δrng*, *ΔrnlA2*, *Δrnz*, *rnc-14*, *ΔrppH* and *hfq-10* alleles all marginally affected the RQ of the two SibC RNAs (Figure 3.4, lanes 1, 3, 4, 5, 6, 7, 11, 12, 22, 23, Table 3.5, columns 3, 4, 5, 6, 11, 12 and Table 3.6, column 3). In contrast, the SibC RNAs accumulated

significantly at 44°C and in the *rne-1* and *pnp-7* single mutants at 30°C (Figure 3.4, lanes 1, 2, 8, 11, 12, 13, 14, Table 3.5, columns 2, 8 and Table 3.6, columns 1, 2). Decreases occurred in the *Δrnr* and *Δrnb* single mutants (Figure 3.4, lanes 1, 9, 10 and Table 3.5, columns 9, 10). The significant decrease observed in the *Δrnb* mutant but relatively minor change in the α values indicated that a change in the RQ did not inherently affect the α values.

Exoribonuclease involvement in SibC metabolism

Since the two SibC RNAs, SibC141 and SibC109, observed in wild type cells at 30°C differ with respect to their 3' ends (Figures 3.3-3.4, lane 1) (Fozo *et al*. 2008), it was possible that exoribonuclease(s) participated in the conversion of the SibC141 species to produce SibC109. However, none of the exoribonuclease mutants, *pnp-7*, *Δrnr* or *Δrnb*, caused an increase in αSibC141 (Figure 3.4, lane 1, 8, 9, 10 and Table 3.5, columns 1, 8, 9, 10). Surprisingly, in the *Δrnb* mutant, SibC141 was absent, and in its place, a species of ~145 nt was observed (Figure 3.4, lane 9). If these extra nucleotides were at the 3' end, then transcription normally terminated approximately four nucleotides downstream of the most distal mapped 3' end (Figure 3.3.B). Transcription terminating at this site would generate an eight nucleotide single-stranded region to which RNase II could bind and then remove these additional nucleotides back to the mapped 3' end (Cheng and Deutscher 2002). However, removal of these additional nucleotides was not necessary for the generation of SibC109, because the α values were not significantly altered (Figure 3.4, lanes 1, 9 and Table 3.5, columns 1, 9). In the *pnp-7* mutant, αSibC109 increased, which implicated PNPase in the decay of SibC109 (Figure 3.4, lanes 1, 8 and Table 3.5, columns 1, 8). The α values in the *Δrnr* mutant were essentially the same as in the wild type control, which implied that RNase R was not involved in SibC metabolism (Figure 3.4, lanes 1, 10 and Table 3.5, columns 1, 10).

Endoribonuclease involvement in SibC metabolism

The results from the exoribonuclease mutants suggested endoribonuclease(s) played the primary role in the generation of SibC109 from SibC141. Neither RNase LS nor RNase Z appeared to be involved in SibC metabolism because the α values in the *ΔrnlA2* and *Δrnz* mutants were not significantly different from the wild type control (Figure 3.4, lanes 1, 4, 5 and Table 3.5, columns 1, 4, 5). In contrast, αSibC141 increased in the *Δrng*, *rnc-14* and *rne-1* mutants at 30°C (Figure 3.4, lanes 1, 3, 6, 11, 13, 15 and Table 3.5, columns 1, 2, 3, 6). The effect of the *rne-1* and *rnc-14* alleles were greater than that of the *Δrng* allele, suggesting a greater involvement of RNase E and RNase III in SibC metabolism. Interestingly at 30°C, RNase E and RNase III appeared to be in the same metabolic pathway since the effect of combining the alleles was not additive (Figure 3.4, lanes 11, 13, 15, 16 and Table 3.5, columns 1, 2, 6, 7).

Since both the *rne-1* and *rnpA49* alleles encode proteins that are inactivated at 44°C, a second set of experiments were performed at this temperature. As was observed for GadY (Figure 3.1, lanes 11, 12, Table 3.2, column 1 and Table 3.3, column 1), the higher temperature affected the RQ and α values of the SibC RNAs. In contrast to what was observed with GadY, not only did the total amount of SibC RNAs increase over four-fold in the wild type control grown at 44°C, but the cleavage pattern was altered with the appearance of a new ~111 nt species (SibC111) (Figure 3.4, lanes 11, 12, Table 3.5, column 1 and Table 3.6, column 1). In fact, under these conditions the majority of the shorter SibC RNAs were SibC111. Interestingly, both the *rne-1* and *rnpA49* alleles at 44°C resulted in a significant increase in αSibC141 with the effect of the *rne-1* mutation being greater than that of the *rnpA49* allele (Figure 3.4, lanes 2, 7, 12, 14 and Table 3.6, columns 1, 2, 3). In the *rne-1* mutant, SibC109 was absent but a small amount of SibC111 was still visible (Figure 3.4, lanes 2, 14 and Table 3.6, columns 1, 2). In the

rnpA49 mutant there was a marginal decrease in αSibC109 and a significant decrease in αSibC111 (Figure 3.4, lanes 7, 12 and Table 3.6, columns 1, 3). When taken together, the *rne-1* and *rnpA49* results suggested both RNase E and RNase P were necessary for the production of SibC111, but RNase E was primarily responsible for generating SibC109. Surprisingly, in the *rne-1 rnc-14* double mutant at 44°C, both SibC109 and SibC111 were largely absent (Figure 3.4, lanes 17, 18 and Table 3.6, columns 1, 4, 5). Since RNase P was presumably present in this genetic background, the absence of SibC111 implies RNase III was necessary for RNase P activity.

Three of the single mutants tested, *hfq-10*, *ΔrppH* and *pnp-7*, caused an increase in αSibC109 (Figure 3.4, lanes 1, 8, 11, 22, 23 and Table 3.5, columns 1, 8, 11, 12). In *E. coli*, both SibC forms copurified with Hfq (Zhang *et al.* 2003). Unlike what was observed for the GadY RNAs, there was only a marginal reduction in the RQ of SibC RNAs in the absence of Hfq (Figure 3.4, lanes 11, 23 and Table 3.5, column 12). Typically, Hfq is thought to stabilize sRNAs (Masse *et al*. 2003a). Therefore, the increase in αSibC109 could represent stabilization of SibC109 and/or destabilization of SibC141.

The results from the *rne-1* and *rnpA49* mutants implicated both RNase E and RNase P in the metabolism of SibC141. Although both of these enzyme's activities can be inhibited by a 5' triphosphate (Mackie 1998) (Bowden and Kushner, unpublished results), it was surprising that in the *ΔrppH* strain there was an increase in αSibC109 (Figure 3.4, lanes 11, 22 and Table 3.5, columns 1, 11). Since the SibC RNAs differ with respect to their 3' ends, and the data described above suggested SibC109 arose from an endoribonucleolytic cleavage of SibC141, it is not clear why the absence of RppH should stabilize the smaller species. It would appear, however, that RppH mediated removal of the triphosphate may not be necessary for RNase E and RNase P

activity on SibC141. Furthermore, the decay of the SibC109 species may involve more than Hfq and RppH, since in the *pnp-7* mutant an increase in αSibC109 was also observed (Figure 3.4, lanes 1, 8 and Table 3.5, columns 1, 8). The *pnp-7* allele also caused an increase in αGadY59 (Figure 3.1, lanes 1, 8 and Table 3.2, columns 1, 8).

The effect of RNase E alleles on SibC metabolism

At the permissive temperature, 30°C, the *rne-1* allele caused an increase in both αSibC141 and the RQ of SibC RNAs (Figure 3.4, lanes 11, 13 and Table 3.5, columns 1, 2). To further investigate the function of RNase E in SibC metabolism, three truncation alleles were tested at 30°C. In the *rneΔ91* mutant, there was an increase in αSibC109 (Figure 3.4, lanes 11, 19 and Table 3.7, columns 1, 3). If RNase E was involved in the decay of SibC109, this result may be correlated with the increase in αSibC109 detected in the *ΔrppH* mutant (Figure 3.4, lanes 11, 22 and Table 3.5, columns 1, 11). In the *rneΔ374* mutant, there was an increase in αSibC141 (Figure 3.4, lanes 11, 20 and Table 3.7, columns 1, 4). Interestingly, the opposite result was observed in the *pnp-7* mutant (Figure 3.4, lanes 1, 8 and Table 3.5, columns 1, 8). These results may indicate that RNase E and PNPase were functioning in a degradosome independent manner in SibC metabolism. In the *rneΔ645* mutant, there was an increase in αSibC141 that was greater than the increases observed in the *rne-1* and *rneΔ374* mutants (Figure 3.4, lanes 1, 13, 20, 21 and Table 3.7, columns 1, 2, 4, 5).

Metabolism of SibC's target, *ibsC*

As shown in Figure 3.3, SibC completely overlaps its target, *ibsC*. Accordingly, there is the potential for extensive duplex formation between SibC and *ibsC* (Figure 3.3.A). Han *et al.* (2010) found using *in vitro* footprinting assays that there are two target recognition domains in SibC, which likely initiate pairing with *ibsC*. Since of the endoribonuclease mutants tested in this

study only RNase III is capable of cleaving double-stranded RNA, it was expected that inactivation of RNase III should have a significant impact on the steady-state level of full-length *ibsC*. In fact, in wild type cells *ibsC* was not detected [Figure 3.5, lane 1, the ~74 nt band present in all lanes was a hybridization product not related to *ibsC* (Roche-Rios and Kushner, unpublished results)]. This result was in agreement with previous data showing *ibsC* was only visible if *sibC* was deleted (Fozo *et al*. 2008). Consistent with this study's hypothesis, of all the endoribonuclease and exoribonuclease mutants tested, only the *rnc-14* single mutant caused a significant accumulation of full-length *ibsC*160 (Figure 3.5, lane 6). These results suggested RNase III was necessary for initiating the decay of *ibsC*160. In the *rne-1 rnc-14* double mutant at 30°C, there was a significant increase in the RQ of *ibsC*160 and a modest increase in α*ibsC*160 (Figure 3.5, lanes 5, 6 and Table 3.8, columns 1, 2). However, neither of these effects were exacerbated by the inactivation of RNase E activity at 44°C (Figure 3.5, lanes 5, 6, 7, 8 and Table 3.8, columns 1, 2, 3, 4). The slight decreases in the α*ibsC*160 values were due to stabilization of some of the *ibsC* breakdown products. Thus, RNase E appeared to play a limited role in the metabolism of *ibsC* decay products but not in the decay of the full-length *ibsC*160 transcript.

In the *hfq-10* mutant, *ibsC*160 was visible on Northern blots (Figure 3.5, lane 23). Since, the full-length *ibsC* mRNA and SibC RNAs were present in the mutant, it appears that Hfq was necessary for initiating SibC/RNase III mediated decay of *ibsC*160.

Discussion

The data presented in Figures 3.1, 3.4 and Tables 3.2-3.7 suggested RNase E, RNase III, RNase P, RNase G, RNase II, PNPase, RppH and Hfq play some role in GadY and SibC metabolism *in*

vivo. In contrast, neither RNase LS, RNase Z nor RNase R participate in either sRNA's metabolism. Although previous work has implicated either RNase E or RNase III as being the primary ribonucleases mediating general sRNA metabolism (Masse *et al*. 2003a; Moll *et al.* 2003a; Vogel *et al.* 2004; Afonyushkin *et al.* 2005; Viegas *et al.* 2007), our work suggests the metabolism of both SibC and GadY is much more complicated. The *rne-1* and *rnc-14* single mutants caused alterations to the relative stabilities to the major GadY and SibC species. When combined in the double mutant, these two alleles resulted in a significant reduction to the shorter species. Thus, both RNase E and RNase III are involved in initiating the processing/decay of these sRNAs. However, although RNase E and RNase III appear to be functioning independently in GadY metabolism, in the case of SibC, they appear to work cooperatively. With respect to SibC, the interdependence of RNase E and RNase III may not result from hierarchical processing of SibC141. Rather, the apparent interdependence may result from a required RNase III cleavage of *ibsC* within the sRNA:target duplex with the RNase E cleavage site being occluded in the duplex structure.

In the absence of RNase G, both the full-length GadY105 and SibC141 species appeared to be stabilized. As compared with the effects of the *rne-1*, *rnc-14* and *rnpA49* alleles, the *Δrng* allele caused the least amount of change in the α values, suggesting a more limited, and most likely a secondary, role of RNase G in GadY and SibC metabolism. Given that these sRNAs appear to be highly dependent upon RNase E, an involvement of RNase E's homolog, RNase G, is not particularly surprising. However, this the first time that RNase G has been implicated in the metabolism of cis-encoded sRNAs. In contrast, Viegas *et al.* (2007) reported that the rifampicin determined half-lives of trans-encoded sRNAs, CsrB, CsrC and MicA, in a *Δrng*

strain were not significantly different from wild type, although the metabolism of these sRNAs was dependent upon RNase E.

Like RNase G, RNase P has not previously been implicated in regulatory sRNA metabolism. RNase P is primarily known for its role in the maturation of tRNAs (Mohanty and Kushner 2007), but it also participates in the 5' end maturation of 4.5S (Bothwell *et al*. 1976), and tmRNA (Komine *et al.* 1994). Although both 4.5S and tmRNA are small, noncoding RNAs, they are not considered regulatory sRNAs like GadY and SibC. Yet, the results of this study are consistent with a stabilization of both the GadY105 and SibC141 species in the *rnpA49* strain. The involvement of RNase P in sRNA metabolism expands the substrate repertoire of this enzyme to include the processing of regulatory RNAs as well as housekeeping RNAs.

In vivo RNase II plays a limited role in the 3' end processing of 6S RNA (Li *et al.* 1998), a regulatory sRNA that sequesters the σ^{70} holoenzyme by mimicking an open promoter structure (Wassarman 2007). The involvement of RNase II in SibC metabolism is readily apparent from the results of this study. In the *Δrnb* mutant, SibC141 was absent and a longer SibC RNA ~145 nt accumulated. In contrast, the variation in the results obtained for GadY makes its involvement in the metabolism of GadY unclear.

A deficiency in PNPase activity is known to increase the rifampicin measured half-lives of MicA, CsrB, CsrC and SraL (Viegas *et al.* 2007). Furthermore, breakdown products of these sRNAs accumulated in the PNPase mutants (Suzuki *et al.* 2006; Viegas *et al.* 2007; Andrade and Arraiano 2008). In this study, αGadY59 and αSibC109 increased in the *pnp-7* mutant, which may suggest PNPase is necessary for the decay of GadY59 and SibC109. When these results are combined with previously published work (Suzuki *et al.* 2006; Viegas *et al.* 2007; Andrade and Arraiano 2008), it is clear that PNPase mediated decay of sRNAs is a somewhat common feature

of sRNA metabolism. PNPase along with the RNA helicase, RhlB, and enolase can associate with RNase E to form the degradosome (Carpousis 2007). In GadY metabolism, the *pnp-7* and *rneΔ374* phenotypes were similar, which may indicate that PNPase was functioning in a degradosome-dependent manner. In contrast, the effects of these alleles on SibC α values were almost opposite suggesting, in this case, PNPase was functioning in a degradosome-independent manner.

Both GadY105 and SibC141 are thought to be the primary transcripts (Opdyke *et al.* 2004; Fozo *et al*. 2008). The metabolism of both primary transcripts appeared to be dependent in part on both RNase E and RNase P. Both of these enzymes can be inhibited by the presence of a 5' triphosphate group on a primary transcript (Mackie 1998) (Bowden and Kushner, unpublished results). The metabolism of GadY105 seemed to be dependent in part on RppH activity, while the metabolism of SibC141 did not. These results suggested a 5' triphosphate inhibits the activity of RNase E and/or RNase P when acting on GadY105 but not on SibC141.

The major species of GadY and SibC copurify with Hfq (Zhang *et al*. 2003; Opdyke *et al.* 2004), indicating that complexes are formed *in vivo*. Similar to other sRNAs that interact with Hfq, the absence of Hfq led to the total loss of all three predominant GadY RNAs (Masse *et al*. 2003a; Papenfort *et al.* 2009). Although SibC also interacts with Hfq, and the results presented by this study suggest Hfq is necessary for SibC function, there was only a modest reduction in the relative amount of SibC RNAs in the *hfq-10* strain. This result was similar to what was observed for another sRNA, MicM, which requires Hfq for its function, but is not significantly destabilized by the absence of Hfq (Rasmussen *et al.* 2009). Unlike other sRNAs that are thought to function stoichiometrically, MicM probably acts catalytically (Overgaard *et al.* 2009), raising the possibility that SibC might also act catalytically.

Collectively, our results demonstrate complex pathways for GadY and SibC metabolism *in vivo*. It is clear that no one enzyme mediates all aspects of either sRNA's metabolism, rather, the cell employs a combination of enzymes. Furthermore, this study clearly demonstrates both RNase III and RNase E are necessary for GadY and SibC metabolism, and implicates for the first time, an involvement of RNase G, RNase P, RNase II and RppH in sRNA decay. However, there are several outstanding issues/questions uncovered by this study that warrant further investigation. These include: (1) the interpretation of changes in the α values; (2) manifestation of aberrant metabolic defects and the accumulation of minor species and breakdown products; (3) dramatic changes in the RQ values of the sRNAs in the different genetic backgrounds, (4) the potential for multiple, complex pathways; and (5) the possibility of target-dependent versus target-independent metabolic pathways.

Interpretation of changes to α values

The primary limitation of this work was that the actual stability of each RNA species was not determined in the various loss-of-function strains. Rather, the relative stability was inferred based on the calculation and comparison of α values. In cases where αGadY105 and αSibC141 approached one, like what was observed in the *rne-1 rnc-14* double mutant, it was assumed that GadY105 and SibC141 were stabilized resulting in the absence of the shorter species. It became less clear how to interpret changes in the α values when the α value of a shorter species increased and the α value of a longer species decreased. This could be due to stabilization of a shorter species, destabilization of a longer species or a forced reciprocal change that did not result form a change in stability. For example, in the *hfq-10* mutant, αSibC109 increased and αSibC141 decreased. Hfq is known to stabilize sRNAs (Masse *et al*. 2003a; Moll *et al.* 2003a), therefore in

this example, this result may be due to greater destabilization of SibC141 relative to SibC109 in the absence of Hfq.

In the GadY analysis, there was a significant decrease in αGadY105 in the *pnp-7* mutant. Does this mean that GadY105 was destabilized or was the decrease a result of a forced reciprocal change due to the increase in αGadY90 and αGadY59? Although uncommon, some RNAs do become less stable in the absence of a ribonuclease (Mohanty and Kushner 2003). It is known that in PNPase defective cells, there is an increase in RNase II activity (Zilhão *et al*. 1996). It is difficult to determine from our data if in the *Δrnb* mutant GadY105 was stabilized, but if RNase II were involved in the decay of GadY105, then the decrease in αGadY105 observed in the *pnp-7* mutant may have been due to destabilization of GadY105. Accordingly, in the *pnp-7* mutant the rate of GadY105 turnover may have increased because of more RNase II activity.

To bolster or refute the interpretations of changes to the α values, the chemical half-lives of the major GadY and SibC RNAs need to be determined. This could be done by treating the cells with rifampicin. However, given the potential caveats of rifampicin treatment by causing increased half-lives because of depletion of the target pool (Masse *et al*. 2003a), the half-lives need to be determined by the method employed by Overgaard *et al.* (2009). In this approach, expression of the sRNA and target are induced, the inducer is removed from the medium to terminate transcription of both the sRNA and target, and then the rate of sRNA turnover is calculated.

Aberrant metabolic defects and accumulation of minor species/breakdown products

In the *rnc-14* single mutant and the *rne-1 rnc-14* double mutant at 30°C, there was a smear above GadY105 on Northern blots, indicating GadY RNAs of ~106 to ~110 nt, but GadY90 and GadY59 were still present. In the double mutant at 44°C, GadY105, GadY90 and GadY59 were

absent and in their place were GadY RNAs that were approximately three nucleotides longer than each of the normal GadY RNAs. It is possible that these differences in size corresponded to changes at the 5' end, but it is more likely that these differences occurred at the 3' ends. If the changes occurred at the 5' ends, then the cleavage sites generating GadY90 and GadY59 were shifted to a more upstream position. If the changes occurred at the 3' ends, then the cleavage sites generating GadY90 and GadY59 remained intact. It is possible that when normal metabolism was sufficiently hampered, like in the *rne-1 rnc-14* double mutant, that one or more of the three nucleotidyl transferases, poly(A) polymerase, PNPase and tRNA nucleotidyltransferase, were adding these additional nucleotides (Kushner 1996). Therefore, comparing double mutants of RNase III and one of the three nucleotidyl transferases and monitoring for the loss of the smear could indicate if these enzymes were responsible for the addition of these nucleotides.

For both GadY and SibC, minor species intermediate in size to the major forms of the RNAs were detected in one or more of the strains tested. These species raise the possibility that cleavage events may not always occur at the 5' end of GadY90 or GadY59 or at the 3' end of SibC111 or SibC109, but may proceed through a series of cleavage events. In the *Δrng* mutant, a GadY RNA ~96 nt accumulated. This species was not detected in the other mutants, whereas, a GadY RNA ~86 nt and a SibC RNA ~116 nt were visible in all of the strains. It remains unclear what enzymes were generating these products. These species made up such a small proportion of total GadY and SibC RNAs that these RNAs may have arisen through minor, secondary metabolic pathways. Another possibility is that the GadY86 and SibC116 species were due to nonspecific hybridization.

In the *rnc-14* mutant and the *rne-1 rnc-14* double mutant, several 3' *ibsC* decay products accumulated. The accumulation of these species indicated that the breakdown of these species were largely dependent upon RNase III, and to a limited extent RNase E, but some other, yet unidentified, enzyme was generating them. It is possible that this activity represented another double-stranded endoribonuclease, or single-stranded endoribonuclease that was cutting within non-duplexed regions. On overexposed images of the Northern blots, three 5' SibC decay products were present within these same strains: ~92, ~67 and ~49 nt (Figure S.3.2). These species were similar in size to some of the *ibsC* decay products. Therefore, these SibC and *ibsC* RNAs may represent SibC:*ibsC* duplexes that normally were substrates for RNase III.

The manifestation of putative aberrant metabolic events and the accumulation of minor species and breakdown products revealed additional layers of complexity and subtly to both GadY and SibC metabolism *in vivo*. Yet, it remains unclear if these events or species have much biological relevance in wild type physiology.

Dramatic changes in the RQ

For both GadY and SibC, the RQ of these RNAs were significantly affected by both temperature and the mutants tested in this study. In some cases, the RQ increased at 44°C or in a particular mutant, while in other cases, the RQ decreased at 44°C or in a particular mutant. For example, in the *rne-1* strain at both 30°C and 44°C, there was a dramatic increase in the RQ of SibC RNAs. Since the metabolism of SibC141 appeared to be highly dependent upon RNase E, an increase in the amount of SibC RNAs in this strain was consistent with a stabilization of SibC141. However, as the GadY results clearly demonstrated, there were instances of a mutation causing both a decrease in the RQ and a change to the α values. These observations raise the question, were the observed changes in the RQ due to synthesis or stability effects, e.g. was the reduction in the RQ

of GadY RNAs observed in the *rne-1* strain at both 30°C and 44°C due to inhibition of transcription or global destabilization? Use of transcriptional fusions in these genetic backgrounds would help to answer this type of question. Having this information would bolster the α analysis, because it would establish if changes in the RQ and α values are correlated or are independent.

The potential for multiple, complex metabolic pathways

The data generated by this study suggested multiple, complex pathways mediating GadY and SibC metabolism. There is the potential for independent pathways, the involvement of multiple enzymes in a hierarchical processing pathway, as well as, pathways that generated the shorter, stable RNAs and those that did not. These possibilities are exemplified by the GadY analysis. In the *rne-1 rnc-14* double mutant at 44°C, the shorter, stable GadY RNAs were largely absent, which indicated that these two enzymes were primarily responsible for GadY processing. Both the single mutants affected the α values. RNase E appeared to be necessary for the generation of GadY59 from GadY90, and RNase III was necessary for the production of GadY90 and GadY59 from GadY105. When combined in the double mutant, the effects on GadY metabolism were additive and resulted in a more exacerbated *rnc-14* mutant-like profile. These results suggested RNase E and RNase III were functioning independently in two major pathways that initiated and mediated GadY metabolism. GadY has two putative targets, *gadX* and *gadXW*, and affects the metabolism of the two targets differently (Opdyke *et al.* 2004; Tramonti *et al.* 2008). GadY is thought to stabilize *gadX* and stimulate processing of *gadXW*. If the metabolism of GadY is coupled to that of its targets, then presumably the metabolism of GadY when bound to *gadX* is different from its metabolism when bound to *gadXW*. Since RNase E and RNase III have

completely different substrate specificities, it is possible that the metabolic pathways mediated by these two endoribonucleases correlate with GadY interacting with different targets.

In addition to RNase E and RNase III, RNase P and RNase G also appeared to be involved in GadY metabolism. This raises the question, are RNase G and RNase P mediating separate pathways, or if in combination with either RNase E or RNase III, functioning in hierarchical processing pathways? On overexposed images of the Northern blots, there were smears and faints bands of GadY RNAs intermediate in length to GadY105 and GadY59. The presence of these intermediates was consistent with hierarchical processing pathways. Therefore, addressing this question *in vivo* by testing all permutations of the *rne-1*, *rnc-14*, *rnpA49* and *Δrng* alleles is critical for elucidating the detailed mechanisms of GadY metabolism.

The participation of RNase E, RNase III, RNase P and RNase G in GadY metabolism only applied to the generation of GadY90 and GadY59, but there may be pathways that do not result in the generation of either. RNase II and PNPase may participate in the decay of GadY105 and GadY90, respectively. RNase II and PNPase activity cannot result in the production of either GadY90 or GadY59, because these RNAs share the same 3' end. Thus, in addition to independent pathways and pathways involving more than one enzyme, there could be decay pathways independent of processing pathways.

Target-dependent versus target-independent metabolic pathways and stoichiometric versus catalytic mechanisms of action

SibC and *ibsC* are part of a type I toxin-antitoxin system, in which SibC, the antitoxin, promotes the rapid decay of *ibsC*, which encodes a short toxic peptide (Fozo *et al*. 2008). In early stationary phase, *ibsC* was not detected in wild type cells, which suggested SibC mediated regulation was robust, presumably because the concentration of SibC was significantly greater

than that of *ibsC* (Levine *et al.* 2007; Fozo *et al*. 2008). This study extends the hypothesis of SibC mediated regulation of *ibsC* to include Hfq as being necessary for SibC mediated destabilization of *ibsC* by RNase III. The results also indicated that RNase E was the primary enzyme necessary for the processing of SibC141 into SibC111/109. The putative RNase E cleavage site falls within a predicted single-stranded region (Figure 3.3.B). Han *et al.* (2010) reported that this region is one of the two target recognition domains of SibC that likely initiates contact with *ibsC*. Thus, the dependence of RNase E and RNase III in SibC141 processing observed in the *rne-1 rnc-14* double mutant may have resulted from stabilization of the SibC:*ibsC* duplex, such that, in the absence of RNase III, *ibsC* in the SibC:*ibsC* duplex was not cleaved and the RNase E cleavage site on SibC was occluded in the duplex molecule.

This possibility raises two questions: does RNase E process unbound SibC only or does cleavage of *ibsC* by RNase III facilitate the subsequent cleavage of SibC by RNase E? Normally, the concentration of SibC is probably greater than that of *ibsC*. Thus, there is the potential for an unbound population of SibC. When unbound, the putative RNase E cleavage site(s) in SibC should be single-stranded and accessible. However, RNase E and RNase III may form a hierarchical processing pathway of the SibC:*ibsC* duplex. Using *in vitro* footprinting, Han *et al.* (2010) reported that a perfect duplex between SibC and *ibsC* does not form. However, these assays were done in the absence of Hfq.

Since Hfq is known to induce structural rearrangements in both the sRNA and target (Moller *et al.* 2002a; Zhang *et al.* 2002; Moll *et al.* 2003b; Geissmann and Touati 2004; Antal *et al.* 2005), the experiments should be redone in the presence of Hfq. If an imperfect duplex forms, RNase III could cut a stem-loop structure in *ibsC*, but if a more extensive duplex is generated, then RNase III could cleave asymmetrically only within *ibsC*. Normally, RNase III cuts both

strands of a duplex, but in some cases, it is known to cut only one strand of a duplex (Kramer and Rosenberg 1976). If RNase III cleaves only *ibsC*, cleavage of *ibsC* might promote structural rearrangement of the duplex such that the RNase E processing site becomes accessible. Another possibility, and probably more likely, is that RNase III cleaves both SibC and *ibsC* within the context of the duplex. RNase III mediated cleavage of SibC within a duplex may be largely invisible to this study's analysis if cleavage by RNase III does not result in the generation of SibC109 or if only a small proportion of SibC is bound to *ibsC*. If only a small proportion of SibC interacts with *ibsC*, then stabilization of SibC141 in the *rnc-14* mutant may not have been significant enough to be detected by this study.

Questions concerning whether RNase E or RNase III cut SibC within the context of the duplex or if RNase E cleaves unbound SibC only, speak to the mechanism of action of SibC, i.e. is SibC acting stoichiometrically or catalytically? If either RNase E or RNase III is cleaving SibC within the duplex, this would indicate that SibC is functioning stoichiometrically. Whereas, if SibC is not cleaved within the context of the duplex and is recycled, then it is possible that RNase E processing of unbound SibC is the metabolic consequence of a catalytic mechanism of action. Most sRNAs are thought to act stoichiometrically, but SibC does share some similarities to a catalytic sRNA, MicM. Both SibC and MicM appear to require Hfq for their function, but in the absence of Hfq, neither is significantly destabilized (Rasmussen *et al.* 2009).

Adaptation of an experimental system to study sRNA metabolism *in vivo*

This study revealed several phenomena that can complicate the study of sRNA metabolism *in vivo*. These include dramatic changes in the RQ of a sRNA in ribonuclease deficient strains that may be due to changes in transcription initiation rates, the potential for multiple, independent metabolic pathways, multiple enzymes participating in a hierarchical processing pathway,

pathways that generate shorter, stable RNAs versus those that do not, as well as, target-independent verse target-dependent metabolic events.

To circumvent and unravel these complications, the metabolism of sRNAs *in vivo* may be best analyzed by using a modified version of the assay employed by Overgaard *et al.* (2009). By deleting the chromosomal copy of both the sRNA and target and expressing each from an inducible plasmid, this group was able to determine the chemical half-life of the sRNA in the absence of rifampicin and determine the mechanism of action of the sRNA, i.e. catalytic versus stoichiometric. By engineering this system into single mutants and multiple mutants defective in ribonuclease activity, it would be possible to establish a correlation between enzyme(s), metabolic pathway, target and mechanism of action.

This system can be adapted to study the metabolism of any cis-encoded or trans-encoded sRNA. It could also be used to study the metabolism of a sRNA during different phases of growth or under different growth conditions. Furthermore, the metabolism of shorter, stable RNAs can be studied independent of their generation by expressing the shorter form(s) from the vector. To provide a more comprehensive picture of sRNA metabolism, probes that span the sRNA can be used in Northern blotting and cleavage sites can be mapped by a combination of 5' RACE, primer extension and 3' RACE. In total, this system may provide a versatile and elegant way to investigate all possible metabolic fates of any sRNA.

Table 3.1. Bacterial strains used in this study.

Strain Designation	Strain Number	Genotype	Source or Reference
wild type	MG1693*	*thyA715, λ-, rph-1*	*E. coli* Genetic Stock Center, Yale University
rne-1	SK5665	*thyA715, λ-, rph-1, rne-1*	Arraiano *et al.* (1988)
rneΔ91	SK2684	*thyA715, λ-, rph-1, srlD300::*Tn*10, recA56, rneΔ1018::bla, pDHK4* (*rneΔ91*)	Chung and Kushner (unpublished results)
rneΔ374	SK2683	*thyA715, λ-, rph-1, srlD300::*Tn*10, recA56, rneΔ1018::bla, pDHK3* (*rneΔ374*)	Chung and Kushner (unpublished results)
rneΔ645	SK2685	*thyA715, λ-, rph-1, srlD300::*Tn*10, recA56, rneΔ1018::bla, pDHK6* (*rneΔ645*)	Ow *et al.* (2000)
rnpA49	SK2525	*thyA715, λ-, rph-1, rbsD296::*Tn*10, rnpA49*	Ow *et al.* (2002)
Δrng	SK2538	*thyA715, λ-, rph-1, rng::cat*	Ow *et al.* (2003)
ΔrnlA2	SK3170	*thyA715, λ-, rph-1, rnlA2::kan*	Perwez and Kushner (unpublished results)
Δrnz	SK4477	*thyA715, λ-, rph-1, Δrnz::apr*	Maples and Kushner (unpublished results)
rnc-14	SK4455	*thyA715, λ-, rph-1, rncΔ14::*Tn*10*	Stead *et al.* (in press)
rne-1 rnc-14	SK5173	*thyA715, λ-, rph-1, rne-1, rncΔ14::*Tn*10*	Stead *et al.* (in press)
pnp-7	SK5691	*thyA715, λ-, rph-1, pnp-7*	Arraiano *et al.* (1988)
Δrnb	CMA201	*thyA715, λ-, rph-1, Δrnb::*Tn*10*	Piedade *et al.* (1995)
Δrnr	SK2059	*thyA715, λ-, rph-1, rnr::kan*	Mohanty *et al.* (2010)
ΔrppH	SK4390	*thyA715, λ-, rph-1, ΔrppH::kan*	Mohanty *et al.* (2010)
hfq-10	SK10246	*thyA715, λ-, rph-1, hfq-10::cat*	Mohanty *et al.* (2004)

*MG1693 is a *thyA715* derivative of MG1655

Table 3.2. Analysis of GadY levels in various strains of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*Δrng*	*ΔrnlA2*	*Δrnz*	*rnc-14*	*rne-1 rnc-14*	*pnp-7*	*Δrnb*	*Δrnr*	*ΔrppH*	*Δhfq*
Species												
αGadY105	0.41 (±0.02)	0.50 (±0)	0.50 (±0.04)	0.43 (±0.02)	0.40 (±0.01)	0.57 (±0.03)	0.74 (±0.02)	0.25 (±0.01)	0.48 (±0.07)	0.41 (±0.01)	0.65 (±0.01)	ND
αGadY90	0.48 (±0.02)	0.30 (±0.04)	0.39 (±0.03)	0.47 (±0.02)	0.50 (±0.01)	0.39 (±0.03)	0.21 (±0.02)	0.53 (±0.01)	0.44 (±0.03)	0.46 (±0.01)	0.27 (±0.02)	ND
αGadY59	0.11 (±0.02)	0.20 (±0.04)	0.11 (±0.01)	0.10 (±0)	0.10 (±0)	0.04 (±0)	0.05 (±0)	0.22 (±0.02)	0.08 (±0.04)	0.13 (±0.02)	0.08 (±0)	ND
Relative Quantity (RQ)	1.00 (±0)	0.41 (±0.06)	1.76 (±0.14)	1.37 (±0.10)	1.30 (±0.11)	0.62 (±0.12)	0.23 (±0.03)	0.46 (±0.07)	1.35 (±0.22)	1.23 (±0.21)	0.86 (±0.05)	ND
Column	1	2	3	4	5	6	7	8	9	10	11	12

The relative frequency (α) of each GadY RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total GadY RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments. ND = Not detected.

Table 3.3. Analysis of GadY levels in various strains of *E. coli* at 44°C.

Genotype	wild type	*rne-1* 60'	*rnpA49* 60'	*rne-1 rnc-14* 30'	*rne-1 rnc-14* 60'
Species					
αGadY105	0.44 (±0.02)	0.44 (±0.04)	0.54 (±0.01)	0.94 (±0.01)	0.96 (±0.01)
αGadY90	0.39 (±0.03)	0.54 (±0.04)	0.32 (±0.01)	0.04 (±0)	0.03 (±0.02)
αGadY59	0.17 (±0.01)	0.02 (±0.01)	0.14 (±0)	0.02 (±0)	0.01 (±0)
Relative Quantity (RQ)	0.14 (±0)	0.03 (±0)	0.04 (±0.01)	0.05 (±0)	0.04 (±0.01)
Column	1	2	3	4	5

The relative frequency (α) of each GadY RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total GadY RNAs was determined relative to the amount detected in the wild type control (Table 3.2, column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 3.4. Analysis of GadY levels in various RNase E mutants of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*rneΔ91*	*rneΔ374*	*rneΔ645*
Species					
αGadY105	0.41 (±0.02)	0.50 (±0)	0.43 (±0.01)	0.27 (±0.03)	0.41 (±0.02)
αGadY90	0.48 (±0.02)	0.30 (±0.04)	0.43 (±0)	0.62 (±0.04)	0.33 (±0.02)
αGadY59	0.11 (±0.02)	0.20 (±0.04)	0.14 (±0.02)	0.11 (±0.01)	0.26 (±0.01)
Relative Quantity (RQ)	1.00 (±0)	0.41 (±0.06)	0.88 (±0.06)	1.22 (±0.12)	0.30 (±0.01)
Column	1	2	3	4	5

The relative frequency (α) of each GadY RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total GadY RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 3.5. Analysis of SibC levels in various strains of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*Δrng*	*ΔrnlA2*	*Δrnz*	*rnc-14*	*rne-1 rnc-14*	*pnp-7*	*Δrnb*	*Δrnr*	*ΔrppH*	*hfq-10*
Species												
αSibC141	0.69 (±0.04)	0.84 (±0.02)	0.76 (±0.01)	0.67 (±0.01)	0.68 (±0.01)	0.86 (±0.01)	0.82 (±0.06)	0.53 (±0.04)	0.72 (±0.03)	0.70 (±0.03)	0.55 (±0.07)	0.50 (±0.07)
αSibC109	0.31 (±0.04)	0.16 (±0.02)	0.24 (±0.01)	0.33 (±0.01)	0.32 (±0.01)	0.14 (±0.01)	0.18 (±0.06)	0.47 (±0.04)	0.28 (±0.03)	0.30 (±0.03)	0.45 (±0.07)	0.50 (±0.07)
Relative Quantity (RQ)	1.00 (±0)	2.05 (±0.53)	1.06 (±0.20)	0.79 (±0.09)	0.99 (±0.26)	0.86 (±0.20)	3.03 (±0.86)	1.99 (±0.19)	0.26 (±0.01)	0.63 (±0.03)	0.99 (±0.39)	0.75 (±0.20)
Column	1	2	3	4	5	6	7	8	9	10	11	12

The relative frequency (α) of each SibC RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total SibC RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 3.6. Analysis of SibC levels in various strains of *E. coli* at 44°C.

Genotype	wild type	*rne-1* 60′	*rnpA49* 60'	*rne-1 rnc-14* 30'	*rne-1 rnc-14* 60'
Species					
αSibC141	0.76 (±0.04)	0.99 (±0)	0.91 (±0.01)	1.00 (±0)	1.00 (±0)
αSibC111	0.15 (±0.03)	0.01 (±0)	0.02 (±0)	0 (±0)	0 (±0)
αSibC109	0.09 (±0.01)	0 (±0)	0.07 (±0)	0 (±0)	0 (±0)
Relative Quantity (RQ)	4.59 (±0.96)	7.64 (±0.39)	4.22 (±0.25)	3.35 (±0.76)	4.02 (±0.85)
Column	1	2	3	4	5

The relative frequency (α) of each SibC RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total SibC RNAs was determined relative to the amount detected in the wild type control (Table 3.5, column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 3.7. Analysis of SibC levels in various RNase E mutants of *E. coli* at 30°C.

Genotype	wild type	*rne-1*	*rneΔ91*	*rneΔ374*	*rneΔ645*
Species					
αSibC141	0.69 (±0.04)	0.84 (±0.02)	0.59 (±0.03)	0.79 (±0.07)	0.90 (±0.01)
αSibC109	0.31 (±0.04)	0.16 (±0.02)	0.41 (±0.03)	0.21 (±0.07)	0.10 (±0.01)
Relative Quantity (RQ)	1.00 (±0)	2.05 (±0.53)	0.59 (±0.06)	0.85 (±0.16)	3.35 (±0.84)
Column	1	2	3	4	5

The relative frequency (α) of each SibC RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of total SibC RNAs was determined relative to the amount detected in the wild type control (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

Table 3.8. Analysis of *ibsC* levels in various strains of *E. coli* at 30°C and 44°C.

Genotype	*rnc-14* 30°C	*rne-1 rnc-14* 30°C	*rne-1 rnc-14* 30' 44°C	*rne-1 rnc-14* 60' 44°C	*hfq-10* 30°C
Species					
α*ibsC*160	0.21 (±0.06)	0.36 (±0.08)	0.34 (±0.06)	0.32 (±0.06)	0.94 (±0)
Relative Quantity (RQ)	1 (±0)	3.8 (±0.02)	3.8 (±0.08)	3.8 (±0.04)	0.67 (±0.07)
Column	1	2	3	4	5

The relative frequency (α) of *ibsC*160 RNA in each strain was determined based on quantitative Northern blotting as described in the Materials and Methods. The Relative Quantity (RQ) of *ibsC*160 was determined relative to the amount detected in the *rnc-14* mutant (column 1) as described in the Materials and Methods. In parentheses below each number is the standard deviation determined from at least two independent experiments.

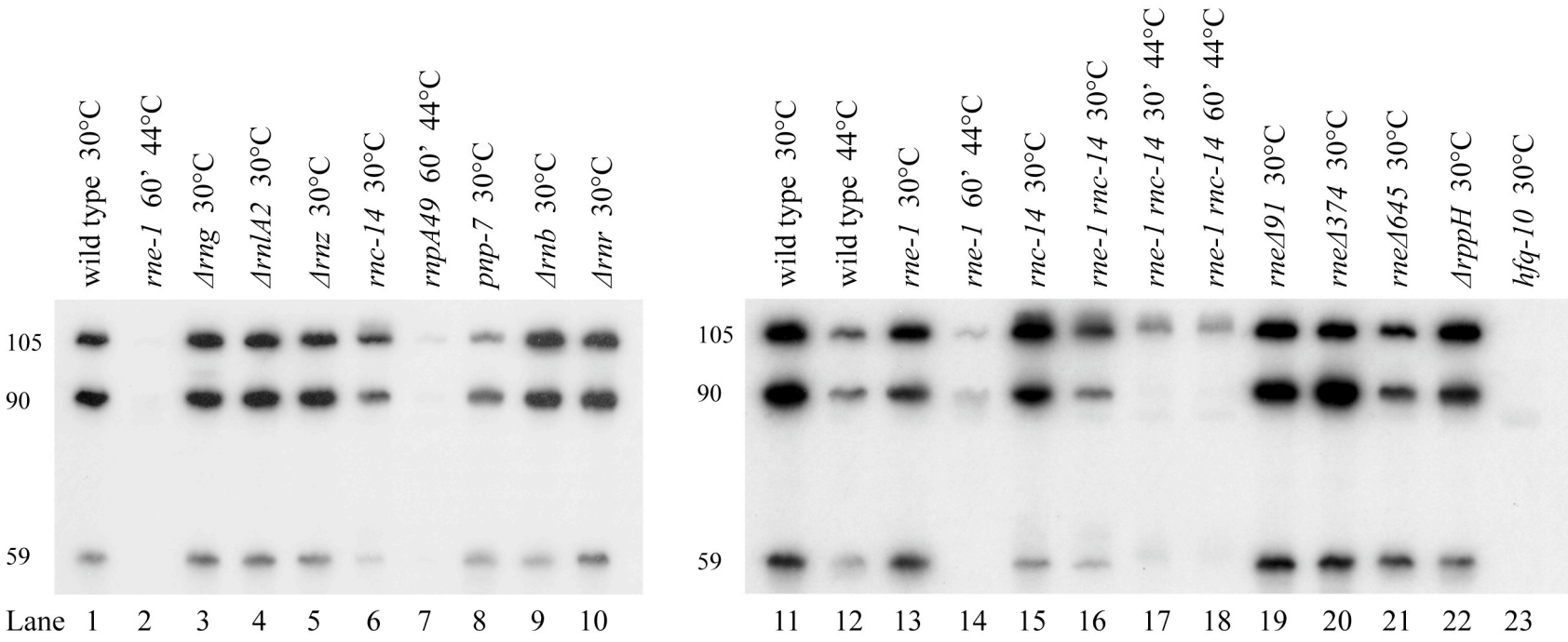

Figure 3.1. Northern blot analysis of GadY. All lanes contain 10 μg of total steady-state RNA harvested from early stationary phase cells except for *hfq-10* (30 μg). The sizes of the three GadY RNAs were estimated from electrophoretic mobility based on the migration of both a RiboRuler low range RNA ladder (Fermentas) and a low molecular weight marker, 10-100 nt (USB). The estimated sizes were in excellent agreement with the ends and lengths reported by Opdyke *et al.* (2004).

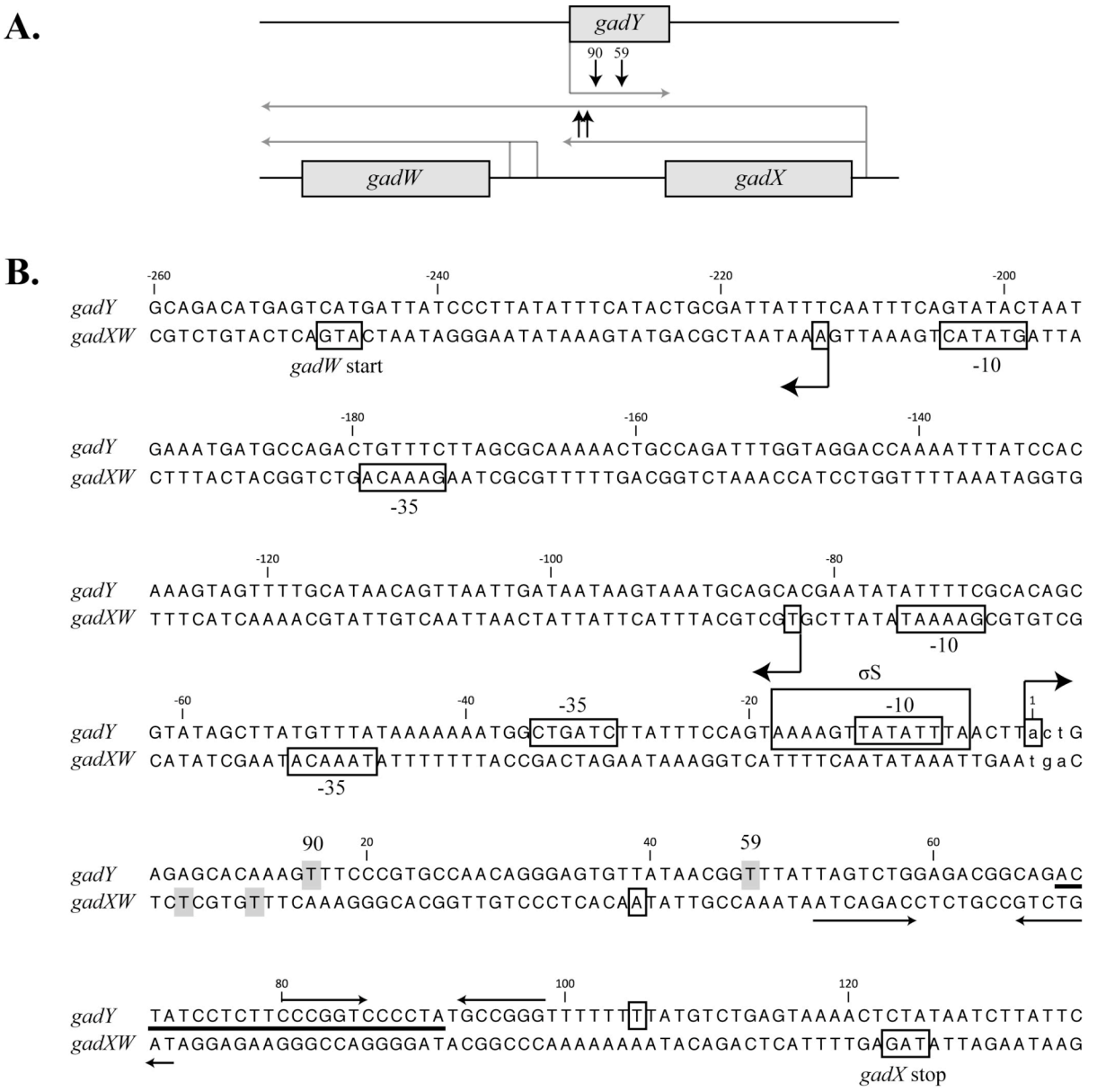

Figure 3.2. The *gadW*/*gadX*/*gadY* region of the *E. coli* genome. (A.) Diagram of the *gadY* and *gadXW* loci. The horizontal arrows represent the transcripts. The vertical arrows represent the mapped ends that likely arise through endoribonucleolytic cleavages (Tramonti *et al*. 2008). (B.) The nucleotide sequences of the *gadY* and *gadXW* genes are annotated with features based on Opdyke *et al*. (2004) and Tramonti *et al*. (2008). The promoters, start and stop codons are boxed in and labeled. The transcriptional start sites are indicated by boxes with bent arrows, and the putative transcription termination elements are shown with inverted arrows. The single boxed nucleotides are the putative 5' and 3' ends of GadY and *gadX*, and the shaded nucleotides are the mapped ends of GadY and *gadX* that presumably arise through processing events. Underlined in bold is the region of *gadY* that the GadY Northern probe was designed against.

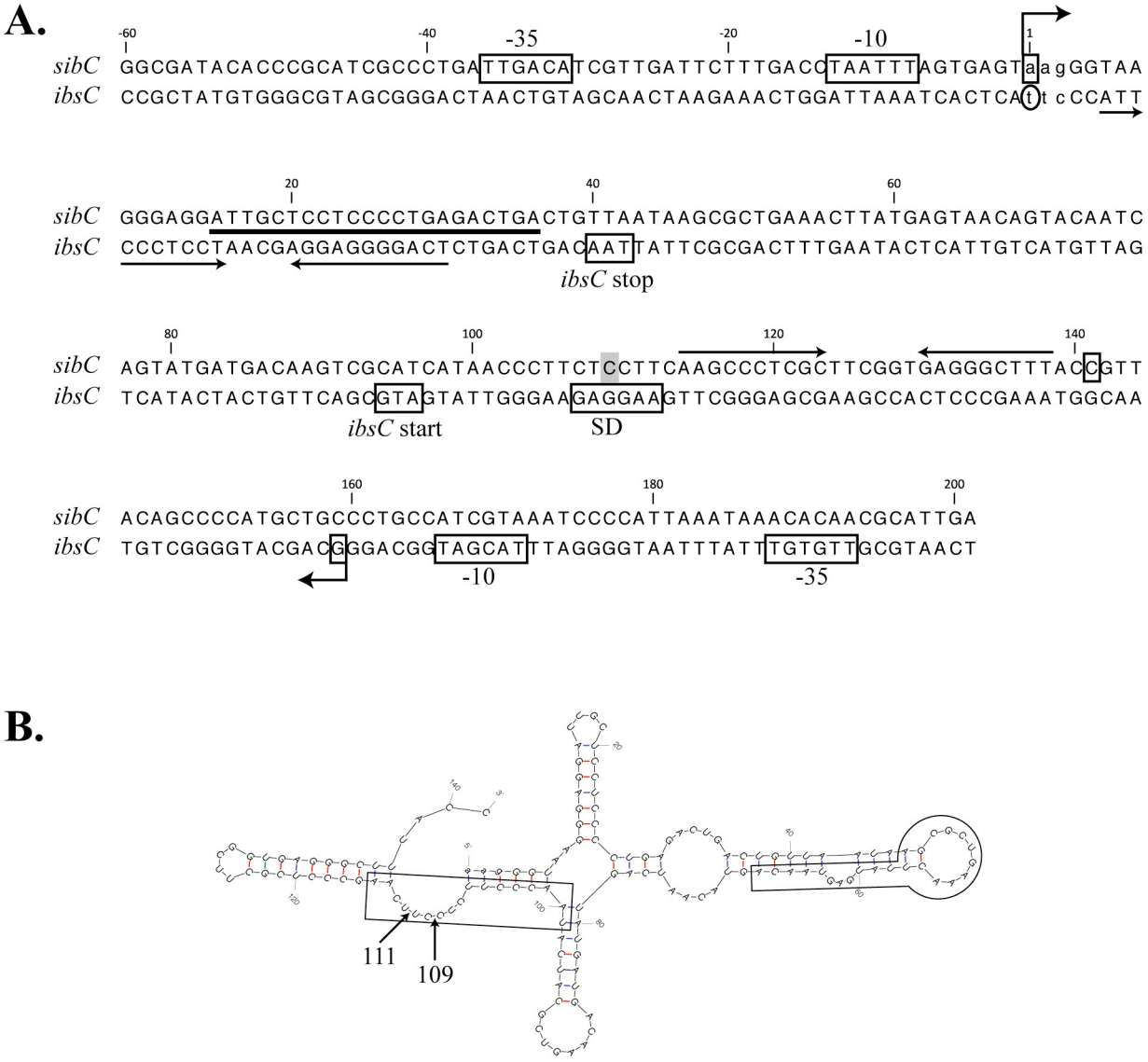

Figure 3.3. The *sibC*/*ibsC* region of the *E. coli* genome. (A.) The nucleotide sequences of the *sibC* and *ibsC* genes are annotated with features based on Fozo *et al*. (2008) and Han *et al*. (2010). The promoters, Shine-Delgarno (SD), start and stop codons are boxed in. The transcriptional start sites are indicated by boxes with bent arrows, and the putative transcription termination elements are shown with inverted arrows. The single boxed nucleotide corresponds to the most distal 3' end of SibC. The shaded nucleotide is the most proximal 3' end of SibC and likely arises through processing. The circled nucleotide corresponds to the putative 3' end of *ibsC*. The region underlined in bold is the region that the SibC and *ibsC* Northern probes were designed against. (B.) The predicted secondary structure of SibC (mfold). The boxed in areas are the target recognition domains identified by Han *et al*. (2010). The arrows show the most proximal 3' ends of SibC.

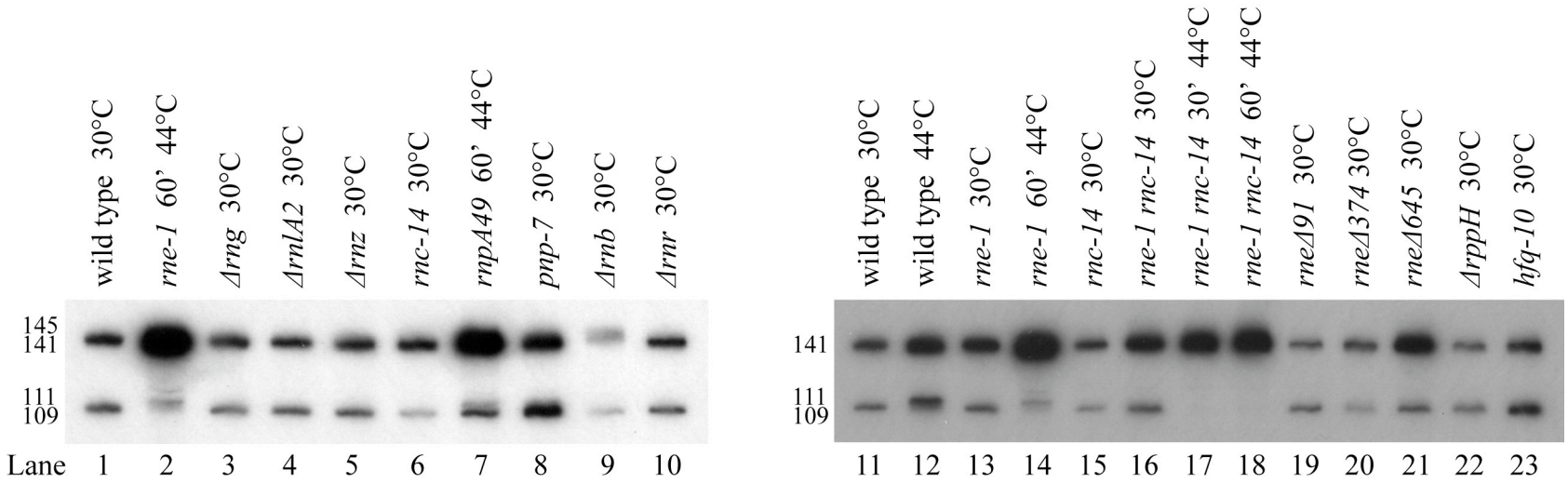

Figure 3.4. Northern blot analysis of SibC. The gels were loaded as described in Figure 3.1. The sizes of the SibC RNAs were estimated from electrophoretic mobility based on the migration of both a RiboRuler low range RNA ladder (Fermentas) and a low molecular weight marker, 10-100 nt (USB). The estimated sizes of the two predominant forms of SibC were in excellent agreement with the ends and lengths reported by Fozo *et al*. (2008).

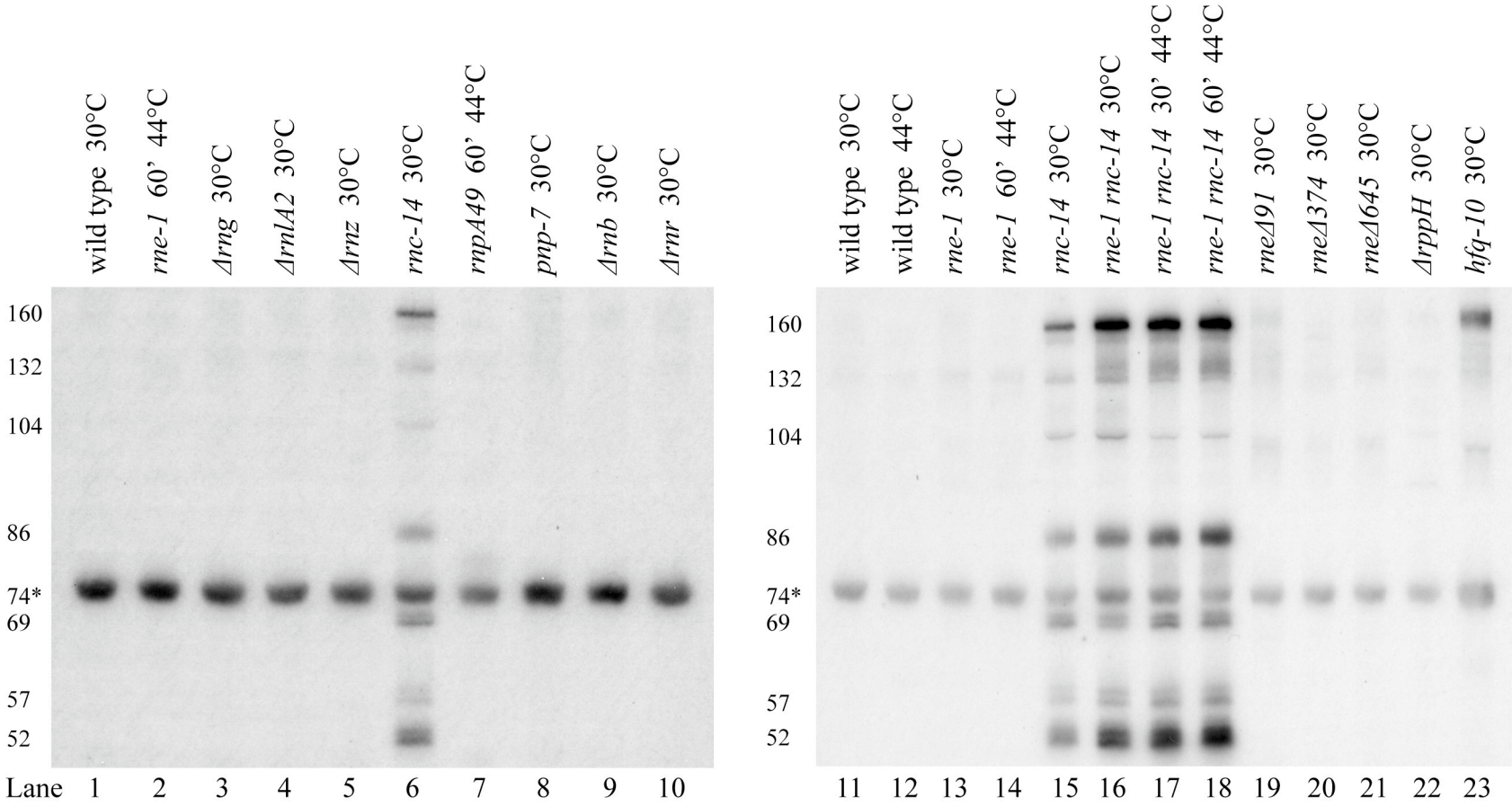

Figure 3.5. Northern blot analysis of *ibsC*. The gels were loaded as described in Figure 3.1. The sizes of the *ibsC* mRNAs were estimated from electrophoretic mobility based on the migration of both a RiboRuler low range RNA ladder (Fermentas) and a low molecular weight marker, 10-100 nt (USB). The estimated size of full-length *ibsC* was in excellent agreement with the ends and lengths reported by Han *et al*. (2010). The band marked with an * represents hybridization to a RNA species that is not related to *ibsC* (Roche-Rios and Kushner, unpublished results).

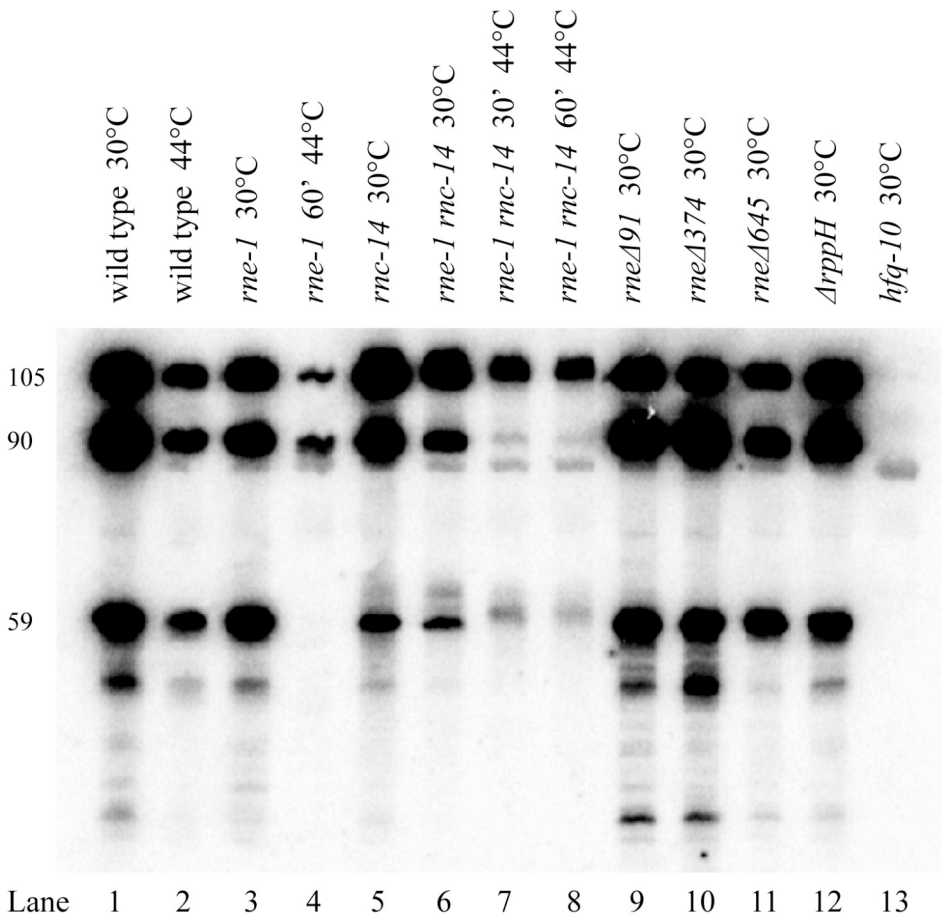

Figure S.3.1. Overexposed Northern blot of GadY shown in Figure 3.1.

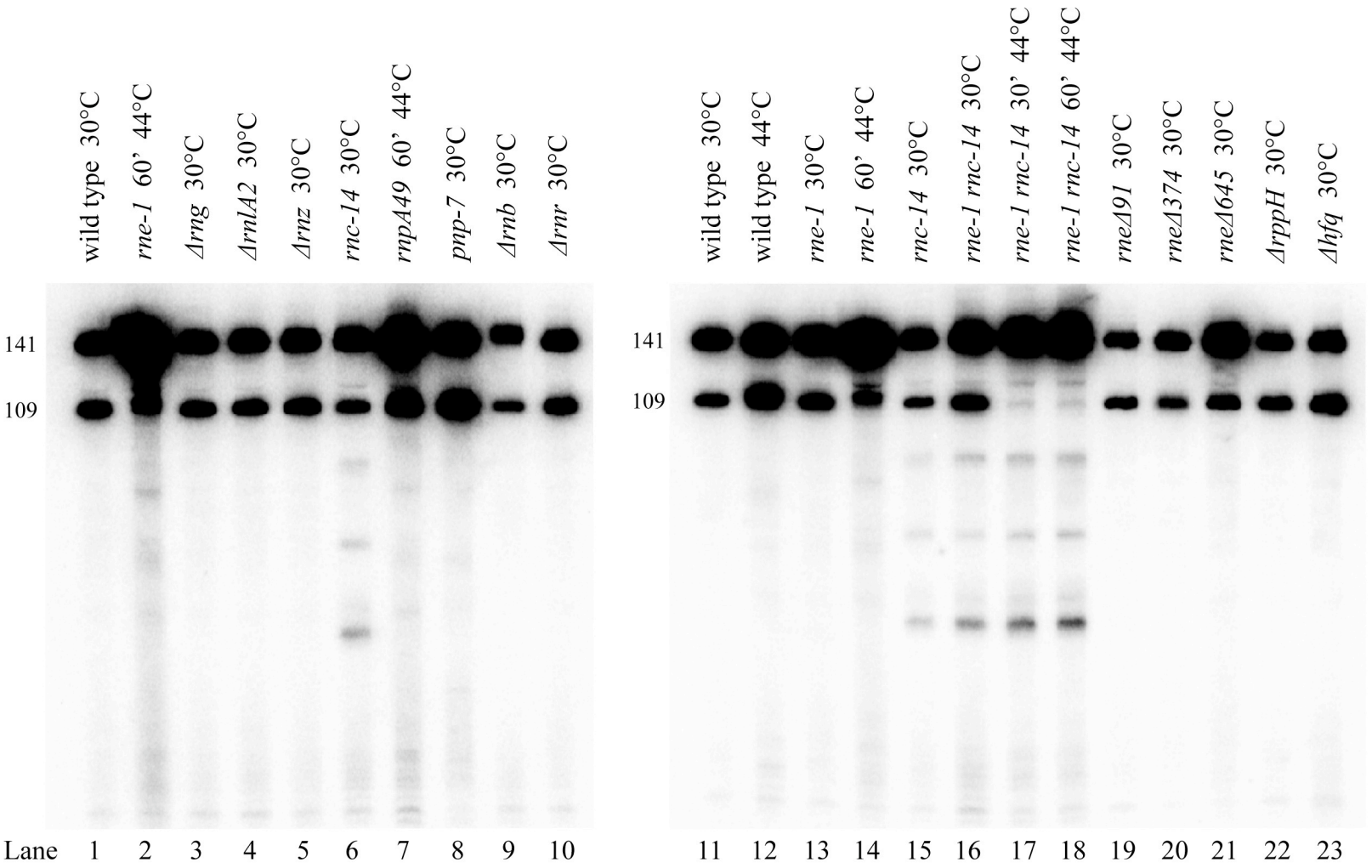

Figure S.3.2. Overexposed Northern blots of SibC shown in Figure 3.4.

CHAPTER 4

Concluding Remarks to the Dissertation

This study was designed to identify genes involved in sRNA metabolism by assaying for the loss of shorter, stable sRNA species in ribonuclease deficient mutants. To assess global ribonuclease involvement in sRNA metabolism, we examined loss-of-function alleles of all the known major cytoplasmic endoribonucleases and exoribonucleases of *E. coli* K-12, including: RNase E, RNase G, RNase LS, RNase Z, RNase P, RNase III, PNPase, RNase II and RNase R (Li and Deutscher 2004). Since none of the single ribonuclease deficient mutants that we tested resulted in the complete loss of the shorter, stable species, a relative frequency (α) analysis was used to estimate the relative stability of the individual sRNA species. We assumed that the relative frequency of each species should be the same as the wild type control, regardless of the total amount of the sRNA, unless the stability of a particular species was affected by the mutation under investigation. Using this type analysis, a block in production of the shorter form(s) could stem from the stabilization of the longer form, which would cause the α value of the longer form to approach one and the α value of the shorter form(s) to approach zero.

The results of this study clearly demonstrated that this approach was successful in identifying genes that were necessary for the metabolism of a particular sRNA independent of changes in steady-state levels or rifampicin measured chemical half-life determinations. Furthermore, by using the same RNA harvested from physiologically equivalent populations of early stationary phase cells, we were able to demonstrate that the changes that we observed with respect to changes in the α values and changes in the total amount of the sRNA (RQ) were

specific for each sRNA. For example, the RNase III (*rnc-14*) allele affected the α values of the GadY and SibC sRNAs (Chapter 3) but not the ArcZ and RprA sRNAs (Chapter 2). Furthermore, although incubation at 44°C caused a reduction in the RQ of total ArcZ and GadY sRNA in wild type cells, under these conditions there was an increase in the RQ of RprA and SibC sRNA. We observed that a change in the RQ of a sRNA did not inherently affect the α values, and that the sometimes extreme variation observed between experiments in the RQ of a particular sRNA was not reflected in a variation of the α values. Thus, a relative frequency based analysis can be a robust and highly reproducible way to study sRNA metabolism *in vivo*.

Collectively, the literature supports two basic models of sRNA decay. The first hypothesizes that trans-encoded sRNAs, like ArcZ and RprA, which typically lack extensive complementarity with their targets and require Hfq for their function, are degraded by RNase E (Masse *et al*. 2003a; Moll *et al.* 2003a; Morita *et al.* 2005). In the case of cis-encoded sRNAs, like GadY and SibC, and sRNAs that extensive complementarity with their targets are degraded by RNase III (Gerdes *et al*. 1992; Franch *et al.* 1999; Vogel *et al.* 2004). The results of this study were largely consistent with these expectations, but also demonstrated that sRNA metabolism is far more complex than either model predicts.

For example, both ArcZ121 and RprA105 are trans-encoded sRNAs that bind Hfq *in vivo* and *in vitro* (Wassarman *et al.* 2001; Zhang *et al*. 2003; Updegrove *et al.* 2008; Soper *et al.* 2010), and the literature supports the expectation that RNase E would be necessary for the metabolism of both (Masse *et al.* 2003a; Moll *et al.* 2003a; Morita *et al.* 2005). Consistent with this hypothesis and published findings (Papenfort *et al.* 2009; Madhugiri *et al.* 2010), our results clearly demonstrated a major involvement of RNase E in the metabolism of both ArcZ121 and RprA105. Although, most of the other endoribonucleases or exoribonucleases tested in this study

did not appear to participate in ArcZ121 metabolism, we observed, a yet understood, role of RNase P in RprA105 metabolism (Chapter 2).

In contrast, both GadY and SibC are cis-encoded sRNA that bind Hfq *in vivo* (Zhang *et al*. 2003; Opdyke *et al.* 2004). In these cases, some published work suggested either RNase III or RNase E would be necessary for the metabolism of these sRNAs (Gerdes *et al*. 1992; Franch *et al.* 1999; Masse *et al*. 2003a; Moll *et al.* 2003a; Vogel *et al.* 2004; Morita *et al.* 2005). However, we observed an involvement of both RNase III and RNase E in the metabolism of the GadY and SibC sRNAs (Chapter 3). In the case of GadY metabolism, RNase E and RNase III appeared to be functioning independently, since the effect of combining the alleles was additive. However, RNase E and RNase III appeared to be functioning in a coordinated manner in SibC metabolism, because the effect of combining the alleles was not additive. The observed dependence between RNase E and RNase III in SibC metabolism may not have resulted from hierarchical cleavage of SibC141. Rather, RNase III was necessary to cleave *ibsC* mRNA with in the context of the SibC141:*ibsC* duplex, and the duplex structure probably occludes the RNase E cleavage site.

Because we took a comprehensive approach in terms of the mutants tested, we observed an involvement of RNase P, RNase G, PNPase, RNase II and RppH in sRNA metabolism, whereas we found no evidence that would suggest RNase LS, RNase Z or RNase R were involved in the metabolism of ArcZ, RprA, GadY or SibC. RNase P has not been implicated in regulatory sRNA metabolism previously. RNase P is primarily known for its role in the maturation of tRNAs (Mohanty and Kushner 2007), but it also participates in the 5' end maturation of 4.5S (Bothwell *et al.* 1976), and tmRNA (Komine *et al.* 1994). Although, both 4.5S and tmRNA are small, noncoding RNAs, they are not considered regulatory sRNAs like RprA, GadY and SibC. However, the results of this study are consistent with a stabilization of

the RprA105, RprA45, GadY105 and SibC141 species in the *rnpA49* strain. At this point, we do not understand the exact role of RNase P in sRNA metabolism. The defects that we observed in the *rnpA49* mutant could result from indirect effects, e.g. the *rnpA49* allele could affect RNase E or Hfq levels. However, the *rnpA49* mutation did not affect the metabolism of ArcZ121, which may suggest the effects of this mutation observed for RprA, GadY and SibC were specific to these sRNAs. To determine if RNase P is directly involved in sRNA metabolism, *in vitro* cleavage assays need to be performed. However, regardless of a direct or indirect role of RNase P in sRNA metabolism, the results of this study clearly demonstrated that the influence of RNase P extends beyond that of processing of housekeeping RNAs to include a possible global effect on regulatory sRNA metabolism as well.

ArcZ121, RprA105, GadY105 and SibC141 are thought to be the primary transcripts (Argaman *et al.* 2001; Opdyke *et al.* 2004; Fozo *et al*. 2008; Papenfort *et al.* 2009; Mandin and Gottesman 2010). Although the metabolism of ArcZ121 appeared to be primarily dependent upon RNase E, we observed a dependence upon both RNase E and RNase P on the metabolism of RprA105, GadY105 and SibC141. Furthermore, RNase E and RNase P can be inhibited by the presence of a 5' triphosphate group on a primary transcript (Mackie 1998) (Bowden and Kushner, unpublished results). Interestingly, the metabolism of RprA105 and GadY105 appeared to be dependent in part on the activity of RppH, the RNA pyrophosphohydrolase (Deana *et al.* 2008), whereas the metabolism of ArcZ121 and SibC141 did not. These results suggest that a 5' triphosphate inhibits the activity of RNase E and/or RNase P when acting on RprA105 and GadY105 but not on ArcZ121 or SibC141. This could mean that RNase E and/or RNase P were using different mechanisms of action when acting on RprA and GadY versus ArcZ and SibC (Kime *et al*. 2009), or that there was another RNA pyrophosphohydrolase in the cell that was

removing these pyrophosphate groups (Deana *et al*. 2008). Like RNase P, this is the first time that RppH has been implicated in sRNA metabolism.

In the absence of RNase G, both the GadY105 and SibC141 species appeared to be stabilized. However, as compared with the effects of the *rne-1*, *rnc-14* and *rnpA449* alleles, the *Δrng* allele caused the least amount of change in the α values, suggesting a more limited and most likely a secondary role of RNase G in GadY and SibC metabolism. Similarly, we observed that in a *rne-1 Δrng* double mutant at 44°C the absence of RprA68 (data not shown). Given that the metabolism of GadY, SibC and RprA appeared to be highly dependent upon RNase E, an involvement of RNase E's homolog, RNase G, was not particularly surprising. However, like RNase P and RppH, this is the first time that RNase G has been implicated in the decay of both trans-encoded and cis-encoded sRNAs.

In vivo RNase II plays a limited role in the 3' end processing of 6S RNA (Li *et al.* 1998), a regulatory sRNA that sequesters the σ^{70} holoenzyme by mimicking an open promoter structure (Wassarman 2007). The involvement of RNase II in SibC metabolism was readily apparent from the results of this study. In the *Δrnb* mutant, SibC141 was absent and a longer SibC RNA ~145 nt accumulated. However, the removal of these additional nucleotides was not necessary for the generation of SibC109, since the α values in the *Δrnb* mutant were not significantly different from the wild type control.

A deficiency in PNPase activity is known to increase the rifampicin measured half-lives of MicA, CsrB, CsrC and SraL (Viegas *et al.* 2007). Furthermore, breakdown products of these sRNAs accumulated in the PNPase mutants (Suzuki *et al.* 2006; Viegas *et al.* 2007; Andrade and Arraiano 2008). In this study, we observed an increase in αRprA45, αGadY59 and αSibC109, as well as, the accumulation of a shorter ArcZ (<55 nt) species in the *pnp-7* mutant, which may

suggest PNPase was necessary for the decay of RprA45, GadY59, SibC109 and shorter ArcZ transcripts. When these results are combined with previously published work (Suzuki *et al.* 2006; Viegas *et al.* 2007; Andrade and Arraiano 2008), it is clear that PNPase mediated decay of sRNAs is a somewhat common feature of sRNA metabolism. PNPase along with the RNA helicase, RhlB, and enolase can associate with the C-terminal scaffolding domain of RNase E to form the degradosome (Carpousis 2007). In GadY metabolism, the *pnp-7* and *rneΔ374* phenotypes were similar, which may indicate that PNPase was functioning in a degradosome-dependent manner. In contrast, the effects of these alleles on both the SibC and RprA α values were almost opposite suggesting, in these cases, PNPase was functioning in a degradosome-independent manner.

Interestingly in the *Δhfq* mutant we observed a different phenotype for each sRNA with respect to changes in the RQ and α values as compared to the wild type control. In the absence of Hfq, the three major GadY RNAs, GadY105, GadY90 and GadY59 were absent. In contrast, although both ArcZ55 and RprA45 were absent, ArcZ121 and RprA105 were retained. Surprisingly, the steady-state level of RprA105 was reduced as compared to the wild type control, whereas the steady-state level of ArcZ121 appeared to increase. In the case of the SibC RNAs both SibC141 and SibC109 were retained and we observed a slight increase in αSibC109. All of the major forms of these four sRNAs, with the exception of RprA45, are known to associate with Hfq *in vivo* (Wassarman *et al.* 2001; Zhang *et al*. 2003; Opdyke *et al.* 2004). Complicating the interpretation of these results, is the fact that Hfq can form a complex with RNase E via the C-terminal scaffolding domain (Morita *et al.* 2005). Thus, it remains unclear if the metabolic defect in the *rneΔ374* mutant, which lacks the C-terminal scaffolding domain, stemmed from, in part, a dependence upon a physical interaction between Hfq and RNase E.

However, it is clear that Hfq appeared to affect the overall metabolism of each sRNA differently, which suggests complex and differential roles for Hfq in the metabolism of these four sRNAs.

When the results of this study are taken together, it is clear that sRNA metabolism involves more than just RNase E and RNase III. We have demonstrated that no one enzyme mediates all aspects of ArcZ, RprA, GadY or SibC metabolism, rather, the cell employs a combination of enzymes. Collectively, our work suggests the potential for multiple, independent metabolic pathways, multiple enzymes participating in a hierarchical processing pathway, pathways that generate shorter, stable RNAs versus those that do not, as well as, target-independent versus target-dependent metabolic events.

To circumvent and unravel these complications, the metabolism of sRNAs *in vivo* may be best analyzed by using a modified version of the assay employed by Overgaard *et al.* (2009). By deleting the chromosomal copy of both the sRNA and target and expressing each from an inducible plasmid, this group was able to determine the chemical half-life of the sRNA in the absence of rifampicin and determine the mechanism of action of the sRNA, i.e. catalytic versus stoichiometric. By engineering this system into single mutants and multiple mutants defective in ribonuclease activity, it would be possible to establish a correlation between enzyme(s), metabolic pathway, target and mechanism of action.

This assay system can be adapted to study the metabolism of any cis-encoded or trans-encoded sRNA. It could also be used to study the metabolism of a sRNA during different phases of growth or under different growth conditions. Furthermore, the metabolism of shorter, stable RNAs can be studied independent of their generation by expressing the shorter form(s) from the vector. To provide a more comprehensive picture of sRNA metabolism, probes that span the sRNA can be used in Northern blotting and cleavage sites can be mapped by a combination of 5’

RACE, primer extension and 3’ RACE. In total, this system may provide a versatile and elegant way to investigate all possible metabolic fates of any sRNA.

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